Orthogonal tools for controlling protein function by post-translational modifications open up new possibilities for protein circuit engineering in synthetic biology. Phosphoregulation is a key mechanism of signal processing in all kingdoms of life, but tools to control the involved processes are very limited. Here, we repurpose components of bacterial two-component systems (TCSs) for chemically induced phosphotransfer in mammalian cells. TCSs are the most abundant multi-component signal-processing units in bacteria, but are not found in the animal kingdom. The presented phosphoregulated orthogonal signal transduction (POST) system uses induced nanobody dimerization to regulate the trans-autophosphorylation activity of engineered histidine kinases. Engineered response regulators use the phosphohistidine residue as a substrate to autophosphorylate an aspartate residue, inducing their own homodimerization. We verify this approach by demonstrating control of gene expression with engineered, dimerization-dependent transcription factors and propose a phosphoregulated relay system of protein dimerization as a basic building block for next-generation protein circuits.
NATURAL PROTEIN CIRCUIT ENGINEERING PROMISES INCREASED CONTROL OVER CELLULAR BEHAVIOR. TECHNOLOGICALLY, HOWEVER, MUCH PROGRESS MUST BE MADE IN DEVELOPING SUITABLE SYSTEMS TO ACHIEVE THIS GOAL. IN NATURE, HIGHLY COMPLEX SIGNALING NETWORKS HAVE EVOLVED TO REGULATE DIVERSE FUNCTIONS IN LIVING CELLS. THESE FUNCTIONS INCLUDE THE ACTIONS OF AUXILIARY REGULATORS, TUNING OF SIGNAL DETECTION WINDOWS, CONTROLLING ON/OFF KINETICS, Enabling DYNAMIC EFFECTS BASED ON FAST NEGATIVE FEEDBACK, AND MODULATING NOISE RESISTANCE AND ULTRASENSITIVITY. SYNTHETIC BIOLOGY AIMS TO CAPITALIZE ON THESE SOPHISTICATED CONTROL SYSTEMS BY TRANSFERRING PATHWAYS FROM ONE SPECIES TO ANOTHER, BY ENGINEERING AND REROUTING EXISTING PATHWAYS, OR EVEN BY DESIGNING NEW PATHWAYS ALTOGETHER. THESE ENGINEERED SYSTEMS HAVE THE POTENTIAL TO FIND APPLICATIONS IN MEDICINE AND BIOTECHNOLOGY, AS WELL AS CONTRIBUTING TO THE UNDERSTANDING OF THE PRINCIPLES THAT UNDERLY NATURAL SIGNALING BY OFFERING A SYNTHETIC PLATFORM FOR DISSECTING THE INTERPLAY AND FUNCTION OF INDIVIDUAL SIGNALING PROTEINS.

THE DEVELOPMENT OF NEW PLATFORMS FOR PROTEIN CIRCUIT DESIGN AIDS IN THESE EFFORTS, AND IN PARTICULAR MODULAR SYSTEMS THAT ARE COMPATIBLE WITH PREVIOUS DESIGNS MAY ENABLE THE CONSTRUCTION OF HIGHER ORDER CIRCUITS.

IN CONTRAST TO SINGLE-STEP INPUT–OUTPUT SYSTEMS, SUCH AS GENE SWITCHES BASED ON THE TETR PROTEIN FAMILY, THE FOCUS OF ENGINEERING PROTEIN CIRCUITS LIES ON SWITCHEABLE PROTEIN–PROTEIN INTERACTIONS (PPIs) THAT REQUIRE POST-TRANSLATIONAL MODIFICATIONS (PTMs). MOST CURRENT DESIGNS FOR SYNTHETIC PTMs RELY ON PROTEIN CLEAVAGE WITH VARIOUS PROTEASES. CONTROLLING PROTEIN FUNCTION BY PROTEOLYTIC CLEAVAGE ENABLES THE CONSTRUCTION OF A VERY DIVERSE SET OF SYSTEMS FOR DIFFERENT APPLICATIONS. THE DEVELOPMENT OF NEW PLATFORMS FOR PROTEIN CIRCUIT DESIGN AIDS IN THESE EFFORTS, AND IN PARTICULAR MODULAR SYSTEMS THAT ARE COMPATIBLE WITH PREVIOUS DESIGNS MAY ENABLE THE CONSTRUCTION OF HIGHER ORDER CIRCUITS.

