CHAPTER TWELVE

Targeted protein degradation through light-activated E3 ligase recruitment

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Abstract

Optical control of protein function through proteasomal degradation benefits from the noninvasive nature and spatiotemporal precision of light as a trigger. In this chapter, light activation of protein degradation with an optically controlled degron, termed optoDeg, is discussed. This method utilizes genetic code expansion to insert a photocaged analog of lysine at the N-terminal position of a protein of interest for spatial and temporal control of the N-end pathway, inducing proteasomal degradation. Methods for the use of optoDeg for degradation of the fluorescent reporter EGFP and the kinase MEK1 are described. The system is fast, with complete degradation of proteins within minutes following irradiation, and highly specific, with genetically directed introduction of the light-activated degron.

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1. Introduction

Our new optically controlled degron, or optoDeg, utilizes a small peptide degron, comprised of 14 amino acids with an N-terminal photocaged lysine, genetically fused to the protein of interest (POI). The small size prevents perturbation of the function of the POI, until a brief irradiation triggers fast POI degradation within seconds to minutes (Ryan, Liu, & Deiters, 2021). Degradation can be controlled with high temporal and spatial resolution using light as an external trigger and degradation is completely specific for the POI. Furthermore, only minimal protein engineering is required and the approach has broad applicability to a variety of POIs, as we have demonstrated optically activated degradation of a fluorescent reporter (EGFP), a bioluminescent reporter (firefly luciferase), a kinase (MEK1), and a phosphatase (MKP3).

The optoDeg design was inspired by the N-end rule, which describes the relationship of the N-terminal residue to the *in vivo* half-life of a protein (Varshavsky, 2011) through recruitment of the ubiquitin-proteasome machinery. Destabilizing N-terminal residues, or N-degrons, are exposed via proteolytic cleavage by proteases such as caspases, separase, and calpains (Varshavsky, 2008). Primary N-degrons, classified as either Type 1 (Arg, Lys, His) or Type 2 (Phe, Leu, Trp, Ile, Tyr) recruit endogenous E3 ligases to ubiquitinate the protein and ultimately label it for proteasomal degradation (Sriram, Kim, & Kwon, 2011).

Replacement of an N-degron residue with a photocaged unnatural amino acid (UAA) via genetic code expansion allows for minimal perturbation of protein function while introducing spatiotemporal control by using light as an external regulatory element (Ankenbruck, Courtney, Naro, & Deiters, 2018; Courtney & Deiters, 2018). UAA mutagenesis utilizes an engineered aminoacyl tRNA synthetase to acylate a cognate tRNA with the specified UAA. The UAA is then inserted into the POI in response to a recoded amber stop codon (UAG), catalyzed by the ribosome (de la Torre & Chin, 2021). Genetic incorporation of a photocaged lysine analog, such as hydroxycoumarin lysine (HCK), in place of the N-terminal residue results in steric and electrostatic disruption of N-degron recognition by the E3 ligase. Following a brief irradiation, the optoDeg is decaged, yielding the destabilizing lysine for E3 ligase recruitment and the subsequent ubiquitination and degradation of the POI (Fig. 1). Other examples of controlling protein-protein interactions with light using genetic code expansion

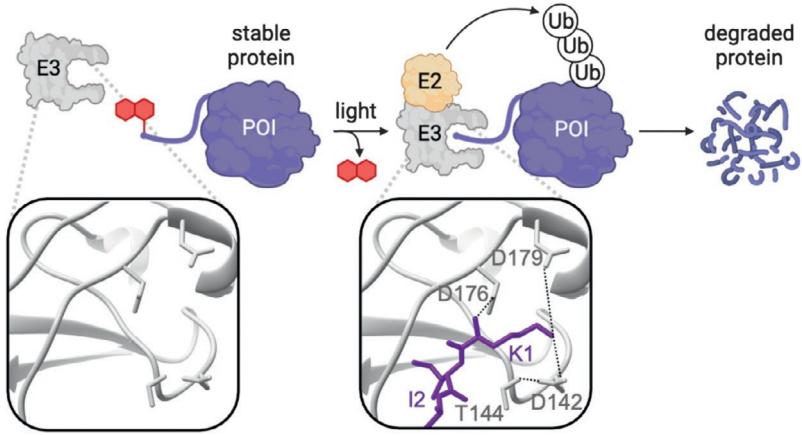

Fig. 1 Light-inducible N-degron design. Residue X (A, K, or HCK) is linked to the POI with a 13-residue peptide linker. The optoDeg containing HCK can be activated via light stimulation to induce E3 ligase recruitment for polyubiquitination and proteolytic degradation of the POI. The shown protein structure depicts the E3 ligase UBR bound to a peptide substrate bearing an N-terminal lysine (purple). Dotted lines represent electrostatic interactions between the lysine and the negatively charged aspartic acid residues in the UBR box (gray).

are still rare and include a caged phosphatase (Courtney & Deiters, 2019), a caged nanobody (Joest, Winter, Wesalo, Deiters, & Tampé, 2021), nuclear localization sequences (Engelke, Chou, Uprety, Jess, & Deiters, 2014; Gautier et al., 2010), and protein SUMOylation (Wesalo, Luo, Morihiro, Liu, & Deiters, 2020). Additionally, optical control of chimeric protein degraders, or PROTACs, has been achieved through insertion of photoswitchable azobenzene linkers (Jin et al., 2020; Pfaff, Samarasinghe, Crews, & Carreira, 2019; Reynders et al., 2020) and through the control of critical protein-small molecule interactions with bulky, light-cleavable caging groups (Liu et al., 2020; Naro, Darrah, & Deiters, 2020; Xue, Wang, Zhou, Zhong, & Pan, 2019).

