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Control of Protein Function through Optochemical Translocation

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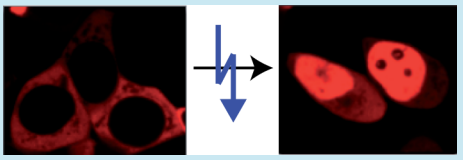
Supporting Information

ABSTRACT: Controlled manipulation of proteins and their function is important in almost all biological disciplines. Here, we demonstrate control of protein activity with light. We present two different applications—light-triggered transcription and light-triggered protease cleavage—both based on the same concept of protein mislocation, followed by optochemically triggered translocation to an active cellular compartment. In our approach, we genetically encode a photocaged lysine into the nuclear localization signal (NLS) of the transcription factor SATB1. This blocks nuclear import of the protein until illumination induces caging group removal and release of the protein into the nucleus. In the first application, prepending this NLS to the transcription factor FOXO3 allows us to optochemically switch on its transcription activity. The second application uses the developed light-activated NLS to control nuclear import of TEV protease and subsequent cleavage of nuclear proteins containing TEV cleavage sites. The small size of the light-controlled NLS (only 20 amino acids) minimizes impact of its insertion on protein function and promises a general approach to a wide range of optochemical applications. Since the light-activated NLS is genetically encoded and optically triggered, it will prove useful to address a variety of problems requiring spatial and temporal control of protein function, for example, in stem-cell, developmental, and cancer biology.



KEYWORDS: *protein control, optogenetics, nuclear import, photocontrolled transcription, photocontrolled TEV-cleavage*

For biological, biophysical, and biochemical studies of the cell it is essential to temporally and spatially control protein function. While drugs have been traditionally used with great success to temporally control protein activity,[1] they are dependent on diffusion and are inherently a bulk technique, which limits temporal control and does not allow for spatial control. In contrast, light offers very precise spatial and temporal control. Hence, optical control of protein function will expand the range of applications already achieved by drug control.[2,3] In general, two concepts have been explored for activity control of proteins with light. One concept makes use of inhibiting a protein's active site by splitting it into two parts, masking it with a caging group, or blocking it in a particular protein conformation. Light-triggered protein dimerization, caging group removal, or conformational change induction, respectively, activate protein function.[4−12] The second method is more indirect and relies on initial mislocalization of the protein, preventing its activity and activation of the protein through optochemical translocation to a specific cellular compartment.[13−15] Blocking the active site of a protein is very specific to the particular protein of interest and often involves optimization via trial and error before it can be adapted to other proteins. Nature controls a multitude of processes in cells via mislocalization and translocation to the active region of a protein (e.g., the cell membrane, the mitochondria, or the nucleus). In many signaling pathways, exclusion from the nucleus keeps key proteins inactive, and they are activated by nuclear import. Guided by Nature's design, we use the nucleus as compartment and mislocalize proteins by retaining them in the cytoplasm, leading to protein inactivity, followed by optochemical translocation into the nucleus and subsequent protein activation. We hypothesize that this represents a more general approach to the conditional control of a wide range of proteins using light as an external trigger, since the same caged nuclear localization signal (NLS) can be readily adapted for various applications. Here, we show two entirely different applications of modifying proteins with spatiotemporal control based on the same mechanism of optochemically triggered nuclear entry. First, we control transcription: nuclear entry of the transcription factor FOXO3 is inhibited by a caged NLS until light-triggered decaging of the NLS releases the transcription factor into the nucleus and switches on transcription and protein expression (Figure 1a). Second, we apply the mechanism to protein cleavage: nuclear entry of TEV-protease is triggered by decaging of the NLS. The protease then translocates into the nucleus, where it cleaves proteins with a TEV-cleavage site. Proteins containing an NLS remain in the