THE DESIGN IS INFORMED BY THE FUNCTION OF NATIVE BACTERIAL TWO-COMPONENT SYSTEMS (TCSSs), WHICH RELY ON THE TRANS-AUTOPHOSPHORYLATION OF A HISTIDINE RESIDUE IN THE HK TO FORM PHOSPHOHISTIDINE. THE PHOSPHOHISTIDINE SERVES AS SUBSTRATE FOR THE SUBSEQUENT PHOSPHOTRANSFER TO AN ASPARTATE RESIDUE IN THE RR TO FORM PHOSPHOASPARTATE. AS A STARTING POINT, WE CHOSE THE PROTO Typical Homodimeric Escherichia Coli HK DcuS, WHICH SENSES C4-DICARBOXYLATES (E.G. FUMARATE) AND CONTROLS THE RR DcuR33 (Fig. 1a). When these proteins are expressed in mammalian cells, phosphotransfer between DcuS and DcuR is constitutively active. THE CYTOPLASMIC DOMAIN OF DcuS (DcuS203–543; NUMBERING ACCORDING TO UNIPROT35) CONTAINS AN N-TERMINAL PER-ARMN-SIM (PAS) DOMAIN CONNECTED BY A LINKER, WHOM FUNCTION IS INCOMpletely UNDERSTOOD, TO A KINASE DOMAIN CONSISTING OF THE DIMERIZATION HISTIDINE PHOSPHOTRANSFER (DHP) AND CATALYTIC ATP-BINDING (CA) DOMAINS. THE KINASE TRANS-AUTOPHOSPHORYLATES ITS DHP DOMAIN UPON RECEPTOR ACTIVATION. THE PAS DOMAIN AND THE LINKER BETWEEN THE PAS AND KINASE DOMAINS ARE LOCATED AT THE DIMER INTERFACE BETWEEN THE RECEPTOR CHAINS AND PLAY A MAJOR ROLE IN REGULATING KINASE ACTIVITY36-38 (Fig. 1a). OUR FIRST AIM WANTED TO GENERATE N-TERMINAL TRUNCATION MUTANTS TO IDENTIFY MINIMAL DOMAINS OF DcuS THAT ARE NOT ACTIVE WHEN EXPRESSED CYTOSOLICALLY, BUT RETAIN INTACT KINASE DOMAINS (Fig. 1b). WE THEN PLANNED TO FUSE THESE MINIMAL DOMAINS TO AN ANTI-Caffeine HEAVY CHAIN NANOBODY (acVHH) FOR CHEMICALLY INDUCED DIMERIZATION (CID)39,40 (Fig. 1c).

WE HYPOTHESIZED THAT CID OF THE KINASE DOMAIN OF A BACTERIAL HK WOULD TRIGGER TRANS-AUTOPHOSPHORYLATION OF THE HOMODIMER, FOLLOWED BY PHOSPHOTRANSFER TO (AND DIMERIZATION OF) THE CORRESPONDING RR (Fig. 1d). TO TEST SYSTEM FUNCTION, WE FUSED THE RR TO A TRANSACTIVATOR DOMAIN AND MONITORED PHOSPHORYLATION-DEPENDENT RR DIMERIZATION BY MEASURING REPORTER GENE EXPRESSION FROM AN INDUCIBLE PROMOTER, ACTIVATED BY THE BINDING OF THE DIMERIZED RR. THE PHOSPHOTRANSFER IS EXPECTED TO BE SPECIFIC FOR THE ENGINEERED HK/RR PAIR, AS THEY HAVE COEVOLVED FOR SPECIFIC BINDING INTERFACES. THE REGULATORY DOMAIN OF THE RR CONTAINS A CATALYTIC CENTER THAT USES PHOSPHOHISTIDINE AS A SUBSTRATE FOR AUTOPHOSPHORYLATION35. PHOSPHOTRANSFER BETWEEN HK AND RR THEREFORE CONSISTS OF TWO AUTOPHOSPHORYLATION EVENTS, WHICH REDUCE THE LIKELIHOOD OF OFF-TARGET PHOSPHORYLATION. IT SHOULD BE NOTED THAT MAMMALIAN ORTHOLOGUES TO BACTERIAL TCSs HAVE NOT BEEN IDENTIFIED, AND HK/RR PAIRS ARE NOT PRESENT IN THE ANIMAL KINGDOM. THEREFORE, WE REFER TO THE ENGINEERED KINASE AS THE ORTHOGONAL RECEPTOR KINASE (ORK; FUSION OF acVHH TO THE TRUNCATION MUTANT OF DcuS; Fig. 1e) AND THE RR AS THE ORTHOGONAL GENE EXPRESSION REGULATOR (OGR; FUSION OF VP16 TO DcuR; Fig. 1e), AND WE DESIGNATE THE FULL SYSTEM (ORK/OGR/REPORTER) AS THE PHOSPHOREGULATED ORTHOGONAL SIGNAL TRANSDUCTION (POST) SYSTEM.

