Bioorthogonal Engineering of Bacterial Effectors for Spatial—Temporal Modulation of Cell Signaling

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ABSTRACT: The complicated and entangled cell signaling network is dynamically regulated by a wide array of enzymes such as kinases. It remains desirable but challenging to specifically modulate individual, endogenous kinases within a cell, particularly in a spatial—temporally controlled fashion. Current strategies toward regulating the intracellular functions of a kinase of interest either lack specificity or require genetic engineering that may perturb its physiological activity. Herein, we harnessed a bacterial effector OspF for optical and chemical modulation of the endogenous mitogen-activated protein kinase (MAPK) cascade in living cells and mice. The phospho-lyase OspF provided high specificity and spatial resolution toward the desired kinase such as the extracellular signal-regulated kinase (ERK), while the genetically encoded bioorthogonal decaging strategy enabled its temporal activation in living systems. The photocaged OspF (OspF©) was introduced into living mice to modulate ERK-mediated gene expression. Finally, our spatially and chemically controlled OspF was further used to precisely tune immune responses in T cells. Together, our bioorthogonal engineering strategy on bacterial effectors offers a general tool to modulate cell signaling with high specificity and spatial—temporal resolution.

INTRODUCTION

Eukaryotic cells have evolved a diverse repertoire of enzymes for catalyzing potent chemical modifications on proteins that dictate diverse signaling events. For example, nearly 600 kinases exist in human cells to control phosphorylation, and dysregulation of this complex and interconnected signaling network is often linked to diseases such as cancer. The endogenous mitogen-activated protein kinase (MAPK) cascade is one of the central signaling pathways that regulates numerous cellular processes. As two essential nodes at the end of the MAPK cascade, extracellular signal-regulated kinase (ERK) and p38 receive upstream signals and shuttle between cytoplasm and nucleus to phosphorylate more than 150 substrates participating in regulation of gene expression, cell proliferation, as well as diverse cellular responses. In addition, ERK can directly phosphorylate almost all the upstream components of the receptor tyrosine kinase (RTK)-RAS-MAPK cascade to mediate the negative feedback regulation. Misfunction of MAPK cascade is considered as a hallmark in cancer cells including melanoma and colorectal cancers, and has also been connected to immune diseases. Nevertheless, methods for precise tuning of the endogenous MAPK cascade are highly challenging, particularly in a spatial—temporally controlled fashion. For example, although small-molecule modulators have been developed for targeting the MAPK pathway, they often have certain off-target effects, particularly among the isoforms of MAPK family enzymes. Meanwhile, optogenetics and chemical genetic strategies require genetic manipulation and overexpression of the kinase of interest that may perturb its native cellular functions.

Diverse effector proteins have been evolved by bacteria to modulate signaling pathways inside host cells with high specificity. For example, OspF is a phospho-lyase from Shigella spp. that can be secreted through a Type III secretion system (T3SS) into host cells to specifically dephosphorylate phosphothreonine at residue 202 on ERK and residue 180 on p38, respectively (Scheme 1 and Figure S1). The resulting dehydrobutyrylase cannot be rephosphorylated by its upstream kinases and thus permanently abrogated ERK and p38 activity (Scheme 1B). Inspired by the high specificity of OspF
(A) Bioorthogonal caging of the phospho-lyase OspF can be used to specifically modulate the endogenous ERK and p38 activity by light or chemical triggers. (B) Structure of caged OspF bearing ONBK (o-nitrobenzyloxy carbonyl-N'-lysine, left) or TCOK-a (axial isomer of trans-cyclooctene lysine, right) at residue 134. The catalytic center of OspF is highlighted. The presence of 365 nm light or the chemical trigger (Me2-Tz) would decage ONBK or TCOK-a, and rescue the phospho-lyase activity of OspF. The in situ rescued OspF can specifically remove the phosphate group on phosphothreonine at residue 202 on ERK and residue 180 on p38, respectively. The resulting dehydrobutyrine at these sites can no longer be rephosphorylated, leading to permanently abrogated ERK and p38 activity.


