# Orthogonal Optogenetic Triple-Gene Control in Mammalian Cells

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ⓈSupporting Information

ABSTRACT: Optogenetic gene switches allow gene expression control at an unprecedented spatiotemporal resolution. Recently, light-responsive transgene expression systems that are activated by UV-B, blue, or red light have been developed. These systems perform well on their own, but their integration into genetic networks has been hampered by the overlapping absorbance spectra of the photoreceptors. We identified a lack of orthogonality between UV-B and blue light-controlled gene expression as the bottleneck and employed a model-based approach that identified the need for a blue light-responsive gene switch that is insensitive to low-intensity light. Based on this prediction, we developed a blue light-responsive and rapidly reversible expression system. Finally, we employed this expression system to demonstrate orthogonality between UV-B, blue, and red/far-red light-responsive gene switches in a single mammalian cell culture. We expect this approach to enable the spatiotemporal control of gene networks and to expand the applications of optogenetics in synthetic biology.

KEYWORDS: *LOV, gene expression, multichromatic, optogenetics, synthetic biology*
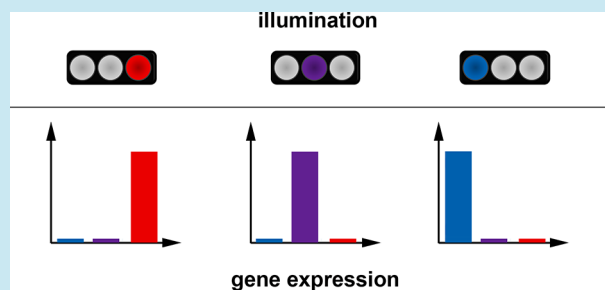
Over the past decades, the naturally occurring diversity in protein−DNA and protein−protein interactions has been mined to construct a growing number of high-precision trigger-inducible transgene switches. These can be further assembled to independently control several transgenes or to build advanced gene expression networks[1,2] and constitute the backbone of synthetic biology.[3,4] First-generation gene switches are controlled by small molecules and are subject to significant limitations regarding the spatiotemporal resolution of gene expression. Consequently, several light-responsive gene expression systems have recently been developed for mammalian cells that can be controlled with the unprecedented resolution of light.[5,6] These respond to the UV-B,[7,8] blue,[9−14] or red/far-red[15] regions of the light spectrum. Most of these systems are characterized by superior regulation characteristics regarding the dynamic range of gene expression and the adjustability of expression levels by tuning of the photon flux. However, the integration of these gene switches into synthetic networks as well as their application in functional genomics requires the orthogonal operation of multiple switches within a single cell and this is challenging due to the overlapping absorbance spectra of the photoreceptors: UV-B-responsive gene switches are only activated by UV-B light, whereas blue light-inducible systems respond to UV-B and to blue light. Further, the phytochrome B-based red/far-red light-responsive toggle switch is activated by UV-B, blue, and red light.[7] We have previously demonstrated that the unique feature of the red/far-red light

responsive toggle switch—its active and rapid switchability between stable ON and OFF states—can be utilized to establish orthogonality between the red/far-red and blue or UV-B light-responsive gene switches.[7] In contrast to the actively reversible red/far-red light-responsive gene switch, the blue light-responsive LightON system that is based on light-induced homodimerization of vivid (VVD)[12] and a UVR8-based UV-B light-responsive gene switch that capitalizes on the UV-B-induced recruitment of CONSTITUTIVELY PHOTO-MORPHOGENIC 1 (COP1) to the UV-B photoreceptor UV resistance locus 8 (UVR8)[7] return to the OFF-state passively in the dark with active-state half-life times of 2 h. Therefore, it was possible to activate these systems by pulses of blue or UV-B light, respectively, while the red/far-red light-triggered gene switch was kept in the inactive state by supplementary far-red light illumination. However, this approach did not allow the orthogonal operation of blue and UV-B light-responsive expression systems (Figure 1A).

