Spatiotemporally confined red light-controlled gene delivery at single-cell resolution using adeno-associated viral vectors

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Methodologies for the controlled delivery of genetic information into target cells are of utmost importance for genetic engineering in both fundamental and applied research. However, available methods for efficient gene transfer into user-selected or even single cells suffer from low throughput, the need for complicated equipment, high invasiveness, or side effects by off-target viral uptake. Here, we engineer an adeno-associated viral (AAV) vector system that transfers genetic information into native target cells upon illumination with cell-compatible red light. This OptoAAV system allows adjustable and spatially resolved gene transfer down to single-cell resolution and is compatible with different cell lines and primary cells. Moreover, the sequential application of multiple OptoAAVs enables spatially resolved transduction with different transgenes. The approach presented is likely extendable to other classes of viral vectors and is expected to foster advances in basic and applied genetic research.

INTRODUCTION

Single-cell sequencing and, more recently, single-cell multimodal omics approaches are uncovering new biology, revolutionizing our understanding of cellular heterogeneity in multicellular systems at unprecedented resolution and scale (1–3). The insight gained at the single-cell level is rapidly increasing because of single-cell analytical techniques becoming more and more broadly accessible, easier to handle, and economically affordable (1). However, the development of technologies for engineering mammalian cells at the single-cell scale is lagging behind.

An ideal system for spatially controlled gene transfer at single-cell resolution would (i) control gene delivery at the level of cell entry to avoid side effects by off-target viral uptake; (ii) exhibit low invasiveness; (iii) allow repetitive application in the same culture for delivering different genes to different cells; (iv) rely on standard laboratory equipment; (v) allow high throughput; and (vi) be compatible with native (unmodified) and optimally also nondividing, primary cells. Spatially controlled nonviral and viral gene delivery is a highly active field of research, and multiple delivery technologies using microinjection, gene guns, magnetic fields, electric fields, sonication, and light have been developed (4, 5). In terms of spatial control, light-responsive delivery systems are the gold standard as light can easily be applied with unmatched spatiotemporal precision.

Previously developed light-controlled gene delivery technologies involve optical transfection (6, 7), photochemical internalization (8), and light-controlled viral transduction (9–20). Although optical transfection based on laser-induced cell membrane perforation allows high spatial control, it is accompanied by high cell toxicity and restriction to small irradiation areas (4, 7). Photochemical internalization relies on endocytosis of nucleic acid or viral vectors and the subsequent light-induced permeabilization of the endosomal membrane by reactive oxygen species (ROS) produced by photosensitizing drugs (8). This method is inherently prone to off-target effects, as endocytosis occurs also in off-target cells and endosomal nucleic acid or viral vectors are also spontaneously released. Furthermore, intracellular generation of ROS may cause cell damage (8, 21).

Viral gene delivery systems have strong advantages compared to nonviral delivery systems (4, 5) such as high efficacy, prolonged transgene expression, and, depending on the vector system, the ability to also efficiently transduce nondividing and primary cells. To date, several light-controlled viral transduction approaches relying on different mechanisms have been developed as detailed in table S1. However, their widespread adoption is limited because these systems either need cytoxic ultraviolet (UV) light for induction (9–14), require viral uptake to both target and off-target cells before optical control (9–11, 14–20), or rely on the pre-engineering of target cells to express the photoreceptor (20).

Here, we overcome the limitations described above by the development of OptoAAV to optically guide the selective transfer of genetic information into single target cells. OptoAAV is based on adeno-associated viral (AAV) vectors that play a key role as gene delivery vehicles in fundamental research and clinically licensed gene therapies (22–24). AAV vectors are nonenveloped, single-stranded DNA vectors that transduce both dividing and nondividing cells and provide an excellent safety profile due to the absence of AAV-associated pathologies and episomal persistence (22, 23). The OptoAAV technology (i) controls transduction at the level of viral cell entry, thus minimizing the impact on off-target neighboring cells; (ii) uses noninvasive, low-intensity, and tissue-penetrating red and far-red light;
The OptoAAV technology comprises an engineered AAV-2 and a light-responsive adapter protein that mediates selective interaction of the AAV with the target cell (Fig. 1). The viral vector is genetically modified to be blind to its natural cellular receptor [heparan sulfate proteoglycan (HSPG)] and to expose the phytochrome-interacting factor 6 (PIF6; amino acids 1 to 100) from Arabidopsis thaliana on the capsid surface (OptoAAV). The adapter protein consists of phytochrome B (PhyB; amino acids 1 to 651) of A. thaliana and a designed ankyrin repeat protein (DARPin) specific for a cell surface protein of the target cell (PhyB-DARPin). Upon illumination with red (~660 nm) light, PhyB of the adapter protein interacts with PIF6 on the viral vector, thus recruiting OptoAAV to the cell surface, which results in transduction of the target cell. In contrast, illumination with far-red (~740 nm) light dissociates the interaction between PhyB and PIF6 and consequently prevents transduction (Fig. 1).

