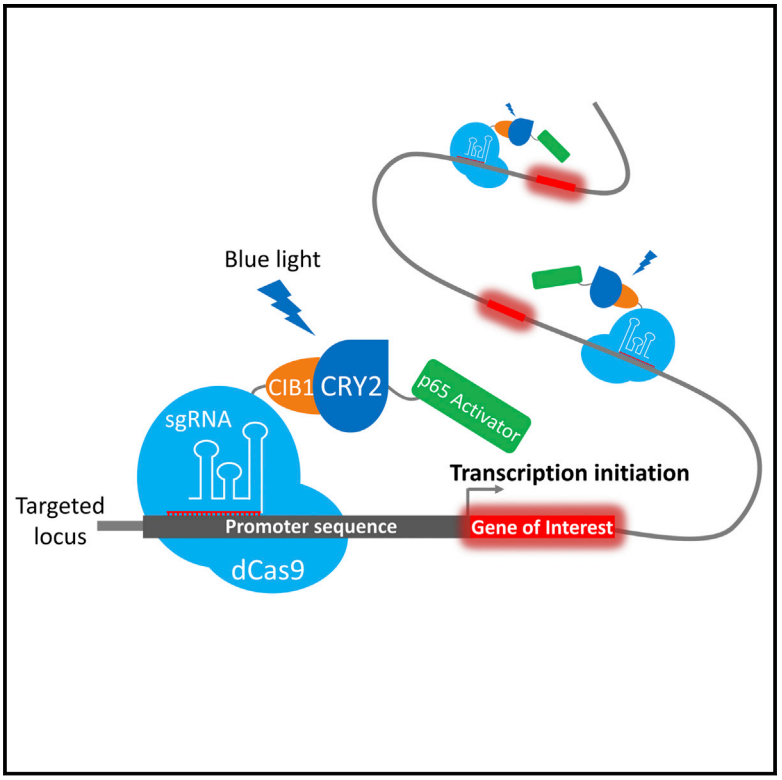
Brief Communication

Chemistry & Biology

CRISPR-Cas9-based Photoactivatable Transcription System

Graphical Abstract



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In Brief

Nihongaki et al. have developed a CRISPR-Cas9-based photoactivatable transcription system. This optogenetic tool allows RNA-guided endogenous gene activation by light. Using this system with multiple guide RNAs, robust and multiplexed endogenous gene photoactivation was achieved with ease.

Highlights

- CRISPR-Cas9-based optogenetic endogenous gene expression system in mammalian cells
- This system allows spatiotemporal gene regulation by light
- Robust endogenous gene photoactivation can be achieved using multiple guide RNA
- This feature enables multiplexed photoactivation of user-defined endogenous genes

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CRISPR-Cas9-based Photoactivatable Transcription System

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SUMMARY

Targeted endogenous gene activation is necessary for understanding complex gene networks and has great potential in medical and industrial applications. The CRISPR-Cas system offers simple and powerful tools for this purpose. However, these CRISPR-Cas-based tools for activating user-defined genes are unable to offer precise temporal control of gene expression, despite the fact that many biological phenomena are regulated by highly dynamic patterns of gene expression. Here we created a light-inducible, user-defined, endogenous gene activation system based on CRISPR-Cas9. We demonstrated that this CRISPR-Cas9-based transcription system can allow rapid and reversible targeted gene activation by light. In addition, using this system, we have exemplified photoactivation of multiple user-defined endogenous genes in mammalian cells. The present CRISPR-Cas9-based transcription system offers simple and versatile approaches for precise endogenous gene activation in basic biological research and biotechnology applications.

INTRODUCTION

Complex gene networks are essential for diverse biological phenomena, such as cellular programming, metabolism, homeostasis, memory formation, and circadian rhythm. To understand gene functions in these phenomena, approaches that enable endogenous gene expression to be regulated at will are required. For targeted endogenous gene regulation, a new class of programmable genome targeting technology, CRISPR (clustered regularly interspaced palindromic repeats)-Cas (CRISPR-associated) system in bacteria, has offered powerful tools (Cheng et al., 2013; Gilbert et al., 2013; Jinek et al., 2014; Maeder et al., 2013a; Nishimasu et al., 2014; Perez-pinera et al., 2013; Qi et al., 2013). By single-guide RNA (sgRNA), the catalytically inactive Cas9 protein (dCas9) derived from *Streptococcus pyogenes* can be bound on a target endogenous genome sequence that is complementary to the first 20 nucleotides (nt) of the sgRNA and is adjacent to a protospacer-adjacent motif (PAM) of the form NGG (Gilbert et al., 2013; Qi et al., 2013). Several studies have shown that dCas9 fused with the transcriptional activator domain enables activation of user-defined endogenous genes (Cheng et al., 2013; Maeder et al., 2013a; Perez-pinera et al., 2013). However, these RNA-guided programmable transcription factors are unable to offer precise spatiotemporal control of gene expression, despite the fact that many biological phenomena are regulated by highly dynamic patterns of gene expression. Therefore, a system that enables precise switching on and off of multiple endogenous gene expression would be indispensable for better understanding complex biological systems.