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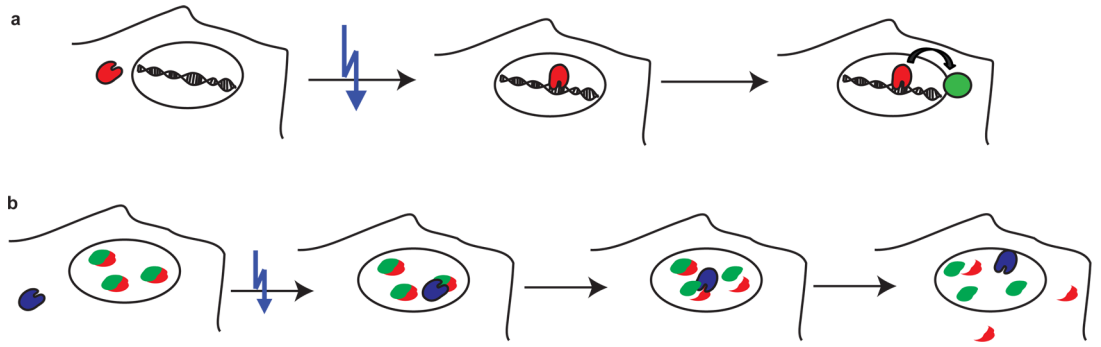

Figure 1. Schematic drawing of the applications: in both cases, light induces nuclear entry of a protein admitting it to its region of activity. (a) A transcription factor (red) is kept in the cytoplasm. Photoactivation releases it to the nucleus where it initiates expression of proteins (green). (b) TEV-protease (blue) is kept in the cytoplasm and enters the nucleus after photoactivation. Proteins in the nucleus (green/red) with a TEV cleavage site will remain intact until the protease enters the nucleus and starts cleavage. Upon cleavage of the protein, its part containing the NLS will remain nuclear (green) whereas the other part (red) will distribute all over the cell.

