JACS
JOURNAL OF THE AMERICAN CHEMICAL SOCIETY

Communication

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Light-Inducible Spatiotemporal Control of Gene Activation by Customizable Zinc Finger Transcription Factors

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Supporting Information

ABSTRACT: Advanced gene regulatory systems are necessary for scientific research, synthetic biology, and gene-based medicine. An ideal system would allow facile spatiotemporal manipulation of gene expression within a cell population that is tunable, reversible, repeatable, and can be targeted to diverse DNA sequences. To meet these criteria, a gene regulation system was engineered that combines light-sensitive proteins and programmable zinc finger transcription factors. This system, light-inducible transcription using engineered zinc finger proteins (LITEZ), uses two light-inducible dimerizing proteins from *Arabidopsis thaliana*, GIGANTEA and the LOV domain of FKF1, to control synthetic zinc finger transcription factor activity in human cells. Activation of gene expression in human cells engineered with LITEZ was reversible and repeatable by modulating the duration of illumination. The level of gene expression could also be controlled by modulating light intensity. Finally, gene expression could be activated in a spatially defined pattern by illuminating the human cell culture through a photomask of arbitrary geometry. LITEZ enables new approaches for precisely regulating gene expression in biotechnology and medicine, as well as studying gene function, cell−cell interactions, and tissue morphogenesis.

Gene regulation systems are prevalent in diverse areas of biotechnology and biological research.[1] The advent of commonly used gene regulation systems, such as the tetracycline-inducible gene expression system,[2,3] has enabled numerous studies of the effects of magnitude and duration of gene expression. Advanced technologies for precise regulation of gene expression in time and space would facilitate the development of safe and effective gene therapies, economical metabolic engineering and biopharmaceutical production, and studies of intercellular interactions, physiology of complex tissues, and dynamics of gene regulation. Current gene regulatory systems cannot be used to realize these goals because they do not address all of the critical requirements of genetic control: tunability, reversibility, spatial control, and temporal control. For example, tetracycline-based systems control gene expression in a dose-specific, tunable manner via the concentration of tetracycline,[2] but because tetracycline is a small, diffusible molecule, it is difficult to spatially control gene expression. Additionally, temporal control requires physical addition or removal of the molecule, which can complicate applications requiring dynamic gene regulation. Other systems based on small inducer molecules, including steroids, antibiotics, metabolites, or immunosuppresive drugs,[4] also suffer from these limitations and may have unintended pleiotropic effects on cellular physiology.

Molecular caging of small molecules, oligonucleotide inhibitors, and proteins can also be used to control gene expression.[5] This involves modifying the target molecule with photolabile groups that inhibit function. Irradiation with UV light releases the caging groups and frees the active effector molecule. Although this technology can be used to control gene expression or protein−protein interactions in a spatiotemporal manner,[6] it is irreversible, it requires potentially harmful UV irradiation of cells, and the photocaged molecules must be chemically synthesized or produced by the cell via technically challenging methods.

A gene expression system that is activated by light addresses many of the shortcomings of current regulatory systems.[7] Cellular systems can be illuminated in any pattern to allow high-resolution spatial genetic control,[8] and the absence of small-molecule inducers reduces the potential for off-target effects. Furthermore, light intensity and duration of illumination can be easily adjusted to tune levels of gene expression. Recent work in this area has used light-inducible protein interactions as a genetic photoswitch by fusing one binding partner to a DNA-binding protein and the other partner to a transcriptional activation domain.[9−12] Illuminating cells expressing these fusion proteins induces the transcription of transgenes that have been placed downstream of target sites for the DNA-binding protein.

The objective of this study was to combine light-inducible gene regulation with synthetic zinc finger protein (ZFP) technology to engineer a system that can be easily modified to target any DNA sequence in response to light. This approach could potentially be used to activate any desired gene in a light-dependent manner. ZFPs are DNA-binding proteins that are assembled from modular zinc finger domains, each of which recognizes a specific 3-base-pair (bp) DNA triplet; thus, a ZFP that contains six zinc finger domains can bind with high specificity to a contiguous 18-bp sequence.[13] The discovery of synthetic zinc finger domains that target each DNA base-pair triplet makes it possible to engineer a ZFP that binds almost any DNA sequence.[14] This technology has been used to engineer promoter-targeted ZFPs fused to transcriptional effector domains that regulate a variety of endogenous target genes in a consitutive[15,16] or chemically-induced manner.[17]

