



Optical Control of Protein Activity by Fluorescent Protein Domains

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chain amino acids, glutamate, and glycine (Fig. 3C, fig. S9, and table S5). Accumulation of these metabolites would be expected if the KGDH, BCKDH, and GCS enzyme complexes, which contain DLD, were inhibited (Fig. 3C, fig. S9, and table S5). In agreement with the orthogonality observed in the PLS-DA (Fig. 3B and fig. S9), in *dld-1(wr4)* nematodes the metabolite changes in response to phosphine (in dimension t[3]) were different for most metabolites feeding into the KGDH, GCS, and PDH enzyme complexes, but not for BCKDH (Fig. 3C, fig. S9, and table S5).

The DLD-1 substitution in *C. elegans* is located at the interface with the E2 subunits, which are unique to each complex and have nonidentical interactions with DLD (fig. S8F), producing complex-specific effects similar to those observed in human DLD deficiencies. (16). In addition, extra-mitochondrial activities of DLD could further contribute to the metabolic effects of the mutation. Interestingly, resistance alleles of DLD in insects do not cause a deficiency in the metabolic activity of the enzyme (fig. S10), indicating that resistance in the field is confined to variants with low fitness effects.

It is known that mutations that perturb the *dld* gene in plants generate sensitivity to arsenic (17) and that an oxidized toxic form of arsenic, arsenite (AsO_2^-), interacts with the dihydro form of the lipamide cofactor that is reoxidized by the active disulfide of DLD (18). Furthermore, phosphine resistance in a genetically uncharacterized insect isolate coincided with increased sensitivity to arsine (AsH_3), a reduced form of arsenic (19). We tested the arsenite and arsine sensitivity of *C. elegans dld-1(wr4)* mutants and found that they had an increased sensitivity to both compounds (Fig. 3D and fig. S11A). In contrast, *dld-1(wr4)* transgenic animals expressing wild-type *dld-1*, together with *alh-6(wr3)* mutants, did not show increased sensitivity to either compound

(Fig. 3D and fig. S11A). Furthermore, five out of six insect strains carrying phosphine resistance substitutions also presented increased sensitivity to arsine (fig. S11, B and C).

The attribution of phosphine resistance to a *dld-1* mutation suggests a specific model to explain sensitivity to arsenic compounds: Mutations in *dld-1* that decrease electron transfer from lipamide increase the amount of the cofactor in the reduced state, a condition that makes it susceptible to arsenite attack, resulting in termination of tricarboxylic acid cycle activity. This insight suggests that chemicals that mimic the reactivity of arsenic compounds with dihydrolipamide will be potent phosphine synergists, and that the mutated proteins we have characterized will provide the tools to identify such compounds. Indeed, one of the most intriguing outcomes of this study is that resistance to a small redox-active gas can exhibit such metabolic specificity, despite its potentially diverse reactivity within cells.

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Supplementary Materials

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Materials and Methods
Figs. S1 to S11
Tables S1 to S7
References (20–29)

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Optical Control of Protein Activity by Fluorescent Protein Domains

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Fluorescent proteins (FPs) are widely used as optical sensors, whereas other light-absorbing domains have been used for optical control of protein localization or activity. Here, we describe light-dependent dissociation and association in a mutant of the photochromic FP Dronpa, and we used it to control protein activities with light. We created a fluorescent light-inducible protein design in which Dronpa domains are fused to both termini of an enzyme domain. In the dark, the Dronpa domains associate and cage the protein, but light induces Dronpa dissociation and activates the protein. This method enabled optical control over guanine nucleotide exchange factor and protease domains without extensive screening. Our findings extend the applications of FPs from exclusively sensing functions to also encompass optogenetic control.

The ability to control protein activity by light is enormously beneficial for studying protein function within physiological contexts and for controlling synthetic biological systems

with spatial or temporal specificity (1, 2). Protein domains that naturally exhibit light-dependent conformational changes or interactions have been adapted to control other proteins (3–10). How-

ever, existing methods require exogenous cofactors (3) or endogenous cofactors that might vary between cells and physiological states (11), exhibit slow kinetics (10), force homodimerization (6, 7), or use toxic blue light (7–10). Furthermore, strategies for optical control of a particular polypeptide require extensive screening and optimization (7, 9, 12, 13). Here, we describe the discovery of an engineered protein interaction that is controlled by less energetic cyan light and requires no cofactors. We use this light-controlled association to develop a simple generalizable design for light-inducible proteins.

