様　式　Ｃ－１９、Ｆ－１９－１、Ｚ－１９　（共通）

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研究課題名（和文）A thermogenetic tool enabling thermodynamic control of cellular protein functions

研究課題名（英文）A thermogenetic tool enabling thermodynamic control of cellular protein functions

研究代表者
ＶＵ　ＱＵＡＮＧ・ＣＯＮＧ（VU, QUANG CONG）

金沢大学・ナノ生命科学研究所・特任助教

研究者番号：５０９６１６２９

交付決定額（研究期間全体）：（直接経費）　　2,200,000円

研究成果の概要（和文）：本研究では、熱によってタンパク質の機能を制御するツール（発熱ツール）を開発した。ヒトのカスパーゼ-8に温度感受性ドメインを融合し、HEK293T細胞で発現させた。加熱すると温度感知ドメインがコアセルベーションされ、カスパーゼ-8が活性化されて細胞死が促進された。一方、カスパーゼ8のC360A変異を導入した細胞は加熱しても生存し、熱活性化ヒトカスパーゼ8の有効性が確認された。さらに、活性化をモニターするためのカスパーゼ-8インジケーターを開発した。

研究成果の学術的意義や社会的意義
Our heat-activated caspase 8 system offers a precise and controllable method to initiate apoptosis by heating, with potential applications in targeted cancer therapies. The successful implementation of this approach validates thermogenetics and opens avenues for novel therapeutic strategies.

研究成果の概要（英文）：This study aimed to develop a thermogenetic tool that utilizes heat to control protein functions for the manipulation of cellular processes. For the proof of concept, we developed a heat-activated human caspase 8 for programmed cell death. We fused a temperature-sensing domain with a human caspase 8 and expressed it in HEK293T cells. Upon heating, the temperature-sensing domain underwent coacervation, bringing two caspase 8 molecules into close proximity to induce dimerization and activation, thereby promoting cell death. For control, we introduced a C360A mutation in caspase 8 to inhibit its activation. When heated, HEK293T cells expressing the caspase 8 mutant remained alive, and cell division occurred. These results indicate the successful development of a heat-activated human caspase 8 for programmed cell death. Additionally, we developed a caspase 8 indicator to monitor its activation.

研究分野：Biotechnology

キーワード：Thermogenetics　Caspase 8

様　式　C－１９、F－１９－１（共通）

１．研究開始当初の背景

Controlling protein function is essential for *in situ* cellular applications, allowing precise temporal and spatial manipulation of protein activities. This capability is crucial for studying and understanding target protein functions. Optogenetics has emerged as a powerful tool for precisely manipulating protein activities using specific wavelengths of light. For example, blue light can open channelrhodopsins to allow ion flow and depolarize neurons (Deisseroth 2010), alter the conformation of light-oxygen-voltage-sensing domains (Niopek et al. 2016), or induce dimerization of the Vidvid fungal photoreceptor (Kawano et al. 2015). However, blue light often struggles with poor tissue penetration. Although near-infrared (NIR) upconversion nanoparticle-mediated optogenetics has achieved stimulation depths of approximately 4.2 mm in mouse brains (Chen et al. 2018), this is inadequate for clinical therapy. Recently, thermogenetics, which utilizes temperature-gated ion channels such as transient receptor potential (TRP) channels, has garnered significant attention as an alternative to optogenetics. For example, Bath et al. achieved rapid thermogenic control of neuronal activity in freely moving Drosophila using the TRPA1 channel (Bath et al. 2014). Temperature can be controlled both globally and spatially at significant depths using technologies such as focused ultrasound or magnetic particle hyperthermia, making thermogenetics a promising candidate for clinical applications. However, the current availability of thermogenetic tools is limited, and existing tools using TRP channels are only capable of manipulating cations (e.g., Na^+, K^+, and Ca^{2+}) to study neural activities. Therefore, there is a pressing need for innovative techniques that leverage temperature changes as an external stimulus to achieve thermodynamic control of cellular proteins. These techniques should allow activation of proteins at varying levels (e.g., 10%, 50%, or 100%) with high spatiotemporal resolution and reversibility.

２．研究の目的

In this study, we aim to develop a platform for thermodynamic control of cellular protein functions through temperature changes. This will be achieved by employing: (1) the phase transition behavior of thermal-responsive elements, which undergo reversible coacervation at temperatures above their phase transition temperature (T_t). This process will bring two fusion proteins into close proximity, inducing dimerization or reconstitution to activate them; and (2) near-infrared (NIR) local heating technology. As a proof-of-concept, a 980 nm laser will be used to spatiotemporally modulate temperature increases at the subcellular level (**Figure 1**).