Implementation of the OptoAAV system

To develop and characterize the OptoAAV system, we selected the DARPin E_01 showing high affinity [dissociation constant (K_d) = 0.5 nM] to the human epidermal growth factor receptor (EGFR) that is overexpressed by many tumor cells (25, 26). This and other DARPin s have previously been used for the retargeting of AAV (27–29), adenoviral (30) and lentiviral (31) vectors, and viruses such as measles virus (32) either by exposing them on the viral surface or by using them as adapter mediating the interaction between the cell and the viral vector (33). We produced the fusion protein of DARPinEGFR and the photosensory domain of PhyB in Escherichia coli and purified it via its hexahistidine tag by immobilized metal affinity chromatography (IMAC; PhyB-DARPinEGFR fig. S1). We verified PhyB photoswitching by acquiring the absorbance spectra upon 660- and 740-nm illumination (fig. S2) and validated the light-dependent interaction of PhyB-DARPinEGFR with PIF6 by size exclusion chromatography (fig. S3). Flow cytometry experiments revealed that PhyB-DARPinEGFR bound specifically via the DARPinEGFR to A-431 cells overexpressing EGFR (fig. S4). To demonstrate that PhyB-DARPinEGFR can recruit PIF6-tagged molecules to cells in a light-dependent manner, we analyzed light-induced recruitment of an mVenus-PIF6 fusion protein to A-431 cells by flow cytometry (Fig. 2A) and confocal microscopy (fig. S5). In addition, the microscopy experiment revealed that mVenus-PIF6 was massively internalized after 20-min incubation at 37°C, whereas it remained mainly membrane-localized when cells were incubated on ice. This observation is in line with internalization reported for EGFR (34).

To display PIF6 on the capsid of AAV-2, we genetically fused the active phytochrome binding domain of PIF6 to the N terminus of the viral capsid protein VP2 (20). We placed the coding sequence of the PIF6-VP2 fusion protein under control of a cytomegalovirus (CMV) promoter (plasmid pMH303) and prevented expression of native VP2 by deletion of its start codon in the plasmid pRCVP2koA (Fig. 2B) (27). To prevent transduction of cells in a light-independent manner, these plasmids additionally contained the mutations R585A and R588A in the cap genes, ablating the natural tropism of AAV-2 for its natural receptor HSPG (Fig. 2B) (35). As model transgene, we selected the green fluorescent protein (GFP) or the red fluorescent protein mScarlet under control of the constitutive CMV promoter encoded on a self-complementary genome. Self-complementary AAV vectors are characterized by a higher transduction efficiency at the expense of only half of the loading capacity (36). We produced our OptoAAVs using the helper-free packaging system in human embryonic kidney (HEK)–293T cells and purified assembled AAV capsids by iodixanol gradient ultracentrifugation (37). We confirmed the incorporation of PIF6-VP2 in OptoAAV_GFP and the ablation of native VP2 by Western blotting against the viral capsid proteins VP1, VP2, and VP3 (Fig. 2C). From one OptoAAV_GFP production (ten 15-cm dishes), we determined an average genomic titer of (1.6 ± 0.6) × 10^11 vector genomes (vg). We next analyzed the light-controlled interaction of OptoAAV_GFP with PhyB. To this aim, we incubated OptoAAV_GFP with PhyB-functionalized agarose beads under 660-nm light and subsequently analyzed the supernatant for unbound viral particles by Western blotting against the viral capsid proteins (Fig. 2D). OptoAAV_GFP did not bind to the PhyB beads, suggesting that PIF6 was not accessible or exposed on the capsid surface. As previous studies showed that the N terminus of VP2 becomes exposed upon limited heat shock (38, 39), we incubated our OptoAAV_GFP for 10 min at 62.5°C and repeated the
Red light-controlled transduction

After the characterization of the individual components, we tested the ability of the system to transduce A-431 cells in a light-dependent manner. For this, we used OptoAAV~GFP~ that had been previously incubated for different periods of time at different temperatures (Fig. 3A and figs. S6 and S7A). We observed a 5.6- to 81-fold increase in the percentage of transduced cells at 660- compared to 740-nm illumination (low light intensity, both 20 \( \mu \text{mol m}^{-2} \text{s}^{-1} \), equals 0.36 and 0.32 mW cm\(^{-2} \), respectively). In agreement with the above-described in vitro binding studies (Fig. 2D), heat treatment of OptoAAV~GFP~ resulted in a strong increase of transduced cells (from ~2 to ~40%). On the basis of these data, we exposed PIF6 on OptoAAVs by a 10-min incubation step at 62.5°C in all further experiments. Transduction experiments with untreated and heated unmodified [wild-type (WT)] AAV-2 vectors revealed that this heat treatment reduced the infectious titer 5.9-fold (fig. S7B). For OptoAAV, a likely similar loss of infectivity is outcompeted by the gain in light-controlled infectivity caused by the heat-induced exposure of PIF6. Control experiments verified that the adapter protein and OptoAAV~GFP~ are both required for transduction and that the transduction efficiency of unmodified AAV-2 vectors in the presence of the adapter protein is not affected by 660- and 740-nm illumination (Fig. 3B). The percentage of transduced A-431 cells reached up to...
While EGFR is known to be internalized by endocytosis (34), we next asked whether active internalization of the cellular target receptor is a prerequisite for the functionality of the OptoAAV system. To this aim, we displayed SpyCatcher on the surface of HEK-293T cells by fusing it to the secretion signal and transmembrane domain of major histocompatibility complex I (MHC I). Afterward, we covalently coupled purified PhyB-mCherry-SpyTag to SpyCatcher (41). The MHC I transmembrane domain has previously been used to anchor proteins stably on the cell membrane (42) and does, to our knowledge, not contain an endocytosis function. We observed only minimal internalization of PhyB-mCherry-SpyTag on the engineered HEK-293T by confocal microscopy in comparison to the massive internalization of EGFR on A-431 cells (fig. S11A). As we were able to transduce the engineered PhyB-displaying HEK-293T cells with OptoAAV GFP in a light-dependent manner with a similar efficiency as for the A-431 cells (fig. S11B), we suggest that active internalization of the cellular target receptor is not required for the OptoAAV GFP system. We hypothesize that viral uptake is induced upon cell attachment by binding of the OptoAAV to co-receptors such as the AAV receptor (43).