Dronpa is a monomeric fluorescent protein (FP) derived from a tetrameric parent (14). Fluorescence of Dronpa switches off under cyan light

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(~500 nm) and switches on under violet light (~400 nm) (14). With off-photoswitching, β strand 7 near the chromophore becomes flexible (15); this strand forms part of the cross-dimer interface in the tetrameric parent (15). A Dronpa mutant with Lys¹⁴⁵ on β strand 7 changed to Asn (Dronpa145N) is tetrameric at low micromolar concentrations, but dilution promotes monomerization and facilitates off-photoswitching (16). This suggests that multimerization inhibits conformation changes associated with off-photoswitching. We hypothesized, conversely, that conformation changes occurring during off-photoswitching might promote monomerization, whereas on-photoswitching might promote multimerization (Fig. 1A).

To determine if light could control Dronpa145N multimerization, we performed native polyacrylamide gel electrophoresis (PAGE) (see supplementary materials and methods). Dronpa145N was tetrameric at concentrations from 10 to 100 μ M in the initial bright state, whereas wild-type Dronpa (Dronpa145K for clarity; K, Lys) was monomeric (fig. S1A). Cyan illumination of 100 μ M Dronpa145N induced a shift from cyan-absorbing to violet-absorbing species (Fig. 1C) and a loss of green fluorescence (fig. S1B), as previously described (14). Simultaneously, Dronpa145N redistributed from tetrameric toward monomeric species (Fig. 1B, lane 2), implying that off-photoswitched Dronpa145N has a dissociation constant exceeding 100 μ M. Violet light restored

cyan absorbance (Fig. 1C) and green fluorescence (fig. S1B) and also induced retetramerization (Fig. 1B, lane 3), indicating that monomerization was not due to irreversible protein damage. These results show that Dronpa145N interactions can be controlled by light.

A dimer-to-monomer conversion might be more easily harnessed to control protein activity than a tetramer-to-monomer conversion. Given the lack of multimerization of Dronpa 145K, we explored whether oligomerization of Dronpa145K and Dronpa145N could be limited to dimerization. To achieve high effective concentrations of Dronpa145K and Dronpa145N without driving Dronpa145N tetramerization, we fused Dronpa145K in tandem to Dronpa145N via a linker (K-N tandem dimer) (Fig. 1D). The effective concentration of one domain relative to another on the same polypeptide has been estimated at ~70 μ M (17). The K-N construct migrated in native PAGE primarily as expected for a tandem dimer (Fig. 1E). If the Dronpa domains were engaged in light-sensitive intramolecular interaction, illumination should induce dissociation, resulting in a more elongated faster-migrating conformation. Indeed, the tandem dimer migrated faster after cyan illumination, and this process was reversed after violet light-induced recovery (Fig. 1E). Expected transitions between cyan- and violet-absorbing forms were again observed (Fig. 1F and fig. S1C). Thus, the K-N tandem dimer undergoes reversible

light-induced conformational changes consistent with dissociation and reassociation of Dronpa domains.

To determine whether light-induced Dronpa145N dissociation could occur in mammalian cells, we created two fusions: N-CAAX, a fusion of Dronpa145N to the membrane-anchoring K-Ras C-terminal farnesylation motif (CAAX box), and mNeptune-N, a fusion of the far-red FP mNeptune to Dronpa145N (Fig. 2A) (18). Upon 10-fold relative overexpression of N-CAAX to insure an excess of membrane-localized Dronpa, some mNeptune-N was membrane-bound through Dronpa145N oligomerization (Fig. 2, C and D). Cyan light switched off Dronpa fluorescence (Fig. 2B) and resulted in the release of mNeptune from the membrane (Fig. 2, C and D). Release required prolonged exposures (2 min, metal halide lamp at 100% neutral density through a 40 \times 1.2-numerical aperture lens) and was only partial, but nevertheless indicated that light could induce Dronpa domain dissociation in cells.

