HHS Public Access

Author manuscript

ACS Chem Biol. Author manuscript; available in PMC 2015 January 17.

Published in final edited form as:

ACS Chem Biol. 2014 January 17; 9(1): 111–115. doi:10.1021/cb400755b.

A General Method for Regulating Protein Stability with Light

Kimberly M. Bonger[*,†], **Rishi Rakhit**, **Alexander Y. Payumo**, **James K. Chen**, and **Thomas J. Wandless**[*]

Department of Chemical & Systems Biology, Stanford University, Stanford CA, 94305, USA

Abstract

Posttranslational regulation of protein abundance in cells is a powerful tool for studying protein function. We here describe a novel genetically encoded protein domain that is degraded upon exposure to non-toxic blue light. We demonstrate that fusion proteins containing this domain are rapidly degraded in cultured cells and in zebrafish upon illumination.

Methods to conditionally control protein function in cells can be valuable for researchers studying complex biological systems. Over the last several years, we and others have developed technologies to regulate proteins post-translationally using small molecule-ligands.[1] However, some experiments require tight spatial or temporal control of protein function, which makes the use of ligands sub-optimal as they can diffuse freely within and between cells and can be difficult to remove. The use of light as a perturbant has several advantages over small-molecule ligands as it can be delivered instantly and can be applied in a spatially restricted manner to cells or organisms.

Several methods employing light-responsive protein domains have recently been described, but significant trial-and-error can be required to make these systems useful for the particular proteins being studied. One strategy employs the Light-Oxygen-Voltage (LOV) domains that are found in plant photoreceptor proteins and respond to blue light via a flavin cofactor.[2] The LOV2 domain of phototropin 1 from *Avena sativa* (AsLOV2) possesses a C-terminal alpha helix that is tightly bound to the LOV core domain in the absence of light. Exposure to blue light induces formation of a flavin-cysteine adduct, resulting in unfolding of the helix. The AsLOV2 domain has been used to regulate the activity of proteins by sterically inhibiting interactions with an effector protein or by conformationally restricting a specific protein state.[3-5] These methods are suitable for the engineered proteins reported, but not generally applicable to any protein-of-interest. Engineered photosensory domains have also been used to establish light-regulated protein-protein interactions.[6-14] Light-induced translocation to the cell or nuclear membrane has been reported to regulate location-specific protein activity and light-induced gene expression, respectively. These light-dependent translocation strategies, however, generally require two or more genetic manipulations. We

[*]**Author information**k.bonger@science.ru.nl and wandless@stanford.edu.

[†]Present Address: Department of Biomolecular Chemistry, Radboud University Nijmegen, PO Box 9101, 6500 HB Nijmegen, The Netherlands.

Supporting information

A detailed description and analysis of all constructs described in the text. This material is available free of charge *via* the Internet at http://pubs.acs.org.

set out to develop a method for posttranslational control of protein levels in mammalian cells that (1) is generally useful for any protein, (2) makes use of a single regulatory domain, and (3) is regulated by non-toxic blue light, so that this technique does not require a small-molecule ligand that may have unintended effects.

We envisioned that a small peptide degron could be fused to the C-terminal alpha helix of the AsLOV2 domain to engineer a conditional Blue-Light Inducible Degradation (B-LID) domain. Renicke et al recently showed that such approach can be applied in *Saccharomyces cerevisiae*.[15] We have previously shown that a small four-amino acid peptide degron, RRRG, fused to the C-terminus of a protein of interest results in fast proteasome-mediated degradation in mammalian cells.[16] We reasoned that the C-terminal alpha helix would interact with the LOV core domain in the dark state, thus sequestering the degron away from cellular quality control proteins and rendering it cryptic. Exposure to blue light should cause the C-terminal helix to dissociate from the LOV core domain, thus revealing the degron and inducing degradation of the B-LID domain and its fusion protein through the processive activity of the proteasome (Figure 1a).

We began by using full length (residues 404-546) as well as truncated versions of the AsLOV2 domain of phototropin 1 with the RRRG degron fused to the C-terminus of the photo-responsive J-alpha helix [Supporting Information (SI) Figure S1]. These constructs were fused to the C-terminus of yellow fluorescent protein (YFP) to facilitate easy analysis of B-LID stability. NIH3T3 cells stably transduced with these constructs were analyzed by analytical flow cytometry. We used a blue LED light source to illuminate the cells. The four constructs tested (LOV5-8, SI Figure S1) displayed low fluorescence in both the dark and illuminated states, likely because the RRRG degron was too strong or insufficiently sequestered by the core LOV2 domain, leading us to investigate the use of the weaker RRRGN degron. Cells expressing YFP fused to LOV2 domains encoding the RRRGN degron displayed fluorescence when not illuminated, and a slight decrease in fluorescence was observed for cells that were exposed to blue light (465 nm) for two hours (LOV1-4, SI Figure S1).

