Auxin-Activated Caspase 9 System (AuxiCasp9), a Suicide Switch for Engineered Cells

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Letter

Auxin-Activated Caspase 9 System (AuxiCasp9), a Suicide Switch for Engineered Cells

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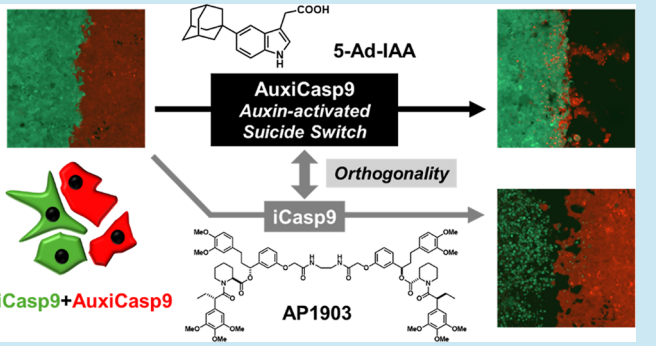
ABSTRACT: Engineered cells incorporating synthetic gene circuits have been developed for therapeutic and diagnostic applications. However, cell proliferation and long-term engraftment may pose a risk of unpredictable toxicities. To address this issue, a suicide switch that can be selectively activated by exogenous stimuli has been developed as a regulatory system for engineered cells. However, the variety of reliable suicide switches is limited and insufficient for the orthogonal integration of multiple functions in engineered cells. This study developed auxin-activated caspase 9 (AuxiCasp9), a novel auxin-based suicide switch, by fusing the auxin receptor protein transport inhibitor response 1 (TIR1) carrying E7K/E10K/F74A mutations and auxin protein (Aux) to caspase 9. AuxiCasp9 exhibited cell death-inducing activity comparable to that of the established systems, inducible caspase 9 (iCasp9) and rapamycin-activated caspase 9 (RapaCasp9). Finally, we confirmed the orthogonality of AuxiCasp9 and iCasp9, suggesting the feasibility of multiplexed control of engineered cells.



KEYWORDS: *auxin, cell death, apoptosis, caspase, suicide switch*

INTRODUCTION

Synthetic gene circuit-incorporating engineered cells have been developed for therapeutic and diagnostic applications.[1,2] Integrating synthetic genetic circuits enables cells to augment their inherent functions and acquire new capabilities. Among these engineered cells, chimeric antigen receptor (CAR)-T cell has shown remarkable therapeutic efficacy in B-cell lymphomas.[3] However, excessive CAR-T cell activation in the body leads to severe side effects, such as cytokine release syndrome.[4] Therefore, developing a regulatory system that eliminates overactive engineered cells from the body in response to exogenous stimuli is necessary. Such exogenous stimuli must be biologically inert so as not to further exacerbate pathological conditions.

A suicide switch is a genetically encoded element that enables the selective depletion of expressing cells in response to chemical compounds.[5] Among the various suicide switches activated by small molecules, inducible caspase 9 (iCasp9; Figure S1E)[6] has been tested in clinical trials (Figure 1A).[7] iCasp9, a fusion protein, consists of a mutated FK506 binding protein (FKBP12) and the catalytic domain of caspase 9 (Casp9). FKBP12 was mutated to allow the binding of AP1903, a chemical inducer of dimerization (CID). By binding AP1903, the homodimerization of iCasp9 induces caspase 9 activation and, subsequently, activates endogenous caspase 3, leading to cell death (apoptosis). As AP1903 cannot bind to wild-type FKBP12, a suicide switch is activated without interference from endogenous FKBP12.[8] Using the same design, Stavrou et al.[9] developed rapamycin-activated caspase 9 (RapaCasp9; Figure S1F), comprising FKBP12, the FKBP12-rapamycin binding domain (FRB), and Casp9. RapaCasp9 exhibits activity comparable to that of iCasp9. However, rapamycin is an immunosuppressive drug that inhibits the mammalian target of rapamycin (mTOR) and affects protein synthesis leading to cytotoxicity.[10,11]

As engineered cells are increasingly designed to integrate multiple synthetic gene circuits,[1,2] safety switches must operate independently of other engineered modules. However, the limited availability of reliable suicide switches constrains the multiplexed control of engineered cells.