To find conditions for Dronpa domain dissociation that require less light, we explored Dronpa145K-Dronpa145N heterodimerization (Fig. 2E). Dronpa145K-CAAX (K-CAAX) was able to recruit mNeptune-N to the membrane (Fig. 2G). Off-photoswitching of membrane fluorescence was faster than with N-CAAX (Fig. 2F), and release of mNeptune required only 20 s of illumination (Fig. 2, G and H). On-photoswitching

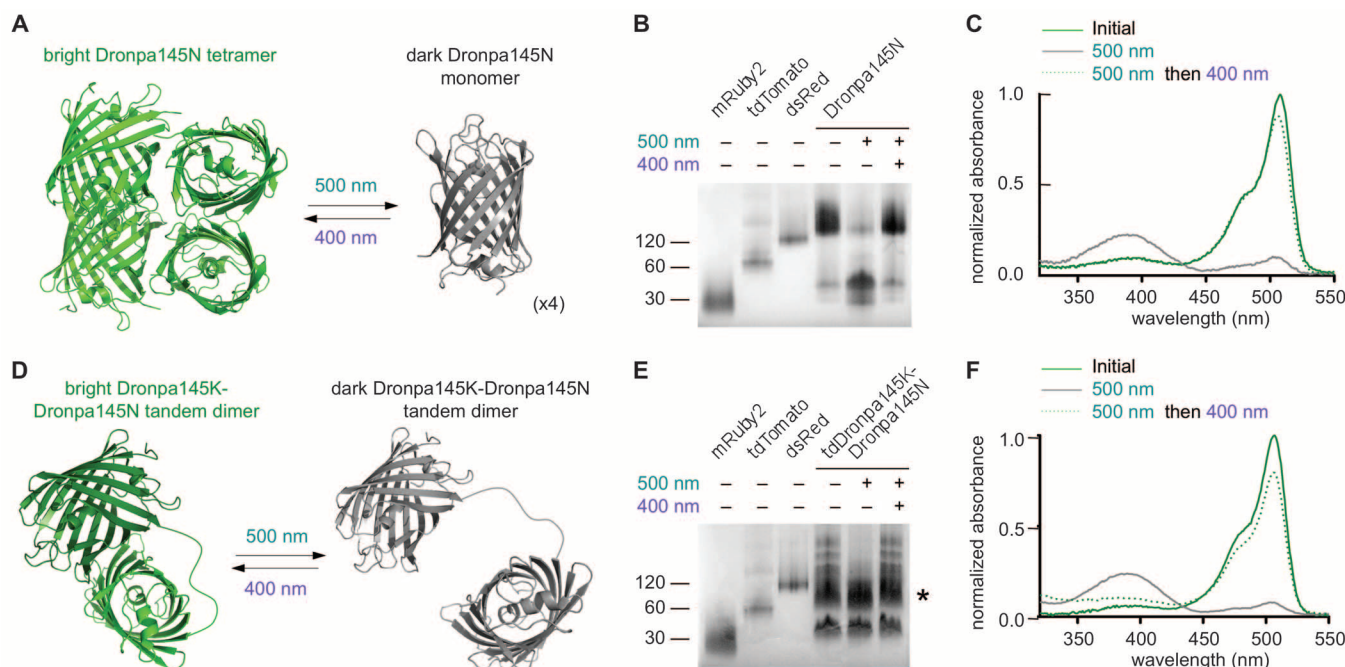


Fig. 1. Control of photochromic FP domain association by light. (A) Hypothesized bidirectional control of Dronpa145N oligomerization state by 500-nm cyan and 400-nm violet light. (B) Native PAGE of Dronpa145N (100 μ M) demonstrated 500-nm-induced dissociation and 400-nm-induced retetramerization. mRuby2 (31), tdTomato, and dsRed2 (20 μ M) served as monomeric, dimeric, and tetrameric standards, respectively. All proteins were polyhistidine-tagged at the NT. (C) Absorbance spectra confirm reversible photoswitching. (D) Hypothesized

bidirectional conformational switching by light in a Dronpa145K-Dronpa145N (K-N) tandem dimer. (E) Native PAGE of the K-N tandem dimer demonstrated faster migration by the K-N tandem dimer (100 μ M) after 500-nm light, an effect reversed by 400-nm light. The asterisk marks the location expected for tandem dimer migration, similar to tdTomato. Some cleavage of the tandem dimer to a monomer in this protein preparation was apparent. (F) Absorbance spectra of K-N tandem dimers confirm reversible photoswitching.

