

Modular engineering of thermoresponsive allosteric proteins

Received: 14 April 2025

Accepted: 14 January 2026

Published online: 12 February 2026



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Thermogenetics enables noninvasive spatiotemporal control over protein activity in living cells and tissues, yet its applications have largely been restricted to transcriptional regulation and membrane recruitment. Here, we present a generalizable strategy for engineering thermosensitive allosteric proteins through the insertion of optimized *Avena sativa* LOV2 domain variants. Applying this approach to a diverse set of structurally and functionally unrelated proteins in *Escherichia coli*, we generated potent, thermoswitchable chimeric variants that can be tightly controlled within narrow temperature ranges (37–41 °C). Extending this strategy to mammalian systems, we engineered CRISPR–Cas genome editors directly modulated by subtle temperature changes within the physiological range. Lastly, we showcase the incorporation of a chemoreceptor domain as an alternative thermosensing module, suggesting thermosensitivity to be a widespread feature in receptor domains. This work expands the toolkit of thermogenetics, providing a blueprint for temperature-dependent control of virtually any protein of interest.

Engineered switchable proteins are widely applied in basic research and biotechnology. They are typically based on naturally occurring proteins that are modified to be activated or deactivated by exogenous stimuli, most commonly light or chemicals, allowing researchers to remotely control various cellular processes. With respect to future biomedical applications, temperature represents a particularly attractive physical cue because of the increased tissue penetration compared to light and the superior spatiotemporal precision compared to chemicals^{1–3}. Efficient approaches for engineering thermoresponsive proteins are lacking, however, and the few existing strategies are mostly limited to the control of gene expression^{4–6}. Recent work used the *Botrytis cinerea* LOV4 (*BcLOV4*) domain for temperature-dependent recruitment of proteins to the plasma membrane^{7,8}. While this method is generally compatible with various different effector proteins, it relies on a large protein domain (*BcLOV4* is approximately 600 amino acids) and is

restricted to effectors, the activity of which depends on plasma membrane localization.

To address these key limitations, we aimed to develop a generalizable approach for thermogenetic control through modular engineering of temperature-dependent protein allostery. By inserting the *Avena sativa* LOV2 (*AsLOV2*) domain from phototropin 1 and improved mutants thereof into effector proteins, we demonstrate the modular engineering of thermoresponsive hybrids. Starting with the bacterial transcription factor AraC as a proof of concept, we achieved potent and tunable activity switching in response to small temperature shifts between 37 °C and 41 °C. We successfully transferred this concept to several additional proteins, including different CRISPR–Cas9 gene editors and transactivators, and showcase the broad applicability of the concept in bacteria and in mammalian cells. Lastly, we extend the method to a second receptor using a glucocorticoid ligand-binding

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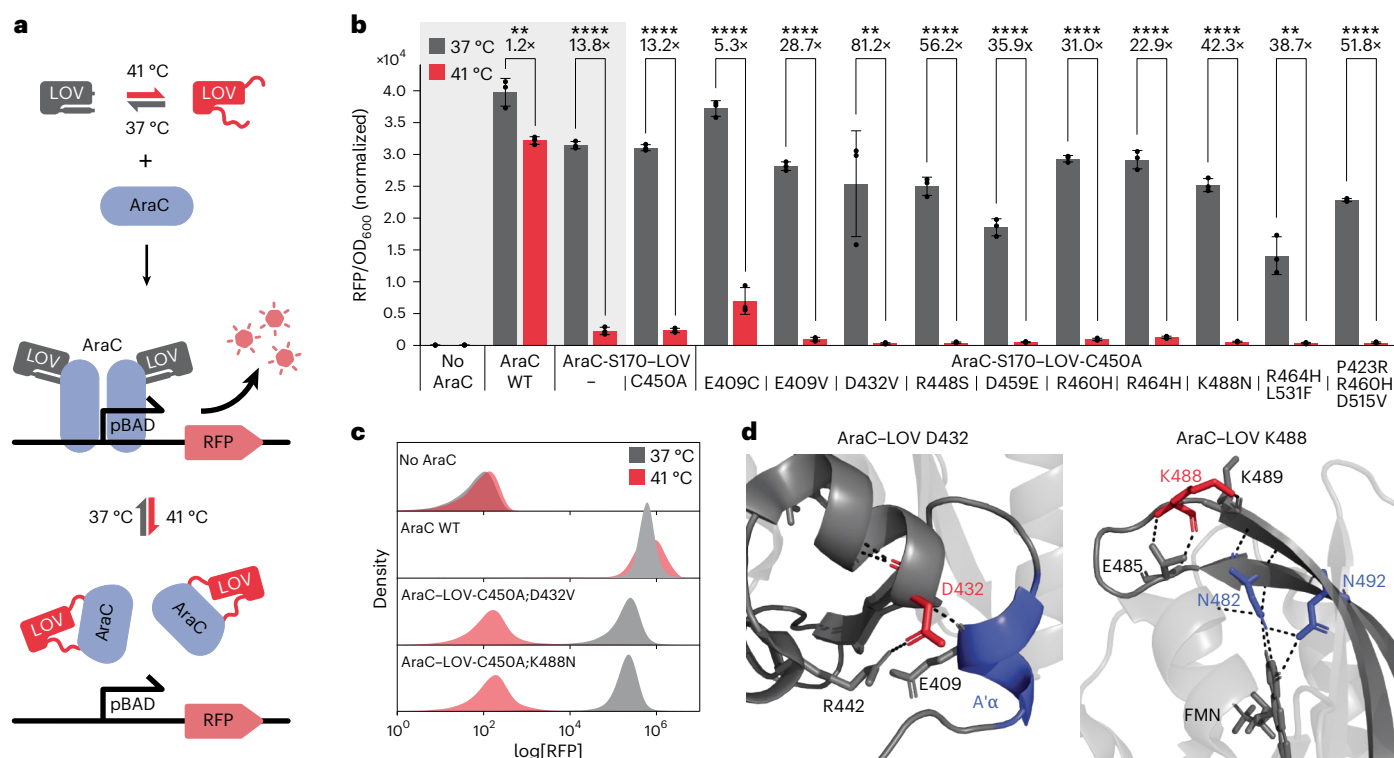


Fig. 1 | Engineering a thermoswitchable *E. coli* transcription factor by AsLOV2 domain insertion. **a**, Assay schematics. **b,c**, *E. coli* transformed with plasmids encoding the indicated AraC-LOV variants and an mRFP reporter were induced with 400 μ M IPTG and 16 mM arabinose and incubated at 37 °C or 41 °C for 16 h followed by measurements of reporter fluorescence (RFP) and culture density (OD₆₀₀) in a plate reader (**b**) or flow cytometry (**c**). **d**, Close-up views of

the AsLOV2 crystal structure (PDB 2V0U). Substituted residues are marked in red and functionally critical elements are marked in blue. Hydrogen bonds are indicated. In **b**, data represent the mean $\pm$ s.d. ($n = 3$ independent experiments). Statistical analysis was conducted using a two-sided Student's *t*-test. ***P* < 0.01 and *****P* < 0.0001. WT, wild-type.

domain and demonstrate the dual regulation of genome editing by the US Food and Drug Administration (FDA)-approved drug cortisol and temperature. Our work overcomes a major bottleneck in thermogenetics by introducing a generalizable approach for engineering thermogenetic switches that can be controlled within the narrow human physiological temperature range and are not limited to specific applications or protein classes.

Results

Engineering a thermoswitchable AraC variant by AsLOV2 insertion

An ideal thermoresponsive module would comprise a compact protein domain that exerts a large conformational rearrangement in response to small changes in temperature. Insertion of such a domain into an effector protein of choice could, thus, couple the activity of the effector to the conformational state of the thermosensor. Intrigued by the relatively low stability of many LOV domains^{7,9,10}, we decided to test the widely applied AsLOV2 for its response to temperature changes. AsLOV2 naturally acts as a photoreceptor, responding to blue-light excitation (450 nm) with conformational changes that result in the reversible undocking of its N-terminal and C-terminal A' α and J α helices, respectively, from the core^{11,12}. Inserting AsLOV2 into effector proteins is a common strategy for engineering light-responsive proteins by reversibly perturbing the effector's structural integrity upon AsLOV2 photoexcitation¹³. To assess whether the same effect can be achieved in the dark by thermal stimulation alone, we used an optogenetic variant of the transcription factor AraC carrying an AsLOV2 insertion after residue S170, which we previously established for coregulating transgene expression by light and arabinose in *E. coli*¹⁴ (Fig. 1a). At 37 °C, both wild-type AraC and the AraC-LOV domain hybrid mediated strong

expression of a pBAD-driven, monomeric red fluorescent protein (RFP) (Supplementary Fig. 1). At 41 °C, however, strongly reduced RFP expression was consistently observed for AraC-LOV, whereas the wild-type AraC control remained unaffected, indicating a potent thermoresponse of AraC-LOV (Fig. 1b and Supplementary Fig. 1). Importantly, cell growth was only slightly affected by the elevated temperature (Supplementary Fig. 1a). Furthermore, substitution of the AsLOV2 C450 residue, which is central to the native AsLOV2 photocycle, to alanine did not affect the temperature switching of the AraC-LOV hybrid and was included in all downstream constructs to avoid cross-activation during short exposure to ambient room light directly before measurements.

