Optogenetic control of cofilin and αTAT in living cells using Z-lock

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Here we introduce Z-lock, an optogenetic approach for reversible, light-controlled steric inhibition of protein active sites. The light oxygen voltage (LOV) domain and Zdk, a small protein that binds LOV selectively in the dark, are appended to the protein of interest where they sterically block the active site. Irradiation causes LOV to change conformation and release Zdk, exposing the active site. Computer-assisted protein design was used to optimize linkers and Zdk-LOV affinity, for both effective binding in the dark, and effective light-induced release of the intramolecular interaction. Z-lock cofilin was shown to have actin severing ability in vitro, and in living cancer cells it produced protrusions and invadopodia. An active fragment of the tubulin acetylase αTAT was similarly modified and shown to acetylate tubulin on irradiation.

The cofilin pathway has been implicated in tumor cell migration during the early stages of metastasis. Rapid cofilin activation in specific cellular compartments results in the severing of actin filaments. Local F-actin severing by cofilin can produce either polymerization or depolymerization of F-actin depending on the location and timing of cofilin activation. Conventional approaches to understand the role of cofilin in cell motility have not been sufficient to decipher important mechanistic questions; cofilin overexpression or suppression are complicated by cellular compensation, lack of kinetic information and a lack of spatial control, which impedes detection of the immediate effects of cofilin activation. To address these concerns, we sought an optogenetic analog of cofilin that could be activated locally with precise kinetics.

We previously produced a photoactivatable cofilin analog by attaching a photocleavable protecting group to a constitutively active cofilin mutant. Irradiation of this analog produced cofilin-dependent localized actin polymerization in living cells, but activation was irreversible and led to accumulation of active cofilin. Furthermore, production of the analog required cofilin isolation, chemical labeling and reinjection. Recently, a genetically encoded photoactivatable cofilin based on the Lifeact peptide was produced. However, a subsequent study demonstrated that Lifeact significantly alters cofilin severing activity, which prevented us from using the probe to study cofilin-mediated F-actin severing during tumor cell migration.

Due to these issues we developed Z-lock, a new optogenetic approach with potentially broad use, and applied it to cofilin. To demonstrate the versatility of the method, we also generated a light-controlled analog of the alpha-tubulin acetylase αTAT. Z-lock was based on the LOV2 domain of Avena sativa Phototropin1, a protein of proven use for optogenetics and the basis of several optogenetic approaches. The C-terminal helix of LOV2 unwinds reversibly in response to irradiation between 400 and 500 nm. Unwinding is rapid (<0.5 s) and the rate of return to the folded state is tunable, with point mutations producing return half lives of 1.7–496 s (ref. 23). Z-lock also made use of Zdk, a protein A fragment we developed previously that binds selectively to the dark conformation of LOV2 (refs. 24,25). As shown in Fig. 1a, Zdk and LOV2 were attached to the C and N termini of cofilin such that they bound to each other in the dark and formed a loop occluding the active site. On irradiation, Zdk and LOV2 unlinked, freeing the active site. Because photocontrol was based on steric inhibition, Z-lock could potentially be applied to diverse proteins or protein fragments with specific activity. The most important requirement was appropriate orientation of the two termini, which we achieved with linker optimization, and which could in some cases benefit from circular permutation.

We previously used Zdk in another optogenetic approach, named LOVTRAP, to sequester molecules at particular subcellular locations. LOVTRAP relied on the binding of Zdk to LOV in the dark, which held the target protein on organelles such as mitochondria until it was released by irradiation. Here, we use LOV and Zdk to build an intramolecular bridge over the target proteins’ active sites. Building this bridge presented challenges in protein engineering different from those encountered with LOVTRAP. We had to adjust the affinity of the binding components to accommodate the intramolecular interaction of Zdk and LOV, and had to properly orient the bridge they formed on binding. Z-lock produced an active protein that could be turned on and off wherever it was, not a protein that was sequestered and reversibly released. The fluorescent protein DRONPA has also been used to control proteins through light-regulated homodimerization over their active site. Z-lock’s most important advantages are the ability to adjust the affinity and kinetics of the Zdk-LOV interaction, and the relatively small size of LOV and Zdk, facilitating engineering.

This paper describes the engineering and placement of a light-modulated bridge over the active sites of both cofilin and α-Tat. We make use of a linker, built into the middle of the bridge, that can be reversibly perturbed by irradiation, and the engineering involved for proper placement and affinity of the linker components.
Zdk1 achieved a modest (≈50%) reduction in F-actin binding in the dark state. They were compared using the cosedimentation assay variants of Zdk (Zdk1 and Zdk2), with different affinity and Zdk-LOV would not reach each other to bind in the dark. We tested two linkers that were too short, the Rosetta algorithm was unable to find appropriate linkers, we performed Rosetta structure prediction for designs incorporating Zdk1 versus Zdk2. Linker residues connecting Zdk and LOV to coflin are shown in red.

This results in a set of tools that can be valuable for similarly controlling other protein active sites.

Results

Development and optimization of Z-lock coflin. Cryo-EM structures of coflin bound to F-actin show that the C terminus is free and solvent exposed, and previous studies have demonstrated that green fluorescent protein fused to coflin’s C terminus does not interfere with coflin function. However, coflin’s N terminus makes several contacts with F-actin. Therefore, we tested whether fusion of Zdk to coflin’s N terminus affected F-actin binding (Supplementary Fig. 1a). For this, we used a well-characterized assay that measures the ability of coflin to cosediment with actin during ultracentrifugation (Supplementary Fig. 1b and Supplementary Fig. 2)\(^\text{19}\). Zdk fusion affected neither F-actin binding nor coflin’s ability to convert F-actin to G-actin (Supplementary Fig. 1c, d and Supplementary Figs. 2a–g and 3a–d).