For this purpose, here we demonstrate a targeted genome photoactivation system based on dCas9 and the light-sensitive cryptochrome 2 (CRY2) and its binding partner CIB1 from *Arabidopsis thaliana* (Kennedy et al., 2010). In our CRISPR-Cas9-based photoactivatable transcription system, targeting dCas9 into given genome sequences just requires designing the first complementary 20 nt of the sgRNA by simple plasmid construction, and therefore this system offers a simple and versatile method for spatiotemporally activating multiple user-defined endogenous genes.

RESULTS

Design and Optimization of the CRISPR-Cas9-Based Photoactivatable Transcription System

This CRISPR-Cas9-based photoactivatable transcription system consists of two fusion proteins and sgRNAs (Figure 1A). The first fusion protein is the genomic anchor probe, containing dCas9 and CIB1. This anchor probe binds to the targeted genome sequence by sgRNAs. The second fusion protein is the activator probe, which includes the photolyase homology region of CRY2 (CRY2PHR) and the transcriptional activator domain. In the absence of blue light, the genomic anchor binds the promoter region of the targeted gene by sgRNAs while the activator probe is freely diffuse within the nucleus. Upon blue light irradiation, CRY2PHR and CIB1 are heterodimerized and consequently the transcriptional activator domain is recruited to the target locus to activate gene expression.

We generated the two fusion proteins and assessed the induction potency of each combination of the two by measuring reporter gene activity in both light and dark state conditions. In this assessment, we constructed the firefly luciferase reporter under the control of the upstream activator sequence (UAS) of Gal4. Several studies have shown that the level of gene activation by synthetic transcription factors based on dCas9 and the activation domain can be synergistically enhanced by using multiple sgRNAs targeting different sites in the promoter region of the same locus (Cheng et al., 2013; Maeder et al., 2013a;

