VIP Optopharmacology

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Light-Regulated Stapled Peptides to Inhibit Protein–Protein Interactions Involved in Clathrin-Mediated Endocytosis**

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Protein–protein interactions (PPIs)[1] are essential in almost every biological process. Recently, PPIs have emerged as therapeutic targets of interest,[2] and ligands directed to interactions mediated by α-helices have been systematically developed.[3] Several linear and cyclic peptides,[4] including stabilized α-helical "stapled" peptides,[5] able to inhibit key cancer-related targets have been reported.

Since PPIs are precisely orchestrated in cells, it would be useful to complement the pharmacological selectivity of peptide inhibitors with a means of controlling their kinetics and site of action. In this regard, one option would be to regulate the activity of PPI inhibitors with light, thus controlling their effects with spatiotemporal patterns of illumination.[6] Photosensitive crosslinkers have been introduced in peptides or small proteins to regulate their structure[7] and binding properties.[8] Here we present a peptide design strategy to obtain photoswitchable inhibitors of protein–protein interactions (PIPPIs) based on helical interaction motifs and apply it to photoregulate clathrin-mediated endocytosis (CME) in living cells.

CME is a key process in all eukaryotic cells. It supports a wide range of functions, including the regulation of surface expression of proteins, the uptake of nutrients, the control of cell signaling, and the turnover of membrane components.[9] CME encompasses a complex network of PPIs involving many and diverse protein families. The main adaptor protein of this machinery is the AP2 complex, which mediates the binding of clathrin to the membrane or to cargo receptors,[9] with or without the help of accessory proteins (such as β-arrestin, AP180, and epsin). In addition to the small-RNA-interference strategy,[10] the screening of small-molecule libraries has led to the identification of new CME inhibitors that target clathrin[11] and dynamin.[12] These inhibitors are membrane-permeable, can be applied directly to cells, and dramatically perturb clathrin-coated pit (CCP) dynamics in minutes; however, their action cannot be localized and is reversible only after a long washing time (1–2 h).

One way to tackle this limitation would be to selectively target specific PPIs to turn CME on and off in designated cell populations or subcellular regions. Here we developed PIPPIs targeted to AP2, the best characterized hub of the CME interactome. These inhibitors, which we call traffic light (TL) peptides, are cell-permeable photoregulators of CME that enable "stop" and "go" signals to control membrane endocytosis, thus allowing the spatiotemporal patterning of membrane receptor internalization in living cells.

Our design strategy (Figure 1 a, b) was based on the structure of the β-arrestin C-terminal peptide bound to the β-appendage of AP2 (β-adaptin).[13] This peptide (BAP-long, Figure 1 c) binds to β-adaptin with an α-helical structure, in which four conserved residues of the DxxFxxFxxxR motif (red in Figure 1 a–c) are aligned along the helix side facing the binding pocket. Since the stability of the secondary structure of helical peptides has been correlated with the capacity to interact with their target protein,[5] we reasoned that it should be feasible to reversibly regulate peptide affinity by using techniques to photocontrol their secondary structure.[7,14] We tested this idea by conjugating the photoisomerizable crosslinker 3,3′-bis(sulfonato)-4,4′-bis (chloroacetamido)azobenzene (BSBCA)[15] between pairs of cysteines introduced in the BAP-long sequence (yellow residues in Figure 1 a–c) in order to reversibly change the stability of the helix by using 380 nm and 500 nm light (wavelengths that favor the *cis* and *trans* isomers, respectively, indicated in purple and blue-green in Figure 1 a,b). The choice of cysteine substitution sites of the TL peptides was based on two criteria. First, we applied the