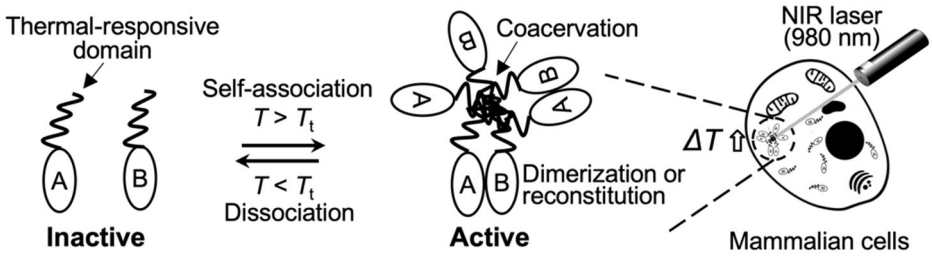

Figure 1. Schematic illustration of the concept of temperature-controlled protein function exploiting thermal-responsive domain and NIR heating technology.

３．研究の方法

To demonstrate the concept of temperature-controlled protein functions, we aimed to develop a heat-activated human caspase 8 system to regulate programmed cell death. Caspase 8 is a critical member of the cysteine protease family and serves as the initiator caspase in the extrinsic apoptosis pathway. Its activation is crucial for the initiation of the apoptotic cascade, and it has been demonstrated to undergo autoactivation through homo-dimerization (Oberst et al. 2010). To utilize this mechanism, we fused a caspase 8 to an end of a temperature-sensing domain. Upon heating, the temperature-sensing domain undergoes coacervation, bringing two caspase 8 molecules into close proximity. This spatial arrangement promotes their dimerization, a necessary step for their autoactivation. The dimerized caspase 8 undergoes autocleavage, producing the active form of the enzyme, which then triggers the apoptotic pathway leading to cell death (**Figure 2**).

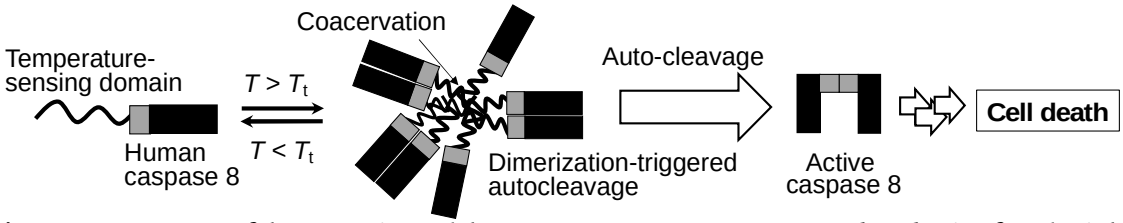

Figure 2. Concept of heat-activated human caspase 8. Caspase 8 molecule is fused with a temperature-sensing domain. Upon increasing the temperature higher than the phase transition temperature T_t, the temperature-sensing domain forms coacervation. This process brings two caspase 8 molecules into close distance, facilitating dimerization-triggered autocleavage of caspase 8 to promote cell death.

4. 研究成果

(1) Development of caspase 8 indicator

To monitor caspase 8 activation, we developed a caspase 8 indicator by fusing a red fluorescent protein mCherry with a caspase 8 substrate (IETD) and a triple nuclear export signal (3xNES) (**Figure 3A**). When expressed in cells, the indicator localized in the cytoplasm. Upon cleavage by caspase 8, mCherry translocated from the cytoplasm to the nucleus. We demonstrated the performance of the caspase 8 indicator in live cells in response to apoptosis-inducing drug staurosporine. To monitor cell apoptosis in real-time, we utilized Annexin XII conjugated with a polarity-sensitive dye that turns on green fluorescence on the cell membrane of apoptotic cells. After treatment with staurosporine, we observed the fluorescence signal in the nucleus and the green fluorescence from Annexin XII on the cell membrane, indicating cell apoptosis (**Figure 3B,C**). This result demonstrated the capability of the caspase 8 indicator to monitor caspase 8 activation in real-time.