of Dronpa by violet light induced membrane re-localization of mNeptune-N (Fig. 2, G and H). Reversing the positions of Dronpa domains by expressing N-CAAX and mNeptune-Dronpa145K (mNeptune-K) did not result in membrane mNeptune signal (fig. S2A), perhaps because tetramerization between concentrated N-CAAX molecules outcompeted weaker heterodimerization with mNeptune-K. Use of only monomeric Dronpa domains (K-CAAX and mNeptune-K) also resulted in no membrane mNeptune (fig. S2B), as expected.

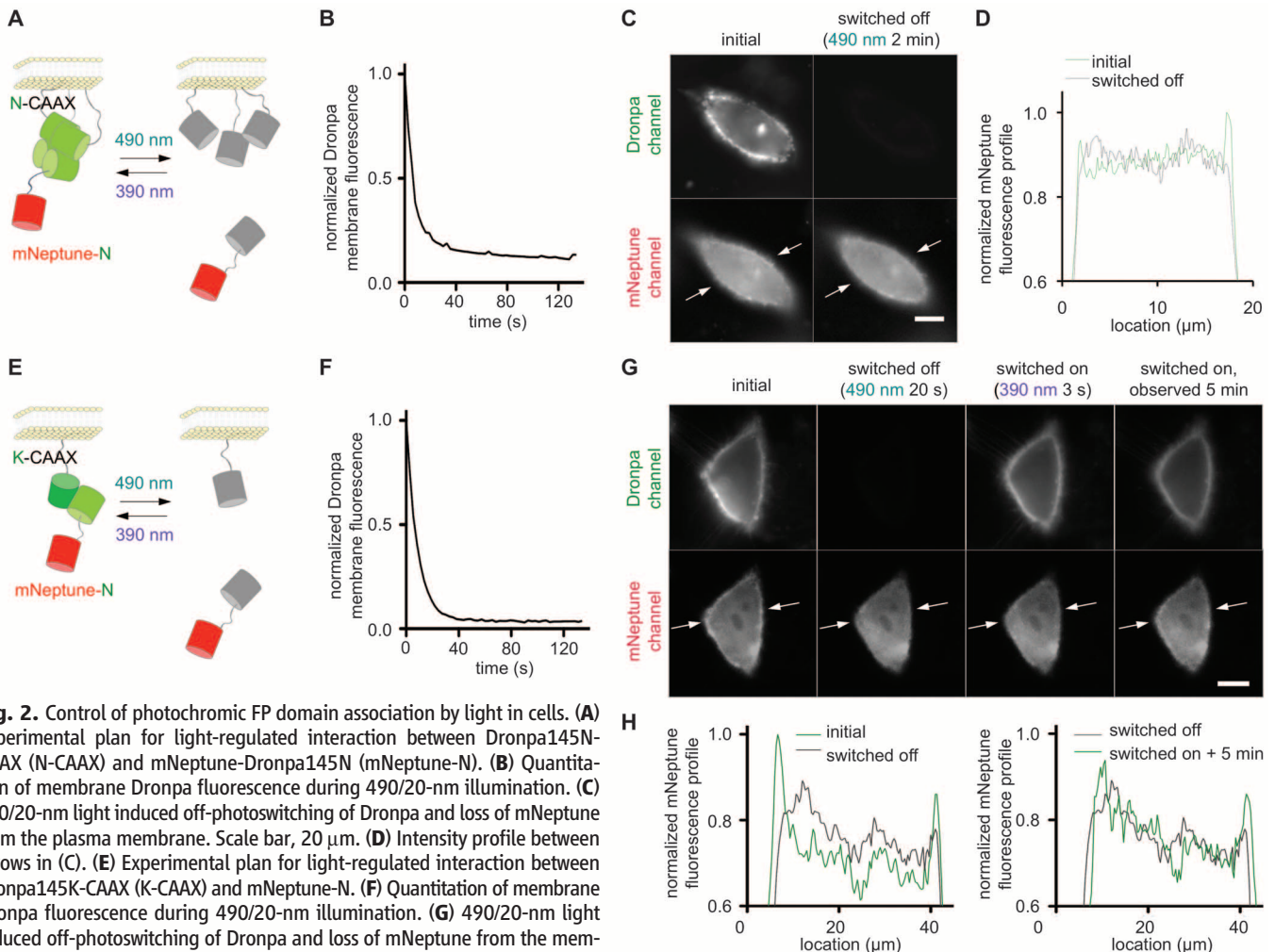
We hypothesized that we could use Dronpa to build light-controllable single-chain proteins. Specifically, we hypothesized that protein functions could be blocked by fusing Dronpa domains to the amino terminus (NT) and the carboxyl terminus (CT) (fig. S3A). Binding of the two Dronpa domains would “cage” the protein in an inactive state by masking surfaces required for binding interaction partners or substrates, similarly to auto-inhibition of many kinases (19), transcription factors (20), and guanine nucleotide exchange factors (GEFs) for monomeric guanosine triphosphatases (GTPases) (21). Protein function could then be