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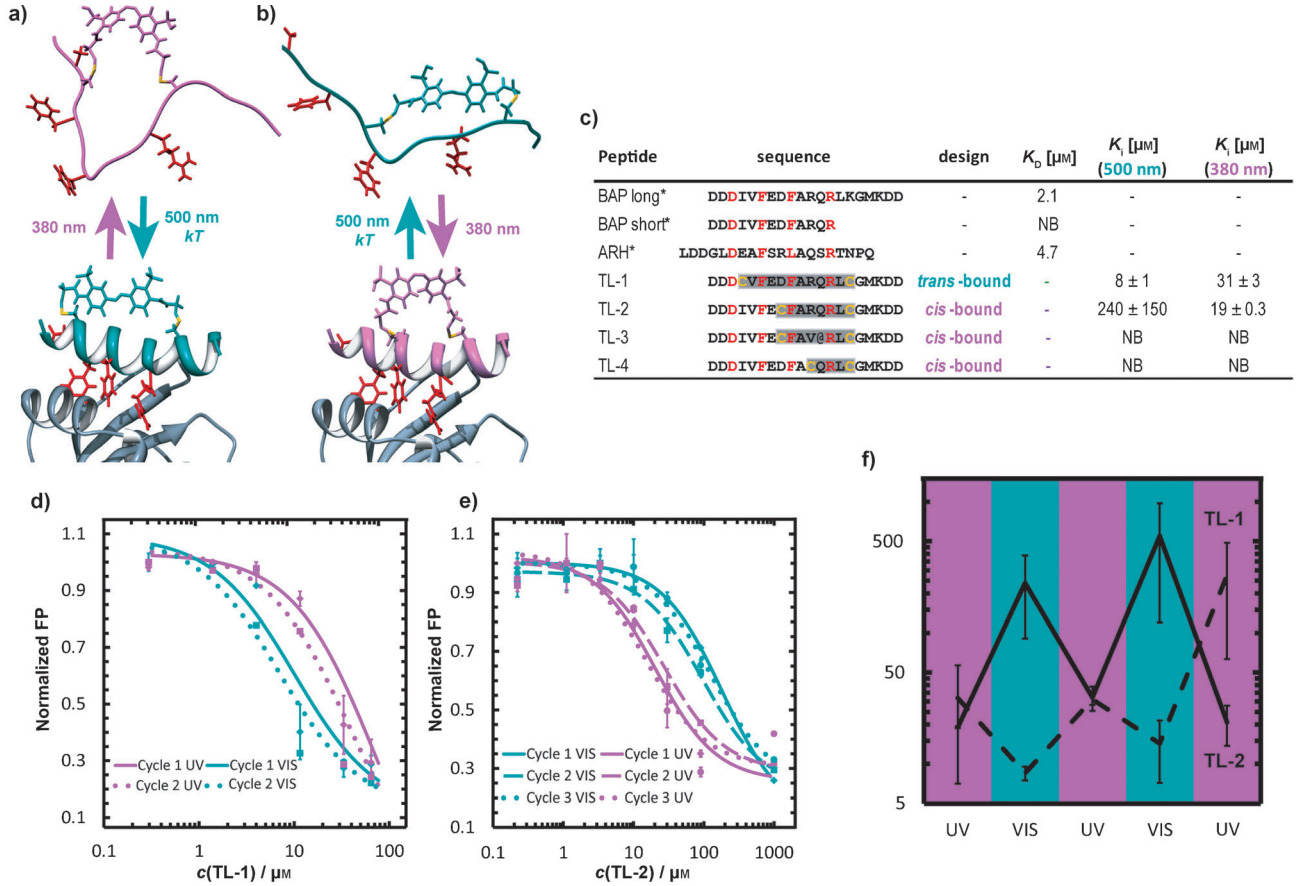

Peptide	sequence	design	K_D [μM]	K_i [μM] (500 nm)	K_i [μM] (380 nm)
BAP long*	DDDIVFEDFARQRLKGMKDD	-	2.1	-	-
BAP short*	DDDIVFEDFARQR	-	NB	-	-
ARH*	LDDGLDEAFSRLAQSRTNPQ	-	4.7	-	-
TL-1	DDD-VFEDFARQRL-GMKDD	*trans*-bound	-	8 ± 1	31 ± 3
TL-2	DDDIVFE-FARQRL-GMKDD	*cis*-bound	-	240 ± 150	19 ± 0.3
TL-3	DDDIVFE-FAV@RL-GMKDD	*cis*-bound	-	NB	NB
TL-4	DDDIVFEDFA-QRL-GMKDD	*cis*-bound	-	NB	NB