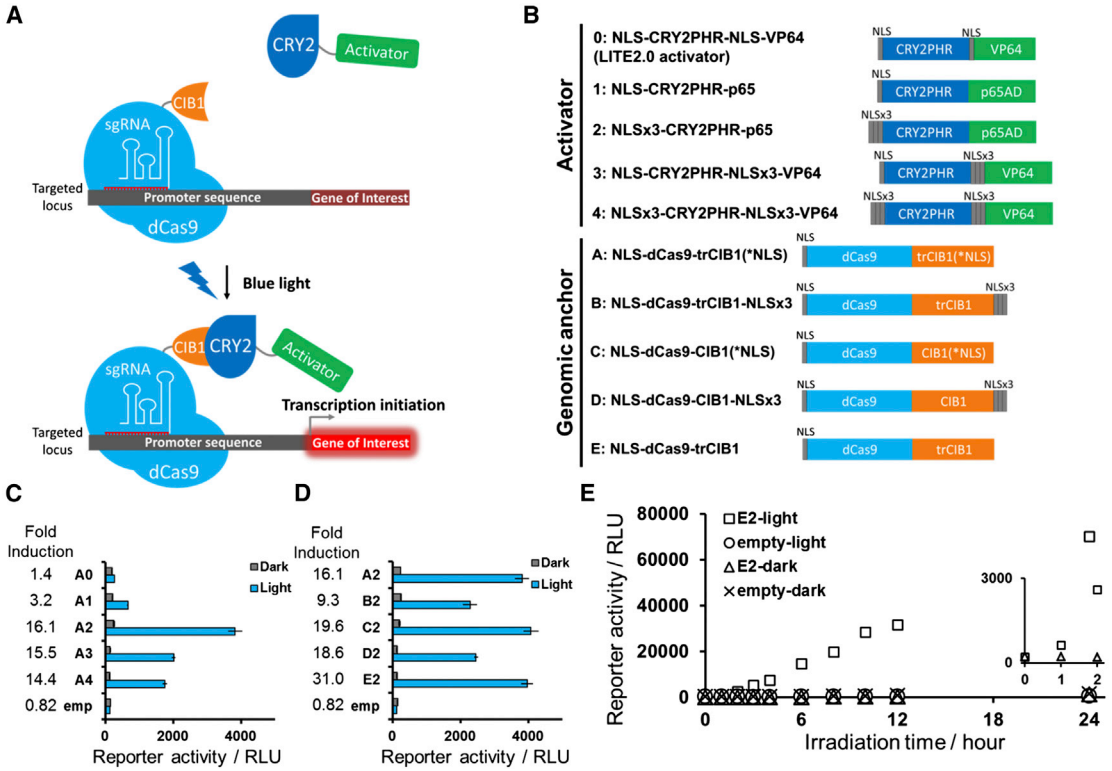

Figure 1. Design and Optimization of the CRISPR-Cas9-Based Photoactivatable Transcription System

(A) Schematic of the CRISPR-Cas9-based photoactivatable transcription system.

(B) Constructs of the genomic anchor probes (A–E) and activator probes (0–4).

(C) Luciferase reporter activity induced by each combination of the genomic anchor probe (A: NLS-dCas9-trCIB1(*NLS)) and activator probes (0–4).

(D) Luciferase reporter activity induced by each combination of the genomic anchor probe (A–E) and activator probes (2: NLSx3-CRY2PHR-p65).

(E) The time course of light-induced reporter gene transcription. The empty vector containing no insert was used as a negative control.

Error bars, SEM (n = 6 from two individual experiments with biological triplicates). See also Figures S1 and S2.

Perez-pinera et al., 2013). To obtain robust reporter gene activation in this experiment, we simultaneously transfected triple sgRNAs targeting different regions of the UAS into HEK293 cells with a genomic anchor probe, an activator probe, and luciferase reporter (Figure S1).

To build the effective light-inducible transcription system, we generated several genomic anchors and activators with different additional motifs (Figure 1B). The nuclear localization signal (NLS) derived from SV40 large T antigen is used to localize these probes in the nucleus. For adequate nuclear localization, we also tested three tandem repeat of the NLS sequence (NLSx3). Furthermore, we made alternations in the CIB1 domain. We tested C-terminal truncated (Δ308–334) CIB1 (trCIB1), reported as the mutant from which the transcriptional factor-like domain has been partially removed (Konermann et al., 2013). We also examined a CIB1 variant that is mutated at the internal NLS (CIB1(*NLS)) (Kennedy et al., 2010).

First, we tested which activator probes could most efficiently induce reporter gene expression with the genomic anchor, NLS-dCas9-trCIB1(*NLS) (Figure 1C; Figure S2A). Four of the five activator units showed light-induced reporter upregulation in HEK293 cells. In particular, NLSx3-CRY2PHR-p65 yielded the highest reporter activation in the light state as well as fold induction (16.1-fold). Conversely, we found that the A0 combination of NLS-dCas9-trCIB1(*NLS) and NLS-CRY2PHR-NLS-VP64, which is similar to the design and combination of the optimized LITE system, an optical gene regulation system based on transcription activator-like effector (TALE), could not significantly induce reporter gene expression (Konermann et al., 2013). This result showed that just replacing TALE with dCas9 in the LITE system is insufficient to build the CRISPR-Cas9-based photoactivatable transcription system.

Next, we optimized the genomic anchor to reduce background activity (Figure 1D; Figure S2B). We compared five genomic anchor units and found that the E2 combination of NLS-dCas9-trCIB1 and NLSx3-CRY2PHR-p65 yielded the lowest background activity and the highest fold induction (31.0-fold). We also made a direct comparison between the LITE system and the CRISPR-Cas9-based photoactivatable transcription system using luciferase reporter containing part of the Neurog2 promoter sequence (Figure S2C). We found that the E2 combination based on dCas9 showed higher reporter activation and fold induction (8.0-fold) than the LITE system based on TALE. Consistent with Figure 1C, we observed that the A0 combination could not induce robust gene expression.