induced by light-mediated dissociation of the Dronpa domains (fig. S3A).

We first controlled the Cdc42 GEF intersectin, which can be inactivated by terminal circularization (22). We fused Dronpa145K or Dronpa145N at the NT of the intersectin Dbl homology (DH) domain and Dronpa145N at the CT followed by the CAAX sequence, creating K-I-N-CAAX and N-I-N-CAAX (fig. S3B). As catalytically active controls, we fused Dronpa145K to either side of intersectin (K-I-CAAX and I-K-CAAX) (fig. S3B). We coexpressed these constructs in fibroblasts with a mNeptune-fascin reporter to mark filopodia and lamellipodia (23). I-K-CAAX or K-I-CAAX robustly induced filopodia and lamellipodia (fig. S3, C and D), as expected for Cdc42 activation, which induces filopodia directly and lamellipodia directly via the formin-family protein FMNL2 (24) and indirectly via Rac (25). Cells expressing N-I-N-CAAX and K-I-N-CAAX produced filopodia or lamellipodia at much lower frequencies than I-K-CAAX or K-I-CAAX (fig. S3, C and D). These experiments were performed by transient transfection, which results in variable expression levels. When designated as low, medium, or high

expressers by Dronpa fluorescence (fig. S4, A and C), low expressers, which included the majority of cells, exhibited basal filopodia or lamellipodia infrequently (0% for N-I-N-CAAX and 8% for K-I-N-CAAX) (fig. S4, B and D). Thus, fusion of flanking Dronpa domains cages intersectin activity effectively as long as higher expression levels are avoided, similar to phototropin-based photoactivable Rac (PA-Rac) (26).

We next asked whether caged intersectins could mediate filopodia or lamellipodia induction by light (Fig. 3, A and E). Illumination with 490/20-nm light for 30 s switched off more than 50% of the fluorescence in both N-I-N-CAAX- and K-I-N-CAAX-transfected fibroblasts (Fig. 3, B and F). This light dose induced abundant filopodia formation within 30 min in 78% of cells expressing N-I-N-CAAX (Fig. 3C and fig. S5D). This response was light-dependent, as only 10% of cells expressing N-I-N-CAAX formed filopodia in the same time interval without illumination ($P < 0.0001$ by Pearson χ^2 test) (fig. S5, A and D). Cells continued to exhibit filopodial mobility throughout 1 hour of observation and did not show blebbing that might indicate phototoxicity