Due to ribosomal infidelity in the early steps of mRNA translation, amber stop codon suppression and resultant incorporation of photocaged UAAs at the N-terminal region of a POI can have decreased efficiency. This obstacle for genetic code expansion pertaining to the N-terminus of a protein has been overcome using a ribosomal skipping site (Joest, Winter, Wesalo, Deiters, & Tampé, 2022). As a complementary approach, we utilized an N-terminal ubiquitin that is cotranslationally cleaved by deubiquitinases (Lévy, Johnsson, Rümenapf, & Varshavsky, 1996) to generate a POI that contains an N-terminal UAA, the caged lysine HCK.

Here, we are describing the application of the optoDeg approach for the optical control of protein function. Using an optimized peptide degron of just 14 amino acids attached to the N-terminus of the POI, the optoDeg approach can easily be applied to any protein target. The extent of POI degradation can be validated through simple and accessible western blot analysis of protein expression before and after irradiation. Further, the degradation of fluorescently labeled POIs can be monitored via fluorescence microscopy. We first describe the validation of the optoDeg technique through optical control of EGFP expressed *in cellulo*. We then present an example of optoDeg for the control and monitoring of cell signaling cascades. To demonstrate this, constitutively active MEK1, a protein kinase central to the Ras/MAPK signaling pathway, can be expressed with an appended optoDeg peptide. The active MEK1 phosphorylates serine/threonine kinases ERK1 and ERK2, inducing nuclear translocation and proliferation of the Raf/Ras pathway. Once MEK1 is degraded, ERK2 is not phosphorylated and thus remains localized to the cytoplasm.

2. Materials and equipment

2.1 Materials

1. EcoRI restriction enzyme (NEB R3101S)
2. BamHI restriction enzyme (NEB R0136S)
3. Antarctic phosphatase enzyme (NEB M0289L)
4. Antarctic phosphatase reaction buffer (10×, NEB M0289L)
5. Agarose, low EEO (Fisher BP160)
6. Ethidium bromide (CAS 1239-45-8, Thermo Scientific AC17096)
7. T4 Ligase (NEB M0202S)
8. rCutSmart Buffer (10×, NEB B6004S)
9. DMSO
10. dNTP mix (Fisher FERR0181)
11. Phusion polymerase kit (Thermo Scientific F350L)
12. DpnI (NEB R0176S)
13. GeneJET Gel Extraction Kit (Thermo Scientific FERK0692)
14. GeneJET PCR Purification Kit (Thermo Scientific FERK0702)
15. Gibson Master Mix (Gibson et al., 2009)
16. Top10 chemically competent cells
17. Agar (Fisher BP1423-2)

18. Tissue culture dish, 100 × 20 mm (Fisher 353,003)
19. Kanamycin (CAS 25389-94-0, VWR 100217-492)
20. LB broth (Fisher BP1426-2)
21. GeneJET Plasmid Miniprep Kit (Thermo Scientific K0503)
22. HEK293T cells (ATCC CRL-11268)
23. NIH 3T3 cells (ATC CRL-1658)
24. Poly-D-lysine hydrobromide (Fisher ICN15017550)
25. 12-Well plate (Greiner 665180)
26. 96-Well plate (VWR 82050-748)
27. Hydroxycoumarin lysine (HCK, 50 mM in DMSO) (Luo et al., 2014)
28. pE323-HCKRS (Schmied, Elsässer, Uttamapinant, & Chin, 2014)
29. Dulbecco's Modified Eagle Medium (DMEM), 10% fetal bovine serum (Sigma-Aldrich, F0926)
30. Penicillin/streptomycin (Cytiva SV30010)
31. Opti-MEM transfection media (Gibco, 22600-050)
32. Linear polyethyleneimine (LPEI, Polysciences, 23966)
33. Lipofectamine 3000 and P3000 (Invitrogen, L3000008)
34. Buffers
    a. 1× TBE (10.8% (*w*/*v*) tris base, 5.5% (w/v) boric acid, 4% (*v*/v) 0.5 M EDTA (pH 8.0), in milliQ water)
    b. 1× SDS buffer (0.3% (w/v) tris base, 1.44% (*w*/*v*) glycine, 0.1% (*w*/*v*) sodium dodecyl sulfate, in milliQ water)
    c. 1× Transfer buffer (0.3% (w/v) tris base, 1.44% (w/v) glycine, in milliQ water)
    d. 1× PBS (0.256% (w/v) $Na_2HPO_4{\cdot}7H_2O$, 0.8% (w/v) sodium chloride, 0.2% (w/v) potassium chloride, 0.2% (w/v) KH_2PO_4, in milliQ water)
    e. RIPA buffer (50 mM Tris-HCl, pH 8.0, 150 mM sodium chloride, 1% (v/v) NP-40, 0.5% (w/v) sodium deoxycholate, 0.1% (w/v) sodium dodecyl sulfate) supplemented with 1× Protease inhibitor (Thermo Scientific 78429)
    f. 4× SDS-PAGE sample loading buffer (2 mL of 1 M Tris-HCl (pH 6.8), 0.8 g sodium dodecyl sulfate, 4 mL glycerol, 400 μL B-mercaptoethanol, 8 mg bromophenol blue, 4 mL milliQ water)
    g. 10× TBS (1.21% (w/v) tris base, 8.76% (w/v) sodium chloride, in milliQ water)
    h. TBST (100 mL 10× TBS, 1 g Tween-20, 1 L milliQ water, stored in fridge)
    i. 5% Milk (Fisher NC9121673) in TBST

35. Antibodies
    a. rabbit pAb anti-GFP (ProteinTech 50-173-5832)
    b. rabbit pAb anti-GAPDH (ProteinTech 50-172-6351)
    c. rabbit pAb anti-HA (Cell Signaling 3724S)
    d. anti-rabbit IgG HRP-linked secondary antibody (Cell Signaling 7074S)
36. PDVF membrane (0.45 μm, Millipore, IPVH00010)
37. PageRuler Prestained Protein Ladder (Thermo Scientific PI26617)
38. SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific 34580)
39. Live cell imaging solution (LCIS, Invitrogen, A14291DJ)
40. Phenol red-free DMEM (Cytiva, SH3028401)
41. Aluminum foil
42. Cycloheximide (Acros, 357420010)
43. Fibroblast Growth Factor (FGF, Gibco PHG0264)