For further functional validation of this combination, we transfected these constructs into HeLa and COS-7 cells (Figures S2D and S2E). All the samples showed light-induced reporter gene

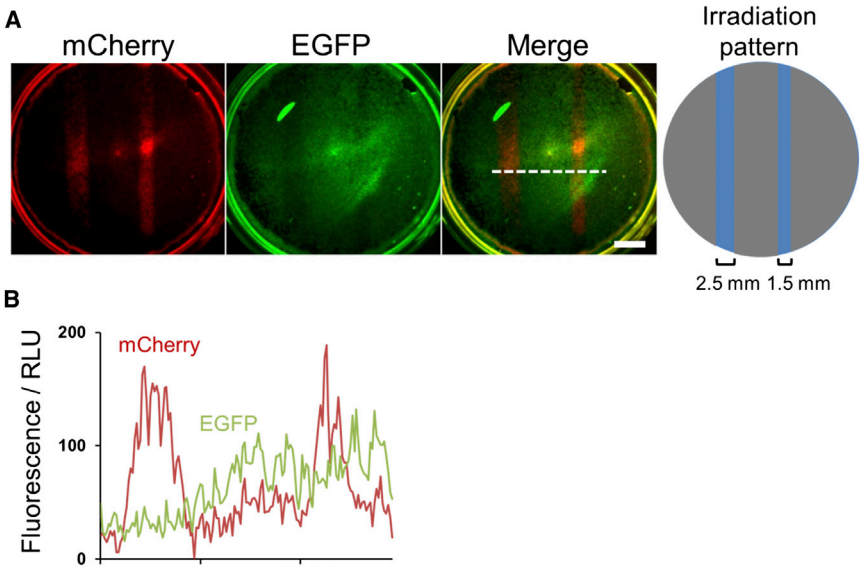

Figure 2. Spatial Gene Activation by CRISPR-Cas9-based Photoactivatable Transcription System

(A) Slit-patterned mCherry expression in HEK293T cells illuminated by blue light with a spatial pattern using a black masking tape. The widths of slits are 2.5 and 1.5 mm, respectively. Scale bar represents 5 mm.

(B) Line scan intensity profile of mCherry (red) and EGFP (green) in (A). See also Figure S1.

expression, demonstrating the wide applicability of this photoactivatable transcription system. We also investigated the time course of light-induced reporter gene expression in HEK293T cells using this system. We found that reporter gene increased as the illumination time increased, and only an hour of blue light irradiation could activate the reporter gene (~2.8-fold) (Figure 1E). In addition, we compared the gene expression kinetics between this Cas9-based photoactivatable transcription system and a tetracycline-inducible system, the Tet-On system (Figures S2F and S2G). We confirmed that, in terms of the gene expression kinetics, our Cas9-based photoactivatable transcription system can match the Tet-On system, which is commonly used in biological sciences. In the following experiments, we used the E2 combination constructs.

Spatial Gene Activation by Patterned Illumination

We tested whether this system offers spatial gene activation by light (Figure 2). To do this, we generated mCherry reporter to visualize the expression pattern of the reporter gene with a fluorescence stereomicroscope. HEK293T cells transfected with NLS-dCas9-trCIB1, NLSx3-CRY2PHR-p65, mCherry reporter, sgRNAs targeting mCherry reporter, and EGFP as transfection marker were irradiated with slit-patterned blue light. After 24 hr, the slit pattern of mCherry-expressing cells according to the irradiation pattern was observed (Figure 2), demonstrating that this system can spatially control gene expression.

Optogenetic Activation of Endogenous Gene by the CRISPR-Cas9-Based Photoactivatable Transcription System

We next tested whether this system can also optically activate endogenous gene expression in HEK293T cells (Figure 3). To do this, we generated four sgRNAs targeting the different sequences in the promoter of the human *ASCL1* gene, which encodes the transcription factor regulating neural differentiation. To determine which sgRNA can most efficiently induce *ASCL1* expression, we transfected NLS-dCas9-trCIB1 and NLSx3-CRY2PHR-p65 with individual sgRNAs into HEK293T cells. By comparative quantitative PCR, we confirmed that *ASCL1* is successfully activated in all the cases using each sgRNA (Figure 3A). When we simultaneously transfected all four sgRNAs targeting the promoter region of *ASCL1*, *ASCL1* expression in the light state was significantly enhanced compared with using individual sgRNAs as expected (Figure 3A) (Cheng et al., 2013; Maeder et al., 2013a; Perez-pinera et al., 2013). It is notable that the transfection of multiple sgRNAs targeting *ASCL1* did not affect *ASCL1* expression in the dark and showed a substantially high induction ratio of *ASCL1* expression by light (~50-fold). These results demonstrate that our photoactivatable transcription system can induce endogenous gene expression by light and the level of gene expression can be synergistically enhanced by multiple sgRNAs.

