

Light inducible protein degradation in *E. coli* with the LOVdeg tag

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Abstract

Molecular tools for optogenetic control allow for spatial and temporal regulation of cell behavior. In particular, light controlled protein degradation is a valuable mechanism of regulation because it can be highly modular, used in tandem with other control mechanisms, and maintain functionality throughout growth phases. Here, we engineered LOVdeg, a tag that can be appended to a protein of interest for inducible degradation in *Escherichia coli* using blue light. We demonstrate the modularity of LOVdeg by using it to tag a range of proteins, including the LacI repressor, CRISPRa activator, and the AcrB efflux pump. Additionally, we demonstrate the utility of pairing the LOVdeg tag with existing optogenetic tools to enhance performance by developing a combined EL222 and LOVdeg system. Finally, we use the LOVdeg tag in a metabolic engineering application to demonstrate post-translational control of metabolism. Together, our results highlight the modularity and functionality of the LOVdeg tag system, and introduce a powerful new tool for bacterial optogenetics.

eLife assessment

This **valuable** study reports on a new tool that allows for light-controlled protein degradation in *Escherichia coli*. With the improved light-responsive protein tag, endogenous protein levels can be reduced severalfold. The methodology is **convincing** and will be of interest to the fields of gene expression regulation in bacteria and, more generally to synthetic biologists.

Introduction

Currently, the most widely used optogenetic systems in bacterial synthetic biology rely on transcription to modulate gene expression. Light responsive transcription factors allow genes of interest to be controlled modularly without having to re-engineer promoters. These tools have provided a valuable means to probe regulatory networks (Dessauges et al., 2022 ; Harrigan et al., 2018 ; Lugagne and Dunlop, 2019 ; Olson et al., 2014 ). However, these networks can be governed by complex control, where post-transcriptional and post-translational mechanisms work in concert with transcriptional regulation (Chubukov et al., 2014 ; Link et al., 2013 ; Mahmoud

and Chien, 2018 [\[10\]](#)). Light responsive regulators that act beyond transcription are necessary to more fully mimic natural biology and have the potential to improve upon current deficits of transcriptional control. In synthetic contexts, transcriptional regulation suffers from limited response dynamics. For example, in the case of gene deactivation, decreasing protein abundance is limited by cell division-based dilution or natural protein half-lives, which are in the range of 5-20 hours for the majority of proteins in *E. coli* (Maurizi, 1992 [\[11\]](#)). When cells are growing in exponential phase the effective degradation rate of these long-lived proteins is controlled by the cell cycle time, which is on the order of 0.5 hours. However, at high cell densities, such as during stationary phase, slow growth rates result in protein half-lives on the scale of tens of hours (Maier et al., 2011 [\[12\]](#)). Subpopulations of cells in stationary phase have been reported to proliferate, however these growing subpopulations are small compared to non-growing cells and add to heterogeneous protein half-lives within the population (Jöers et al., 2020 [\[13\]](#)), making it challenging to reliably remove proteins. It should be noted that a proportion of proteins are actively degraded during slow growth, but these represent the minority in *E. coli* (Gupta et al., 2022 [\[14\]](#)). This is a critical issue for metabolic engineering where chemical production is typically carried out at stationary phase in two-stage fermentations (Lalwani et al., 2018 [\[15\]](#)).

Post-translational optogenetic control has the potential to address some of the shortcomings of transcriptional regulation because post-translational control mechanisms can function independently of growth-based dilution. Existing approaches to post-translational optogenetic control include the use of light inducible dimers in split protein systems (Baumschlager et al., 2017 [\[16\]](#); Kawano et al., 2015 [\[17\]](#); Nihongaki et al., 2015 [\[18\]](#)), domain insertions with light controlled allosteric domains (Dagliyan et al., 2016 [\[19\]](#); Gil et al., 2020 [\[20\]](#); Tague et al., 2023 [\[21\]](#)), and membrane-confined functional control (Strickland et al., 2012 [\[22\]](#); Wang et al., 2016 [\[23\]](#)). These approaches, however, suffer from a lack of modularity, which is a key benefit of the transcriptional control approaches. Split protein systems and domain insertions can require significant engineering for each protein of interest and solutions for a given protein are unlikely to map to proteins with different structures. Likewise, localization-based approaches are only applicable to a small subset of proteins that have position dependent function, such as transcription factors that require nuclear localization (Yumerefendi et al., 2018 [\[24\]](#)).

Protein degradation offers potential as a modular post-translational mechanism of control. Endogenous proteolytic machinery is necessary for proteome homeostasis and acts as a global regulatory system (Mahmoud and Chien, 2018 [\[10\]](#)). Targeted proteolysis has proven useful in many synthetic biology applications in both eukaryotic and prokaryotic systems (Andersen et al., 1998 [\[25\]](#); Cameron and Collins, 2014 [\[26\]](#); McGinness et al., 2006 [\[27\]](#); Morreale et al., 2022 [\[28\]](#); Trauth et al., 2019 [\[29\]](#)). In eukaryotic cells, light dependent protein degradation has been demonstrated using various mechanisms (Bonger et al., 2014 [\[30\]](#); Deng et al., 2020 [\[31\]](#); Liu et al., 2020 [\[32\]](#); Renicke et al., 2013 [\[33\]](#); Xue et al., 2019 [\[34\]](#)). For example, Bonger et al. utilized the LOV2 domain of *Avena sativa* phototropin 1 (AsLOV2), to achieve light dependent protein degradation in mammalian cells (Bonger et al., 2014 [\[30\]](#)). LOV2 is a blue-light responsive protein that is widely used in optogenetic tools (Pudasaini et al., 2015 [\[35\]](#)). LOV2 contains a core Per-Arnt-Sim (PAS) domain surrounded by N- and C-terminal α -helices. Upon blue light illumination, the LOV2 protein undergoes a conformational change where the C-terminal α helix reversibly unfolds and becomes unstructured (Halavaty and Moffat, 2007 [\[36\]](#); Harper et al., 2003 [\[37\]](#); Yamamoto et al., 2009 [\[38\]](#)). Incorporation of a degradation-targeted peptide sequence into the C-terminal results in light dependent protein degradation in yeast (Renicke et al., 2013 [\[33\]](#)).

However, the bacterial proteasome differs from the eukaryotic proteasome in many ways (Mahmoud and Chien, 2018 [\[10\]](#); Schrader et al., 2009 [\[39\]](#)). The bacterial proteasome includes several proteases with divergent targeting behaviors and does not utilize ubiquitin as a generalized modification to trigger degradation (Finley, 2009 [\[40\]](#)). In bacteria, protein targeting for degradation is predominantly dependent on primary amino acid sequence as opposed to a ubiquitin-like appendage and, apart from certain well-studied cases, the rules governing sequence recognition of

bacterial degrons are not fully understood (Baker and Sauer, 2006 [↗](#); Striebel et al., 2009 [↗](#)). Because of these complexities, light dependent degradation systems for bacteria have lagged behind their eukaryotic counterparts, where interaction of a ubiquitin ligase with a defined degron can be used as a control mechanism (Bondeson et al., 2022 [↗](#)). Nevertheless, optogenetic degradation remains a key target for bacterial synthetic biology applications. In principle, an optogenetic degradation system could be fast-acting, modular, and interface with endogenous proteasome machinery. These features would provide a straightforward way of adding dynamic control to a protein of interest, complementing existing transcriptional tools for optogenetic control of gene expression. One recent system developed by Komera *et al.* utilizes a light responsive split TEV protease to expose or remove constitutively active degradation tags in *E. coli* (Komera et al., 2022 [↗](#)). Although this system achieves protein degradation in response to light, it acts indirectly through activation of an exogenous protease. A simpler approach where the degradation tag itself is light responsive would streamline this by eliminating the need for multiple exogenous components.

Here, we develop LOVdeg, a modular protein tag based on the AsLOV2 protein that is conditionally degraded in response to blue light in *E. coli*. We demonstrate that attaching this tag to a protein of interest confers light dependent protein instability. We show the modularity of the LOVdeg tag by incorporating it into multiple proteins with widely varying function, converting them all into optogenetically controlled systems. In addition, through photocycle stabilizing mutations, we create a version of the LOVdeg tag that responds to infrequent exposure to blue light. We also demonstrate that our degradation tag can be used in concert with other optogenetic systems for multi-layer control by using EL222, a blue light responsive system for transcriptional control, together with LOVdeg. Lastly, we incorporate optogenetic degradation into a metabolically engineered strain to control production of octanoic acid. Overall, this work introduces a new bacterial optogenetic tool that overcomes several drawbacks of transcriptional control by providing post-translational degradation, while avoiding the need for substantial protein engineering that can be required for alternative post-translational control mechanisms.

Results

Design and characterization of the AsLOV2-based degradation tag

The *E. coli* proteasome consists of five AAA+ proteases and is continuously active, either degrading misfolded proteins for quality control or balancing regulatory protein levels (Mahmoud and Chien, 2018 [↗](#)). We set out to exploit the endogenous proteasome activity in order to design a light responsive protein tag. To do this, we took insight from studies related to native protein quality control. In bacteria, peptides from stalled ribosomes are targeted for degradation through interaction with a tmRNA that appends a short amino acid sequence, known as an SsrA tag, to the C-terminal end of the incomplete protein (Keiler, 2015 [↗](#)). The *E. coli* SsrA tag has been studied extensively and is known to interact with the unfoldases ClpX and ClpA (Flynn et al., 2001 [↗](#); Gottesman et al., 1998 [↗](#)). Addition of the SsrA peptide sequence to exogenous proteins targets them for degradation by the host proteasome. SsrA mediated degradation has proven useful in synthetic gene circuit function and biochemical production (Elowitz, 2000 [↗](#); Gurbatri et al., 2020 [↗](#); Stricker et al., 2008 [↗](#); Torella et al., 2013 [↗](#); Ye et al., 2021 [↗](#)). A recent structural study of ClpX interacting with the SsrA tag from Fei *et al.* demonstrated that in order for ClpX to unfold a tagged protein, the C-terminal tail needs to be unstructured and sufficiently long to fit into the ClpX pore (Fei et al., 2020 [↗](#)). We reasoned that the mechanism of AsLOV2, in which the C-terminal J α helix becomes unstructured upon blue light absorption, could be utilized to provide light inducible protein degradation.

Biochemical studies have probed the amino acid sequence of the SsrA tag and its role in degradation targeting. Flynn *et al.* demonstrated that ClpA and ClpX unfoldases interact with overlapping residues within the SsrA sequence and that the last three amino acids (L-A-A) are particularly important for successful degradation (Flynn *et al.*, 2001 [DOI](#)). Importantly, in addition they showed that mutation of the leucine in the C-terminal 'L-A-A' lowers unfoldase affinity but does not hinder degradation completely. We noticed that the C-terminal amino acid sequence of the AsLOV2 domain, comprised of residues 404-546 of *Avena sativa* phototropin 1, are 'E-A-A' at positions 541-543 (**Fig. 1a** [DOI](#)). The dark state structure of the native AsLOV2 domain (PDB: 2V1A) shows that these three amino acids and K544 complete the folded Ja helix (**Fig. 1b** [DOI](#)). A truncation of residues 544-546 leaves the Ja helix largely intact and the resulting C-terminal 'E-A-A' remains caged as part of the folded helix as seen upon examination of the dark state structure (**Fig. 1b** [DOI](#)). We hypothesized that this truncation would be stable in the dark state as a consequence of 'E-A-A' caging and unstable in the light state due to Ja helix unfolding and exposure of an unstructured degradation tag.

Optogenetic systems have used the AsLOV2 domain in *E. coli*, however, it is often appended N-terminally or internally to another protein (Li *et al.*, 2022 [DOI](#); Strickland *et al.*, 2008 [DOI](#)). To the best of our knowledge, wild type or truncated AsLOV2, with its C-terminal end exposed, have not been used for proteolytic degradation in bacterial cells. To test the stability of C-terminal AsLOV2 in *E. coli* in response to light, we constructed a plasmid where we used an IPTG-inducible promoter to control the expression of mCherry translationally fused to AsLOV2 (**Fig. 1c** [DOI](#)). In this construct, the full C-terminal sequence is intact so unfoldases are not expected to have good access for protein degradation. Consistent with this, induction of mCherry-AsLOV2(546) with IPTG increased expression of the fusion construct, and 465 nm blue light induction resulted in only a modest decrease in expression (**Fig. 1d** [DOI](#)). Next, we tested a version of the construct where three amino acids were truncated to expose the C-terminal 'E-A-A', AsLOV2(543). This construct destabilized the protein fusion as predicted, resulting in significantly lower protein expression compared to the non-truncated version (**Fig. 1d** [DOI](#)). However, counter to our expectations, we observed only a modest decrease in protein levels upon blue light induction.