Therefore, we developed a suicide switch that utilizes a synthetic derivative of the plant hormone auxin (indole-3-acetic acid, IAA) as a CID. This switch may not interfere with mammalian signaling pathways, and auxin is reported to be non-cytotoxic at high concentrations.[12−14] In this study, we developed an auxin-activated Casp9 (AuxiCasp9) that efficiently induces cell death at low concentrations of auxin derivatives and is orthogonal to iCasp9.

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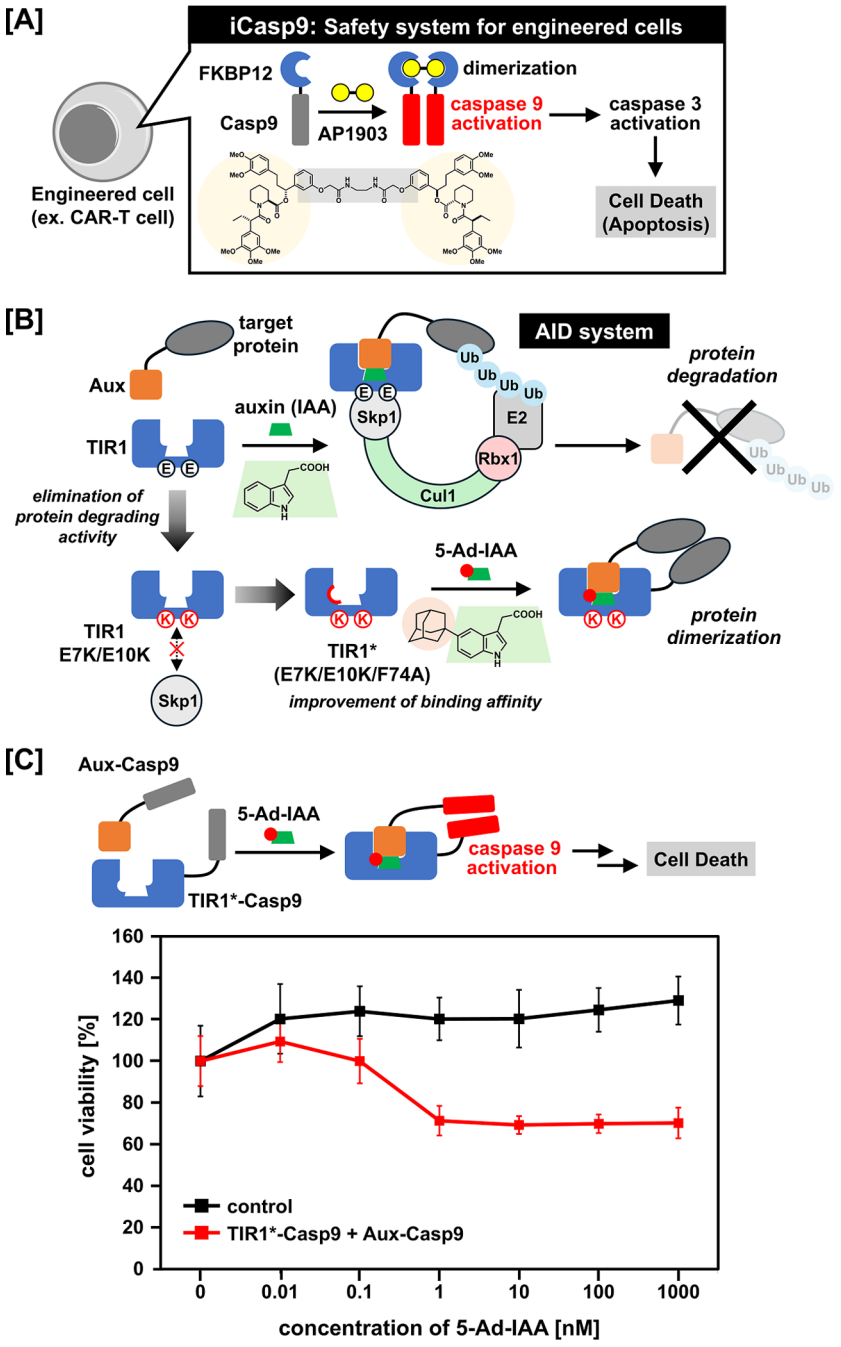