Next, we investigated the time course of light-induced *ASCL1* transcription. We found that 3 hr of blue light irradiation was enough to induce significant *ASCL1* mRNA transcription (~10-fold) (Figure 3B). We also tested whether endogenous gene activation by this system is reversible and repeatable. Incubation for 18 hr in the dark first after blue light irradiation reduced the *ASCL1* mRNA expression level to baseline level and second after light irradiation can induce *ASCL1* mRNA expression again (Figure 3C). These results demonstrate that this system can offer rapid, reversible, and repeatable endogenous gene activation.

Multiplexed Photoactivation of User-Defined Endogenous Genes

To show that this system allows photoactivation of various endogenous genes, we generated four sgRNAs each targeting the promoter regions of human *MYOD1*, *NANOG*, and *IL1RN* genes and tested their light-induced transcription in HEK293T cells (Figures 4A–4C). The light-dependent transcription of each gene was observed when HEK293T cells were cotransfected with the four sgRNAs. Next, we tested multiple photoactivation of *ASCL1* and *MYOD1* genes in HEK293T cells (Figure 4D). In the sample transfected with NLS-dCas9-trCIB1, NLSx3-CRY2PHR-p65, and multiple sgRNAs targeting the promoters of *ASCL1* and *MYOD1*, we observed light-dependent transcription of both *ASCL1* and *MYOD1*. We found no significant difference in *ASCL1* and *MYOD1* expression levels between the multiple and single photoactivation experiments ($p > 0.20$), indicating that *ASCL1* and *MYOD1* activation are saturated under our transfection conditions. We also confirmed that cells transfected with sgRNAs targeting the promoter of *ASCL1* showed light-induced transcription

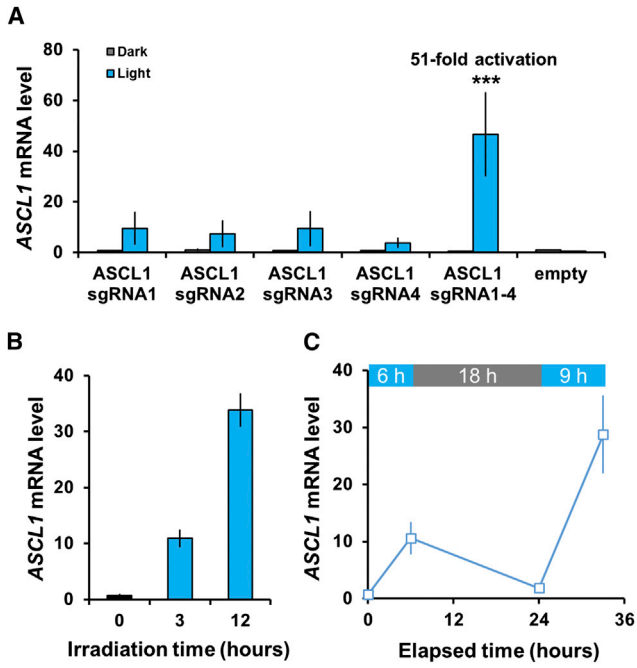

Figure 3. Optogenetic Activation of the Endogenous *ASCL1* Gene in HEK293T Cells by the CRISPR-Cas9-Based Photoactivatable Transcription System

(A) Light-induced *ASCL1* expression in HEK293T cells measured by qRT-PCR. The four sgRNAs targeting the promoter region of *ASCL1* were transfected individually or in combination, as indicated.

(B) The time course of light-induced *ASCL1* transcription.

(C) Reversible and repeatable activation of *ASCL1* transcription. In these experiments, the data are expressed as the amount of mRNA relative to the negative control transfected with empty vector in the dark.

Error bars, SEM. In (A), $n \geq 6$ from at least two individual experiments with biological triplicates. In (B) and (C), $n = 3$ from the same experiment. Student's two-tailed t test was performed. ***$p < 0.001$ versus the sample in the dark. See also Figure S1.

of *ASCL1* without affecting *MYOD1* expression and vice versa. These results demonstrate that this system can be used for multiplexed photoactivation of user-defined endogenous genes.

