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Light-Dependent Cytoplasmic Recruitment Enhances the Dynamic Range of a Nuclear Import Photoswitch

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Cellular signal transduction is often regulated at multiple steps to achieve more complex logic or precise control of a pathway. For instance, some signaling mechanisms couple allosteric activation with localization to achieve high signal to noise. Here, we create a system for light-activated nuclear import that incorporates two levels of control. It consists of a nuclear import photoswitch, light-activated nuclear shuttle (LANS), and a protein engineered to preferentially interact with LANS in the dark, Zdk2. First, Zdk2 is tethered to a location in the cyto-

plasm that sequesters LANS in the dark. Second, LANS incorporates a nuclear localization signal (NLS) that is sterically blocked from binding to the nuclear import machinery in the dark. If activated with light, LANS both dissociates from its tethered location and exposes its NLS, which leads to nuclear accumulation. We demonstrate that this coupled system improves the dynamic range of LANS in mammalian cells, yeast, and *Caenorhabditis elegans* and provides tighter control of transcription factors that have been fused to LANS.

Introduction

In cells, signal transduction is regulated by a variety of mechanisms that are commonly in use at multiple points in a single pathway. For example, the inhibitor of NF- κ B (I κ B) proteins applies two mechanisms to inactivate NF- κ B transcription factors. First, they bind to NF- κ B transcription factors and sterically occlude their nuclear localization signals (NLSs) from interacting with the nuclear import machinery. Second, I κ Bs have nuclear export signals (NESs) that direct NF- κ B/I κ B complexes to

the cytoplasm. NF- κ B pathways are activated if I κ Bs are phosphorylated and then degraded by the proteasome. This degradation event releases NF- κ Bs from the I κ Bs and exposes their NLSs.^[1] The combined effect of losing a NES and gaining a NLS provides a strong driving force to enter the nucleus and initiate gene transcription. The spatiotemporal regulation of NF- κ Bs is an inspiration for developing artificial signaling pathways, and here we employ a similar strategy to an engineered nuclear import photoswitch.

A common approach for studying signaling mechanisms in biology is to perturb a pathway and then to observe how the system responds. Light-activatable proteins are well suited to these types of studies, because they allow pathways to be reversibly activated with high spatiotemporal resolution in living cells and organisms.^[2] Recently, we developed a protein photoswitch, called light-activatable nuclear shuttle (LANS), that resides in the cytoplasm and goes to the nucleus if activated with light.^[3] LANS is based on the naturally occurring light oxygen voltage 2 (LOV2) domain from *Avena sativa*, which undergoes a large conformational change upon activation with blue light.^[4] To create LANS, an NLS motif was embedded at the end of the C-terminal helix (J α -helix) of the AsLOV2 domain.^[5,6] In the dark, the J α -helix is folded and sterically blocks binding to importins, a critical step in nuclear import. Upon light activation, the J α -helix undocks from the AsLOV2 domain, which allows the NLS to bind to importins and enter the nucleus. To maintain LANS in the cytoplasm in the dark, a NES was fused to the switch. Several NESs were tested to find a motif that would keep the switch cytoplasmic in the dark but allow it to be redirected to the nucleus if the embedded NLS was exposed.^[3] We were able to achieve functional changes in nucleocytoplasmic levels by using two different constructs, termed LANS1 and LANS4, that carry two different

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NESs. LANS4 has the higher dynamic range (light vs. dark nuclear localization) of the two constructs, but LANS1 has tighter caging (less nuclear in the dark), allowing different levels of control depending on the demands of the experiment. Despite these results, both LANS switches still enter the nucleus to some extent in the dark. This “leakiness” is not ideal in applications for which it is important to have very little activity in the dark.

LANS only uses a single point of regulation to control nuclear import, the exposure of a NLS. In contrast, NF- κ B includes two control points, the exposure of a NLS and the release of a NES. To mimic the NF- κ B pathway more closely, we decided to incorporate a second layer of control for nuclear import medi-

ated by LANS. Recently, we engineered a system, LOVTRAP, that preferentially interacts with the AsLOV2 domain in the dark.^[7] We showed by anchoring one of the LOVTRAP variants, Zdk2, to mitochondrial, vacuole, and plasma membranes that it was possible to recruit the AsLOV2 domain to the membranes in the dark and to release the domain with blue light. Given that LANS is built from the AsLOV2 domain, we hypothesized that we could use Zdk2 to anchor LANS in the cytoplasm in the dark and thus reduce the amount of LANS found in the nucleus in the dark. Upon light stimulation, LANS should release from the membrane and expose its NLS for translocation to the nucleus (Figure 1 A). We refer to the combined system (an anchored Zdk2 with LANS) as LANSTRAP. To