Figure 1. Design of auxin-activated cell death induction system. (A) Schematic representation of the inducible caspase 9 (iCasp9)-based cell death induction system. (B) Design of protein dimerization system based on the auxin-inducible degron (AID) system. S-phase kinase-associated protein 1 (Skp1), Cullin 1 (Cul1), and RING-box protein 1 (RBX1) are components of the E3 ubiquitin ligase complex. The F-box protein TIR1 forms a complex with these proteins to promote the ubiquitination of Aux proteins. Mutations were introduced to suppress interactions with the ubiquitin ligase complex (E7K/E10K) and to improve affinity for the auxin derivative 5-Ad-IAA (F74A). (C) HEK293T cells expressing mCherry (control) and HEK293T cells co-expressing mCherry, TIR1*-Casp9, and Aux-Casp9 (TIR1*-Casp9 + Aux-Casp9) were treated with various concentrations (0.01−1000 nM) of 5-Ad-IAA. After 24 h of incubation, cell viability was calculated by normalizing the fluorescence intensity at each concentration to that of untreated cells (mean ± SD; $n = 4$).

RESULTS

The auxin-inducible degron (AID) system was established by transplanting the auxin-dependent degradation pathway from plants into mammalian cells.[12,13] In this system, auxin induces heterodimerization between *Oryza sativa* transport inhibitor response 1 (TIR1) and truncated *Arabidopsis thaliana* auxin protein, IAA 17 (Aux; see Supporting Information), leading to the ubiquitin-proteasome degradation of the target protein fused to Aux (Figure 1B). In addition, an auxin-based heterodimerization system that eliminates protein-degrading functions was developed,[14] in which two glutamic acid residues that are important for the interaction between ubiquitin ligase complexes were mutated to lysine residues (Figure 1B, TIR1/E7K/E10K). However, this system requires a high concentration of

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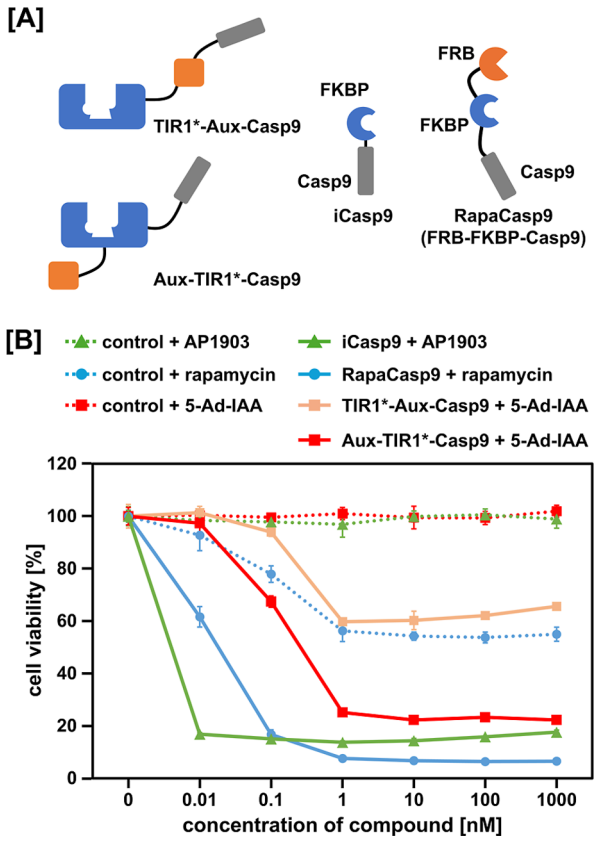

Figure 2. Improved single-construct auxin-activated cell death induction system. (A) Schematic illustration of the single-construct auxin-activated suicide switches, iCasp9, and RapaCasp9. (B) HEK293T cell lines expressing mCherry alone (control), iCasp9, RapaCasp9, TIR1*-Aux-Casp9, or Aux-TIR1*-Casp9 were treated with various concentrations (0.01−1000 nM) of the respective compound (AP1903, rapamycin, or 5-Ad-IAA). After 24 h of incubation, cell viability was calculated by normalizing the fluorescence intensity at each concentration to that of untreated cells (mean ± SD; $n = 4$).