To demonstrate that the OptoAAV system can be expanded to other cell lines with minimal or absent EGFR expression, we modularly...
exchanged the DARPin\textsubscript{EGFR} with DARPins specific for EpCAM (DARPin Ec1) (44), Her2/ErbB2 (DARPin 9\textsubscript{29}) (25), and CD4 (DARPin D55.2) (45) (figs. S1 and S2). Using these adapter proteins, we were able to selectively transduce further cell lines (Fig. 4A) and primary human CD4-positive T lymphocytes (Fig. 4B) in a light-dependent manner.

**Spatiotemporal control of transduction**

Next, we aimed at transducing cells in a spatially resolved manner. To this end, we incubated A-431 cells for 10 min with the adapter protein under 740-nm illumination (Fig. 5A). After a washing step to remove unbound adapter protein, we added OptoAAV\textsubscript{GFP} or OptoAAV\textsubscript{mScarlet} and illuminated the cells spatially resolved with 660-nm light for 9 s using a photomask. Following incubation for 2 hours in the dark, we washed the cells, incubated them for another 46 hours under 740-nm light, and visualized the transduced cells by microscopy (Fig. 5A and fig. S12A). We observed spatially resolved transduction (i.e., expression of the fluorescent proteins) with a spatial resolution in the 100-\mum range, while nontransduced cells were distributed over the whole wells as seen in the 4',6'-diamidino-2-phenylindole (DAPI) images. We next tested whether OptoAAVs encoding different transgenes can be used to sequentially transduce cells in a spatially resolved manner. To this aim, we performed the just described experiment with OptoAAV\textsubscript{GFP} and photomask 1. Following 2-hour incubation in the dark and a subsequent washing step, we repeated the same procedure with OptoAAV\textsubscript{mScarlet} and photomask 2 (Fig. 5B and fig. S12, B and C). This approach enabled the spatially resolved transduction of cells with two different transgenes and can likely be expanded to additional transgenes. In areas illuminated with both photomasks, we observed cells expressing both transgenes, indicating that the OptoAAV system can deliver several transgenes into one target cell (fig. S12B).

We next extended the spatial resolution to the single-cell level by illumination of one selected cell using a conventional confocal microscope equipped with a 633-nm laser. To track the illuminated cell over the course of the experiment (48 hours), we seeded A-431 cells, of which 1% were fluorescently labeled (eFluor670), on a coverslip with a labeled grid (Fig. 6A). Following incubation with the adapter protein and addition of OptoAAV\textsubscript{GFP} under 740-nm light, we illuminated a single isolated eFluor670-labeled cell with low-intensity 633-nm laser light (\sim 120 ms; Fig. 6A). After 48 hours, we analyzed the transduction by microscopy. In 4 of 17 experiments (24%), the single illuminated eFluor670-labeled cell showed GFP expression (for a representative successful experiment, see fig. S13A). As the success rate was lower than transduction of eFluor670-labeled cells at 660 nm in comparable flow cytometry experiments (38%; fig. S13B), we hypothesized that absorption of the 633-nm laser by the eFluor670 dye might have reduced photoactivation of...
PhyB. Therefore, we repeated the experiment with carboxyfluorescein diacetate succinimidyl ester (CFSE)–labeled cells, which have no absorbance at 633 nm and OptoAAV mScarlet. In 9 of 15 experiments (60%), we were now able to transduce the single illuminated CFSE-labeled cell (Fig. 6B and fig. S14A), which is in agreement with the transduction rate at 660 nm of comparable flow cytometry experiments (58%; fig. S14C). From the nonilluminated cells, 1.0% showed mScarlet fluorescence, although mainly with a much lower intensity than the illuminated cell (fig. S14A). This off-target transduction rate is similar with the one under constant 740-nm illumination in comparable flow cytometry experiments (0.6%; fig. S14B).