To produce light-controlled steric inhibition of coflin, we needed to position the Zdk-LOV complex where it would block coflin F-actin binding in the dark state, but not in the lit state. To find appropriate linkers, we performed Rosetta structure prediction simulations and assessed the orientation of the Zdk-LOV complex relative to the active site (Methods)\(^\text{11}\). We were able to achieve correct positioning by combining a 5 amino acid (aa) N-terminal linker and an 8 aa C-terminal linker (Fig. 1b). With longer linkers, the complex failed to reliably orient over coflin’s actin binding site. For linkers that were too short, the Rosetta algorithm was unable to form the LOV-Zdk complex over coflin, indicating that Zdk and LOV would not reach each other to bind in the dark. We tested two variants of Zdk (Zdk1 and Zdk2), with different affinity and Zdk-LOV binding sites, to see which produced light-dependent F-actin interactions. They were compared using the cosedimentation assay and LOV2 mutants that mimicked the lit and dark conformations. Zdk1 achieved a modest (≈50%) reduction in F-actin binding in the dark state that fully recovered in the lit state (Fig. 2a, GSGGGG lane). Zdk2 produced more robust inhibition (≈80%) in the dark state, but activity was not recovered in the lit state (Fig. 2b,c and Supplementary Fig. 4, Cofilin and Zdk2 Cofilin LOV lanes).

We attempted to improve the dark state inhibition of the Zdk1 design. Comparing structural models of the Zdk1 and Zdk2 designs revealed a potentially strained linker conformation connecting Zdk1 to coflin, which we hypothesized was hindering Zdk1 binding to dark state LOV2 (Fig. 1b). To improve the Zdk1 design, we tested whether removing a proline and/or lysine from the C terminus of Zdk1 would enhance linker flexibility and improve binding to dark state LOV2. In rapid optimization studies using single tests of multiple different linkers, one linker was found to produce a threefold reduction in dark state binding, but lit state binding was also reduced (Fig. 2a and Supplementary Fig. 5).

We turned to optimizing the Zdk2 design based on the hypothesis that Zdk2 was binding too tightly to LOV2. This was reasonable given robust inhibition in both the lit and dark conformation, the higher affinity of Zdk2 for lit state LOV (Zdk2: 761 ± 78 nM; Zdk1: 4 ± 4 μM)\(^\text{19}\), and the fact that in our design the two proteins were physically linked together (Fig. 2d). We reasoned that lowering Zdk2 affinity could facilitate dissociation, so we performed Rosetta mutational analysis to identify point mutants that would modestly decrease Zdk2 affinity (Fig. 2e). Mutations were chosen based on two criteria: (1) avoiding mutation of residues mediating contact between Zdk2 and the Jα helix of LOV2, as this could reduce selectivity for dark state LOV2, and (2) mutating nonpolar residues to other nonpolar residues, as this is more accurately modeled by Rosetta\(^\text{19, 22}\). To assess each potential mutation, we used Rosetta to calculate the change in Gibbs free energy (ΔΔG) for Zdk2 in isolation and for the Zdk2–LOV2 complex, which yielded the ΔΔG of binding (Fig. 2f). We selected several Zdk2 mutants for testing (V15A, V15I and I32F) based on their predicted reduction in binding affinity and neutral effect on Zdk2 stability (Supplementary Table 1). All mutants tested displayed increased F-actin binding in the lit state relative to wild-type Zdk2 (Fig. 2g and Supplementary Fig. 6). One mutant, Zdk2 I32F, exhibited a roughly fivefold difference in F-actin binding for the lit versus dark state. This analog was selected for further development and was named Z-lock coflin (Fig. 2b,c, Supplementary Fig. 4, Z-lock coflin (I32F) lane, and Supplementary Table 2). Coassembly by Rosetta\(^\text{23, 24}\). Z-link cofilin (Supplementary Fig. 8a,b).

Z-lock coflin effects on actin in vitro and in cells. To test light-dependent severing of actin by Z-lock coflin, we deposited in vitro polymerized F-actin on cover slips and added lysate from MTLn3 cells expressing Z-lock coflin. Irradiation of these cover slips led to F-actin severing (Supplementary Fig. 9a–c), while lysates containing Z-lock coflin dark state mutant (Supplementary Table 2) had no effect (Supplementary Fig. 9a–c). To confirm that the F-actin severing was caused by coflin, we purified wild-type coflin and Z-lock coflin mutants that mimic the lit and dark conformations (Methods and Supplementary Table 2). Wild-type coflin and the lit state mutant severed F-actin efficiently, but the dark state mutant did not (Supplementary Fig. 9d). Consistent with previous studies of coflin\(^\text{23}\), Z-lock coflin in living cells was sequestered in the cytosol until irradiation, when it translocated to the cell edge and colocalized with actin. When irradiation ceased, the Z-lock coflin returned to its initial distribution (Supplementary Fig. 10).

Previous studies have shown that coflin promotes tumor cell migration and directionality by initiating actin polymerization at the cell edge.\(^\text{30}\). We assessed changes in cell migration and F-actin levels following photoactivation of coflin in live tumor cells. Z-lock coflin or a dark state mutant control were expressed in the MTLn3...
breast cancer cell line, and the effects of cofilin photoactivation were assessed using time lapse fluorescence microscopy. Analysis of cell perimeters before and after photoactivation revealed localized protrusion at the site of photoactivation for Z-lock cofilin but not for the Z-lock cofilin dark state mutant (Fig. 3a, Supplementary Video 1 and Supplementary Table 2). We assessed the directional-ity of cell movement before and after photoactivation by measuring the cosine of the angle between the site of photoactivation and the vector indicating the overall direction of cell movement (Fig. 3b). Photoactivation resulted in reorientation of the vector toward the spot of photoactivation, and a consequent increase in cosine values. No change in cosine values was observed for the dark state mutant control. We next investigated the effect of global photoactivation on F-actin levels in MTLn3 cells (Fig. 3c). The low expression levels of...
Z-lock coflin minimally impacted F-actin levels before irradiation (Supplementary Fig. 11a–d). Photoactivation resulted in a significant increase in F-actin at 3 min post-activation, closely paralleling the timing and level of F-actin increase induced by epidermal growth factor (EGF) stimulation. This suggested that coflin-dependent actin severing and free barbed-end formation is sufficient to induce...