In order to improve the dynamic range of the B-LID domain, we investigated the effects of mutations that are known to modulate the properties of the LOV domain. As the interaction of the C-terminal helix to the LOV2 core domain is in equilibrium with helix undocking, we aim at either stabilizing the conformation in the dark or in the lit state. We first introduced into all eight constructs a mutation (I532A) that results in enhanced helix binding to the core LOV2 domain with the goal of decreasing degron exposure, and thus decreasing degradation, in the unilluminated state.[17]

Additionally, another mutation (V416A) that lowers the rate of flavin deprotonation was explored to increase the time of degron exposure in the lit state.[18] We tested these mutations alone or in combination by transducing cells with each construct and exposing them to blue light. Cells stably expressing these constructs displayed varying levels of light-regulated YFP expression (LOV9-32, SI Figure S1).

The LOV24 construct displayed the best combination of stability in the dark state and instability when illuminated with blue light. The LOV24 domain lacks the last three amino acids of the original AsLOV2 domain, it contains the stronger RRRG degron, and it contains the I532A mutation which results in stronger helix binding to the AsLOV2 core domain. We tested additional mutations that are known to increase the helix binding affinity (e.g., the G528A/N538E tandem mutant)[17] but these did not improve the dynamic range of the LOV24 construct (SI Figure S2).

When illuminated with blue light for two hours NIH3T3 cells stably expressing the YFP-LOV24 fusion protein showed a 5-10 fold decrease in fluorescence as measured by flow cytometry and immunoblotting, whereas YFP alone was not affected by illumination (Figure 1b and SI Figure S3). Addition of the proteasome inhibitor MG132 prevented the light-dependent decrease in fluorescence (Figure 1b), indicating the involvement of the proteasome. The maximum amount of YFP-LOV24 degradation was observed approximately 90 minutes after starting illumination, and no further reduction in YFP levels was observed as illumination was continued (Figure 1c and 1d). Since conformational dynamics of the LOV2 domain is on the millisecond timescale[19] it is likely that the capacity of the proteasome to degrade the large pool of substrate that is generated upon illumination is the rate limiting step. To test the ability of the LOV24 domain to confer light-dependent stability to other proteins we fused the LOV24 domain to a β-actin-mCherry fusion protein as well as to the mCherry fluorescent protein alone. Cells stably expressing these constructs were illuminated with blue light for two hours resulting in low fluorescence levels for both β-actin-mCherry-LOV24 (SI Figure S4) and the mCherry-LOV24 fusion as evidenced by fluorescence microscopy, flow cytometry and immunoblot (Figure 2a-c).

An important feature of light-regulated conditional protein alleles such as the B-LID domain is the ability to perturb these proteins in a temporary or spatially selective manner in living organisms. This can be particularly useful in transparent model organisms such as zebrafish or *C. elegans*. Illumination is instantaneous, which allows for rapid regulation of light-sensitive proteins. The use of small molecule ligands to regulate proteins may be troublesome in these organisms, as ligands may elicit unanticipated or undesired effects (e.g., toxicity) and may not be evenly distributed within the organism. We demonstrated the feasibility of using the B-LID domain *in vivo* by injecting mRNA encoding mCherry-LOV24 into zebrafish embryos. The embryos were then cultured with or without blue light illumination. Fluorescence was detectable 6 hours post-injection for embryos grown without illumination, and mCherry expression became more apparent after 24 hours (Figure 2d). Embryos continually illuminated with blue light displayed low mCherry fluorescence at all time points, and light-induced degradation of mCherry-LOV24 was confirmed by immunoblotting (Figure 2e). We next raised embryos in the dark for six hours, at which point illumination was started to promote degradation of the mCherry-LOV24 fusion protein (SI Figure S5). A significant decrease in fluorescence was observed compared to the embryos that were not illuminated. This confirms the potential of the B-LID domain and light to reversibly regulate a protein of interest in transparent organisms.