**Outlook**

In summary, we demonstrated that the OptoAAV technology enables the spatiotemporally resolved and light dose-dependent selective transduction of native cell lines and primary cells using low-intensity red light. Because of its modular design, the system can be customized to target cell types of choice by switching to an adapter protein with the desired specificity. OptoAAV intrinsically features a two-factor control for specificity. Successful transduction requires both recognition of the target cell type by the adapter protein and the light stimulus, leading to vector binding and internalization. Such AND-type control was shown in previous studies to substantially increase target specificity (46). Using a conventional confocal microscope, OptoAAV allowed the selective transduction of single cells by local illumination. These experiments could allow perturbing biological processes at the single-cell level to reconstruct and understand the implications of cell heterogeneity. In addition, the sequential application of different OptoAAVs enabled the selective transduction of cells with different transgenes within the same culture. This feature may be of particular interest when applying OptoAAV for the delivery of genes encoding differentiation factors in tissue engineering for regenerative medicine. Although we only tested the in vitro functionality of OptoAAV so far, it may also be applied for the site-specific...
in vivo gene delivery in fundamental research or (cancer) gene therapy. Another promising area of application would be in neuroscience. Light-responsive ion channels such as channelrhodopsin (ChR) are routinely used for the light-responsive induction of action potentials in neurons, both in tissue culture and in vivo (47). In living animals, ChRs are often introduced via AAV-based vectors, and the injection of viral vectors can lead to substantial spread of opsin expression, e.g., caused by the backflow of the vector along the injection tract. While this might sometimes be a desired feature, it is often a detrimental complication, hampering conclusions about the role of an activated or deactivated brain area (48). Using OptoAAV, gene delivery could be targeted with high spatial resolution and selectivity to the cells of interest by (i) choosing an appropriate adapter protein and (ii) by local illumination with red light. For transduction, the same waveguide-based illumination hardware as used for activation of ChR could be used. In an in vivo setting, the spatial resolution and tissue penetration may be further increased by two-photon activation of the OptoAAV system as recently shown for a bacterial phytochrome (49). Moreover, OptoAAV may be used to obtain further insights into viral cell entry, e.g., by determining required binding times (50). Because of its modular design, we suggest that the OptoAAV approach could serve as blueprint for rendering further classes of viral vectors light-responsive.

**MATERIALS AND METHODS**

**Cloning of plasmids**

The nucleic acid sequences of all plasmids generated in this study are depicted in table S2. The plasmids were assembled by Gibson (51) or AQUA (52) cloning, and the used templates for polymerase

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**FIGURE 6. Spatially resolved transduction of single cells using a conventional confocal microscope.**

**(A)** Experimental workflow. A-431 cells containing 1% fluorescently labeled (CFSE or eFluor670) cells were seeded on a gridded coverslip. Forty-eight hours after seeding, cells were incubated with 50 nM PhyB-DARPinEGFR in PBS supplemented with 10% FCS under 740-nm light for 10 min. After washing, OptoAAVmScarlet or OptoAAVmScarlet in PBS supplemented with 10% FCS were added and a bright field and CFSE or eFluor670 image was acquired under 740-nm illumination (200 μmol m⁻² s⁻¹). After switching the 740-nm light off, a single CFSE or eFluor670-positive cell was illuminated with the 633-nm laser of the confocal microscope. Following 2-hour incubation in the dark, cells were washed and incubated for 46 hours in medium under 740-nm light. Last, cells were fixed, DAPI-stained, and imaged by confocal microscopy. 

**(B)** Light-controlled transduction of a single CFSE-stained A-431 cell. The experiment was performed as in (A) with OptoAAVmScarlet (MOI: 4.9 x 10⁴). The area illuminated with the 633-nm laser is encircled in red. Representative images from n = 9 successful experiments (out of 15). All experiments are shown in fig. S14A. Scale bar, 100 μm.
chain reaction (PCR) are mentioned in table S2. The codon-optimized (for E. coli) sequences for the DARpins Ec1 (EpCAM) (44, 9, 29 (Her2/ErbB2) (25), and D55.2 (CD4) (45) and the codon-optimized (for human and E. coli) sequence for SpyCatcher003 (41) were ordered as gBlocks from Integrated DNA Technologies (Coralville, IA). All other sequences or mutations were introduced with oligonucleotides and PCR.

**Illumination**

If not indicated otherwise, samples were illuminated with microcontroller-regulated illumination panels containing LEDs with ~660-nm (LED660N-03, Roithner Lasertechnik, Vienna, Austria) peak wavelength: 660 nm; LH W5AM, Osram Opto Semiconductors, Regensburg, Germany; peak wavelength: 660 nm; LST1-01F06-PRD1-00, Opulent Americas, Raleigh, NC; peak wavelength: 655 nm) or 740-nm (LED740-01AU, SMB1N-740D, both Roithner Lasertechnik; LZ4-00R308, LED Engin, San Jose, CA) peak wavelengths. For spatially resolved illumination, custom laser photoplot films (4000 DPI, JD Photo Data, Herts, UK) were used as photomask. The experiment depicted in Fig. 3C was performed with optoPlate-96 (53) equipped with 630-nm (150141R737100, Würth Elektronik, Niedernhall, Germany) and 780-nm (SM'T780-27, Marubeni, Tokyo, Japan) LEDs, and illumination protocols were defined with optoConfig-96 (54). For illumination at the confocal microscope, a P-4000 LED light source (CoolLED, Andover, UK) with 740 nm was used. After illumination of the samples with the indicated wavelengths, they were only handled under dim green safe light until the end of the experiment to prevent photoswitching of PhyB. Light intensities were measured with an AvaSpec-ULS2048 fiber-optic spectrometer (Avantes BV, Apeldoorn, The Netherlands). Where indicated, pulsed 660-nm illumination (5 min ON, 55 min OFF) was used to prevent continuous cycling of PhyB between the binding and nonbinding state as occurring under continuous 660-nm light (50). If not indicated otherwise, samples were illuminated at an intensity of 20 μmol m⁻² s⁻¹.