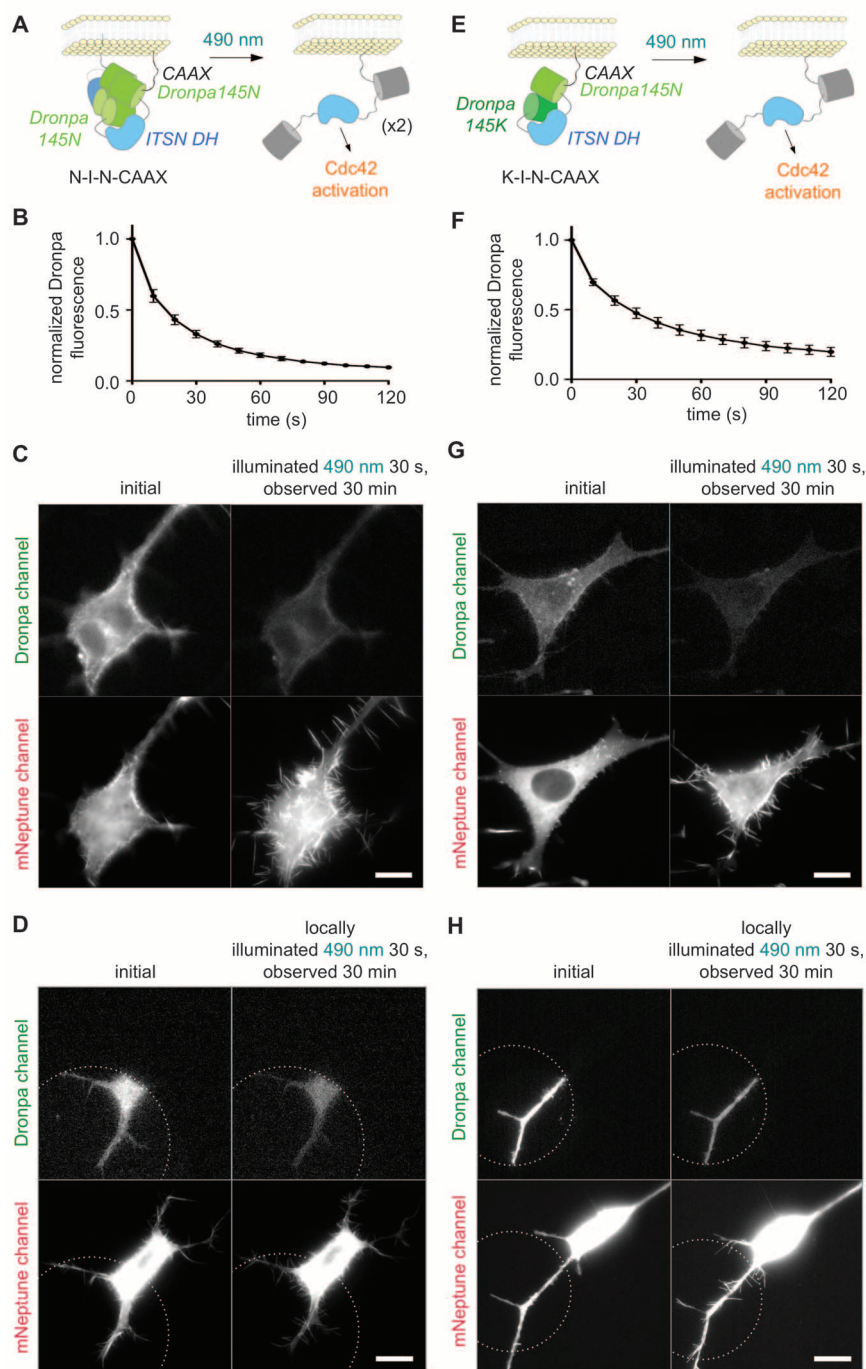


Fig. 3. A light-inducible single-chain GEF. (A) Proposed mechanism for photo-uncaging of N-I-N-CAAX activity. ITSN, intersectin. (B) Off-photoswitching of Dronpa fluorescence in N-I-N-CAAX versus 490/20-nm light dosage during microscopy. Whole-cell fluorescence results from five cells were quantified and normalized to the initial value. Error bars represent SD. (C) In NIH 3T3 cells expressing N-I-N-CAAX, 490/20-nm illumination for 30 s (off-switching) followed by incubation at 37°C for 30 min resulted in robust induction of filopodia, as revealed by mNeptune-Fascin. (D) Local illumination by 490/20-nm light locally induced filopodia, marked by mNeptune-fascin, in NIH 3T3 cells expressing N-I-N-CAAX. The dotted curves indicate the area of illumination. (E) Proposed mechanism for photo-uncaging of K-I-N-CAAX activity. (F) Off-photoswitching of Dronpa fluorescence in K-I-N-CAAX versus 490/20-nm light dosage during microscopy. The experiment was performed as in (B). (G) In NIH 3T3 cells expressing K-I-N-CAAX, exposure to 490/20-nm light for 30 s (off-switching) followed by incubation at 37°C for 30 min resulted in robust induction of filopodia. (H) Local illumination by 490/20-nm light locally induced filopodia, marked by mNeptune-fascin, in NIH 3T3 cells expressing K-I-N-CAAX. The dotted curves indicate the area of illumination. Scale bars in (C), (D), (G), and (H), 20 μ m.