Here, we describe the mathematical model-based analysis of the requirements for orthogonal triple-wavelength expression control that prompted us to develop a novel blue light-responsive gene switch that can be combined with existing UV-
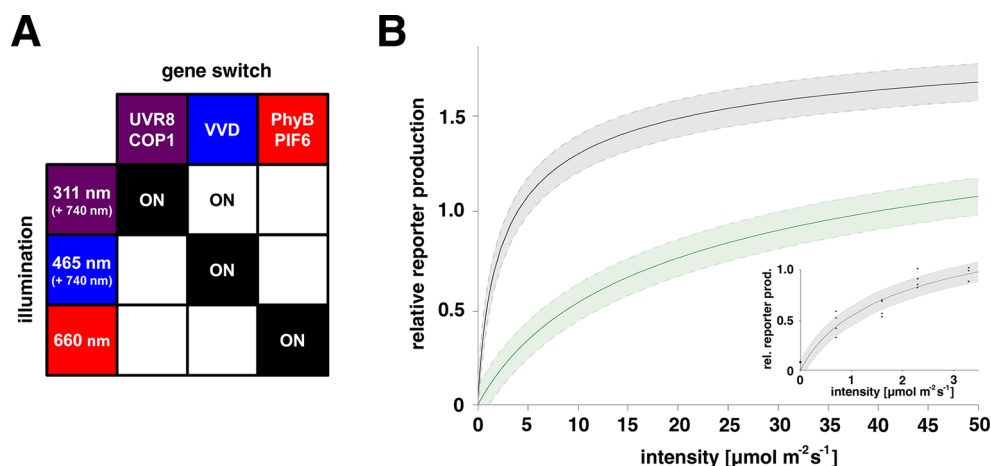
Figure 1. Requirements for orthogonal multichromatic expression control. (A) Response matrix for the existing multichromatic gene expression setup. By combining pulsed UV-B and blue light-illumination with continuous 740 nm illumination, the orthogonal operation of the UV-B light-responsive UVR8-COP1 or the blue light-inducible VVD-based LightON system with the PhyB-PIF6-based red/far-red light-controlled toggle switch has been attained. However, the UVR8-COP1 and the LightON system cannot be operated in an orthogonal manner. (B) Dose response curve of the blue light-regulated LightON system and model-prediction for orthogonal blue light control. CHO-K1 cells were transfected with the LightON system for expression of FLuc (pKM085, pFR-Luc). 24 h post-transfection, the cells were illuminated for 24 h with 450 nm light. The gray curve represents the model of the LightON system, and the inset shows the fitted data that was used to calibrate the model. The green curve shows the predicted response profile if the association constant of the system is lowered 10-fold. The shaded error bands are estimated by a simple error model with a constant Gaussian error.

B and red/far-red light gene expression technology for fully orthogonal trichromatic triple-gene control in mammalian systems.

■ RESULTS AND DISCUSSION

Previously, the orthogonal operation of optogenetic gene switches has been prevented by the activation of the blue light-inducible LightON system by low-intensity (2.5 μmol m^{-2} s^{-1}) UV-B light.[7] We examined the response of the LightON system to 450 nm light of increasing intensities and observed significant activation of the system at intensities as low as 0.7 μmol m^{-2} s^{-1} (Figure 1B, inset), which is in agreement with previously reported data.[12]

Next, we developed a mathematical model of the intensity-dependent activation of the LightON system. The LightON system is based on inactive monomers that are homodimerized by the illumination of light with the on-rate $k_{on}I$ where I is the light intensity. These homodimers activate the expression of the target gene and are dissociated with a constant off-rate k_{off}. Since the time scale of association and dissociation is fast compared to the time scale of gene expression, the light-dependent homodimerization is in a chemical equilibrium that depends only on the light intensity, on the association constant $K_b = k_{on}/k_{off}$, and on the total number of monomers. This model was fitted to the intensity response data by using a maximum likelihood method (the detailed model derivation and the calibration of the model can be found in Mathematical Model S1). The parametrized model was used to determine parameters that could be changed for engineering a system with a lower sensitivity toward activating light. This analysis revealed that a decrease of the association constant would result in a system that would be activated only at higher intensities and should therefore be less sensitive toward illumination with UV-B light of moderate intensities (Figure 1B). This behavior is a general property of the system and does not depend on a specific parameter set.