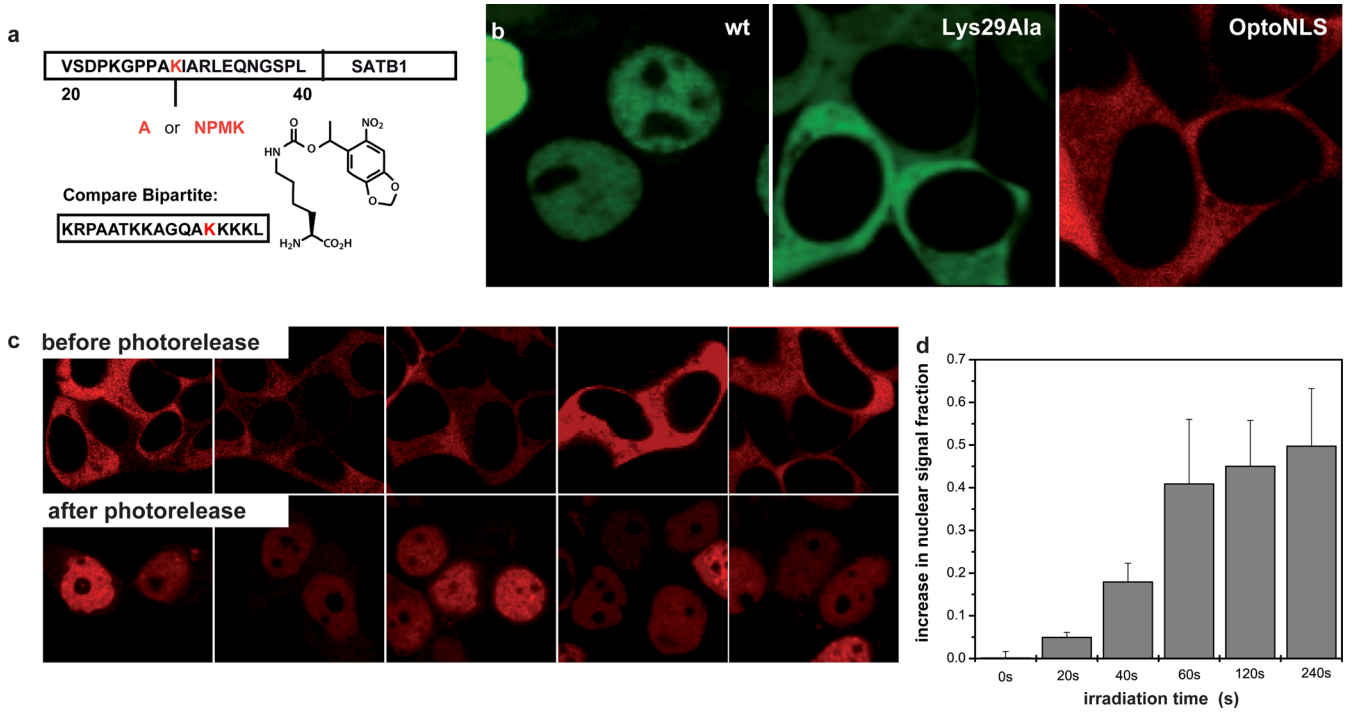

Figure 2. Photocontrol of SATB1 transcription factor nuclear import. (a) Schematic of light-activated nuclear translocation. The protein of interest is excluded from the nucleus by the photocaged NLS. Upon photorelease of the caging group, the protein enters the nucleus. Lys29 in the SATB1-NLS (aa 20–40) is replaced by alanine or lysine, which is photocaged by the nitropiperonylmethyloxycarbonyl group, blocking nuclear import. For comparison the sequence of the bipartite NLS is shown. (b) SATB1 with wildtype NLS (wt) is located in the nucleus (nuclear signal fraction: 0.950 ± 0.087). eGFP-Lys29Ala-SATB1 and eGFP-OptoNLS-SATB1-mCherry are excluded from the nucleus (nuclear signal fractions: 0.037 ± 0.037 and 0.049 ± 0.026, respectively). (c) Before photolysis, eGFP-OptoNLS-SATB1-mCherry is restricted to the cytoplasm (upper panel). Seven hours after illumination (lasting 120 s) with 350 nm UV light, SATB1 is found predominantly in the nucleus. (d) Dose dependence of the optically gated nuclear import. Difference of nuclear intensity of eGFP-OptoNLS-SATB1-mCherry before illumination and 2 h after a range of UV illumination times.

nucleus, but others diffuse throughout the cell after cleavage by TEV (Figure 1b). We believe that these initial applications lay the foundations for broad usage of the presented optochemical NLS control.

RESULTS AND DISCUSSION

Construction of Optochemically Controlled Nuclear Import System. In order to realize these applications through controlling protein function by optochemically triggered translocation into the nucleus, a tight photoactivatable nuclear entry mechanism is needed. The absence of any background activity or leakiness is very important, since a single transcription factor in the nucleus could start protein expression. Similarly, with time a single protease in the nucleus can cleave a detectable amount of proteins. Recently, we have reported a technique that employs site specific-genetic encoding of a photocaged lysine (NPMK, nitropiperonylmethyloxycarbonyl lysine) into a bipartite NLS to allow for nuclear entry upon illumination and decaging.[13] Although the system shows prompt import after photoactivation, leakage into the nucleus before activation is significant. These properties render the method useful for nuclear import studies but not for

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protein manipulation. However, the rapid response upon illumination encouraged us to further investigate this approach. A less promiscuous NLS that is completely deactivated by mutation of one single amino acid combined with the photocaged amino acid could yield tight optical control of nuclear entry and provide the basis for a wide range of applications.