To optimize the dynamic range of thermal control, we performed error-prone PCR on LOV-C450A and screened the resulting library of *E. coli* (complexity: $\sim 5 \times 10^6$) for temperature-dependent reporter expression on agar plates (Methods and Supplementary Fig. 2). We identified several AsLOV2 point mutants, including D432V, R448S, K488N and P423R/R460H/D515V, that showed up to 82-fold reduction in activity upon heat induction compared to only a 13.2-fold change observed for the parental hybrid protein (Fig. 1b,c and Supplementary Fig. 3). This improvement was primarily caused by a strongly reduced reporter expression in the 41 °C off state, as indicated by plate reader measurements and flow cytometry. To investigate potential temperature effects on protein stability, we performed western blots of wild-type AraC, AraC-LOV-C450A and two mutants (E409C and R464H/L531F) that showed unexpected bimodal distributions in the cytometry assay. The results revealed strongly reduced protein levels of the AraC-LOV variants compared to wild-type AraC under both conditions (Supplementary Fig. 4). In contrast, increased temperature had no significant effect on the protein levels, suggesting that the

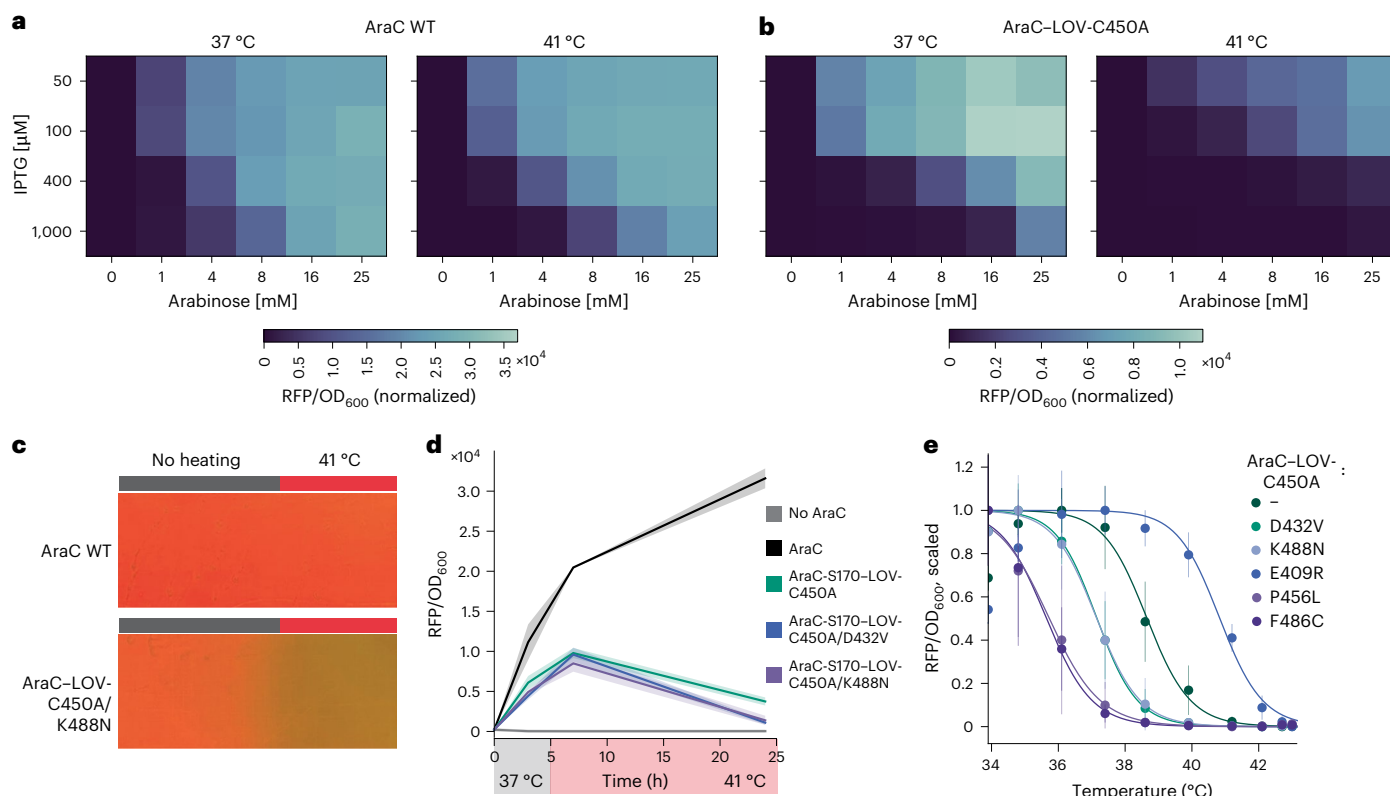


Fig. 2 | Thermosensitive AraC-LOV hybrids are precisely tunable and enable spatiotemporal control of gene expression. **a, b**, *E. coli* carrying a pBAD-mRFP reporter and wild-type AraC (**a**) or AraC-LOV-C450A (**b**) were incubated for 16 h at 37 °C or 41 °C in the presence of inducers at the indicated concentration. Expression of the AraC variants is IPTG inducible, while AraC activity is arabinose dependent. RFP fluorescence and OD₆₀₀ were evaluated in a plate reader. **c**, Photographs of spatial gene expression control. AraC and AraC-LOV reporter strains were plated on agar and one half of the tray was heated while the other half was kept at room temperature for 16 h. **d**, *E. coli* containing a pBAD-mRFP

reporter and expressing the indicated AraC variant or a dummy control protein were incubated at 37 °C. After 5 h, the temperature was increased to 41 °C followed by another 19 h of incubation. OD₆₀₀ and RFP expression (normalized to OD) were periodically assessed in a plate reader. **e**, Temperature response profiles of AraC-LOV variants. Samples were prepared as in **a**, but incubated for 16 h at different temperatures. Data were min-max-normalized for each sample (raw data in Supplementary Fig. 7). **d, e**, Data represent the mean ± s.d. (*n* = 3 independent experiments).

observed activity changes were not caused by temperature-dependent protein degradation.

From the best-performing AraC-LOV hybrids, we selected the mutants D432V and K488N for further characterization. Both substitutions are likely to have destabilizing effects. D432 forms a hydrogen bond with E409 in the mechanistically important A α helix (Fig. 1d), while K488 stabilizes a loop connecting two β -sheets, both in direct contact with the flavin mononucleotide chromophore (Fig. 1d). Consistent with this hypothesis, a D432V/K488N double mutant resulted in strongly reduced activity already at 37 °C, indicating that these substitutions synergistically impair stability (Extended Data Fig. 1). When comparing temperature response to blue-light-induced transcriptional deactivation, we found that all AraC-LOV hybrids were inactive both at 41 °C in the dark and at 37 °C under blue-light exposure, in line with previous reports on the LOV-C450A mutant acquiring a constitutive lit state during prolonged illumination^{15,16} (Extended Data Fig. 1). To further investigate the similarity between heat-induced and light-induced deactivation, we compared multiple A α and J α helix-stabilizing substitutions that qualitatively reduced the responsiveness to both stimuli^{17,18}. Notably, the LOV-T406A/T407A double mutant showed a markedly stronger loss of thermal than light responsiveness whereas LOV-G528A/N538E displayed the opposite trend (Extended Data Fig. 1). These findings indicate that helix undocking in response to light versus temperature may not necessarily proceed through the identical molecular mechanism.

Continuing with a detailed characterization of our thermogenetic system, inducer dose escalation experiments revealed that reporter activation by AraC-LOV-C450A at 37 °C and its thermal response at 41 °C are expression dependent and tunable over a wide range of concentrations (Fig. 2a,b and Supplementary Fig. 5). However, very high inducer levels (that is, 1,000 μ M IPTG and certain conditions with 25 mM arabinose) resulted in compromised thermal switching, likely because of AraC accumulation in the cells. For all subsequent experiments, we chose the 400 μ M IPTG and 16 mM arabinose condition, consistent with our previous work on optogenetic AraC variants¹⁴. Next, focusing on our lead candidates, we showcased efficient spatial control when *E. coli* expressing AraC-LOV-C450A/K488N were grown on agar plates and a heat gradient was applied (Fig. 2c). Moreover, we demonstrated reversible temperature control through timed incubation of liquid cultures at different temperatures for both the D432V and the K488N mutant (Fig. 2d and Supplementary Fig. 6). Subsequent experiments, in which identical samples were incubated at several temperatures between 34 and 43 °C in parallel, revealed a sharp decrease in transcriptional activation within a range of only 3–4 °C for the improved AraC-LOV hybrids (Fig. 2e and Supplementary Fig. 7). Using our screening pipeline, we further identified three single-point substitutions, E409R, P456L and F486C, with shifted transition temperatures in the range of 34–37 °C, which allowed even cold-induced protein activation at temperatures below 37 °C (Fig. 2e and Supplementary Fig. 7).

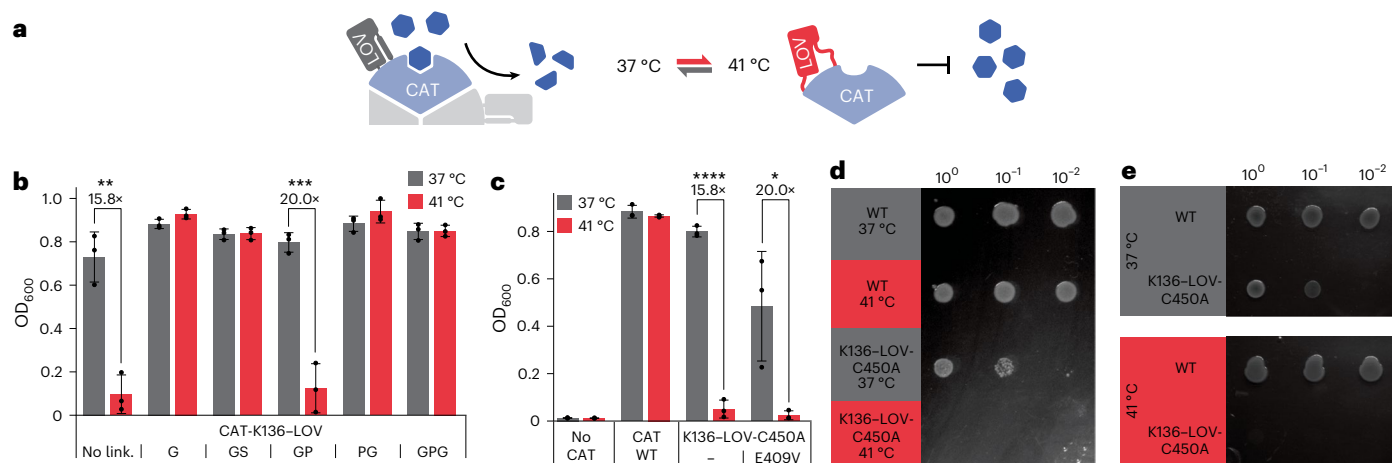


Fig. 3 | An engineered CAT-LOV hybrid acts as a heat-inducible microbial kill switch. **a**, Assay schematics. **b,c**, *E. coli* cultures expressing either wild-type CAT, no CAT, the indicated CAT-K136-LOV linker variants (**b**) or CAT-K136-LOV variants with point substitutions in the LOV domain (**c**) were grown in the presence of chloramphenicol. Samples were incubated at either 37 °C or 41 °C for 16 h, followed by measurement of the OD₆₀₀. **d**, Serial dilutions of the cultures from **c** (previously incubated at either 37 °C or 41 °C) were spotted onto LB

agar supplemented with chloramphenicol and incubated at 37 °C overnight before the image was taken. **e**, Serial dilutions of precultures expressing either wt CAT or CAT-K136-LOV-C450A were spotted onto LB agar supplemented with chloramphenicol and the plates were incubated overnight at the indicated temperature. **b,c**, Data represent the mean $\pm$ s.d. ($n = 3$ independent experiments). Statistical analysis was conducted using a two-sided Student's *t*-test. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ and **** $P < 0.0001$.