Our approach builds on previous studies showing blue light-inducible heterodimerization of the proteins GIGANTEA (GI) and the light oxygen voltage (LOV) domain of FKF1.[10] GI and

Received: July 5, 2012
Published: September 10, 2012

   dx.doi.org/10.1021/ja3065667 | *J. Am. Chem. Soc.* 2012, 134, 16480−16483

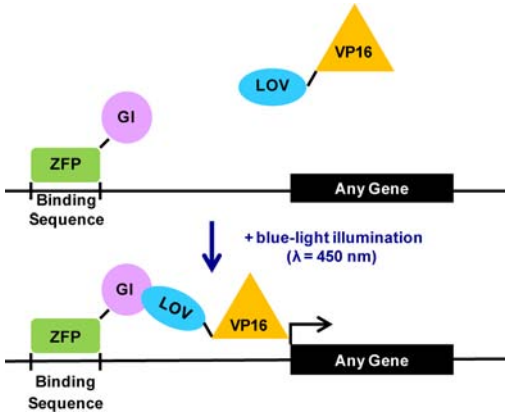

Figure 1. The GI-ZFP fusion protein localizes to the ZFP DNA binding sequence upstream of the transgene. Blue light initiates heterodimerization between GI and LOV, which translocates VP16 to the gene of interest and activates transcription.

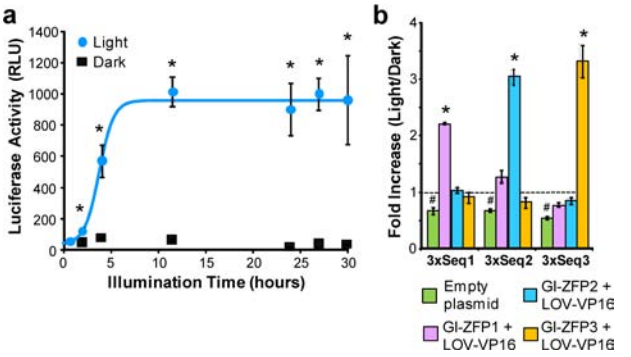

Figure 2. (a) Luciferase activity increases with blue-light illumination time in HeLa cells transfected with LOV-VP16, GI-ZFP2, and a luciferase reporter containing 9 copies of the ZFP2 binding site upstream of luciferase (*$p < 0.0001$ vs dark). (b) In cells transfected with LOV-VP16, a GI-ZFP, and a luciferase reporter, significant reporter activation was only observed when a GI-ZFP was paired with a luciferase reporter containing three copies (3x) of its corresponding ZFP binding site. Cells were illuminated for 30 h. A significant decrease in luciferase activity was observed in cells transfected with only a luciferase reporter and junk DNA (#$p < 0.05$).

FKF1 are naturally found in the plant species *Arabidopsis thaliana* and are responsible for activating the CONSTANS pathway that controls phototropic flowering in response to day length.[18] Light-induced heterodimerization occurs via a riboflavin molecule that non-covalently occupies a binding pocket in the LOV domain. In response to blue light (450 nm), the flavin mononucleotide transitions into an excited state and forms a cysteinyl-flavin adduct with residue 91 of FKF1, a cysteine that is highly conserved across the LOV domain family.[19]

*L*ight-*i*nducible *t*ranscription using *e*ngineered *z*inc finger proteins (LITEZ) consists of two fusion proteins: (1) the LOV domain of FKF1 fused to three repeats of the transcriptional activation domain VP16,[10] and (2) GI fused to a zinc finger protein (GI-ZFP) which localizes GI to the ZFP target DNA sequence (Figure S1). Blue light illumination initiates heterodimerization between LOV and GI, which translocates LOV-VP16 to the target gene and activates transcription (Figure 1).

LITEZ has several advantages over current inducible gene expression systems. First, no addition or removal of an inducer molecule is required for gene activation, which eliminates the possibility of off-target effects due to the inducer molecule. Furthermore, unlike light-inducible systems that require adding an exogenous cofactor,[9] the flavin mononucleotide that is required for light-induced GI-LOV binding is naturally present in mammalian cells. This is particularly useful for applications in which purification is required to remove supplement molecules, such as biopharmaceutical production.