**Fig. 3 | Effect of Z-lock coflin activation on leading edge protrusions and invadopodium formation in tumor cells.**

| a | b | c |
|---|---|---|
| Pre-PA | Post-PA | Pre | Post | Pre | Post |
| Z-lock | Control | Z-lock | Control | Z-lock | Control |

- **a**: Cell perimeter before (left) and after (right) photoactivation. Retraction = red; protrusion, green; no change, gray. The site of photoactivation is indicated by the yellow circle. The dark state mutant of Z-lock coflin is shown as a control (Z-lock, n = 8 cells; Control, n = 9 cells) (three independent experiments).
- **b**: Analysis of directional migration versus irradiation of either Z-lock coflin or Z-lock coflin dark state mutant (Supplementary Table 2). The cosine of the angle between the site of photoactivation and the vector of cell movement were calculated for 2 min before (pre-PA) and after photoactivation (post-PA). Photoactivation led to an increase in the cosine value for Z-lock coflin (P = 0.0271; n = 8 cells, paired two-tailed t-test) but not for the Z-lock coflin dark state mutant (P = 0.5782; n = 9 cells, paired two-tailed t-test). Cosine value for pre-PA Z-lock coflin and Z-lock coflin dark state mutant were not significantly different (P = 0.3523, unpaired two-tailed t-test). Tukey box-and-whisker plot shown with outliers displayed as dots (three independent experiments).
- **c**: F-actin content of MTLn3 cells expressing Z-lock coflin that were either unstimulated (n = 34 cells), stimulated with 5 nM EGF (n = 56 cells) or photoactivated (PA) (n = 51 cells). F-actin content was assessed following fixation and phalloidin staining. PA cells were irradiated for 1 min and fixed 3 min after photoactivation. Phalloidin intensity was significantly different for both EGF (P < 0.0001) and PA (P < 0.0001) relative to unstimulated cells. EGF and PA phalloidin intensity were not significantly different from one another (P = 0.0521). The normalized phalloidin intensity for Z-lock coflin versus a dark state mutant. Data is shown as mean ± s.e.m. (three independent experiments).

- **d**: Cell area change within the PA spot for cells expressing the dark mutant of Z-lock coflin (n = 9) (Supplementary Table 2). The cosine of the angle between the site of photoactivation and the vector of cell movement were calculated for 2 min before (pre-PA) and after photoactivation (post-PA). Photoactivation led to an increase in the cosine value for Z-lock coflin (P = 0.0271; n = 8 cells, paired two-tailed t-test) but not for the Z-lock coflin dark state mutant (P = 0.5782; n = 9 cells, paired two-tailed t-test). Cosine value for pre-PA Z-lock coflin and Z-lock coflin dark state mutant were not significantly different (P = 0.3523, unpaired two-tailed t-test). Tukey box-and-whisker plot shown with outliers displayed as dots (three independent experiments).
- **e**: Cell area change within the PA spot. Red arrow indicates the start of photoactivation. Cells were irradiated for 60 s using a 500 ms pulse of blue light every second (n = 9). A spot opposite the site of PA is monitored (n = 9).
- **f**: Cell area change within the PA spot for cells expressing the dark mutant of Z-lock coflin (n = 9) (Supplementary Table 2). Data is shown as mean ± s.e.m. (three independent experiments).
- **g**: Representative immunofluorescence images of MTLn3 cells stained for Tks5 and cortactin to identify invadopodium precursors (indicated by the white arrow). Scale bar, 10 μm.
- **h**: Change in number of invadopodium precursors per cell following photoactivation, based on the data in Fig. 3g. Photoactivation resulted in a significant increase of invadopodium precursors for Z-lock coflin (P = 0.0332, Mann–Whitney test, two-sided) but not for the Z-lock coflin dark state mutant (P = 0.6001, Mann–Whitney test, two-sided) at 5 min post-photoactivation. Tukey box-and-whisker plot shown with outliers displayed as dots.
increases in F-actin following EGF stimulation. Irradiation of the cell edge produced protrusions that retracted when the light was turned off, indicating that effects were reversible (Fig. 3d). During light-induced protrusion, cells retracted at positions away from the site of irradiation (Fig. 3e). Control cells expressing the dark state mutant of Z-lock coflin showed no response to light (Fig. 3f). Sequential photoactivation at the cell edge sometimes induced protrusions multiple times, but this was inconsistent (Supplementary Fig. 12a–d and Supplementary Video 2).

Z-lock induces invadopodia. Metastasis requires dissemination of primary tumor cells to distant organs, where they form secondary tumors. A key step in this process is tumor cell invasion into blood vessels, which is enabled by matrix-degrading protrusions termed “invadopodia”. Previous studies indicate that this process is dependent on coflin; coflin depletion by RNAi inhibits invadopodium precursor stabilization and results in the slow accumulation of actin cytoskeletal defects that directly affect precursor stabilization and maturation. Transient coflin activation, such as that observed following EGF stimulation, directly contributes to invadopodia precursor stabilization and maturation by driving localized actin polymerization. We therefore assessed the effect of coflin photoactivation on invadopodium precursor assembly (Fig. 3g). MTLn3 cells expressing either Z-lock coflin or the Z-lock coflin dark state mutant were globally irradiated for 1 min to mimic the kinetics of coflin activation following EGF stimulation. The number of invadopodium precursors at different time points was measured by quantifying the number of cortactin- and Tks5-positive puncta, made visible by immunostaining (Fig. 3g). Photoactivation led to a significant increase in invadopodium precursors 5 min after photoactivation (Fig. 3h,i). No significant change was observed for the Z-lock coflin dark state mutant control at any time point. Notably, our results closely match previous findings that precursor formation peaks at 5 min following EGF stimulation.

