The authors note that Fig. 3 appeared incorrectly. As originally published, the color scale in Fig. 3C was inadvertently reversed (with red indicating minimal chemical shift changes and blue indicating maximal changes upon illumination). The corrected figure and its legend appear below.

**Fig. 3.** Solution NMR data suggests EL222 undergoes light-induced rearrangement of two ordered domains. (A) Superposition of $^{15}$N/$^1$H HSQC spectra of EL222 acquired under dark (black) or lit (red) conditions show light-induced changes in peak location and intensity. (B) Chemical shift difference analysis of $^{15}$N/$^1$H HSQC spectra shown in Fig. 3A indicate significant changes occurring in both domains, including the HTH 1α-2α loop, 3α-4α loop, and 4α-helix located at the interface with the LOV domain. Secondary structure elements as indicated by the NMR data and X-ray structure are indicated. (C) Mapping values from Fig. 3B onto the dark-state crystal structure illustrates the pattern of chemical shift perturbations at the interdomain interface. Chemical shift differences are mapped in color, with a red to blue gradient indicating maximal to minimal changes upon illumination (and gray indicating sites without unambiguously assigned $^{15}$N/$^1$H signals in both dark and lit states). Side chains are indicated for 1α-2α loop, 3α-4α loop, and 4α-helix residues in the HTH domain with $^{15}$N/$^1$H HSQC chemical shift changes upon illumination. (D) $^2$H exchange protection factor analyses (32) of EL222 conducted in the dark (black) and lit (red) states show similar protection, but to a lower overall degree upon illumination, consistent with reorganization of two ordered domains. Protection factors $>10^6$ are lower bound estimates because these sites did not sufficiently exchange for robust fitting of the time-dependent peak intensity changes.
APPLIED BIOLOGICAL SCIENCES


The authors note that Anne E. Carpenter and Matthias Wessling should be added to the author list between Roman K. Truckenmüller and Gerhard F. Post. Anne E. Carpenter should be credited with analyzing data. Matthias Wessling should be credited with designing research. The corrected author and affiliation lines, and author contributions appear below. The online version has been corrected.

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The authors note that the author name Chad S. Seigel should instead appear as Chad S. Siegel. The corrected author line appears below. Both the online article and the print article have been corrected.

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The authors note that the title appeared incorrectly. The title should instead appear as “Protein disulfide isomerase homolog PDILT is required for quality control of sperm membrane protein ADAM3 and male fertility.” The online version has been corrected.

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Structural basis of photosensitivity in a bacterial light-oxygen-voltage/helix-turn-helix (LOV-HTH) DNA-binding protein

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Light-oxygen-voltage (LOV) domains are blue light-activated signaling modules integral to a wide range of photosensory proteins. Upon illumination, LOV domains form internal protein-flavin adducts that generate conformational changes which control effector function. Here we advance our understanding of LOV regulation with structural, biophysical, and biochemical studies of EL222, a light-regulated DNA-binding protein. The dark-state crystal structure reveals interactions between the EL222 LOV and helix-turn-helix domains that we show inhibit DNA binding. Solution biophysical data indicate that illumination breaks these interactions, freeing the LOV and helix-turn-helix domains of each other. This conformational change has a key functional effect, allowing EL222 to bind DNA in a light-dependent manner. Our data reveal a conserved signaling mechanism among diverse LOV-containing proteins, where light-induced conformational changes trigger activation via a conserved interaction surface.

Environmental sensory proteins play a crucial function for cellular adaptation in response to changing conditions. These proteins frequently contain effector domains whose activity is regulated by specialized sensory domains sensitive to various stimuli. One widely distributed class of such sensory domains is the PAS (PER-ARNT-SIM) family, whose members typically regulate protein/protein interactions in response to changing environmental cues (1). A subset of PAS domains, called light-oxygen-voltage (LOV) domains, use flavin cofactors to detect changes in blue light intensity or redox state (2). LOV domains are found in regulatory proteins for phototropism (3), seasonal gene transcription (4), bacterial stress responses (5, 6), and many other diverse biological responses. Within these pathways, LOV domains control a wide range of effector domains, including kinases, F boxes, and DNA-binding domains (7). Recently, these natural proteins have been joined by engineered LOV fusions that confer in vitro and in vivo LOV-based photoregulation to a range of protein targets (8–11).