While AsLOV2 switches from mostly folded in the dark state to mostly unfolded in the light state, both states are present at an equilibrium with or without light (Yao *et al.*, 2008 [DOI](#)). Due to the suboptimal equilibrium of native AsLOV2, dark state undocking and unfolding of the Ja helix is a common issue in AsLOV2-based optogenetic tools, and many studies have aimed at improving the dynamic range of AsLOV2 (Guntas *et al.*, 2015 [DOI](#); Lungu *et al.*, 2012 [DOI](#); Strickland *et al.*, 2012 [DOI](#), 2010 [DOI](#); Wang *et al.*, 2016 [DOI](#)). Guntas *et al.*, performed phage display on an AsLOV2-based protein that incorporates a caged peptide sequence in the Ja helix. They identified a variant with 11 amino acid substitutions with much tighter dark state caging, called iLID. Interestingly, several of these mutations do not have a direct interaction with the caged peptide, but instead are located at the hinge loop connecting the PAS domain to the Ja helix or in the PAS domain itself (**Fig. S1**). Guntas *et al.* further characterized amino acid substitutions and determined several that did not affect caging or dynamic range if reverted (F502Q, H521R, and C530M). We focused on the mutations found to result in tighter dark state caging (L493V, H519R, V520L, D522G, G528A, E537F, N538Q, and D540A), and reasoned that this set should stabilize the Ja helix irrespective of the caged peptide. Thus, we next constructed a variant of AsLOV2 with these eight mutations, which we denote as AsLOV2*.

To test whether these mutations are beneficial in the context of protein stabilization, we fused AsLOV2*(546) or AsLOV2*(543) to mCherry (**Fig. 1c** [DOI](#)). mCherry with non-truncated AsLOV2*(546) expressed highly with the addition of IPTG, but blue light did not result in a decrease in mCherry levels, consistent with lack of access by proteases (**Fig. 1d** [DOI](#)). In contrast, the truncated version containing the mutations, AsLOV2*(543), displayed the desired light induced degradation, expressing highly in the dark and exhibiting 11.5x lower expression in response to blue light (**Fig. 1d** [DOI](#)). We also confirmed that AsLOV2*(543) expression was decreased in response to light

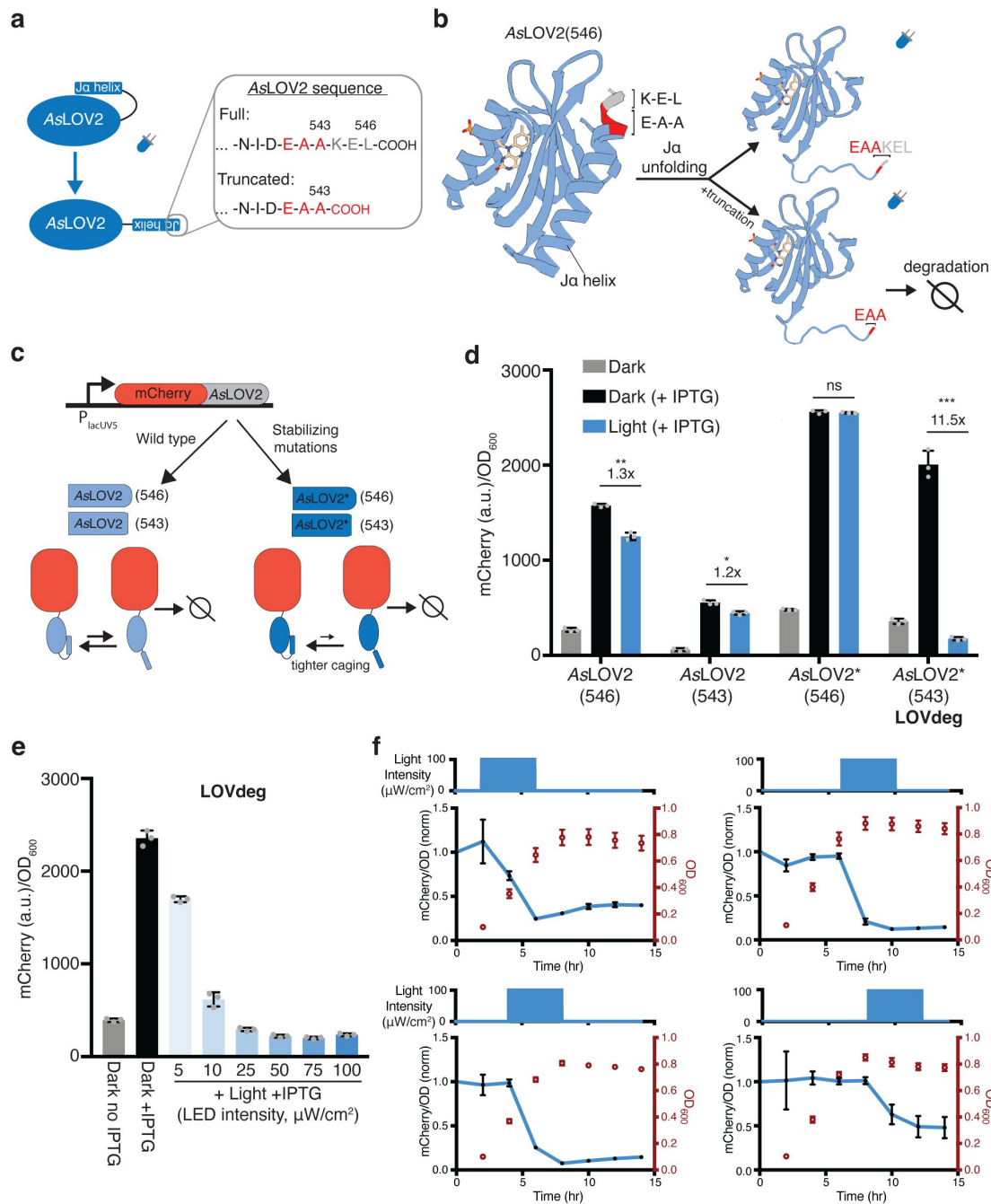


Figure 1.

Design of AsLOV2-based degradation tag. **(a)** Primary sequence of AsLOV2(546) C-terminal sequence. A three amino acid truncation exposes E-A-A. **(b)** Structure of AsLOV2 (aa404-546, PDB: 2V1A). Amino acids 541-543 (E-A-A) are red and 544-546 (K-E-L) are gray at the C-terminal of the Jα helix. **(c)** Construct used to characterize optogenetic control using AsLOV2 variants. Each variant is translationally fused to mCherry expressed from an IPTG inducible promoter. Variants include wild type AsLOV2 (light blue) and a dark state stabilized version, AsLOV2* (dark blue), with and without the three amino acid truncation. **(d)** mCherry protein levels in response to 465 nm blue light for wild type AsLOV2, and mutated AsLOV2* fusions with and without truncation. AsLOV2*(543) is the variant we denote the 'LOVdeg' tag. (***p < 0.0001; **p < 0.001; *p < 0.01; n.s., not significant; two tailed unpaired t-test; n = 3 biological replicates). **(e)** mCherry-LOVdeg in response to variable light intensities. **(f)** mCherry fluorescence levels and optical density of mCherry-LOVdeg with 4 hours of 465 nm blue light exposure applied at different points in the growth cycle. Light exposure programs are plotted above each subplot and are staggered 2 hours apart (starting at 2, 4, 6, or 8 hours), all lasting 4 hours. Expression levels are normalized to the dark state control (Fig. S8). Error bars show standard deviation around the mean.

without any IPTG induction (Fig. S2). Interestingly, AsLOV2*(543) is degraded more in response to light relative to AsLOV2(543). We conducted experiments to identify the mechanism underlying AsLOV2*(543) degradation and found evidence that ClpA is involved, and other proteases and unfoldases play complementary roles (Supplementary Text, Fig. S3-6). We also verified that light exposure itself did not affect mCherry protein levels or growth by conducting experiments with an IPTG inducible mCherry without any AsLOV2 variant fused and exposing cells to blue light (Fig. S7). We chose to move forward with the AsLOV2*(543) variant due to its light inducible properties and we denoted this variant as the ‘LOVdeg’ tag.

Next, we used the LOVdeg tag to test the impact of light intensity and the timing of light exposure. We found that the LOVdeg tag is destabilized in proportion to light intensity (**Fig. 1e** [↗](#)), making it straightforward to tune degradation by adjusting light levels. In addition, a key advantage of protein degradation-based mechanisms is that they should function at a range of growth phases. We tested this by varying when we applied blue light, ranging from early exponential to stationary phase. We found that light-dependent decreases in protein levels can be achieved at various stages of growth (**Fig. 1f** [↗](#), Fig. S8).

To benchmark the degradation capacity of the LOVdeg tag, we compared it to protein levels of mCherry subject to constitutive degradation via addition of an SsrA tag. In order to maintain comparable transcription and translation, we appended an iLID AsLOV2 derivative, which was modified to contain a full length SsrA sequence, to the IPTG inducible mCherry. As expected, mCherry-iLID-SsrA expressed poorly independent of IPTG induction (Fig. S9). To quantify the light-dependent degradation of the LOVdeg tag, we compared its dark state expression level to that of mCherry-iLID-SsrA. Using this comparison, light induced degradation of the LOVdeg tag reached 6x degradation compared to 14x for the SsrA tag. However, the comparison between these two is convoluted by the time required to degrade the protein in response to light, making it challenging to directly compare these numbers.

A potential concern is that light induced disorder of the J α helix could result in a decrease in solubility and aggregation of the LOVdeg tag. To rule this out as the cause of the fluorescence decrease, we captured microscopy images of cells in dark and light conditions. The imaging confirmed a light-dependent decrease in mCherry expression without the formation of visible protein aggregates (Fig. S10). Thus, the LOVdeg tag variant provides blue light dependent protein degradation.

Modularity of the LOVdeg tag

Post-translational control of protein function can require significant protein engineering for each use case (Sheets et al., 2020 [↗](#); Tague et al., 2023 [↗](#); Zhu et al., 2023 [↗](#)). Degradation tags, by contrast, offer post-translational control that theoretically requires little to no protein engineering and is protein agnostic. To test the modularity of the LOVdeg tag, we incorporated optogenetic control into three systems with highly diverse functions and relevance to synthetic biology and biotechnology applications: the LacI repressor, CRISPRa activation, and the AcrB efflux pump.

First, we sought to test whether the LOVdeg tag could be fused to transcription factors to enable light dependent regulation. The LacI repressor is a widely used chemically inducible system in synthetic biology. We translationally fused the LOVdeg tag to LacI and paired it with a reporter where the LacUV5 promoter controls expression of mCherry (**Fig. 2a** [↗](#)). Our results show that light exposure successfully increased mCherry expression (**Fig. 2b** [↗](#)). Light induced mCherry expression did not achieve the full levels provided with saturating IPTG induction, however we still observed a notable increase. We tested an alternative strategy for further improving de-repression, which suggested that the discrepancy between IPTG versus light dependent induction likely stems from the delay in LacI degradation compared to the rapid allosteric action of IPTG (Supplementary Text, Fig. S11).