Canonical NLSs like bipartite or SV40 are all fairly promiscuous.[16] Out of the manifold of native NLSs of proteins[17] that of the transcription factor SATB1[18,19] has properties that appear promising for successful photocaging. It is a single 20 amino acid domain that lacks the clusters of basic residues associated with the classical NLS (Figure 2a).[20] Furthermore, it can be prepended to heterologous proteins such as GFP to control their nuclear import indicating its potential multifunctional use for import of a variety of proteins. Most importantly, Nakayama et al. demonstrated that upon mutation of one single lysine to alanine in SATB1s NLS no nuclear import was detectable (Figure 2b), whereas the native NLS without mutation led to almost complete nuclear import.[20] This discovery was further validated by us. The sensitivity of the SATB1 NLS to a single amino acid change at position 29 makes it a good candidate for leakage-free photocaging as needed for applications of protein activity control. Thus, we constructed a plasmid that encodes both parts needed for the site-specific incorporation of photocaged lysine, the engineered pyrrolysyl tRNA synthetase and its cognate tRNA from *M. barkeri*. Furthermore, we altered the SATB1 gene by mutating Lys29 in the SATB1 NLS to the TAG amber stop codon and flanked the peptide-coding region with sequences for eGFP and mCherry. Without amber suppression, only eGFP should be expressed, whereas upon successful suppression, photocaged lysine should be incorporated, yielding an eGFP-K29NPMK-SATB1-mCherry fusion protein. The chosen experimental design ensures that full-length SATB1 is only expressed in the caged form, since the stop codon is located at the very beginning of SATB1's coding sequence. For simplicity, we will refer to the SATB1-derived K29-NPMK-NLS as the "OptoNLS".

As shown in Figure 2c, transfected cells display an mCherry signal, denoting successful incorporation of photocaged lysine and expression of eGFP-OptoNLS-SATB1-mCherry. Most importantly, eGFP-OptoNLS-SATB1-mCherry is restricted to the cytoplasm and no leakage into the nucleus is detectable. As expected, eGFP can be found in the cytoplasm as well as in the nucleus, since the truncated eGFP protein (28 kDa) resulting from incomplete suppression of the TAG codon is below the size cutoff for passive diffusion into the nucleus (Supporting Information Figure S1).[21] Irradiation with UVA light of about 350 nm (DAPI filter cube) decaged the lysine, generating the native SATB1 sequence, which then entered the nucleus (Figure 2c). The amount of nuclear SATB1 transport was dependent on the illumination time, and there was no import without illumination (Figure 2d). After sufficient illumination and reaction time, the fraction of nuclear SATB1 was almost that of the protein with a native NLS as displayed in Figure 2c, which was imaged 7 h after a 120 s UV exposure. Since we could not detect any leakage but could recover nuclear import with illumination, the OptoNLS seemed to represent a good basis for our applications of optochemical protein function control through protein mislocalization. The only drawback of the SATB1 NLS was that SATB1 import took several hours to saturate post illumination. Other import cargoes with a canonical NLS usually take only minutes.[22,23] The slow import does not necessarily need to originate from the NLS though—it could also be due to the protein's properties.

Optochemically Controlled Transcription Factor Activity. First, we designed an optochemical control of transcription. We chose FOXO3, a forkhead transcription factor implicated in regulation of apoptosis and in tumorigenesis, as a target for OptoNLS control.[24,25] A well-established GFP reporter system for detecting FOXO3 activity exists, based on a minimal promoter and tandem repeats of the FOXO transcriptional response element.[26] We replaced FOXO3's native NLS with the OptoNLS and fused mCherry to FOXO3's C-terminus, ensuring that mCherry is expressed only after successful incorporation of the photocaged lysine (Figure 3a).