The association constant can be decreased by lowering the on-rate (k_{on}) and/or by increasing the off-rate (k_{off}). Since the off-rate does not depend on the activation wavelength, the sensitivity of the system to light of any activating wavelength (e.g., blue or UV-B light for photoreceptors that use flavin chromophores) can be reduced by increasing k_{off}. Therefore, to set up a blue light-responsive gene switch with a significantly reduced association constant, we turned to the TULIPs system that has been shown to be rapidly reversible with an active-state half-life time of only 17 s.[16] This optogenetic protein interaction module consists of an engineered variant of the LOV2 domain from *Avena sativa* phototropin 1 that carries a C-terminal epitope tag fused to its LOV2 Jα-helix (LOVpep). The tag is caged in the dark but becomes accessible for an engineered, high-affinity PDZ domain upon blue light illumination. We constructed the gene switch following the two-hybrid principle: LOVpep was fused to the Gal4 DNA-binding domain (Gal4BD) thus tethering it to a response construct harboring 5 repeats of the Gal4 binding motif (UAS$_G$) upstream of a minimal TATA promoter, while the PDZ interaction domain was fused to the VP16 transactivation domain. In the dark, the epitope tag of the LOVpep fusion protein is inaccessible for the PDZ domain. Consequently, gene expression is shut off. Upon illumination with blue light, unraveling of Jα makes the epitope tag accessible for the PDZ domain and gene expression is initiated via VP16. Upon termination of illumination, gene expression is rapidly shut off as caging of the epitope tag is re-established (Figure 2A).