Allosteric thermocontrol of diverse bacterial protein classes

Next, to test the modularity of our thermogenetics approach, we transferred the concept to different effector proteins, starting with a chloramphenicol acetyltransferase (CAT) (Fig. 3a). Building on an existing optogenetic AsLOV2 insertion variant at K136 of the antibiotic resistance enzyme¹⁹, we evaluated effects of different flexible and rigid interdomain linkers around the AsLOV2 insertion site and assessed these variants using cell growth assays in the presence of chloramphenicol as a readout (Fig. 3b and Extended Data Fig. 2). Cultures expressing CAT-LOV hybrids reliably reached stationary phase at 37 °C, albeit at a slower growth rate than the wild-type CAT control (Extended Data Fig. 2a). Notably, for the CAT-LOV-C450A hybrid with LOV-flanking GP linkers and some AsLOV2 mutants thereof, antibiotic resistance was effectively switched off at 41 °C resulting in cell death, as indicated by optical density at 600 nm (OD₆₀₀) measurements (Fig. 3c and Extended Data Fig. 2b). We next verified effective bacterial killing at 41 °C by performing dilution spot assays on cultures preincubated at different temperatures (Fig. 3d). Comparable results were obtained when a preculture grown at 37 °C was spotted onto replica plates and subsequently incubated at either 37 °C or 41 °C (Fig. 3e). In the context of CAT, the K488N and E409V mutants exhibited strong thermal responses, whereas the D432V variant failed to confer antibiotic resistance at 37 °C (Extended Data Fig. 2b). As observed for AraC, CAT-LOV hybrid protein levels were substantially lower than those of wild-type CAT but were not affected by temperature, as determined by plate reader measurements of RFP-tagged CAT variants (Extended Data Fig. 2c). This finding corroborates that thermal response is driven by conformational adaptations, rather than temperature-dependent protein degradation.

To apply our method to an additional structurally unrelated protein in *E. coli*, we focused on a *Streptococcus pyogenes* Cas9 (SpyCas9) CRISPR activation (CRISPRa) system, in which the transcriptional activator SoxS is recruited to a modified sgRNA scaffold by the MS2 aptamer-binding domain MCP^{20,21} (Supplementary Fig. 8a). We inserted the AsLOV2-C450A domain into MCP at three different sites and tested these variants at 37 °C using RFP expression as a readout. A hybrid carrying the LOV insertion after N27 resulted in strong reporter expression (Supplementary Fig. 8b) and was selected for further characterization. Building on these results, we repeated the CRISPRa experiment at 37 °C and 41 °C and included the AsLOV2-K488N mutant, which showed

reduced leakiness in the context of AraC (Supplementary Fig. 8c). As expected, the LOV insertion variants were effectively inactivated at 41 °C, while the constitutively active system based on wild-type MCP was only slightly affected by the temperature increase. Consistent with our previous results, the K488N mutant reduced leakiness in the off state (41 °C), albeit at the expense of weaker activity at 37 °C (Supplementary Fig. 8c). We note that, across all systems described in this study, control samples were not affected by temperature changes, except for MCP-SoxS and AraC, where controls exhibited small but reproducible activity changes at 41 °C versus 37 °C (Fig. 1b and Supplementary Figs. 1 and 8). The readouts underlying these two cases depend on multiple protein-protein and protein-DNA interactions (DNA binding, AraC dimerization and MCP binding to MS2), which may be mildly affected by elevated temperature. This likely explains the observed, minor temperature effects in the MCP-SoxS and AraC control samples.

Importantly, our thermogenetically regulated MCP could be easily adapted to the various applications of the MS2 aptamer system such as mRNA tagging²² and purification²³ in a plug-and-play manner. Collectively, these data demonstrate the general applicability of the AsLOV2 insertion strategy for temperature-dependent allosteric protein regulation.

Temperature-induced peptide uncaging and temporal protein control

In addition to allosteric protein control through AsLOV2 domain insertion, we investigated the conditional uncaging of signaling peptides in the AsLOV2 J α helix. The light-dependent exposure of functional peptides caged within the C-terminal J α helix is a commonly used strategy in optogenetics^{24–27}. In this case, the peptide is appended to or integrated into the J α helix, resulting in peptide caging in the AsLOV2 dark-adapted state because of the tight association of J α with the LOV protein core. In turn, light-induced unfolding of the J α helix exposes the peptide. To assess whether this concept could be adapted for thermoregulation, we fused RFP to several modified variants of AsLOV2 in which the C terminus of the J α helix was modified to resemble an SsrA degradation tag on the basis of a previously reported design²⁶ (Extended Data Fig. 3a). Constitutive expression of these constructs in *E. coli* revealed a strong heat-dependent decrease in RFP levels at

41 °C and even more pronounced at 42 °C, while RFP expression in controls lacking the LOV-caged SsrA peptide remained unaffected by the temperature condition (Extended Data Fig. 3b).

Complementary to the LOV–SsrA peptide degron strategy in bacteria, we implemented LEXY, a peptide-uncaging approach, for the control of protein nucleocytoplasmic trafficking in mammalian cells²⁵. LEXY consists of AsLOV2, with a nuclear export signal (NES) peptide embedded into the α helix, thereby enabling inducible nuclear export. When fused to a nuclear import signal (NLS)-tagged mCherry, LEXY mediates reversible nucleocytoplasmic shuttling that can be monitored in real time by fluorescence microscopy (Extended Data Fig. 4a). To assess thermal control of protein shuttling, we transiently transfected HEK293T with several LEXY variants, including the canonical LEXY, a corresponding C450A/K488N double mutant and AsLOV2-NES16-C450A, a LEXY derivative carrying the C450A mutant and a different, functionally caged NES (Extended Data Fig. 4b). We further included a negative control construct comprising NLS–mCherry fused to wild-type AsLOV2 instead of LEXY. After 24 h, mCherry fluorescence was imaged during repeated heating and cooling cycles consisting of 20 min of heating from 36 °C to 40 °C followed by 20 min of cooling back to 36 °C. Overall, temperature-dependent effects were substantially weaker than the light-induced nuclear export with LEXY (Extended Data Fig. 4c). Nevertheless, all three NLS–mCherry–LEXY variants consistently showed reversible heat-induced translocation to the cytoplasm, while the wild-type AsLOV2 control remained unaffected (Extended Data Fig. 4d–g and Supplementary Data 1). The observed responses occurred on the timescale of minutes and remained reversible over three consecutive induction cycles. Together with our degron-caging experiments, these findings suggest that AsLOV2-mediated thermoswitching is generally compatible with peptide-caging approaches but the dynamic control range is low compared to both thermocontrol through AsLOV2 domain insertion and light-dependent peptide uncaging.

Thermocontrol of CRISPR effectors in mammalian cells

Thermogenetic gene editors responding to narrow temperature changes within the human physiological range could fuel various applications in biomedical research and, eventually, precision gene therapy. Thus, we sought to transfer our concept to CRISPR–Cas9 in human cells. First, to create temperature-activated genome editors, we build upon the CASANOVA strategy for Cas9 control with light-switchable anti-CRISPR (Acr) proteins previously developed by us^{28,29}. We inserted AsLOV2-C450A or AsLOV2-C450A/K488N after position E76 into the broad-spectrum Cas9 inhibitor AcrIIA5 (Fig. 4a) and coexpressed the resulting AcrIIA5–LOV hybrid with *Staphylococcus aureus* Cas9 (Sau-Cas9) and an sgRNA targeting the endogenous *EMX1* or *GRIN2B* locus in HEK293T cells, followed by 72 h of incubation at 37 °C, 40 °C and, for *EMX1*, also 38.5 °C. We observed an up to 3.4-fold increase in indel rates between 37 °C and 40 °C at the targeted sites as determined by deep amplicon sequencing and T7 endonuclease I (T7EI) assay (Fig. 4b,c and Supplementary Fig. 9). In control samples expressing wild-type AcrIIA5 or no Acr at all, indel rates were low (<5%) or high (~60–75%), irrespective of the temperature applied (Fig. 4b,c). Consistent with our previous work on switchable Acrs, a Cas9-to-Acr vector mass ratio of 2:1 yielded the largest stimulus-dependent change in editing activity^{28–30} (Fig. 4b,c), while the flexibility of the inhibitor would also allow for case-specific optimization.

To invert this mechanism of action and demonstrate control of a second Cas9 ortholog, we directly inserted AsLOV2 variants into *SpyCas9* at two allosteric sites (E566 and T1048) that we recently identified in the context of light-mediated regulation¹² (Fig. 4d). Both Cas9–LOV hybrids mediated potent, heat-inactivated editing (Supplementary Fig. 10) with the *SpyCas9*-E566–LOV-C450A hybrid and its AsLOV2-D432V and AsLOV2-K488N point mutants resulting in particularly effective thermocontrol across multiple target sites (Fig. 4e and Supplementary Fig. 11). We next assessed the effect of

increased temperatures on cell viability using an MTT assay, with 72 h of incubation at 37 °C or 40 °C after transfection. As expected, cells were noticeably stressed by the transfection itself. On the contrary, the temperature condition had only minor additional effects on cell viability (Supplementary Fig. 12a), confirming that the higher temperature was, overall, well tolerated. Moreover, shortening the incubation period to 48 h further attenuated the effect of temperature on cell fitness, while still maintaining potent, temperature-dependent Cas9 activity switching (Supplementary Fig. 12b,c).

Remarkably, Cas9 thermoregulation could also be plug-and-play applied for CRISPRa simply by inserting LOV mutants at E566 into a dCas9–VPR transcriptional activator (Supplementary Fig. 13a). As a side note, an alternative CRISPRa approach using previously published ssrA-based LOV dimerization systems (iLID–ssrA, oLID–ssrA and LOV–ssrA)^{31,32} showed negligible heat-induced gene induction and only the oLID fusion responded primarily to light (Supplementary Fig. 13b).

Lastly, we hypothesized that temperature sensitivity may not be a specific feature of LOV domains only, but rather a widespread phenomenon of sensory domains. Sensory domains need to be capable of transitioning between different conformational states and may become conformationally unstable when temperature rises above their native working temperature. To investigate this hypothesis experimentally, we selected a circularly permuted glucocorticoid receptor (cpGR2) domain recently reported by us, which adopts a more compact conformation upon cortisol addition²⁹. We inserted cpGR2 into *SpyCas9* at positions E566 and T1048 and tested the resulting hybrid effectors for temperature-dependent and ligand-dependent genome editing in HEK293T cells. In the presence of cortisol, both Cas9–cpGR2 hybrids mediated potent *CCR5* editing at 37 °C. By contrast, no editing was observed at 40 °C, irrespective of cortisol addition (Supplementary Fig. 14). The T1048 variant showed a particularly strong thermal control of genome editing across different target loci with up to 28-fold changes between the temperature conditions, whereas control samples remained unaffected (Fig. 4f). These experiments demonstrate that, like AsLOV2, cpGR2 is inherently temperature sensitive in its cortisol-induced state.