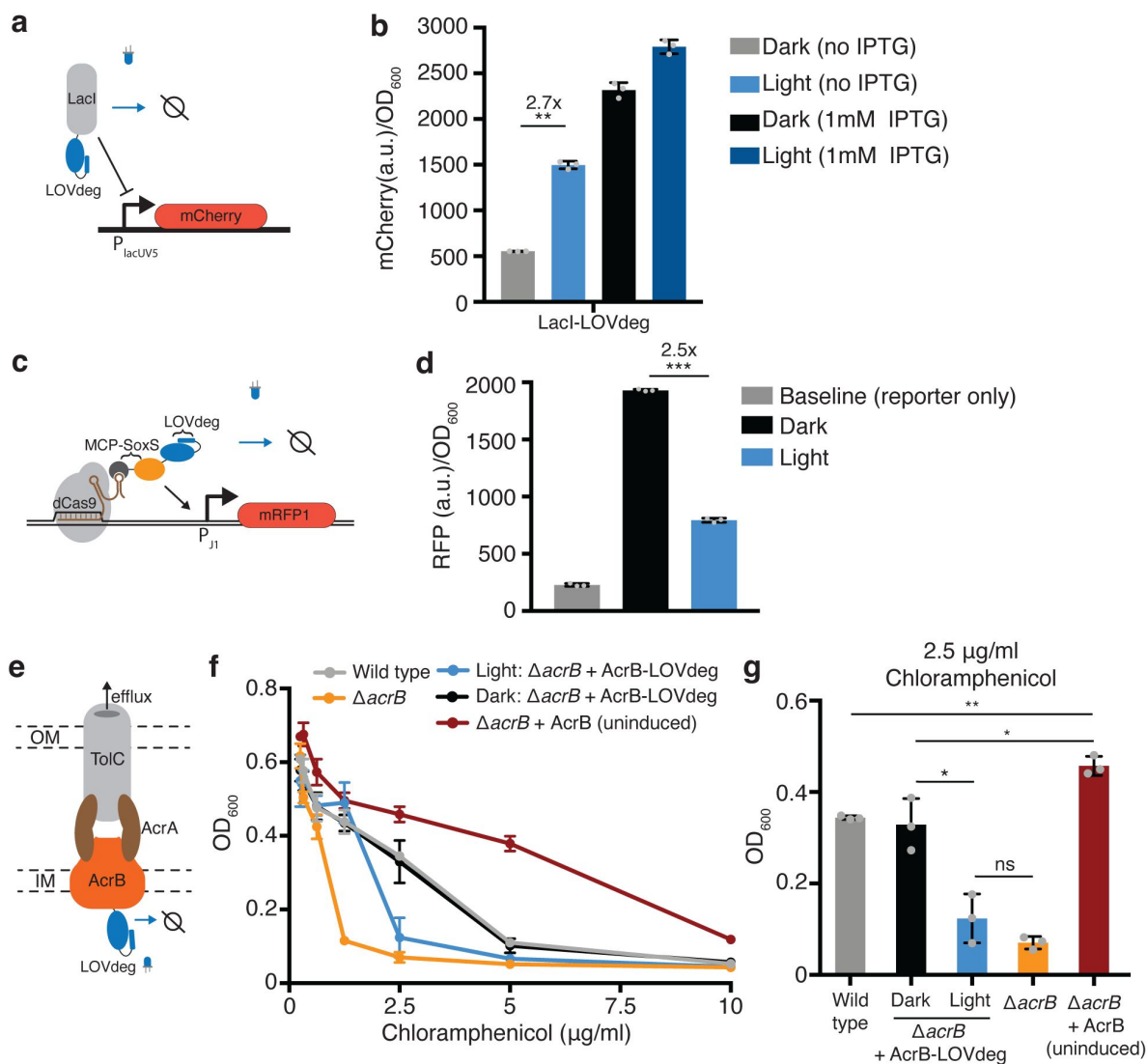


Figure 2.

Incorporating light responsiveness into diverse proteins with the LOVdeg tag. **(a)** Control of mCherry repression using a LacI-LOVdeg fusion. **(b)** mCherry expression in response to light exposure for strains with LacI-LOVdeg compared to IPTG induction (** $p < 0.001$, two tailed unpaired t-test). **(c)** Schematic of SoxS-based CRISPRa activation with a LOVdeg tag appended to the MCP-SoxS activator. **(d)** CRISPRa control of mRFP1 expression in response to light (*** $p < 0.0001$, two tailed unpaired t-test). **(e)** Schematic of the LOVdeg tag appended to AcrB of the AcrAB-TolC efflux pump. IM, inner membrane; OM, outer membrane. **(f)** Chloramphenicol sensitivity tests. Wild type cells (BW25113) are compared to a Δ acrB (BW25113 Δ acrB) strain, Δ acrB complemented with AcrB-LOVdeg (Δ acrB + AcrB-LOVdeg) exposed to light or kept in the dark, and Δ acrB strain complemented with an IPTG-inducible AcrB (Δ acrB + AcrB). No IPTG was added to Δ acrB + AcrA or Δ acrB + AcrB-LOVdeg. **(g)** OD₆₀₀ of strains shown in (f) at 2.5 µg/ml chloramphenicol (** $p < 0.001$; * $p < 0.05$; ns, not significant; two tailed unpaired t-test). Error bars show standard deviation around the mean ($n = 3$ biological replicates).

Next, we incorporated the LOVdeg tag into the SoxS-based bacterial CRISPRa activation system (Dong et al., 2018). In this system, a scaffold RNA, which is a modified gRNA containing an MS2 stem loop, is used to localize dCas9 and the transcriptional activator SoxS, which is fused to an MS2 coat protein (MCP). We translationally fused the LOVdeg tag to the MCP-SoxS protein, such that in the dark CRISPRa will be active and light exposure relieves activation (Fig. 2c). In the original system, MCP-SoxS expression is anhydrotetracycline (aTc) inducible. This induction system is not amenable to blue light stimulation because aTc is photosensitive (Baumschlager et al., 2020). Thus, we changed the MCP-SoxS construct to an IPTG inducible promoter prior to blue light experiments (Fig. S12a, Table S1, Table S2). We confirmed that the promoter switch maintained CRISPRa activity (Fig. S12b). Fusing the LOVdeg tag to the activator component indeed relieved CRISPRa activity, resulting in a decrease in expression under blue light stimulation (Fig. 2d). However, reversal of activator activity was not complete and expression during light stimulation remained above the baseline levels from the reporter only control, which could be due to the presence of low levels of the MCP-SoxS activator.

We also added the LOVdeg tag to the endogenous membrane protein AcrB. This represents a challenging test case for degrading a native protein. The AcrAB-TolC complex is a multidrug efflux pump with clinical relevance due to its role in antibiotic tolerance and resistance acquisition (El Meouche and Dunlop, 2018; Lizarralde-Guerrero and Taraveau, 2021; Okusu et al., 1996). AcrAB-TolC has also been utilized in metabolic engineering as a mechanism to pump out toxic chemical products and boost strain performance (Dunlop et al., 2011; Fisher et al., 2014). However, inducible control of AcrB is challenging because cell viability is sensitive to overexpression (Turner and Dunlop, 2015). Optogenetic transcriptional control systems have high dynamic ranges but do not operate in the low expression ranges relevant to very potent protein complexes such as AcrAB-TolC. Light based degradation is well suited for this challenge because it works by decreasing protein levels, allowing the upper bound of expression to be determined by the promoter.

Previous work from Chai *et al.* demonstrated that AcrB can be targeted for proteolysis by fusing an SsrA tag to the C-terminal (Chai et al., 2016). Degradation is possible because the C-terminal end of AcrB is on the cytoplasmic side of the inner membrane and can interact with cytoplasmic unfoldases (Du et al., 2014). Therefore, we reasoned that fusing the LOVdeg tag to AcrB would result in light inducible degradation that disrupts activity of the AcrAB-TolC complex (Fig. 2e). To determine if activity of the AcrAB-TolC complex was successfully disrupted by light, we performed an antibiotic sensitivity test using chloramphenicol, which is a known substrate of the AcrAB-TolC pump (Okusu et al., 1996). We transformed a construct containing *acrB-LOVdeg* into cells lacking the endogenous *acrB* gene (Δ *acrB*). When kept in the dark, Δ *acrB* cells with *acrB-LOVdeg* showed comparable chloramphenicol tolerance to wild type cells (Fig. 2f-g). Blue light stimulation, in contrast, sensitized the Δ *acrB* + AcrB-LOVdeg strain to chloramphenicol compared to both wild type and Δ *acrB* + AcrB-LOVdeg kept in the dark. This suggests that the LOVdeg tag successfully targets AcrB for degradation in a light dependent fashion. The blue light exposed cells still retain modest levels of chloramphenicol sensitivity when compared to Δ *acrB* cells without any AcrB complementation, likely due to basal levels of efflux pump expression from AcrB-LOVdeg relative to the knockout. As a point of comparison, we also tested chloramphenicol sensitivity of Δ *acrB* cells with IPTG-inducible AcrB expression (Δ *acrB* + AcrB) without IPTG. With no induction, this represents the lowest expression levels that can be achieved with a traditional IPTG-inducible system. The uninduced Δ *acrB* + AcrB cells displayed significantly higher tolerance to chloramphenicol when compared to wild type and other experimental groups. This underscores the challenge of controlling potent complexes like AcrAB-TolC through the use of chemical inducers alone and demonstrates how post-translational control, such as that provided by LOVdeg, is a viable and necessary strategy to decrease expression to levels approaching those in the Δ *acrB* knock out strain.

Tuning frequency response of the LOVdeg tag

Light inducible systems have the potential to respond to the frequency of light exposure. Frequency dependent tools can allow optogenetic circuits to be multiplexed beyond limited wavelength options or to add a layer of logic to optogenetic circuits (Benzinger et al., 2022 [↗](#)). With added logic operations, optogenetic circuits could perform complex signal processing, analogous to those demonstrated with multiplexed chemically inducible circuits (Shin et al., 2020 [↗](#)), while allowing dynamic light inputs. Additionally, higher sensitivity LOVdeg tags would also be useful in bioreactor settings, where poor light penetration into dense cultures is a feasibility concern. With these use cases in mind, we sought to characterize and alter the LOVdeg frequency response.

LOV domains utilize a flavin cofactor to absorb light. In the case of AsLOV2, the cofactor responsible for light absorption is flavin mononucleotide (FMN). A cysteine residue in AsLOV2 forms a reversible covalent bond with FMN, which initiates broader conformational change. A full photocycle of AsLOV2 consists of absorption of a photon, covalent bond formation with cysteine 450, Jα helix destabilization and unfolding, decay of the cysteine-FMN bond, and Jα helix refolding (Swartz et al., 2001 [↗](#)). Previous studies have determined the dynamics of bond formation and the time delay of Jα helix refolding. Further, mutations have been found that stabilize or destabilize the light state conformation (Christie et al., 2007 [↗](#); Kawano et al., 2013 [↗](#); Zayner et al., 2013 [↗](#)). In the context of the LOVdeg tag, the time needed for the Jα helix to refold, known as the reversion time, likely determines degradation characteristics (Fig. 3a [↗](#)). In principle, the time spent in the light state dictates the amount of time the protein is susceptible to degradation and, therefore, would impact the frequency response given variable light inputs.

To test the effect of LOVdeg tag refolding dynamics, we illuminated cells with 5 second pulses of blue light followed by variable length dark periods to allow Jα helix refolding (Fig. 3b [↗](#)). While holding the blue light duration fixed, we tested dark periods ranging from 475 seconds (5:475 seconds on:off, frequency of 0.002 seconds⁻¹ since there is 1 pulse every 480 seconds) to 5 seconds (5:5 seconds on:off, frequency of 0.1 seconds⁻¹) (Fig. 3b [↗](#)). We used 5 seconds for the pulse length because it is markedly shorter than the overall degradation dynamics for the LOVdeg tag, which ensures that a single pulse is not long enough to induce significant degradation. We first tested the frequency response of the original LOVdeg tag (Fig. 3c [↗](#)). The response is in line with known refolding dynamics of AsLOV2 (Li et al., 2020 [↗](#)), where over 50% degradation is only achieved at high frequencies (0.1 seconds⁻¹). Next, we tested a LOVdeg tag variant that contains a slow-photocycle mutation, V416I (Zoltowski et al., 2009 [↗](#)). This amino acid substitution has been shown to increase the dark state reversion time from 8 seconds to 84 seconds *in situ* (Li et al., 2020 [↗](#)). Indeed, for LOVdeg tag (V416I) over 50% degradation was achieved at medium frequencies (0.008 seconds⁻¹, which corresponds to 5:120 seconds on:off) (Fig. 3c [↗](#)). This variant offers the potential for better performance in settings where increased light sensitivity is preferred, such as within bioreactors.

Integrating LOVdeg with EL222 for multi-layer control

Another attractive aspect of post-translational optogenetic control is that it can integrate with existing systems that act at the transcriptional or translational level. Adding control at multiple layers has been shown to enhance the performance and robustness of natural and synthetic systems (Alon, 2007 [↗](#); Hasenjäger et al., 2019 [↗](#); Szydlowski et al., 2022 [↗](#)). One commonly used system for optogenetic transcriptional control is EL222. EL222 is a blue light responsive LOV protein that dimerizes and binds DNA upon light exposure (Zoltowski et al., 2013 [↗](#)). In bacteria, EL222 can be used as a transcriptional repressor or activator depending on the placement of its binding site in the promoter (Ding et al., 2020 [↗](#); Jayaraman et al., 2016 [↗](#)). We chose to combine the transcriptional repression of EL222 with the LOVdeg tag. In this arrangement, the systems work synergistically to decrease gene expression in response to blue light using simultaneous transcriptional repression and protein degradation (Fig. 4a [↗](#)).