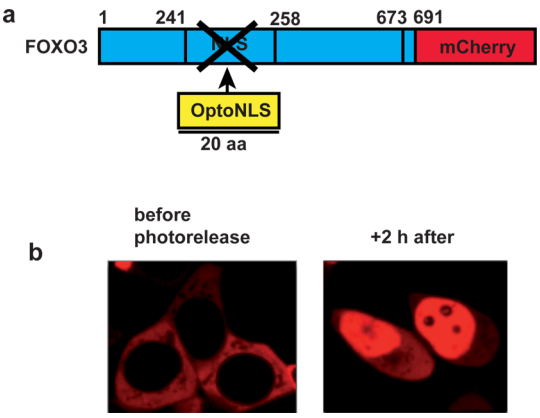

Figure 3. Photocontrol of transcription factor FOXO3. (a) The construct of OptoNLS-FOXO3-mCherry has the OptoNLS inserted at the site of the native NLS. (b) OptoNLS-FOXO3-mCherry (left) is initially excluded from the nucleus but efficiently enters the nucleus after photorelease (right, 120 s illumination, mean nuclear signal fractions: 0.06 ± 0.03 and 0.64 ± 0.16, respectively).

As shown in Figure 3b, suppression and incorporation of photocaged lysine was achieved and OptoNLS-FOXO3-mCherry was efficiently excluded from the nucleus before irradiation. Importantly, brief illumination of the cells with UV light led to rapid entry of OptoNLS-FOXO3-mCherry into the nucleus (Figure 3b). Hence, the photocaged SATB1-NLS retains its desirable properties when prepended to heterologous proteins. The import of OptoNLS-FOXO3-mCherry took only ~5 min to fully equilibrate (Supporting Information Figure S2, Movie S3), showing that the origin of OptoNLS-SATB1's slow import was indeed the SATB1 protein and not an inherent deficiency of the OptoNLS. Next, we tested whether OptoNLS-FOXO3 retained its ability to activate transcription and thus light-control of the activity of FOXO3 could be obtained. We transfected cells with OptoNLS-FOXO3-mCherry and the GFP transcription reporter system that leads to GFP expression upon FOXO3 activity. A functional optochemical control of FOXO3 transcription activity should thus lead to cytoplasmic FOXO3 and absence of GFP expression before illumination and nuclear entry followed by GFP expression after illumination (Figure 4a). As expected, the OptoNLS-FOXO3-mCherry was cytoplasmic before illumination (Figure 4b, left panel). Without illumination, even >50 h after transfection, no GFP expression was detected, showing that the OptoNLS allows tight control of gene expression. After illumination, FOXO3 entered the nucleus (Figure 4b), and about 15 h later, GFP expression was detected (Figure 4c). This demonstrates

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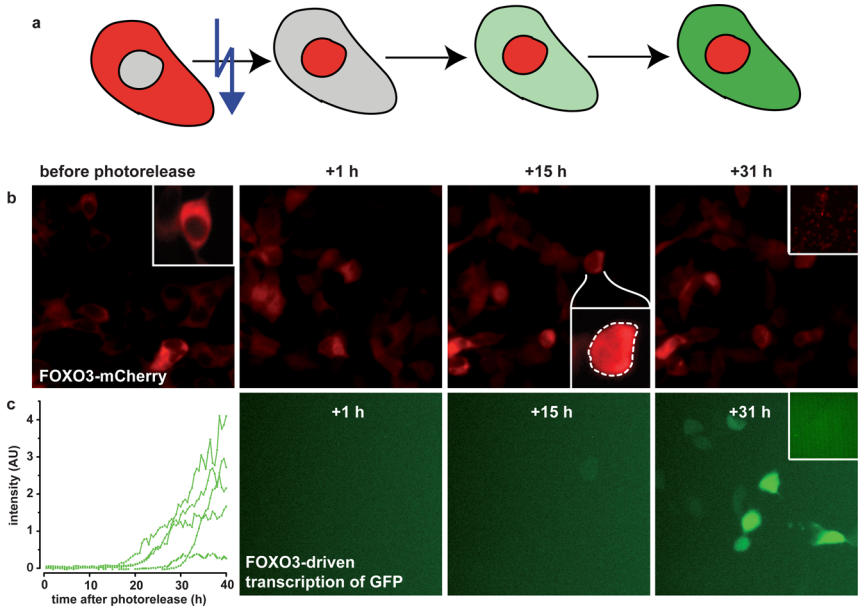