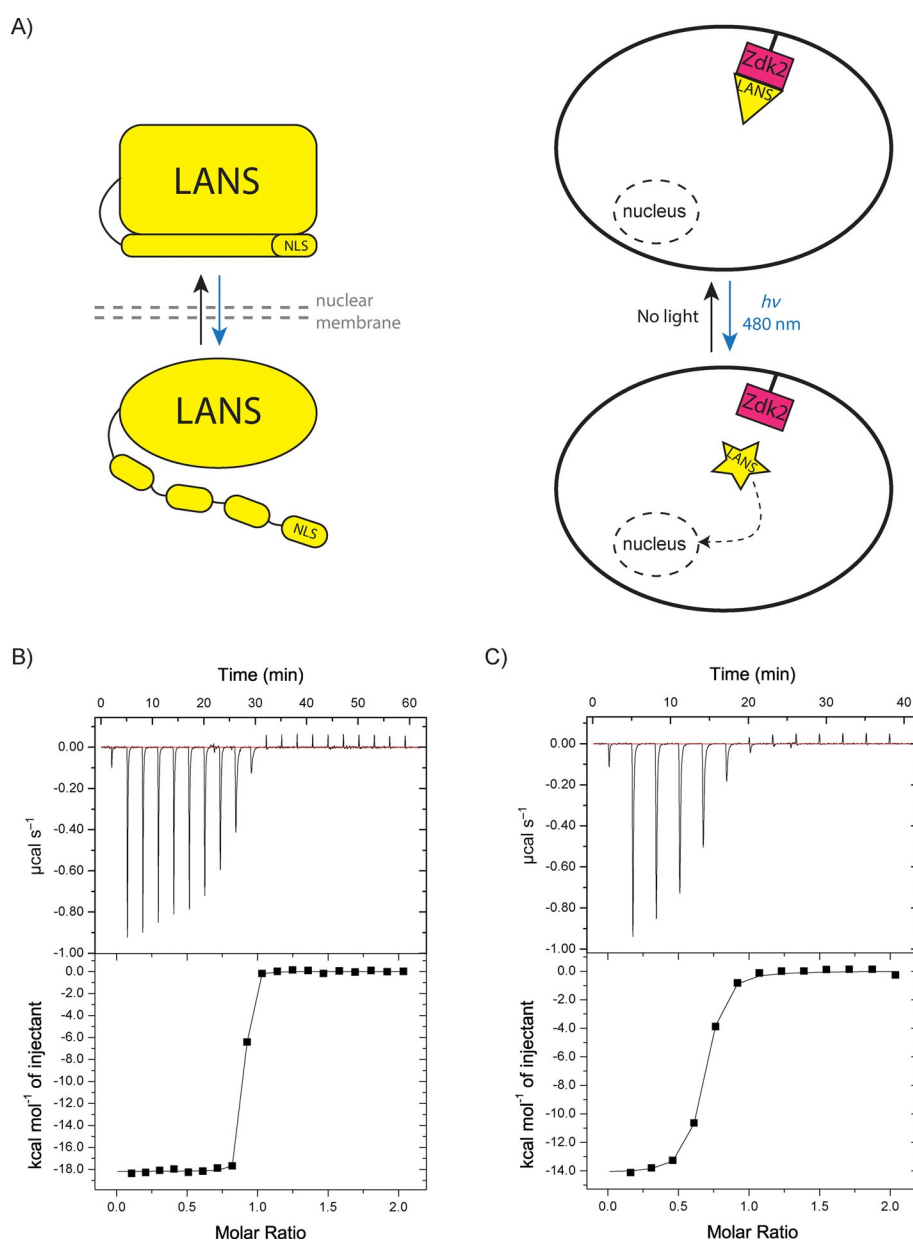


Figure 1. LANSTRAP system schematic and in vitro characterization. A) Schematics of LANS alone (left) and LANSTRAP (right), in which LANS binds Zdk2 from LOVTRAP in the dark and sequesters it in the cytoplasm (top) until blue-light illumination triggers its dissociation and translocation to the nucleus (bottom). The blue arrow indicates light illumination and activation, and the black arrow indicates its reversion if the stimulus is absent in the dark. B) ITC binding measurements for LANS wild-type with Zdk2 and C) LANS lit mimic (I539E) to Zdk2.

test our hypothesis, we performed in vitro binding studies, localization studies in mammalian cells, and activity assays in yeast and *Caenorhabditis elegans*.