This raises the question: How can a class of light-regulated domains with similar tertiary structures control such a wide variety of effectors? What is clear is that LOV domains all share similar architectures and photochemical responses to illumination, harnessing the energy of incoming blue light photons to form a covalent adduct between the S$_\beta$-sheet surface of the LOV domain, suggesting a site for signal propagation common between them. The functional importance of regulated interactions at this site have been validated by the ability of point mutations on the S$_\beta$-sheet or interacting effector surfaces to decouple changes in effector activity from adduct formation (18, 19).

Among the known LOV-containing proteins are several transcription factors, such as the zinc-finger containing N. crassa white collar-1 (WC-1) (20) and the algal basic leucine zipper AUREOCHROMES (21). Although light controls the binding of these proteins to DNA, the mechanism(s) of this regulation is not understood at a molecular level. Here we address this shortcoming by examining how a LOV domain directly regulates DNA binding, establishing the generality of LOV signaling. Our studies focus on EL222, a 222 amino acid protein isolated from the marine bacterium Erythrobacter litoralis HTCC2594. In addition to an N-terminal LOV domain, EL222 also contains a C-terminal helix-turn-helix (HTH) DNA-binding domain representative of LUXR-type DNA-binding proteins (22). Combining regulatory models from a diverse group of LOV-based photosensors (15) and LUXR-type proteins (23), we hypothesized that the EL222 N-terminal LOV domain represses DNA-binding activity of the C-terminal domain in the dark, and that this inhibition would be released with blue light illumination.

Results

Dark-State Crystal Structure of EL222 Suggests Mode to Inhibit DNA Binding. As an initial step to examining this model, we solved the 2.1-Å resolution crystal structure of EL222 in the dark state (Table S1), observing interactions between the LOV and HTH domains consistent with our hypothesis (Fig. 1). The EL222 structure contains both of the two expected domains, an N-terminal α-helix, β-strand extension of its LOV domain upon illumination (18). In both cases, the external structures interact with the β-sheet surface of the LOV domain, suggesting a site for signal propagation common between them. The functional importance of regulated interactions at this site has been validated by the ability of point mutations on the β-sheet or interacting effector surfaces to decouple changes in effector activity from adduct formation (18, 19).

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α/β LOV domain and a C-terminal all-helical HTH domain. A single FMN chromophore was observed within the LOV domain, orienting the critical isoalloxazine C4a atom only 3.9 Å from the cysteine (Cys) 75 Sγ atom that is expected to form the photochemical adduct. The LOV domain is followed by a C-terminal Jα-helix as observed in other LOV structures (15, 24), but here serves as an interdomain linker that associates more closely with the HTH effector domain rather than docking onto the LOV β-sheet surface as in AsLOV2 (15). This arrangement allows the EL222 LOV β-sheet surface to directly interact with the 4α-helix and 1α-2α loop of the HTH domain. This β-sheet interface is analogous to that used by other LOV and PAS domains to bind their effectors (25) (Fig. S1), burying approximately 700 Å² of surface area between the EL222 LOV and HTH domains. Notably, we observed differences in the relative arrangement of the LOV and HTH domains in the two molecules of EL222 found in the asymmetric unit, due to the translation of the HTH domain by approximately 2.5 Å parallel to the axis of helix 4α (Fig. S1E). Although this translation slightly alters the particular interactions between domains (Table S2), both molecules still fundamentally use a similar mix of hydrophobic and polar contacts at the LOV/HTH interface (Fig. 1B). The plasticity of this interface is consistent with a signaling role, poised for the facile conversion between conformations via allosteric change within the LOV domain (26). As in the structures of NarL and DosR (27, 28), the regulatory LOV domain of EL222 contacts the HTH dimerization helix (4α), but it does not also directly contact with the DNA-binding recognition helix (3α) as observed with the regulatory domains of these other structures. Such structural comparisons supported our hypothesis that EL222 fails to bind DNA in the dark by both sequestering the LOV domain interfering with HTH-DNA interactions (Table S2).