2.2 Equipment

1. PCR thermocycler (T100 Thermal Cycler, Bio-Rad)
2. Incubator (VWR, Symphony)
3. Shaker (New Brunswick Scientific, Excella E24 Incubator Shaker Series)
4. Sterile laminar flow hood (ThermoFisher Scientific, 1300 Series A2)
5. Water jacketed CO_2 incubator (HEPA class 100, Forma Series II, Thermo Electron Corporation)
6. Transilluminator (VWR Dual Transilluminator)
7. LED (405 nm, Mouser, LZ1-10UB00-01U7)
8. Electrophoresis power supply (Power-Pac 1000, Bio-Rad)
9. Electrophoresis chamber (Bio-Rad)
10. ChemiDoc Imaging System (Bio-Rad)
11. Zeiss Axio Observer Z1 with an AzioCam MRm camera and Plan-Apochromat 10×/0.45 objective and Plan-Neofluar 40× objective
    a. FITC filter set (38 HE, Ex. BP 470/40, Em. BP 525/50)
    b. dsRed filter set (43 HE, Ex. BP 550/25, Em. BP 605/70)
12. Tokai Hit incubated stage (Inu)
13. ZEN 2 (blue edition) software package (Zeiss)
14. ImageJ software (National Institutes of Health)

3. Step-by-step method details

3.1 Plasmid construction: pUb-X-EGFP-P2A-mCherry

Timing: 4–5 days

3.1.1 Digest the backbone of pEGFP-HA (8000 ng in 43 μL of water and 5 μL of 10× rCutSmart Buffer) with 1 μL of EcoRI and 1 μL of BamHI restriction enzymes for 3 h at 37 °C

3.1.2 Prevent ligation of the linearized backbone by combining 44 μL of the digested backbone with 5 μL of 10× Antarctic phosphatase buffer and 1 μL of Antarctic phosphatase enzyme. Incubate for 1 h at 37 °C

3.1.3 Run the digested product (50 μL) on a 0.8% agarose gel containing ethidium bromide (80 V for 20–30 min) in 1× TBE. Identify the band corresponding to the ~4500 bp backbone through brief exposure on a transilluminator, then cut out the band, taking care to excise only the band of interest without excess agarose. Extract the backbone from the gel using the GeneJET Gel Extraction Kit following the manufacturer's protocol

3.1.4 PCR amplify the Ub insert from the pET-Ub plasmid (Braxton, Quartner, Pawloski, Fushman, & Cropp, 2019): 2 μL of 10 ng/μL template DNA, 20 μL of 5× GC buffer, 3 μL of DMSO (to reduce secondary structures and increase primer annealing (Varadharajan & Parani, 2021)), 2 μL of 10 mM dNTP mix, 2 μL of a solution containing 10 μM each of the primers P1 and P2 (Table 1), 70 μL of water, and 1 μL of Phusion polymerase (added last) with total volume of 100 μL. For the thermocycler settings, use an annealing temperature of 67.8 °C and an elongation time of 30 s. DpnI treat the PCR product for 1 h with 0.5 μL of DpnI and 5 μL of 10× rCutSmart Buffer

Thermocycler settings:

1. Initial denature step at 95 °C for 3 min—1 cycle
2. Denature at 95 °C for 30 s, then anneal at 5 °C below the primer melting temperature for 30 s, then elongate at 72 °C for 30 s per kilobase—29 cycles
3. Final elongation step at 72 °C for 10 min—1 cycle
4. Cool to 12 °C for 10 min

3.1.5 Digest the insert PCR product by diluting 43 μL of the PCR with and 5 μL of 10× rCutSmart Buffer. Add 1 μL each of EcoRI and

Table 1 Primer list.

P1	**gcggaattcaccatgcagatttttgtgaaaacc**
P2	gcgggatcctgtcgaccaagcttgccgccacgcagacgcagc
P3	gcgtggcggcgccggcggccttggtcgacaggatcc
P4	caaggccgccggcgccgccacgcagacgcagcacc
P5	gcgtggcggctagggcggccttggtcgacaggatcc
P6	caaggccgccctagccgccacgcagacgcagcacc
P7	gagctgtacaagtcaggaagcggagctactaacttcagc
P8	agcggccgcgactctagatcataatcagccata
P9	agcggccgcgactctagatcataatcagccata
P10	gggatcactctcggcatggacgagctgtacaag
P11	gtcgccaccgccccgaagaagaaaccgaccccgatccagctg
P12	cccgacccacgctgctggtgtgtacccatacgatgttccagattacgcttaatgataaagcggccgcgactct
P13	cacgctgctggtgtgtacccatacgatgttccagattacgcttaatgataaagcggccgcgactctagatcat aatcag
P14	gacagaagccaccggtcgccaccccgaagaagaaaccg
P15	cgcaaatgggcggtaggcgtg
P16	cgtcgccgtccagctcgacca
P17	catggtcctgctggagttcgtg

BamHI restriction enzymes and incubate the mixture for 2 h at 37 °C. Purify the entire PCR product using the GeneJET PCR Purification Kit following the manufacturer's instructions

3.1.6 Combine 1 equivalent (around 100 ng) of the backbone from step 3.1.3 with 10 M equivalents (about 50 ng) of the insert from step 3.1.5, keeping total DNA volume under 8.5 μL. If necessary, add water to bring the volume to 8.5 μL. Combine the DNA mixture with 1 μL of T4 DNA Ligase buffer, and 0.5 μL of T4 DNA ligase. Ligate by incubating overnight at 16 °C. This will yield the pUb-K-EGFP-HA plasmid