DISCUSSION

In conclusion, we have developed the CRISPR-Cas9-based photoactivatable transcription system. Previously, it was reported that optical endogenous gene activation can also be achieved by the LITE system based on TALE (Konermann et al., 2013). Unlike the LITE system, which requires complex and time-consuming DNA assembly to target a given sequence, our CRISPR-Cas9-based transcription system provides easy-to-use, user-defined endogenous gene activation. The capacity of our CRISPR-Cas9-based photoactivatable transcription system was exemplified by synergistic photoactivation of endogenous genes. In addition, we have demonstrated multiplexed photoactivation of user-defined endogenous genes. These features of the present photoactivation system are easy programmability and highly parallel applicability of the CRISPR-Cas technology. We exemplified multiplexed gene expression using eight different sgRNAs. The number of sgRNAs is readily increased to a large extent, according to the recent report by Chen et al. (2013), in which ~70 sgRNAs were used for imaging specific genomic loci based on dCas9 tagged with EGFP. Thus, the present optogenetic system based on the CRISPR-Cas technology with easy programmability and highly parallel applicability could achieve highly synergistic and massively multiplexed spatiotemporal activation of endogenous genes with light.

Konermann et al. (2013) mentioned that the TALE DNA-binding domain of the LITE system might be replaced with dCas9. However, we found that replacing TALE with dCas9 in the LITE system could not provide dCas9-based optical gene activation. One possible explanation for this is that the larger and more complex structure of dCas9 than that of TALE may weaken the localization and dimerization of the two fusion proteins. To overcome this, we generated an originally designed molecular probe and finally established the robust dCas9-based optical gene activation system. The present CRISPR-Cas9-based photoactivatable transcription system is an optogenetic tool employing Cas9 technology. Furthermore, by replacing transcriptional activator p65 with other domains, such as epigenetic-modifying enzymes (de Groote et al., 2012; Maeder et al., 2013b), nucleases (Miller et al., 2011), and recombinases (Mercer et al., 2012), this system could also offer diverse types of Cas9-guided genome regulation with high spatiotemporal resolution. The present CRISPR-Cas9-based photoactivatable transcription system will contribute to expanding the possibilities of optogenetic regulation, mammalian genome engineering, and biotechnology applications (Brieke et al., 2012; Lienert et al., 2014).

SIGNIFICANCE

CRISPR-Cas9 provides a powerful tool for targeted endogenous gene regulation. To expand the scope of Cas9 technology, here we created an optogenetic tool based on dCas9, enabling light-inducible user-defined endogenous gene activation. It has been reported that optical endogenous gene activation can also be achieved with the LITE system based on TALE (Konermann et al., 2013). However, we found that replacing TALE with dCas9 in the LITE system could not provide dCas9-based optical gene activation. Therefore, we designed a new molecular probe and finally established the robust dCas9-based optical gene activation system. We demonstrated that this Cas9-based transcription system can allow rapid and reversible targeted gene activation by light and exemplified photoactivation of multiple user-defined endogenous genes in mammalian cells. The present system will contribute to expand the possibilities of optogenetic regulation, mammalian genome engineering, and biotechnology applications.

EXPERIMENTAL PROCEDURES

Bioluminescence Assay

For the reporter gene expression assay, HEK293, HeLa, COS-7, and HEK293T cells were plated at approximately 2.0×10^4 cells/well in 96-well black-walled plate (Thermo Fisher Scientific), and cultured for 24 hr at 37°C in 5% CO_2. The cells were then transfected with Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocols. cDNAs encoding genomic anchor probes, activator probes, sgRNAs, and reporter were transfected at a 1:1:1:1 ratio. In this experiment, the ratio of three sgRNAs was 1:1:1. The total amount of DNA was