The spatial regulation of specific genes in zebrafish embryos have shown to be a promising approach to investigate the spatiotemporal differences in gene function.[20] Here, uncaging of

a small molecule by a UV pulse generated the active molecules instantly allowing spatial activation. The disadvantage of the B-LID system may be the long-term illumination that is needed to ensure complete spatial degradation of the fusion protein. In some cases however, reversible gene regulation is desired which is difficult to achieve in life organisms with the uncaging strategy thereby making the B-LID strategy more appropriate.

This new method allows protein levels to be rapidly and reversibly controlled by light on a posttranslational level. The B-LID is simple to use through fusion to the 3'-end of any gene under study. Additionally, only one genetic manipulation is required, which makes this strategy attractive for use in organisms that are not easily amenable to high-efficiency gene targeting. We have demonstrated the utility of the B-LID domain in cultured cells and in zebrafish embryos, but we envision its use in other organisms.

Methods

Blue-Light Induced Degradation

For blue-light induced degradation experiments we used a commercially available blue LED light source (TaoTronics TT-AL02 Aquarium Coral Reef Tank LED Grow Light 120W Output, Blue/White Ratio 30:25). The lamp was set so only the blue LEDs (460-470 nm, 30 × 3W) were used and a green filter (roscolene #781) was placed between the light source and the culture dishes. Cells were cultured approximately 15 cm from the light source in normal growth media. The measured powers at 465 nm at a distance of 15 cm from the light source was 0.65 mW/cm^2 with the green filter. No extra measures were taken to protect cells from ambient light in the dark-state. However, to ensure similar cell growth conditions between the samples, the cell culture dishes that were cultured without illumination were wrapped with aluminum foil and placed the dishes in the path of the light source next to the illuminated cells.

Cloning, cell culture, transfections and transductions

Genes encoding the proteins tested as fusions to the B-LID domain were cloned in the multiple cloning site by standard techniques in the retroviral pBMN vector. For screening, the 3'-terminus of cDNA encoding YFP was fused to the various full-length AsLOV2 domains or c-terminal truncations thereof. I532A, V416A, G528A and N538E mutations were introduced with a Quikchange Site-Directed Mutagenisis Kit (Stratagene).

The ΦNX ecotropic packaging cells were cultured in DMEM supplemented with 10% heat-inactivated fetal bovine serum, 2 mM glutamine, 100 U/mL penicillin and 100 μg/mL streptomycin. The NIH3T3 cell line was cultured in DMEM supplemented with 10% heat-inactivated donor bovine serum, 2 mM glutamine, 100 U/mL penicillin and 100 μg/mL streptomycin. The ΦNX cells were transfected using standard Lipofectamine 2000 protocols. Viral supernatants were harvested 48 h posttransfection and filtered through a 0.45 μm nylon syringe filter. NIH3T3 cells were incubated with the retroviral supernatants supplemented with 4 μg/mL polybrene for 4-6 h at 37 °C. Cells were cultured in growth media for 24 to 36 h to allow for viral integration, then assayed as described.

Flow cytometry

Transduced NIH3T3 cells were plated at 1×10^5 cells per well of a 12-well plate and treated with vehicle or blue light for indicated time points prior to analysis. Cells were detached from wells using trypsin-EDTA and quenched with 1 mL of growth medium. No extra measures were taken to keep cells in dark during sample preparation. Samples were analyzed at the Stanford Shared FACS Facility with 10,000 events typically represented.

Immunoblotting

NIH3T3 cells were washed in PBS and lysed on ice in MIPP buffer containing protease inhibitors. Protein concentration was measured by Bradford assay. A sample containing 5-10 μg of total protein was boiled with 5% (v/v) 2-mercaptoethanol. Samples were resolved using 9% SDS-PAGE gels and transferred to PVDF. Blots were blocked in 5% dried non-fat milk in TBS buffer containing 0.05% Tween-20, and proteins were visualized by antibodies detecting YFP, the HA epitope or β-tubulin using standard protocols.

Zebrafish

HA-mCherry-LOV24 mRNA was *in vitro* transcribed from a HA-mCherry-LOV24 PCR product with the mMessage Machine SP6 kit (Ambion) according to manufacturer's directions. For injections of mRNA, a 150 ng/μL solution containing 100 mM KCl and 0.1 % (w/v) phenol red was prepared. One-cell stage zebrafish embryos were microinjected with this solution (1-2 nL/embryo) and embryos were divided. One dish was covered in foil (dark) and one dish was uncovered (light). Embryos from both dishes were cultured 10 cm from the light source in E3 media to ensure a similar temperature. Embryos in which mature protein was evaluated for degradation were cultured in the dark for 6 hours and then uncovered to allow illumination.