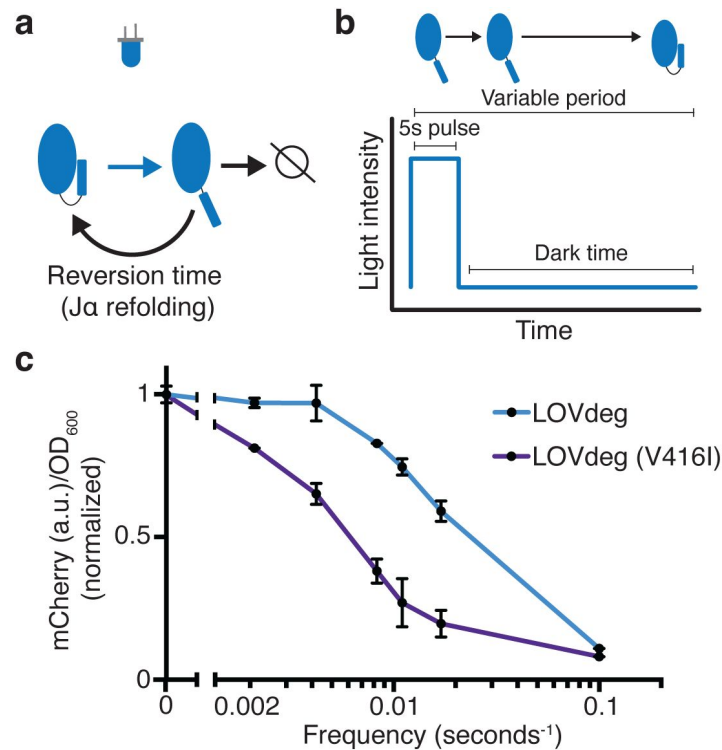


Figure 3.

Modulating LOVdeg frequency response with photocycle mutations. **(a)** Photocycle of AsLOV2. Upon light absorption, the Ja helix unfolds for a period of time dictated by the stability of the light state conformation. If not degraded, the Ja helix refolds, blocking degradation. **(b)** The light program used to test frequency responses of LOVdeg photocycle variants in (c). A constant pulse of 5 sec is followed by a variable dark time that allows for Ja helix refolding. **(c)** Expression of mCherry-LOVdeg and variant mCherry-LOVdeg (V416I) in response to different light exposure frequencies. Fluorescence values are normalized to dark state expression. Error bars show standard deviation around the mean ($n = 3$ biological replicates).

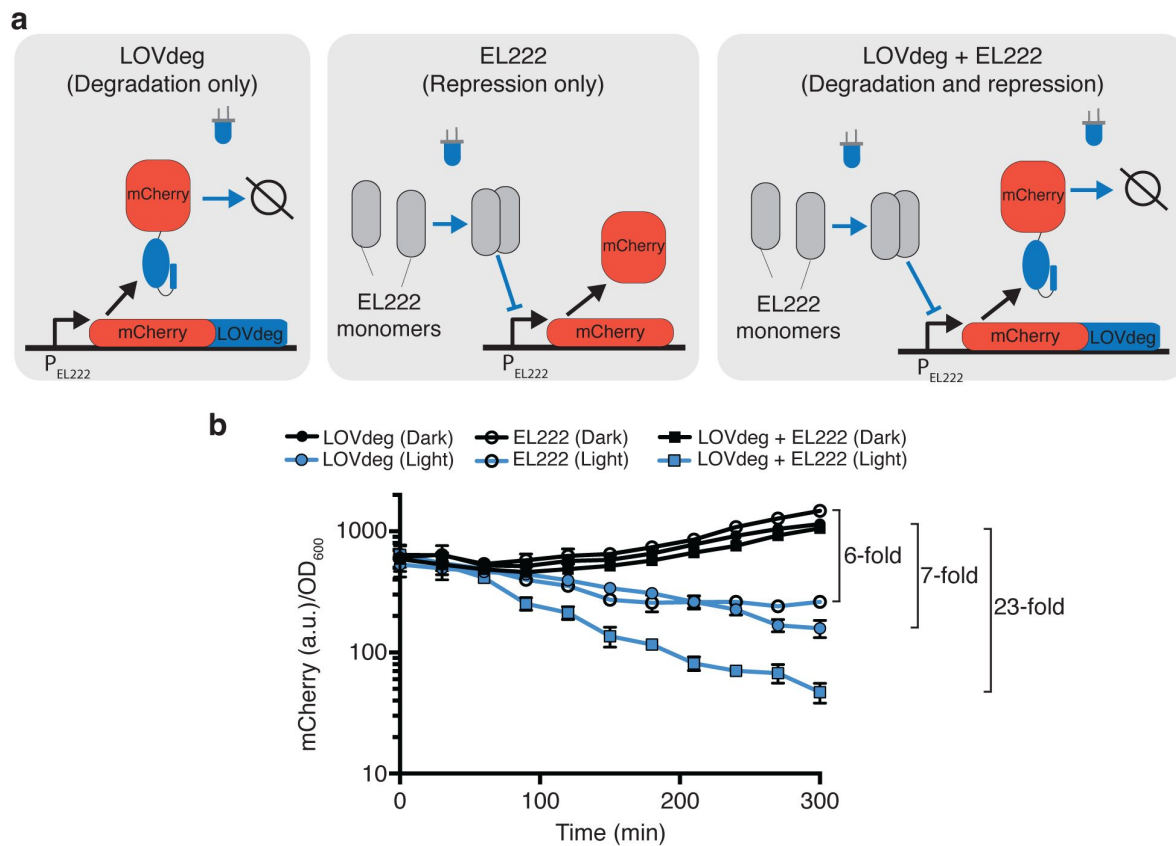


Figure 4.

Enhanced light response using EL222 transcriptional control together with LOVdeg. **(a)** mCherry-LOVdeg expressed from an EL222 responsive promoter that is constitutively active in the absence of EL222. Addition of EL222 represses mCherry production. These two forms of regulation are combined when mCherry-LOVdeg is expressed from an EL222 responsive promoter, resulting in a circuit that both degrades and represses in response to light. **(b)** Light and dark expression of mCherry in the 'degradation only' (closed circles), 'repression only' (open circles), or 'repression + degradation' (squares) strains. Error bars show standard deviation around the mean ($n = 3$ biological replicates).

To test the performance of this combined optogenetic circuit, we created a construct in which the mCherry-LOVdeg fusion protein is driven by a promoter containing an EL222 binding site (P_{EL222}). We tested the light response of mCherry-LOVdeg expression with and without EL222 present, as well as including EL222 control of mCherry without the LOVdeg tag fused (**Fig. 4b**). As expected, mCherry-LOVdeg expression decreased in response to light even without EL222 present, representing the sole action of the degradation tag. Similarly, EL222-only mCherry decreased in response to light due to repression by EL222. However, multi-layer control resulted in a faster decrease in expression in response to light and reached lower levels compared to either LOVdeg or EL222 alone. The fold change decrease in expression was improved from 7x and 6x for just the LOVdeg or EL222, respectively, to 23x when both systems were combined.

Optogenetic control of octanoic acid production

We next sought to apply the LOVdeg tag to a metabolic engineering task, as we envision that post-translational control will be especially advantageous in these biotechnology applications. Transcriptional control alone is particularly problematic in a metabolic engineering setting because chemical production is typically carried out at stationary phase with slow growth rates, meaning that proteins expressed at basal levels will accumulate in production settings. Therefore, dynamic control using transcriptional optogenetic systems alone will only allow protein levels to increase or plateau. However, a prerequisite for dynamic control of metabolic pathways is that enzyme levels can be modulated to turn off production.

As a proof of concept, we chose to control the enzyme *CpFatB1* with LOVdeg, EL222, or the EL222-LOVdeg circuit. *CpFatB1* is an acyl-ACP thioesterase from *Cuphea palustris* that primarily catalyzes octanoyl-ACP to produce octanoic acid. Octanoic acid is a valuable medium chain oleochemical with limited natural sources (Sarria et al., 2017). Specifically, we expressed the catalytically enhanced mutant from Hernandez-Lozada, et al., *CpFatB1.2-M4-287*, which we denote here as *CpFatB1** (Hernández Lozada et al., 2018). This enzyme interfaces with the endogenous fatty acid synthesis pathway in *E. coli* to produce free fatty acids (**Fig. 5a**). In this pathway, the carbon tail of an acyl-ACP moiety is elongated two carbons at a time. *CpFatB1** specifically catalyzes C8-ACPs, which results in production of free octanoic acid. Importantly, since *CpFatB1** is very catalytically active, low levels of expression are optimal for production and strains with high expression exhibit a growth defect (Hernández Lozada et al., 2018). Therefore, to dynamically regulate *CpFatB1** activity in cells, expression must be controlled in a low range, which is particularly challenging in stationary phase.

A first step towards optogenetically controlling this metabolic pathway is to demonstrate that enzyme levels can be modulated between protein levels that are relevant to endpoint titers. If expression can only oscillate between high and medium protein levels, dynamic light control will not be effective. To test whether we could effectively stunt *CpFatB1** activity, we controlled enzyme expression using only LOVdeg tag degradation, only EL222 repression, or combined LOVdeg and EL222 throughout at 24 hour fermentation period (**Fig. 5b**). LOVdeg alone successfully decreased octanoic acid in the light condition. The EL222-only condition did not significantly decrease octanoic acid production with light compared to conditions in the dark, demonstrating the shortcomings of solely transcriptional control at stationary phase. In contrast, the LOVdeg tag with EL222 resulted in a significant decrease in octanoic acid in the continuous light condition. Thus, protein degradation is needed to effectively shunt the metabolic pathway during stationary phase.

Interestingly, the LOVdeg-only strain, in which *CpFatB1**-LOVdeg is expressed through the IPTG-inducible LacUV5 promoter, exhibits higher dark state octanoic acid production compared to *CpFatB1** expressed under the EL222 promoter. The dark state production of the LOVdeg-only strain is similar to that of *CpFatB1** without the LOVdeg tag, both expressed via the same LacUV5 promoter. This is likely due to limited constitutive expression from P_{EL222} . This limitation further demonstrates the utility of post-translational control where the promoter is not involved in the

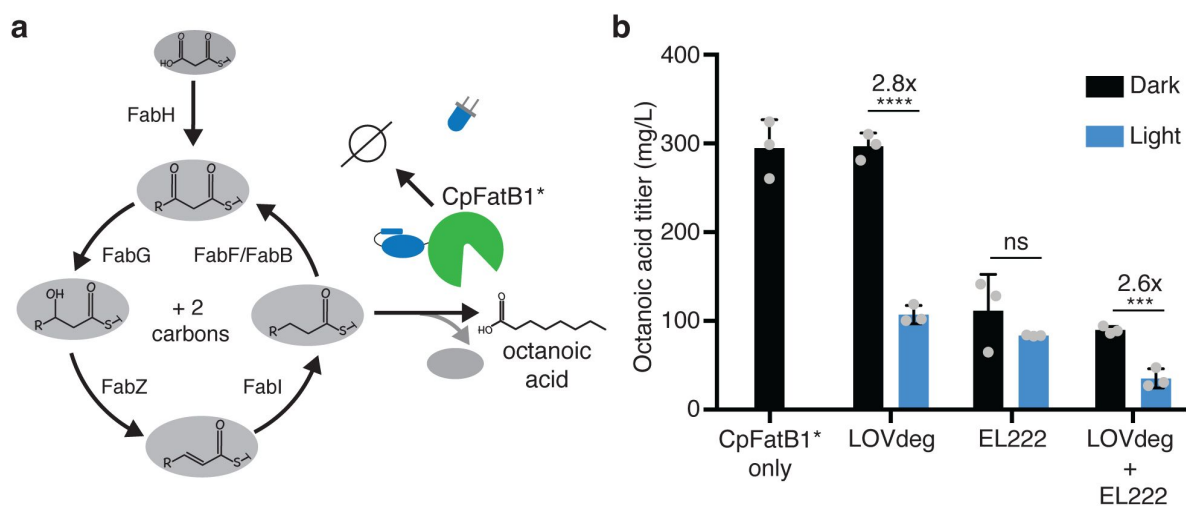


Figure 5.