Figure 4. Photocontrol of FOXO3-mediated transcription. (a) Schematic drawing: A transcription factor (red) is kept in the cytoplasm. Photoactivation releases it to the nucleus where it initiates expression of proteins (green). (b) Nuclear import of OptoNLS-FOXO3-mCherry (red). Before photorelease, OptoNLS-FOXO3-mCherry is excluded from the nucleus. Inset, zoom of single cell. Dashed line denotes nuclear envelope; inset colorscale cropped from 0 to 255 to 0−100 to emphasize absence of detectable leakage into nucleus. After photorelease, OptoNLS-FOXO3-mCherry is actively transported into the nucleus. Inset at t = +15 h: zoom of single cell showing accumulation in nucleus. Dashed line denotes nuclear envelope; inset colorscale cropped as before. (c) Transcriptional activation of a FOXO3-driven GFP reporter (green) upon optically triggered nuclear import of OptoNLS-FOXO3-mCherry. The GFP signal is readily detectable at t > 17 h after photorelease of OptoNLS-FOXO3-mCherry. Each trace in the intensity vs time plot represents one nucleus. The images in parts b and c are the mCherry- and GFP-channel of the same image. The inserts in the last image are a control without UV illumination, where even after 39h no GFP could be detected.

the successful application of the OptoNLS for photochemical control of the transcription activity of FOXO3. The fraction of GFP expressing cells is fairly low due to the fact that only cells, which received all three plasmids, (NPMK-pylRS/pyltRNA, OptoNLS-FOXO3, and the FOXO3-driven reporter) express GFP. However, in future applications, this can be improved by constructing stably expressing cell lines.

Optochemically Controlled Protease Cleavage. Since the OptoNLS allowed for tightly controlled transcriptional activity showing no leakage, we designed another application: optochemically controlled protease cleavage. This allows specific target complexes in the nucleus to be cleaved upon optically triggered protease translocation. We employed TEV as the light-triggered protease and SATB1 as the target. To construct the photocontrolled protease, we prepended the OptoNLS to TEV protease (Figure 5a). Then, we appended two CFP proteins to increase its molecular weight beyond the passive diffusion limit[21] and thus reduced potential leakage of TEV into the nucleus prior to decaging. Next, we generated the target. We introduced a TEV cleavage site into SATB1 immediately following its PDZ-like domain, the region of natural sumoylation-triggered SATB1 cleavage.[27] Additionally, we flanked SATB1 with GFP and mCherry (Figure 5a). Thus, before optochemical activation the TEV protease should remain cytoplasmic and the mCherry-SATB1^{TEV}-GFP fusion protein should be localized in the nucleus. Upon illumination, TEV should enter the nucleus and cleave SATB1. The part of SATB1 fused to GFP retains its NLS and should stay nuclear whereas the mCherry part without NLS will be distributed all over the cell via passive diffusion from the nucleus (Figure 5b).

As shown in Figure 5c, the GFP-SATB1^{TEV}-mCherry target responded to optical decaging and translocation of the OptoNLS-TEV as expected. Before photorelease of TEV, both GFP (green) and mCherry (red) were colocalized in the nucleus, yielding a yellow signal. No cleavage of SATB1 could be detected, demonstrating that the OptoNLS offers background-free protease cleavage. After light-induced translocation of OptoNLS-TEV, mCherry (red) was increasingly detected in the cytoplasm, whereas the GFP (green), which is attached to the NLS containing part of SATB1, remained confined to the nucleus. This successful optochemical trigger of TEV protease activity can now be used to spatiotemporally control cleavage of any nuclear protein that can be constructed with a TEV cleavage site. It allows for switching off protein function in the nucleus for cell biology studies as well as releasing protein parts that show cytoplasmic activity into their active compartment. Furthermore, the light-triggered system can be used to create well-defined conditions for studies of the effect of cleavage by enzymes such as caspases by inserting a TEV cleavage site into the cleaved target protein at the original cleavage site.