Z-lock αTAT. To probe the generality of the Z-lock approach, we used it to control a second target, the alpha-tubulin acetyl transferase (αTAT). The level of tubulin acetylation is regulated by the antagonistic actions of αTAT and the tubulin deacetylases, histone deacetylase 6 (HDAC6) and sirtuin type 2 (SIRT2). SIRT2 has been studied extensively and is known to deacetylate substrates other than tubulin, including the HAT domain of p300 histone acetyltransferase and a histone H4 peptide. αTAT, on the other hand, is highly specific for microtubules and preferentially acetylates microtubules, for example tubulin. The functional consequences of alpha-tubulin acetylation have been investigated primarily through overexpression or knockdown of the deacetylase or the acetyltransferase. We sought to help define the role of this dynamic modification by producing a tool that can generate acetylation in living cells.

αTAT catalyzes the transfer of an acetyl moiety from acetyl CoA to tubulin. Full-length αTAT consists of a catalytic core, a C-terminal extension and a tail domain. We used the functional core domain (2–236 a.a.), for which structural information is available. The flexible, unstructured tail was excluded because it contains multiple phosphorylation sites that could affect activity independent of the Z-lock steric block. The N and C termini of the αTAT core are on opposite sides of both the tubulin and acetyl coenzyme A (CoA) binding sites. Blocking either site could effectively inhibit αTAT activity. Fusing the LOV domain alone to the N terminus of αTAT did not effectively block tubulin binding. To engineer a Z-lock analog of αTAT, we fused Zdk1 to the N terminus of the αTAT core, and LOV2 to the C terminus (Fig. 4a). Western blotting showed that acetylation levels in cells were elevated about twofold when expressing αTAT core relative to a dominant negative, kinase-dead mutant αTAT (DN αTAT) (Fig. 4b,c and Supplementary Fig. 13, Supplementary Table 2). Using a fixed 10 a.a. (5GS) linker between Zdk1 and αTAT, we tested three different linkers between αTAT and LOV2 (Supplementary Table 2; 4, 6, and 8 a.a., all 5GS repeats). We found that caging was optimal using the 6 a.a. (3GS) linker. Longer linkers between αTAT and LOV2 could fully recover αTAT activity, but were not as effective at reducing activity in the dark state. Further reduction of linker length resulted in lower light-induced activity. (Fig. 4b,c and Supplementary Table 2) Varying the linker between Zdk1 and αTAT had little effect. The 3GS version was named Z-lock αTAT, and was used in all subsequent experiments.

An initial examination of the αTAT structure indicated three potential inhibition mechanisms. The Z-lock components could sterically occlude the binding site for tubulin or for acetyl CoA, or the binding of Zdk to LOV could distort the αTAT structure without occluding these sites. To examine which of these three mechanisms is most relevant to our designed switch, we performed structure prediction simulations with Rosetta (Supplementary Table 2). The starting models for the simulations were Zdk fused to the N terminus of αTAT with the linker (Zdk-GSGSGSGSGS-αTAT) between the two domains in a random conformation and the LOV domain (in the dark state) fused to the C terminus of αTAT with the linker (αTAT-GSGS-LOV) also in a random conformation. Monte Carlo optimization of the backbone torsion angles in the two linkers was then used to search for low energy models in which Zdk was appropriately docked against the LOV domain. Distance constraints derived from the crystal structure of Zdk bound to the LOV domain were used to direct the docking between Zdk and the LOV domain. Then, 20,000 independent simulations were performed and the models output from each simulation were examined to identify what surfaces of αTAT are occluded when Zdk binds to the LOV domain. In more than 90% of the models the Zdk/LOV complex was adjacent to the tubulin binding on αTAT and is predicted to sterically occlude binding to tubulin, while the Zdk/LOV complex never came within six angstroms of the acetyl CoA site (Fig. 4d). This result indicates that the Z-lock switch is reducing activity in the dark state by blocking binding to tubulin. To also test whether Z-lock may be reducing αTAT activity by placing strain on the structure we performed the same structure prediction simulations (sampling different linker conformations to dock Zdk against LOV), but with different starting models for αTAT. The αTAT starting models were derived from an elastic network model (elNemo) that predicts conformational changes αTAT is likely to undergo in the folded state. We found that, even with the αTAT models that most dramatically perturbed its structure, no changes were observed when simulating Zdk docking with the LOV domain. This indicates that the engineered linkers have enough flexibility to accommodate natural structural perturbations αTAT may undergo, and that the Z-lock switch is not functioning by placing strain on the αTAT structure.

To examine the ability of Z-lock αTAT to acetylate tubulin in living cells, we quantified immunofluorescence staining of acetyl-tubulin in COS7 cells transfected with Z-lock αTAT or controls (Fig. 4e). Immunofluorescence images showed increased tubulin acetylation in cells expressing full length αTAT and αTAT core, relative to αTAT dominant negative or untreated cells. Irradiation of Z-lock αTAT induced a twofold increase in acetylation (Fig. 4f and Supplementary Table 2).