**Protein production and purification**

For production of the different PhyB-DARPin proteins and PhyB-mCherry-SpyTag, the corresponding plasmid (see table S2) encoding the fusion protein and the biosynthesis enzymes for the chromophore phycocyanobilin (PCB) was transformed into E. coli BL21 Star (DE3)pLysS (Thermo Fisher Scientific, catalog no. C602003) at 30°C for 5 hours and purified by IMAC using buffers without TCEP followed by a buffer exchange to PBS as described above. PhyB was produced from plasmid pMH1105 in E. coli and purified by IMAC as described previously (56). FRB-mCherry-DARPin was produced from plasmid pMH212 in E. coli and purified by IMAC as described previously (28). Afterward, the buffer of PhyB and FRB-mCherry-DARPin was exchanged to PBS supplemented with 0.5 mM TCEP as described above.

**Protein characterization**

The identity and purity of the proteins was analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) followed by Zn²⁺ staining of the chromophore PCB [incubation in 1 mM zinc acetate for 10 min followed by imaging of fluorescence under UV light (312 nm) using an agarose gel documentation system (Intas, Göttingen, Germany)] and by Coomassie staining of proteins. As protein size standard, a PageRuler prestained protein ladder (Thermo Fisher Scientific, catalog no. 26616) or a Pierce prestained protein molecular weight marker (Thermo Fisher Scientific, catalog no. 26612) was used. Protein concentration was determined by Bradford assay (Bio-Rad, Hercules, CA, catalog no. 500-0006) using BSA (Sigma-Aldrich, St. Louis, MO, catalog no. 50479) as standard. The absorbance spectra were acquired with an Infinite M200 Pro microplate reader (Tecan, Männedorf, Switzerland). The interaction of PhyB-DARPin Ec1 and mVenUS-PiF6 was analyzed by size exclusion chromatography on a light-protected Superdex 200 10/300 GL column (GE Healthcare, Freiburg, Germany, catalog no. 17-5175-01) connected to the Äkta Explorer Fast Protein Liquid Chromatography System (GE Healthcare) using PBS as running buffer at a flow rate of 0.5 ml min⁻¹. The column was calibrated with a gel filtration standard (Bio-Rad, catalog no. 151-1901).

**Cell culture**

A-431 [human epidermoid carcinoma; German Collection of Microorganisms and Cell Cultures (DSMZ), Braunschweig, Germany, catalog no. ACC 91; cell line identity verified by short tandem repeat (STR) profiling], A549 [human lung carcinoma; CLS, Eppelheim, Germany, catalog no. 300114), HeLa [human cervix adenocarcinoma; American Type Culture Collection (ATCC), Manassas, VA, catalog no. CCL-2], MDA-MB-231 (human breast adenocarcinoma, obtained from Signalling Factory Core Facility, University of Freiburg, Germany; cell line identity verified by STR profiling), and HEK-293T (HEK, DSMZ, catalog no. ACC 635) cells were cultivated in Dulbecco’s modified Eagle’s medium (DMEM) complete [DMEM (PAN Biotech, Aidenbach, Germany, catalog no. P04-03550) supplemented with 10% (v/v) FCS (PAN Biotech, catalog no. P30-3602), penicillin (100 U ml⁻¹), and streptomycin (100 μg ml⁻¹)]. SK-OV-3 cells (human ovary adenocarcinoma; ATCC, catalog no. HTB-77) were maintained in McCoy’s 5A medium (Sigma-Aldrich, catalog no. M8403) supplemented with 10% (v/v) FCS, 2 mM l-glutamine.
(Thermo Fisher Scientific, catalog no. 25030-024), penicillin (100 U ml\(^{-1}\)), and streptomycin (100 µg ml\(^{-1}\)). MDA-MB-453 cells (human breast metastatic carcinoma; ATCC, catalog no. HTB-131) were cultivated in RPMI 1640 medium (Thermo Fisher Scientific, catalog no. 61870-010) supplemented with 10% (v/v) FCS, penicillin (100 U ml\(^{-1}\)), and streptomycin (100 µg ml\(^{-1}\)). CHO-K1 (Chinese hamster ovary; DSMZ, catalog no. ACC 110) cells were maintained in HTS medium (Cell Culture Technologies, Gravesano, Switzerland, catalog no. CHTS) supplemented with 10% (v/v) FCS, 2 mM glutamine, penicillin (100 U ml\(^{-1}\)), and streptomycin (100 µg ml\(^{-1}\)). All cells were cultivated at 37°C in a humidified atmosphere containing 5% CO\(_2\) and passed upon reaching a confluence of ~80%.