The LOVpep-PDZ interaction can be modulated by point mutations in the LOV domain that alter the docking equilibrium of the Jα-helix to the core domain[16] and by choosing PDZ domains with varying affinities for the epitope tag.[17] To find the configuration with the best dynamic range of gene expression, we initially screened several previously described LOVpep-PDZ pairs[16] and found that the combination of Gal4BD-LOVpep[T406A,T407A,I532A] (pKM292) with ePDZb-VP16-NLS (pKM297) yielded the best expression
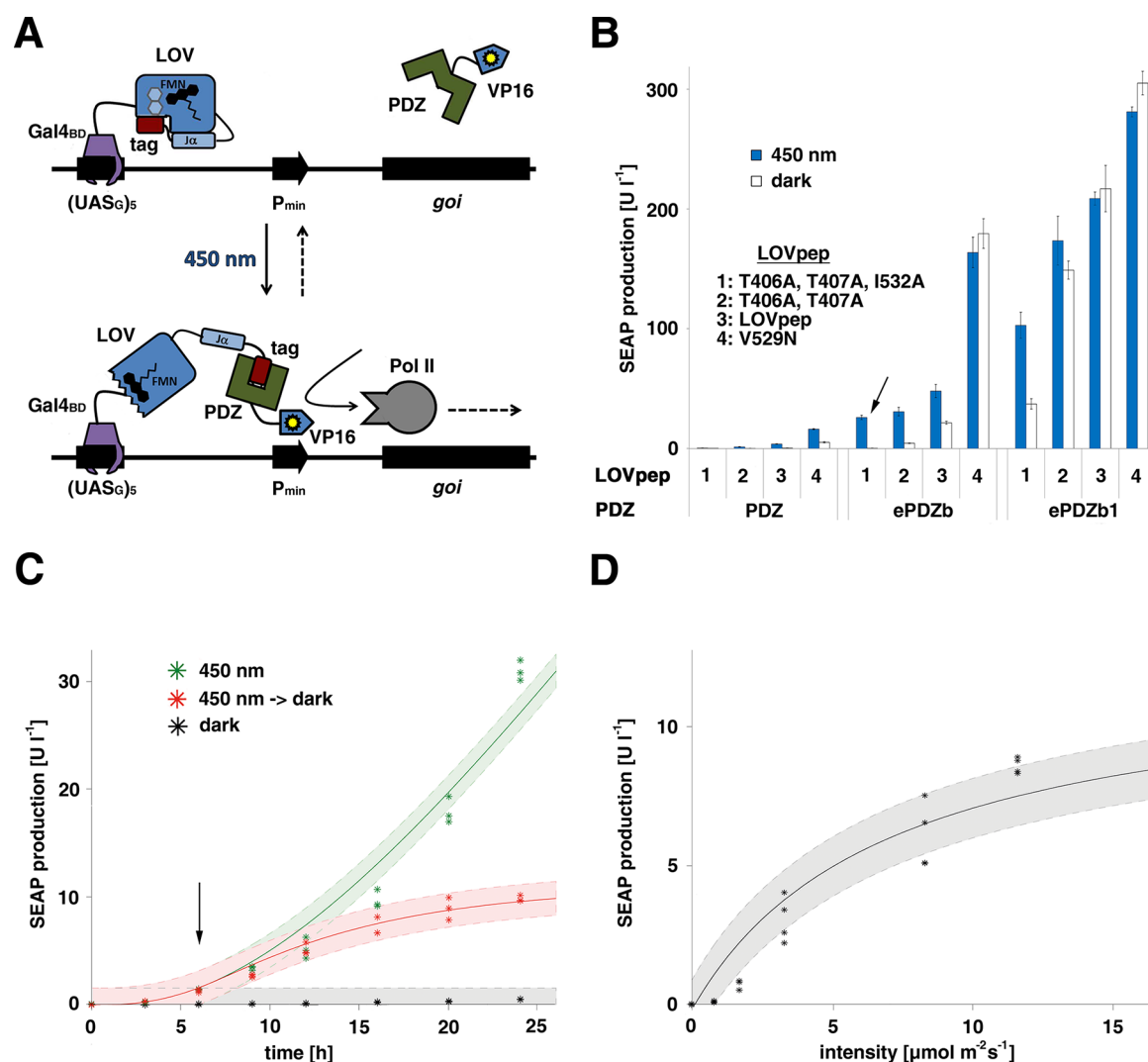
Figure 2. Rapidly reversible blue light-induced gene expression. (A) Mode of function. The Gal4(BD)-LOVpep fusion protein is tethered to a response construct harboring five (UAS_G) repeats upstream of a minimal promoter (P_{min}), while the PDZ interaction domain is fused to the VP16 transactivation domain. In the dark, the epitope tag of the LOVpep fusion protein is caged, resulting in the shut-off of gene expression. Blue light-illumination triggers uncaging of the epitope tag. This results in recruitment of PDZ-VP16 to trigger reporter (*goi*) expression. Upon illumination shut-off, gene expression is rapidly terminated as caging of the epitope tag is re-established. (B) Screen of LOVpep and PDZ variants. CHO-K1 cells were transfected with four LOVpep variants combined with three PDZ fusions and a reporter plasmid (pKM084). 24 h post-transfection, the culture medium was replaced with fresh medium and the cells were illuminated with 450 nm light (15 μmol m^{-2} s^{-1}) for 24 h. The arrow indicates the best configuration regarding the dynamic range of reporter expression (pKM292, pKM297, pKM084). Data are means ± SD ($n = 4$). (C) Light-inducible expression kinetics. CHO-K1 cells were transfected for blue light-responsive SEAP production (pKM516, pKM084). 24 h post-transfection, the culture medium was replaced with fresh medium and the cells were either illuminated with 450 nm light (10 μmol m^{-2} s^{-1}) for 24 h, incubated in the dark or first illuminated for 6 h with 450 nm light and then further incubated in the dark. The arrow indicates the switch from illumination to darkness. (D) Dose−response curve. CHO-K1 cells were transfected for blue light-inducible SEAP production (pKM516, pKM084). After 24 h, the culture medium was replaced with fresh medium and the cells were illuminated for 24 h with 450 nm light of increasing intensities. In C and D, the curves represent the model fitted to the data and the shaded error bands are estimated by a simple error model with a constant Gaussian error.

characteristics as indicated by an induction of over 100-fold in blue light-illuminated compared to dark-incubated CHO-K1 cells (Figure 2B).