Discussion

In summary, Z-lock provides a versatile means to place a light-controlled, reversible block over important protein sites. Because it is based on steric inhibition, it should be applicable to a wide variety of protein fragments and in some cases complete proteins. We believe the technique will be most useful where a single active site, that is a single activity, needs to be controlled and where other regulatory sites are either removed or mutated away. We envision Z-lock will be used to control protein fragments that modulate endogenous
targets, such as the αTAT fragment used here. Cofilin is an example of an intact protein, but is a special case in that it has essentially one important active site. The modifications that we made to cofilin had little effect on the protein’s affinity for actin (Supplementary Fig. 7). As with most optogenetic analogs, upstream regulation was eliminated, so that activity was affected only by irradiation.
The successful completion of the Z-lock analogs here required protein modeling and engineering, primarily to adjust affinities and linkers for intermolecular interactions. There was a trade-off between residual dark state activity and maximal activation. This residual activity is an Achilles heel of most nonchannel optogenetics techniques and often requires careful control of expression level to find conditions where the cell is impacted only on irradiation. Adjusting Zdk affinity as exemplified here could tune the balance between ‘leakiness’ and activity. Expression of a relatively small amount of Z-lock cofilin was sufficient to achieve optogenetic control (Supplementary Fig. 11).

Z-lock cofilin was used to control actin dynamics in vitro and in live cells, and supports a role for cofilin in initiating actin polymerization and in generating invadopodia in tumor cells. Precise control of activation kinetics enabled us to show that cofilin activation alone can generate the actin assembly steps produced by EGF stimulation. In the future, we hope to use the technique in live animals to assess cofilin’s contribution to tumor cell migration.

Online content
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Methods

Antibodies, DNA constructs and transfection. Antibodies were from the following sources: Cofilin (D3F9) XP Rabbit mAb (Cell Signaling no. 5175), β-actin (8H10D10) Mouse mAb (Cell Signaling no. 3700), Tk3 (Santa Cruz Biotechnology, sc-9012), Cortactin (Abcam, ab33333). The complementary DNA of the LOV2 domain from A. sativa (oat) Phototropin1 (L-I04-L547) was used to generate photosensitive constructs. Three variants of LOV2 were used: wild-type, dark state mutant (C450A, L514K, G532A, L531E and N538E) and lit state mutant (I510E/I539E) (Supplementary Table 2). The cDNA of full length rat cofilin was used for all constructs. The Z-domains that selectively bind dark state LOV2 have been described elsewhere. For transient expression in mammalian cells, constructs were cloned into pmCherry-C1 (Clontech Laboratories, Inc.). Cells were transfected with Lipofectamine 2000 (Life Technologies) using the manufacturer’s protocol 24 h before cloning into pmCherry-C1 (Clontech Laboratories, Inc.). Cells were transfected with actin polymerization buffer (500 mM KCl, 20 mM MgCl2 and 10 mM ATP) to a final 1X concentration. Following polymerization, the pH of the polymerized actin solution was adjusted to pH 6.8 to limit cofilin-mediated actin depolymerization. After pH adjustment, 30 µl of the polymerized actin solution was added to 20 µl of purified cofilin in storage buffer (10 mM Tris, 50 mM NaCl, pH 7.4) via gel filtration using a spin column packed with a 7 kDa molecular weight cut-off size exclusion resin (Zeba Spin Desalting Columns, Fisher Scientific).

Actin co sedimentation assay. This assay was performed using the Actin Binding Protein Spin-Down Assay Kit (Invitrogen, Inc.) according to the manufacturer’s protocol with minor modifications. Briefly, lyophilized rabbit muscle actin was resuspended to 1 mg ml⁻¹ in 5 mM Tris–HCl pH 8.0 with 0.2 mM CaCl2. The resuspended solution of rabbit muscle actin was polymerized by adding 10X actin polymerization buffer (500 mM KCl, 20 mM MgCl2 and 10 mM ATP) to a final 1X concentration. Following polymerization, the pH of the polymerized actin solution was adjusted to pH 6.8 to limit coflin-mediated actin depolymerization. After pH adjustment, 30 µl of the polymerized actin solution was added to 20 µl of purified cofilin in storage buffer (10 mM Tris, 50 mM NaCl, pH 7.4). The reaction mixture contained test protein at a final concentration of 8 µM and F-actin at a final concentration of 12 µM. Reactions were incubated at room temperature for 1 h, and centrifuged. The supernatant was removed and pellets were resuspended in 50 µl Milli-Q water. Samples were combined with Laemmli sample buffer, boiled for 5 min and stored at 4°C. Samples were resolved by SDS–PAGE followed by western blot using the above-mentioned cofilin and β-actin antibodies. The results were normalized by first measuring the band density of coflin in the pellet and supernatant fractions. The relative percentage of coflin bound to F-actin was then calculated by dividing the band density of coflin in the pellet fraction by the sum of the band densities for both the pellet and supernatant fractions. The average relative percentage of coflin bound to F-actin for wild-type coflin was then used to normalize other measurements.

F-actin severing with Z-lock coflin. F-actin was polymerized using a mixture of unlabeled-actin (1.4 µM), biotin-actin (0.2 µM) and rhodamine-actin (0.4 µM) in actin polymerization buffer (40 mM pH 7.5 Tris–HCl, 10 mM MgEGTA, 4 mM MgCl2, 100 mM KCl) for 2 h at room temperature. MatTek glass bottomed petri dishes were coated with a layer of PEG/PEG–biotin mixture, dried and stored at 4°C before use. Before imaging, the coated dishes were incubated with 0.5 mg ml⁻¹ streptavidin for 5 min, and washed five times with Tris–HCl. F-actin (0.033 µM) was deposited on the coated dishes for 15 min and rinsed twice with wash buffer (actin polymerization buffer, 3X) before imaging. Wash buffer was removed and purified coflin or its mutants was added (Z-lock coflin lit, Z-lock coflin dark, see Supplementary Table 2). This was diluted in assay solution (10 mM pH 7.5 Tris–HCl, 2.5 mM EGTA, 0.25 mM MgCl2, 5 mM MgCl2, 0.5 mM BSA, GLOX, 0.5 mM, 10 mM DTT, protease inhibitors). Polymerization of F-actin was examined immediately after adding the protein and after indicated times using total internal reflection microscopy.

