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Reporting Summary

Nature Research wishes to improve the reproducibility of the work that we publish. This form provides structure for consistency and transparency in reporting. For further information on Nature Research policies, see our Editorial Policies and the Editorial Policy Checklist.

Statistics

For all statistical analyses, confirm that the following items are present in the figure legend, table legend, main text, or Methods section.

n/a	Confirmed
☐	☒ The exact sample size (*n*) for each experimental group/condition, given as a discrete number and unit of measurement
☐	☒ A statement on whether measurements were taken from distinct samples or whether the same sample was measured repeatedly
☐	☒ The statistical test(s) used AND whether they are one- or two-sided *Only common tests should be described solely by name; describe more complex techniques in the Methods section.*
☒	☐ A description of all covariates tested
☐	☒ A description of any assumptions or corrections, such as tests of normality and adjustment for multiple comparisons
☐	☒ A full description of the statistical parameters including central tendency (e.g. means) or other basic estimates (e.g. regression coefficient) AND variation (e.g. standard deviation) or associated estimates of uncertainty (e.g. confidence intervals)
☐	☒ For null hypothesis testing, the test statistic (e.g. *F*, *t*, *r*) with confidence intervals, effect sizes, degrees of freedom and *P* value noted *Give P values as exact values whenever suitable.*
☒	☐ For Bayesian analysis, information on the choice of priors and Markov chain Monte Carlo settings
☒	☐ For hierarchical and complex designs, identification of the appropriate level for tests and full reporting of outcomes
☒	☐ Estimates of effect sizes (e.g. Cohen's *d*, Pearson's *r*), indicating how they were calculated

Our web collection on statistics for biologists contains articles on many of the points above.

Software and code

Policy information about availability of computer code

Data collection	For UV-vis analysis, UV WinLab (ver. 2.85.04) Software was used for acquisition. For experiments measuring yeast two hybrid bgal signals, MARS Data Analysis software (BMG labtech) was used for acquisition. For absorbance measurement for phage ELISAs, Gen5 software was used for acquisition. For size exclusion chromatography experiments, BioLogic DuoFlow Version 5.0 was used. For fluorescence measurements, MARS Data Analysis software 3.33 (BMG labtech) was used. For ITC, VPViewer2000 Ver: 1.30.0 was used for acquiring data. For live-cell imaging and photomask experiments, Nikon NIS-Elements AR5.11.03 was used. For quantification of reporter gene expression (Figs. 2e,f; 3e,f; 4b,c; 5g; Extended Fig.1d; Supplementary Fig. 15b,c; 16; 22a; 26 and 27) , the software used for acquisition in the plate reader is MikroWin (version5.18, Berthold) and MARS Data Analysis (version 3.31, BMG Labtech). For RT-qPCR (Supplementary Fig.17, 20 and 21b), the software used for acquisition is Applied Biosystems® Real-Time PCR Software and qPCRsoft4.1(Analytik-Jena).
Data analysis	All data analysis, graph display, and statistics of reporter gene expression were performed with GraphPad (version 3.31) software. The raw data of RT-qPCR experiments were analyzed by the LinRegPCR (2020.2) program. The analysis of DNA sequences and the construction of plasmid maps were performed with Geneious(9.1.8) and Benchling. The composition of microscopy Figures was performed with ImageJ (ver. 1.53a) and OMERO. Mitochondrial-to-cytoplasmic ratio quantification was done with ImageJ (ver. 1.53a). For the analysis of ITC data, NITPIC (ver. 1.2.7) and SEDPHAT/ITCsy (ver. 1.0a) were used.

For manuscripts utilizing custom algorithms or software that are central to the research but not yet described in published literature, software must be made available to editors and reviewers. We strongly encourage code deposition in a community repository (e.g. GitHub). See the Nature Research guidelines for submitting code & software for further information.

Data

Policy information about availability of data

All manuscripts must include a data availability statement. This statement should provide the following information, where applicable:

- Accession codes, unique identifiers, or web links for publicly available datasets
- A list of figures that have associated raw data
- A description of any restrictions on data availability

Source data for the figures are available (Source Data .xls files). The raw and associated data that support the findings of this study is available from the corresponding author upon request. The plasmids used in the experiments of this study will be available on Addgene. The plasmid maps at the public repository JBEI-ICE (https://public-registry.jbei.org).

Field-specific reporting

Please select the one below that is the best fit for your research. If you are not sure, read the appropriate sections before making your selection.