Photoactivation of EL222 Leads to Adduct Formation and Domain-Scale Rearrangements. Turning from structure to function, we examined light-induced changes in the visible absorbance spectrum to establish that EL222 can undergo LOV photochemistry. As expected for a flavin-containing LOV domain, we observed significant absorbance around 450 nm with vibrational fine structure (29) (Fig. 2A). This absorbance diminished significantly after illuminating samples, with three isobestic points at 330, 384, and 407 nm, consistent with formation of the Cys-FMN adduct. After ceasing illumination, we observed subsequent dark-state recovery of the characteristic absorbance profile with first order exponential kinetics (r = 25.5 s for 25 °C, pH 6.0).

Having established the photosensitivity of EL222, we probed the ability of adduct formation to generate large-scale conformational changes using limited proteolysis. Both dark and lit EL222 treated with chymotrypsin demonstrated an initial cleavage re-moving the N-terminal His₆-tag within the first 5 min (Fig. 2F), but exhibited different behavior with extended incubation times. Dark-state samples underwent little additional proteolysis, consistent with a well-folded, compact protein. In contrast, lit-state samples were more quickly and extensively proteolized, with little full-length protein remaining intact after 60 min. Notably, chymotrypsin treatment of lit-state samples generated stable fragments, one of which was consistent with an intact LOV domain (Fig. 2B, species C). Mass spectrometry established that this fragment was generated by cleavage within the interdomain Jα-helical linker at Met159, which packs against the HTH 4α-helix in the dark-state structure. These data, together with our observation of protease-resistant fragments in dark conditions, suggest that light-induced conformational changes increase the accessibility of the Jα-linker via reorientation of the ordered LOV and HTH domains. This is supported by limited differences between CD spectra recorded under dark and lit conditions (Fig. S3).

NMR Studies of EL222 Photoactivation Establish Long-Range Light-Induced Conformational Changes. To probe these light-induced changes at higher resolution, we used solution NMR spectroscopy. Using a combination of triple resonance and NOESY data, we assigned 15N, 13C, and 1H chemical shifts of EL222 in the dark state (81% of the backbone, 40% of the side chain). TALES analyses of these chemical shifts (30), combined with through-space 1H-1H NOE data, let us confirm that EL222 has very similar secondary and tertiary structures in the crystal and solution states. Notably, solution measurements confirmed that EL222 is monomeric under these conditions. Upon illumination, we observed chemical shift and peak intensity changes at many backbone and side chain positions as observed in 15N/H Heteronuclear single quantum coherence (HSQC) (Fig. 3A) and 13C/H HSQC spectra (Fig. S4). Such changes reflect alterations in the local electronic environments around NMR-active nuclei. Critically, all spectra maintained comparable chemical shift dispersion in the dark and lit states, consistent with LOV photochemistry inducing a domain reorientation, but not unfolding as observed in AsLOV2 (15).

To identify which sites experienced significant changes, we compared 15N/H HSQC spectra recorded under dark and lit conditions (Fig. 3A), using 15N/H Scotch exchange spectroscopy to assign lit-state chemical shifts by correlating dark-state 15N shifts with lit-state 1H shifts (31). From the 109 pairs of dark- and lit-state chemical shifts unambiguously assigned with this analysis, we established that chemical shift changes occur throughout the length of the protein (Fig. 3B). Although clusters of perturbed residues in the LOV domain likely report on adduct-induced configurational changes in the FMN chromophore and resulting conformational changes in the surrounding protein, we also clearly observed long-range (>15 Å from the flavin C4a atom) effects at sites outside the LOV domain as well. These include changes in...
To complement this view from chemical shift changes, we used NMR-based measurements of backbone amide deuteration exchange rates to establish light-induced changes in domain structure and stability. We obtained these data by resuspending uniformly $^{15}$N-labeled samples in $D_2O$-containing buffer, monitoring exchange by loss of intensity in consecutively recorded $^{15}$N/$^2$H HSQC spectra. As we have not assigned the lit-state chemical shifts, $^2$H exchange measurements under illumination relied on duty-cycling the sample between the dark and lit states, using assigned dark-state spectra to measure rates. Converting these exchange rate data into protection factors (32), we found that numerous sites across the protein exchanged very slowly in the dark state, consistent with stable hydrogen bonding as expected from regular secondary structure (Fig. 3D). Many amides within the LOV domain $\beta$-sheet surface are very well protected as expected for PAS domains (15, 33) and specific residues within the first and fourth helices of the HTH (1α and 4α) appear refractory to exchange. Upon illumination, these highly protected regions showed an overall decrease in protection factor, suggestive of distortion in the LOV structure as previously observed in AsLOV2 (15). The fact that these sites remained protected from exchange overall is consistent with both the LOV and HTH domains remaining stably folded, and with light inducing a separation or relative reorientation of the LOV and HTH domains as suggested by limited proteolysis and chemical shift analyses.