To monitor F-actin severing by Z-lock coflin with light, we transiently transfected MTLn3 cells with Z-lock coflin or Z-lock coflin dark (see Supplementary Table 2). Cell lysate was prepared at 4°C and then loaded onto F-actin coated coverslips. PA-cofilin was photoactivated by pulsing blue light (405 nm, 1 s/1 s) (Chroma filter HQ470/40x) for 1 min. The images were taken immediately after and the indicated times.

Western blot quantification. Cofilin and β-actin band densities were quantified using ImageStudio, with local background subtraction. To calculate F-actin binding, the cofilin band density for the pellet fraction was divided by the sum of the cofilin band intensities for the pellet and supernatant fractions.

Whole-cell photoactivation. Cells were plated on glass bottomed dishes (MatTek Corporation) and allowed to spread overnight protected from light. For F-actin staining cells were plated on gelatin coated dishes. For invadopodium precursor staining cells were plated on gelatin coated dishes. All work was performed under red light to prevent unintentional photoactivation. Cells were seeded starved 4 h before photoactivation in Leibovitz’s L-15 media (Gibco) containing 0.35% BSA. Photoactivation was accomplished with a 470 nm LED array (Mouser Electronics, Inc. part no. 828-OVQ12580/B7). The surface of the tissue culture plate was positioned approximately 1 cm away from the LED array, which resulted in a measured power density of 0.064 nW µm⁻² at 445 nm. During photoactivation, cells were maintained in a cell culture incubator set to 37°C and 5% CO₂.

Immunofluorescence. For F-actin and invadopodia analysis MTLn3 cells were fixed with 3.7% paraformaldehyde for 20 min at room temperature and washed three times with wash buffer (actin polymerization buffer, 3X) before fixation. For MTLn3 cells that selected invadopodia (PBG) or MTLn3 cells ΔgαS (GαS) potassium dihydrogen phosphate 0.2 g l⁻¹, sodium chloride 8 g l⁻¹, disodium phosphate 1.15 g l⁻¹). Cells were permeabilized with Triton-X 100.0.1% solution in PBS for 5 min and washed three times with PBS. Cells were blocked with 1% BSA and 1% FBS in PBS for 1 h at room temperature. For invadopodia analysis, cells were stained with primary and secondary antibodies at the manufacturer’s suggested dilutions in blocking buffer for 1 h and washed three times with PBS before photoactivation. For F-actin analysis, DyLight 488 Phalloidin (Cell Signaling, 12935) was incubated with cells at once concentration for 20 min and washed three times with PBS.

Preparation of fluorescent gelatin coated dishes. Gelatin was labeled with Alexa-405 dye and glass bottomed MatTek dishes were coated with the fluorescent gelatin as described earlier. Briefly, dishes were acid-washed (1 N HCl for 10 min) and coated with poly-l-lysine (50 µg ml⁻¹ for 20 min) followed by Alexa-405–gelatin coating (0.2% gelatin for 10 min). Gelatin matrix was then crosslinked (0.1% glutaraldehyde for 15 min) and inactivated (5 mg ml⁻¹ NaBH4 for 15 min). After each wash, gelatin was washed with PBS (3X) and stored at 4°C in 10X Pen-Strep (1,000 IU ml⁻¹ penicillin, 1,000 µg ml⁻¹ streptomycin; Thermofisher Scientific). All solutions were prepared fresh immediately before use.

Live cell imaging. Cells were plated on acid-washed glass bottomed MatTek dishes and allowed to spread overnight, protected from light. Before imaging, cells were serum-starved in Leibovitz’s L-15 media supplemented with 5% FBS. A closed heated chamber was used during live cell imaging. Imaging was performed using a Zeiss LSM 880 confocal microscope equipped with a Plan-Apochromat x63 oil objective (numerical aperture (NA) 1.40). ZEN software (Zeiss) was used to control the microscope and acquire images at each time point. A GaAsP detector with tunable emission collection windows (Zeiss) was used for detection. Yellow fluorescent protein images were acquired using a 514 nm Argon laser (25% power) with a collection window of 525–580 nm. mCherry images were acquired using a 561 nm diode–pumped solid-state laser (20% power laser) with a collection window of 580–650 nm. LOV2 photoactivation was accomplished with a 488 nm
argon laser (1% power) that irradiated a preselected region every 10 s. Images were acquired every 2.5 s.

Directionality analysis. Changes in cell directionality were quantified using the directionality index, which is defined as the cosine of the angle between the site of photoactivation and the vector direction of cell movement \(\phi\). The vector direction of cell movement was determined by measuring the cell centroid at two different time points. We used Fiji/ImageJ to define the cell centroids, following thresholding of each cell. Fiji/ImageJ calculates the centroid by taking the average of the \(x\) and \(y\) coordinates of all of the pixels for the thresholded cell. The site of photoactivation was determined by measuring the centroid of the photoactivation region of interest using Fiji/ImageJ. The directionality index was assessed for two intervals. First, 2 min before photoactivation until the time of photoactivation, which measured cell movement before photoactivation. Second, from the time of photoactivation until 2 min after, which assessed potential changes in directionality in response to photoactivation.

Measurement of F-actin content. Cells fixed and stained with phalloidin were imaged on an Olympus IX-81 microscope equipped with a UPlanFLN 40× objective (Pil, NA 1.30). Metaphorm software (Molecular Devices) was used to control the microscope and acquire images. Dylight 488 and mCherry images were acquired using a 100 W mercury arc lamp with a 1% ND filter and a 500–550 or 563–595 nm band-pass filter, respectively, with 1 s exposure for each channel. Flat field correction was applied to each image using a custom MATLAB script\(^5\). Corrected images were thresholded with Otsu's method\(^5\) using Fiji/ImageJ to generate masks for individual cells. For each image, a region without cells was used to determine background intensity for background subtraction. Mean phalloidin intensity (corrected and background subtracted) was measured for individual cells using previously generated masks. Experimental replicas were imaged on the same day to enable comparison based on signal intensity. Cell intensities for each condition were averaged for the sake of comparison.