auxin (>100 μM), and its application to an auxin-activated suicide switch may fail to achieve sufficient activity. Subsequently, the AID system was further improved by using a synthetic auxin analog, 5-adamantyl-IAA (5-Ad-IAA), along with a modified TIR1 with a complementary auxin-binding pocket (TIR1/F74A).[15,16] This combination enabled efficient protein degradation at nanomolar concentrations. Therefore, we applied a combination of TIR1 mutants (TIR1*, TIR1/E7K/E10K/F74A) and 5-Ad-IAA to the auxin-activated suicide switch (Figure 1B). By fusing Casp9 to the C-terminus of TIR1* and Aux, heterodimerization between the two proteins induces Casp9 homo-dimerization, subsequently activating endogenous caspase 3, leading to cell death (Figure 1C). To confirm whether this design induced cell death, we generated human embryonic kidney 293T (HEK293T) cells co-expressing Aux fused to Casp9 (Aux-Casp9; Figure 1C and S1A) and TIR1* fused to Casp9 (TIR1*-Casp9; Figure 1C and S1B). Control and transfected cells were treated with different concentrations of 5-Ad-IAA. After 24 h, cell viability was determined using the alamarBlue assay. 5-Ad-IAA selectively decreased the viability of transfected cells; however, the reduction plateaued at approximately 30%, starting from 1 nM 5-Ad-IAA (TIR1*-Casp9 + Aux-Casp9, Figure 1C). Thus, an auxin-activated suicide switch can function, although its efficiency is moderate.

This system has a drawback in that it requires simultaneous high-level expression of two constructs, one of which is relatively long. To generate HEK293T cells stably co-expressing the two constructs, cells were first transduced with lentivirus encoding Aux-Casp9 (Figure S1A). Subsequently, the cell population was subjected to a second transduction with the TIR1*-Casp9 lentiviral vector (Figure S1B) to achieve dual expression (Figure S2). If the expression level of either construct is insufficient, auxin-induced dimerization does not occur efficiently. Therefore, we designed and generated two single constructs, each containing TIR1*, Aux, and Casp9 (Figure 2A and S1C,D). iCasp9- and RapaCasp9-expressing cells were used as positive controls (Figure 2A and S1E,F). We evaluated the cell death-inducing activity of these systems using various concentrations of the respective compounds in the alamarBlue assay (Figure 2B). The iCasp9 system induced the most effective cell death (~80%) at the lowest concentration (0.01 nM). In contrast, rapamycin showed moderate cytotoxicity in control cells (~40% reduction), whereas RapaCasp9-expressing cells exhibited enhanced cell death (~90%), confirming its function. The Aux-TIR1*-Casp9 system reduced cell viability by approximately 80% upon treatment with 1 nM 5-Ad-IAA, indicating cell death induction sufficient to function as a suicide switch for engineered cells (Figure 2B). Furthermore, 5-Ad-IAA showed no cytotoxicity at concentrations up to 1 μM, similar to AP1903, suggesting superior selectivity compared with rapamycin. The TIR1*-Aux-Casp9 system, a reverse configuration of Aux and TIR1*, moderately induced cell death, suggesting the importance of construct orientation. These results demonstrated the successful development of a single-construct auxin-activated caspase 9 system, termed AuxiCasp9.

Next, we examined whether AuxiCasp9 (Figure S1D) induced cell death via caspase activation and compared its activity with that of iCasp9 (Figure S1E) and RapaCasp9 (Figure S1F) using Caspase-Glo 9 or 3/7 assays. In AuxiCasp9 cells, caspase 9 and caspase 3 activities gradually increased over a 120 min period after treatment with 5-Ad-IAA (Figure 3A). In contrast, iCasp9 and RapaCasp9 cells exhibited rapid and strong activation of caspase 9 and caspase 3 within 30 min (Figure 3B,C). Although the magnitude and kinetics of activation were lower for AuxiCasp9 than for iCasp9 and RapaCasp9, AuxiCasp9 induced caspase 9 and caspase 3 activation sufficient to trigger apoptosis.

Finally, we confirmed the orthogonality between AuxiCasp9 (Figure S1D) and iCasp9 (Figure S1E). HEK293 cell lines expressing either iCasp9 with EGFP or AuxiCasp9 with mCherry were generated (Figure 4A). When the two cell lines were co-cultured, AP1903 treatment selectively induced cell death in iCasp9 cells (Figure 4B and S3), whereas 5-Ad-IAA treatment selectively eliminated AuxiCasp9 cells (Figure 4C and S3). These data demonstrate that AuxiCasp9 functions orthogonally to the iCasp9 system.