Results and Discussion

Although LANS preserves all of the residues from the AsLOV2 domain that interact with Zdk2, it has additional residues at its C terminus, the effects of which on this interaction have not been established. Therefore, we first tested whether LANS would preferentially bind to Zdk2 in the dark by using isothermal titration calorimetry (ITC) to measure the binding affinity between LANS and Zdk2. Measurements were made with wild-type LANS (without a NES) and a mutant form (I539E) that mimics the lit state of the switch.^[6] Binding with wild-type LANS represents dark-state binding, as the ITC experiments were performed in an enclosed cell without any light source. Wild-type LANS bound to Zdk2 with an affinity of 3.9 nM, whereas the lit-state mimic bound 36 times more weakly with an affinity of 138 nM (Figure 1 B and C). The change in binding affinity might be even larger with actual blue-light stimulation, as there are contacts between Zdk2 and LANS that are unlikely to be directly impacted by the I539E mutation but may be disrupted by light activation. Nevertheless, these results indicated that Zdk2 binds tighter to the dark state of LANS and that this may be useful for cytoplasmic sequestering of LANS in the dark.

We performed two sets of microscopy experiments to characterize the behavior of the switches in living cells. Spinning disk confocal microscopy was performed in HeLa cells with LANS4 and its lit mimetic (I539E) fused to mCherry fluorescent protein. Nuclear/cytoplasmic levels of LANS were measured with and without cotransfection of a TOM20-Zdk2 fusion. TOM20 anchors Zdk2 at the cytoplasmic face of the mitochondrial membrane. Whereas the dark-state nuclear/cytoplasmic fluorescence intensity for the LANS4 switch alone was 0.45,^[3] upon cotransfection with TOM20-Zdk2 it was 0.21. The lit-state nuclear/cytoplasmic fluorescence was similar with (2.7) and without (2.8) the coexpression of TOM20-Zdk2. Thus, an enhanced dark/light dynamic range of 12.5 was observed by confocal microscopy for LANSTRAP (LANS4 coexpressed with TOM20-Zdk2) compared to 6.2 for LANS alone (Figure 2B). The latter is due to a decrease in the dark-state nuclear/cytoplasmic fluorescence by a factor of approximately two for LANSTRAP ($p < 0.0001$, unpaired two-tailed student's *t*-test).

We also used epifluorescence microscopy to monitor photo-induced nuclear localization in real time with cells cotransfected with the TOM20-Zdk2 fusion. Upon blue-light illumination, LANS4 released from the mitochondria and entered the nucleus. Switching the light off resulted in the protein returning to the cytoplasm and the mitochondrial membrane (Movie S1 in the Supporting Information). Nuclear fluorescence was quantified over ten different cells. The normalized fluorescence intensity peaked at a value that was approximately 8.4 ± 0.54 times higher than the starting fluorescence, and we observed a half-life ($t_{1/2}$) of (2.9 ± 1.5) min for import and $t_{1/2} = 2.5 \pm 0.5$ min for export (Figure 2A and C). In experiments without