Figure 1. a, b) Representation of the AP2 β-appendage (gray) and photoswitchable peptide inhibitors displaying key interacting residues in red (modified from [13b], PDB ID 2V8): a) when the azobenzene crosslinker is conjugated at cysteine residues (yellow) at positions *i* and *i*+11, the binding would be favored in *trans* configuration; b) the case would be the opposite by conjugating the crosslinker at positions *i* and *i*+7. c) Sequences and binding constants of unmodified (*) peptides[13b] and designed TL peptides. K_i values of TL peptides were obtained from a FP competition assay. d, e) Displacement of BAP-long from the binding domain of β-appendage by TL-1 (d) and TL-2 (e) under the conditions described in the legend. f) Plot of K_i in μM obtained from FP assays during the illumination cycles performed on TL-1 and TL-2. FP experiments were performed in duplicate, and error bars represent the standard deviation (s.d.).

reported design rules to photocontrol the helix propensity of the peptide alone.[14a] We then considered the structure of the peptide bound to β-adaptin[13] to select sites that could drive large conformational changes of the interactive residues upon photoisomerization, while minimizing steric clash of the azobenzene crosslinker with the adaptin binding domain. We placed one of the cysteines at a fixed position, replacing the lysine K15, and we introduced the other cysteine on the 11th, 7th, or 4th position to the left (sequences indicated in Figure 1c by TL-1, TL-2, TL-4, respectively). Thus, cysteine pairs were separated by approximately 3, 2, or 1 helix turns, respectively and lay on the same side of the helix, opposite the interacting face. This set of peptide mutants was completed with TL-3, in which an additional structural constraint (@ = α-aminoisobutyric acid in Figure 1c) was introduced.[14a] Thus, according to the separation between the cysteine pairs of the photocontrolled peptides,[14a] we predicted that TL-1 would be able to adopt a helical structure more easily when azobenzene was in the *trans* configuration (in the dark-relaxed state or under 500 nm light), than in the *cis* configuration (under 380 nm light). Conversely, TL-2, TL-3, and TL-4 would have higher helix propensity in the *cis* configuration than in *trans*. TL-1 could therefore be an active inhibitor under visible light and should be inactivated by UV light (Figure 1a). In contrast, TL-2, TL-3, TL-4 could behave as inhibitors under UV light and should be inactive in the dark or under visible light (Figure 1b). We synthesized the dithiol linear peptides by classical solid-phase peptide synthesis, as detailed in the Supporting Information. The intramolecular crosslinking with BSBCA was performed by following reported procedures[15] (Figure S1 in the Supporting Information). All completed TL peptides were purified and their photoisomerization properties (absorption spectra and relaxation kinetics) were verified by UV/Vis spectroscopy (Figure S2 in the Supporting Information). We evaluated whether modified peptides retained the capacity to bind to β-adaptin and specifically looked for light-regulated binding. For this purpose, we designed a fluorescence polarization (FP) competition assay[3b,8a,d] (Figure 1e, f, see the Supporting Information for details). This test was validated with unlabeled BAP-long and BAP-short, both of which showed binding strengths comparable to reported results[13b] (K_i = 5.51 μM and no binding, respectively; see Figure S3 in the Supporting Information). TL peptides were then assayed by

recording FP signals both in the dark and after a 3 min exposure to 380 nm and 500 nm light. TL-1 showed satisfactory binding in the *trans* state and lost binding strength in response to 380 nm (Figure 1 c, d and Figure S3). TL-2 bound weakly in *trans* and more tightly in *cis* configuration (Figure 1 c,f and Figure S3). In general, the results for TL-1 and TL-2 are consistent with the scheme shown in Figure 1 a,b. However, TL-3 and TL-4 did not bind β-adaptin in any of the conditions assayed (Figure 1 c and Figure S3). We further tested the reversibility of TL-1 and TL-2 binding by measuring K_i in cycles of illumination at 380 nm and 500 nm. Switching of the wavelengths led to an almost complete recovery of the K_i values of each peptide under the same light conditions (Figure 1 d–f). Although azobenzene-crosslinked homologue peptides (Figure 1 a–c) had displayed a limited photocontrol of the helical structure by itself as determined by circular dichroism (data not reported), FP experiments confirmed our assumptions and allowed the identification of active PIPPIs, as well as the characterization of their optopharmacological performance in vitro. It has been proposed that TL peptides adopt an α-helical structure upon binding to the β-appendage, as shown by BAP-long,[13] suggesting an "induced-fit" process.[16] In this case, the crucial point of photocontrol is the ability of the crosslinker to allow or not the formation of helical structures in the presence of the target protein, rather than the amount of helicity that it can induce in the peptide alone.