LITEZ is designed to target any DNA sequence for light-inducible gene regulation by engineering the synthetic ZFP DNA-binding domain. Three different GI-ZFPs were created that target 12-bp (GI-ZFP1 and GI-ZFP2) or 18-bp (GI-ZFP3) sequences (Table S1). These ZFPs were chosen because they have been well-characterized in previous studies.[15,20] Reporter plasmids were created that contain 3−9 copies of each GI-ZFP's respective binding sequence upstream of the gene encoding *luciferase* (Luc). The reporter 3xSeq1-Luc contains 3 repeats of the binding site for GI-ZFP1; 3xSeq2-Luc, 6xSeq2-Luc, 7xSeq2-Luc, and 9xSeq2-Luc contain 3, 6, 7, and 9 repeats, respectively, of the binding site for GI-ZFP2; and 3xSeq3-Luc contains 3 copies of the binding site for GI-ZFP3. All sequences of these constructs are available in the Supporting Information.

HeLa cells were transfected with LOV-VP16, GI-ZFP2, and 9xSeq2-Luc, and luciferase activity was measured over time in cells illuminated with blue light and in cells incubated in the dark. For illuminated cells, light was continuously pulsed for 3 s every 3 min using a custom-built 3×4 LED array. As illumination time increased, there was an increase in luciferase activity that plateaued after 12 h (Figure 2a). Nonlinear regression yielded a sigmoidal curve ($R^2 = 0.992$) with an inflection point at 3.8 h, which represents the illumination time at which luciferase levels are increasing most rapidly. There was a significant ($p < 0.0001$) 2.7-fold increase in luciferase activity between illuminated and nonilluminated cells after only 2 h of pulsing blue light exposure and a maximum increase of 53-fold at 24 h.

To demonstrate that LITEZ is specific for its target sequence, HeLa cells were transfected with LOV-VP16, one of the three GI-ZFPs, and either the GI-ZFP's corresponding 3xSeq-Luc reporter that contains the correct GI-ZFP binding sequence or one of the two 3xSeq-Luc reporters that contains the incorrect GI-ZFP binding sequence (Figure 2b). Three-factor ANOVA (factors: reporter, GI-ZFP, and illumination) indicated a significant interaction of reporter×GI-ZFP ($p < 0.0001$) and reporter×GI-ZFP×illumination ($p < 0.0001$). Among cells that expressed the same 3xSeq-Luc reporter, pairwise comparisons of each member of the group to a fold-increase of one showed significantly higher fold-increase (light/dark) luciferase activity in cells that contained the correct GI-ZFP/3xSeq-Luc reporter pair ($p < 0.0001$). Illuminated cells that were transfected with only a reporter plasmid and junk DNA showed a significant decrease ($p < 0.05$) in luciferase activity. This may be due to slight toxicity as a result of the light exposure. An MTT toxicity assay showed a modest but significant decrease in metabolic activity when transfected or non-transfected cells were illuminated with blue light compared to cells incubated in the dark (Figure S2). LITEZ is also functional in multiple human cell lines, including HeLa, MCF-7, and HEK 293T cells (Figure S3).

Gene expression levels can be tuned by changing the number of ZFP binding sites upstream of the target transgene (Figure 3a). HeLa cells were co-transfected with LOV-VP16, GI-ZFP2, and a luciferase reporter containing 3, 6, 7, or 9 ZFP2 binding sites upstream of luciferase. A large range of expression was observed; illuminated cells that received the 3xSeq2-Luc reporter exhibited a 3.6-fold increase in luciferase activity compared to

dx.doi.org/10.1021/ja3065667 | J. Am. Chem. Soc. 2012, 134, 16480−16483

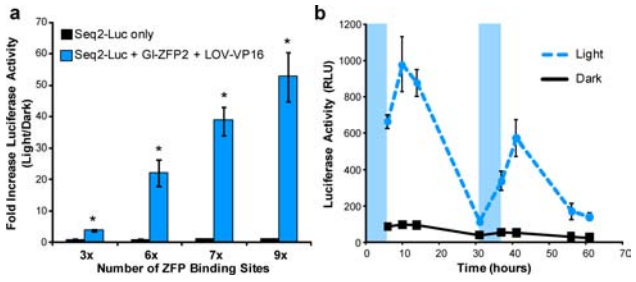

Figure 3. (a) Light-induced luciferase activity increases with the number of upstream GI-ZFP binding sites. HeLa cells were transfected with the Seq2-Luc reporter with either 3, 6, 7, or 9 copies of the ZFP2 binding site and either junk DNA or LOV-VP16 and GI-ZFP2. Transfected cells were illuminated with pulsing blue light for 30 h or incubated in the dark. (*$p < 0.0001$ vs reporter only). (b) LITEZ is reversible and repeatable. HeLa cells were transfected with LOV-VP16, GI-ZFP2, and 9xSeq2-Luc. Cells were either incubated in the dark for the entire experiment (solid line) or illuminated with pulsing blue light (dotted line) for two separate 6-h periods (shaded areas) ($p < 0.0001$ vs dark).

cells incubated in the dark, whereas illuminated cells that received the 9xSeq2-Luc reporter showed a 53-fold increase in luciferase activity. Gene expression levels can also be controlled by varying light intensity with neutral density filters (Figure S4).