Zebrafish imaging

Embryos were manually dechorinated and immobilized in E3 medium containing 0.5% (w/v) low-melt agarose and 0.05% (w/v) Tricaine mesylate. Images were acquired using a Leica M205FA fluorescence stereoscope equipped with a Leica DFC500 digital camera or a Leica DMI 6000B inverted microscope equipped with a coolsnap HQ digital camera.

Zebrafish western blot analysis

Injected embryos were manually decorionated and de-yolked in Ringers buffer containing 1 mM EDTA and 0.3 mM PMSF using a glass pipet that has been drawn to have a tip diameter approximately the size of the yolk. The embryos were washed with cold Ringers containing 1 mM EDTA and 0.3 mM PMSF twice. As much liquid as possible was removed and the embryos were frozen in liquid nitrogen. The embryos were next homogenized in cold SDS-PAGE loading buffer (75 μL/50 embryos; 63 mM Tris-HCl pH 6.8, 3.5% (w/v) SDS, 10% (v/v) glycerol) with a microfuge pestle. The sample was microfuged and the pellet was discarded. A solution of 5% (v/v) aqueous 2-mercaptoethanol and added to the supernatant and boiled for 10 minutes. Samples were resolved using 9% SDS-PAGE gels and transferred to PVDF. Blots were blocked in 5% dried non-fat milk in TBS buffer

containing 0.05% Tween-20. Proteins were visualized by antibodies detecting HA epitope and β-tubulin using standard protocols.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This research was supported by the NIH (R01 GM073046 to T.J.W. and DP1 HD075622 to J.K.C.). K.M.B. gratefully acknowledges the support of a Human Frontiers Science Program (HFSP) fellowship.

References

1. Ausländer S, Fussenegger M. From gene switches to mammalian designer cells: present and future prospects. Trends Biotechnol. 2013; 31:155–168. [PubMed: 23245728]
2. Herrou J, Crosson S. Function, structure and mechanism of bacterial photosensory LOV proteins. Nat. Rev. Microbiol. 2011; 9:713–723. [PubMed: 21822294]
3. Wu YI, Frey D, Lungu OI, Jaehrig A, Schlichting I, Kuhlman B, Hahn KM. A genetically encoded photoactivatable Rac controls the motility of living cells. Nature. 2009; 461:104–108. [PubMed: 19693014]
4. Lee J, Natarajan M, Nashine VC, Socolich M, Vo T, Russ WP, Benkovic SJ, Ranganathan R. Surface Sites for Engineering Allosteric Control in Proteins. Science. 2008; 322:438–442. [PubMed: 18927392]
5. Strickland D, Moffat K, Sosnick TR. Light-activated DNA binding in a designed allosteric protein. Proc. Natl. Acad. Sci. USA. 2008; 105:10709–10714. [PubMed: 18667691]
6. Shimizu-Sato S, Huq E, Tepperman JM, Quail PH. A light-switchable gene promoter system. Nat. Biotechnol. 2002; 20:1041–1044. [PubMed: 12219076]
7. Levskaya A, Weiner OD, Lim WA, Voigt CA. Spatiotemporal control of cell signalling using a light-switchable protein interaction. Nature. 2009; 461:997–1001. [PubMed: 19749742]
8. Yazawa M, Sadaghiani AM, Hsueh B, Dolmetsch RE. Induction of protein-protein interactions in live cells using light. Nat. Biotechnol. 2009; 27:941–945. [PubMed: 19801976]
9. Kennedy MJ, Hughes RM, Peteya LA, Schwartz JW, Ehlers MD, Tucker C. Rapid blue-light–mediated induction of protein interactions in living cells. Nat. Methods. 2010; 7:973–975. [PubMed: 21037589]
10. Strickland D, Lin Y, Wagner E, Hope CM, Zayner J, Antoniou C, Sosnich TR, Weiss EL, Glotzer M. TULIPs: tunable, light-controlled interacting protein tags for cell biology. Nat. Methods. 2012; 9:379–384. [PubMed: 22388287]
11. Wang X, Chen X, Yang Y. Spatiotemporal control of gene expression by a light-switchable transgene system. Nat. Methods. 2012; 9:266–269. [PubMed: 22327833]
12. Polstein LR, Gersbach CA. Light-Inducible Spatiotemporal Control of Gene Activation by Customizable Zinc Finger Transcription Factors. J. Am. Chem. Soc. 2012; 134:16480–16483. [PubMed: 22963237]
13. Lungu OI, Hallett RA, Choi EJ, Aiken MJ, Hahn KM, Kulman B. Designing Photoswitchable Peptides Using the AsLOV2 Domain. Chem. Biol. 2012; 19:507–517. [PubMed: 22520757]
14. Zhou XX, Chung HK, Lam AJ, Lin MZ. Optical Control of Protein Activity by Fluorescent Protein Domains. Science. 2012; 338:810–814. [PubMed: 23139335]
15. Renicke C, Schuster D, Usherenko S, Essen L-O, Taxis C. A LOV2 Domain-Based Optogenetic Tool to Control Protein Degradation and Cellular Function. Chem. Biol. 2013; 20:619–626. [PubMed: 23601651]
16. Bonger KM, Chen LC, Liu CW, Wandless TJ. Small-molecule displacement of a cryptic degron causes conditional protein degradation. Nat. Chem. Biol. 2011; 7:531–537. [PubMed: 21725303]

