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Technical Note

Analysis of Slow-Cycling Variants of the Light-Inducible Nuclear Protein Export System LEXY in Mammalian Cells

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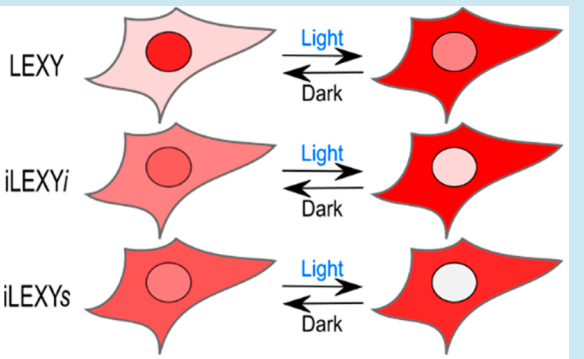
ABSTRACT: The optogenetic tool LEXY consists of the second light oxygen voltage (LOV) domain of *Avena sativa* phototropin 1 mutated to contain a nuclear export signal. It allows exporting from the nucleus with blue light proteins of interest (POIs) genetically fused to it. Mutations slowing the dark recovery rate of the LOV domain within LEXY were recently shown to allow for better depletion of some POIs from the nucleus in *Drosophila* embryos and for the usage of low light illumination regimes. We investigated these variants in mammalian cells and found they increase the cytoplasmic localization of the proteins we tested after illumination, but also during the dark phases, which corresponds to higher leakiness of the system. These data suggest that, when aiming to sequester into the nucleus a protein with a cytoplasmic function, the original LEXY is preferable. The iLEXY variants are, instead, advantageous when wanting to deplete the nucleus of the POI as much as possible.



KEYWORDS: *LEXY, iLEXY, LOV domain, optogenetics, nuclear protein export, NES*

Optogenetics is a technique particularly suited to control protein localization with high spatiotemporal precision.[1] An exemplary optogenetic tool useful in regulating the localization of a protein of interest (POI) in and out of the eukaryotic cell nucleus is the blue light-inducible nuclear protein export system LEXY.[2] Like many other optogenetic tools based on the second light oxygen voltage (LOV) domain of *Avena sativa* phototrophin 1 (*As*LOV2 domain), LEXY exploits the conformational change that this domain undergoes upon exposure to blue light. In the dark, the nuclear export signal (NES) embedded within the Jα helix of the *As*LOV2 domain is concealed from the endogenous CRM1 receptors and, as a consequence, the POI fused to LEXY is retained in the nucleus due to a nuclear localization signal (NLS) added to the fusion construct or present in the POI itself (Figure 1A). Upon absorption of a blue light photon, the Jα helix undocks from the core LOV domain and unfolds, leaving the NES free for recognition, followed by the export of the POI fused to LEXY into the cytoplasm (Figure 1A). Quickly after blue light illumination is ceased, the POI accumulates back into the nucleus because the *As*LOV2 domain reverts back to the dark state within about 80 s[3] making the NES again inaccessible and letting the NLS take over. LEXY has been shown to work robustly in many mammalian cell lines,[4,5] in *Xenopus* epithelial cells and mouse zygotes,[6] and *Drosophila*.[7,8]

In a recent study, focused on understanding how the transcription factor Twist functions in *Drosophila* embryos, the LOV domain within LEXY was mutated, and two variants, iLEXY*i* and iLEXY*s*, were obtained.[9] The authors chose these names to indicate that these variants are an improvement to the original LEXY, allowing for a more complete depletion of Twist from the nucleus and the usage of lower light intensities or longer intervals between light pulses. The mutations (V416I in iLEXY*i* and V416L in iLEXY*s*; Figures 1B and 1C) slow down the dark reversion rate of the LOV domain.[10] iLEXY*i* is *intermediate* between the original LEXY and the *slow* iLEXY*s* variant.[9]

Here we show that iLEXY*i* and iLEXY*s* do not represent an improvement to the original LEXY when wanting to control proteins with cytoplasmic rather than nuclear functions. Nonetheless, iLEXY*i* can perform better than LEXY in mammalian cells, depending on the POI, when low light intensities for the illumination are used.