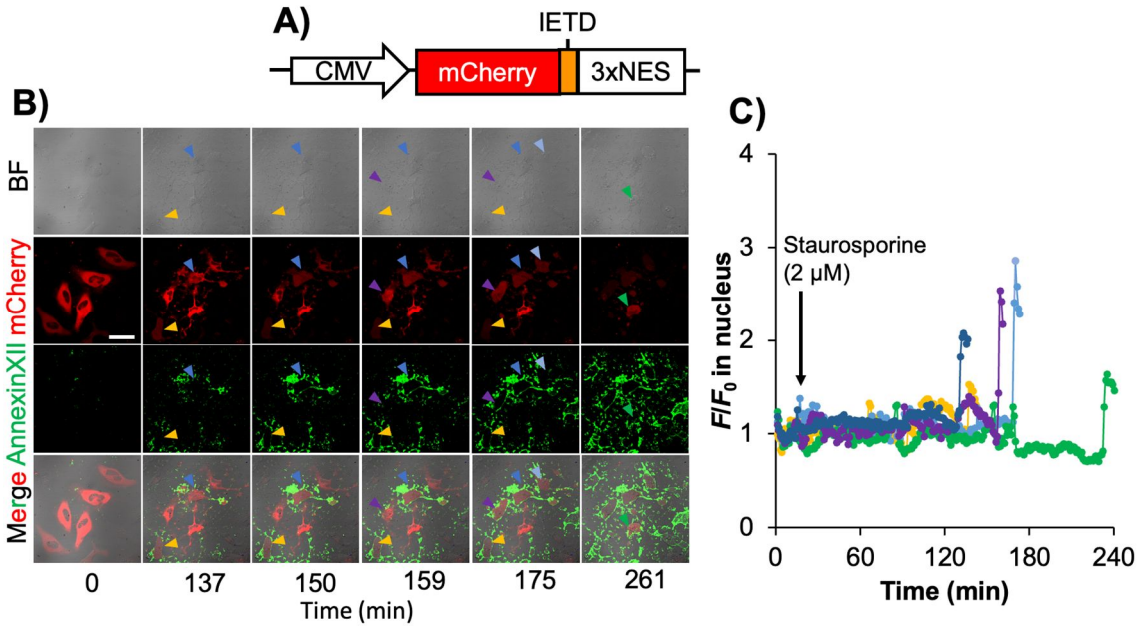

Figure 3. Development of the caspase 8 indicator. (A) Gene design. A red fluorescent protein mCherry is fused with a caspase 8 substrate (IETD) and a triple nuclear export signal (3xNES). Upon cleavage by caspase 8, mCherry translocated from the cytoplasm to the nucleus. (B) Fluorescence images of mCherry, Annexin XII, and bright field (BF) of HeLa cells expressing the caspase 8 indicator. (C) Normalized nucleus fluorescence against time in response to apoptosis-inducing drug staurosprorine (2 μM). Arrows indicate caspase 8-activating cells. Scale bar, 50 μm.

(2) Heat-modulated programmed cell death in mammalian cells

We transfected HEK293T cells with the heat-activated caspase 8 system. To track the expression, we introduced a fluorescent marker, mTagBFP2, along with a self-cleavage peptide, P2A (**Figure 4A,B**). This was done to ensure that we could monitor the expression of the system without interfering function of caspase 8. As shown in **Figure 4C**, we detected no green fluorescence signal from Annexin XII, and the caspase 8 indicator resided in the cytoplasm, indicating that the cells were healthy and caspase 8 had not

been activated. Upon heating, we observed cell apoptosis through the Annexin XII fluorescence signal on the cell membrane, as well as the translocation of the caspase 8 indicators into the nucleus. Furthermore, for control experiment, we introduced C360A mutation in caspase 8 to inactivate its function. As shown in **Figure 4D, E,F**, no green fluorescence signal from Annexin XII was detected, and the caspase 8 indicator remained in the cytoplasm, indicating the cells were healthy. These results collectively demonstrate that caspase 8 was activated by heat, leading to induced cell apoptosis.