In contrast to photocaging-based regulation systems, transcriptional activation by LITEZ is both reversible and repeatable without exchanging culture media or replenishing caged molecules (Figure 3b). HeLa cells were transfected with LOV-VP16, GI-ZFP2, and 9xSeq2-Luc, illuminated 12 h later with pulsing blue light for 6 h, and then incubated in the dark for 25 h before being illuminated again for another 6 h. At various time points, luciferase activity in illuminated cells was compared to control cells that were incubated in the dark throughout the entire experiment. Luciferase activity increased 8-fold in illuminated cells following the first 6 h of illumination. After illumination concluded, luciferase activity increased ~10-fold over cells incubated in the dark within the next 4 h. This may be due to delayed dissociation of the GI-LOV heterodimer and/or slow decay of luciferase mRNA and protein. Twenty-five hours after the first period of illumination ended, luciferase activity decreased to 1.9-fold over average control cell luciferase activity. After these 25 h of incubation in the dark, cells were re-illuminated with pulsing blue light for 6 h. This caused luciferase activity to increase to ~60% of the maximum activity level observed after the first illumination regimen. Failure to achieve similarly high levels of activity after the first round of illumination is likely due to plasmid degradation and dilution as the cells continued to divide. Subsequent removal of blue light decreased activity to 2.4-fold over average control cell luciferase activity within 15 h. Cells incubated in the dark for the entire experiment exhibited a constant low, basal level of luciferase activity.

The relatively rapid kinetics of induction and silencing are a unique characteristic of LITEZ. Only 2 h of pulsed illumination were required to achieve a 2.7-fold induction of luciferase activity over background (dark) levels, and induction saturated by 12 h (Figure 2a). Upon removal of light, luciferase activity continued to increase for ~4 h before declining to near-background levels within the next 24 h (Figure 3b). This observation agrees with previous co-localization studies showing that the GI-LOV heterodimer remains associated for at least 90 min after removal of blue light.[10] This is in contrast to other small-molecule- or light-inducible gene regulation systems that are irreversible[5,6] or have slower activation or de-induction kinetics. For example, a recently reported light-inducible system showed prolonged transgene activation (>45 h) after removal of light.[12] Furthermore, *in vitro* and *in vivo* experiments with the rapamycin-inducible system have shown that expression levels can take up to 3 days after rapamycin withdrawal to begin to decline.[21] Thus, LITEZ may be particularly useful for applications that require faster dynamics of regulation. Reversibility also allows this system to be used to finely regulate toxic protein products and minimize cell death, thereby increasing product yield and quality.

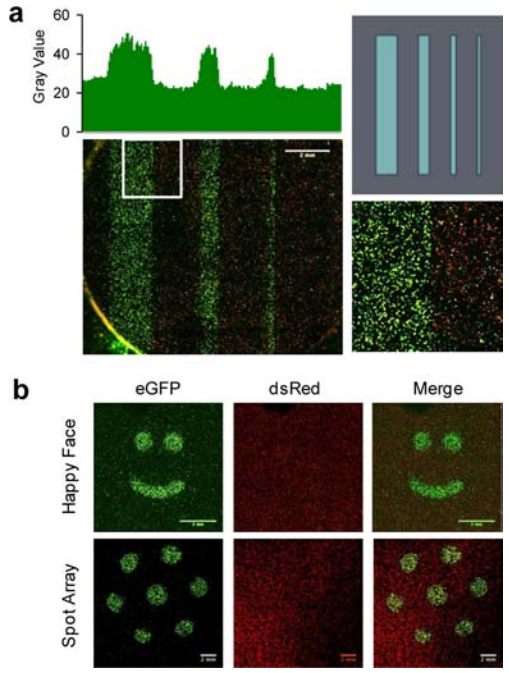

Figure 4. LITEZ can be used to spatially control gene expression in human cells. (a) HEK 293T cells were co-transfected with LOV-VP16, GI-ZFP2, 9xSeq2-eGFP, and a constitutive dsRed expression plasmid and illuminated through a photomask (upper right) containing (a) rectangular slits with widths of 2, 1, 0.5, and 0.3 mm (left to right) or (b) happy face or spot array patterns. The 0.3 mm slit was too narrow to result in an observable pattern. eGFP intensity across the pattern was quantified by image analysis (a, top left). Inset (a, lower right) shows the sharp contrast between eGFP-positive and -negative cells at the border of the 2 mm slit. Scale bars = 2 mm.