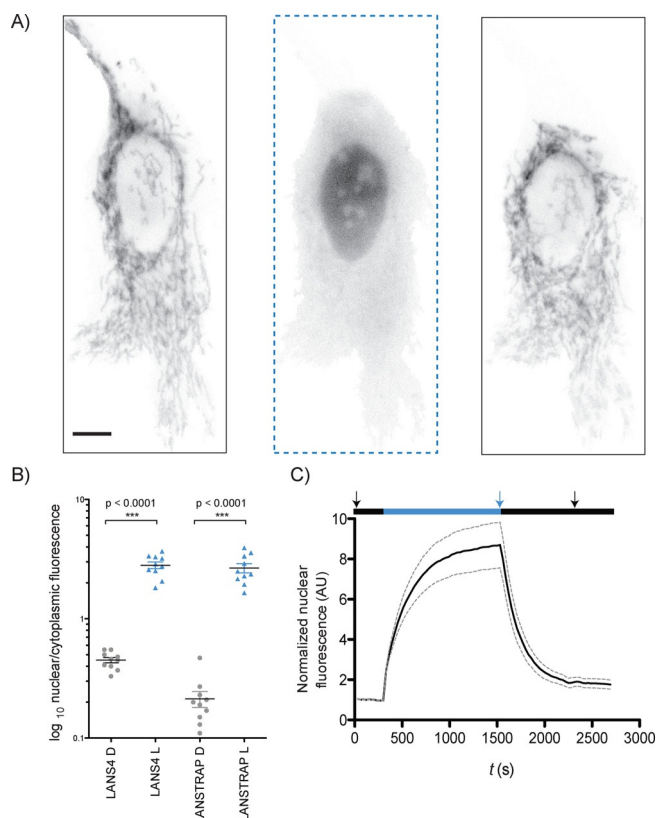


Figure 2. Microscopy characterization of LANSTRAP in HeLa tissue culture cells. A) Snapshots of real-time activation and reversion of LANSTRAP with epifluorescence microscopy with starting dark state (left), full activation (middle), and reversion (right) images. The arrows in panel C correspond to the time points of these images. B) Confocal microscopy quantification of LANS4^[3] and LANSTRAP. All the imaging was performed in the dark with “D” corresponding to wild-type LANS4 switches and “L” to the LANS4 (I539E) lit mimetic. Ten cells for each state were used for quantification. Statistical significance was measured with unpaired two-tailed student's *t*-test. C) Quantification of real-time light-activated nuclear import and reversion by using epifluorescence microscopy ($n = 10$, mean reported \pm SEM with dashed line). The black (dark) and blue (intermittent blue light) indicates the condition applied during the time course. Scale bars: 10 μ m.

coexpression of TOM20-Zdk2, the change in nuclear fluorescence was (2.8 ± 0.6) -fold with time constants for import and export similar to those observed with Zdk2: $t_{1/2} = 3.3 \pm 0.02$ min for import and $t_{1/2} = 2.5 \pm 0.01$ min for export.^[3] To test if the improved dynamic range observed in the microscopy experiments translated to tighter control over function, we used LANSTRAP to control gene transcription in yeast. For this experiment, we fused LANS4 to the LexA DNA binding domain and the Gal4 activation domain and used a yeast strain with the β -galactosidase reporter gene downstream of the LexA binding site. Previously, upon using LANS4 alone, we observed that we also needed to add a larger protein, maltose binding protein (MBP), as a bulky add-on to the LANS4 construct (total protein size = 98 kDa) to mitigate passive diffusion through the nuclear pore. For experiments performed here with LANS4 and LANSTRAP, we used a smaller fusion partner, the fluorescent protein Venus (total protein size = 84 kDa), as we expected that cytoplasmic anchoring with Zdk2 should counteract leakiness due to passive diffusion. With these constructs, the use of