Discussion

We introduced the modular engineering of allosteric thermogenetic protein switches through receptor domain insertion as a powerful method for tailored protein regulation and demonstrated its applicability to diverse effectors in both bacteria and mammalian cells. Our approach leverages a commonly used photoreceptor domain and suggests that numerous other light-regulated proteins that are based on AsLOV2 insertion could easily be adapted for temperature-dependent control. We observed multiple parallels in AsLOV2 behavior under both stimuli, including similar mutational effects on thermal and light activation (Extended Data Fig. 1). Likewise, the same linkers between the LOV domain and the effector protein were suited for both light-driven and heat-driven control in the cases of AraC, Cas9 and AcrIIA5. However, we also noted case-specific differences, for example, the requirement for a specific linker configuration (GP instead of GS) in the CAT–LOV hybrids (Fig. 3b) and distinct effects of mutations that stabilize the native state of AsLOV2 (Extended Data Fig. 1). These findings suggest that, although the conformational changes during light and heat induction are likely similar, subtle mechanistic differences exist that may be relevant for tailored applications.

The success of our approach raises the question of whether the temperature-dependent effects originate from intrinsic properties of the sensory domain and/or the mutual structural strain imposed by the insertion architecture. While this cannot be fully resolved on the basis of our data, the significant thermal responses observed in both tested peptide-caging configurations (LOV–SsrA and LEXY), which rely on terminal fusion of short peptides to AsLOV2 rather than AsLOV2 domain insertion, indicate that AsLOV2 itself responds considerably to

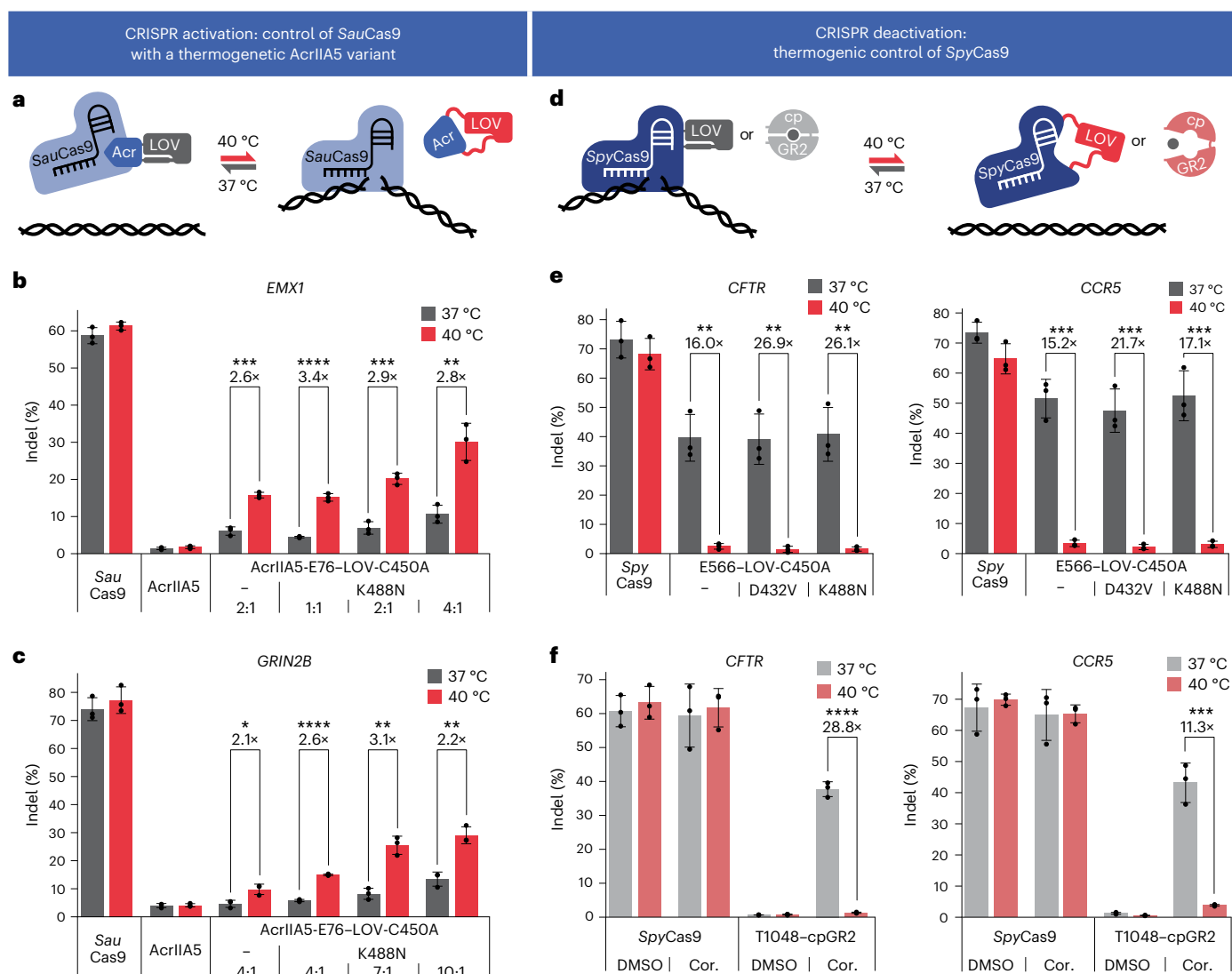


Fig. 4 | Engineering of potent thermally regulated CRISPR effectors.

a,d, Assay schematics. **b,c,e,f**, HEK293T cells were transiently transfected with plasmids encoding *Sau*Cas9 (**b,c**) or the indicated *Spy*Cas9 variant (**e,f**), and an sgRNA targeting the indicated endogenous locus. In **b,c**, the respective AcrIIA5 or AcrIIA5-LOV variant or an empty stuffer plasmid was cotransfected at the

indicated Cas-to-Acr vector mass ratios. Samples were incubated at 37 °C or 40 °C and editing efficiency was assessed by deep amplicon sequencing 72 h after transfection. In **b,c,e,f**, data are the mean $\pm$ s.d. ($n = 3$ independent experiments). Statistical analysis was conducted using a two-sided Student's *t*-test. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ and **** $P < 0.0001$.

temperature changes (Extended Data Figs. 3 and 4). The substantially larger dynamic control ranges seen with domain insertions, however, suggest that the structural strain introduced by the insertion amplifies this thermal response (Figs. 1–4).

The fact that not only AsLOV2 but also cpGR2 can effectively mediate thermal control may further reflect domain-specific effects but could also point toward thermosensitivity being a more widespread property among sensory domains in nature. To date, only very few proteins or domains are known to undergo changes in activity and/or conformation in response to small temperature changes close to the human physiological temperature optimum³³. We speculate that the apparent suitability of sensory receptor domains for thermoregulation shown in our study may be explained by their specific structural and functional properties. To respond to any kind of stimulus, sensory domains must be capable of (reversibly) adopting different conformations. From a conformational energy landscape perspective, this means that the stimulus shifts the conformational equilibrium between at least two very different structural states³⁴. The AsLOV2 domain, for instance, is well known to coexist in a dark-adapted and light-adapted

state but light induction strongly shifts the equilibrium toward the light-adapted conformation³⁵. Importantly, the thermodynamic conformational equilibrium inherently depends on temperature. In other words, temperature changes can profoundly reshape the conformational energy landscape. This may explain why conformationally promiscuous receptor domains, especially those undergoing order–disorder transitions, may be particularly well suited for adaption as thermosensors.

In addition, the domain insertion strategy used here imposes structural strain on both the receptor domain and the effector protein. While usually not favorable for optogenetic or chemogenetic applications, the resulting destabilization of the hybrid protein is likely beneficial for the engineering of protein thermosensors, where a structural change is intended to occur at the low physiological temperatures of most biological systems³⁶. This insight not only opens exciting possibilities for modularly engineering thermogenetic tools by leveraging existing optogenetic or chemogenetic switches or protein models³⁷ but also underscores the importance of considering temperature effects in experimental design across biological research.

From an application perspective, the thermoregulation of CRISPR tools is of great biomedical interest but only few approaches for CRISPR–Cas effector control have been reported. While Cas9 orthologs with different thermal stabilities are known³⁸, the effect of temperature changes within the physiological range on CRISPR–Cas activity tends to be negligible³⁹. Similarly, the only well-characterized naturally thermosensitive Cas9 inhibitor, AcrIIA2, requires large temperature changes (that is, between 22 °C and 37 °C), which is not easily compatible with mammalian cells⁴⁰. The only example of a directly thermocontrolled Cas9 was reported by the group of A. Möglich and is a hybrid of *Spy*Cas9 and the LOV domain from *Rhodobacter sphaeroides*⁴¹. Of note, this variant has been applied in bacteria and switches below 37 °C. Lastly, a common workaround represents the thermocontrolled expression of CRISPR–Cas using heat-shock promoters or delivery by photothermally regulated nanoparticles^{6,42–44}. These systems, however, require several components and cannot be combined with, for example, cell-type-specific promoters; furthermore, heat-shock promoters are prone to crosstalk with other cellular processes.

Our system, in contrast, combines several highly advantageous features, including precise response within the small physiological temperature window, direct control of Cas9 rather than indirect transcriptional regulation, tunability of the transition temperature and the ability to provide both heat-activated and heat-inactivated control of genome editing by integrating AsLOV2 into either Cas9 or an Acr. In addition, our strategy is modular with regard to the promoter used for expression; for example, it would be compatible with cell-type-specific promoters and should even be compatible with non-DNA-based delivery strategies (mRNA). In light of these advancements, we strongly believe that our method solves several long-standing challenges regarding thermoregulation of proteins.

Online content

Any methods, additional references, Nature Portfolio reporting summaries, source data, extended data, supplementary information, acknowledgements, peer review information; details of author contributions and competing interests; and statements of data and code availability are available at <https://doi.org/10.1038/s41589-026-02151-y>.

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Methods

Molecular cloning

The plasmids generated and used in this study are listed in Supplementary Table 1 and the corresponding GenBank files are provided in Supplementary Data 1. The amino acid sequences of the relevant proteins are shown in Supplementary Table 2 and the engineered variants and mutants thereof are shown in Supplementary Table 3. The constructs were generated by Golden Gate assembly, Gibson assembly or kinase, ligase and DpnI treatment. Linker sequences and type IIS restriction enzyme recognition sites were introduced as 5' primer overhangs. Oligonucleotides were purchased from Merck and PCRs were performed using Q5 high-fidelity DNA polymerase (New England Biolabs) under standard conditions. PCR products were analyzed on agarose gels and the correct bands were excised and purified using the QIAquick gel extraction kit (Qiagen). Assembly reactions were performed using enzymes and buffers purchased from New England Biolabs and Thermo Fisher Scientific. Chemically competent *E. coli* Top10 cells (Thermo Fisher Scientific) were transformed with the assembled constructs and plated on agar supplemented with the appropriate antibiotic. Lastly, plasmid DNA was purified from the liquid cultures using the QIAprep Spin Miniprep or Plasmid Plus Midi kit (Qiagen). All constructs were verified by Sanger sequencing using Microsynth SeqLab. The *Renilla* luciferase expression plasmid was purchased from Promega. H2B-GFP was a gift from G. Wahl (Addgene, plasmid 11680). pX601-AAV-CMV::NLS-SaCas9-NLS-3xHA-bGHpA;U6::BsaI-sgRNA was a gift from F. Zhang (Addgene, plasmid 61591) and sgRNA1_Tet-inducible luciferase reporter and Tet-inducible mCherry reporter were gifts from M. Sato (Addgene, plasmids 64161 and 64128).