(movie S1). Similarly, 90% of cells expressing K-I-N-CAAX formed abundant filopodia within 30 min after illumination (Fig. 3G, fig. S5D, and movie S2), compared with 25% not exposed to light ($P < 0.0001$ by Pearson χ^2 test) (fig. S5, B and D). Illumination of K-CAAX-expressing cells did not induce filopodia (fig. S5, C and D), confirming that the effect is not due to light alone. These results demonstrate that a protein caged by Dronpa fusion can be uncaged by light.

We investigated whether caged intersectin constructs could control filopodia formation with spatial or temporal specificity. First, we performed local illumination (490/20-nm light for 30 s) to portions of cells expressing N-I-N-CAAX or K-I-N-CAAX and observed that filopodia appeared specifically in the illuminated regions (Fig. 3, D and H). We next tested whether light could induce filopodia in different locations at different times in one cell. We applied a 30-s uncaging pulse of cyan light at one subcellular region, a 3-s global recaging pulse of violet light, and finally another 30-s uncaging pulse at a different subcellular region. After the first uncaging pulse, filopodia appeared in the first region, whereas after the global recaging and second uncaging pulse, filopodia appeared in the second region simultaneously with retraction in the first region (fig. S6).

Whether Cdc42 activation can lengthen existing filopodia has been unclear, as Cdc42 effectors that promote filopodia extension rather than initiation have not been found. Rapid induction of intersectin activity by light allowed us to address this question. We observed that photo-uncaging of intersectin caused lengthening of many preexisting filopodia (fig. S7). This suggests that models in which Cdc42 governs only filopodia initiation are incomplete (27) and that effectors may exist that promote filopodia extension analogous to how FMNL2 promotes lamellipodia extension downstream of Cdc42 (24).

An attractive feature of our design is potential generalizability. Other methods for optical control of single polypeptides, such as fusion to xanthopsin or phototropin, require extensive screening to achieve coupling of light-induced conformational changes to protein activation and, thus, have been applied to only a few targets (7, 9, 13). Our caged protein design does not require precise linkages; therefore, it should be more easily generalizable. Proteases are a class of enzymes for which light activation has not yet been achieved. Unlike GTPases or kinases, proteases are not naturally regulated by membrane recruitment, preventing the use of reversible membrane targeting methods to control them. Hence, we investigated whether we could create a light-inducible protease by fusion to Dronpa domains. We chose to regulate the hepatitis C virus (HCV) NS3-4A protease because its high sequence specificity and lack of overt toxicity allows assessment of function in mammalian cells (28). Furthermore, it is composed predominantly of β strands and loops (29), providing a structural contrast to the completely α -helical DH domain (30).