**Photoactivation of EL222 Promotes DNA-Binding Activity.** These light-induced structural changes imply a corresponding functional change, which we presumed to be a light-activated DNA-binding activity, given our data above and the domain architecture of EL222. Without a preestablished biological role of this protein, we started without any validated DNA-binding site. To address this issue, we used a candidate-based approach, assuming that EL222 might be autoregulatory and bind to a DNA sequence upstream of its own coding sequence. Scanning through the 350-bp region located 5′ to the start of EL222 translation with a series of 21 overlapping 45-bp candidate sequences tested, none bound EL222 as assessed by gel shift assays conducted under dark conditions. However, all of the candidate sequences bound EL222 under illumination at or above 70-μM protein (Fig. S5A), suggesting light-dependent activation of nonspecific DNA binding. Titrating to lower protein concentrations, we found two sequences that bound EL222 at concentrations as low as 7 μM (Fig. 4 for results of one of these sequences, oligomer 1). In both instances, DNA binding only occurred when the protein:DNA mix was incubated under white light. Binding was cooperative with respect to protein concentration, with a Hill coefficient of approximately four, suggesting that a pair of dimers bound within this 45-bp section. No binding occurred under dark-state conditions, even at protein concentrations capable of nonspecific DNA binding in the light. Protein previously exposed to bright light, then allowed to recover to dark state overnight at 4°C also demonstrated the same minimal residual DNA-binding activity as protein that was not exposed to light, indicating the activity is reversible and light dependent (Fig. S5B). From these data, we can conclude that EL222 demonstrates light-dependent DNA-binding activity. Although the DNA sequence used in these gel shift experiments bound with the highest affinity of all sequences tested, we suspect that this is not an optimal binding sequence for EL222 based on the affinities of similar HTH-containing proteins for their cognate DNA sequences (34, 35). Nevertheless, these data suggest that this DNA sequence retains its utility for assaying protein activity in future structural and/or functional experiments.

Taken together, our data demonstrate that conformational changes propagate through the LOV domain upon illumination, disrupting inhibitory LOV-HTH interactions mediated by the LOV $\beta$-sheet. To test this, we mutated several sites to constitut-
and several interhelical loops (Figs. 1, 3, and 5). Disruption of
between the protein in the dark or lit state (Fig. S6) proteolysis of L120K using chymotrypsin showed little difference
binding in the dark state via interactions with the HTH 4
reorganization of two ordered domains. Protection factors
are consistent with the EL222 LOV domain inhibiting DNA
Within the context of regulation of HTH-containing proteins, our
ture that constitutively binds DNA.
that the L120K mutation forces EL222 into a lit-state-like struc-
tively break the LOV/HTH dark-state interaction and generate
proteins locked in the DNA-binding conformation. One of these
mutations, L120K, targeted a hydrophobic patch between the
β-sheet surface of the LOV domain and the HTH 4α-helix (Fig. S6B). Gel shift assays conducted under dark-state condi-
tions demonstrated that EL222 L120K bound DNA with similar
affinity to wild type under lit-state conditions (Fig. S6C). Limited
proteolysis of L120K using chymotrypsin showed little difference
between the protein in the dark or lit state (Fig. S6D), with both
resembling the lit state of wild-type protein. These results suggest
that the L120K mutation forces EL222 into a lit-state-like struc-
ture that constitutively binds DNA.