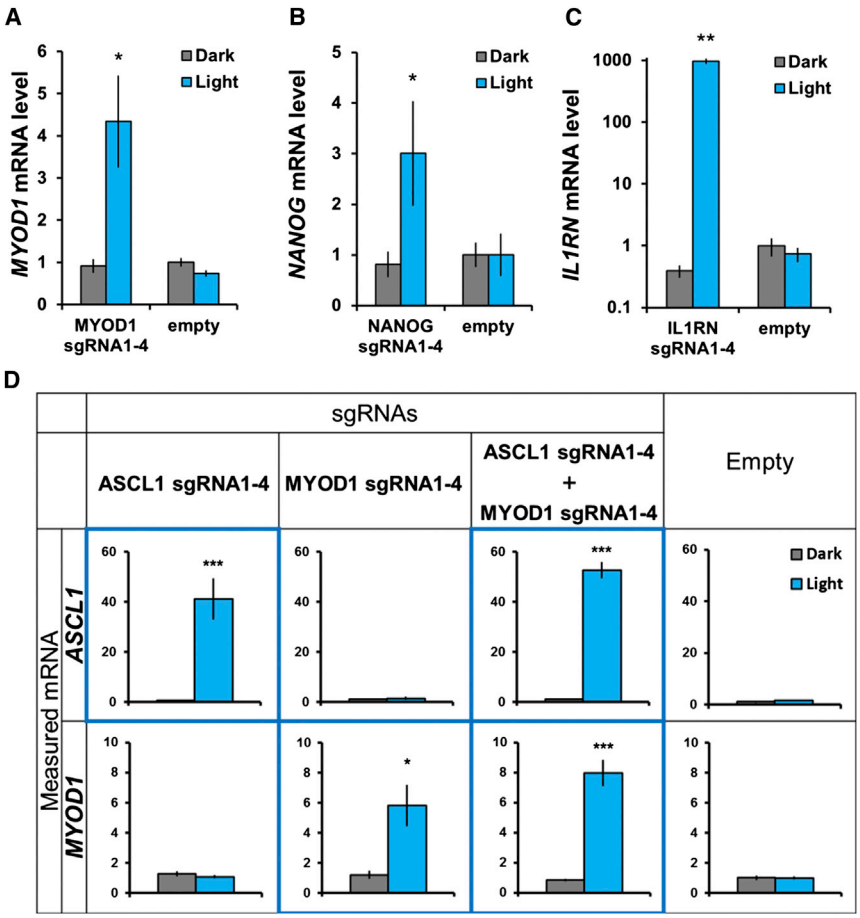

Figure 4. Optogenetic Activation of Various Endogenous Genes in HEK293T Cells by the CRISPR-Cas9-Based Photoactivatable Transcription System

(A–C) Light-induced *MYOD1* (A), *NANOG* (B), and *IL1RN* (C) expression in HEK293T cells measured by qRT-PCR. The four sgRNAs targeting the promoter region of each gene were transfected in combination.

(D) Multiplexed endogenous gene photoactivation. HEK293T cells were transfected with cDNAs encoding NLS-dCas9-trCIB1, NLSx3-CRY2PHR-p65, and the indicated sgRNAs. In these experiments, the data are expressed as amount of mRNA relative to the negative control transfected with empty vector in the dark.

Error bars, SEM ($n \geq 6$ from at least two individual experiments with biological triplicates). Student's two-tailed t test was performed. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ versus the sample in the dark. See also Figure S1.

0.2 μg/well. Twenty hours after the transfection, the culture medium was replaced with 100 μl of phenol red-free DMEM (Sigma Aldrich) containing 500 μM of D-luciferin (Wako Pure Chemical Industries) as a substrate. After incubation for 24 hr at 37°C in 5% CO_2 under continuous blue light irradiation or in the dark, bioluminescence measurements were performed using a Centro XS^3 LB 960 plate-reading luminometer (Berthold Technologies). In the time course experiment, bioluminescence measurements were performed at the indicated time points. For Tet-inducible expression, 1.0 μg/ml doxycycline (Dox) was used. Blue light irradiation was performed using a 470 ± 20 nm LED light source (CCS Inc.). The intensity of the blue light was 1.5 W/m^2.

Quantitative Real-Time PCR Analysis

Total RNA isolation and reverse transcription PCR were performed using the Cells-to-CT kit (Life Technologies). qRT-PCR was performed by the StepOnePlus system (Life Technologies) using TaqMan probes.

Additional experimental procedures can be found in the Supplemental Experimental Procedures.

SUPPLEMENTAL INFORMATION

Supplemental Information includes two figures, Supplemental Experimental Procedures, and a supplementary note on the amino acid sequences of the genomic anchor probes (A–E) and activator probes (0–4) and can be found with this article online at http://dx.doi.org/10.1016/j.chembiol.2014.12.011.

AUTHOR CONTRIBUTIONS

Y.N., S.Y., F.K., H.S., and M.S. designed the experiments. Y.N. and S.Y. performed the experiments. Y.N. analyzed the data. Y.N. and M.S. wrote the manuscript.

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