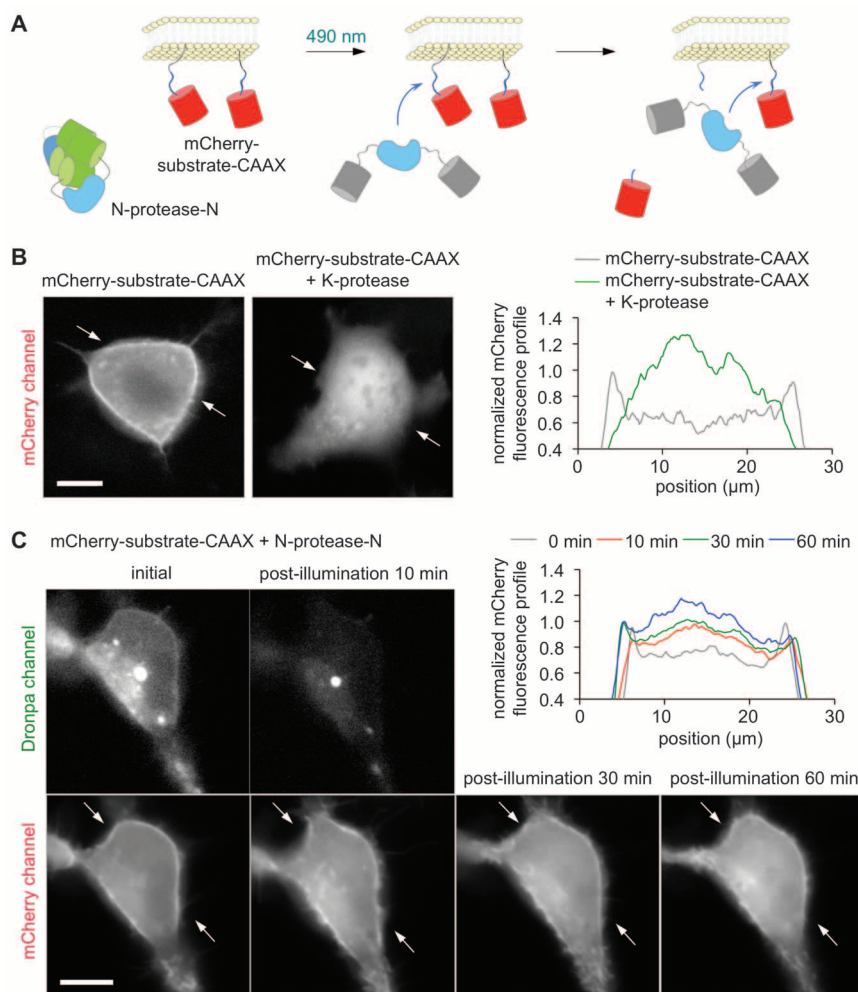


Fig. 4. A light-inducible single-chain protease. **(A)** Strategy for sensing activity of the N-protease-N protein with mCherry-substrate-CAAX. **(B)** Distribution of mCherry in cells expressing mCherry-substrate-CAAX in the absence (left) or presence (middle) of cotransfected K-protease. The chart at right shows the fluorescence intensity profile along the line between the arrows in the images. **(C)** As expected from its size (81 kD), N-protease-N was excluded from the nucleus (left). 490/20-nm light for 15 s induced off-photoswitching of Dronpa fluorescence (Dronpa channel) and induced release of mCherry from the membrane (mCherry channel). The chart at right shows the intensity profile along the line between the arrows in the images, which confirmed that mCherry fluorescence decreases from the membrane and increases in the cytosol and nucleus after illumination. Scale bars in **(B)** and **(C)**, 20 μm.

We constructed a Dronpa145N-protease-Dronpa145N fusion (N-protease-N) and, as a protease reporter, a fusion of mCherry, the NS4A/NS4B cleavage site of HCV polypeptide, and the CAAX-box farnesylation signal (mCherry-substrate-CAAX) (Fig. 4A). We expected that mCherry fluorescence would be released from the membrane into the cytosol by protease activity. Indeed, mCherry signal was membrane-bound in cells expressing mCherry-substrate-CAAX alone and cytoplasmic in cells coexpressing a positive control Dronpa145K-protease (Fig. 4B). We then used mCherry-substrate-CAAX to report light induction of N-protease-N. After off-switching of Dronpa fluorescence, cells showed an increase in cytosolic mCherry within 10 min, which continued to increase over 60 min (Fig. 4C). This response required illumination (fig. S8A) and pro-

tease (fig. S8B). Thus, the caged protein design can be used to control an enzyme domain that is not easily regulated by relocalization within the cell.

Since their discovery, FPs have seen widespread use exclusively as sensing tools. We discovered that photochromic FPs can have dual identities as optical sensors and light-controlled actuators. We have translated this discovery into a simple design for optically controllable proteins, which we propose to call FLIPs, for fluorescent light-inducible proteins. FLIPs also serve as their own reporters, as the photochromic FP domains report both protein localization and activity state. Thus, our results place photochromic FPs in a distinct central location in the optogenetic toolbox, integrating both sensing and controlling functions in a single protein class.

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Supplementary Materials

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Materials and Methods

Figs. S1 to S8

References

Movies S1 and S2

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