Discussion
Within the context of regulation of HTH-containing proteins, our
data are consistent with the EL222 LOV domain inhibiting DNA
binding in the dark state via interactions with the HTH 4α-helix and several interhelical loops (Figs. 1, 3, and 5). Disruption of
these interdomain contacts by light-induced conformational changes in the LOV domain (or mutagenesis of residues at the
LOV/HTH interface) induces DNA-binding activity. A similar regulatory model is used by other two-domain response regulator proteins, including the *Escherichia coli* nitrate/nitrite
response protein NarL. In this case, transfer of a phosphate group
to the N-terminal receiver domain disrupts inhibitory contacts of
this domain with the C-terminal LuxR-type HTH domain, allow-
ing dimerization and DNA binding (27, 36, 37). Studies of
response regulator proteins from NarL and other LuxR family
members indicate that their regulatory domains also contact the
HTH 1α-2α loop and 4α-helix (28, 36, 37), similar to EL222.
Although this aspect of regulation shows strong parallels between
NarL and EL222, we note that they are activated quite differ-
ently. In contrast with the intramolecular mechanism that we
describe for EL222, NarL activation is entirely dependent on a
separate sensor protein (NarQ or NarX) that detects an en-
vironmental signal (nitrate or nitrite) (38, 39) and initiates an
intermolecular phosphotransfer to NarL. Finally, although the
combination of N-terminal sensory and C-terminal HTH DNA-

![Fig. 3. Solution NMR data suggests EL222 undergoes light-induced rearrangement of two ordered domains. (A) Superposition of 15N-1H HSQC spectra of EL222 acquired under dark (black) or lit (red) conditions show light-induced changes in peak location and intensity. (B) Chemical shift difference analysis of 15N-1H HSQC spectra shown in Fig. 3A indicate significant changes occurring in both domains, including the HTH 1α-2α loop, 3α-4α loop, and 4α-helix located at the interface with the LOV domain. Secondary structure elements as indicated by the NMR data and X-ray structure are indicated. (C) Mapping values from Fig. 3A onto the dark-state crystal structure illustrates the pattern of chemical shift perturbations at the interdomain interface. Side chains are indicated for 1α-2α loop, 3α-4α loop, and 4α-helix residues in the HTH domain with 15N-1H chemical shift changes upon illumination. (D) 1H exchange protection factor analyses (32) of EL222 conducted in the dark (black) and lit (red) states show similar protection, but to a lower overall degree upon illumination, consistent with reorganization of two ordered domains. Protection factors >106 are lower bound estimates because these sites did not sufficiently exchange for robust fitting of the time-dependent peak intensity changes.](image-url)
expose the 4 conformational changes that release inhibitory LOV/HTH interactions and mical formation of a cysteinyl/flavin adduct in the LOV domain generates steric conflicts with DNA if it could bind in monomeric form. The photocheable of binding DNA as the LOV domain sequesters the HTH 4 Fig. 5. Some of these interactions can be modulated by co-N- and C-terminal segments that are essential to signaling hetero- or homodimerization, whereas others bind different β domain and, more generally, PAS domain signaling via the controlled through ligand-induced protein folding (35) rather (35, 40), we note that some of these proteins may likely be controlled through ligand-induced protein folding (35) rather than covalent bond formation as seen in NalL and EL222. Our results also further validate a conserved aspect of LOV domain and, more generally, PAS domain signaling via the β-sheet surface. Many PAS and LOV domains use this surface for hetero- or homodimerization, whereas others bind different N- and C-terminal segments that are essential to signaling (15, 18, 25). Some of these interactions can be modulated by cofactors within the PAS/LOV domain, providing a ligand-regulated binding domains may suggest that EL222 resembles response regulators that directly detect diffusible small ligands in the cell (35, 40), we note that some of these proteins may likely be controlled through ligand-induced protein folding (35) rather than covalent bond formation as seen in NalL and EL222. Our results also further validate a conserved aspect of LOV domain and, more generally, PAS domain signaling via the β-sheet surface. Many PAS and LOV domains use this surface for hetero- or homodimerization, whereas others bind different N- and C-terminal segments that are essential to signaling (15, 18, 25). Some of these interactions can be modulated by cofactors within the PAS/LOV domain, providing a ligand-regulated environmental switch. EL222 extends this paradigm by demonstrating that fully folded effector domains can bind to this surface, harnessing conformational changes within the LOV domain to rearrange the LOV-effector complex (without unfolding the effectors, as seen with the isolated Jα-helix in AsLOV2; ref. 15). Notably, these effector domains have different structures but appear to work through a common mechanism involving the β-sheet, potentially explaining how a single type of sensory domain can regulate a diverse group of effectors (7). Such information is particularly useful for both understanding naturally occurring LOV-regulated proteins and engineering light-regulated systems. These currently include LOV fusions to small GTPases, metabolic enzymes, DNA-binding domains, and other enzymes (8–10). All of these designed proteins have taken advantage of the well-characterized signaling mechanism of AsLOV2, including the PA-Rac1 light-activated GTPase (8). This fusion protein tethers the photosensory LOV domain closely to the effector GTPase when the Jα-helix is bound by the LOV domain, inhibiting enzymatic activity. With the knowledge of the broader principles provided here by EL222, such engineering may well be extended to an even larger range of target effectors as part of the rapidly growing toolbox of “optogenetic” tools (41) that offer precise spatial and temporal control of protein activity in vitro and in vivo.