toward ERK and p38 as opposed to other kinases including other members of the MAPK family, we decided to employ this phospho-lyase to modulate the endogenous MAPK signaling cascade. However, since the constitutively active form of OspF will irreversibly inhibit ERK and p38 activity and thus permanently turn off the MAPK signaling cascade, temporally controlled activation of OspF is highly desired. Previous methods relying on controlled expression of OspF protein have a poor temporal resolution, and are exceedingly difficult for applications in live animals. We envisioned that our recently developed genetically encoded bioorthogonal decaging strategy would enable precise and temporally controlled activation of OspF under living conditions. Herein, by bioorthogonal engineering and targeting of the bacterial effector OspF to different subcellular compartments (e.g., cytosol versus nucleus), we report spatial–temporally controlled modulation of the endogenous MAPK signaling cascade in living systems by small-molecule triggers or light.

■ RESULTS AND DISCUSSION

Engineering an Optically Controlled OspF (OspF*) for Living Cells. We started by engineering an optically controlled OspF in living cells based on the genetic code expansion system. The photocaged lysine analogues such as ONBK and other o-nitrobenzyl caged or coumarin lyses have been previously developed for controlling lysine-dependent protein activity inside living cells. As lysine 134 (K134) is the catalytic residue for OspF’s phospho-lyase activity, we used ONBK to replace OspF’s K134 residue (Scheme 1B). The resulting protein OspF-* completely lost its phosphothreonine lyase activity similar to the catalytically inactive OspF-K134A mutant until the 365 nm light-mediated photo-decaging (Scheme 1B). The expression of OspF* was first verified by multiple methods including immunoblotting, flow cytometry analysis, as well as mass spectrometry analysis (Figure 1A and

![Figure 1](https://example.com/figure1.png)

**Figure 1.** Optical modulation of the endogenous MAPK signaling cascade by OspF* in living cells. (A) Immunoblotting analysis of expression and activation of OspF* in living cells. OspF activity was detected by phosphorylation of ERK and p38 using specific antibodies. The phosphorylation of JNK and IκB was also analyzed to confirm the specificity of OspF. The expression of OspF* was verified by using anti-Myc tag antibody. OspF-WT and actin were used as controls. (B) Variation of photoactivation time from 0 to 5 min was performed on cells harboring OspF*, and a decreasing of ERK/p38 phosphorylation was detected with increasing photo-activation time. Cells with no ONBK supplementation were used as a control. (C) Dual-luciferase analysis of activation of OspF* in living cells. The relative luminescence activity is proportional to the phosphorylation of JNK and IκB before and after photoactivation (Figure 1A and Figure S5). Finally, we found that expression of OspF* did not affect the phosphorylation status of JNK (another MAPK that is not OspF’s substrate) as well as other kinases we tested including IκB and Akt before and after photoactivation. (Figure 1A and Figure S6), which further confirmed the high specificity toward ERK/p38. We also constructed a stable HEK293T cell line that constitutively
expresses the MmPyfRSLNBK-1/IRNAPyl CUa pair, the key component for UAA incorporation, for production and optical rescue of OspF*. Immunoblotting results confirmed the expression and photoactivation of OspF*, and flow cytometric analysis further showed the expression efficiency in cells (Figures S3GJ and S7).

We then applied OspF* for temporal modulation of the MAPK pathway. First, we showed that, upon photoactivation of OspF* inside cells, the attenuation of ERK/p38 phosphorylation was observed as fast as 5 min (Figure S8) while the protein level of OspF* was not changed even 60 min after photoactivation. We then employed a luciferase reporter, SRE-luc, to monitor OspF activity in living cells.31 SRE-luc activity is known to shuttle between the nucleus and cytoplasm and mediate biological processes such as negative feedback regulation. Modulating ERK activity with spatial resolution may allow modulation of ERK's activity in the nucleus as opposed to cytoplasm with high spatial resolution.