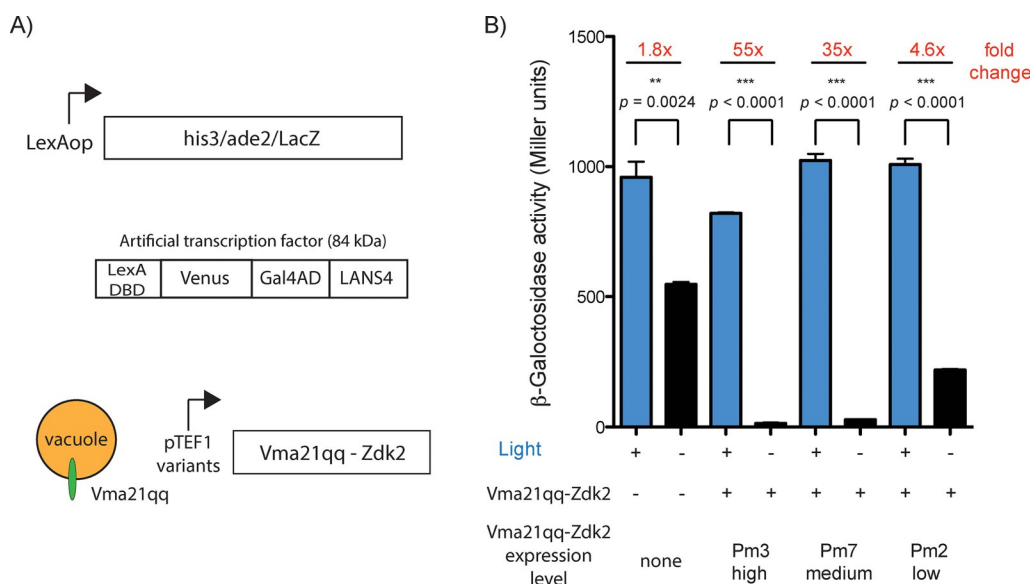


Figure 3. Control of transcription in yeast using LANSTRAP. A) Schematic of the NMY51 reporter genes (top), LexA artificial transcription factor used in these experiments (middle), and Vma21qq-Zdk2 construct schematic and illustration (bottom). B) Assessment of transcriptional control by LANSTRAP by β -galactosidase assays in NMY51 with and without Zdk2 co-expression.

LANS4 alone did not provide tight control of β -galactosidase expression. We only observed a 1.8-fold increase in expression under blue-light stimulation, and relatively high transcription levels were observed in the dark (Figure 3B). To test if LANSTRAP could improve the dynamic range, we generated three yeast strains by using the reporter strain (NMY51) as the parent. We fused Zdk2 to the C terminus of Vma21qq, a small protein localized to the vacuole, and modulated the expression of Vma21qq-Zdk2 by using three variants of the TEF1 constitutive promoter (Figure 3A). The three promoter variants used here, Pm3, Pm7, and Pm2, exhibit decreasing levels of expression for their downstream open reading frame.^[9] All three of the strains expressing Vma21qq-Zdk2 fusion exhibited improved light/dark photoswitching dynamic range in the β -galactosidase assays. Although the lit-state expression levels of β -galactosidase were not perturbed, dark-state transcription decreased in response to the increasing Vma21qq-Zdk2 expression level. With Pm2, a 4.6-fold increase in expression was observed with blue-light stimulation, whereas with Pm7 a 35-fold change and with Pm3 a 55-fold light/dark transcriptional response were observed (Figure 3B). Notably, the dark-state transcription levels with Pm3 decreased to 14.8 Miller units, which constitutes a 37-fold decrease relative to the dark-state transcription in the absence of the cytoplasmic anchor. In comparison, the original transcription factor alone (LexA-MBP-Gal4AD-LANS4), in the absence of Zdk2 coexpression, exhibited a 21-fold dynamic range between the dark and light β -galactosidase expression levels.

Finally, we investigated the applicability of LANSTRAP to influence *C. elegans* vulval development through light-mediated control of the LIN-1/ETS transcription factor. The *C. elegans* vulva develops from a group of six precursor cells, all of which are competent to initiate vulval development.^[10] LIN-1/ETS is expressed in all six precursor cells and acts to repress the

primary vulval cell fate.^[11] In wild-type animals, an EGF signal from a neighboring cell, called the anchor cell, triggers MAPK-mediated phosphorylation and inactivation of LIN-1 in one of the vulval precursor cells.^[12] As a result, that cell can adopt the primary vulval fate and initiate vulval development (Figure 4A). LIN-1 remains active in the other vulval precursor cells that do not initiate vulval development. Given that LIN-1 is a repressor of the vulval fate, loss of LIN-1 activity leads to a multivulval phenotype in which more than one vulval precursor cell initiates vulval development.^[11] Conversely, gain-of-function mutations in LIN-1 result in a vulvaless phenotype.^[12] In previous work, we used CRISPR/Cas9-triggered homologous recombination to re-engineer the *lin-1* gene, replacing the normal EGF-mediated regulation of LIN-1 with LANS-based light-dependent activity.^[3] *lin-1::lans* animals grown in the dark exhibited a multivulval phenotype, consistent with LIN-1 sequestration in the cytoplasm, whereas *lin-1::lans* animals grown in the light exhibited vulvaless phenotypes, thus suggesting constitutive nuclear localization and activity of LIN-1. Although these data showed that LANS could influence developmental signaling through control of an endogenous transcription factor, the multivulval phenotype observed for animals raised in the dark was mild compared to complete loss of *lin-1* function, which suggested that LIN-1::LANS retained some ability to enter the nucleus and activate transcription even in the dark. We therefore asked whether adding the Zdk2 anchor to this system would enhance light-mediated control of development.