Methods

Protein Expression and Solution Characterization. EL222 protein samples were obtained using standard E. coli heterologous expression and affinity purification methods as detailed in SI Methods. Thin layer chromatography established that EL222 bound FMN, not FAD or riboflavin. Additional solution characterization included UV-visible absorbance spectroscopy (60 μM sample; Varian Cary 50 spectrophotometer), CD spectroscopy (15 μM sample; AVIV 62DS), and limited proteolysis (1:43 wt:wt ratio of cystatin:EL222). Photoexcited adduct-containing states were generated using a photographic flash (UV-vis absorbance, CD) or filtered mercury lamp (limited proteolysis).

Crystallographic Structure Determination. Crystals of EL222 were grown using the hanging drop method, using equal volumes of 8 mg/ml EL222 (1–222) and a reservoir of 20% (wt/vol) PEG 8K, 0.1 M β-mop (pH 7.5), 0.1 M ammonium acetate. X-ray diffraction data were collected from a single crystal on beam line 7-1 at Stanford Synchrotron Radiation Laboratory. The structure was solved by four-step molecular replacement using PHASER (42), with independent search models for the LOV and HTH domains (without a Jα-helix for the LOV domain). The structure of the Jα-interdomain helix was built manually as supported by difference density. The initial model of EL222 was subjected to iterative cycles of model building with COOT (43) and subsequent refinement with REFMACS (44) and PHENIX (45). Final R and Rfree values were 26.3% and 32.9%, respectively, with further statistics of the refinement available in Table S1.

Solution NMR Studies. Solution NMR data were collected at University of Texas Southwestern using Varian 600 and 800 MHz spectrometers equipped with cryogenically cooled probes and laser illumination as previously described (15), with samples between 250–650 μM. NMR data were processed using NMRpipe (46) and analyzed with NMRView (47). Backbone and limited side-chain chemical shift assignments of dark-state EL222 were obtained using 1H-15N(H) (32), U-15N(H), 15C, 13C-N-labeled protein and a combination of 2H-modified triple resonance and NOESY experiments. Lit-state chemical shift differences were determined using 15N/1H Scotch data to correlate dark- and lit-state chemical shifts (31), whereas lit-state 1H exchange rates were determined using interleaved dark/lit acquisition (15).

DNA-Binding Studies. DNA-binding activity was assessed using gel shift assays using 32P-labeled dsDNA 45-bp oligonucleotide fragments of DNA located to the 5’ end of the EL222 gene, as detailed in SI Methods. Gel shift results presented in Fig. 4 used one of these fragments (oligomer 1, genomic position 983532–983577), using a photographic flash to generate the photoexcited adduct state.

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