Simultaneous Modulation of OspF* by Light. ERK is known to shuttle between the nucleus and cytoplasm and phosphorylate a seres of substrates with different physiological outputs.3 ERK can translocate into the nucleus to phosphorylate transcription factors such as Myc proto-oncogene protein (c-MYC) to induce gene expression and cell proliferation. Meanwhile, phosphorylated ERK remains in the cytoplasm to phosphorylate over 50 substrates and mediate biological processes such as negative feedback regulation. Modulating ERK's activity with spatial resolution may help to dissect its functions in different subcellular regions. Small-molecule ERK inhibitors will block all of 's activity that lacks spatial resolution. In contrast, subcellular-targeted OspF* may allow modulation of ERK's activity in the nucleus as opposed to cytoplasm with high spatial resolution. We first generated nucleus-located OspF* (nu-OspF*) by fusing a bipartite nuclear localization sequence (NLS) from

Figure 2. Optical control of subcellular-targeted OspF* enables spatial–temporal modulation of the MAPK signaling cascade. (A) Diagram of the nucleus- and cytosol-located OspF* (nu-OspF* and cyt-OspF*). (B) Immunofluorescent staining and flow cytometry quantification of the expression of subcellular-located OspF*. Cells expressing nu-OspF* or cyt-OspF* were stained using an anti-Myc antibody followed by an Alexa fluor 488-conjugated antimouse IgG before being evaluated by flow cytometry. Histogram showing percentage of transfected cells that harbor full-length nu-OspF* or cyt-OspF*. Data were analyzed by FlowJo software. (C) Immunofluorescence imaging of the subcellular-located OspF*. Cells expressing nu-OspF* or cyt-OspF* were fixed and evaluated by immunofluorescence imaging using an anti-Myc antibody followed by an Alexa fluor 488-conjugated antimouse IgG. The nucleus was stained with Hoechst 33342. Overlay of fluorescence image and DIC image is shown, and all fluorescence images are shown in Figure S11. Scale bars represent 10 μm. (D, E) Schematic illustration and immunoblotting results of the effects on ERK substrates after rescue of nu-OspF*. Phosphorylation of c-MYC was attenuated within 5 min in nu-OspF* expressed cells after photoactivation, followed with dramatic protein degradation. No obvious variation on p90RSK phosphorylation was detected. (F, G) Schematic illustration and immunoblotting results of the effects on ERK substrates after rescue of cyt-OspF*. Phosphorylation of p90RSK was attenuated within 5 min in cyt-OspF* expressed cells after photoactivation. Less decrement of c-MYC phosphorylation and protein abundance was detected at the same time point (5 min, 60 min) in comparison with nu-OspF* harboring cells. Data shown in parts E and G are representative of at least three independent experiments.

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nucleoplasm to the N-terminal of OspF*, while two K to A mutations were introduced to this NLS to render the resulting OspF* exclusively cytosol-located (cyt-OspF*) (Figure 2A).33,34 Subcellular fractionation and immunoblotting analysis confirmed the proper subcellular localization as well as the similar expression levels of nu-OspF* and cyt-OspF* in the nucleus and cytoplasm of HEK293T cells (Figure S10). Flow cytometric analysis verified the expression of both nu-OspF* and cyt-OspF* in most cells (Figure 2B and Figure S3B,C,E,F), while immunofluorescence analysis also verified the adequate expression as well as the proper subcellular localization of nu-OspF* and cyt-OspF* in the majority of cells (Figure 2C and Figure S11). Next, a significantly decreased ERK phosphorylation was observed in cells after photoactivation of either nu-OspF* or cyt-OspF* (Figure S12). Furthermore, we analyzed the extent of ERK phosphorylation in the nucleus and cytoplasm after activation of subcellular-targeted OspF*. Immunoblotting results showed that phosphorylation of ERK in the nucleus was significantly decreased after nu-OspF* activation, but negligible variation was detected on cytosolic ERK. In contrast, a significant decrease of ERK phosphorylation in the cytoplasm was detected after optical rescue of cyt-OspF* inside cells (Figure S10). These results proved that our subcellular-targeted OspF* could modulate the extent of ERK phosphorylation in different cellular compartments.

To demonstrate the spatial modulation capability of our subcellular-located OspF*, we monitored the phosphorylation states of ERK’s nuclear substrate c-MYC as well as the cytoplasmic substrate p90 ribosomal S6 kinase (p90RSK), respectively. As an oncogenic transcriptional factor, the phosphorylation (5 min) and protein abundance (60 min) was observed within 30 min, and a decreased phosphorylation was detected only in the nu-OspF* expressed cells within 5 min after optical rescue (Figure S15). Taken together, our engineered OspF can modulate endogenous ERK functions with high spatial—temporal resolution.