Optogenetic control of octanoic acid production. **(a)** Schematic of fatty acid synthesis in *E. coli*. CpFatB1* catalyzes elongating C8-ACP molecules from this pathway to produce free octanoic acid. CpFatB1* is tagged with a LOVdeg tag to create optogenetic control. **(b)** Octanoic acid titer from strains that express CpFatB1* only, CpFatB1*-LOVdeg only, EL222 regulated CpFatB1* only, or CpFatB1*-LOVdeg + EL222. Octanoic acid is quantified by GC-MS. Strains were kept either in the dark or with continuous blue light exposure for the duration of the production period. Error bars show standard deviation around the mean (****p < 0.0001; ***p < 0.0001; ns, not significant; two tailed unpaired t-test; n = 3 biological replicates).

light response. Previous efforts aimed at increasing the strength of EL222 responsive promoters have been carried out ([Ding et al., 2020](#)), and further promoter engineering would be needed to increase EL222 promoter strength to increase octanoic acid production in the dark state. Clearly, decreasing titers is not the overall goal in metabolic engineering. However, the ability to control expression at relevant ranges throughout stationary phase represents an important stepping stone to investigate dynamic control schemes or feedback control that may lead to enhanced strain performance.

Discussion

Here, we developed and characterized the LOVdeg tag, which provides blue light inducible protein degradation, offering unique advantages as a tool for bacterial synthetic biology. Protein degradation offers a mode of control that is currently limited in the bacterial optogenetic toolkit. The bacterial light responsive degradation system from Komera *et al.* accomplishes protein degradation indirectly through an exogenous split protease ([Komera et al., 2022](#)). In comparison, in our approach the degradation tag itself is light responsive and does not require extra components, making it straightforward to incorporate. In this study, we showed that the truncated AsLOV2 protein can be fused to the C-terminal end of various proteins to destabilize them and then target them for degradation. The addition of stabilizing mutations from iLID (to create AsLOV2*) result in low basal activity in the dark, with strong switching in response to light. Aside from creating a light-responsive degradation tag, the effectiveness of these stabilizing mutations may serve to improve the switch-like behavior in many other AsLOV2-based systems.

We demonstrated the modularity of the LOVdeg tag by incorporating it into three distinct contexts with little to no fine tuning. Each system, the LacI repressor, CRISPRa activation, and the AcrB efflux pump, was converted to optogenetically controlled by simply tagging the protein with LOVdeg. This modularity mimics the ease of use of transcriptional optogenetic systems, which only require a change of the promoter. However, incorporating light control at the protein level simplifies this process because protein levels can remain in their native, or previously fine-tuned, context. This configuration is especially beneficial for proteins that require low expression, as transcriptional optogenetic systems often suffer from leaky basal expression. The LOVdeg tag provides a design that can work with endogenous protein levels, circumventing this issue. In this study, we focused on proteins encoded on plasmids expressed from synthetic promoters. However, we anticipate the LOVdeg tag can be useful in systems biology contexts where natural promoters linked to endogenous gene networks are best left untouched. In this case, the LOVdeg tag can add the ability to optogenetically manipulate genes while keeping them in their native gene regulatory context.

Since degradation occurs post-translationally, the LOVdeg tag can easily be integrated with existing optogenetic systems to enhance their function. We demonstrated this by combining the LOVdeg tag with the EL222 repression system, showing that the synergistic action of transcriptional repression and degradation results in an increase in the dynamic range, owing to the lower off-state under light illumination. In this configuration, EL222 and the LOVdeg tag work coherently to decrease expression. However, the LOVdeg tag can potentially be incorporated into activating systems incoherently to create dynamic functionality, such as pulse generators and inverters ([Benzinger et al., 2022](#)).

Throughout this study, we performed experiments using low copy number plasmids that result in moderate levels of a given protein of interest. While this is a common use case for synthetic biology, degradation as the sole mode of gene expression control may be limiting when proteins are at very high levels. As an ATP-dependent process that utilizes a finite pool of proteolytic machinery, degradation rates can saturate at sufficiently high protein levels ([Cookson et al., 2011](#)). Furthermore, using protein degradation as the sole mode of gene expression control is

wasteful in some contexts. Constitutive transcription and translation of a gene followed by degradation utilizes valuable cellular resources, which is an important consideration in metabolic engineering. To avoid this type of energetic waste, we envision the LOVdeg tag could be used in concert with other modes of regulation, as we demonstrated with EL222. In addition, it is unclear whether the LOVdeg tag is compatible with other prokaryotic (including mitochondrial) proteasomes. Future studies focused on the portability of the LOVdeg tag may address this question and potentially lead to further mechanistic insight.

In summary, the LOVdeg tag offers a straightforward route for introducing optogenetic control of protein degradation in *E. coli*. By lowering the barrier to entry for incorporating light responsiveness into a protein of interest, we envision that systems typically studied with chemical induction or constitutive expression can now be controlled optogenetically without extensive fine-tuning. Furthermore, the LOVdeg tag can act as a circuit enhancer when incorporated into existing optogenetic systems to increase functionality and robustness.

Methods

Strains and plasmids

We used *E. coli* BW25113 as the wild type strain. All knockout strains are from the Keio collection (Baba et al., 2006 [DOI](#)), which were derived from BW25113. We used golden gate cloning to create all plasmid constructs (Engler et al., 2008 [DOI](#)) (Table S1). The IPTG inducible constructs were derived from pBbS5c-mRFP1 from the BglBrick plasmid library (Lee et al., 2011 [DOI](#)). In the constitutive version of the mCherry-AsLOV2 variants, we swapped the IPTG inducible promoter with a constitutive promoter, P_{W7} (5'-ttatcaaaaagagtattgaaataaagtctaacctataggaagattacagccatcgagagggacacggcgaa-3'). We used this constitutive version for microscopy and the protease knockout studies; all other experiments used the IPTG inducible promoter P_{lacUV5}.

In all cases of protein fusions to AsLOV2 variants, a five amino acid GS linker of 'S-S-G-S-G' was used between the protein of interest and the LOVdeg tag. In the LacI-LOVdeg experiments, pBbS5c-mCherry was also used as the backbone, but with the LOVdeg sequence cloned after the *lacI* gene instead of *mCherry*. In the LacI-LOVdeg experiment to test the impact of basal LacI, pBbS5c-LacI-LOVdeg-mCherry was co-transformed with pBbAa-LacI-Decoy from Wang and Tague *et al.* (Wang et al., 2021 [DOI](#)). CRISPRa constructs were based on the MS2-SoxS system from Dong *et al.* (Dong et al., 2018 [DOI](#)). The J106 gRNA sequence from Dong *et al.* was used to target dCas9 upstream mRFP1 under control of the minimal J1 promoter. This gRNA plasmid was constructed using the golden gate assembly method to replace the targeting sequence in the pCD061 backbone (Addgene #113315). The mRFP1 reporter plasmid was derived from pJF076Sa (Addgene #113322) by replacing the ampicillin resistance gene with a kanamycin resistance gene from the BglBrick library. MS2-SoxS-LOVdeg was expressed from a variant of pJF093 (Addgene #113323). TetR and its corresponding promoter driving expression of MS2-SoxS were replaced with the LacI-P_{Trc} inducible system from pBbA1c-mRFP1 from the BglBrick library (Lee et al., 2011 [DOI](#)). The inducer was changed to IPTG because aTc is sensitive to blue light (Baumschlager et al., 2020 [DOI](#)). The LOVdeg tag was added to this construct with a C-terminal fusion to SoxS. Plasmids containing *acrAB* were built from pBbA5k-acrAB from El Meouche *et al.* (El Meouche and Dunlop, 2018 [DOI](#)). The FLP recombination protocol from Datsenko and Wanner was used to cure the *kanR* cassette from the genome of the Δ *acrB* strain (Keio collection, JW0451) (Datsenko and Wanner, 2000 [DOI](#)).

The slow photocycle variant of the LOVdeg tag, V416I, was constructed using site directed mutagenesis of Valine at amino acid position 416.

EL222 was synthesized by IDT (Coralville, IA) and plasmids were constructed to mimic EL222 repression systems from Jayaraman *et al.* (Table S1) (Jayaraman *et al.*, 2016 [DOI](#)). A variant of the promoter $P_{BLrep-v1}$ from Ding *et al.*, P_{raB} was used in all EL222 experiments and we refer to it in figures as P_{EL222} (Ding *et al.*, 2020 [DOI](#)). Plasmid pBbE5k- P_{EL222} -mCherry-LOVdeg was co-transformed with pEL222, which constitutively expresses EL222 (Table S2).

For octanoic acid production experiments, the coding sequence of CpFatB1.2-M4-287 derived from Hernández Lozada *et al.* was synthesized by Twist Biosciences (South San Francisco, CA) and cloned after the P_{EL222} or P_{lacUV5} promoter (Hernández Lozada *et al.*, 2018). Plasmid pBbE5k- P_{EL222} -CpFatB1*-LOVdeg was co-transformed with pEL222 for LOVdeg + EL222 production control (Table S2).

Plasmids expressing ClpA, ClpX, and HslU were constructed by amplifying the unfoldase gene from the wild type genome and inserting it into the pBbA8k backbone from the BglBrick library (Lee *et al.*, 2011 [DOI](#)).

Constructs from this work are available on AddGene: https://www.addgene.org/Mary_Dunlop/ [DOI](#)

Blue light stimulation

Unless otherwise noted, bacteria were cultured in Luria broth (LB) with appropriate antibiotics for plasmid maintenance at 37°C with 200 rpm shaking. Antibiotic concentrations used for plasmid maintenance were 30 µg/mL for kanamycin, 100 µg/mL for carbenicillin, and 25 µg/mL for chloramphenicol. All light exposure experiments were carried out with a light plate apparatus (LPA) (Gerhardt *et al.*, 2016 [DOI](#)) using 465 nm blue light. Overnight cultures of light sensitive strains were diluted 1:50 and precultured in the dark for 2 hours. For IPTG inducible constructs, 1 mM IPTG was added when the cells were diluted. After 2 hours in the dark, cells were exposed to blue light in the LPA at a setpoint of 100 µW/cm². Red fluorescence (excitation 560 nm, emission 600 nm) and optical density (OD) readings were taken using a BioTek Synergy H1m plate reader (BioTek, Winooski, VT) after 5 hours of incubation unless otherwise noted. For experiments testing degradation at various growth phases, a 4 hour light window was introduced at variable times, as shown in **Fig. 1** [DOI](#). For CRISPRa experiments, light stimulation was continued for 8 hours prior to RFP and OD readings. For frequency response experiments, the LPA was programmed using its Iris software (<https://taborlab.github.io/Iris/> [DOI](#)) to pulse blue light at varying frequencies. A 5 second light pulse was kept constant for each experiment while the time between pulses was varied (5s, 55s, 85s, 115s, 235s, and 480s).

Chloramphenicol sensitivity testing

Chloramphenicol sensitivity experiments were performed in M9 minimal media (M9 salts, 2 mM MgSO₄, 100 µM CaCl₂) with 1% glucose at 37°C with 200 rpm shaking. Overnight cultures in LB were initially diluted 1:50 into M9 media for 4 hours. The M9 conditioned cultures were then diluted again 1:20 into 24 well plates containing M9 media with varying levels of chloramphenicol (0, 0.3125, 0.625, 1.25, 2.5, 5, and 10 µg/ml) and grown for 20 hours before measuring optical density (OD) using a BioTek Synergy H1m plate reader (BioTek, Winooski, VT). We also conducted experiments with the *E. coli* BW25113 $\Delta acrB$ strain (referred to as $\Delta acrB$). For these, $\Delta acrB$ was transformed with pBbA5k-AcrAB-LOVdeg (Table S2) and chloramphenicol sensitivity experiments were carried out either in the LPA with constant light illumination or kept in the dark for the duration of growth. No IPTG was added to the $\Delta acrB$ + AcrAB-LOVdeg cultures because basal expression is enough to recover wild type resistance. The same chloramphenicol sensitivity protocol was performed in the dark using BW25113, $\Delta acrB$ + AcrB without induction, and $\Delta acrB$ as controls.

Microscopy

Strains were grown overnight in LB medium. Cultures were refreshed 1:100 in M9 minimal media for microscopy (M9 salts supplemented with 2 mM MgSO₄, 0.1 mM CaCl₂) with 0.4% glucose for two hours. Samples were then placed on 1.5% low melting agarose pads made with M9 minimal media for microscopy with 0.4% glucose. Samples were grown at 30°C. Cells were imaged at 100x using a Nikon Ti-E microscope. Blue light exposure was provided by a LED ring (Adafruit NeoPixel 1586), which was fixed above the microscope stage and controlled by an Arduino using a custom Matlab script. Blue light was kept constant except during image acquisitions.