To test the activity of Zdk2 in *C. elegans* development, we generated extrachromosomal arrays expressing Zdk2 in vulval precursor cells. Extrachromosomal arrays are semistable in *C. elegans*, so that within a population of animals, some carried the array and some did not. By following the presence of the array with a fluorescent marker, we were able to score vulval development with or without the Zdk2 anchor in worms raised

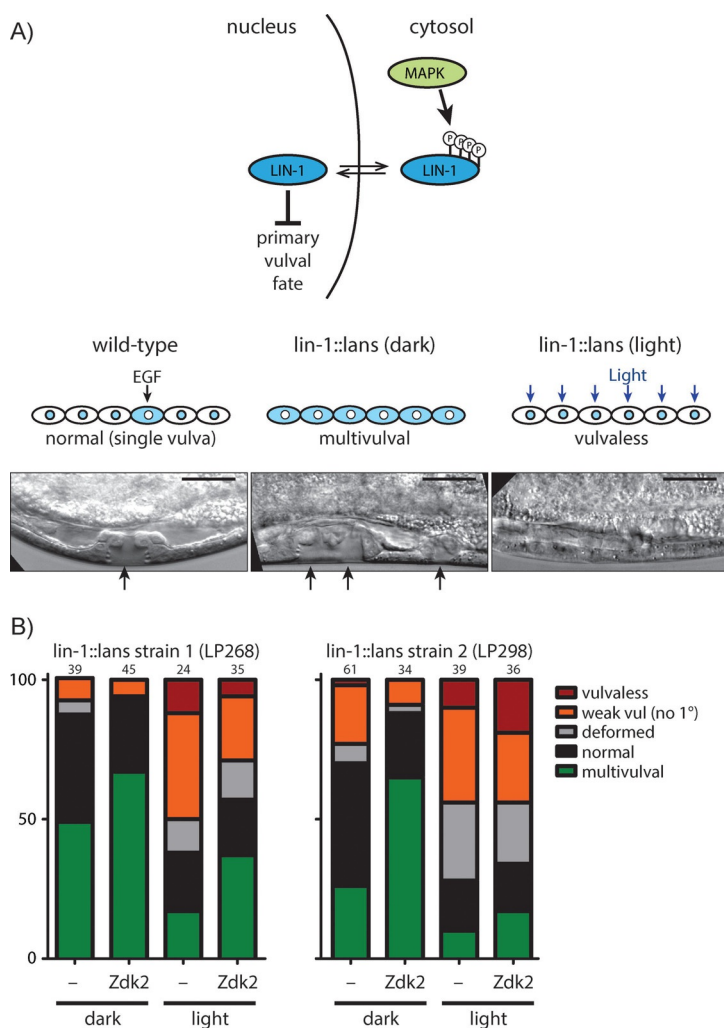


Figure 4. Control of vulval development in *C. elegans* by using LANSTRAP. A) LIN-1 role in primary vulval fate determination (top), and schematic of the effect on phenotype of wild-type LIN-1 and LIN-1 constitutively active form controlled by LANS (bottom). B) Quantification of phenotypes in the indicated strains and conditions. Numbers at the top of each bar indicate the total number of animals scored in this experiment.

under identical conditions on the same plate. We performed this experiment in parallel in two independently constructed *lin-1::lans* strains. Consistent with our previous findings,^[3] animals lacking the array exhibited low penetrance (< 50%) of multivulval phenotypes in the dark, whereas light activation suppressed the multivulval phenotype and induced a vulvaless phenotype (Figure 4B). Expression of the Zdk2 anchor increased the penetrance of multivulval phenotypes in the dark in both strains, consistent with our prediction that Zdk2 would sequester LIN-1::LANS in the cytoplasm and reduce its activity in the dark. However, even in the presence of Zdk2, the multivulval phenotype was weaker in both severity and penetrance than that produced by a *lin-1* null allele,^[11] which suggests that LIN-1::LANS retains some signaling activity even under these conditions. We next examined whether expression of the Zdk2 anchor still allowed for activation of LIN-1::LANS by blue light. Zdk2 expression did not prevent light induction of vulvaless phenotypes (Figure 4B); this suggests that the anchor could

be released upon light activation as expected. However, we did observe a higher penetrance of “residual” multivulval phenotypes in light-stimulated Zdk2-expressing animals, and this suggests that Zdk2 might have inhibited light activation of LIN-1::LANS to some extent. This might be partially a consequence of expressing Zdk2 from an extrachromosomal array, as transgenes generated in this manner typically show high levels of overexpression. In summary, these results suggest that expression of Zdk2 improves the caging of LANS while still allowing for light-stimulated nuclear import in vivo.