3.1.7 Perform site directed mutagenesis via PCR with 1 μL of template DNA (10 ng/μL), 10 μL of 5× GC buffer, 1.5 μL of DMSO, 1 μL of 10 mM dNTP mix, 0.5 μL of Phusion polymerase (added last) with 1 μL of forward and reverse primers (P3 and P4, respectively,

each diluted to 10 μM in 10 μL of water) to create pUb-A-EGFP-HA. Repeat the same PCR with primers P5 and P6 to create pUb-TAG-EGFP-HA. Conduct each PCR with an elongation time of 3 min and an annealing temperature of 57 °C. DpnI treat the PCR product for 1 h with 0.5 μL of DpnI and 5 μL of 10× rCutSmart Buffer

3.1.8 Amplify the P2A-mCherry gene from pWnt3A-P2A-mCherry (unpublished) using primers P7 and P8, an annealing temperature of 59.8 °C, and an elongation time of 30 s using the thermocycler settings from step 3.1.4. Amplify the pUb-X-EGFP backbone with primers P9 and P10 using an annealing temperature of 60.5 °C and an elongation time of 2.5 min

3.1.9 Anneal the amplified P2A-mCherry insert from step 3.1.8 and the pUb-X-EGFP backbone from step 3.1.6 following the published Gibson assembly protocol (Gibson et al., 2009). This will yield the pUb-X-EGFP-P2A-mCherry plasmid (Fig. 2)

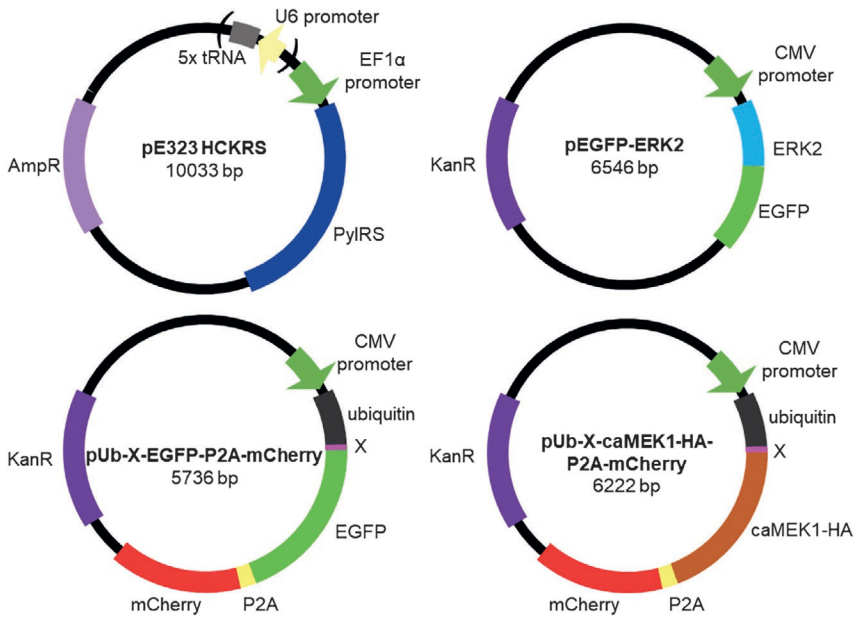

Fig. 2 Plasmid maps depicting pE323-HCKRS, pEGFP-ERK2, pUb-X-EGFP-P2A-mCherry, and pUb-X-caMEK1-HA-P2A-mCherry. The pE323 HCKRS was selected for encoding HCK because of its superior incorporation efficiency for UAAs (Zhou, Wesalo, Liu, & Deiters, 2020). X indicates the position of the key residue for optoDeg design: stabilizing A, destabilizing K, or optoDeg HCK.

3.1.10 Thaw Top10 competent cells (50 μL) on ice for 3–5 min, then add 5 μL of the DNA product to the cells under a flame. Incubate the cells on ice for 30 min, then heat shock at 37 °C for 30–45 s. Return the cells to ice and incubate for 2 min. Under sterile conditions, add 200 μL of SOC media to the cells. Incubate with agitation for 45 min at 37 °C. Use 4–8 Rattler Plating Beads to spread the mixture evenly on a freshly prepared agar plate containing 50 μg/mL kanamycin in 10 mL of agar. Incubate the plate overnight at 37 °C. The following day, inoculate a single colony into LB broth containing 50 μg/mL kanamycin by scooping the selected colony with a sterile pipet tip and dropping the tip into the LB broth mixture (5 mL). Incubate the inoculated colony with shaking for 16–20 h at 37 °C

3.1.11 Pellet the cells via centrifugation (4500 *g* for 10 min), then isolate the DNA using a GeneJET Plasmid Miniprep Kit

3.1.12 Confirm the DNA sequence with Sanger sequencing (Genewiz). Use universal primers CMV forward (P15), EGFP-N reverse (P16), and EGFP-C (P17) to sequence the plasmid

3.2 Plasmid construction: pUb-X-caMEK1-P2A-mCherry

Timing: 3–4 days

3.2.1 PCR amplify the caMEK1 insert from the pMEK-DD-HA plasmid (Gautier, Deiters, & Chin, 2011) using primers P11 and P12, an annealing temperature of 61.5 °C, and an elongation time of 1.5 min

3.2.2 Amplify the pUb-X-P2A-mCherry backbone from pUb-X-EGFP-P2A-mCherry (from step 3.1.9) with primers P13 and P14, with annealing temperature 61.5 °C and a 2.5 min elongation time

3.2.3 Assemble the plasmid using 75 ng of the backbone from step 3.2.2 and 3 M equivalents of the insert from step 3.2.1, using 15 μL of Gibson Master Mix (add water to bring the final volume to 20 μL). This will yield the pUb-X-caMEK1-P2A-mCherry plasmid (Fig. 2)

3.2.4 Transform and inoculate the DNA product from step 3.2.3 in Top10 competent cells as described in step 3.1.10

3.2.5 Pellet the cells via centrifugation (4500 *g* for 10 min), then isolate the DNA with the GeneJET Plasmid Miniprep Kit following the manufacturer's protocol