RESULTS

We introduced the V416I (iLEXY*i*) and V416L (iLEXY*s*) single point mutations individually in a plasmid encoding the

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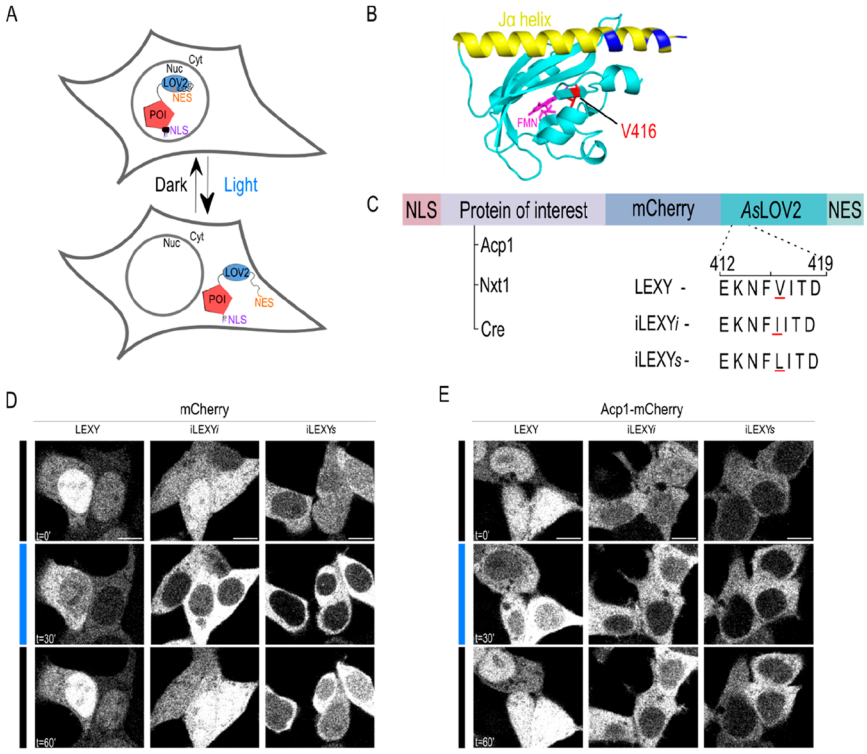

Figure 1. Comparison between LEXY and iLEXY in mammalian cells. (A) Schematic representation of the mechanism of action of LEXY: POI, protein of interest; LOV2, second light oxygen voltage domain of *Avena sativa* phototropin 1; NLS, nuclear localization signal; NES, nuclear export signal; Nuc, nucleus; Cyt, cytoplasm. (B) 3D model structure of LEXY obtained from AlphaFold2 using ColabFold.[12] The Jα helix is colored in yellow, the mutations introduced to incorporate the NES into the Jα helix[2] are colored in blue (A542L, A543L, P547A, A549L, 552D), V416 is colored in red and the rest of the protein is shown in cyan. The position of the chromophore flavin mononucleotide (FMN; shown in magenta) is aligned from PDB id: 2v0u. (C) Schematic representation of the constructs used in this study. The components (NES, NLS, and proteins) are not drawn to scale. (D,E) Representative confocal fluorescence microscopy images of HEK 293E cells transiently transfected with the indicated constructs. Cells were imaged prior to the illumination (upper row), after 30 min of blue light illumination (second row), and after recovery in the dark for additional 30 min (third row). Scale bar, 10 μm.

red fluorescence protein mCherry fused to LEXY under the strong CMV promoter (Figure 1C). The N-terminal NLS ensures nuclear localization of mCherry in the dark. We then transiently transfected each construct in HEK 293E cells and performed optogenetic stimulation of live cells under the microscope. While all constructs were light-responsive and reversible, iLEXY*i* and iLEXY*s* were characterized by a marked cytoplasmic localization of mCherry *prior* to blue light illumination (Figures 1D and 2A). This feature was observed regardless of the POI being controlled (Figures 1C−E and 2A and S1 and Table S1). Because the proteins were much more cytoplasmic when fused to iLEXY*i* and iLEXY*s* than to LEXY, they could be depleted from the nucleus during the blue light illumination phase to a higher extent (Figure 2A); this was particularly true for the iLEXY*s* constructs. However, they took much longer to accumulate back into the nucleus after blue light illumination was stopped ($\tau_{1/2} \sim 269.5$ s for mCherry-LEXY vs $\tau_{1/2} \sim 636.6$ s for mCherry-iLEXY*s*; Table S2). When normalizing the data to the localization at the start of the experiment, the difference between the LEXY and iLEXY*i* constructs disappeared in most cases, with the exception of the Acp1-iLEXY*i* construct, which was worse in terms of both light/dark fold change and kinetics of recovery (Figures 2C and S2). All constructs were always worse in terms of nuclear/cytoplasmic ratio at the start of the experiment and in terms of kinetics of recovery when fused to iLEXY*s* (Figure 2A). The nuclear/cytoplasmic ratio for the iLEXY variants at the beginning of the experiment was lower in all analyzed cells, regardless of the expression levels of the POI (Figure S3).