Octanoic acid production experiment

For octanoic acid production experiments, strains expressing *CpFatB1** under various modes of control (Table S2) were cultured in LB overnight with light illumination to maintain low *CpFatB1** expression. Overnight cultures were diluted 1:20 into M9 minimal media with 2% glucose and kept in the light until they reached early stationary phase (OD₆₀₀ of 0.6) unless otherwise noted. The LPA was then programmed to either maintain light for low octanoic acid production or turn off light exposure to induce octanoic acid production for 24 hours prior to fatty acid extraction and quantification.

Fatty acid quantification

Samples for GC-MS quantification were taken at 24 hours. 400 µL of vortexed culture was used for fatty acid extraction and derivatization into fatty acid methyl esters as described by Sarria *et al.* (Sarria *et al.*, 2018 [DOI](#)) with the following minor modifications: An internal standard of nonanoic acid (C9) was added to the 400 µL sample at a final concentration of 88.8 mg/L and vortexed for 5 sec. The following was then added to the sample for fatty acid extraction and vortexed for 30 sec: 50 µL 10% NaCl, 50 µL glacial acetic acid, and 200 µL ethyl acetate. The sample was then centrifuged at 12,000 g for 10 mins. After centrifugation, 100 µL of the ethyl acetate layer was mixed with 900 µL of a 30:1 mixture of methanol:HCl (12N) in a 2 mL microcentrifuge tube. The solution was vortexed for 30 sec followed by an incubation at 50°C for 60 mins for methyl ester derivatization. Once cooled to room temperature, 500 µL hexanes and 500 µL water were added to the 2 mL microcentrifuge tube, vortexed for 10 sec, and allowed to settle. 250 µL of the hexane layer was mixed with 250 µL ethyl acetate in a GC-MS vial for quantification.

The samples were analyzed with an Agilent 6890N/Agilent 5973 MS detector using a DB-5MS column. The inlet temperature was set to 300°C with flow at 4 mL/min. The oven heating program was initially set to 70°C for 1 min, followed by a ramp to 290°C at 30°C/min, and a final hold at 290°C for 1 min. GLC-20 and GLC-30 FAME standard mixes (Sigma) were tested using this protocol to ensure proper capture of all chain lengths and to gauge retention times. Internal standards were used for quantification, with chain lengths C8-C12 quantified with the nonanoic acid internal standard and C14-C18 quantified with the pentadecanoic internal standard.

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Conflict of Interest

The authors declare no competing interests.

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Reviewer #1 (Public Review):

Specifically controlling the level of proteins in bacteria is an important tool for many aspects of microbiology, from basic research to protein production. While there are several established methods for regulating transcription or translation of proteins with light, optogenetic protein degradation has so far not been established in bacteria. In this paper, the authors present a degradation sequence, which they name "LOVdeg", based on iLID, a modified version of the blue-light-responsive LOV2 domain of *Avena sativa* phototropin I (AsLOV2). The authors reasoned that by removing the three C-terminal amino acids of iLID, the modified protein ends in "-E-A-A", similar to the "-L-A-A" C-terminus of the widely used SsrA degradation tag. The authors further speculated that, given the light-induced unfolding of the C-terminal domain of iLID and similar proteins, the "-E-A-A" C-terminus would become more accessible and, in turn, the protein would be more efficiently degraded in blue light than in the dark.

Indeed, several tested LOVdeg-tagged proteins show clearly lower cellular levels in blue light than in the dark. Depending on the nature and expression level of the target protein, protein levels are reduced modestly to strongly (2 to 20x lower levels upon illumination). Accordingly, the authors propose to use their system in combination with other light-controlled expression systems and provide data validating this approach. The LOVdeg system allows to modulate protein levels to a similar degree and with comparable kinetics as optogenetic systems controlling transcription or translation of protein, and can be combined with such systems.

The manuscript and the figures are generally very well-composed and follow a clear structure. The schematics nicely explain the underlying principles. Besides the advantages of the LOVdeg approach, including its complementarity to controlled expression of proteins, the revised version of the manuscript also highlights the limitations of the method more clearly, e.g., (i) the need to attach a C-terminal tag of considerable size to the protein of interest, (ii) the limited efficiency (slightly less efficient and slower than EL222, a light-dependent transcriptional control mechanism), and (iii) the incompletely understood prerequisites for

its application. Taken together, this manuscript describes the LOVdeg system as a valuable addition to the tool box for controlling protein levels in prokaryotic cells.

- <https://doi.org/10.7554/eLife.87303.2.sa2>

Reviewer #2 (Public Review):

In this manuscript the authors present and characterize LOVdeg, a modified version of the blue-light sensitive AsLOV2 protein, which functions as a light-inducible degron in *Escherichia coli*. Light has been shown to be a powerful inducer in biological systems as it is often orthogonal and can be controlled in both space and time. Many optogenetic systems target regulation of transcription, however in this manuscript the authors target protein degradation to control protein levels in bacteria. This is an important advance in bacteria, as inducible protein degradation systems in bacteria have lagged behind eukaryotic systems due to protein targeting in bacteria being primarily dependent on primary amino acid sequence and thus more difficult to engineer. In this manuscript, the authors exploit the fact that the J-alpha helix of AsLOV2, which unwinds into a disordered domain in response to blue light, contains an E-A-A amino acid sequence which is very similar to the C-terminal L-A-A sequence in the SsrA tag which is targeted by the unfoldases ClpA and ClpX. They truncate AsLOV2 to create AsLOV2(543) and combine this truncation with a mutation that stabilizes the dark state to generate AsLOV2*(543) which, when fused to the C-terminus of mCherry, confers light-induced degradation. The authors do not verify the mechanism of degradation due to LOVdeg, but evidence from deletion mutants contained in the supplemental material hints that there is a ClpA dominated mechanism. The LOVdeg is able to target mCherry for protein degradation across different phases of bacterial growth, which is important for regulating processes at stationary phase and a potential additional advantage over transcriptional repression systems. They demonstrate modularity of this LOVdeg by using it to degrade the LacI repressor, CRISPRa activation through degradation of MCP-SoxS, and the AcrB protein which is part of the AcrAB-TolC multidrug efflux pump. In all cases, measurement of the effect of the LOVdeg is indirect as the authors measure reduction in LacI repression, reduction in CRISPRa activation, and drug resistance rather than directly measuring protein levels. Nevertheless the evidence is convincing, although seemingly less effective than in the case of mCherry degradation, although it is hard to compare due to the different endpoints being measured. The authors further modify LOVdeg to contain a known photocycle mutation that slows its reversion time in the dark, so that LOVdeg is more sensitive to short pulses of light which could be useful in low light conditions or for very light sensitive organisms. They also demonstrate that combining LOVdeg with a blue-light transcriptional repression system (EL222) can decrease protein levels an additional 23-fold (relative to 7-fold with LOVdeg alone). Finally, the authors apply LOVdeg to a metabolic engineering task, namely reducing expression of octanoic acid by regulating the enzyme CpFatB1, an acyl-ACP thioesterase. The authors show that tagging CpFatB1 with LOVdeg allows light induced reduction in octanoic acid titer over a 24 hour fermentation. In particular, by comparing control of CpFatB1 with EL222 transcriptional repression alone, LOVdeg, or both the authors show that light-induced protein degradation is more effective than light-induced transcriptional repression. The authors suggest that this is because transcriptional repression is not effective when cells are at stationary phase (and thus there is no protein dilution due to cell division). Overall, the authors have generated a modular, light-activated degron tag for use in *Escherichia coli* that is likely to be a useful tool in the synthetic biology and metabolic engineering toolkit.

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Reviewer #3 (Public Review):

The authors present the mechanism, validation, and modular application of LOVtag, a light-responsive protein degradation tag that is processed by the native degradosome of *Escherichia coli*. Upon exposure to blue light, the c-terminal alpha helix unfolds, essentially marking the protein for degradation. The authors demonstrate the engineered tag is modular across multiple complex regulatory systems, which shows its potential widespread use throughout the synthetic biology field. The step-by-step rational design of identifying the protein that was most dark-stabilized as well as most light-responsive for degradation, was useful in terms of understanding the key components of this system. The most compelling data shows that the engineered LOVTag can be fused to multiple proteins and achieve light-based degradation, without affecting the original function of the fused protein.

- <https://doi.org/10.7554/eLife.87303.2.sa0>

Author Response

The following is the authors' response to the original reviews.

Note to all Reviewers

We appreciate the reviewers' comments and suggestions for improving the manuscript. Below is a summary of new data added and a brief description of the major new results. A detailed point-by-point response follows.

New data:

- Figure 1f
- Figure 2b, f, g
- Figure 4b
- Figure S7 • Figure S8
- Figure S9

Summary of major new results/edits:

- At the request of Reviewer #1 we have updated the name of the degradation tag to be more specific and we now call it the "LOVdeg" tag.
- We have added new controls demonstrating that light stimulation does not cause photobleaching or toxicity issues (Fig. S7).
- We now show that LOVdeg can function at various points in the growth cycle, demonstrating robust degradation (Fig. 1f, Fig. S8).
- We have included relevant controls for the AcrB-LOVdeg efflux pump results (Fig. 2f-g).
- We have included important benchmarking controls, such as an EL222-only control and SsrA tag control to provide a clearer view of how LOVdeg performance compares to other systems (Fig. S9, Fig. 4b).

Additional note:

- While repeating experiments during the revision process we found that the results for the combined action of EL222 and the LOVdeg tag were not as dramatic as in our original

measurements, though the overall findings are consistent with our original results. Specifically, we still find that the combination of EL222 and the LOVdeg tag produces a lower signal than either on their own. We have updated these data in the revised manuscript (Fig. 4b).

Reviewer #1:

Public Review:

Specifically controlling the level of proteins in bacteria is an important tool for many aspects of microbiology, from basic research to protein production. While there are several established methods for regulating transcription or translation of proteins with light, optogenetic protein degradation has so far not been established in bacteria. In this paper, the authors present a degradation sequence, which they name "LOVtag", based on iLID, a modified version of the blue-light-responsive LOV2 domain of Avena sativa phototropin I (AsLOV2). The authors reasoned that by removing the three C-terminal amino acids of iLID, the modified protein ends in "-E-A-A", similar to the "-L-A-A" C-terminus of the widely used SsrA degradation tag. The authors further speculated that, given the light-induced unfolding of the C-terminal domain of iLID and similar proteins, the "-E-A-A" C-terminus would become more accessible and, in turn, the protein would be more efficiently degraded in blue light than in the dark.

Indeed, several tested proteins tagged with the "LOVtag" show clearly lower cellular levels in blue light than in the dark. While the system works efficiently with mCherry (10-20x lower levels upon illumination), the effect is rather modest (2-3x lower levels) in most other cases. Accordingly, the authors propose to use their system in combination with other light-controlled expression systems and provide data validating this approach. Unfortunately, despite the claim that the "LOVtag" should work faster than optogenetic systems controlling transcription or translation of protein, the degradation kinetics are not consistently shown; in the one case where this is done, the response time and overall efficiency are similar or slightly worse than for EL222, an optogenetic expression system.

The manuscript and the figures are generally very well-composed and follow a clear structure. The schematics nicely explain the underlying principles. However, limitations of the method in its main proposed area of use, protein production, should be highlighted more clearly, e.g., (i) the need to attach a C-terminal tag of considerable size to the protein of interest, (ii) the limited efficiency (slightly less efficient and slower than EL222, a light-dependent transcriptional control mechanism), and (iii) the incompletely understood prerequisites for its application. In addition, several important controls and measurements of the characteristics of the systems, such as the degradation kinetics, would need to be shown to allow a comparison of the system with established approaches. The current version also contains several minor mistakes in the figures.

We thank reviewer #1 for the feedback and suggestions to strengthen the manuscript. We have addressed these comments in the points that follow and now include important controls and benchmarks for our molecular tool.

Major points

- 1. The quite generic name "LOVtag" may be misleading, as there are many LOV-based tags for different purposes.*

We appreciate that it would be beneficial to have a more specific name. We have updated the name to "LOVdeg" tag, which captures both the inclusion of LOV and the degradation function of the tag.

Updated throughout the manuscript and figures

1. *Throughout the manuscript, the authors use "expression levels". As protein degradation is a post-expression mechanism, "protein levels" should be used instead.*

We have transitioned to using "protein levels" at many points in the manuscript.