**Engineering a Chemically Controlled OspF (OspFc) for Living Animals.** We have shown that our engineered OspF* could be applied for modulation of endogenous MAPK signaling cascade in living cells, which provided high spatial—temporal resolution desired for mechanistic study in vivo. Meanwhile, small-molecule-mediated chemical-rescue strategies possess higher penetration capability in deep tissue and are thus more compatible for kinase signaling modulation at the tissue and animal level. Since we have recently developed the small-molecule-mediated chemical-decaging strategy to rescue lysine-dependent protein activity in living animals,38,39,42 we created a chemically rescued OspF (termed OspF*) for applications in living animals. The inverse electron demand Diels–Alder reaction (iDA) between TCOK-a and Me2-Tz (3,6-dimethyl-1,2,4,5-tetrazine) has been shown as a rapid and efficient bioorthogonal cleavage reaction for intracellular protein activation in both prokaryotic and eukaryotic systems;38,39,42 we site-specifically incorporated the chemically caged lysine-TCOK-a into OspF at residue K134, and the resulting OspF* (OspF-K134TCOK) exhibited no phospho-lyase activity (Scheme 1B and Figure S1). The addition of the decaging reagent Me2-Tz would regenerate free lysine at K134 with its phospho-lyase activity rescued (Scheme 1B and Figure S1). Indeed, our immunoblotting results showed that the lyase activity of OspF* against ERK and p38 can be effectively rescued after treatment with 100 μM Me2-Tz (Figure 3A). We also performed a time course study to show the stability of OspF* and its effect on endogenous MAPK signaling after chemical decaging. Immunoblotting results showed that an obviously attenuated phosphorylation level of ERK and p38 was observed within 10 min after Me2-Tz treatment and can sustain for over 60 min (Figure S16). Meanwhile, no apparent change of OspF* abundance was detected even 60 min after chemical decaging (Figure S16). We then employed the aforementioned luciferase reporter, SRE-luc, to monitor OspF* activity in living cells. Indeed, an obvious decrease of bioluminescence signal was detected in PMA stimulated cells harboring OspF* after 100 μM Me2-Tz treatment, which was similar to the effect of OspF-WT (Figure 3B). In contrast, no bioluminescence variation was observed in the same batch of cells without Me2-Tz treatment (Figure 3B). These results showed that our chemical-rescue strategy can rescue OspF* for MAPK modulation in living cells.
the leg injected with OspF-K134CbzK-expressing cells with and without Me₂-Tz treatment. Therefore, our chemical-decaging strategy on OspF can be applied to living animals.

Precise Tuning of T Cell Responses by Chemically Controlled, Subcellular-Targeted OspFc. We further expanded our chemically rescued OspFc to tune immune responses. T cell receptor (TCR) stimulation can lead to T cell activation with a series of immune responses such as cytokine production and release (Figure 4A). As a key component in TCR signaling, activated ERK will translocate into the nucleus and regulate the expression of cytokines such as IL-2 and IL-8. Meanwhile, ERK also phosphorylates and inhibits the activity of a panel of upstream components in cytosol including MEK1, BRAF, SOS, and Lck to avoid over-stimulation and hyperactivation of TCR signaling (Figure 4B). For immunotherapeutic strategies such as adoptive T cell therapy, overstimulation of the MAPK cascade on native or engineered T cells may lead to cytokine release syndrome (also called cytokine storm), a life-threatening immune response. To this end, specific silencing of ERK-induced cytokine expression in the nucleus without affecting the cytosolic signaling loops may become a viable strategy for suppressing cytokine release without affecting T cell activity. We envision that our subcellular-targeted OspFc can be used to specifically inhibit ERK’s activity in the nucleus with the cytosolic feedback regulation unaffected (Figure 4B).