Last, we tested if the iLEXY variants would perform better than the original LEXY in mammalian cells when using light regimes featuring less light, in terms of either intensity or frequency of delivery, as shown by Kögler and colleagues in *Drosophila* embryos.[9] In this case, we only employed the mCherry and Nxt1-mCherry constructs. Nxt1 was selected because it was the POI for which iLEXY*i* performed most similarly to LEXY (Figure 2A). We applied two light regimes: one in which the interval between successive illuminations was longer (60 s instead of 30 s used in the first set of experiments), and one in which the light intensity was reduced 5 times (2% (~5.7 W/m^2) vs 10% (~28.5 W/m^2) used in the first set of experiments). Compared to the light regime we used in Figure 2A, there was only a slight increase in the light/dark fold change for the Nxt1-mCherry-iLEXY*i* construct for the first light regime (Figures S4A and 2B); the increase was more pronounced for the second light regime, characterized by 5 times lower light intensity (Figures 2B, 2D, and S4B).

DISCUSSION

LEXY is an optogenetic tool that allows reversibly controlling with blue light the nuclear export of POIs. Here we compared the behavior in mammalian cells of the original LEXY[2] to that of two slow-cycling variants, iLEXY*i* and iLEXY*s*, which have been analyzed in *Drosophila* cells and embryos.[9] The data

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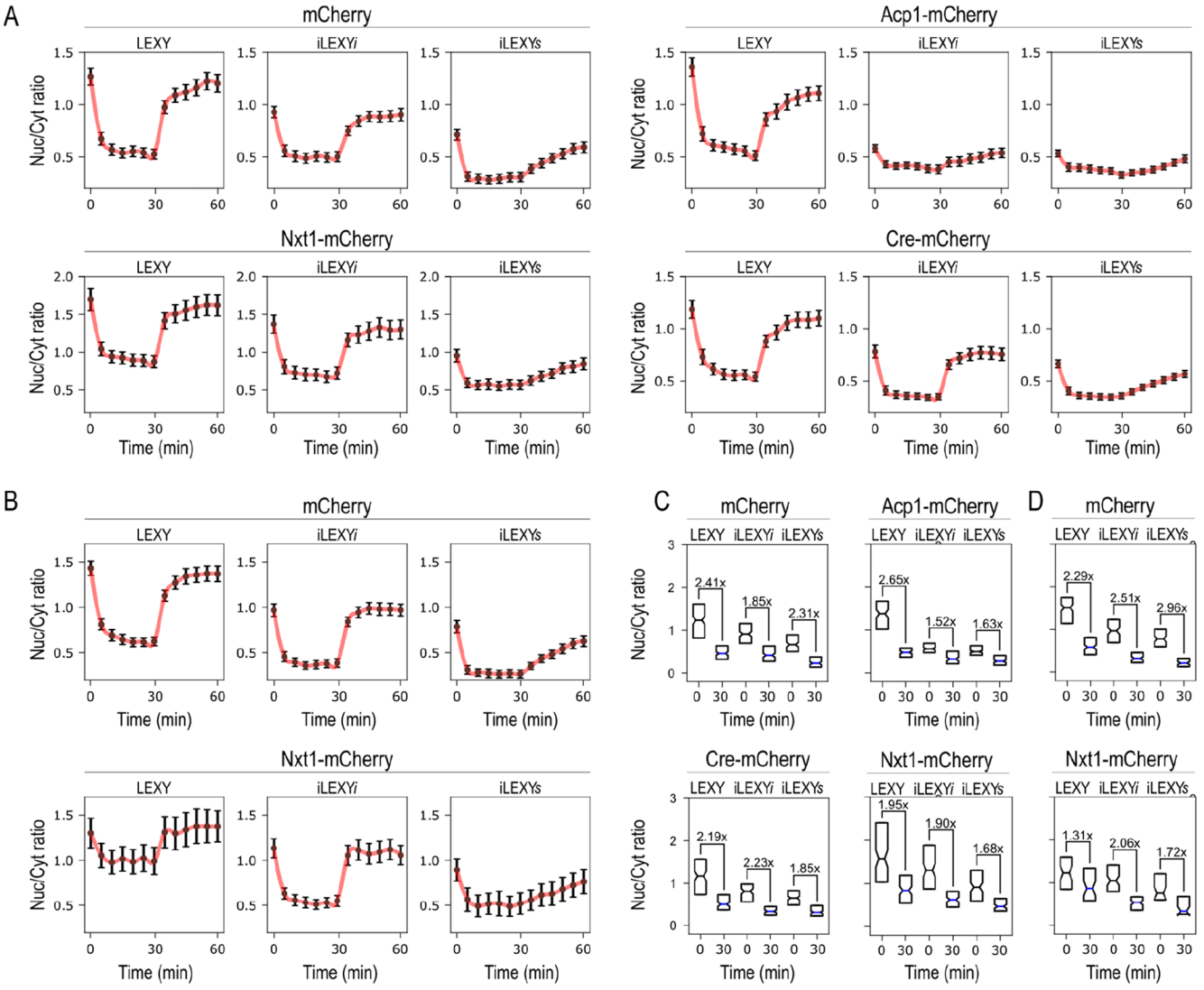