DISCUSSION

Chemically induced protein dimerization systems enable the control of various cellular processes, including protein degradation, protein transport, gene regulation, and induction of cell death.[17] Auxin has been previously used as a CID to induce degradation of target proteins.[12] This system induced auxin-responsive cell death via the targeted degradation of Inhibitor of Caspase-activated DNase.[18] However, our study represents the first application of an auxin-based system for a suicide switch

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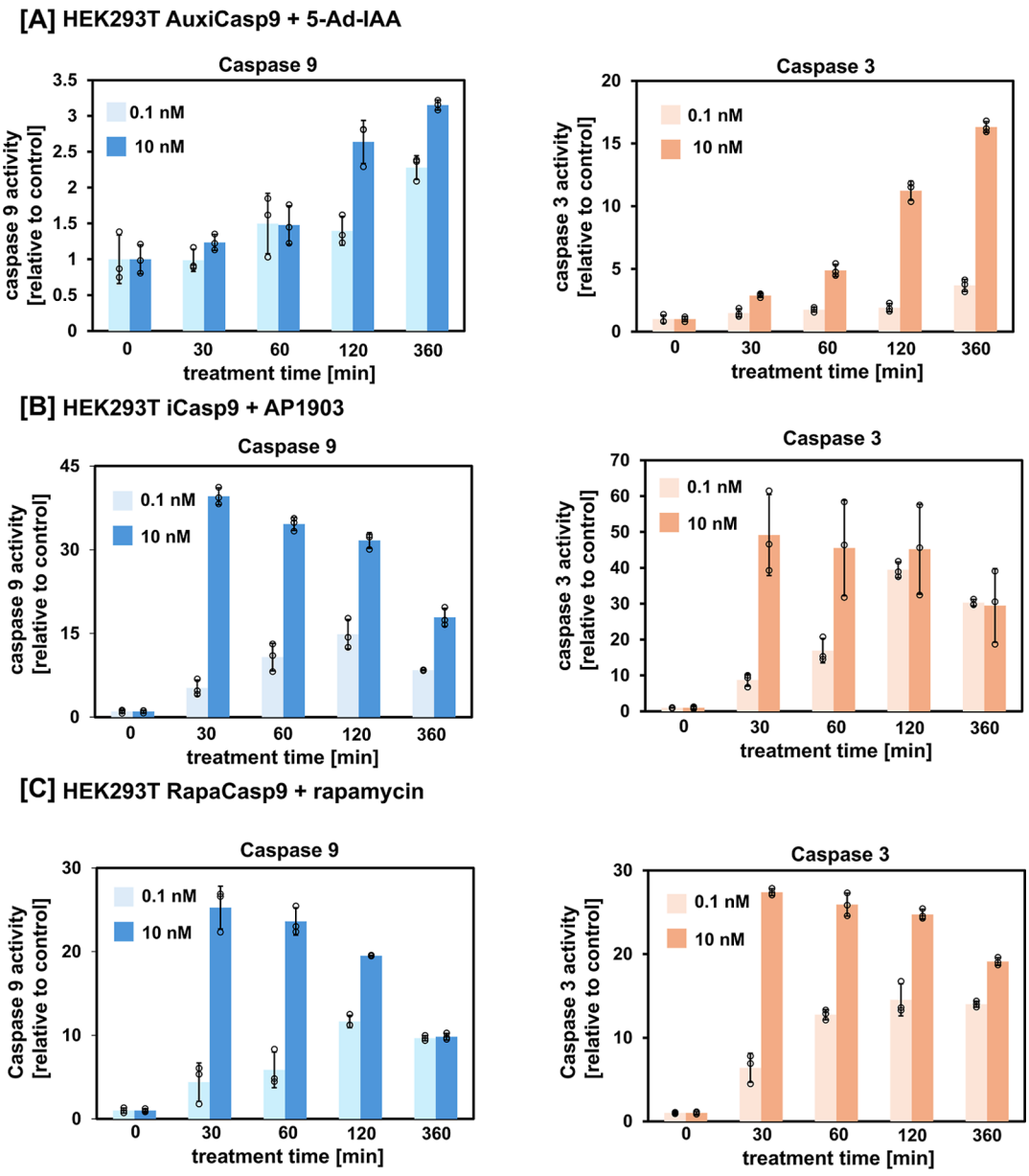

Figure 3. Activation levels of caspase 9 and caspase 3 in HEK293T cells. (A) HEK293T cells expressing AuxiCasp9, (B) iCasp9, or (C) RapaCasp9 were treated with the indicated concentration (0.1 or 10 nM) of the respective compounds (5-Ad-IAA, AP1903, or rapamycin). After incubation for the indicated times, Caspase-Glo 9 or Caspase-Glo 3/7 reagent was added to the medium, and luminescence was measured using a microplate reader. The blank value from the wells containing medium and reagent only was subtracted from each measurement. Caspase 9 (left panels) and caspase 3 (right panels) activities at each time point were normalized to those of untreated cells (mean ± SD, $n = 3$). Individual data points are plotted as open circles.