We next pursued the chemical rescue of OspFc in living mice. A previously developed xenograft model was used to demonstrate the chemical activation of OspFc in vivo. Stimulated HEK293T cells expressing OspFc and SRE-luc reporter were injected into living mice subcutaneously, followed by tail vein injection of Me₂-Tz (50 μL, 300 mM) to activate OspFc in living mice. HEK293T cells harboring OspFc were subcutaneously injected into mice followed by tail vein injection of Me₂-Tz (50 μL, 300 mM). Mice were fed for another 24 h for luciferase expression, and luciferin was injected 10 min before bioluminescence imaging. OspFc by Me₂-Tz treatment would attenuate ERK activity and thus reduce luciferase expression and bioluminescence signal. Representative images of rescued OspFc activity measured by bioluminescence after OspFc treatment. An obvious decrease of bioluminescence was observed only in the leg injected with cells harboring OspFc after Me₂-Tz treatment.

Figure 3. Chemical rescue of OspFc in living cells and mice. (A) Immunoblotting analysis of expression and activation of OspFc in HEK293T cells. After expression, cells were incubated with 100 μM Me₂-Tz for another 1 h. OspFc activity was detected by phosphorylation of ERK and p38 using specific antibodies. The phosphorylation of JNK was also analyzed to confirm the specificity of OspFc. Cells expressing OspFc-WT or transfected with empty vector were used as controls, and actin was used as loading controls. (B) Dual-luciferase analysis of activation of OspFc in living cells. The relative luminescence activity is proportional to the endogenous activity of OspFc-WT. OspFc-WT were used as controls. Data are presented as mean ± SD (n = 3). (C) Schematic flow showing the chemical rescue of OspFc in living mice. HEK293T cells harboring OspFc were subcutaneously injected into mice followed by tail vein injection of Me₂-Tz (50 μL, 300 mM). Mice were fed for another 24 h for luciferase expression, and luciferin was injected 10 min before bioluminescence imaging. OspFc by Me₂-Tz treatment would attenuate ERK activity and thus reduce luciferase expression and bioluminescence signal. Representative images of rescued OspFc activity measured by bioluminescence after OspFc treatment. An obvious decrease of bioluminescence was observed only in the leg injected with cells harboring OspFc after Me₂-Tz treatment.

We next pursued the chemical rescue of OspFc in living mice. A previously developed xenograft model was used to demonstrate the chemical activation of OspFc in vivo. Stimulated HEK293T cells expressing OspFc and SRE-luc reporter were injected into living mice subcutaneously, followed by tail vein injection of Me₂-Tz (50 μL, 300 mM), equal to 66 mg/kg body weight). As a control, a noncleavable lysine analogue, CbzK (benzyloxy carbonyl caged lysine), was incorporated into OspF at the same residue K134 to generate an inactivated counterpart (OspF-K134CbzK). Cells harboring OspF-K134CbzK and SRE-luc were subcutaneously injected into the other leg on the same mouse (Figure 3C). Bioluminescence imaging showed that the lyase activity of OspFc can be effectively rescued with Me₂-Tz treatment, as made evident by the significantly attenuated bioluminescence signal in mice upon Me₂-Tz addition (Figure 3D and Figure S17). In contrast, a similar bioluminescence level remained in...
Me\textsubscript{2}-Tz did not interfere with cytokine secretion in Jurkat cells (Figure S20). The nu-OspFc-expressing Jurkat cells were then stimulated by PMA and ionomycin for 10 min, followed by the addition of 100 μM Me\textsubscript{2}-Tz to rescue nu-OspFc activity. The secretions of IL-2 and IL-8 were found completely inhibited by chemically rescued nu-OspFc even 6 h after PMA and ionomycin stimulation (Figure 4D, red line), whereas a continuous IL-2 and IL-8 secretion was detected in cells without nu-OspFc activation (Figure 4D, blue line). Furthermore, the same batch of Jurkat cells was stimulated by PMA and ionomycin for 3 h before the addition of 100 μM Me\textsubscript{2}-Tz, which also led to a decrease of IL-2 and IL-8 secretion levels (Figure 4D, purple line). Taken together, we showed that our OspFc-enabled spatial−temporal modulation of ERK−signaling would allow the in situ suppression of cytokine secretion without affecting other functions of T cells.