3.2.6 Confirm the DNA sequence as described in step 3.1.12

3.3 Western blot validation of optically controlled pUb-X-EGFP-P2A-mCherry

Timing: 6–7 days

3.3.1 Dilute 65 μL of poly-D-lysine hydrobromide (PDK, 10 mg/mL) into 6435 μL sterile water. In a laminar flow hood, add 500 μL of the PDK mixture to each well of a 12-well plate. Incubate the plate at room temperature for 10–20 min, then aspirate the PDK mixture. Wash each well with 500 μL of sterile water, then allow the plate to air dry for 45–60 min before plating cells in DMEM containing penicillin and streptomycin antibiotics

3.3.2 Grow HEK293T cells to 80–90% confluency in a water jacketed CO2 incubator at 37 °C

3.3.3 For wells where cells will express plasmids without a TAG mutant, gently lift the old media and replace it with 900 μL of fresh, prewarmed (37 °C) antibiotic-free DMEM

3.3.4 For wells where cells will express plasmids containing a TAG mutant, gently remove the old media and replace it with 900 μL of prewarmed (37 °C) antibiotic-free DMEM containing 500 μM of HCK. To make the UAA-containing media, dilute 10 μL of a 50 mM stock of HCK in DMSO into 890 μL of antibiotic-free DMEM

3.3.5 For each designated well, combine 800 ng of pE323-HCKRS (Fig. 2), 800 ng of pUb-X-EGFP-P2A-mCherry (from Section 3.1), and 8 μL of LPEI (1 mg/mL), then add Opti-MEM for a final volume of 100 μL. Allow the mixture to incubate at room temperature for 10 min, then gently add 100 μL of the transfection mix dropwise to each well

3.3.6 Incubate the plate for 24 h at 37 °C and 5% CO_2

3.3.7 Remove the media and wash the cells with 500 μL of prewarmed (37 °C) PBS, incubating each wash for 10 min at room temperature. Repeat for a total of three PBS washes

3.3.8 To the washed cells, add 990 μL of fresh phenol red-free DMEM with 10% fetal bovine serum and without antibiotics. Expose the appropriate wells to light by irradiating the plate with 365 nm light for 3 min (VWR Dual Transilluminator, 25 mW/cm^2) through the bottom of the plastic plate, about 3 mm below the cells. Use aluminum foil to shield non-irradiated wells from light exposure

3.3.9 After irradiation, add 10 μL of cycloheximide (5 mg/mL in water) to each well to inhibit further protein expression, then incubate the plate overnight at 37 °C with 5% CO_2

3.3.10 The following day, wash each well once with 500 μL of ice-cold PBS to remove residual DMEM

3.3.11 For each well, dilute 1.5 μL of 100× protease inhibitor cocktail into 149 μL of ice-cold RIPA buffer. Lyse the cells by adding 125 μL of the lysis buffer directly to the well then shake the plate on ice at 250 rpm for 15 min. Transfer the lysates to 1.7 mL microcentrifuge tubes and pellet the cell debris through centrifugation (13,000 *g* for 10 min)

3.3.12 Combine 60 μL of the lysate with 20 μL of 4× SDS-PAGE sample loading buffer and heat the samples at 95 °C for 10 min

3.3.13 Load 11 μL of each sample onto two duplicate 10% *v*/v SDS-PAGE gels and separate via electrophoresis (60 V for 20 min, 150 V for 100 min, or until the dye front runs off the gel)

3.3.14 Transfer the separated proteins from each gel to a PDVF membrane via protein transfer (80 V for 2 h) in ice-cold 1× transfer buffer

Keep transfer cold throughout by placing chamber in a large ice bath

3.3.15 Block the membrane by rocking with 5 mL of 5% milk in TBST at room temperature for 2 h

3.3.16 Make the antibody solutions by diluting rabbit pAb anti-GFP (2 μL) or rabbit pAb anti-GAPDH (2 μL) in TBST (4 mL, each solution). Replace the blocking buffer with the antibody solution and incubate overnight at 4 °C with rocking

3.3.17 Wash out the primary antibody by replacing the buffer with 5 mL of fresh, ice-cold TBST and incubating for 10 min at room temperature with rocking. Repeat for a total of three washes

3.3.18 Make a master mix of the secondary antibody solution by diluting 2 μL of the anti-rabbit IgG HRP-linked secondary antibody in 5 mL of TBST. Incubate each blot in 5 mL of the solution for 1 h at room temperature with rocking

3.3.19 Wash out the secondary antibody by incubating the membrane with 5 mL of fresh, ice-cold TBST for 10 min at room temperature with rocking. Repeat for a total of three washes

3.3.20 Develop the blots with SuperSignal West Pico Chemiluminescent Substrate by mixing 2 mL of the luminol/enhancer solution with

2 mL of the peroxide solution and incubating the membrane in the resulting mixture with rocking at room temperature for 5 min

3.3.21 Image the blots using a BioRad ChemiDoc system with automated exposure times. Use the Cy5 and Cy3 channels to detect the PageRuler Pre-stained Protein Ladder, and the Chemi channel to detect the chemiluminescent substrate. A representative western blot of XDeg-EGFP-P2A-mCherry is shown in Fig. 3

3.4 Live cell fluorescence imaging of optically controlled XDeg-EGFP-P2A-mCherry

Timing: 3–4 days

3.4.1 Grow cells to 80–90% confluency in a PDK treated 96-well (see step 3.4.1, adding 50 μL of diluted PDK (0.1 mg/mL) to the 60 internal wells of the plate), clear bottom plate

3.4.2 For wells where the cells will express proteins without a TAG mutant, aspirate the old media and replace it with 90 μL of fresh, prewarmed (37 °C) antibiotic-free DMEM

3.4.3 For wells where cells will be transfected with plasmids containing a TAG mutation, aspirate the old media and replace it with 90 μL of prewarmed (37 °C) antibiotic-free DMEM supplemented with 50 μM HCK (dilute 1 μL of a 50 mM HCK stock solution in DMSO into 89 μL of antibiotic-free DMEM)

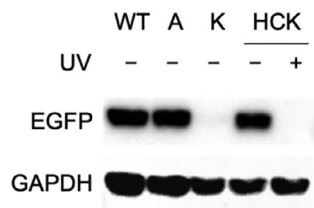

Fig. 3 Western blot analysis of pUb-X-EGFP-P2A-mCherry. In comparison to wild type (WT) pEGFP-HA, N-terminal alanine (A) represents a negative control for the N-end pathway. The destabilizing N-terminal lysine (K) is used as a positive control for degradation. HCK decaging and protein stability can be directly compared with and without UV irradiation. Caging of the N-terminal lysine of pUb-optoDeg-EGFP-P2A-mCherry prevents recognition by an E3 ligase and degradation of the protein via the N-end pathway. Irradiation and subsequent decaging of pUb-optoDeg-EGFP-P2A-mCherry yields the destabilizing N-terminal lysine and resultant protein degradation comparable to the positive control.