using dimerization of caspase 9. The single-vector design composed of three components, TIR1*, Aux, and Casp9, is also the first example of a fully integrated auxin-responsive system that induces cell death (Figure 2). The TIR1*-Aux-Casp9 construct exhibited lower activity than that of Aux-TIR1*-Casp9 (Figure 2B), which may be attributable to differences in dimerization topology. Linker optimization may improve cell death-inducing activity, as suggested by previous studies on RapaCasp9.[9] Compared with RapaCasp9, AuxiCasp9 can be activated by 5-Ad-IAA, which is substantially less cytotoxic than rapamycin. Although the use of rapalog (rapamycin analogs) and FRB mutation can reduce the intrinsic cytotoxicity of rapamycin, the effective concentrations of rapalog tend to be higher than that of rapamycin.[19,20]

We demonstrated that AuxiCasp9 is orthogonal to iCasp9 (Figure 4), indicating that FKBP-based systems can function independently of AuxiCasp9. Accordingly, AuxiCasp9 can serve as a safety switch for engineered cells, while FKBP-based systems can be used simultaneously to control other cellular functions. The importance of developing orthogonal control channels has already been emphasized in the context of multifunctional CAR-T cell engineering.[21] As engineered cells continue to increase in functional complexity, the development of orthogonal regulatory systems has become a central challenge in synthetic biology.[1−3] Therefore, AuxiCasp9 expands the

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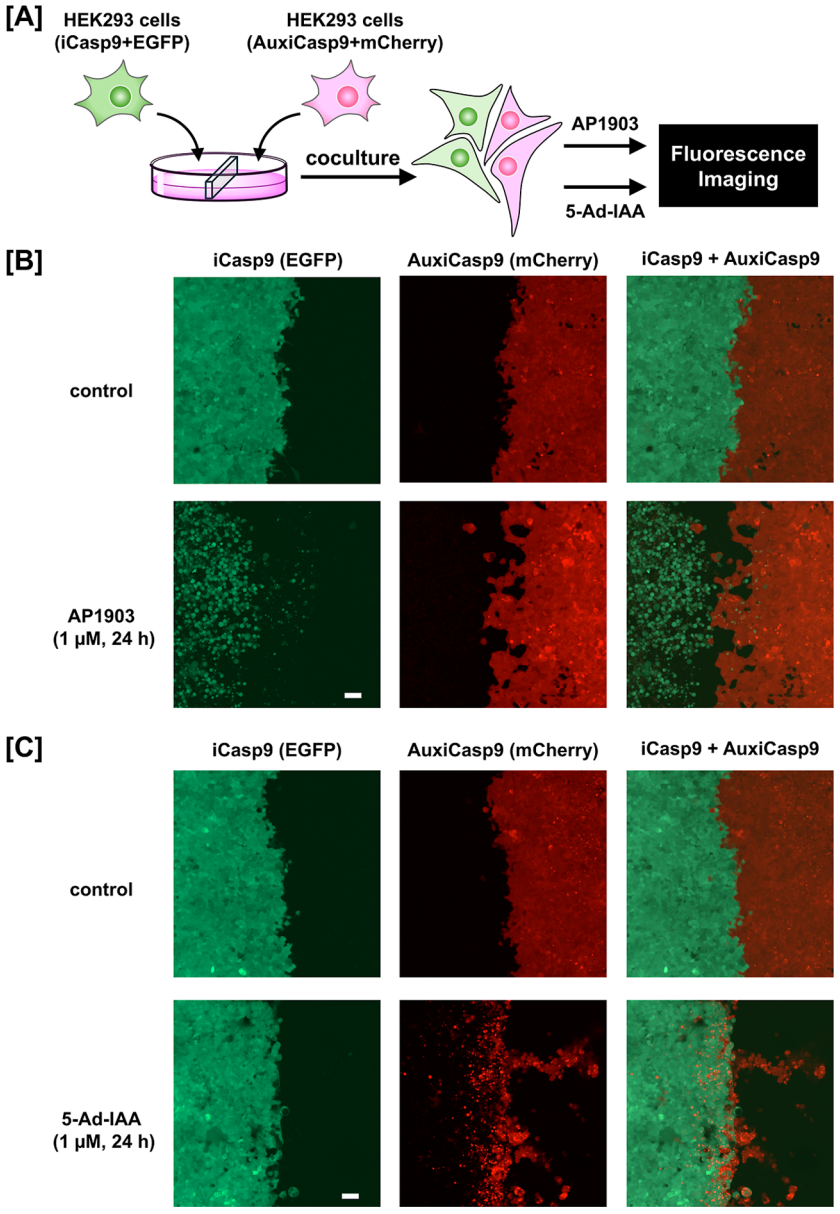