DEFINING THE DCUS MINIMAL KINASE DOMAIN. WE COMPARED THE KINASE ACTIVITY OF SEVERAL N-TERMINALLY TRUNCATED VARIANTS OF THE DcuS INTRACELLULAR DOMAIN (Fig. 2a, b). THE DcuS N-TERMINAL TRUNCATION VARIANTS WERE CYTOSOLICALLY EXPRESSED IN HEK-293T CELLS, TOGETHER WITH THE RESPONSE REGULATOR DcuR FUSED TO THE TRANSACTIVATOR VP16 (ORTHOGONAL GENE EXPRESSION REGULATOR; OGR;
The design of ORK and OGR proteins. The regulatory domain catalyzes the transfer of the phosphoryl group from phosphohistidine to one of its own aspartate residue (2) kinase trans autophosphorylation and (3) phosphotransfer to an engineered effector protein, such as the orthogonal gene expression regulator (OGR).

The effector dimerizes upon phosphotransfer to perform its function, i.e., DNA binding (5), leading to (6) activation of gene expression.

Fig. 1 Design of the phosphoregulated orthogonal signal transduction (POST) system. a Mechanism of the native signal cascade activated by the bacterial histidine kinase DcuS. (1) Upon activation, (2) the homodimeric histidine kinase DcuS trans-autophosphorylates a histidine residue in its kinase domain, consisting of the dimerization and histidine-containing phosphotransfer (DHP) domain and the catalytic and ATP-binding (CA) domain. (3) This phosphohistidine is the substrate for the response regulator DcuR that catalyzes the autophosphorylation of an aspartate residue in its dimerization domain. (4) Phosphorylated DcuR dimerizes and (5) binds response elements in its operator site to control (6) gene expression. b Linear schematic depiction of the N-terminal truncation constructs. The constructs start with the amino acid number indicated to the left and end with amino acid number 543 (numbered according to Uniprot ID: P0AEC8). c The camelid heavy chain nanobody acVHH dimerizes in the presence of caffeine. d Schematic illustration of the POST system design. (1) Caffeine induces dimerization of acVHH domains in engineered orthogonal receptor kinase (ORK), causing (2) kinase trans autophosphorylation and (3) phosphotransfer to an engineered effector protein, such as the orthogonal gene expression regulator (OGR). (4) The effector dimerizes upon phosphotransfer to perform its function, i.e., DNA binding (5), leading to (6) activation of gene expression. e Detailed design of ORK and OGR proteins. The regulatory domain catalyzes the transfer of the phosphoryl group from phosphohistidine to one of its own aspartate residues and subsequently dimerizes.

Fig. 2 Reducing DcuR autodimerization by generating truncation mutants. a Schematic of DcuS truncation mutants. b Reporter gene expression in response to expression of different DcuS N-terminal truncation variants together with DcuR-VP16. The bar chart shows the mean ± s.d. of n = 3 biologically independent samples overlaid with a scatter dot plot of the original data points, measured 24 h after transfection, and the results are representative of three independent experiments. Source data are provided as a Source Data file.