Thermal control setup

All experiments in which samples were cultured at up to three different temperatures were performed in standard shaking incubators for bacteria or humidified incubators for mammalian cells. The incubators were operated with identical settings except for temperature, which was adjusted as indicated. Temperature settings were validated periodically using a Xintest HT-9815 thermometer and K-type thermocouples.

AraC reporter assays

Precultures of *E. coli* Top10 cells transformed with constructs encoding the respective AraC variant and the mRFP reporter were inoculated from glycerol stocks and cultured overnight in 4 ml of LB medium supplemented with chloramphenicol (25 $\mu\text{g ml}^{-1}$) and kanamycin (50 $\mu\text{g ml}^{-1}$) at 37 °C and 220 rpm. The next day, main cultures were prepared by inoculating LB medium supplemented with chloramphenicol (25 $\mu\text{g ml}^{-1}$), kanamycin (50 $\mu\text{g ml}^{-1}$), 400 μM IPTG and 16 mM arabinose (unless otherwise specified) with the precultures. Cultures were grown for 16 h at 37 °C in the dark, at 37 °C under constant blue-light exposure or at 41 °C in the dark before mRFP fluorescence and OD₆₀₀ were measured in a Tecan infinite 200 plate reader using the i-control software (Tecan, version 2.0). Except for the blue-light condition, samples were always incubated in the dark. The blue-light setup consisted of adhesive light-emitting diode strip lights (Yunbo, B50W1-B) with a wavelength of 460–465 nm attached to the metal rack tube holders in the incubator to provide continuous illumination at an intensity of 5.5–5.9 W m⁻² as previously described⁴⁵. For specific experiments, RFP levels of 20,000 cells were measured in a Cytoflex S flow cytometer (Beckman Coulter, Cytexpert 2.5.0.77) and analyzed using Cytoflow (version 1.3.0; <https://github.com/cytoflow/cytoflow>). The gating strategy is shown in Supplementary Fig. 15a,b. Initial experiments and characterization of the thermoresponsive variants were performed in 15-ml culture tubes using 4 ml of medium inoculated with 40 μl of a preculture. Dose escalation and reversibility assays were performed in 48-well plates in a 500- μl culture volume inoculated with 5 μl of preculture.

To measure the reversibility of reporter expression, precultures were diluted 1:30 in 500 μl of LB medium supplemented with all

antibiotics and inducers in 48-well plates and incubated at 37 °C or 41 °C for 5 h before they were moved to the other temperature condition and incubated for another 19 h. After 3 h and 7 h of incubation, the samples were diluted 1:20 and 1:30 in fresh medium. RFP fluorescence and OD₆₀₀ were measured at the beginning of the experiment and after 3, 7 and 24 h.

For simultaneous measurements at different temperatures, samples were incubated along a heat gradient in a thermal cycler as previously described⁴. Precultures were diluted to an OD₆₀₀ of 0.25, inducers (400 μM IPTG and 16 mM arabinose) were added and 25- μl aliquots of the culture were divided into 12 0.2-ml PCR tubes. The tubes were placed in a thermal cycler (Eppendorf) and the gradient function was used to incubate each sample at a different temperature. After 18 h of incubation, 75 μl of LB medium was added to each sample, 90 μl of which was transferred to a 96-well microtiter plate. Fluorescence measurements were performed as described above. To infer transition temperatures, the mean reporter values for each AraC-LOV variant were min-max-normalized and fitted to the Hill equation using the Neutcurve Python package⁴⁶.

To evaluate the spatial control of gene expression, 2.5% LB agar supplemented with chloramphenicol (25 $\mu\text{g ml}^{-1}$), kanamycin (50 $\mu\text{g ml}^{-1}$), 400 μM IPTG and 16 mM arabinose was prepared. Bacteria from precultures were streaked onto the agar, which was then placed in a metal tray. One half of the tray was heated from below to -41 °C for 16 h, with the temperature of the agar monitored using an Xintest HT-9815 thermometer and a K-type thermocouple. The entire setup was kept at room temperature (24 °C) so that a steep thermal gradient was established between the heated and nonheated halves of the tray. Images were captured under blue light.

Directed evolution experiments

To create an AsLOV2 mutant library, the LOV domain was amplified by error-prone PCR using the GeneMorph II random mutagenesis kit (Agilent) according to the manufacturer's protocol for medium mutation rates. The AraC expression plasmid was opened at position S170 by around-the-horn PCR using Q5 HotStart high-fidelity DNA polymerase (New England Biolabs). The primers carried type IIS restriction enzyme recognition sites as overhangs enabling the efficient assembly of the AraC-S170-LOV fusion sequence through Golden Gate cloning. After Golden Gate assembly, electrocompetent *E. coli* Top10 cells carrying a pBAD-driven mRFP reporter were transformed with the plasmid library using a Gene Pulser Xcell (Biorad) at 1,800 V and 200 Ω . The transformed cells were allowed to recover for 1 h in 1 ml of SOC medium without antibiotics while incubating at 37 °C and 800 rpm in a thermoshaker (Eppendorf). Next, a 10-ml culture of LB medium supplemented with chloramphenicol (25 $\mu\text{g ml}^{-1}$) and kanamycin (50 $\mu\text{g ml}^{-1}$) was inoculated with the whole 1 ml of culture and grown overnight. In parallel, serial dilutions of the transformants were plated on agar and used to determine a transformation efficiency of 5.2 million colony-forming units. Sanger sequencing of randomly picked colonies revealed an amino acid substitution rate of -1.3 per clone. Considering a theoretical library complexity of 2,679 possible single amino acid mutants and 7.2 million double mutants, all single mutants and many of the possible double mutants were covered. Lastly, glycerol stocks of the library were prepared in aliquots and stored at -80 °C until further use.

To select the library for thermoresponsive candidates, a preculture was grown from the glycerol stocks overnight at 37 °C and 220 rpm. The next day, the cultures were diluted 1:1,000,000 and 400 μl were plated onto 20 large LB agar petri dishes (20-cm diameter) supplemented with chloramphenicol (25 $\mu\text{g ml}^{-1}$), kanamycin (50 $\mu\text{g ml}^{-1}$), 400 μM IPTG and 16 mM arabinose. Plates were incubated overnight at 40 °C and photographed under blue light. The plates were then incubated at 37 °C for an additional 2 h and imaged again. Next, pictures were analyzed computationally the 'colony counter' package (<https://github.com/morris-lab/Colony-counter>). Colonies that were nonfluorescent

after the initial incubation at 40 °C but showed bright fluorescence after the 37 °C incubation step were considered promising candidates. These clones were picked and their performance was tested using the quantitative reporter assay described above. Mutations in the verified lead candidates were determined by Sanger sequencing.

Western blot

Cultures were prepared as described for AraC reporter assays. Then, 1 ml of culture was pelleted at 20,000g for 2 min and resuspended in 300 µl of water. The samples were then mixed with 100 µl of 4× Laemmli sample buffer (BioRad) supplied with 10% β-mercaptoethanol and incubated at 95 °C for 10 min to lyse the cells, followed by centrifugation at 20,000g for 2 min. Next, 25 µl of the sample was loaded onto a NuPAGE 12%, Bis–Tris gel (Thermo Fisher Scientific) and PAGE was performed at 200 V for 35 min in MES buffer. The proteins were transferred onto a PVDF membrane overnight at 100 V and 10 °C. The membrane was blocked for 1 h at room temperature with 4% milk powder in Tris-buffered saline with Tween-20 (TBS-T), followed by an overnight incubation with a mouse anti-His IgG primary antibody (Proteintech, 66005-1-Ig; 1:50,000) at 10 °C. The next day, the membrane was washed three times for 10 min with TBS-T and was subsequently incubated with a goat anti-mouse IgG secondary antibody (Jackson Immuno Research, 115-035-068; 1:10,000) for 1 h at room temperature, followed by three additional TBS-T washes. The western blot was developed using Super-Signal West Pico PLUS (Thermo Fisher Scientific) for 5 min before images were acquired (LAS 3000 Imaging System, Fuji).

Antibiotic resistance assays

Precultures of *E. coli* Top10 cells transformed with the respective CAT-encoding construct were inoculated from glycerol stocks and grown overnight in 4 ml of LB medium supplemented with ampicillin (100 µg ml⁻¹) at 37 °C and 220 rpm. The next day, main cultures were prepared by adding 5 µl of precultures to 48-well plates containing 500 µl of LB medium supplemented with 100 µg ml⁻¹ ampicillin and 25 µg ml⁻¹ chloramphenicol in technical duplicates. Two plates were prepared, one incubated at 37 °C and 220 rpm and the other incubated at 41 °C and 220 rpm. After 16 h of incubation, the OD₆₀₀ was measured in a Tecan infinite 200 plate reader. The values of an LB-only control were subtracted from each sample. For quantitative assessment of cell survival, serial dilutions of main cultures grown at different temperatures were subsequently spotted onto LB agar supplemented with 100 µg ml⁻¹ ampicillin and 25 µg ml⁻¹ chloramphenicol and grown overnight at 37 °C before images were taken. Thermally induced killing of bacteria growing on agar was performed in a similar manner. In this case, serial dilutions of a preculture were spotted onto two separate replicate LB agar plates supplemented with 100 µg ml⁻¹ ampicillin and 25 µg ml⁻¹ chloramphenicol, one incubated overnight at 37 °C and the other incubated at 41 °C.

To measure growth curves, cultures were prepared identically but incubated in the plate reader at 37 °C for 16 h, while shaking at a 4-mm radius. The OD₆₀₀ was measured every 15 min. To measure RFP fluorescence, precultures of *E. coli* Top10 cells transformed with constructs encoding the respective CAT–RFP variant were inoculated from glycerol stocks. These cultures were then grown overnight in 4 ml of LB medium supplemented with ampicillin (100 µg ml⁻¹) at 37 °C and 220 rpm. The next day, main cultures were prepared by inoculating 4 ml of LB medium supplemented with ampicillin (100 µg ml⁻¹) with 40 µl of the precultures. After 16 h of incubation at either 37 °C or 41 °C, mRFP fluorescence and OD₆₀₀ were measured in a Tecan Infinite 200 plate reader. All incubation steps were performed in the dark.