Conclusion

Light-mediated nuclear import and export is a generalizable approach for controlling a variety of biological functions.^[13] We and others have used it to control transcription factors as well as enzymes that modify the epigenome.^[3,14] In these studies, it was important to tune the relative strength of the nuclear localization signals (NLSs) and nuclear export signals (NESs) embedded in the switches to maximize the dynamic range, and it was challenging to minimize dark-state activity. Here, we showed that by adding an additional mode of regulation to the pathway it was possible to lower dark-state activity and increase the dynamic range. Our approach was inspired by the multiple mechanisms used to regulate NF- κ B activity and should be applicable to other light-activatable switches. Redchuk et al. also recently proposed the use of a similar mode of control by multiplexing BphP1-Q-PAS1 with light-activatable nuclear shuttle (LANS), thus using near-infrared and blue light as stimuli.^[15] The main distinction between our approach and that of Redchuk et al. is the number of stimuli required. For instance, we also developed LINX—a variant of the AsLOV2 domain that cages a NES in its α -helix and transports from the nucleus to the cytoplasm with the application of light.^[14a] In this case, anchoring Zdk2 in the nucleus should provide a way to hold LINX more tightly in the nucleus in the dark.

The LANS coupled with Zdk2 (LANSTRAP) system is compatible with a variety of organisms and is fully genetically encoded. In previous work, we showed that lasers could be used to activate LANS in specific cells in the *C. elegans* embryo.^[3] LANSTRAP will allow even tighter control of transcription factors or the epigenome in these types of studies and should provide a valuable approach for studying animal development.

Experimental Section

DNA cloning: All cloning PCR amplifications were performed by using high-fidelity Q5 polymerase, and all preliminary construct screens were performed by colony PCR by using Taq polymerase. All enzymes were purchased from New England Biolabs (NEB). All plasmids were verified by DNA sequencing.

Light illumination setup: The illumination setup for the fluorescence polarization assays was as described in Lungu et al.^[5] Briefly, collimated blue LED with a maximum emission at $\lambda = 455$ nm (Thorlabs) provided 6 mW cm^{-2} illumination to a sample in a 1 cm quartz cuvette.

For the *Saccharomyces cerevisiae* and *C. elegans* experiments, an LED strip with maximum emission at $\lambda = 465$ nm (Mouser Electronics, cat. #: 901-SB-0465-CT) was placed in a 25×35 cm array 15 cm above the samples in an incubator set at 30°C for yeast and 20°C for *C. elegans*, thus obtaining even illumination of 1 mW cm^{-2} .

Isothermal titration calorimetry: Zdk2 was cloned in pQE-80 L vectors by restriction digest with BamHI and HindIII. All purifications were performed as in Yumerefendi et al.^[3] Purified proteins were dialyzed together against 5 L of 20 mM $\text{Na}_2\text{HPO}_4/\text{NaH}_2\text{PO}_4$, pH 7, 100 mM NaCl, and 1 mM dithiothreitol (DTT) overnight at 4°C and automatic titrations performed with MicroCal Auto-iTC200 (GE Healthcare). Each titration consisted of 20 injections, for which $30 \mu\text{M}$ of Zdk2 was in the cell and $250 \mu\text{M}$ of LANS wild-type and lit mimetic (I539E) in the syringe. Measurements were taken at room temperature, 26°C . The baseline of each titration was determined and subtracted from all data points. A heat-change titration curve was fitted to a one-site binding model by using Origin software (OriginLab).

Mammalian cell culture: HeLa and HEK293T (ATCC) tissue cultures were grown at 37°C , 10% CO_2 in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (FBS) and passaged every 2–3 days usually in Nunc T-75 culture flasks (Thermo Scientific).

Mammalian cell imaging: The constructs used in this work were produced and reported by Yumerefendi et al and Wang et al.^[3,7] Coverslips were washed with phosphate-buffered saline (PBS) (GIBCO) and were coated with fibronectin ($10 \mu\text{g mL}^{-1}$) at room temperature for a minimum of 1.5 h. Cells were seeded for 3 h to overnight and were then transfected by using FuGENE 6 (Promega) and imaged after approximately 18 h post-transfection in Ham's F-12 K medium free of Phenol red (Caisson) and containing 10% FBS buffered with HEPES (10 mM, pH 8). Coverslips were mounted in an Attofluor live-cell chamber (Invitrogen) placed in a microscope stage with a heated stage adaptor (Warner) and an objective temperature controller (Biopatch).