3.4.4 For each designated well, combine 100 ng of pE323-HCKRS (Fig. 2), 100 ng of pUb-X-EGFP-P2A-mCherry (Section 3.1), 1 μL of LPEI (1 mg/mL) in Opti-MEM for a final volume of 10 μL. Allow the mixture to incubate at room temperature for 10 min, then gently add 10 μL of the transfection mix dropwise to the specified well

3.4.5 Incubate the plate for 24 h at 37 °C and 5% CO_2

3.4.6 Aspirate the media and wash the cells with 50 μL of prewarmed (37 °C) PBS, incubating each wash for 10 min. Repeat for a total of three PBS washes for each well

3.4.7 Add 90 μL of fresh live cell imaging solution (LCIS) to each well

3.4.8 Prepare the microscope by setting up the Tokai Hit incubated stage with a blank plate using the following settings: 5% CO_2 and 37 °C. Add ~25 mL of water to the insulating jacket and turn on the CO_2 regulator. Allow the apparatus to equilibrate for 10–15 min. Then, carefully place the experiment plate in the incubated stage and cover it with the lid. Allow the final assembly to equilibrate for another 5–10 min

3.4.9 Select and save three distinct fields of view within a single well, for each of the conditions being analyzed: pUb-K-, pUb-A-, or pUb-optoDeg-EGFP-P2A-mCherry. Image the cells for initial timepoints before irradiation using the DsRed and FITC filter sets. Export the images as JPEG files

3.4.10 Leaving the plate inside the incubated stage and being careful not to move it, irradiate the designated optoDeg wells with a 405 nm LED (700 mA) for 15 s from the top of the well (about 12 mm directly above the cells). Use aluminum foil to block wells that are not irradiated. Immediately image the cells (with the same saved positions and filters from step 3.5.9) and export the file as a JPEG file

Be sure to wear proper UV safety glasses for the duration of irradiation.

3.4.11 Image the cells at 10, 20, and 30 min post-irradiation. Export the image files as JPEG files

3.4.12 To determine the extent of degradation over time, process the images in ImageJ. Open the mCherry image in two separate windows. Apply a mask based on the mCherry fluorescence to remove background

On MacOS: Open the Threshold Color Panel (control + shift + T). Leaving the Hue and Saturation parameters unchanged, slide the top Brightness bar all the way to zero (left), then adjust the bottom brightness bar until all fluorescent cells, including the lighter contrast cells, are visible. This will create the threshold. To outline cells expressing mCherry at or above the specified threshold, click Select in the Threshold Color Panel. To create a mask based on the applied threshold, go to Process > Binary > Convert to Mask. Once generated, outline the mask by going to Edit > Selection > Create Selection. Copy the generated outline (command + C) and paste (command + V) it onto the mCherry and EGFP images of the same position. Click and drag the outline to adjust the position if needed. Calculate the mean fluorescence (mean gray value) in the designated outline by clicking inside one of the highlighted cells (the yellow outline will disappear), then press "m." A window will appear and populate with the data for the selected image. To save and analyze the data, copy it from the results window and paste it an Excel spreadsheet or other analysis software

On PC: Open the Threshold window (control + shift + T) and slide the top brightness bar all the way to the left, then adjust the bottom brightness bar until all fluorescent cells, including the lighter contrast cells, are visible. Make sure the "Dark background," "Stack histogram," and "Don't reset range" boxes remain unchecked and click Apply. This will create a mask based on the applied threshold for cells expressing mCherry. To outline the mask, go to Edit > Selection > Create Selection. Copy and paste the selection onto the mCherry and EGFP images of the same position. Pasting will yield the outlines filled in with black, so remove the outline by clicking within the bounds of a cell. To calculate the mean fluorescence in the outline, go to Results > Mean Gray Value, then press "m" and a window will open with the calculated mean fluorescence of the outlined area. Copy and paste the data into an Excel spreadsheet or other software to save and analyze

For single cell analysis: After applying the mask to both images, for PC, synchronize the mCherry and EGFP windows by going to Analyze > Tools > Synchronize Windows and selecting the file names associated with the mCherry and EGFP images. Ensure that

"Sync cursor" is checked. For MacOS, open both mCherry and EGFP windows, then synchronize by opening Analyze > Tools > Synchronize Windows, selecting only "sync cursor" then clicking "Synchronize All." Select a single cell in the mCherry channel and outline with the Freehand selections tool on ImageJ. This will simultaneously outline the same area in the EGFP image. Calculate the mean fluorescence of the outlined cell by keying "m" on both images and copy the populated data into an Excel spreadsheet or other software. Using this data, calculate the EGFP/mCherry ratio. Repeat this process for 100 cells between 9 fields of view through each of the three replicate wells

3.4.13 Calculate the standard deviation from the values generated from analyzing 100 cells

3.4.14 Calculate the statistical significance with a two-way ANOVA test comparing the mean EGFP/mCherry fluorescence values from each sample over different time points. Representative single cell analyses of light titration of XDeg-EGFP-P2A-mCherry is shown in Fig. 4