Updated throughout the manuscript

1. *Degradation dynamics (time course experiments) should be shown. The only time this is done in the current version (in Fig. 4), degradation appears to be in the same range (even a bit slower) than for EL222, which does not support the claim that the "LOVtag" acts faster than other optogenetic systems controlling protein levels.*

In the revised manuscript, time course data are now shown at multiple points. These include new data in Fig. 1f and Fig. S8 that demonstrate degradation at various stages of growth. Fig. S4 also shows the dynamics of degradation when comparing to the addition of exogenously expressed ClpA. We have added text in the results section to point the reader to these data. In addition, we have made minor modifications to the text in the Introduction to avoid making claims about speed comparisons. Fig. 1f, Fig. S8, Fig. S4

Results: Design and characterization of the AsLOV2-based degradation tag, Introduction

1. *"Frequency" is used incorrectly for Fig. 3. A series of 5 seconds on, 5 seconds off corresponds to a frequency of 0.1 Hz (1 illumination round / 10 s), not of 0.5 Hz. What the authors indicate as "frequency" is the fraction of illumination time. However, the (correct) frequency should be given, as this is likely the more important factor.*

We have changed how we calculate frequency to use the proposed definition of one pulse per time period. We updated the values in the text and in the figure. Fig. 3c

Results: Tuning frequency response of the LOVdeg tag

1. *To properly evaluate the system, several additional controls are needed:*
 - a. *To test for photobleaching of mCherry by blue light illumination, untagged controls should be shown for the mCherry-based experiments. Fluorescence always seems to be lower upon illumination, except for the AsLOV2*(S46) data, where it cannot be excluded that fluorescence readings are saturated. Relatedly, the raw data for OD and fluorescence should be included. Showing a Western blot against mCherry in at least one case would allow to separate the effects of photobleaching and degradation.*

We appreciate the suggestion and have conducted these important controls. We now include new data demonstrating that light induction does not change fluorescence levels using an untagged mCherry control, nor does it significantly affect endpoint OD levels. Based on these results, we did not perform a Western blot because there were no effects to separate. Fig. S7

- b. *In Fig. 2b, light + IPTG should be shown to estimate the activity of the system at higher expression levels.*

We have added these to the figure. Light + IPTG modestly increases expression compared to IPTG only, likely due to the saturating level of IPTG added, which achieves near full induction. Fig. 2b

c. In Fig. 4, EL222 alone should be shown to allow a comparison with the LOVtag. From the data presented, it looks like EL222 is both slightly faster and more efficient than the LOVtag.

We have added the EL222-only case for comparison with LOVdeg only and EL222 + LOVdeg. We note that Reviewer #3 raised a similar concern. Fig. 4b

d. The effect of the used light on bacterial viability under exponential and stationary conditions should be shown.

In this revision, we have added new data on light exposure at various points during exponential and stationary phase (Fig. 1f, Fig. S8). These OD data show that growth curves are similar for all cultures, regardless of the time light is applied during the growth phase. Additionally, we also now include ODs for the photobleaching experiments. These data also show that growth is not significantly altered under continuous light exposure. Figure 1f, Fig. S7b

1. The claim that "Post-translational control of protein function typically requires extensive protein engineering for each use case" is not correct. The authors should discuss alternative options, e.g. based on dimerization, more extensively and in a less biased manner.

We have toned down the language in this location and at other points in the manuscript. However, we maintain that other types of post-translational control, such as dimerization or LOV2 domain insertion, require more protein engineering than inserting a degradation tag. For example, we and others have directly demonstrated this in previous work (e.g. DOI: 10.1021/acssynbio.9b00395, 10.1101/2023.05.26.542511, 10.1038/s41467-023-38993-6), where numerous split site or insertion variants need to be screened and fine-tuned for successful light control. In contrast, a degradation mechanism has the potential to require less fine tuning to achieve a light response. We have included the above sources to clarify this point. Introduction, Results: Modularity of the LOVdeg tag

Minor points

1. In Suppl. Fig. 1, amino acid numbers seem to be off. Also, the alterations in iLID (compared to AsLOV2) that are not used in "LOVtag" appear to be missing and the iLID sequence incorrect, as a consequence.

Thank you for catching this. The number indices in Fig. S1 have been corrected. We also realized we were reporting the iLID(C530M) variant in our amino acid sequence and have reverted the 530M back to C. Fig. S1

1. Why is AsLOV2(543) more efficiently degraded than AsLOV2(543) (blue column in Fig. 1d) when the dark state should be stabilized in AsLOV2(543)?

We are not sure of the exact reason for the increased degradation response in the AsLOV2* (543) variant. It may be that the dark-state stabilizing mutations introduced also have more favorable interactions with degradation machinery, although this is highly speculative.

1. Why does the addition of EL222 reduce protein levels so strongly in the dark for CpFatB1 (Fig. 5)?*

We believe this effect stems from the EL222 responsive promoter (PEL222). With LOVdeg only, CpFatB1* is expressed from an IPTG inducible promoter (PlacUV5) whereas EL222 responsive constructs necessitate a promoter switch containing an EL222 binding site. We have clarified this point and expanded our discussion of these results.

Results: Optogenetic control of octanoic acid production

1. *Fig. 2f / S10 are difficult to interpret. Why does illumination only lead to a significant effect at 2.5 and 5 µg/ml and not at lower concentrations, where the degradation system would be expected to be most efficient?*

We have expanded our discussion on these results to explain that this likely stems from basal protein levels of AcrB-LOVdeg in the light that can provide resistance at low antibiotic concentrations. We have also added new controls to this figure to show the chloramphenicol sensitivity of a ΔacrB strain and a ΔacrB strain with an IPTG-inducible version of acrB with no induction, demonstrating the lowest achievable chloramphenicol resistance from a standard inducible system.

Results: Modularity of the LOVdeg tag, Fig. 2f-g

1. *Fig. 2f / S10 do not measure the MIC (which is a clearly defined value), but the sensitivity to Chloramphenicol.*

We have changed the text to use the term chloramphenicol sensitivity instead of MIC. Results: Modularity of the LOVdeg tag

1. *"***" in Fig. S1 should be explained.*

We have removed the "***" to avoid confusion. Fig. S1

1. *The fold-change differences between light and dark, indicated in some selected cases, should be listed for all figures.*

We have added fold-change values where appropriate. Fig 1d, Fig. 2b

Reviewer #2:

Public Review:

In this manuscript the authors present and characterize LOVtag, a modified version of the bluelight sensitive AsLOV2 protein, which functions as a light-inducible degron in Escherichia coli. Light has been shown to be a powerful inducer in biological systems as it is often orthogonal and can be controlled in both space and time. Many optogenetic systems target regulation of transcription, however in this manuscript the authors target protein degradation to control protein levels in bacteria. This is an important advance in bacteria, as inducible protein degradation systems in bacteria have lagged behind eukaryotic systems due to protein targeting in bacteria being primarily dependent on primary amino acid sequence and thus more difficult to engineer. In this manuscript, the authors exploit the fact that the J-alpha helix of AsLOV2, which unwinds into a disordered domain in response to blue light, contains an E-A-A amino acid sequence which is very similar to the C-terminal L-A-A sequence in the SsrA tag which is targeted by the unfoldases ClpA and ClpX. They truncate AsLOV2 to create AsLOV2(543) and combine this truncation with a mutation that stabilizes the dark state to generate AsLOV2(543) which,*

when fused to the C-terminus of mCherry, confers light-induced degradation. The authors do not verify the mechanism of degradation due to LOVtag, but evidence from deletion mutants contained in the supplemental material hints that there is a ClpA dominated mechanism. They demonstrate modularity of this LOVtag by using it to degrade the LacI repressor, CRISPRa activation through degradation of MCP-SoxS, and the AcrB protein which is part of the AcrAB-TolC multidrug efflux pump. In all cases, measurement of the effect of the LOVtag is indirect as the authors measure reduction in LacI repression, reduction in CRISPRa activation, and drug resistance rather than directly measuring protein levels. Nevertheless the evidence is convincing, although seemingly less effective than in the case of mCherry degradation, although it is hard to compare due to the different endpoints being measured. The authors further modify LOVtag to contain a known photocycle mutation that slows its reversion time in the dark, so that LOVtag is more sensitive to short pulses of light which could be useful in low light conditions or for very light sensitive organisms. They also demonstrate that combining LOVtag with a blue-light transcriptional repression system (EL222) can decrease protein levels an additional 269-fold (relative to 15-fold with LOVtag alone). Finally, the authors apply LOVtag to a metabolic engineering task, namely reducing expression of octanoic acid by regulating the enzyme CpFatB1, an acyl-ACP thioesterase. The authors show that tagging CpFatB1 with LOVtag allows light induced reduction in octanoic acid titer over a 24 hour fermentation. In particular, by comparing control of CpFatB1 with EL222 transcriptional repression alone, LOVtag, or both the authors show that light-induced protein degradation is more effective than light-induced transcriptional repression. The authors suggest that this is because transcriptional repression is not effective when cells are at stationary phase (and thus there is no protein dilution due to cell division), however it is not clear from the available data that the cells were in stationary phase during light exposure. Overall, the authors have generated a modular, light-activated degen tag for use in *Escherichia coli* that is likely to be a useful tool in the synthetic biology and metabolic engineering toolkit.

We thank Reviewer #2 for the constructive feedback. In the updated manuscript, we now include data demonstrating degradation at different growth stages and address other points brought up in the review to improve understanding of the degradation tag.

Overall, the authors present a well written manuscript that characterizes an interesting and likely very useful tool for bacterial synthetic biology and metabolic engineering. I have a few suggestions that could improve the presentation of the material.

Major Comments:

- *Could the authors clarify, perhaps through OD measurements, that the cultures in the octanoic acid experiment are actually in stationary phase during the relevant light induction. It isn't clear from the methods.*

We have updated the Methods to clarify that the cells are entering stationary phase (OD600 = 0.6) when light is either kept on or turned off for production experiments. Production is continued for the following 24 hours. Note that we now show OD measurements in a separate set of experiments (Fig. 1f, Fig. S8).

Methods: Octanoic acid production experiment. Fig. 1f, Fig. S8

- *Can the authors clarify why there is an overall decrease in protein in the clpX deletion? And is it this initial reduction that is the source of the change in fold in 1C? Similarly, for hslU is it because overall protein levels are higher with the tag? In general, I feel that the interpretation of Supplemental Figures S6-S10 could be moved in more detail to the main*

text, or at least the main takeaway points. But this is a personal preference, and not necessary to the major flow of the story which is about the utility of the LOVtag tool.

As shown in Fig. S5, expression of mCherry without any degradation tag is decreased in a clpX knockout strain compared to wild type. This difference may be the result of reduced cell health, and we now note this in the text. The strains shown in Fig. 1c are in wild type cells with normal expression, so this is not the source of the fold change. As for hslU, we agree it is interesting that expression seems to increase. However, the increase is modest and could stem from gene network regulation differences in that strain compared to wild type and may not be related to LOVdeg tag degradation. Each endogenous protease is involved in a wide range of functions within the cell, and it is unknown how global gene expression is impacted. We acknowledge the suggestion of moving the protease results to the main text, but we have ultimately elected to keep these data in the Supplementary Information to maintain the flow in the manuscript. However, we have added additional text pointing the reader to the Supplemental Text and include a brief summary of the findings in the main text.

Results: Design and characterization of the AsLOV2-based degradation tag

• What is the source of the poor repression in Figure 2D?

Presumably, this stems from low levels of the CRISPRa MCP-SoxS activator, even in the presence of light. We have added this point to the text.

Results: Modularity of the LOVdeg tag

• In general, it would be nice to have light-only controls for many of the experiments to validate that light is not affecting the indicated proteins or their function.

We thank the reviewer for this suggestion and note that Reviewer #1 raised a similar concern. We have now included light-only data for a strain containing IPTG-inducible mCherry without the LOVdeg tag (Fig. S7). These data show that light itself, at the levels used in this study, does not affect mCherry expression or cell growth. This strain serves as a direct control for data presented in Fig. 1 and Fig. 2b, as the systems are identical except for the addition of the LOVdeg tag onto either mCherry or the LacI repressor. Additionally, the control translates to other experiments since mCherry is used as a reporter for other systems in this study. Fig. S7

• It would be nice to directly measure the function of the tool at different phases of E. coli growth to show directly that protein degradation works at stationary phase, rather than the more indirect measurements used in the octanoic acid experiment.

We thank the reviewer for this suggestion, which significantly strengthens our results. We have added an experiment that tests the LOVdeg tag at different phases of growth (Fig. 1f, Fig. S8). In this experiment, cultures are grown from early exponential to stationary phase, and light is introduced at various points. Exposure windows of 4 hours, ranging from early exponential to stationary phase, all show functional light inducible degradation. Fig. 1f, Fig. S8.