**Generation of stable HEK-293T–SpyCatcher cell line**

To generate HEK-293T cells stably expressing SpyCatcher-MHC-moxBFP, we used lentiviral transduction as described previously (50). Briefly, HEK-293T cells were transfected with the lentiviral packaging plasmid pCMV 

**Analysis of protein binding to cells**

To analyze the binding of PhyB and PhyB-DARPin\(_{\text{EGFR}}\) to A-431 cells, A-431 cells were detached with trypsin/EDTA solution (PAN Biotech, catalog no. P10-023500) and washed once with DMEM complete. Afterward, 8 \times 10^5 cells ml\(^{-1}\) were incubated with 1 µM PhyB or PhyB-DARPin\(_{\text{EGFR}}\) in DMEM complete for 2 hours at 37°C in the dark. Next, cells were washed with PBS, resuspended in PBS supplemented with 2% (v/v) FCS, and analyzed for PhyB fluorescence with a Gallios flow cytometer (Beckman Coulter, Brea, CA) using a 638-nm laser for excitation and a 660/20-nm bandpass filter for emission.

To analyze the recruitment of mVenus-PIF6 to the cell surface by flow cytometry, 5 \times 10^4 A-431 cells were seeded per well of a 24-

**AAV vector production and purification**

AAV vectors were produced using the adenovirus helper-free packaging system (37). For production of OptoAAVs, HEK-293T cells were transfected with the plasmids pMH303, pRCVP2koA (27; gift from H. Büning), pHelper (Cell Biolabs, San Diego, CA, catalog no. VKP-402) and the self-complementary vector plasmid pCMVgfp or pCMV\(_{\text{mscarlet}}\) (both plasmids were gifts from D. Grimm) in an equimolar ratio. For production of WT AAV-2 vectors encoding GFP, cells were transfected with the plasmids pAAV-RC2 (Cell Biolabs, catalog no. VKP-402), pHelper and the self-complementary vector plasmid pCMVgfp in an equimolar ratio. Briefly, 8 \times 10^6 HEK-293T cells were seeded per 15-cm cell culture dish, and after 24 hours, cells were transduced with 60 µg of total plasmid DNA mixed with 200 µg of PEI (M\(_\text{w}\), 25,000) in 3 ml Opti-MEM (Thermo Fisher Scientific, catalog no. 22600-134). After 72 hours, cells were scraped from the cell culture plates, pelleted by centrifugation (400 g for 15 min), washed once with PBS, and resuspended in virus lysis solution [50 mM tris-HCl and 150 mM NaCl (pH 8.5); 500 µl per 15-cm dish]. After lysing the cells by five freeze-thaw cycles, the lysate was incubated with benzozone (50 U ml\(^{-1}\); Merck Millipore, Darmstadt, Germany, catalog no. 70664-3) at 37°C for 1 hour before cell debris were removed by centrifugation at 4000g for 15 min. Last, AAVs were purified from the supernatant by discontinuous iodixanol density gradient centrifugation (57). Centrifugation was performed for 2 hours at 171,000g and 4°C using the Optima L-90 K ultracentrifuge equipped with a Type 70.1 Ti fixed-angle rotor (Beckman Coulter). OptoAAVs from 10 plates were purified per Quick-Seal polypropylene tube (Beckman Coulter, catalog no. 342413). After centrifugation, the 40% iodixanol fraction containing OptoAAVs or WT AAVs was aliquoted, shock-frozen in liquid nitrogen, and stored at ~80°C.

**AAV characterization and quantification**

Purified AAV vectors were analyzed by Western blotting against the viral capsid proteins. To this aim, iodixanol-purified AAVs were mixed with SDS loading buffer [final concentration: 62 mM tris-HCl (pH 6.8), 10% (v/v) glycerol, 2% (w/v) SDS, 2.5% (v/v) 2-mercaptoethanol, and 0.01% (w/v) bromophenol blue sodium salt] and incubated at 95°C for 5 min. After separating the samples by SDS-PAGE, the proteins were transferred onto a polyvinylidene difluoride membrane, and the membrane was blocked with blocking buffer [PBS containing 3% (w/v) milk powder] at 4°C overnight. Following incubation of the membrane for 2 hours with a hybridoma supernant containing AAV-2–specific B1 antibody (58; gift from D. Grimm) diluted 1:10 in blocking buffer, the membrane was washed with PBS-T [PBS supplemented with 0.05% (v/v) Tween 20] and incubated for 1 hour with secondary anti-mouse horseradish peroxidase (HRP)–conjugated antibody (GE Healthcare, catalog no. NA931) diluted 1:2000 in blocking buffer. After washing the membrane with PBS-T, ECL Prime Western blotting detection reagent (Thermo Fisher Scientific, catalog no. T3605; 0.5 µM in PBS for 15 min), and cells were imaged on a Zeiss LSM 880 laser scanning confocal microscope (Zeiss, Oberkochen, Germany) using a 63× Plan Apochromat objective [numerical aperture (NA): 1.4]. mVenus, mCherry, and TO-PRO-3 were excited with a 514-, 561-, and 633-nm laser and detected between 517 to 543 nm, 570 to 597 nm, and 643 to 720 nm, respectively (pinhole was adjusted to image a 1.0-µm section for each channel).
Analysis of EGFR activation