Inducible protein degradation

E. coli Top10 cells were transformed with plasmids encoding different mRFP–LOV fusion reporter proteins. Precultures were inoculated from glycerol stocks and grown overnight at 37 °C and 220 rpm in 4 ml of

LB medium supplemented with (50 µg ml⁻¹) kanamycin. The next day, three identical main cultures were prepared by inoculating 4 µl of the respective precultures into 4 ml of LB medium supplemented with the same antibiotic and 1 mM arabinose, followed by incubation at 220 rpm and 37, 41 or 42 °C. After 16 h of incubation, mRFP fluorescence and OD₆₀₀ were measured in a Tecan infinite 200 plate reader. All incubation steps were performed in the dark.

SoxS-mediated CRISPRa in *E. coli*

E. coli Top10 cells were cotransformed with plasmids encoding an mRFP reporter and dSpyCas9, an sgRNA with an MS2 stem loop integrated into its scaffold and an MCP–SoxS fusion protein or variants thereof. Precultures were inoculated from glycerol stocks and grown overnight at 37 °C and 220 rpm in LB medium supplemented with 100 µg ml⁻¹ ampicillin and 25 µg ml⁻¹ chloramphenicol. The next day, two identical main cultures were prepared by inoculating 4 ml of LB medium supplemented with the same antibiotics with 100 µl of the respective precultures, followed by incubation at 220 rpm and 37 or 41 °C. All incubation steps were performed in the dark. After 16 h of incubation, mRFP fluorescence and OD₆₀₀ were measured in a Tecan infinite 200 plate reader. The values of an LB-only control were subtracted from each sample.

Human cell culture

HEK293T cells were maintained in 1× DMEM without phenol red (Thermo Fisher Scientific) supplemented with 10% (v/v) FBS (Thermo Fisher Scientific), 2 mM L-glutamine, 100 U per ml penicillin and 100 µg ml⁻¹ streptomycin (all from Thermo Fisher Scientific). Cells were cultured at 37 °C with 5% CO₂ in a humidified incubator and passaged at a confluency of 70–80%. Authentication of the cell line was performed before use and routine testing for *Mycoplasma* contamination was conducted. Cell growth at 40 °C was visually inspected with a Keyence BZ-9000 microscope using the Keyence BZ-II Viewer (Keyence, version 1.01) and BZ-II Analyzer software (Keyence, version 1.01) (Supplementary Fig. 16). Cells were always incubated in the dark, only being exposed to ambient light during transient transfection and immediately before lysis. HEK293T cells were provided by E. Wiedtke (Heidelberg University clinics and BioQuant). The cell line was authenticated by single-nucleotide polymorphism profiling (Multiplexion) in March 2025.

Transient transfection

HEK293T cells were seeded at a density of 12,500 cells per well in 100 µl of medium into a 96-well plate. After 24 h, cells were transfected with a total of 150 ng of DNA using 0.5 µl of Lipofectamine 2000 (Thermo Fisher Scientific) per well according to the manufacturer's instructions. For genome editing using SpyCas9, cells were cotransfected with 75 ng of a construct expressing either wild-type SpyCas9 or a SpyCas9–LOV or SpyCas9–cpGR2 hybrid and 75 ng of the corresponding sgRNA expression vector. For AcrIIA5-dependent genome editing with SauCas9, cells were cotransfected with 150 ng of (1) an all-in-one construct encoding SauCas9 and an EMX1-targeting or GRIN2B-targeting sgRNA and (2) AcrIIA5 wild type or the AcrIIA5-E76–LOV2 mutant at the vector mass ratios indicated in the figures. Cas9 without sgRNA was transfected as a negative control. The sgRNA sequences used in this study are listed in Supplementary Table 4. After transfection, all samples were either incubated for 72 h at 37 °C or 40 °C with 5% CO₂ in a humidified incubator. For the chemical induction, 1 µl of 20 µM cortisol was added per well after 2 h, where 200 µM of cortisol dissolved in DMSO was prediluted tenfold in DMEM.

Imaging of nuclear export using LEXY

HEK293T cells were seeded into four-compartment 35-mm dishes at a density of 75,000 cells per chamber in 500 µl of medium. After 24 h, each chamber was transfected with 250 ng of DNA using 0.75 µl of

Lipofectamine 2000 (Thermo Fisher Scientific). Cells were cotransfected with 20 ng of a construct encoding H2B–EGFP, 10 ng of a construct encoding mCherry fused to AsLOV2 or a LEXY variant, as well as 220 ng of a stuffer plasmid. Then, 24 h after transfection, 200 μ l of medium was added to each chamber.

Fluorescence imaging was performed at the Nikon Imaging Center at Heidelberg University with a Nikon total internal reflection fluorescence microscope, equipped with a Nikon S Fluor $\times 40$ objective, an Andor Neo 5.5 scientific complementary metal–oxide–semiconductor camera and an on-stage incubation chamber (TokaiHit). To prevent exposure to ambient light, cells were kept in the dark and focused on the mCherry channel. For time-lapse microscopy, an incubation chamber was gradually heated from 36 °C to 40 °C over a period of 20 min, followed by 20 min of cooling back to 36 °C. CO₂ levels were constantly kept at 5%. mCherry images were taken every 2 min (excitation wavelength: 560 nm, emission wavelength: 620 nm, 27% laser intensity) with a 400-ms exposure time. Subsequently, LEXY samples were exposed to 1 s of blue-light irradiation every minute for 5 min (excitation wavelength: 475 nm, emission wavelength: 525 nm, 100% laser intensity) to measure blue-light response. EGFP imaging was performed in the same FITC channel with 17% laser intensity and 400-ms exposure time.

To quantify the nuclear and cytoplasmic mCherry fluorescence, images were first bleach-corrected using the simple ratio method in Fiji 2.16.0. Background fluorescence was subtracted using the mean intensity of three regions of interest (ROIs) with a 100-pixel diameter. Subsequently, 30-pixel diameter ROIs were manually assigned in the nucleus and cytoplasm to calculate fluorescence intensities. Statistical tests for all groups are provided in Supplementary Data 1.

Genome editing of endogenous loci

Editing efficiency at endogenous loci was assessed by deep amplicon sequencing or T7EI assay, as indicated. In both cases, samples were processed 72 h after transfection. Media were aspirated and cells were lysed in DirectPCR lysis reagent (PeqLab) supplemented with 200 μ g ml^{−1} proteinase K (Roche Diagnostics). Lysis was performed in a shaking incubator at 120 rpm and 55 °C for at least 6 h, followed by heat inactivation of proteinase K at 85 °C for 45 min. Targeted genomic loci were PCR-amplified using Q5 high-fidelity 2 $\times$ master mix (New England Biolabs) with the primers listed in Supplementary Table 5. Dual-barcoded versions of these primers were used for next-generation sequencing. Correct amplicon length and purity were determined by running samples on a 1% 0.5 $\times$ Tris–acetate–EDTA agarose gel. Barcoded samples were pooled in equimolar ratios and Illumina sequencing was performed by GENEWIZ using the Amplicon-EZ service. Indel frequencies were assessed using CRISPResso 2.0 (version 2.3.1) as previously described (<https://github.com/pinellolab/CRISPResso2>, <https://github.com/mjendrusch/acr-dms>)^{47,48}. For T7EI assays, 5 μ l of PCR amplicons were annealed in 20 μ l of 1 $\times$ NEB buffer 2 by heating the samples to 95 °C for 5 min, followed by gradual cooling to 25 °C. The annealed samples were incubated with 0.5 μ l of T7EI at 37 °C for 15 min. Gene editing efficiency was quantified by running samples on a 2% 1 $\times$ Tris–borate–EDTA agarose gel and analyzing band intensities using Fiji 2.16.0. The editing efficiency was calculated as $100 \times (1 - \sqrt{1 - \text{cleaved fraction}})$, where the cleaved fraction is the sum of the intensities of the cleaved bands divided by the total band intensities. Uncropped gel images are provided in Supplementary Fig. 17.

dSpyCas9–VPR-mediated CRISPRa in mammalian cells

HEK293T cells were seeded at a density of 75,000 cells per well in 600 μ l of medium into a 24-well plate. After 24 h, cells were transfected with a total of 600 ng of DNA using 1.8 μ l of Lipofectamine 3000 (Thermo Fisher Scientific) according to the manufacturer's instructions. Cells were cotransfected with (1) 240 ng of a construct encoding dSpyCas–VPR wild type or dSpyCas–LOV–VPR hybrid; (2)

120 ng of a TetO-targeting sgRNA expression vector; (3) 120 ng of a 13 $\times$ TetO–mCherry–MODC reporter plasmid coencoding a constitutively expressed EGFP–MODC as transfection control; and (iv) 120 ng of stuffer plasmid. For the iLID-based transcriptional activation assay, the cells were cotransfected with (1) 360 ng of a plasmid encoding dSpyCas–VPR or 180 ng of plasmids encoding dSpyCas–LOV2ssrA/oLID/iLID and sspB–VPR; (2) 120 ng of a TetO-targeting sgRNA expression vector; and (3) 120 ng of a 13 $\times$ TetO–mCherry–MODC reporter construct. Cells were incubated at either 37 °C or 40 °C for 48 h. For the iLID experiments, a third condition was included, in which the cells were incubated at 37 °C under blue-light illumination. The light setup was controlled by a Raspberry Pi running a custom Python script that applied an illumination duty cycle of 5 s of light followed by 10 s of darkness. The light intensity was set to 30 μ mol m^{−2} s^{−1}. For the flow cytometry assay, the cells were washed with 1 $\times$ PBS, trypsinized and resuspended in 1 $\times$ DMEM without phenol red supplemented with 10% (v/v) FBS, 2 mM L-glutamine, 100 U per ml penicillin and 100 μ g ml^{−1} streptomycin (all from Thermo Fisher Scientific). The cells were transferred to 2-ml tubes and kept on ice from this stage onward. The samples were washed with 800 μ l of PBS, resuspended in 250 μ l of PBS and passed through a 0.45- μ m cell strainer. Flow cytometry was carried out on a Cytoflex S (Beckman Coulter, using Cytexpert 2.5.0.77.). Cells were identified using the forward scatter and side scatter channels as indicated in Supplementary Fig. 15c,d. For each sample, 20,000 cells were recorded. The mCherry fluorescence signal was measured with a yellow laser (excitation: 561 nm, emission: 610 nm). The collected data were subsequently processed and analyzed using the Cytoflow software package (version 1.3.0; <https://github.com/cytoflow/cytoflow>).

Cell viability assay

HEK293T cells were seeded and transfected in 96-well plates, as described above. Then, 48 h or 72 h after transfection, Triton X-100 was added to the untreated wells at concentrations ranging from 1% to 0.001% as a positive control for cell death.