Invadopodium precursor analysis. MTLn3 cells transfected with light-insensitive control or mCherry Z-lock coflin were irradiated for 1 min and fixed at 1, 3, 5, and 30 min post-photoactivation. Cells were stained with anti-Cortactin and anti-TRAP antibodies and imaged on a Delta Vision epi-fluorescence microscope (Applied Precision Inc.), equipped with a CoolSNAP HQ2 camera and a x60, NA 1.42 objective lens. Invadopodium precursors were identified as TRAP- and cortactin-positive puncta. The number of invadopodium precursors per cell were quantified in Fiji/ImageJ.

Modeling of linkers connecting Zdk and LOV2 to coflin. Structural models were generated using the RosettaRemodel package with the Rosetta3.5 series software\(^1\). This package was designed to provide a framework for flexible protein design using the loop modeling tools in Rosetta. In this case, we used the domain insertion protocol to model the orientation of the Zdk-LOV complex relative to coflin with Zdk and LOV attached to the N and C termini of coflin (PDB 4BEX), respectively\(^5\). For both Zdk1 (PDB 4FEX) and Zdk2 (PDB 4DFT), we modeled linkers of different length and composition and assessed whether the Zdk-LOV complex reliably oriented over the actin binding interface of coflin\(^6\).

Computational identification of Zdk2 point mutants. The change in binding energy for Zdk2 point mutants was calculated using the dG monomer package with the Rosetta3.5 series software\(^1\). The package was designed to predict the change in stability (the dG or ΔΔΔG) of a protein induced by a point mutation. In this case, we calculated the ΔΔΔG induced by several point mutants for both Zdk2 and the Zdk-LOV complex, using the Protein Database structure 4DFT as a starting point. Subtracting the ΔΔΔG for Zdk2 from the ΔΔΔG for the Zdk-LoV complex yielded the change in binding energy. The shift in binding curve was calculated using the following equation:

\[
\Delta \Delta G = -RT \ln \left( \frac{k_{d1}}{k_{d2}} \right)
\]

where \(\Delta \Delta G\) is the change in binding energy, \(R\) is the gas constant, \(T\) is the temperature and \(k_{d1}/k_{d2}\) is the shift in the binding curve.

Statistical analysis. Statistical significance was determined by either \(t\)-test (normally distributed dataset) or Mann–Whitney test (nonnormally distributed dataset). Normality of each dataset was tested using the D’agostino and Pearson (normally distributed dataset) or Mann–Whitney test (nonnormally distributed dataset). Normality test. For selected datasets, we used a Tukey box-and-whisker plot. The ends of the box denote the interquartile range (IQR) and the median is marked as a line across the box. Whiskers represent either the largest data point less than or equal to 1.5 times the IQR (upper whisker) or the smallest data point more than or equal to −1.5 times the IQR (lower whisker). Outliers (any value larger than 1.5 times the IQR or smaller than −1.5 times the IQR) are displayed as dots. All statistical analysis was conducted with GraphPad Prism 7. During optimization of linkers and Zdk2 mutants, some constructs were modified once (Fig. 2a-g). The final design used for cell-based experiments was retested by actin cosedimentation assay in three independent experiments and the data is shown in Fig. 2c. Microscopy images are representative of three independent experiments.

Biochemical validation of Z-lock αTAT. A 5 s on −5 s off regime of blue light exposure was achieved using an LED-panel constructed for placement in a 37 °C, 5% CO2 incubator and controlled with an Arduino board. The blue light intensity on the cells was approx. 0.05 mW μm\(^{-2}\). Cells were kept under blue light for 30 min and then lysed in lysis buffer on ice for 20 min. Lysates were loaded on SDS–PAGE gels and transferred to polyvinylidene difluoride membranes for western blotting. The samples were stained with monoclonal anti-Acetyl-Tubulin antibody (Sigma, 6-11B-1) for acetylated alpha-tubulin and anti-FLAG antibody (Abcam, ab9763) for either αTAT or Z-lock αTAT at 4 °C overnight. The samples were then washed and stained with dye-labeled secondary antibodies (ThermoFisher, Dylight 800; Bio-Rad, Starbright 700) at room temperature for 1 h.

Immunofluorescence of microtubule acetylation. Cells were fixed with ice-cold 100% methanol for 3 min before permeabilizing with 0.5% Triton-X100. Staining was performed with the same primary antibodies as above and with a pair of dye-conjugated secondary antibodies (Abcam: ab150105, ab175471). All immunofluorescence buffers were made from a 10X stock of BB80 with 0.1% Triton-X100. Stained cells were mounted in prolong gold (ThermoFisher Scientific) and imaged on an Olympus spinning disk confocal microscope with a x60 objective. Intensity measurements were made and masks were applied to allow for masking the cells in Fiji/ImageJ and normalizing acetylation intensity to expression level.

Reporting Summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability. The data that support the findings of this study are available from the corresponding authors upon request.

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Author contributions

J.S.C. and K.M.H. conceived of the project. O.I.S., B.L. and N.P. carried out the experiments, with assistance and advice from V.P.S. and R.J.E. in live cell imaging and assay of effects on coflin. K.M.H. and J.S.C. supervised the project. O.I.S., F.D.T. and B.K. performed Rosetta modeling. T. Watanabe for assistance with local photoactivation experiments and C. Onyeji for help with cloning and biochemical assays. N.P. was supported by grant PF-16-18-01-CSM from the American Cancer Society.

Competing interests

The authors declare no competing interests.

Additional information

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- [ ] Clearly defined error bars
- [ ] State explicitly what error bars represent (e.g. SD, SE, CI)

Our web collection on statistics for biologists may be useful.