To quantify DcuS activity, we designed a reporter plasmid containing DcuR-specific-binding sites (DcuR response elements; RE) upstream of a minimal promoter that drives expression of the reporter protein, secreted alkaline phosphatase (SEAP; DcuR-RE\textsubscript{DK}-PhCMV\textsubscript{min}-SEAP-pA; Fig. 1d). The complete intracellular domain of DcuS (P\textsubscript{SV40-DcuS\textsubscript{203–543}}) led to high reporter gene expression. Variants lacking the PAS domain (P\textsubscript{SV40-DcuS\textsubscript{324–543}}pA, P\textsubscript{SV40-DcuS\textsubscript{330–543}}pA, P\textsubscript{SV40-DcuS\textsubscript{334–543}}pA, P\textsubscript{SV40-DcuS\textsubscript{340–543}}pA) had reduced activity (Fig. 2b). The basal reporter activity can likely be attributed to a combination of promoter leakiness, residual DcuS dimerization, and uninduced DcuR binding to its response elements.
parameters may be improved in future studies by identifying and optimizing minimal promoters and response elements, and by mutagenesis of DcuR and DcuS.

Generating caffeine-inducible HKs. We excluded the constitutively active variant DcuS203–543 from further analysis and examined whether CiD could restore the activity of DcuS324–543, DcuS330–543, DcuS334–543, and DcuS340–543. We fused these truncated variants via a (GGGGS)-linker to the nanobody acVHH, which dimerizes in the presence of caffeine39,40. The N-terminal (PSV40-acVHH-DcuSx-543-pA) and C-terminal (PSV40-DcuSx-543-acVHH-pA) fusions of acVHH to truncated DcuS intracellular domain variants expressed together with the OGR DcuR-VP16 in HEK-293T cells induced reporter gene expression in response to caffeine. Cells transfected with the reporter plasmid alone or with the reporter plasmid and the expression plasmid for the OGR but not for ORKs did not respond to caffeine (Fig. 3a, b). The POST system containing ORKs with DcuS324–543 or DcuS330–543 had the highest signal-to-noise ratio of induced vs. noninduced SEAP expression under the tested conditions; these constructs were used for further analysis.

We also fused the dimerization domains FRB/FKBP via a (GGGGS)-linker to the identified minimal domain of DcuS (PSV40-FRB-DcuS324-543-pA; PSV40-FKBP-DcuS324-543-pA) to test if the POST design strategy can be extended to other CiD systems. FRB/FKBP ORKs together with the OGR robustly induced SEAP expression from the reporter plasmid. We confirmed that FRB ORKs alone, used as the negative control, did not respond to rapamycin (Supplementary Fig. 1).

While the signal-to-noise ratio and sensitivity of the system are not superior to those of endogenous signaling cascades40, these results indicate the successful implementation of an inducible phosphorylation-dependent protein switch based on the phosphorylation of histidine and aspartate residues.

Using POST as a dimerization relay system. Next, we sought to use the phosphorylation-dependent dimerization mechanism of DcuR to establish OGRs as a modular phosphorylation-dependent effector. A phosphoregulated relay system of protein dimerization can serve as a basic building block that is compatible with other synthetic biology systems, such as split transcription factors (Fig. 4a). Phosphorylated DcuR forms homodimers and...
containing the full linker domain (DcuS324–543)

acVHH-DcuS324-543-pA

the strong transactivation domain VPR (PhCMVmin-SEAP-pA).

Phosphorylation by ORKs controls the dimerization of TetR-DcuR and DcuR-VPR and only dimerized TetR-DcuR/DcuR-VPR will induce reporter gene expression from a TetR operator. ORKs, although the mechanism might be complex. The observed effect is likely influenced by the phosphatase activity of the HK and may be affected by asymmetric phosphotransfer (i.e., one phosphorylated monomer per dimer at a time, resulting in one active kinase domain per dimer) and symmetric phosphatase activity, which is a well-investigated effect in several native HKs.

The increase of the Hill coefficient of the dual ORK POST system ranged between 0.8 and 1.5, whereas the Hill coefficients of single-ORK POST ranged between 0.4 and 0.8. Hill coefficients <1 indicate negative cooperativity of single acVHH ORKs, although the mechanism might be complex. The observed effect is likely influenced by the phosphatase activity of the HK and may be affected by asymmetric phosphotransfer (i.e., one phosphorylated monomer per dimer at a time, resulting in one active kinase domain per dimer) and symmetric phosphatase activity, which is a well-investigated effect in several native HKs.