Next, we examined whether our new gene switch met the predicted requirements to serve as a building block for orthogonal multichromatic gene expression control. First, we analyzed the kinetics of expression shut-off after termination of 450 nm illumination. To this end, CHO-K1 cells transfected for blue light-responsive SEAP production were illuminated for 6 h and then moved to the dark for another 24 h while control cells were either illuminated for 24 h or kept in the dark for the entire experiment (Figure 2C). Next, the analysis of the

system's response to illumination with activating 450 nm light of increasing intensities confirmed that the system requires a higher intensity for full activation compared to the LightON system (Figure 2D). The time course of the reporter levels and the intensity response data were used to parametrize a quantitative mathematical model based on ordinary differential equations by maximizing the likelihood of the parameters using the experimental data (the detailed derivation, analysis, and the calibration of the model can be found in Mathematical Model S2).

After confirming that the new blue light-responsive gene switch met the predicted requirements for orthogonal multi-
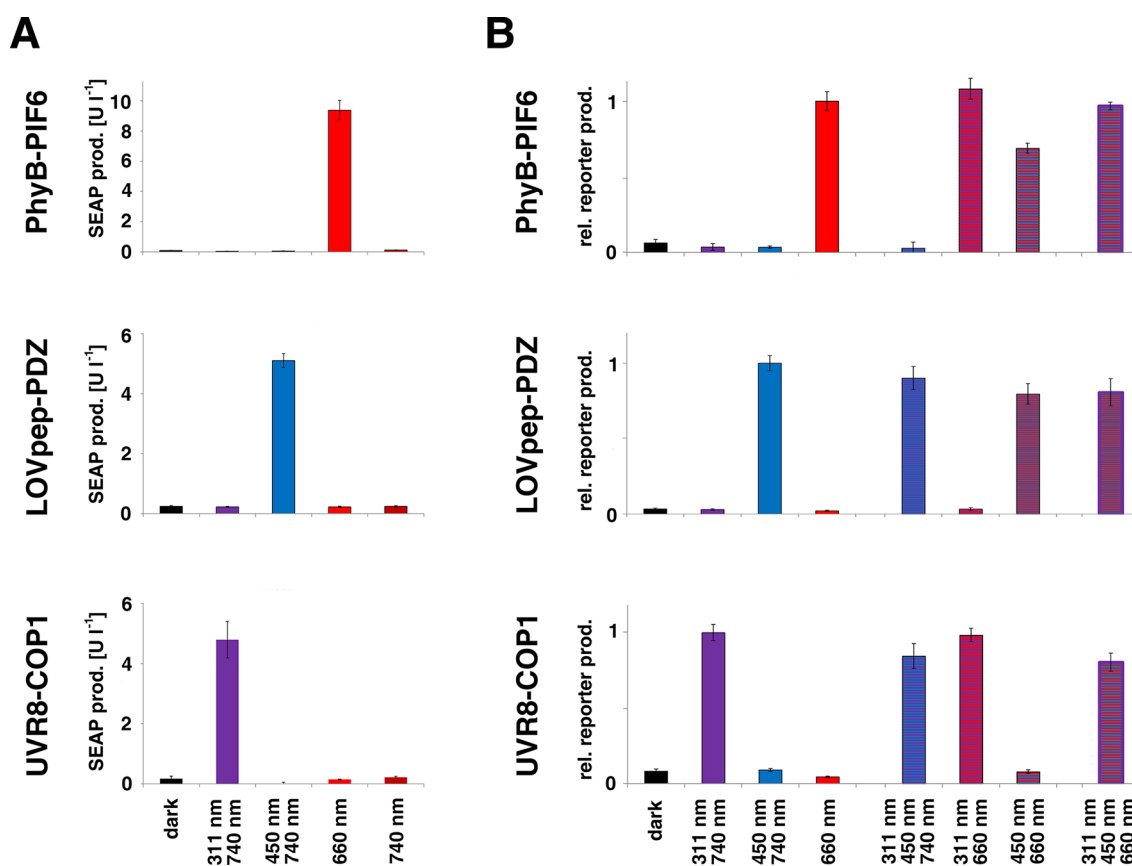
Figure 3. Multichromatic multigene expression control. (A) Spectral responses of the PhyB-PIF6, LOVpep-PDZ, and UVR8-COP1 systems. CHO-K1 cells were transfected for red/far-red light- (pKM301, pMF199), blue light- (pKM516, pKM084), or UV-B light- (pKM279, pKM081) controlled SEAP expression. 24 h post-transfection, the culture medium was replaced with fresh PCB-supplemented medium and the cells were incubated in the dark for 1 h. Subsequently, the cells were incubated in the dark or illuminated as indicated for 24 h. (B) Multichromatic multigene control in a single cell culture. CHO-K1 cells were simultaneously transfected for red light-inducible SEAP production (pKM301, pMF199), blue light-responsive FLuc expression (pKM516, pFR-Luc), and UV-B light-controlled angiopoietin 1 (Ang1) production (pKM279, pKM172). 24 h post-transfection, the culture medium was replaced with PCB-supplemented medium. After 1 h in the dark, the cells were either incubated in the dark or illuminated as indicated for 24 h before reporter quantification. Illumination conditions: 311 nm (2 μmol m^{-2} s^{-1}, 2 min ON/28 min OFF), 450 nm (10 μmol m^{-2} s^{-1}), 660 nm ((A) 2 μmol m^{-2} s^{-1}, (B) 10 μmol m^{-2} s^{-1}), 740 nm (20 μmol m^{-2} s^{-1}). Data are means ± SD (n = 4).