To analyze EGFR activation by Western blotting, 6.5 × 10⁴ A-431 cells were seeded per well of a 24-well plate. After 24 hours, the medium was exchanged to starvation medium (DMEM complete without FCS), and after cultivation for another 24 hours, the cells were incubated as indicated (recombinant human EGFR was purchased from Sigma-Aldrich; catalog no. E9644). Following a washing step with PBS, cells were incubated with 100 µl of lysis buffer [20 mM tris-HCl (pH 7.5), 1 mM EDTA, 100 mM NaCl, 0.5% (v/v) Triton X-100, 0.1% (w/v) SDS, protease inhibitor (complete protease inhibitor cocktail tablets, Roche, Basel, Switzerland, catalog no. 04693116001), 10 mM β-glycerophosphate, 50 mM sodium fluoride, 1 mM sodium orthovanadate, and 10 mM sodium pyrophosphate] for 10 min on ice. Next, the plate was incubated for 30 min at −20°C, and after subsequent thawing on ice, the lysed cells were transferred into microcentrifuge tubes and centrifuged at 10,000g at 4°C for 10 min. Afterward, the supernatants were mixed with SDS loading buffer and incubated at 95°C for 5 min. The samples were separated by SDS-PAGE and analyzed by Western blotting as described above for the AAV samples using the following buffers and antibodies: washing buffer: TBS-T [TBS (50 mM tris-HCl and 150 mM NaCl; pH 7.4) with 0.1% (v/v) Tween 20]; blocking buffer: TBS-T with 5% (w/v) BSA; primary antibodies (diluted 1:1000 in blocking buffer; 1-hour incubation): anti-rabbit immunoglobulin G, HRP-linked (CST, catalog no. 7074).

Light-controlled transduction

If not indicated otherwise, OptoAAVs were incubated at 62.5°C for 10 min in a heat block and stored afterward on ice (up to 6 hours) before use in the experiment. For the light-controlled transduction experiments that were analyzed by flow cytometry, 5000 cells were seeded per well of a 96-well plate in 100 µl of the corresponding medium. For experiments with the optoPlate-96, cells were seeded in a black 96-well plate with transparent bottom (Greiner, Frickenhausen, Germany, catalog no. 655090). After 24 hours, OptoAAVs were mixed with the indicated PhyB-DARPIn protein in the indicated buffer/medium, illuminated for 5 min with 740-nm light, and added to the corresponding wells after washing cells once with PBS. After incubation for the indicated period under the indicated illumination condition, wells were washed with PBS and the cells were further incubated in their corresponding medium under the indicated illumination. Forty-eight hours after addition of the AAVs to the cells, the wells were washed with PBS and the cells were detached by the addition of 50 µl of trypsin/EDTA solution per well. Afterward, 200 µl of PBS supplemented with 5% (v/v) FCS was added to each well and the cells were analyzed for transgene expression using an Attune NxT flow cytometer (Thermo Fisher Scientific). BFP, GFP, CFSE, mScarlet, and eFluor670 were excited with a 405-, 488-, 561-, and 637-nm laser and detected using a 440/50-, 530/30-, 620/15-, and 670/14-nm emission filter, respectively. Autofluorescence of the cells was measured in the unused BFP or eFluor670 channel. Flow cytometry data were analyzed with FlowJo (v10.6.1, Becton, Dickinson and Company, Franklin Lakes, NJ), and the gating strategy used throughout this study is depicted in fig. S6. For experiments with the HEK-293T–SpyCatcher cell line, only BFP-positive cells (~95%) were used for the transduction analysis.

For spatially resolved transduction experiments using a photomask, 1 × 10⁴ or 4 × 10⁴ A-431 cells were seeded in 750 or 300 µl of medium per well of a µ-Slide four-well (ibidi, Gräfelfing, Germany, catalog no. 80426; Fig. 5) or µ-Slide eight-well (ibidi, catalog no. 80826; fig. S12, A and B) chambered coverslip, respectively. After 24 hours, cells were incubated as described sequentially with PhyB-DARPInEGFR and OptoAAV under the indicated illumination regime. Twenty-four hours after addition of OptoAAVs to the cells, cells were transferred from 37°C to 30°C to reduce proliferation and were incubated for another 24 hours. Afterward, cells were fixed with 4% (w/v) PFA in PBS for 20 min, DAPI-stained (1 µg ml⁻¹ in PBS), and imaged on a Zeiss LSM 880 laser scanning confocal microscope using a 10x EC Plan Neofluar objective (NA, 0.3). DAPI was excited with a 405-nm laser and detected between 417 and 470 nm. GFP and mScarlet were excited with 488- and 561-nm lasers and detected in lambda scanning mode (GFP: 499 to 695 nm, bin width: 8.9 nm; mScarlet: 570 to 695 nm, bin width: 8.9 nm), respectively. Afterward, GFP and mScarlet signals were separated from autofluorescence by linear unmixing using Zen Black (v2.3 SP1, Zeiss), tiles were stitched using Zen Blue (v3.1, Zeiss) and median-filtered in Fiji (59).