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To test how rapidly the engineered signaling pathway can respond to caffeine stimulation, we quantified SEAP production every 2 h from cells transfected with the POST system containing the dual-ORK and the DcuR-VP16 OGR. Starting at 4 h after induction, reporter gene expression in the induced group was significantly upregulated compared to the non-induced control (Supplementary Fig. 3).

**Probing for orthogonality.** To look for possible influences of cellular contexts, we tested the POST system ($P_{SV40\text{-}acVHH-}$DcuS$_{324-543}$–VP16–PhCMVmin–SEAP–P$_{CMV\text{-}min}$) in HeLa cells, bone marrow-derived immortalized mesenchymal stem/stromal cells (hMSC-TERT), and human-induced pluripotent stem cells (hiPSCs). The POST system was functional in all tested cell types and performed with a uniform dynamic range of induced vs. noninduced SEAP expression in response to caffeine (Supplementary Fig. 4a–c). Next, we examined the effect of strong activation of endogenous signaling pathways on the DcuR-operon containing reporter plasmid in cells expressing the complete POST system (ORK/OGR/reporter). KCl-induced cell depolarization and induction with TNFα led to noticeable reporter gene expression, but little or no effect was observed upon induction with forskolin, FGF, or IL-6 (Supplementary Fig. 4d). We speculate that the $P_{CMV\text{-}min}$ promoter used in the DcuR reporter displays increased leakiness in KCl-stimulated or TNFα-stimulated cells or that the DcuR reporter contains cryptic transcription factor-binding sites for the respective endogenous transcription factors. Similar strategies as used in previous studies to reduce leaky $P_{CMV\text{-}min}$ expression, by removing cryptic-binding sites and reducing the likelihood of long-range effects of enhancer elements, might reduce this effect. Finally, we investigated whether POST is orthogonal to a number of major endogenous signaling pathways. In synthetic biology, the term orthogonality is used to describe the situation where minimal interactions occur between engineered and native components of a biological system and for the POST system, we primarily use this definition to indicate the low probability of POST-induced changes in endogenous signaling cascades. We cotransfected HEK-293T cells with the ORK/OGR ($P_{SV40\text{-}acVHH-}$DcuS$_{324-543}$–acVHH–PhCMVmin–SEAP–P$_{CMV\text{-}min}$) expression plasmids but without the OGR reporter. Instead we used endogenous pathway-specific reporter plasmids for the NF-κB, CAMP, NFAT, STAT3, and MAPK pathways and measured reporter gene expression in response to caffeine-induced ORK/OGR activation compared to known activators of respective pathways. ORK/OGR activity had no observable effect on any of the tested pathways, indicating orthogonality (Supplementary Fig. 4e–i). Further, expression or activation of the POST system had no detectable effect on the proliferation of HEK-293T cells (Supplementary Fig. 5).

**POST based on EnvZ/OmpR and NarL/NarX.** We tested if the strategy of generating POST systems by identifying minimal domains of a bacterial HK and subsequent nanobody fusion for induced dimerization can be generalized to other HKs. We focused on the well-investigated TCSs NarX/NarL, and EnvZ/OmpR. Both TCSs have similar receptor architecture to DcuS/DcuR, but have a HAMP (present in HKs, adenylate cyclases, methyl accepting proteins, and phosphatases) domain instead of a PAS domain. For EnvZ, we based the design on a reported crystal structure of the intracellular domain (protein data bank ID: 4CTI) and generated a truncation mutant without HAMP, keeping only a conserved motif, involving the amino acids DRT, that is important for EnvZ activation. We tested this construct ($P_{40\text{-}EnvZ}$–$P_{322-450}$–acVHH–PhCMVmin–SEAP–P$_{CMV\text{-}min}$; numbering according to the full-length EnvZ; UniProt ID: P0A8J4) in combination with a VP16 fusion of the response regulator OmpR ($P_{40\text{-}OmpR}$; $P_{40\text{-}OmpR}$–VP16–PhCMVmin–SEAP–P$_{CMV\text{-}min}$; Fig. 7a) and a reporter plasmid with OmpR response elements (OmpR–$P_{CMV\text{-}min}$–SEAP–P$_{CMV\text{-}min}$), and observed very high basal gene expression (Supplementary Fig. 6). We speculated that the shortening of the connector domain between the DHp and CA domains could reduce the autophosphorylation activity, even in formed EnvZ dimer...
complexes. In this setup, activity might be restored with acVHH dimerization by the formation of functional tetramers (dimers of dimers, or higher-order oligomers) that could trans-phosphorylate each other, or by breaking the OFF-conformation of mutated EnvZ dimers. However, both proposed mechanisms would require an acVHH fusion with a very short or rigid linker domain (Fig. 7a). We generated an EnvZ mutant with short-side-chain amino acids (AS) connecting the DHp and the CA domain instead of the connector domain (GQEMP; EnvZ291–295) to provide a minimum of flexibility and rotational freedom, but reducing its length (Psv40-EnvZ232-450; GQEMP:AS-pA). Expression of this EnvZ mutant together with the