In conclusion, we have demonstrated the optochemical control of two different processes—gene transcription and protease cleavage—through the application of tightly regulated light-controlled nuclear import. We used a genetically encoded photocaged lysine at position 29 of the SATB1 NLS to control nuclear import of proteins that were originally mislocalized to the cytoplasm. The developed methods should leverage studies in developmental, stem cell, and cancer biology. They can be used to trigger expression or cleavage of a manifold of proteins. The small size of the OptoNLS (20 amino acids) minimizes its impact upon insertion and promises use of the system for nuclear entry control of a wide range of proteins. The OptoNLS approach should also function in transgenic animals

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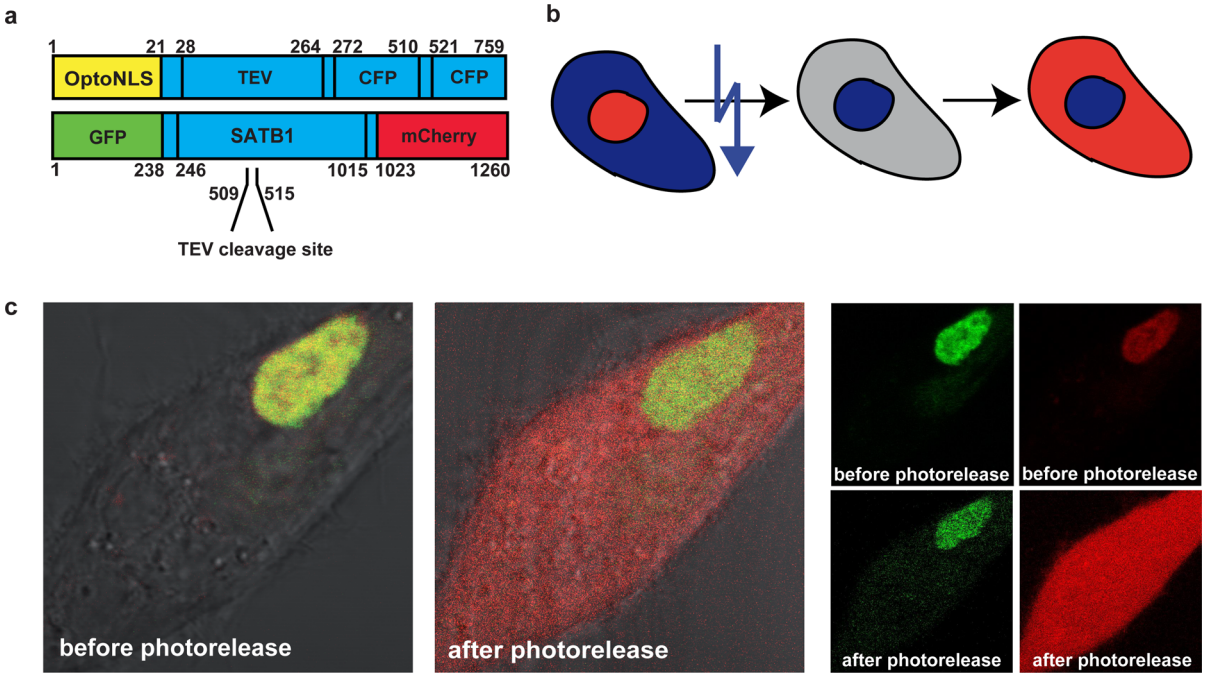