17. Strickland D, Yao X, Gawlak G, Rosen MK, Gardner KH, Sosnick TR. Rationally improving LOV domain–based photoswitches. Nat. Methods. 2010; 7:623–626. [PubMed: 20562867]
18. Zoltowski BD, Vaccaro B, Crane BR. Mechanism-based tuning of a LOV domain photoreceptor. Nat. Chem. Biol. 2009; 5:827–834. [PubMed: 19718042]
19. Yao X, Rosen MK, Gardner KH. Estimation of the available free energy in a LOV-2 Jα photoswitch. Nat. Chem. Biol. 2008; 4:491–497. [PubMed: 18604202]
20. Shestopalov IA, Pitt CLW, Chen JK. Spatiotemporal resolution of the Ntla transcriptome in axial mesoderm development. Nat. Chem. Biol. 2012; 8:270–276. [PubMed: 22286130]

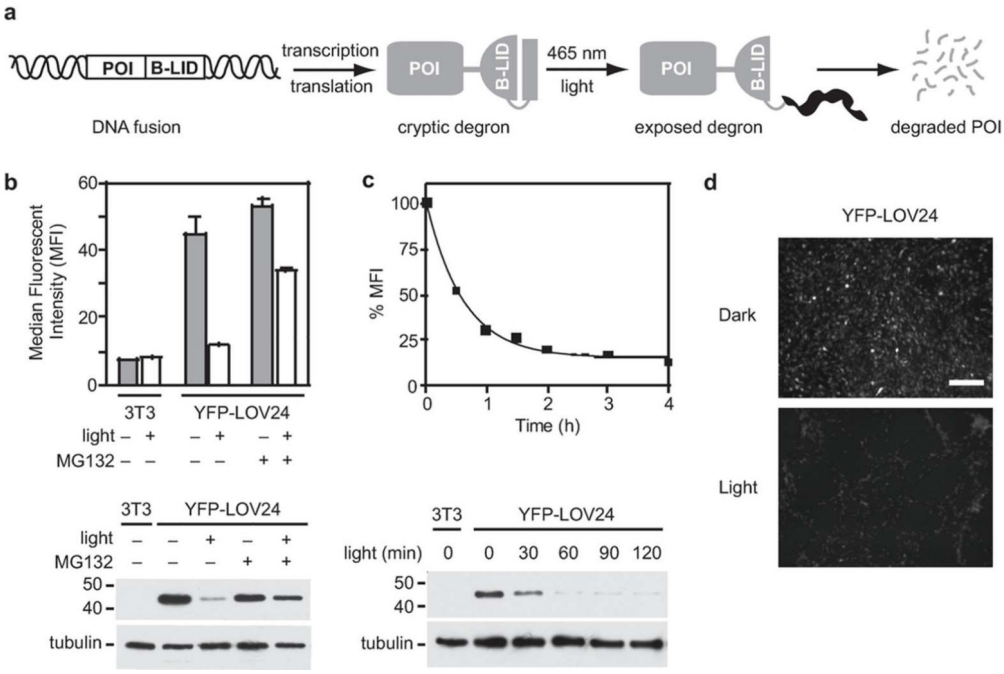

Figure 1.