One of the most powerful aspects of LITEZ is the ability to spatially control the transcriptional state of a target gene. *Luciferase* was exchanged for *enhanced green fluorescent protein* (eGFP) in the 9xSeq2-Luc reporter to make 9xSeq2-eGFP. HEK 293T cells co-transfected with 9xSeq2-eGFP, LOV-VP16, GI-ZFP2, and a constitutive dsRed expression plasmid were illuminated through a photomask for 20 h to create patterns of GFP expression (Figures 4 and S5). Patterns of cells expressing GFP as small as 500 μm were readily achievable.

Spatial patterning of gene expression via light allows for unique applications of inducible gene regulatory systems. This approach can be applied to the field of tissue engineering to create complex constructs that recapitulate the morphology and functionality of natural tissues.[22] Spatial activation of key morphogenetic factors could be used to precisely engineer tissues patterned with multiple cell types. As a result, engineered tissues will more closely mimic native tissues, increasing the probability of implant survival and improving tissue functionality. The spatial control

provided by LITEZ, combined with reversible and repeatable gene activation, will also enable novel basic science studies of gene function, gene regulation, and cell−cell interactions.

As a step toward achieving endogenous gene activation using LITEZ, we created an HEK 293T cell line using the FlpIn system (Invitrogen) that contains a single integrated chromosomal copy of the 9xSeq2-eGFP reporter. Cells were co-transfected with LOV-VP16 and GI-ZFP2 and illuminated with pulsing blue light for 30 h. Flow cytometry showed a significant 4-fold increase in the percentage of eGFP-positive cells and 30 ± 3% (SEM) increase in the geometric mean of eGFP fluorescence in illuminated cells compared to non-illuminated cells (Figure S6, $p < 0.0001$).

Although several light-inducible gene expression systems have recently been described,[9−12,23] LITEZ is the first to report customizable DNA-binding specificity. Previous systems have relied on common DNA-binding domains, so all regulated transgenes must contain the upstream binding sequence that matches that particular DNA-binding protein. In contrast, LITEZ provides the freedom to target almost any sequence with engineered ZFPs, facilitating the use of diverse promoter sequences for combinatorial control of many transgenes in areas such as synthetic biology and metabolic engineering.[24] Further refinements of LITEZ may enable regulating endogenous genes in their natural chromosomal context by engineering ZFPs targeted to promoter sequences. In the current system, it was necessary to include three repeats of the ZFP binding site to achieve 3.6-fold increase of luciferase activity in response to light. When only a single ZFP target site was included, there was no detectable increase in activation in response to light. Thus, improvements to this system, such as increased affinity between the GI and LOV domains in response to light, may be necessary to activate endogenous promoters that do not contain repetitive ZFP binding sites.

Continued development and characterization of light-inducible systems are necessary to understand the optimal properties for precise control of gene expression magnitude and dynamics. There are numerous opportunities for the broad adoption of LITEZ as a powerful research tool, as it enables control of transgene expression with high resolution in both space and time. This technology also has the potential to be incorporated into many medical and industrial applications, including gene therapy, metabolic engineering, synthetic biology, biopharmaceutical production, and tissue engineering.

■ ASSOCIATED CONTENT

Supporting Information

Materials and methods, protein and gene sequences, Figures S1−S6. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

Ricardo E. Dolmetsch provided the GI and LOV-VP16 plasmid constructs. Carlos F. Barbas III provided the construct containing ZFP3. We thank Chandra Tucker, Robert Hughes, Adam Wax, and Pablo Perez-Pinera for valuable discussions about this project. This research was funded by NSF CAREER Award CBET-1151035, NIH Director's New Innovator Award 1DP2-OD008586, a Ralph E. Powe Junior Faculty Enhancement Award from Oak Ridge Associated Universities, and the Duke Biomedical Engineering McChesney Fellowship.

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