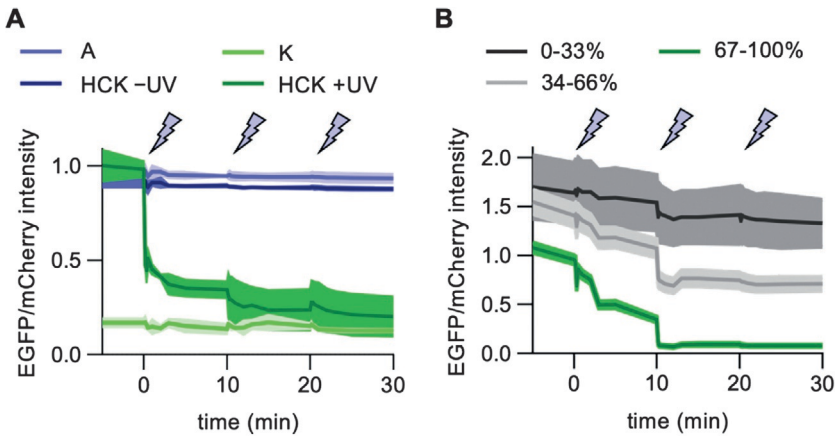

Fig. 4 XDeg-EGFP-P2A-mCherry fluorescence pre- and post-irradiation. (A) EGFP fluorescence relative to mCherry. $p < 0.0001$ from two-way ANOVA between HCK (+UV) and HCK (−UV) at $t = 30$ min. (B) Single cell analysis of EGFP fluorescence relative to mCherry internal control. Cells binned according to percent decrease in EGFP intensity relative to pre-irradiation intensity. A = ADeg-EGFP-P2A-mCherry; K = KDeg-EGFP-P2A-mCherry; HCK = optoDeg-EGFP-P2A-mCherry. Analysis via fluorescence images of optoDeg-EGFP-P2A-mCherry demonstrates rapid protein degradation following decaging, with ~65% EGFP degradation observed after one irradiation and 85% after two or three further irradiations (A). The efficiency of degradation is inversely correlated to the initial protein levels, where cells expressing the lowest amount of optoDeg-EGFP-P2A-mCherry exhibited the highest degradation efficiency (B).

3.5 Validation of XDeg-caMEK1-HA expression through western blot

Timing: 6–7 days

3.5.1 Follow the same steps used in Section 3.3 to express pUb-X-caMEK1-HA in HEK293T cells and analyze the resultant protein stability with and without irradiation via western blot. Use pUb-K-caMEK1-HA and pUb-A-caMEK1-HA as positive and negative controls for degradation, respectively

3.5.2 Probe the resulting blots with primary antibodies: rabbit pAb anti-HA (2 μL), or rabbit pAb anti-GAPDH (2 μL) in TBST (4 mL, each solution). A representative western blot of XDeg-caMEK-HA is shown in Fig. 5

3.6 EGFP-ERK2 translocation assay

Timing: 3–4 days

3.6.1 Grow NIH 3T3 cells to 80–90% confluency in a PDK-treated (see step 3.5.1) black, clear-bottom 96-well plate

3.6.2 For wells where cells have been transfected with plasmids without a TAG mutation, aspirate the media and replace it with 90 μL of prewarmed (37 °C) antibiotic-free DMEM

3.6.3 For wells where cells have been transfected with plasmids containing a TAG mutation, aspirate the old media and replace it with 90 μL of prewarmed (37 °C) antibiotic-free DMEM supplemented with 500 μM HCK (dilute 1 μL of 50 mM HCK stock solution in DMSO into 89 μL of antibiotic-free DMEM)

3.6.4 Transfect each well with 75 ng of pE323-HCKRS (Fig. 2) and 25 ng of pEGFP-ERK2 (Fig. 2) with or without 100 ng of pUb-X-caMEK1-P2A-mCherry (from Section 3.2), using Lipofectamine

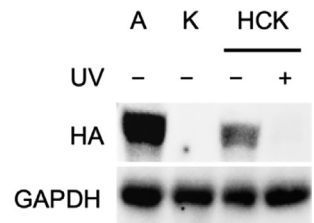

Fig. 5 Western blot analysis of XDeg-caMEK1-HA stability. N-terminal alanine (A) represents a negative control for the N-end pathway. The destabilizing N-terminal lysine (K) is used as a positive control for degradation. HCK decaging and protein stability can be directly compared with and without UV irradiation. A- and optoDeg-caMEK1-HA are expressed as stable proteins, while decaging of optoDeg-caMEK1-HA yields complete protein degradation, comparable to the positive control.

3000. For each well, prepare two microcentrifuge tubes. To tube A, add 5 μL of Opti-MEM, DNA (totaling 200 ng DNA), and 0.4 μL of P3000. To tube B, add 5 μL of Opti-MEM and 0.4 μL of Lipo3000. Flick each tube to mix, then spin down, and incubate the mixture for 5 min at room temperature. Add tube A to tube B (order of addition is important). Mix by slowly pipetting up and down only *one* time. Allow the mixture to incubate at room temperature for 20 min, then add the mixture dropwise to the specified well

3.6.5 Incubate the transfected cells for 36 h at 37 °C and 5% CO_2

3.6.6 Wash the cells with 50 μL of prewarmed (37 °C) PBS, incubating for 10 min. Repeat for a total of three washes

3.6.7 Serum starve the cells by replacing the media with 90 μL of prewarmed (37 °C) LCIS. Incubate for 6 h at 37 °C with 5% CO_2

3.6.8 Prepare the microscope and Tokai Hit incubated stage as described in step 3.5.8

3.6.9 Select and save three fields of view per well for each pUb-X-caMEK1-P2A-mCherry mutant. Once positions are selected, the plate should not be moved so as to preserve the precise fields of view throughout the experiment

3.6.10 Image cells for initial values using the DsRed and FITC filter sets 20 min before starting the experiment

3.6.11 Without removing the plate from the stage, stimulate cells expressing the reporter-only control by adding 10 μL of FGF (1 μg/mL in LCIS) to the appropriate wells

3.6.12 Treat the specified wells with light by irradiating the wells from the top with a 405 nm LED (700 mA) for 1 min (about 12 mm directly above the cells), as described in step 3.5.10. Use aluminum foil to protect non-irradiated wells

Be sure to wear proper UV safety glasses for the duration of irradiation.