Figure 5. Photocontrol of intranuclear SATB1 cleavage. (a) Photocontrolled delivery of the TEV protease into the nucleus of MCF10A cells and subsequent specific cleavage of a transcription factor. The TEV protease has a prepended OptoNLS and two attached CFPs, to block passive diffusion into the nucleus. A TEV protease site was engineered into the SATB1 transcription factor, which was also flanked by two fluorophores to allow intranuclear cleavage to be visualized. (b) TEV-protease (blue) is kept in the cytoplasm and enters the nucleus after photoactivation. SATB1 in the nucleus (yellow) containing a TEV target site will remain intact until the protease enters the nucleus and starts cleavage. Upon cleavage of the protein, its part containing the NLS will remain nuclear whereas the other part (red) will distribute all over the cell. (c) Fluorescence images: before photorelease of the TEV protease, GFP (green), and mCherry (red) are colocalized and fully contained in the nucleus (left panel), confirming that an intact SATB1 is expressed and localized in the nucleus. After photorelease of TEV, it enters the nucleus and there it cleaves SATB1. The green N-terminal fragment stays in the nucleus since it has a functional NLS; the red C-terminal fragment transitions to the cytoplasm (middle). (right) Images split into single color channels.

such as worms and flies, since their genetic code has recently been expanded with unnatural amino acids.[28,29]

METHODS

Cloning. The PCKRS-PyltRNA plasmid was constructed from PCKRS and PyltRNA plasmids[13] using NheI-MfeI and SpeI-*Eco*RI, respectively. The two fragments were then religated. Mutants of Lys29 in the SATB1 NLS were generated with the QuikChange method introducing either the TAG or alanine codon. GFP-SATB1 was a gift from the T. Kowhi-Shigematsu lab. FOXO3 (Addgene plasmid 14937) was obtained from the J. Massagué lab[30] eGFP-OptoNLS-SATB1-mCherry, OptoNLS-FOXO3-mCherry, OptoNLS-TEV-CFP-CFP, and GFP-SATB1-mCherry with a TEV cleavage site in a Cumate expression vector were generated using the SLIC technique.[31]

Transfection and Tissue Culture. Hek 293T cells were grown in DMEM with 10% FBS and plated on 35 mm dishes (MatTek corporation). Transfection was performed with Lipofectamine (Invitrogen) according to manufacturer's instructions using 4 μg of each plasmid required for the specific experiment. After transfection, caged lysine was added yielding a final concentration of 2 mM. Cells were incubated for 24 h until imaging. For the TEV cleavage experiment, MCF10A cells stably selected for the Cumate inducible expression system (System Biosciences) were grown in growth medium[32] and transfected using the neon electroporator (Invitrogen) with 3 μg of each plasmid. Cumate was added for the first 10 h to allow for SATB1 expression. After ~10 h, caged lysine was added yielding a final concentration of 2 mM to allow for TEV expression and Cumate was removed to stop SATB1 expression and avoid premature cleavage in the cytoplasm before import into the nucleus. Cells were then incubated for another ~6 h. Before imaging, in all experiments, medium was replaced with DMEM containing no caged lysine. Nuclear import of the FOXO3 transcription factor was monitored with the Cignal FOXO GFP Reporter system (CCS-6022, www.sabiosciences.com), which consists of the GFP gene under the control of a minimal (m)CMV promoter and tandem repeats of the FOXO transcriptional response element.

Imaging and Analysis. Confocal imaging was performed on an Axiovert 700 (Zeiss) using 488 and 555 nm lasers and a 63× oil immersion objective (NA = 1.4, Zeiss). Time lapse imaging and photolysis were performed on an inverted epifluorescence microscope (Olympus, IX81) at an intensity of about 50 μW in the back aperture of the objective. Photorelease was performed using a 20× objective (Olympus, AchN) and a DAPI filter (Ex: 350/50 nm, Chroma), and imaging was conducted with a GFP filter (Ex: 470/40 nm. Em: 525/50 nm, Chroma) and an mCherry filter (Ex: 560/40 nm, Em: 630/75 nm, Chroma). Image analysis was performed using ImageJ and Matlab.

ASSOCIATED CONTENT

Supporting Information

Figures on suppression efficiency and FOXO3 import kinetics, movies on SATB1 import and control, FOXO3 import, and FOXO3 transcription, description of the calculation of the

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nuclear signal fraction. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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