For spatially resolved transduction experiments using a confocal microscope, A-431 cells were stained with 1.5 or 5 µM cell proliferation dye CFSE (Thermo Fisher Scientific, catalog no. C34554) or eFluor670 (Thermo Fisher Scientific, catalog no. 65-0840-90) according to the manufacturer’s instructions, respectively. The stained...
cells were mixed with unstained cells in a ratio of 1:99, and 1.5 × 10^4 cells were seeded in 300 μl of medium per well of a μ-Slide eight-well grid-500 (ibidi, catalog no. 80826-G500) gridded (with lettered and numbered squares) and chambered coverslip. After 48 hours, cells were incubated as described sequentially with PhyB-DARPineGFR and OptoAAV mScarlet or OptoAAV GFP within a stage-tapecubator (Tokai Hit, Fujinomiya, Japan) installed on a Zeiss LSM 880 laser scanning confocal microscope. CFSE/ transmission and eFluor670 images were acquired using a 25x LD LCI Plan Apochromat water objective (NA, 0.8) and a 488- and 633-nm laser, respectively. CFSE and eFluor670 fluorescence was detected at 490 to 570 nm and 651 to 740 nm, respectively. During imaging, cells were constantly illuminated with 740-nm light (200 μmol m⁻² s⁻¹). After selecting a single, isolated CFSE or eFluor670-positive cell for light-controlled transduction, the 740-nm light was switched off and the cell was illuminated spatially resolved with the 633-nm laser using the bleaching function of the microscope (pixel dwell time: 0.77 μs; pixel size: 0.554 μm; 633-nm laser intensity: 0.5%; 15 iterations). Afterward, the samples were processed and imaged as described above for the spatially resolved transduction experiments using photomasks. The grid was plotted into the fluorescence images based on its weak autofluorescence.

Transduction experiment with T cells
Peripheral blood mononuclear cells (PBMCs) were isolated from healthy human donors by density gradient centrifugation (Ficoll-Paque). Afterward, PBMCs were resuspended in RPMI complete medium [RPMI 1640 medium supplemented with 10% (v/v) FCS, 10 mM Heps (Thermo Fisher Scientific, catalog no. 15630-080), 10 μM sodium pyruvate (Thermo Fisher Scientific, catalog no. 11360-039), 1x minimum essential medium nonessential amino acids (PAN Biotech, catalog no. P08-32100), penicillin (50 U ml⁻¹), and streptomycin (50 μg ml⁻¹)] supplemented with interleukin-2 (IL-2; 500 U ml⁻¹; PeproTech, Hamburg, Germany, catalog no. 200-02) and activated with anti-CD3/CD28 (1 μg ml⁻¹) antibodies. After 72 hours, the remaining PBMCs were mostly T cells (60%), which were used for light-controlled transduction using PhyB-DARPineGFR and OptoAAV GFP as indicated. Six hours after addition of OptoAAV GFP, the cells were incubated in RPMI complete medium supplemented with IL-2 (100 U ml⁻¹) under the indicated illumination. After 42 hours, the cells were stained for CD4 expression by incubation with CD4-V450 antibody (BD Biosciences, San Jose, CA, catalog no. 560345) diluted 1:200 in PBS supplemented with 2% (v/v) FCS at 4°C for 15 min. After washing, the cells were resuspended in PBS supplemented with 2% (v/v) FCS and analyzed using an Attune NxT flow cytometer as described above. V450 fluorescence and autofluorescence were measured in the BFP and mScarlet channel, respectively. Autofluorescent cells (<1.7%) were excluded from the analysis.

Statistical analysis
Statistical significance was tested with unpaired two-sided t tests (no assumption of consistent standard deviations) with correction for multiple comparisons (Holm-Sidak method) using GraphPad Prism (v8.4.3, GraphPad Software, San Diego, CA).

SUPPLEMENTARY MATERIALS
Supplementary material for this article is available at http://advances.sciencemag.org/cgi/content/full/7/25/eabf0797/DC1

View request a protocol for this paper from Bio-protocol.

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Acknowledgments: We are grateful to M. R. Russ (University of Freiburg) for generation of the HEK-SPyCatcher cell line and providing Phyll-bmCherry-SpyTag. We would like to thank R. M. Velasco-Cárdenas, S. Brandl, K. Raute, and S. Minguet (University of Freiburg) for providing the human primary T cells. We thank the technical workshop of the Faculty of Biology for the design and construction of the illumination devices. We acknowledge the excellent scientific and technical assistance of the Signalling Factory Core Facility staff of the University of Freiburg for help on flow cytometry and providing cell lines. We thank the staff of the Life and technical assistance of the Signalling Factory Core Facility staff of the University of Freiburg for help with microscopy resources, and the excellent support in image recording.

Funding: This work was supported by the Deutsche Forschungsgemeinschaft (DFG, German Research Foundation) under Germany’s Excellence Strategy CIBSS, EXC-2189, Project ID: 390939984 and under the Excellence Initiative of the German Federal and State Governments EXC-294 and GSC-4, and in part by the Ministry of Science, Research, and Arts of the State of Baden-Württemberg.

Sci. Adv. 2021; 7 : eabf0797
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Sci Adv 7 (25), eabf0797.
DOI: 10.1126/sciadv.abf0797