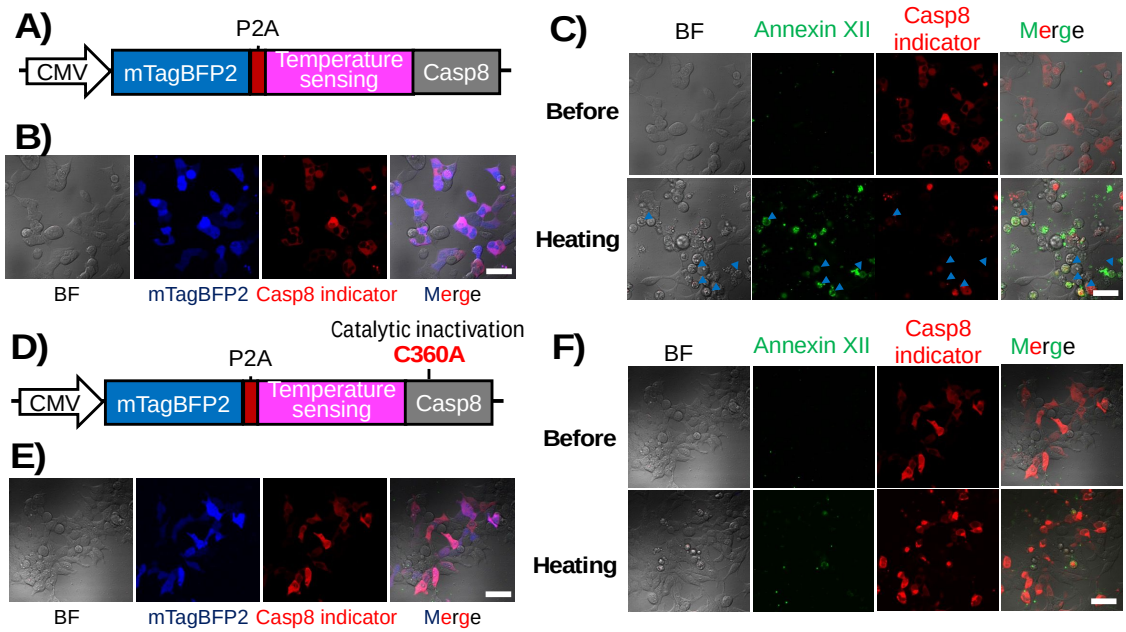

Figure 4. Heat-activated human caspase 8 in HEK293T cells. (A) Gene design; a fluorescent marker, mTagBFP2, along with a self-cleavage peptide, P2A, are fused upstream the temperature-sensing domain and caspase 8 (casp 8). (B) BF and fluorescence images of HEK293T cells co-expressing the system and the caspase 8 indicator. (C) BF and fluorescence images before and after heating. (D) Gene design; a C360A mutation of casp 8 is introduced to inhibit its activation.(E) BF and fluorescence images of HEK293T cells co-expressing the control system and the caspase 8 indicator. (F) BF and fluorescence images before and after heating. Annexin XII indicates cell apoptosis. Arrows indicate caspase 8-activating cells. Scale bar, 50 μm.

(3) Conclusion and future perspectives

In this study, we successfully developed heat-activated human caspase 8 as a thermogenetic tool and demonstrated its functionality in live cells by simply changing the medium temperature. Our heat-activated caspase 8 system provides a precise and controllable method to initiate apoptosis, offering potential applications in targeted cancer therapies where controlled induction of cell death is desirable. By changing the temperature, we can modulate the activation of caspase 8, thereby inducing cell death. This method also exemplifies the broader potential of thermogenetics for regulating various protein functions through temperature-induced structural changes. The successful implementation of this approach not only validates the concept of temperature-controlled protein functions or thermogenetics but also opens avenues for developing novel therapeutic strategies. By employing similar thermosensitive elements, we can design a wide range of protein-based systems that respond to temperature changes, providing high specificity and control in both research and clinical applications.

５．主な発表論文等

〔雑誌論文〕　計3件（うち査読付論文　1件／うち国際共著　1件／うちオープンアクセス　1件）

１．著者名 Vu Cong Quang、Arai Satoshi	４．巻 13
２．論文標題 Quantitative Imaging of Genetically Encoded Fluorescence Lifetime Biosensors	５．発行年 2023年
３．雑誌名 Biosensors	６．最初と最後の頁 939～939
掲載論文のDOI（デジタルオブジェクト識別子） 10.3390/bios13100939	査読の有無 有
オープンアクセス オープンアクセスとしている（また、その予定である）	国際共著 該当する

〔学会発表〕　計0件

〔図書〕　計0件

〔産業財産権〕

〔その他〕

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６．研究組織

	氏名 （ローマ字氏名） （研究者番号）	所属研究機関・部局・職 （機関番号）	備考

７．科研費を使用して開催した国際研究集会

〔国際研究集会〕　計0件

８．本研究に関連して実施した国際共同研究の実施状況

共同研究相手国	相手方研究機関