Fig. 6 Modulating cooperativity. a Schematic of the dual-ORK with an N-terminal and C-terminal acVHH fusion. Synergistic activation of ORK dimers by acVHH nanobodies or ORK oligomerization both have the potential to promote positive cooperativity. Both proposed mechanisms for the observed increase in cooperative behavior remain speculative at this point. b The dose-response curve of the dual-ORK. The plot shows the mean ± s.d. of n = 3 biologically independent samples, measured at 24 h after induction, and the results are representative of three independent experiments. Only error bars larger than the symbol size are displayed. Source data are provided as a Source Data file.

Fig. 7 EnvZ/OmpR and NarX/NarL POST. a Schematic of the engineered ORK/OGR proteins based on EnvZ/OmpR. In the ORK, short linkers consisting only of the two amino acids AS were used to reduce interdomain flexibility between the acVHH and the kinase domain and between the CA and DHp domains by replacing the native GQEMP linker. We hypothesize that this change results in inactive dimers. b Induction of EnvZ/OmpR POST with caffeine. The EnvZ mutant without acVHH (EnvZ232–450,GQEMP,AS) was included as a negative control. c Schematic of the engineered ORK/OGR proteins based on NarX/NarL. d Induction of NarX/NarL POST with caffeine. The NarX truncations without acVHH were included as negative controls. The bar charts show the mean ± s.d. of n = 3 biologically independent samples overlaid with a scatter dot plot of the original data points, measured at 24 h after induction, and the results are representative of three independent experiments. Source data are provided as a Source Data file.
OmpR-OGR resulted in greatly reduced activity (Fig. 7b). We fused the acVHH with only a minimal (AS)-linker to EnvZ232–450, GQEM.PAS to generate an EnvZ-OR (P env40–acVHH–EnvZ232–450; GQEM.PAS). Expression of the EnvZ-OR and the EnvZ-OGR resulted in caffeine-inducible gene expression from the EnvZ reporter plasmid (Fig. 7b). These results demonstrate the functionality of an EnvZ/OmpR-based POST system and add shortening of the connector domain between the DHp and CA domains to the repertoire of strategies for HK autoactivity reduction.

NarX has an additional domain with largely unknown function between the HAMP and kinase domains. We tested two truncation mutants devoid of the HAMP domain (Psv40–NarX233–598–P; P sv40–NarX263–598–P; NarX numbering according to the full-length NarX; UniProt ID: P0AFA2) in combination with a NarL-OR (P sv40–NarL-VP16–P) and a reporter plasmid with NarL response elements34 (NarL-RE2x-PhCMVmin-SEAP-pA). Neither truncation mutant was constitutively active. We chose the shorter construct to generate a NarL-OR by N-terminal fusion to acVHH. Expression of the NarX-OR (P sv40–acVHH–NarX233–598–P) and NarX OGR resulted in caffeine-inducible gene expression from the NarL reporter plasmid, confirming that the NarL/NarX-based POST system is functional (Fig. 7d).

**Discussion**

This work provides a proof-of-concept that engineered components derived from bacterial HKs and RRs can serve as modular and orthogonal signaling units in mammalian cells. In addition to employing the POST system to drive transgene expression from a DcuR-responsive synthetic operon, we show that DcuR phosphorylation causes protein dimerization. We propose that such a relay system of protein dimerization can serve as a basic building block for generating protein circuits, as demonstrated for the two-hybrid OGR design.