## CONCLUSION AND OUTLOOK

Bacterial effector proteins have been previously applied for modulation of cellular signaling pathways but often lack desired spatial−temporal resolution in living systems.\textsuperscript{23} In this work, we engineered optically and chemically controlled phospho-lyase OspF (OspF\textsuperscript* and OspFc) by masking its key lysine residue K134 with photocaged and chemically caged lysine analogue. Subcellular targeting of these bioorthogonal-engineered OspF variants allowed us to specifically modulate the endogenous ERK and p38 activity and thus the native MAPK signaling cascade with high spatial−temporal resolution. While the nucleus- and cytosol-located OspF\textsuperscript* allowed modulation and dissection of endogenous ERK activity in space and time, the chemically caged OspFc enabled modulation of endogenous ERK activity in living mice, making our strategy compatible with various living systems. Given the versatility of bacterial effectors that have been evolved to manipulate diverse host signaling pathways, our bioorthogonal engineering strategy on OspF may be extended to its homologues (e.g., SpvC) and other effector molecules to modulate or rewire cell signaling with high specificity and spatial−temporal resolution.

Dysregulation of the MAPK signaling cascade occurs frequently in melanoma, colorectal, and breast cancers. Off-target activation or overstimulation on MAPK cascade also induces the overproduction of proinflammatory cytokines which contributes to autoimmune diseases. Inhibitors against components in this signaling pathway, particularly RAF and MEK, have been developed or are undergoing clinical trials.\textsuperscript{12} As key nodes of the MAPK signaling cascade, ERK and p38 receive all upstream signals and regulate a number of substrates involved in different cellular processes including gene expression, cell proliferation, and immune response. Selective and spatially specific inhibition of ERK and p38 provides an attractive choice for targeting the MAPK cascade that may have lower adverse effects or can overcome adaptive drug resistance.\textsuperscript{51,52} Given the central role of ERK and p38 at the terminal of the MAPK cascade, our engineered OspF variants offer a powerful toolset for perturbing endogenous MAPK activity with high selectivity, subcellular specificity, as well as minute-scale temporal resolution. Finally, by introducing our nucleus-located OspFc variant into T cells, we selectively disabled ERK's nuclear function on stimulating interleukin

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**Figure 4.** Precise tuning of T cell activation and immune responses by subcellular-targeted, chemically controlled OspFc. (A) Stimulation on T cells will induce immune responses and cytokine release, while overstimulation on T cells can induce cytokine release syndrome. (B) Schematic illustration of the effects on cytokine expression and ERK-mediated feedback regulations after nu-OspFc activation in T cells upon TCR activation. (C) Immunoblotting analysis of expression and activation of nu-OspFc in Jurkat T cells. After expression, cells were incubated with 100 μM Me\textsubscript{2}-Tz for another 1 h. OspFc activity was detected by phosphorylation of ERK using specific antibodies, and actin was used as loading controls. No obvious variation on BRAF phosphorylation and Lck was observed after nu-OspFc activation. (D) Schematic illustration and ELISA results on cytokine secretion in Jurkat cells after chemical rescue of nu-OspFc. Cells transfected with nu-OspFc were stimulated by PMA and ionomycin (time = 0 h), followed by the addition of Me\textsubscript{2}-Tz at time = 10 min (red) or time = 3 h (purple) for nu-OspFc activation. Secretion of IL-2 and IL-8 was detected at indicated time points by ELISA assay. Data are presented as mean ± SD (n = 3).
expression without interrupting its feedback regulation on the cytosolic signaling network. This chemical-decaging strategy may provide promising feasibilities to precisely tune the timing and strength of interleukin secretion in activated T cells under clinically relevant settings.

**ASSOCIATED CONTENT**

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acscentsci.8b00751.

Detailed experimental procedures for plasmid construction and cell biological experiments and additional figures including structures, mass spectrometry, immunofluorescent staining, flow cytometry quantification, immunoblotting analyses, substrate specificity, temporal modulation, subcellular fractionation, optical rescue, densitometric analysis, time-dependent alternation, and cytotoxicity study (PDF).

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**Notes**
The authors declare no competing financial interest. Safety statement: no unexpected or unusually high safety hazards were encountered.

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