Software and code

Policy information about availability of computer code

**Data collection**
- Image Studio (Version 5.2.5) and Image lab (Version 6.0) was used for collecting Western blot and stain-free gel images. Metamorph (Version 7.8.12.0) was used to control Olympus IX-81 microscope and Zen (Version 2.5) was used to control the Zeiss LSM 880.

**Data analysis**
- Prism 7 for Mac OS X (Version 7d) was used for all statistical analysis. Image Studio (Version 5.2.5) and Image Lab (Version 6.0) was used for analyzing Western blot data. Fiji/ImageJ (Version 1.0) was used for microscopy image analysis. Molecular modeling was accomplished with dg monomer and Rosetta Remodel applications contained within the Rosetta 3.5 molecular modeling suite. For measurement of F-actin content, a flat field correction was applied to all images using a custom script contained within the Hahn Lab biosensor processing package. Code is available from the authors upon request or at hahnlab.com.

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Data

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All manuscripts must include a data availability statement. This statement should provide the following information, where applicable:
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- A list of figures that have associated raw data
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Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

Sample size

Sample sizes were chosen to determine whether significant differences existed between groups based on past results. For actin co-sedimentation, directionality index, and change in cellular F-actin content: see Ghosh M. et al., Science, 2004, 743-6. For invadopodia analysis: see Oser M. et al., J. Cell Biology, 2009, 571-587.

Data exclusions

No data was excluded from analysis except cells in a non-physiological state.

Replication

All attempts at replication were successful. During optimization of linkers and Zdk2 mutants, some constructs were tested only once by actin co-sedimentation assay (Figure 2b and 2f). The final design used for cell-based experiments was retested by actin co-sedimentation assay in three independent experiments and the data is shown in Figure 2b.

Randomization

No randomization was performed since data was collected from individual cells from one cell line by electrical setups (i.e. live cell microscopy).

Blinding

With the exception of invadopodia counting, analysis of data was performed by automated protocols implemented in the software listed above, therefore blinding was not necessary. Due to the complexity of invadopodia experiments and analysis, both were routinely performed by the same person with relevant knowledge. Although we attempt to analyze data without knowledge of experimental conditions, blinding for data analysis cannot be assured, and therefore we do not consider the analysis of invadopodia numbers to be blinded.

Reporting for specific materials, systems and methods

Materials & experimental systems

| n/a | Involved in the study |
|-----|-----------------------|
| ☑️ | Unique biological materials |
| ☑️ | Antibodies |
| ☑️ | Eukaryotic cell lines |
| ☑️ | Palaeontology |
| ☑️ | Animals and other organisms |
| ☑️ | Human research participants |

Methods

| n/a | Involved in the study |
|-----|-----------------------|
| ☑️ | ChIP-seq |
| ☑️ | Flow cytometry |
| ☑️ | MRI-based neuroimaging |

Unique biological materials

Policy information about availability of materials

Obtaining unique materials

Cofilin and caged cofilin designs are available as E. coli expression vectors from Novagen (EMD Millipore, 69660). MTLn3 cells are available from the corresponding authors upon reasonable request.
Antibodies

Antibodies used

Antibodies were from the following sources: Cofilin (D3F9) XP® Rabbit mAb (Cell Signaling #5175), β-Actin (B10D10) Mouse mAb (Cell Signaling #3700), Tks5 (Santa Cruz Biotechnology; sc-30122), Cortactin (Abcam; ab33333), DyLight™ 488 Phalloidin (Cell Signaling, 12935). Secondary antibody: ThermoFisher: Dylight 800; Bio-rad: Starbright 700; Alexa Fluor® 488 Donkey Anti-Mouse (Abcam: ab150105); Alexa Fluor® 568 Goat Anti-Rabbit (Abcam: ab175471). The primary antibodies were diluted 1000X for Western blot and 100X for immunostaining. The secondary antibodies were diluted 20000X for Western blot and 200X for immunostaining.

Validation

All antibodies from Cell Signaling were validated by the manufacturer via Western blot and immunofluorescence staining. Validation via Western blot is done for multiple cell lines to confirm the presence of a single band at the appropriate molecular weight. Validation via immunofluorescence confirms appropriate subcellular localization. Tks5 antibody was validated by Western blot and immunofluorescence in multiple publications (Chan K. et al., J. Cell Biol., 2009 and Oser M. et al., J. Cell Biology, 2009). Cortactin antibody was validated by manufacturer by Western blot in multiple cell lines which produced a single band at the correct molecular weight, as well as by immunostaining a cortactin KO cell line. Validated species for the primary antibodies:
Cofilin: Human, Mouse, Rat, Monkey, Dog; β-Actin: Human, Mouse, Rat, Hamster, Monkey, Dog; Tks5: mouse, rat, human; Cortactin: Mouse, Rat, Chicken, Hamster, Cow, Human; Phalloidin: all species above.

Eukaryotic cell lines

Policy information about cell lines

Cell line source(s)

MTLn3 cells were obtained from Dr. G. Nicolson (MD Anderson Cancer Center, Houston, TX, USA). COS7, Hela cells were obtained from ATCC (https://www.atcc.org/). LinXe cells were obtained from www.bioxs.com.

Authentication

MTLn3 cells were not authenticated as they were obtained from the lab that originally isolated the cells as a single cell clone from a lung metastasis of the 13762NF rat mammary adenocarcinoma (see Neri A. et al., J Natl Cancer Inst., 1982 and Segall JE. et al., Clin Exp Metastasis., 1996). COS7, Hela were authenticated by ATCC. LinXe cells were authenticated by the merchant.

Mycoplasma contamination

MTLn3 cells were routinely tested for mycoplasma contamination using PCR detection method, all results were negative.

Commonly misidentified lines (See CLAC register)

No commonly misidentified cell lines were used.