The stability of the B-LID domain is regulated by light. a) Schematic of the B-LID domain genetically fused to a protein of interest. Expression of the protein and exposure to blue light results in degradation of the fusion construct. b) NIH3T3 cells stably expressing the YFP-LOV24 fusion protein were treated with either vehicle or 10 μM MG132 and were kept in the dark or irradiated with blue light for 2 hours and analyzed by flow cytometry and immunoblot using anti-YFP antibody. Tubulin is the loading control. The error bars represent the standard deviation of the mean based on at least two experiments. c) NIH3T3 cells expressing the YFP-LOV24 fusion protein were illuminated with blue light, and degradation of the fusion protein was monitored at various times using flow cytometry and anti-YFP immunoblotting. d) Fluorescence micrograph of NIH3T3 cells stably expressing the YFP-LOV24 fusion protein. Cells were kept in the dark or illuminated with blue light for 2 hours. The scalebar represents 100 μm.

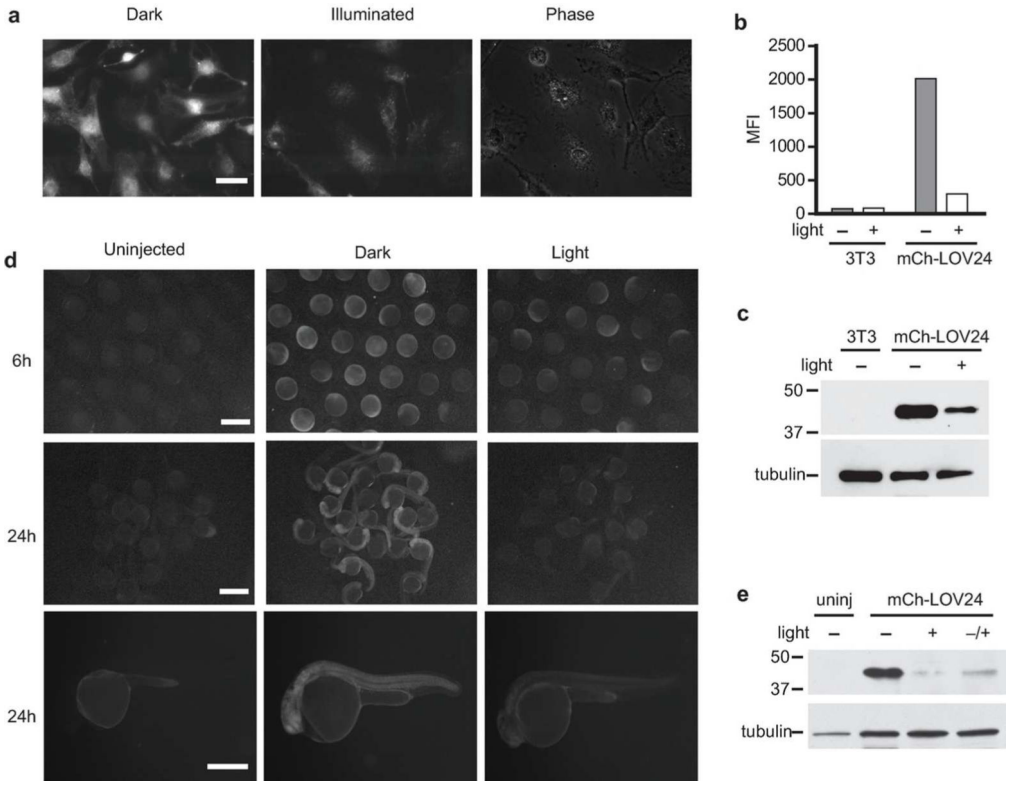

Figure 2.

The B-LID fusion is general and can be applied in zebrafish embryos. a) NIH3T3 cells were stably transduced with HA-mCherry-LOV24 and cells were illuminated with blue light for 2 hours and protein degradation was analyzed by fluorescence microscopy and b) analyzed by flow cytometry and c) confirmed by immunoblotting using an anti-HA antibody. The scalebar in panel a) represents 25 μm. d) Zebrafish embryos were microinjected with mRNA encoding HA-mCherry-LOV24. Embryos were raised in the dark or exposed to blue light for the indicated times, and degradation was observed by fluorescence microscopy and e) evidenced by immunoblotting using an anti-HA antibody. Scalebars represent 1 mm for images of multiple embryos and 250 μm for images of single embryos.