The cell viability assay was performed using the MTT cell proliferation assay kit (Cayman Chemicals) following the manufacturer's instructions. Absorbance was measured at 570 nm using an Infinite M Plex microplate reader (Tecan).

Reporting summary

Further information on research design is available in the Nature Portfolio Reporting Summary linked to this article.

Data availability

Additional information, including relevant amino acid sequences, targeted genomic loci and PCR primers for indel quantification are provided in the Supplementary Information. GenBank files of the DNA constructs are provided in Supplementary Data 1. Important constructs will be shared through Addgene (250776–250780). Source data are provided with this paper.

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Acknowledgements

We sincerely thank U. Engel at the Nikon Imaging Center at Heidelberg University for her support regarding fluorescence microscopy. We thank all members of the D.N. laboratory and the J.M. group for helpful discussions. J.M. is grateful for funding by the German Research Foundation (DFG; project no. 520612620). J.M. is grateful for funding from the Baden-Württemberg Stiftung. This work was supported by the European Union (European Research Council, DaVinci-Switches, project no. 101041570, to J.M.). Views and opinions expressed are, however, those of the authors only and do not necessarily reflect those of the European Union or the European Research Council Executive Agency. Neither the European Union nor the granting authority can be held responsible for them. D.N. is also grateful for funding from the Aventis Foundation. D.N. is grateful for funding by the DFG (project no. 453202693). We gratefully acknowledge the data storage service SDS@hd supported by the Ministry of Science, Research and the Arts Baden-Württemberg and the DFG through grant INST 35/1503-1 FUGG. A.-S.K. is grateful for support by a doctoral scholarship from the German Academic Scholarship Foundation. A.-L.v.B. is supported by the Konrad Zuse School of Excellence in Learning and Intelligent Systems through the German Academic Exchange Service program Konrad Zuse Schools of Excellence in Artificial Intelligence, sponsored by the Federal Ministry of Education and Research.

Author contributions

J.M. conceptualized the study and directed the work with support from D.N. K.H.H., A.-S.K., N.A.M., N.L., N.H. and S.A. performed the experiments. K.H.H., A.-S.K., N.A.M., N.L., N.H., D.N. and J.M. designed

and interpreted the experiments. B.W. predicted the domain insertion sites. N.S. established the CRISPRa assay. A.-L.v.B. and F.V. performed the initial experiments. J.M. and D.N. secured funding. J.M. wrote the manuscript with support from D.N. All authors approved the final manuscript.

Competing interests

K.H.H., A.-S.K., B.W., D.N. and J.M. are coinventors on a patent application related to this work. The remaining authors declare no competing interests.

Additional information

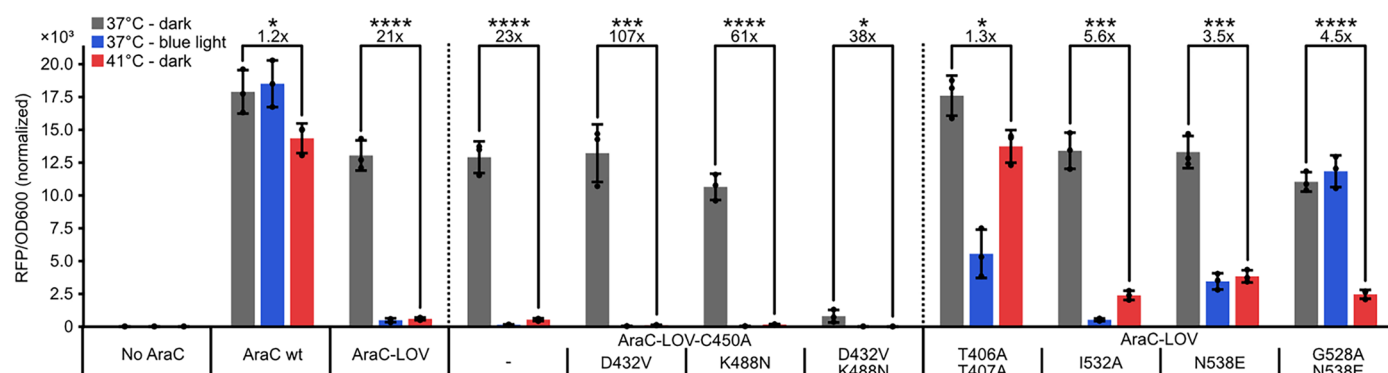
Extended data is available for this paper at <https://doi.org/10.1038/s41589-026-02151-y>.

Supplementary information The online version contains supplementary material available at <https://doi.org/10.1038/s41589-026-02151-y>.

Correspondence and requests for materials should be addressed to Dominik Niopek or Jan Mathony.

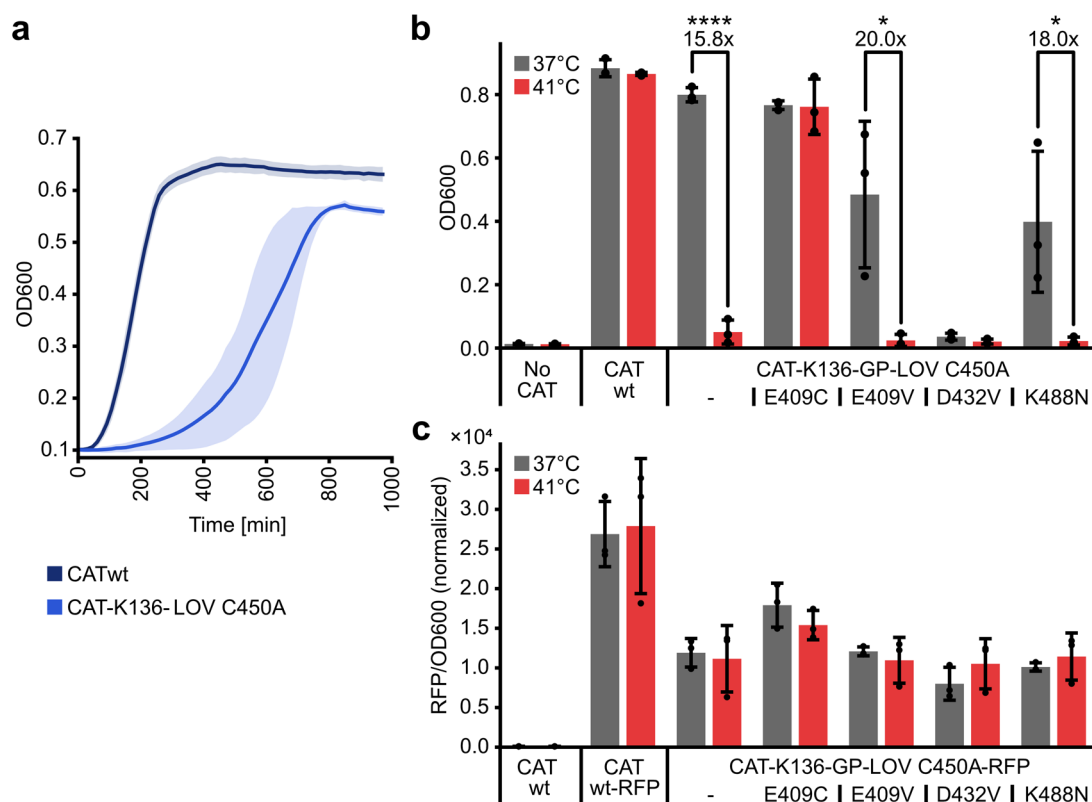
Peer review information *Nature Chemical Biology* thanks Won Do Heo and the other, anonymous reviewer(s) for their contribution to the peer review of this work.

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Extended Data Fig. 1 | Improved AraC-LOV variants respond similarly to the light and temperature stimuli whereas AsLOV2 stabilizing mutations exhibit stimulus-specific effects. *E. coli* carrying a pBAD-mRFP reporter and expressing the indicated AraC variant or a dummy control protein were incubated for 16 h at the indicated light and temperature conditions. RFP expression

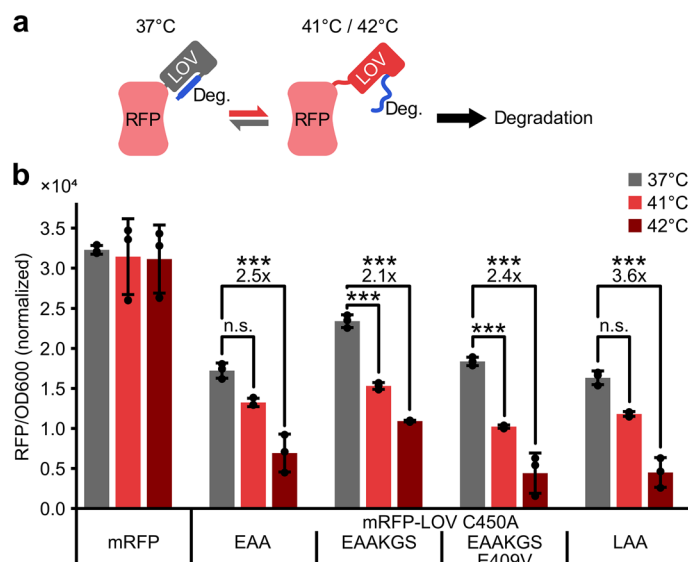
(normalized to OD) was assessed in a plate reader. The dotted line separates the previously reported stabilizing mutations. Data represent the mean $\pm$ SD, $n = 3$ independent experiments. Fold changes are indicated. * $P < 0.05$, *** $P < 0.001$, **** $P < 0.0001$, two-sided Student's t -test. wt, wild-type.



Extended Data Fig. 2 | Characterization of thermosensitive CAT variants.