Having identified two TL peptides with in vitro activity against β-adaptin and opposed photocontrol, we tested them in living cells. Since all the PPIs involved in CME are cytosolic, TL-1 and TL-2 must be targeted intracellularly. Cultured HEK293 cells were incubated for 30 min at 37 °C with fluorescently labeled TL-1 and TL-2. Surprisingly, the spontaneous uptake of these two peptides was observed by using flow cytometry (FACS) and confocal microscopy imaging in living cells. TL-1 and TL-2 internalized in a dose-dependent manner (Figure S4a, b in the Supporting Information), the latter showing stronger cell-penetrating peptide ability (Figure S4c). Membrane permeability was enhanced by the presence of the crosslinker (Figure S4c), but the conformation switching did not influence the uptake (Figure S4d). Confocal microscopy imaging performed in the same cell line and under the same conditions confirmed the FACS results (Figure S4e). The permeability of the TL peptides was observed in several cell lines (Figures S4 f–h and S5 in the Supporting Information) and with distinct fluorescent dyes (Figure S4h). Treatment with the TL peptides did not affect cell viability in our experimental conditions, and low cytotoxicity was observed after 24 h of exposure (Figure S6 in the Supporting Information). Since PIPPIs are targeted intracellularly after incubation, photocontrol of endocytosis can be tested directly by using a CME assay. The transferrin receptor (TfR) is one of the best characterized cell-surface transmembrane proteins that undergo CME. TfR is constitutively expressed in many cell types and is overexpressed in cancer cells.[17] To evaluate the capacity of the TL peptides to inhibit CME, we used confocal microscopy to observe TfR uptake in living cells (see the Supporting Information for details). In the absence of CME inhibitors, cells internalized fluorescent transferrin (Figure 2 a). Cells that had been pre-incubated with TL-1 in the dark accumulated transferrin on the membrane, thereby indicating CME inhibition (Figure 2 b). The same result was

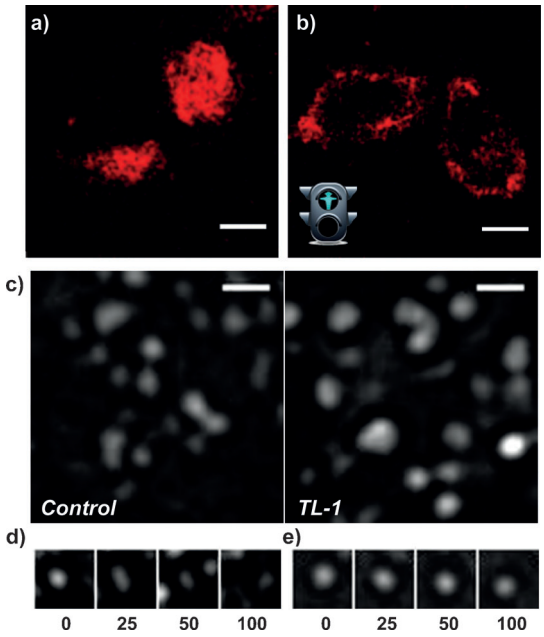

Figure 2. a, b) Transferrin uptake assay by confocal microscopy: after incubation, internalization, and final acid-wash, Alexa647–transferrin is diffused in untreated HeLa cells (a), whereas it is accumulated at the membrane of cells incubated with TL-1 in the dark (b); scale bars, 10 μm. c) Magnified TIRFM images at 37 °C of living HeLa cells transfected with a plasmid encoding clathrin light chain fused with the monomeric red fluorescent protein (LCa–mRFP). The cells shown are untreated (left) and incubated for 30 min with TL-1 in the dark (right). Scale bar, 1 μm. Time-lapse TIRFM sequences of these regions are presented for untreated cells (d) and after incubation with TL-1 (e). The time in seconds is indicated under each frame.