chromatic gene expression control, we determined its spectral response along with those of the PhyB-PIF6-based red/far-red light-controlled toggle switch and the UVR8-COP1-based UV-B-responsive expression system (Figure 3A). For all systems, only basal reporter levels were detected for cells incubated in the dark or under 740 nm illumination. Pulses of 311 nm light (2 min every 30 min) with supplementary 740 nm illumination exclusively activated the UVR8-COP1-based system, while the less-sensitive LOVpep-PDZ system was not activated and expression from the PhyB-PIF6 system was repressed by supplementary 740 nm light. Constant 450 nm illumination supplemented with 740 nm light solely induced expression from the LOVpep-PDZ-controlled reporter, while 740 nm supplementation effectively kept the red/far-red light-responsive toggle switch in the OFF state. Finally, 660 nm illumination exclusively activated the PhyB-PIF6-based toggle switch (Figure 3A).

Having confirmed that our new blue light-inducible gene switch displayed the predicted spectral response, we moved on to express three reporter genes under control of the PhyB-PIF6, LOVpep-PDZ, and the UVR8-COP1 systems in a single cell culture. After 24 h of illumination, the UVR8-COP1 system was only induced in the presence of activating pulses (2 min every 30 min) of 311 nm illumination and the LOVpep-PDZ-based

system was exclusively activated in the presence of activating 450 nm light (Supporting Information Figure S1). The red/far-red light-responsive toggle switch was activated by 660 nm light, but expression levels dropped in the presence of 450 nm illumination (450/660 nm and 311/450/660 nm) (Supporting Information Figure S1). This property of the PhyB-PIF6 system is based on the distinct absorbance spectra of the active FR-form and the inactive R-form of the PhyB photoreceptor.[18] At 660 nm illumination, the PhyB photoequilibrium favors the active FR-from. However, at 450 nm, the photoequilibrium is shifted toward the inactive R-form, explaining the drop of expression levels in the presence of 450 nm illumination. To optimize expression from the PhyB-PIF6 system in the presence of 450 nm light, we cotransfected CHO-K1 cells for blue light-induced FLuc expression and for red light-responsive SEAP production and titrated the 660 nm intensity in the presence of constant 450 nm light (Supporting Information Figure S2). Expression from the LOVpep-PDZ-controlled reporter was not affected by increasing 660 nm intensities, but expression by the red/far-red light-controlled toggle switch could be increased from the previously applied intensity of 2 μmol m^{-2} s^{-1}, reaching saturation at a 660 nm intensity of about 10 μmol m^{-2} s^{-1}.