3.6.13 Image the cells at 20 min intervals following the initial irradiation, up to 80 min. Representative micrographs are shown in Fig. 6

3.7 Quantification of nuclear localization for XDeg-caMEK1

Timing: 1–2 h

3.7.1 After imaging the cells over the designated period of time (80 min), export the images as JPEG files to analyze in ImageJ

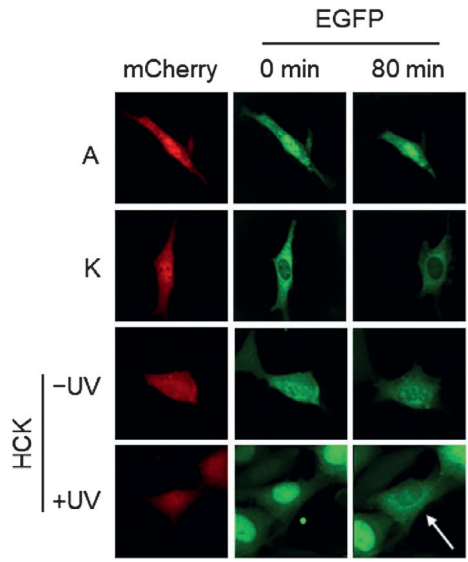

Fig. 6 Representative micrographs of XDeg-caMEK1-P2A-mCherry expressed in NIH 3T3 cells. The stable ADeg- and optoDeg-caMEK1-P2A-mCherry (−UV) fusion proteins are enzymatically active as seen by translocation of EGFP-ERK2 to the nucleus. The unstable KDeg-caMEK1-P2A-mCherry, however, results in cytoplasmic localization of the EGFP-ERK2 reporter since the Ras/MAPK signaling pathway is not activated. optoDeg-caMEK1-P2A-mCherry (+UV) shows the translocation of the reporter from the nucleus, back to the cytoplasm over 80 min post irradiation (arrow).

3.7.2 To determine the ratio of nucleus to cytoplasm localization (N/C ratios), first create a selection using the Oval Selection tool within the appropriate subcellular region—nucleus or cytoplasm. Outlined areas should be between 200 and 1000 μm^2

3.7.3 Measure the mean fluorescence intensity of the outlined area by pressing "m" and populating a results window with the calculated mean. Copy and paste the results into a spreadsheet and average the individual N/C values. A representative quantification of EGFP-ERK2 localization via XDeg-caMEK1-P2A-mCherry activity is shown in Fig. 7

3.7.4 Determine the standard error of the mean from 10 individual cells from three biological replicates

3.7.5 Calculate the statistical significance with a two-way ANOVA test comparing N/C values of each sample across different time points

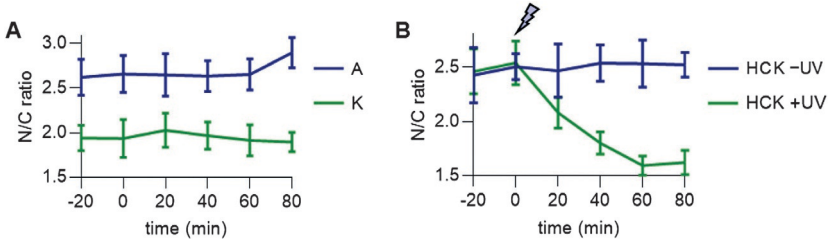

Fig. 7 Quantification of nuclear vs cytoplasmic (N/C) localization of EGFP-ERK2 over an 80 min time-course in 10 cells for each condition. The entire experiment was repeated in triplicate. (A) Negative control ADeg-caMEK1-P2A-mCherry and positive control KDeg-caMEK1-P2A-mCherry co-expressed with the EGFP-ERK2 reporter without external stimulation. (B) Time-course quantification of N/C ratio for cells expressing optoDeg-caMEK1-P2A-mCherry with the EGRP-ERK2 reporter 20 min before and up to 80 min after irradiation, compared to non-irradiated optoDeg-caMEK1-P2A-mCherry. Light-induced degradation of optoDeg-caMEK1 stopped phosphorylation and subsequent nuclear translocation of the EGFP-ERK2 reporter, thus restoring cytoplasmic localization with N/C ratios similar to the positive control, the unstable KDeg-caMEK1.

4. Safety considerations and standards

Common security measures for a biosafety level-2 laboratory must be followed, including waste disposal. Personal protective equipment: safety glasses, lab coat, and nitrile gloves are required for performing experiments. Sterile techniques should be maintained while using a laminar flow tissue culture hood. Cells should be used between passage number 3 and 26. Cell lines should be tested for mycoplasma contamination every 6 months. Eye protection should be worn during irradiation experiments.

5. Summary

An optically controlled N-degron, termed optoDeg, was generated through incorporation of a photocaged amino acid, HCK, at the endogenously exposed N-terminus of a POI. The optoDeg allows for light-mediated induction of POI degradation with complete temporal control, excellent degradation efficiency and kinetics, and design flexibility that requires minimal protein engineering. Here, we describe the methods for applying the optoDeg to two distinct proteins, the fluorescent protein EGFP and the kinase MEK1. The acute triggering of target protein degradation upon irradiation (365 nm or 405 nm) is analyzed by fluorescence imaging and western blots.

Utilizing the methods described here, any protein of interest tolerating a small N-terminal peptide tag can be engineered with the optoDeg for precise spatiotemporal control. We expect our optoDeg to aid in the investigation of dynamic cell signaling networks and the molecular mechanisms that govern cellular processes such as development and proliferation. Genetic code expansion has been achieved in animals (Brown, Liu, & Deiters, 2018), which further broadens the utility of optoDeg.

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