Figure 4. Orthogonality of the AuxiCasp9 system. (A) Schematic representation of the experiment. HEK293 cells co-expressing AuxiCasp9 and mCherry (AuxiCasp9) and HEK293 cells co-expressing iCasp9 and EGFP (iCasp9) were co-cultured and treated with the respective compounds, followed by fluorescence imaging. (B,C) Co-cultured cells were treated with 1 μM AP1903 (B) or 1 μM 5-Ad-IAA (C). After 24 h of incubation, cells were observed using confocal laser scanning microscope. Red indicates AuxiCasp9, and green indicates iCasp9. Scale bar:50 μm.

repertoire of available suicide switches by introducing an auxin-responsive control channel that operates independently of established FKBP-based systems. In addition, when manipulating two or more types of engineered cells, such as therapeutic and diagnostic cells, independent control through distinct suicide switches may enable precise and selective regulation of each cell type.

AuxiCasp9 exhibited high cell death-inducing activity; however, it failed to eliminate all engineered cells (Figure 2B). A similar limitation has been reported for iCasp9, in which a subset of engineered cells evades elimination and regrows, allowing long-term survival.[22,23] To address this limitation, multiple suicide switches have been incorporated into engineered cells. Co-expression of iCasp9 and an inducible caspase 8 markedly suppressed the regrowth of engineered cells and enhanced elimination efficacy.[24] In addition, a strategy combining suicide switches with distinct mechanisms of action, including herpes simplex virus thymidine kinase and RapaCasp9, has been proposed to target diverse cell types.[25] Similarly, combining AuxiCasp9 with suicide switches based on distinct mechanisms of action may provide a route toward improving the completeness of engineered-cell elimination. As AuxiCasp9 is orthogonal to iCasp9, their combined use is expected to minimize leakage and unintended activation. Notably, AuxiCasp9 exhibited cell-death kinetics distinct from those of other inducible suicide-switch systems. Such kinetic differences may be advantageous for future combinatorial strategies using multiple orthogonal switches to achieve more tunable cell ablation in engineered-cell applications.

When applying AuxiCasp9 in cell therapy, immunogenicity associated with the use of plant-derived proteins remains a risk.[26] However, target protein degradation by the AID system

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has been successfully achieved in mice without detectable toxicity,[27] supporting further evaluation of AuxiCasp9 in therapeutic contexts. Future studies should evaluate AuxiCasp9 in clinically relevant cell types, including T cells, and explore its combinatorial use with other orthogonal suicide switches as a step toward more robust and controllable safety-switch designs.

In conclusion, we developed AuxiCasp9 as an auxin-activated cell suicide switch in a single-vector construct, in which the catalytic domain of caspase 9 was fused to Aux and TIR1*. AuxiCasp9 induced sufficient cell death to function as a suicide switch in engineered cells (Figure 2A,B). AuxiCasp9 can be activated by 5-Ad-IAA, a small and chemically simple CID. Moreover, the orthogonality between AuxiCasp9 and iCasp9 was demonstrated (Figure 4B and S3). Collectively, AuxiCasp9 provides an orthogonal and alternative suicide-switch platform, expanding the available toolkit for future development of multifunctional engineered cells.

METHODS

Cell Culture

HEK293, HEK293T, and HEK293LTV cells were cultured in high-glucose Dulbecco's modified Eagle medium (DMEM; FUJIFILM Wako Pure Chemical Corporation) supplemented with 10% (v/v) fetal bovine serum (BioSera), 100 U/mL penicillin, and 100 μg/mL streptomycin at 37 °C in a humidified incubator with 5% CO_2.

Lentiviral and Plasmid Construction

Lentiviral vectors encoding iCasp9/EGFP (VB900129-0975xaa), iCasp9/mCherry (VB230522-1511pzs), mCherry (VB010000-9390nka), or EGFP (VB010000-9389rbj) were purchased from VectorBuilder Inc. pAIDFA-EF1a-NmScarlet-mAID[15] was provided by RIKEN BRC through the National BioResource Project of the MEXT, Japan (cat. RDB19229). psPAX2 plasmid (Addgene plasmid #12260; https://www.addgene.org/12260; RRID:Addgene_12260) and pMD2.G plasmid (Addgene plasmid #12259; https://www.addgene.org/12259; RRID:Addgene 12259) were gifts from Didier Trono.