☒ Life sciences ☐ Behavioural & social sciences ☐ Ecological, evolutionary & environmental sciences

For a reference copy of the document with all sections, see nature.com/documents/nr-reporting-summary-flat.pdf

Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

Sample size	The sample size was determined based on our experience of optogenetic experimental setups, and the exact sizes(n) are provided in each figure legends. (references: Nat Methods. 2020 Jul;17(7):717-725. and Nat Biotechnol. 2022 Feb;40(2):262-272.) Screening of hit clones using phage ELISA (Fig. 1c,d) and yeast two hybrid (Fig. 1f), each single clone was inoculated and either screened for binding/beta-galactosidase expression. One replicate (n=1) was performed as this was a screening process to search for mutants that were worth carrying forward with in vitro characterization. Isothermal titration calorimetry (Fig. 1g-i, Fig. S5a-c) and size exclusion chromatography (Fig. S4) for the validation of in vitro binding as well as absorbance measurements of Amg2 were performed once for each BAm binding protein. Yeast two hybrid experiments for validation of BAm binders were done with at least four cultures with each light conditions, consistent with previous optogenetic tools tested in yeast two hybrid settings. see ref. 4 (Hallet, R. A. et al.) in the main text. Measurement of Amg2 fluorescence (Fig. S6a) was done four times on the same sample and averaged. Amg2 fluorescence experiments for measuring binding affinity of BAmRed binding proteins (Fig. S6b-d) were performed in quadruplicate. Measurement of Amg2 dark reversion rates in vitro was performed once (Figure S18). Gene expression experiments where reporters (absorbance of SEAP activities and luminescence of FLuc or GLuc) were measured in CHO-K1 cells. Three distinct wells were taken for each light condition and/or time point. (Fig. 2e,f; 3e,f; 4b,c; 5g; Extended Fig.1d; Supplementary Fig. 15b,c; 16; 22a; 26 and 27) Transcript/mRNA quantifications (Supplementary Fig.17, 21 and 22b): for each illumination/time condition, cell samples were taken, RNA extracted and cDNA generated. For each sample, three technical replicates were used for the quantification of each transcript (Amg2, BAms, SEAP, and GAPDH). Fluorescence microscopy determinations in HEK293T cells (Figs. 2,3,4 and Supplementary Fig. S7-14, S19, 20, 24, 25): 2-4 independent experiments for each construct combination and illumination condition were used for microscopy observation. Images (n shown in the figure legends) were taken with a Nikon microscope for each condition. Representative images are shown. Fluorescence microscopy determinations in photomask (Fig. 4g and Supplementary Fig. 23): 2-3 independent experiments for each combination and illumination condition were used for microscopy observation. Images were taken with a Nikon microscope for each condition. Representative images are shown.
Data exclusions	Outliers in MCR measurements were excluded, but shown in the box plots. Otherwise, no data was excluded.
Replication	All cellular experimental data shown was produced experimentally in at least two independent experiments (different biological material, plasmids, cells). Data shown are representative. All attempts at replication were successful. In vitro experiments involving purified Amg2 protein and screening binding proteins were performed once.
Randomization	Not relevant to our study as the operator cannot influence the outcome of the measurement.
Blinding	No blinding was carried out as the experiments were designed to test under different conditions, i.e. illumination treatments, times, transfection batches, etc. For example, different illumination conditions and times cannot be blinded.

Reporting for specific materials, systems and methods

We require information from authors about some types of materials, experimental systems and methods used in many studies. Here, indicate whether each material, system or method listed is relevant to your study. If you are not sure if a list item applies to your research, read the appropriate section before selecting a response.

Materials & experimental systems

n/a	Involved in the study
☒	☐ Antibodies
☐	☒ Eukaryotic cell lines
☒	☐ Palaeontology and archaeology
☒	☐ Animals and other organisms
☒	☐ Human research participants
☒	☐ Clinical data
☒	☐ Dual use research of concern

Methods

n/a	Involved in the study
☒	☐ ChIP-seq
☒	☐ Flow cytometry
☒	☐ MRI-based neuroimaging

Eukaryotic cell lines

Policy information about cell lines

Cell line source(s)	HEK-293T, Human embryonic kidney, epithelial and adherent cells was a gift from Prof. Allison McGuigan. CHO-K1, Chinese hamster ovary, fibroblastoid and adherent cells. Stable cell lines were generate into CHO-K1 cells.
Authentication	Cell line information can find: American Type Culture Collection (ATCC), www.atcc.org German Collection of Microorganisms and Cell Cultures (DSMZ), www.dsmz.de Cell lines were authenticated by ATCC and DSMZ, not by us.
Mycoplasma contamination	All cell lines were tested monthly for mycoplasma contamination. Mycoplasma contamination of HEK293T cells are tested monthly by the Allison McGuigan lab. No mycoplasma contamination was detected.
Commonly misidentified lines (See ICLAC register)	No commonly misidentified cell lines were used in this study.