Results: Design and characterization of the AsLOV2-based degradation tag

Minor Comments:

• It would be nice to make clear that the data in S6d and S7 is repeated, but with the HslUV data in S7.

We clarified this point in the caption of Fig. S4 (the former Fig. S7 in the original manuscript).
Fig. S4 caption

• *Why was 5s picked for the frequency response in Figure 3*

We picked 5s because 1) it is a substantially shorter timescale than overall degradation dynamics seen for the LOVdeg tag, and 2) we found that shorter pulses could not be reliably achieved with the light stimulation hardware and software we used (Light Plate Apparatus with Iris software). To ensure high fidelity pulses, we opted for 5 second pulses that we empirically determined to be stable throughout long experiments. We have added text clarifying this. Results: Tuning frequency response of the LOVdeg tag

Reviewer #3:

Public Review:

The authors present the mechanism, validation, and modular application of LOVtag, a light-responsive protein degradation tag that is processed by the native degradosome of Escherichia coli. Upon exposure to blue light, the c-terminal alpha helix unfolds, essentially marking the protein for degradation. The authors demonstrate the engineered tag is modular across multiple complex regulatory systems, which shows its potential widespread use throughout the synthetic biology field. The step-by-step rational design of identifying the protein that was most dark stabilized as well as most light-responsive for degradation, was useful in terms of understanding the key components of this system. The most compelling data shows that the engineered LOVTag can be fused to multiple proteins and achieve light-based degradation, without affecting the original function of the fused protein; however, results are not benchmarked against similar degradation tagging and optogenetic control constructs. Creating fusion proteins that do not alter either of the original functions, is often difficult to achieve, and the novelty of this should be expanded upon to drive further impact.

We appreciate the feedback from Reviewer #3 to improve the manuscript. We have included important controls and benchmarking experiments to address the reviewer's concerns, which are detailed in the points below.

Benchmarking:

The similarity between the L-A-A sequence of SsrA and the E-A-A sequence of LOVtag is one of the pieces of evidence that led the authors to their current protein design. The differences in degradation efficiency between the SsrA degradation tag and LOVtag are not shown, and benchmarking against SsrA would be a valuable way to demonstrate the utility of this construct relative to an established protein tagging tool.

We thank the reviewer for suggesting an experiment to benchmark performance. We have added new experimental data where a full length SsrA tag is added to a fusion protein of nearly identical size (mCherry-iLID), allowing us to directly compare performance to mCherryLOVdeg (Fig. S9). These results show that light inducible control with LOVdeg tag decreases protein expression levels to near those achieved with the native SsrA tag. Fig. S9.

Results: Design and characterization of the AsLOV2-based degradation tag

Additionally, there is a lack of an EL222-only control presented in Figure 4b and in the results section beginning with "Integrating the LOVtag with EL222...". Without benchmarking against this control the claim that "EL222 and the LOVtag work coherently to decrease expression" is unsubstantiated. No assumptions of synergy can be made.

We appreciate this comment and note that Reviewer #1 raised a similar concern. We have added data to Fig. 4b with an EL222-only control for comparison. Fig. 4b

The dramatic change in dark octanoic acid titer between the EL222, LOVtag and combined conditions are surprising, especially in comparison to the lack of change in the dark mCherry expression shown in Figure 4b. This data is the only to suggest that LOVtag may perform better than EL222. However, the inconsistencies in dark state regulation presented in the two experiments, and between conditions in this experiment bring the latter claim to question. A recommendation is that the authors either repeat this experiment, or comment on the observed discrepancy in dark state octanoic acid titers in their discussion.

First, a key difference between the data presented in Fig. 4 and Fig. 5 is that the production experiment is conducted over a long time period (24 hours) and the EL222/LOVdeg reporter experiment is conducted over 5 hours. Likely, performance differences between EL222 and the LOVdeg tag become more pronounced as protein accumulation occurs. Second, the LOVdeg only construct is expressed from a non-EL222 promoter which is able to achieve higher expression (see response to Reviewer #1, Minor point #3). Lastly, a convoluting factor is that the relationship between expression of CpFatB1* and octanoic acid production is not completely linear, and there are likely thresholds or expressions windows that result in similar endpoint titers. We agree a more detailed examination of how CpFatB1* changes over the course of the production period would be very interesting. However, this is beyond the scope of the present study, whose goal is to introduce and showcase the utility of the LOVdeg tag as a tool. We have added new discussion on this in the Results section to clarify some of these points. We have also repeated all experiments in Fig. 4 and consistently see the LOVdeg tag performing as well as or better than EL222. As noted in the remarks to all reviewers, these data have been updated in the revised manuscript.

Results: Optogenetic control of octanoic acid production. Fig. 4d

Based on the methodology presented, no change in the duration in light exposure was tested, even though this may be an important part of the system response. The on/off, for example in Figure 4b, is either all light or all dark, but they claim that their system is beneficial especially at stationary phase. The authors should consider showing the effects of shifting from dark to light at set intervals. (i.e. 1 hr dark then light, 2hr dark until light, etc.) This data would also aid in supporting the utility of this tag for controlling expression during different growth phases, where light may be used after the cells have reached a certain phase.

We have added new data showing the effect of light stimulation at different times in the growth cycle (see response to Reviewer #2, bullet point #5). These data demonstrate that the LOVdeg tag performs well at various points in the growth cycle. Fig. 1f, Fig. S8.

Results: Design and characterization of the AsLOV2-based degradation tag

Minor Revisions Figures:

- *Figure 1:*
- *More clarity is needed in the naming conventions for this figure and in the body of the text. For example, a different convention than 546 and 543 should be used to refer to the full and truncated lengths of the tag. It would greatly aid understanding for this to be made more clear. The authors could simply continue to use "full" and "truncated" to refer to them. In addition, the term "stabilizing*

mutations" in 1c could be changed to read "dark state stabilizing mutations" to aid in clarity.

When describing the design of the LOVdeg tag, we opted towards a more technically accurate description over clarity in order to make our engineering process easily comparable to other LOV2 systems. As such, we kept the number-based nomenclature (543 or 546) to represent the domain within the phototropin 1 protein from *Avena sativa* (AsLOV2). The domain used in this study, and many other studies, are only amino acids 404-546, i.e. not the full sequence, thus saying simply 'full' or 'truncated' is not technically accurate. We believe the detailed nomenclature, which is limited to one section, is important to provide clarity on exactly what we used for protein engineering. In the revised version we introduce the nickname "LOVdeg" tag earlier and use it throughout the rest of the manuscript.

Results: Design and characterization of the AsLOV2-based degradation tag

- *1b It is not clear that this is the dark state stabilized structure in the figure, but is referred to as such only in the body of the text.*

We have added text in the manuscript to clarify this is AsLOV2, not iLID, and have labeled it in the figure caption as well.

Results: Design and characterization of the AsLOV2-based degradation tag

- *1d. Fold change is reported in Figure 2d, and may be relevant to include those values in 1d as well.*

Done. Fig. 1d

- *1e. It is not clear which tag is being used in this bar plot. Please specify that this is the dark state stabilized, truncated tag.*

We have added a title to the plot and language to the caption, both of which clarify this point. Fig. 1e

- *In addition, the microscopy images provided in supplemental material should be included in the first figure as it adds a compelling observation of LOVtag activity.*

We are pleased to hear that the microscopy results are beneficial, however we elected to leave them in Supplementary to preserve the flow of the manuscript in the text surrounding Fig. 1.

- *Figure 2:*
- *2d. It is unclear what the 2.5x fold change is relative to (the baseline or the dark)*

We have added a line in the figure to clarify the comparison being made. Fig. 2d

- *2f. More discussion can be added to describe what concentration of chloramphenicol is biologically/bioreactor relevant.*

Our previous studies on the relationship between AcrAB expression and mutation rate (cited in the text) were carried out at a concentration within the range in which the LOVdeg tag is

effective (5 µg/ml), suggesting this range to be relevant to tolerance and resistance.

- *Figure 3:*
- *We recommend that this data and discussion are better suited for supplementary figures. The results shown here essentially recapitulate the same findings of Zoltowski et al., 2009. In addition, the paper describing this mutation should be cited in this figure caption in addition to the body of the text*

Although these results are in line with previous findings, we believe this dataset is important for several reasons. First, the agreement with known mutations validates the unfolding-based mechanism for degradation control. Second, degradation that is contingent on unfolding of LOV2 offers a direct actuating mechanism of photocycle properties. Other systems, like that in Zoltowski et al., examine properties of purified proteins but lack the mechanism to translate its effect in live cells. This figure demonstrates how degradation can do so and lays the groundwork for degradation-based frequency processing circuits. Last, there are discrepancies between photocycle kinetics in situ, as reported by Li et al. (DOI: 10.1038/s41467-020-18816-8), and in cell-free studies such as in Zoltowski et al. The studies use different methods of measuring photocycle kinetics (in situ vs cell-free). This dataset substantiates relaxation times from Li et al. and suggests cell-free relaxation time constants are over estimated relative to our live cell results.

- *Figure 4:*
- *There is a lack of an EL222-only control presented in Figure 4b. Without this data present, the claim that "EL222 and the LOVtag work coherently to decrease expression" is unsubstantiated. No assumptions of synergy can be made.*

We have added EL222-only data to the figure; we note that Reviewer #1 made a similar request. Figure 4b

Manuscript

Results

- *Design and characterization...*
- *Due to the extensive discussion of ClpX at the beginning of this section, more of the results on evaluating the binding partners and mechanism of LOVtag degradation should be presented in the main body of the manuscript and not in supplementary materials.*

To maintain flow of the manuscript and focus on how the LOVdeg tag works as a synthetic biology tool, we have opted to keep this section in the Supplement Information, but have several lines in the text related to Fig. 1 that point the reader to this material. Results: Design and characterization of the AsLOV2-based degradation tag

- *In the second paragraph of this section, the authors theorize that the C-terminal truncated E-AA sequence will "remain caged as part of the folded helix". How did the authors determine this? Was there any evidence to suggest that the truncated state would be any more responsive than the full length sequence? More data or rationale may need to be introduced to support the overall hypothesis presented in this paragraph.*

We determined this by examining the crystal structure which shows that the E-A-A sequence is part of the folded helix. As seen in Fig. 1b, addition of amino acids after the EAAKEL sequence would not be part of the folded helix which ends prior to the terminal leucine. We added text to clarify our logic.

Results: Design and characterization of the AsLOV2-based degradation tag

- *The similarity between the L-A-A sequence of SsrA and the E-A-A sequence of LOVtag is one of the pieces of evidence that brought the authors to their current protein design. The differences in degradation efficiency between the SsrA degradation tag and LOVtag are not clear, and benchmarking against SsrA would be a valuable way to demonstrate the utility of this construct relative to an established protein tagging tool.*

We added an SsrA comparison to benchmark the system. Fig. S9

Results: Design and characterization of the AsLOV2-based degradation tag

- *Tuning frequency and response...*
- *Overall the results presented in this section essentially recapitulate the effects that mutation presented in Zoltowski et. al., 2009 have on AsLOV2 dark state recovery and although this is a useful observation of LOVtag performance, a recommendation is to move this into a supplementary section.*

See above response to Fig. 3 comment.

- *Integrating the LOVtag with EL222...*
- *The claim is made in this section that LOVtag and EL222 work synergistically, however the experiments presented do not test repression due to EL222 activity alone. Without benchmarking against this control, the claim of synergy is not supported and we recommend that the authors perform this experiment again with the EL222-only control.*

We have added this important control. Fig. 4b

Discussion

- *The statement "the LOVtag can easily be integrated with existing optogenetic systems to enhance their function" is not substantiated without benchmarking LOVtag against an EL222- only control. As mentioned above this condition should be included in the experiments discussed in Figure 4 and in the section "Integrating the LOVtag with EL222.."*

We added EL222-only regulation to benchmark the LOVdeg tag and LOVdeg + EL222 experiments. Fig. 4b

Experiments

Applications:

The application of this tag to the metabolic control of octanoic acid production could be more impactful. For instance, using the LOVtag with two different enzymes to change the composition of long/short chain fatty acids with light induction., Or possibly integrating the tag into a switch to activate production. However, the authors address that "decreasing titers is not the overall goal in metabolic engineering" in their discussion, and therefore the pursuit of this additional experiment is up to the authors' discretion.

We appreciate the suggestions for further applications of the LOVdeg tag. We envision that follow up studies will focus on the application of the LOVdeg tag in metabolic engineering. However, this will require significant development of production systems. We believe this to be out of the scope of this work, where the goal is to present the design and function of the LOVdeg tag as a tool.