observed in the time-lapse confocal microscopy experiment (Figure S7a-b and Supporting Movie M1). To further characterize the inhibition of CME by TL peptides in living cells, we monitored the dynamics of CCPs at high resolution by using total internal reflection fluorescence microscopy (TIRFM).[18] Individual HeLa cells transfected with a plasmid encoding clathrin light chain fused with mRFP and displaying a homogeneous footprint were observed at 37 °C for 15 min and compared to cells preincubated with TL-1 for 30 min in the dark (Figure 2 c). Changes in the morphology and dynamics of the CCPs are shown in the magnified image (control versus TL-1), and in the time-sequenced frame tracks (Figure 2 d, e). Quantification by image analysis yielded a larger average size of CCPs in the presence of TL-1 (Figure S8a, b in the Supporting Information). After having shown that TL peptides inhibited CME, we addressed whether the peptide activity could also be photocontrolled in living cells, as suggested by the in vitro results. For this purpose, we set up a FACS-based quantitative TfR uptake assay by adapting the conditions previously used in the fluorescence imaging of TfR uptake.[12b] By using this method, we verified that TL-1 in the

dark reduces the internalization of Alexa647–transferrin in various cell lines constitutively expressing TfR (HeLa, HEK293, and MCF7, see Figure 3 and Figure S9 in the Supporting Information). We compared HeLa cells incubated with TL-1 or TL-2 at 37 °C, both in the dark and under 380 nm light (2 min every 15 min to reduce thermal relaxation). After removing the solution containing the inhibitor, cells were exposed to Alexa657–transferrin, following the specific procedure (see the Supporting Information), and then analyzed by FACS. While TL-1 in the dark inhibited TfR uptake, a reduction of TL-1 inhibitory capacity was observed under UV illumination (Figure 3). In contrast, TL-2 did not alter TfR uptake in the dark, but strongly inhibited this process after irradiation at 380 nm. These effects confirmed what was previously shown by confocal microscopy imaging and are in agreement with the in vitro results, indicating that TL peptides allow photocontrol of the inhibition of CME in living cells. Thus, TL-1 and TL-2 can be envisaged as traffic lights that direct one pathway of cellular communication with the exterior. TL-1 slows down membrane traffic ("stop" signal) in the dark or under 500 nm light, while it slightly interferes with this process ("go" signal) under 380 nm light. In contrast, TL-2 does not alter traffic in the dark or at 500 nm (go), and strongly reduces membrane internalization at 380 nm (stop).

To visualize at high resolution how TL peptides switch CME on and off in living cells, we monitored CCP dynamics by TIRFM in the presence of TL-1 or TL-2, during illumination at 380 nm or 500 nm for 2.5 min (Figure 4). By using a customized particle tracking software (see the Supporting Information), we studied how the number of detected CCPs, their average speed and lifetime distribution changed with the illumination conditions (dark, 380 nm, and 500 nm). In the case of cells treated with TL-1, exposure to 380 nm released the inhibited CME, and individual cells showed an increase in the number of CCPs (Figure 4a). This increment was accompanied by an increase in their speed average (Figure S10a in the Supporting Information) and a decrease in their lifetime, measured over three cells (Figure S11a in the Supporting Information). Illumination at 500 nm partially reversed these effects (Figure 4b, Figures S10a and

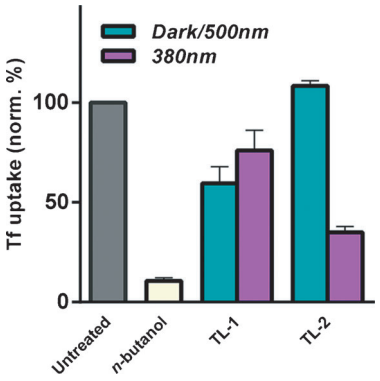

Figure 3. TL peptides inhibit CME in a light-dependent manner, as shown by FACS quantification of transferrin uptake in HeLa cells after incubation with TL-1 and TL-2 under 380 nm and 500 nm light. The results correspond to the average of three independent experiments and are normalized to the fluorescence signal of untreated cells (100% uptake).