The differential expression of three genes under control of the PhyB-PIF6, LOVpep-PDZ, and UVR8-COP1 systems in a single cell culture with the optimized intensity for 660 nm illumination showed a marked increase in expression of the PhyB-PIF6-controlled reporter when 450 nm illumination was applied alongside activating 660 nm light (Figure 3B). This optimized setup allowed the differential expression of one, two, or three genes in response to light of the activating wavelengths.

In summary, we have employed a mathematical model-guided approach to develop a method for the orthogonal control of up to three genes in a single cell. To this end, we developed a novel blue light-responsive gene switch with fast inactivation kinetics and combined it with existing red/far-red and UV-B-controlled gene expression technology to differentially control the expression of three genes within a single cell culture. We expect that this approach will pave the way for broader applications of optogenetic tools in synthetic biology and enable the control of gene networks with the spatiotemporal precision of light.

■ METHODS

DNA Cloning. The construction of expression vectors is given in detail in Supporting Information Table S1.

Cell Culture and Transfection. Chinese hamster ovary cells (CHO-K1, ATCC CCL 61) were cultivated in HTS medium (Cell Culture Technologies) supplemented with 10% fetal bovine serum (FBS) (PAN, cat. no. P30-3602, batch no. P101003TC), 2 mM L-glutamine (Sigma), 100 U mL^{-1} of penicillin and 0.1 mg mL^{-1} of streptomycin (PAN). Cells were transfected, using an optimized polyethylene-imine-based method (PEI, linear, MW: 25 kDa) (Polyscience).[19] All plasmids were transfected in equal amounts (w/w) and only the red/far-red-responsive split transcription factor on pKM301 was transfected in 2-fold excess (w/w).

Illumination Conditions. UV-B illumination was performed using a UV-B narrowband lamp (Philips, prod. no. PL-S 9W/01) in combination with 310 nm bandpass filters (Ashai Spectra, prod. no. ZBPA310) to eliminate light of higher wavelengths. To modulate light intensity, the UV-B light was attenuated by Plexiglas Alltop layers (Evoniks, prod. no. 29080). Blue (450 nm), red (660 nm), and far-red (740 nm) illuminations were performed by custom-made LED arrays.[19] Light intensity was adjusted using neutral density filters (Schott) that were placed on top of the culture dishes and the intensity was measured, using quantum sensors (LI-COR, prod. no. Q45045 or LTF Labortechnik, prod. no. CX-312). The PhyB/PIF6-based red light-controlled gene switch was rendered light-sensitive by the addition of 15 μM phycocyanobilin (PCB; LivChem, cat. no. P14137) from a 25 mM stock in DMSO to the culture medium 1 h before illumination. All cell-handling involving the blue and red light-inducible expression systems was done under safe 522 nm light.

Reporter Gene Assays. The reporter SEAP was quantified in the cell culture medium, using a colorimetric assay as described elsewhere,[19] and firefly luciferase was quantified in cell lysates, as detailed before.[7] Ang1 was quantified using the DuoSet ELISA Development system (R&D systems, cat. no. DY923).

■ ASSOCIATED CONTENT

⬡ Supporting Information
Figure S1, Figure S2, Table S1, Mathematical Model S1, Mathematical Model S2. This material is available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions
K.M. designed and performed the experiments, analyzed the data and wrote the paper; R.E. and J.T. developed the mathematical models and wrote the paper; M.D.Z. and W.W. designed the experiments, analyzed the data and wrote the paper.

Notes
The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

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