Figure 2. Quantification of the extent and kinetics of nuclear export obtained with LEXY and iLEXY in mammalian cells. (A,B) Graphs showing the nuclear/cytoplasmic (Nuc/Cyt) ratio of the mCherry fluorescence intensity over time for the indicated constructs in HEK 293E cells when using 10% (A) and 2% (B) light intensity to activate the export. 2% light corresponds to ~5.7 W/m^2; 10% to ~28.5 W/m^2. The data represent the mean ± standard error of the mean for n = 3 independent experiments. (C,D) Box plots showing the nuclear/cytoplasmic (Nuc/Cyt) ratio *prior* to the illumination (time 0) and after 30 min of blue light (time 30 min; in blue) for the indicated constructs. The interval between successive illuminations was 30 s. The light intensity was 10% in (C) and 2% in (D). The light/dark fold change is indicated in the graph for each construct.

indicate that iLEXY is not "generally" an *improved* version of LEXY, since the mutations increase the leakiness of the system (Figures 1D,E and 2 and Figures S1 and S2), a clear disadvantage if the POI to be controlled has a cytoplasmic function. In this case, the original LEXY is actually preferable. The iLEXY variants are better suited to obtain as complete a nuclear depletion as possible, which can be crucial when aiming to regulate proteins with nuclear functions such as transcription factors. Moreover, the iLEXY variants performed better in terms of light/dark fold change than LEXY when we used lower light intensity (Figure 2D). This is in agreement with the results obtained by Kögler and colleagues in *Drosophila*[9] and suggests that they might be preferable when low light intensities are required.

Mutating V416 to I or L slows down the time taken by the Jα helix to return to the docked, folded state.[10] Another mechanism that could also explain the data is that V416 affects the frequency at which the Jα helix undocks and unfolds. While V416 is not located within the Jα helix, residues 406 and 407, also located outside of the Jα helix, have been reported to increase the frequency of Jα helix undocking,[11] thus suggesting that position 416 might exert a similar role.

To explore the molecular effects of the two mutations at position 416 on the intramolecular signal propagation within the LOV domain, we conducted noncovalent bond network analysis of WT and mutant proteins (see Materials and Methods). While the noncovalent bond network formed by residues in the Jα helix is unchanged for the mutants compared with the WT, having I or L at position 416 causes the disappearance of a hydrogen bond from the network of noncovalent bonds formed by the residue at position 416 (Figure S5). Potentially, the loss of this bond might lead to a conformational change that could allosterically affect the frequency of the Jα helix undocking. At this stage this is a mere speculation; detailed molecular dynamics simulations or NMR experiments are required to clarify whether this allosteric

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effect is present in iLEXY. Regardless whether due only to slower reversion rate or to a mixture of this and more frequent helix undocking, the macroscopic consequence of the mutations is that the NES is exposed for a longer time in the absence of light. It is not surprising, therefore, that these variants have higher cytoplasmic accumulation in the dark than the original LEXY.

In conclusion, we recommend potential users of LEXY to carefully analyze their specific biological question and/or experimental requirements before deciding between LEXY and iLEXY*i*/*s*.

MATERIALS AND METHODS

The materials and the methods used in this study are described in the Supporting Information.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acssynbio.2c00232.

Materials and methods. Supplementary results (Figures S1−S5, Table S3). Key features of the proteins used in this study (Table S1). List of primers and plasmids used in this study (Tables S3 and S4) (PDF)

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Author Contributions

#G.F., E.B.A., and D.W. contributed equally to this work. B.D.V. conceived the study. E.B.A., B.Q., and D.B. cloned the constructs. G.F. performed fluorescence microscopy. D.W. and E.B.A. analyzed the data. M.A.O. performed computational analyses. G.F. and E.B.A. prepared the figures. E.B.A. and B.D.V. supervised the study. B.D.V. wrote the manuscript.

Notes

The authors declare no competing financial interest.

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