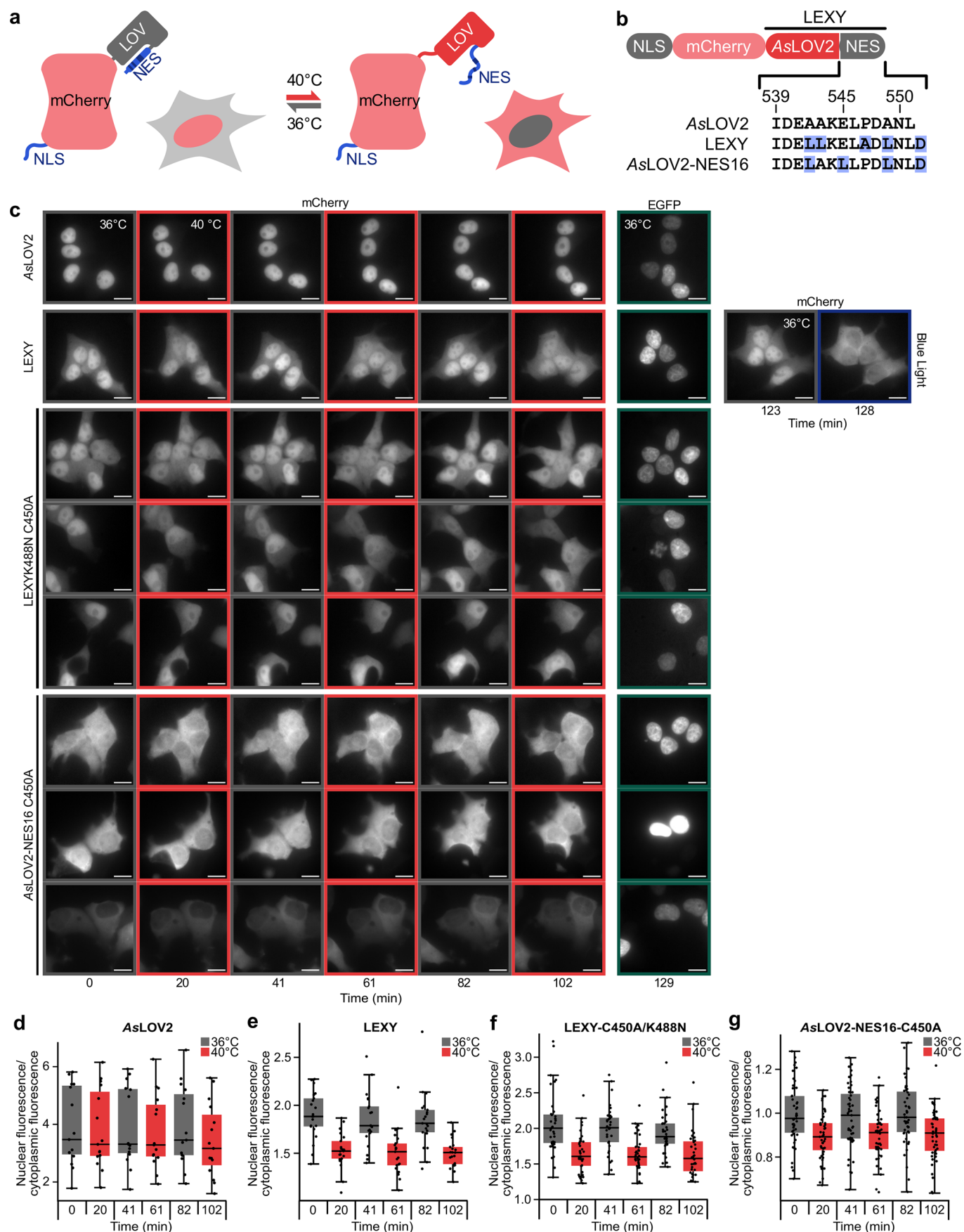
a, Time course showing the growth of *E. coli* cultures expressing the indicated CAT variants at 37 °C in the presence of chloramphenicol. OD₆₀₀ was measured every 15 minutes for 16 h using a plate reader. Lines indicate the mean of *n* = 3 independent replicates and shaded areas show the standard deviation. **b**, *E. coli* cultures expressing either wild-type CAT, no CAT or the indicated CAT-K136-GP-LOV mutants were grown in the presence of chloramphenicol. Replicate samples were incubated at either 37 °C or 41 °C for 16 h, followed by measurement of

the OD₆₀₀. The graph is an expansion of the dataset shown in Fig. 3c. **c**, *E. coli* cultures expressing either wild-type CAT, wild-type CAT fused to RFP or the indicated CAT-K136-GP-LOV mutants fused to RFP were grown in the absence of chloramphenicol. Replicate samples were incubated at either 37 °C or 41 °C for 16 h. RFP expression (normalized to OD) was assessed in a plate reader. **b,c**, Data points indicate *n* = 3 independent experiments and bars represent the mean. Error bars indicate the SD. Fold changes are indicated. wt, wild-type; **P* < 0.05, *****P* < 0.0001, two-sided Student's *t*-test.



Extended Data Fig. 3 | Temperature-responsive uncaging of an SsrA degron peptide in the LOV2 α helix. **a**, Schematic of thermo-switchable RFP degradation. **b**, *E. coli* cultures expressing either mRFP or the indicated mRFP-LOV-C450A-degron fusions were grown for 16 h at the indicated temperatures followed by measurement of RFP fluorescence and OD₆₀₀. Letters

indicate the C-terminal degron motif starting at amino acid 541 of the AsLOV2 domain. Data points indicate $n = 3$ independent experiments and bars represent the mean. Error bars indicate the SD. Fold changes are indicated. n.s. $P > 0.05$, *** $P < 0.001$, one-way ANOVA with Bonferroni correction.



Extended Data Fig. 4 | See next page for caption.

Extended Data Fig. 4 | Reversible nuclear export using temperature-regulated LEXY variants. **a**, Assay schematic of LEXY system mediating temperature-regulated nuclear export. **b**, Schematic of the NLS-mCherry-LEXY and NLS-mCherry-AsLOV2-NES16 constructs as described by Niopek et al.². Mutations introduced in the J α helix to incorporate a nuclear export signal are highlighted in blue. Residue numbers refer to full-length phototropin-1. **c**, Fluorescence microscopy images of HEK293T cells transiently transfected with plasmids encoding H2B-EGFP and mCherry fused to the indicated LEXY or AsLOV2 variants. Post transfection, cells were incubated for 24 h at 37 °C, followed by fluorescence microscopy. Imaging was conducted during three consecutive

temperature cycles, in which samples were gradually heated from 36 °C to 40 °C over a period of 20 min, followed by 20 min of cooling back to 36 °C. For canonical LEXY control images in darkness and after blue light illumination (1 s of illumination, every 60 s for 5 min) were acquired. EGFP images were acquired at the end of the experiment to visualize the nuclei. Scale bar, 13.3 μ m. **d-g**, Boxplots quantifying nuclear-to-cytoplasmic fluorescence ratios of individual cells. Boxes indicate the median and interquartile range, with whiskers extending to the 1.5-fold IQR and individual data points shown. Data corresponds to n = 15 cells (**d**), n = 21 cells (**e**), n = 35 cells (**f**) and n = 47 cells (**g**). For statistical analysis of the results, see Supplementary data 1.

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☐	☒ For null hypothesis testing, the test statistic (e.g. <i>F</i> , <i>t</i> , <i>r</i>) with confidence intervals, effect sizes, degrees of freedom and <i>P</i> value noted <i>Give P values as exact values whenever suitable.</i>
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Software and code

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Data collection	Luciferase data, fluorescence, absorbance and optical density data were obtained using the i-control software (Tecan, version 2.0). Light microscopy images were acquired and processed with the Keyence BZ-II Viewer (Keyence, version 1.01) and BZ-II Analyzer software (Keyence, version 1.01). Fluorescence microscopy images were acquired with a Nikon TIRF microscope. Western blot images were captured with a LAS 3000 Imaging System, Fuji. Flow cytometry was performed using a Cytoflex S cytometer using Cytexpert 2.5.0.77.
Data analysis	Analysis of NGS data was performed using and CRISPResso2 2.3.1. Fluorescence microscopy images were analyzed using Fiji 2.16.0. Cytometry data was analyzed using the Cytoflow v1.3.0 software package.

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- A description of any restrictions on data availability
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Additional information, including relevant amino acid sequences, targeted genomic loci and PCR primers for InDel quantification are provided in the Supplementary Information. Genbank files of the DNA constructs are provided as Supplementary Data files. Important constructs will be shared on Addgene (Addgene ID: xxxx, xxxx). Additional data will be shared upon reasonable request.

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Population characteristics	n/a
Recruitment	n/a
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Life sciences study design

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

Sample size	Sample sizes were chosen on the basis of an initial pilot experiment and further based on similar experiments reported in previous publications.
Data exclusions	No experimental data were excluded from the analysis.
Replication	All attempts for replication were successful. The number of replicates performed is indicated in each figure legend, where applicable.
Randomization	No randomization was used, as samples and controls were treated side-by-side using the identical protocols and workflows for analysis. Also, the study did not involve human or animal subjects.
Blinding	No blinding was used, as the majority of data was automatically collected by machines and analyzed by standard workflows.

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Materials & experimental systems

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☐	☒ Eukaryotic cell lines
☒	☐ Palaeontology and archaeology
☒	☐ Animals and other organisms
☒	☐ Clinical data
☒	☐ Dual use research of concern
☒	☐ Plants

Methods

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

Antibodies

Antibodies used	mouse anti-His IgG primary antibody (Proteintech, 66005-1-Ig, 1:50,000); goat anti-Mouse IgG secondary antibody (Jackson Immuno Research, 115-035-068, 1:10,000)
Validation	Both antibodies have been validated for Western Blot in various dilutions.

Eukaryotic cell lines

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Cell line source(s)	HEK 293T cells were kindly provided by Ellen Wiedtke (Heidelberg University clinics and BioQuant, Heidelberg, Germany).
Authentication	The cell line was authenticated via Single Nucleotide Polymorphism Profiling using a commercially available service (Multiplexion, Heidelberg, Germany) in March 2025.
Mycoplasma contamination	The cell line was tested negative for mycoplasma contamination via a commercially available service (Multiplexion, Heidelberg, Germany) prior to usage.
Commonly misidentified lines (See ICLAC register)	HEK (293T) is a standard cell line widely used for transient transfection and CRISPR/Cas9 experiments.

Plants

Seed stocks	<i>Report on the source of all seed stocks or other plant material used. If applicable, state the seed stock centre and catalogue number. If plant specimens were collected from the field, describe the collection location, date and sampling procedures.</i>
Novel plant genotypes	<i>Describe the methods by which all novel plant genotypes were produced. This includes those generated by transgenic approaches, gene editing, chemical/radiation-based mutagenesis and hybridization. For transgenic lines, describe the transformation method, the number of independent lines analyzed and the generation upon which experiments were performed. For gene-edited lines, describe the editor used, the endogenous sequence targeted for editing, the targeting guide RNA sequence (if applicable) and how the editor was applied.</i>
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Flow Cytometry

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Methodology

Sample preparation	E. coli were diluted in PBS prior to measurement. HEK293T cells were washed with 1x phosphate-buffered saline (PBS), trypsinized and resuspended in 1x DMEM without phenol red supplemented with 10% (v/v) fetal bovine serum, 2 mM L-glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin (all from Thermo Fisher Scientific). The cells were transferred to 2 ml tubes and kept on ice from this stage onward. The samples were washed with 800 µL PBS, resuspended in 250 µL PBS
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and passed through a 0.45 μ M cell strainer.

Instrument

Cytoflex S (Beckman Coulter)

Software

CytExpert (Beckman Coulter)

Cell population abundance

20,000 cells

Gating strategy

E. coli or HEK293T cells were gated based on a forward vs. side scatter area plots. Subsequently, single cells were gated within the SSC height and SSC area channels.

☒ Tick this box to confirm that a figure exemplifying the gating strategy is provided in the Supplementary Information.