POST also provides a complementary method to study histidine kinase activation in the absence of the pathway interactions that can be encountered in bacteria.55 Our results confirm the importance of the linker domain for signal transduction between the PAS and kinase domains of DcuS, in accordance with research on similar linker domains in other bacterial HKs86,57, and suggest that the linker domain has a role beyond purely mechanical signal transduction. Reducing the linker length reduced the HK activity in all constructs, including ORKs activated by an unnatural C-terminal dimerization mechanism. These results show that even in the absence of any N-terminal sensor domain, DcuS-signaling activity in mammalian cells still requires N-terminal amino acids of the linker domain.

The presented ORKs, OGRs, and two-hybrid OGRs were developed by isolating and repurposing functional elements of the bacterial HKs and RRs to serve a predictable biological function in a non-native context. Most analyses were performed with DcuS/DcuR-derived constructs, but the functionality of EnvZ/OmpR-based and NarX/NarL-based systems suggests that the approach is generalizable. These results complement and extend other recent research20. POST is likely complementary to most other designs of gene or protein circuits. As exemplified with TetR, POST can be integrated into existing systems based on bacterial transcription factors. Similarly, it should be complementary to protease-based approaches for protein circuit design. Exchanging the acVHH for other modules from the synthetic biology toolbox is expected to yield systems that respond to other inducers. Our findings represent a proof-of-concept of POST as a basic building block for modular phosphoregulated protein effectors, which may be the basis for controlling increasingly complex systems in mammalian synthetic biology.

POST may therefore contribute to the generation of synthetic protein circuits that approach the versatility of native signal transduction pathways and enable tightly regulated behavior of engineered cells with elaborate functions.

**Methods**

**Cell culture and transfection.** Human embryonic kidney cells (HEK-293T, German Collection of Microorganisms and Cell Cultures (DSMZ): ACC 635), HeLa cells (HeLa, ATCC: CCL-2), and bone marrow-derived immortalized mesenchymal stem/dermal cells (hMSC-TERT) cells59 were cultivated in Dulbecco's modified Eagle's medium (DMEM, 31966-021, Life Technologies Europe BV) supplemented with 10% (v/v) fetal bovine serum (FBS, F7524, lot BCB0318V, Sigma-Aldrich) and 1% (v/v) streptomycin/penicillin (L0022, Biowest) at 37 °C in a humidified atmosphere with 5% CO2 in air. For in vitro experiments, 1.4 × 10⁶ cells in 12 mL DMEM were seeded into 96-well plates (167008, Thermo Fisher Scientific) 24 h before transfection. The transfection mix was prepared, vortexed, incubated for 20 min and then added to the inner 60 wells of the cell culture plate. Medium with the transfection mix was exchanged after 15 h for 125 µL/well medium with different concentrations of the appropriate inducer or no inducer as a negative control. Supernatant for quantification of the secreted reporter protein SEAP was taken after 24 h. The supernatant was taken from separate wells at each time point in the time course experiments.

**hiPSC preparation, culture, and transfection.** hiPSCs were derived in a previous study59. They originate from the adipose tissue of a 50-year-old patient using an mRNA-based reprogramming technique under feeder-free conditions. hiPSCs were cultivated in mTeSR medium (85850, Stem Cell Technologies) supplemented with 100 ng/mL basic FGF (aCSF, 08986, Stem Cell Technologies) and 100 µg/mL Flt3L (08989, Stem Cell Technologies). hiPSCs were transfected with 1–2 µg of plasmid DNA mixed with 50 µL DMEM without any supplements and 600 µL polyethyleneimine (24765-1, Polysciences Inc.). The exact amounts are given in Supplementary Table 4. The transfection mix was prepared, vortexed, incubated for 20 min and then added to the inner 60 wells of the cell culture plate. Medium with the transfection mix was exchanged after 15 h for 125 µL/well medium with different concentrations of the appropriate inducer or no inducer as a negative control. Supernatant for quantification of the secreted reporter protein SEAP was taken after 24 h. The supernatant was taken from separate wells at each time point in the time course experiments.