An Olympus DSU-IX81 Spinning Disk Confocal coupled with Andor solid-state lasers (Andor) was used to perform the confocal microscopy quantification. Z-stacks of 12 at $0.5 \mu\text{m}$ steps were acquired with a PlanApo 60 \times objective (Oil, N.A. 1.42) by using a $\lambda = 561$ nm laser set at 20% intensity (150 EM gain and 300 ms exposure). Two confocal optical slices ($0.5 \mu\text{m}$) each sectioning through the nucleus were combined and mCherry; fluorescence was quantified for 10 cells of each condition.

Live-cell time-lapse series were collected with an Olympus IX81 epifluorescence microscope equipped with a ZDC focus drift compensator and a Photometrics CoolSnap ES2 CCD camera (Roper Photometrics). A UPlanFLN 40 \times objective (Oil, N.A. 1.30) was used with an ET572/35 \times filter for mCherry detection and 1% (UVND 2.0, ET430/24 \times) for blue-light activation of LANS.

Yeast transcription: The transcription factor used in this study was derived from the pNIA-CEN-MBP plasmid constructed by Yumerefendi et al.,^[3] substituting the MBP gene for that of the Venus fluorescent protein by using restriction digest cloning. The Zdk2 was cloned in yeast vectors designed to integrate at the HO gene locus. After sequence verification of the generated plasmids, they

were digested with NotI, and the product of the reaction was transformed with high-efficiency lithium acetate transformation into the NMY51 strain.

β -Galactosidase assays were performed as follows: Fresh colonies were grown overnight at 30°C in SC-leucine (5 mL). On the next day, the cell density was measured at OD_{600} and the cultures (2 mL) were diluted to $\text{OD}_{600} = 0.2$ in duplicate—one for a light and another for a dark condition (falcon tubes were wrapped in aluminium foil). Cultures were grown at 30°C in a shaking incubator (250 rpm) until they reached $\text{OD}_{600} \pm 0.8$ in the presence or absence of blue light ($\lambda = 465$ nm) at $500 \mu\text{W cm}^{-2}$ by a LED strip wrapped around the tube rack. The resulting cultures were pelleted in triplicate and the β -galactosidase assay with CPRG for a substrate was performed according to the manufacturer's instructions (Clontech).

C. elegans culture and strain construction: The two independent lin-1::lans strains used here were LP268: lin-1(cp70[lin-1(Δ CT)::mkate2::lans1 + LoxP HygR unc-58(e665) LoxP]) IV^[3] and LP298: lin-1(cp72[lin-1(Δ CT)::mkate2::lans1 + LoxP HygR unc-58(e665) LoxP]) IV. LP268 and LP298 are two independent isolates of molecularly identical lin-1::lans alleles. These strains were maintained at 20°C on nematode growth medium by using *Escherichia coli* strain OP50 as a food source. To express Zdk2 in vulval precursor cells, the Zdk2 sequence was codon-optimized for *C. elegans* expression^[16] and inserted into the vector pB255^[17] containing the lin-31 promoter for expression in vulval precursor cells. To generate extrachromosomal arrays, the resulting construct was injected into young adults of strain LP268 or LP298 at a concentration of $50 \text{ ng } \mu\text{L}^{-1}$; $5 \text{ ng } \mu\text{L}^{-1}$ pCFJ104 (Pmyo-3::mCherry^[18]) was included in the injection mix as a marker for extrachromosomal arrays.

Scoring of vulval phenotypes: Scoring of vulval phenotypes was performed exactly as previously described.^[3] Briefly, animals were synchronized by bleaching and allowed to develop under blue LED illumination (see above). Dark controls were placed in the same incubator but wrapped in aluminium foil to prevent light exposure. Mid-L4 animals were imaged by using DIC (for scoring of phenotypes) and mCherry fluorescence (to determine which animals carried the extrachromosomal array). To avoid bias, samples were blinded before imaging. After image acquisition, we first scored each image for phenotype without viewing the mCherry channel; then, we examined the mCherry channel to determine whether each animal was carrying the array. Finally, after scoring was complete, strain background and dark/light condition were revealed. Detailed descriptions of phenotypic categories are given in ref. [3]. Data were plotted by using GraphPad Prism software.

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Conflict of Interest

The authors declare no conflict of interest.

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