RapaCasp9 vector was amplified from RapaCasp9/Halo-NLS (P240702-1008gme) and subcloned into the mCherry vector. RapaCasp9/Halo-NLS vector was generated from the iCasp9/EGFP vector (VB900129-0975xaa) by replacing the EGFP expression cassette with Halo-NLS, followed by assembly of the BsrGI-digested vector with a synthesized RapaCasp9 gene fragment (Twist Biosciences). AuxiCasp9 vector (Figure S1D) is available from RIKEN Bioresource Center, Japan (BRC, Gene engineering division: https://dna.brc.riken.jp/en/) under the identifier RDB21303.

Transduction

Lentiviral particles were generated by transient transfection of 293LTV cell line (Cell Biolabs) using the Lipofectamine 3000 Reagent (Thermo Fisher Scientific). The psPAX2 and pMD2.G plasmids were co-transfected along with the lentiviral transfer vectors. After 24 h, the medium was removed, cells were washed with phosphate-buffered saline and cultured in DMEM for an additional 48 h. The conditioned medium containing lentiviral particles was collected and used to infect HEK293 or HEK293T cells, followed by incubation for 24 h.

Establishment of Cell Death Induction System-Expressing Cell Lines

To generate HEK293T cells expressing TIR1*-Casp9 and Aux-Casp9, lentiviral transduction was performed as described above. HEK293T cells were transduced with a lentiviral vector carrying the Aux-Casp9 vector, followed by selection with 2 μg/mL puromycin (FUJIFILM Wako Pure Chemical Corporation). Next, the cells were transduced with a lentiviral vector carrying the TIR1*-Casp9 vector, and EGFP-positive cells were sorted using FACS Aria (Becton Dickenson) at the Support Unit for Bio-Material Analysis in the RIKEN Center for Brain Science, Research Resources Division. HEK293 and HEK293T cell lines expressing other cell death induction systems were established using the same lentiviral transduction and puromycin selection procedures.

Cell Viability Assay

Cell viability was evaluated using the alamarBlue reagent (Thermo Fisher Scientific). Briefly, 1×10^4 cells/100 μL were seeded into 96-well black plates (Corning Inc.) and incubated at 37 °C for 24 h. Cells were treated with AP1903 (Selleck Chemicals), rapamycin (Tokyo Kasei Co., Ltd.), or 5-Ad-IAA (Tokyo Kasei Co., Ltd.) and incubated for 22 h. AlamarBlue reagent was then added to the medium at a final concentration of 10%. Before and after 2 h of incubation, the increase in fluorescence intensity was measured using a SpectraMax M2 microplate reader (Molecular Devices) at excitation and emission wavelengths of 560 and 590 nm, respectively.

Caspase Activity

Caspase 9 and caspase 3 activities were measured using Caspase-Glo 9 and Caspase-Glo 3/7 Assay Kits (Promega Corporation). Cells (1×10^4 cells/100 μL) were seeded into 96-well white plates (Thermo Fisher Scientific) and incubated at 37 °C for 24 h. Cells were treated with the respective compounds, and 100 μL of Caspase-Glo 9 and 3/7 reagent was added to each well. After 50 min of incubation at room temperature, luminescence was measured using a SpectraMax M2 microplate reader.

Orthogonality of the AuxiCasp9 System

Dual-color live-cell imaging of iCasp9- or AuxiCasp9-expressing cells was performed using an LSM900 confocal laser scanning microscope (Carl Zeiss AG). Cells were co-cultured using a Culture-Insert 2 Well in a μ-Dish 35 mm High (ibidi GmbH). After 24 h, cells were treated with the respective compounds for 24 h, and then fluorescence images were acquired using an LSM900 microscope.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acssynbio.6c00010.

Supporting information (amino acid sequences of each suicide-switch construct and Figures S1−S3) (PDF)

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Author Contributions

The manuscript was written with contributions from all authors. S.Y. and K.D. designed the experiments; S.Y. and S.S.S. performed the experiments; S.Y., S.S.S., S.S., and K.D. analyzed the data; S.Y. and K.D. wrote the original manuscript; S.S. and Y.Y. contributed to the conceptualization and project administration; and K.D. directed the project.

Notes

The authors declare no competing financial interests.

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