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**Inducer preparation.** All stock solutions were prepared at 1000× concentration. 100 mM caffeine (108160100, ACROS Organics) was prepared as an aqueous solution and was stored at 4 °C for up to 4 weeks. The solution was warmed to 37 °C before experiments to dissolve precipitates. For dose–response experiments, working solutions were prepared by serial dilution in DMEM with 10% (v/v) FBS (F7524, Sigma-Aldrich) and 1% (v/v) streptomycin/penicillin (L0022, Biowest), prewarmed to 37 °C. 10 µg/mL recombinant human TNFα (300-01A, Peprotech), Aquous solutions of 10 µg/mL recombinant human FGF-basic (154 a.a.) (100-14B, Peprotech) and 10 µg/mL IL-6 (200–06, PeproTech) were prepared and stored at –20 °C prior to use. A solution of 10 mM forskolin (F9197, Sigma-Aldrich) in ethanol was prepared and stored at –20 °C prior to use. An aqueous solution of 4 M KCl (P9914, Sigma-Aldrich) was prepared and stored at 4 °C. A solution of 10 mM rapamycin (AC-CN2-0025-C100, AdiopoGen) in DMSO was prepared and stored in aliquots at –80 °C.

**Plasmid preparation.** Plasmids were generated by means of molecular cloning with the aid of restriction endonucleases (New England Biolabs, HF enzymes were used whenever possible), and ligations were done with T4 DNA ligase (M0202L, New England Biolabs) prior to ligation. PCRs were performed with Q5 high-fidelity DNA polymerase (M0491L, New England Biolabs).

**Oligo annealing.** For reporter plasmids, oligos were designed to contain operator sequences of DNA ligase (M0202L, New England Biolabs). Plasmid backbones were dephosphorylated with Quick CIP phosphatase (50508L, New England Biolabs) prior to ligation. PCRs were performed with Q5 high-fidelity DNA polymerase (M0491L, New England Biolabs). Details of all restriction enzymes and primers used for each plasmid are presented in Supplementary Table 1. The plasmids were amplified with XL10 gold chemically competent cells (C2992, New England Biolabs) and DNA was purified with a purification miniprep kit (D4054, Zymo Research).

**PCR amplification of bacterial genes.** The genes for DcuS and NarX intracellular domain, DcuR, NarL, and OmpR were directly PCR-amplified from XL10 gold...
chemically competent cells (C2992, NEB). EnvZ intracellular domain was amplified from BL21 (C3010, NEB; BL21(DE3)pLYS-S-TIR) cells. 0.2 μL of bacteria were added to the PCR mix, based on the assumption that the initial denaturation step of 98°C for 30 s is sufficient for bacterial lysis.

**SEAP measurements.** SEAP activity (U/L) in cell culture supernatants was quantified by kinetic measurements (1 measurement/min for 30 min at 37°C) of absorbance increase due to phosphatase-mediated hydrolysis of para-nitrophenyl phosphate (pNPP). 4–80 μL of supernatant was adjusted with water to a final volume of 90 μL, heat-inactivated (30 min at 65°C, then centrifuged for 1 min, 1000×g), and mixed in a 96-well dish with 100 μL of 2 × SEAP buffer (20 mM homoarginate, 1 mM MgCl₂, 21% (v/v) diethanolamine, pH 9.8) and 20 μL of substrate solution containing 20 mM pNPP (Acros Organics BVBA). Absorbance was measured at 405 nm with a Tecan Genios PRO multistate reader (Tecan AG).

**Sequence upload to GenBank.** GenBank files and a multi-FASTA file were generated with Benchling. GenBank (gb) files were converted to feature tables with GB2Seq61 for sequence submission with BankIt.

**Data availability**

All data is available in the main text or the supplementary information materials. Sequence data of original plasmids are deposited in GenBank (MT267299-MT267334) and Benchling. Accession codes are provided in Supplementary Table 1. Source Data are provided with this paper.

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**Author contributions**

L.S. developed the project. L.S., M.S., M.M, A.B., P.S. designed and performed the experiments. L.S., M.S., M.F. analyzed the results. L.S., M.F. wrote the manuscript.

**Competing interests**

The authors declare no competing interests.

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