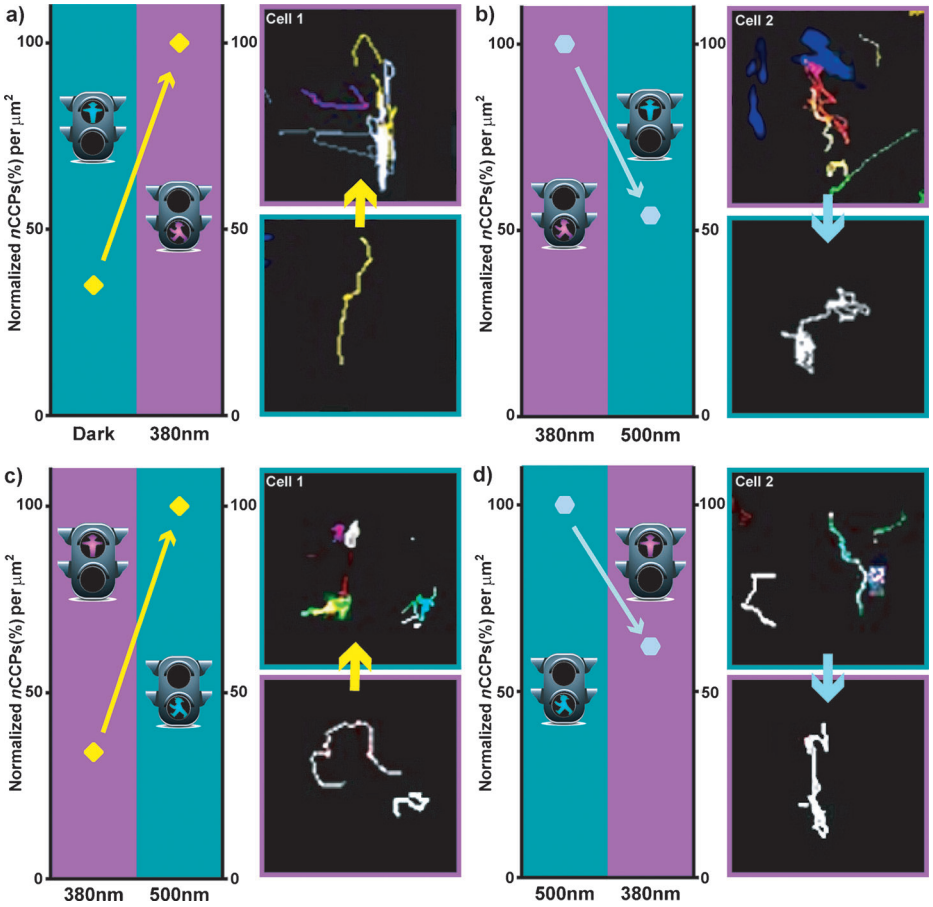

Figure 4. a–d) TL peptides provide stop and go signals to CME during TIRFM imaging of clathrin dynamics. Plots show the number of CCP events per square micron on the cell membrane surface. For each data point under visible and UV light, representative CCP tracks detected with a particle-tracking software are presented in the magnified cell images with lines of different colors (see the Supporting Information for details). a) Using TL-1, the go signal is given by 380 nm light, which increases the number of CCPs per square micron. b) CCP events are reduced with the stop signal (500 nm). c) Using TL-2, the go signal (500 nm light) produces an increase in the number of CCP events and d) the stop signal (380 nm) correspondingly reduces them. The results are representative of three independent experiments.

S11a). Correspondingly, TL-2 arrested CME inhibition at 500 nm, which increased the number of events detected (Figure 4c), increased their speed (Figure S10b), and decreased their lifetime (Figure S11b). These effects were partially reversed at 380 nm (Figure 4d, Figures S10b and S11b). It should be noted that in untreated cells the distribution of speed averages of the tracks detected was consistent with reported results,[18a] while in the presence of TL peptides, it changed significantly depending on light: CCPs globally slowed down during inhibition conditions (stop signals), and the regular rates were almost completely recovered upon release of the inhibition (go signals; Figure S10). Changes in the lifetime distribution with illumination were less pronounced but in complete agreement with the photocontrol of CME inhibition induced by TL-1 and TL-2 (Figure S11). In conclusion, here we report two cell-permeable molecules, TL-1 and TL-2, that are able to disrupt a key PPI for CME in a photocontrolled manner. The evaluation of the strength of CME inhibition and of the capacity to turn this process on and off with light in living cells renders the TL peptides light-responding traffic regulators that allow CME control in spatiotemporally defined patterns. This chemical discovery, applied to a biological process of great interest such as endocytosis, is expected to have a significant impact in the scientific community devoted to cell biology. In addition the TL peptides provide an example of a direct optopharmacological strategy to manipulate the assembly and activity of endogenous protein complexes, without requiring chemical or genetic modification of the target, and can be extended to further applications for the inhibition of other protein–protein interactions.

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