ONLINE METHODS

Analytical model of photoswitching

Under our experimental conditions, $K_{\text{lit}}^\text{obs} \approx K_{\text{DNA}}$ because $K_{\text{helix}}^\text{lit} \approx 10$ and $x_{\text{fus}}$ generally is below 10. For subsequent calculations, we assigned $K_{\text{DNA}}$ to be the measured lit state DNA affinity of LovTAP. Values of $K_{\text{dark}}^\text{helix}$ and $K_{\text{lit}}^\text{helix}$ are taken from this work and previous work\textsuperscript{17} respectively. We note that if $K_{\text{dark}}^\text{helix}$ and $K_{\text{lit}}^\text{helix}$ are very large or very small (i.e., the helix is never docked or is always docked), the Eq. 6 will still hold, but the effector will effectively be unregulated by light. In the former and latter cases, it will be constitutively active and inactive, respectively.

The model presented in Eqs. 1-6 should be valid for a monomeric system where only one helix undocking event is required for full activation. Because LovTAP binds DNA as a homodimer, an alternative model was also investigated where full activation requires undocking of both helices from their respective LOV domains. In this model the helices undock independently with equilibrium constants $K_{\text{helix}} / x_{\text{eff}}$.

$$K_{\text{obs}} = \frac{K_{\text{DNA}}}{\left(1 + \frac{x_{\text{eff}}}{K_{\text{helix}}}\right)^2}$$

Eq. 7

This model also agrees with the lit-state DNA binding data for the accessible range of $x_{\text{eff}}$ values in this study (Supplementary Fig. 5). However, in the dark state, the experimental DNA binding data can only be accurately predicted by the the monomeric model (Supplementary Fig. 5). Although LovTAP binds DNA as a dimer, for unclear reasons, it follows a model of photoactivation that is characteristic of a monomeric protein.
**Computational prediction of stabilizing mutations**

We followed two strategies to identify mutations that increase the probability of LOV-Jα docking. First, simulated mutations were introduced in the 27-residue Jα helix to increase its intrinsic helical propensity or create favorable tertiary contacts between it and the LOV core. We submitted point-mutated Jα (AsLOV2 523-543) to AGADIR\(^1^9\) (http://www.embl-heidelberg.de/Services/serrano/agadir/agadir-start.html). Helicities were scored with the following settings: pH = 7, T = 278 K, ionic strength = 0.1, N\text{term} = acetylated, C\text{term} = amidated. We identified three mutations, Gly528 to Ala (G528A), Ile532 to Ala (I532A), and Asn538 to Glu (N538E), that increase Jα's average intrinsic helicity from 1.5% to 6.5% (G528A), 2.1% (I532A) and 2.0% (N538E). Several other mutations that were predicted to increase helicity by AGADIR either had no effect on photoswitching (e.g. R526D) or constitutively undocked the Jα helix (G528L) (data not shown). Gly528 and Ile532 are in the hydrophobic interface between the LOV core and Jα so that changes to either may improve tertiary packing (or perhaps destabilize it in the case of G528L) (Supplementary Fig. 1). The N538E mutation may create a salt bridge with Lys413 (Supplementary Fig. 1).

Second, we used the program HyPARE to screen potentially stabilizing mutations \textit{in silico}\(^2^0\). HyPARE performs electrostatic calculations on a pair of known structures to predict how the introduction of a novel charge would affect the bimolecular association rate constant (\(k_{\text{on}}\)). We divided the dark-state AsLOV2 crystal structure\(^1^6\) into two parts: the LOV core (residue 404–519) and the Jα helix (residue 520–546). HyPARE predicted that the introduction or change of
charged residues at four sites (D419, F429, Y508 and I510) would increase $k_{on}$ between these two segments.

**AsLOV2-Jα protein sample preparation**

An AsLOV2–Jα construct containing residues 404-546 of *Avena sativa* phototropin 1 was used in this study. We used the QuikChange site-directed mutagenesis kit (Stratagene) to introduce the D419K, F429K, Y508K, I510K, G528A, I532A and N538E mutations. Expression and purification of the wild-type and mutant proteins was performed as described previously with minor modifications.

For NMR experiments, the proteins were expressed from plasmid pHis$_6$-GB1 in *E. coli* strain BL21(DE3) and grown in M9 minimal media containing 1 g/L $^{15}$NH$_4$Cl as the sole nitrogen source for uniform $^{15}$N labeling. Uniform $^{13}$C labeling is achieved by using $^{13}$C$_6$-Glucose (2 g/L) as the sole carbon source in M9 media. Cultures were grown at 37 ºC to $A_{600} = 0.8-1.0$ and induced with 0.5 mM IPTG at 20ºC and kept in dark. After 14 hours of induction, the cells were harvested in 50 mM pH 7.5 phosphate buffer containing 0.1 M NaCl, 5 mM betamercaptoethanol and 1 mM phenylmethylsulphonyl fluoride. The cells were lysed by extrusion and clarified by centrifugation at 26,000 g for 20 minutes at 4ºC. The supernatant was loaded onto a Ni$^{2+}$-NTA affinity column (QIAGEN) for rapid purification of the His$_6$–GB1–AsLOV2–Jα protein. The His$_6$–GB1 tag was removed by TEV protease and AsLOV2–Jα protein was purified from the cleavage mixture by anion exchange chromatography (Source 15Q, GE Healthcare). Purified
proteins were exchanged into 50 mM phosphate buffer (pH 6), 0.1 M NaCl. NMR samples contained ~ 1 mM protein, 10% D₂O, 1 mM DTT and a protease inhibitor cocktail.

For CD experiments, the proteins were prepared as described, except that E. coli were grown in LB media and final purification was done by gel filtration chromatography using a HiPrep 16/60 Sephacryl S-100 HR column (GE Healthcare) equilibrated with 10 mM NaPO₄, pH 8.0, 65 mM NaCl, 0.1 mM EDTA.

LovTAP sample preparation

A PCR fragment encoding the LOV2–TrpR fusion protein was subcloned into the expression vector pCal-n (Stratagene) so as to be in frame with the amino terminal calmodulin binding peptide. BL21(DE3) E. coli (Novagen) carrying this vector were grown in LB media at 37° C to an OD₆₀₀ ~ 0.5 and induced at 20° C by the addition of 0.3 mM IPTG. After ~ 16 hours, the cells were harvested by centrifugation and resuspended in B-buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 1 mM imidazole, 1 mM MgCl₂ and 2 mM CaCl₂, 10 mM β-mercaptoethanol and EDTA-free protease inhibitor cocktail (Roche)). The cells were lysed by the addition of lysozyme and sonicated. The lysate was cleared by centrifugation and applied to equilibrated calmodulin affinity resin (Stratagene). The resin was washed with W-buffer (50 mM Tris, pH 8.0, 500 mM NaCl, 1 mM imidazole, 1 mM MgCl₂ and 2 mM CaCl₂) and the protein eluted in 3-5 mL with E-buffer (50 mM Tris, pH 8.0, 500 mM NaCl, 2 mM EGTA). The eluate was applied to a HiPrep 16/60 Sephacryl S-100 HR column (GE Healthcare) wrapped with aluminium foil and equilibrated with G-buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 1 mM EDTA) running at 1 mL/min. Fractions with significant absorbance at 447 nm (A₄₄₇) were collected and analyzed by SDS-PAGE. Concentration was determined by A₄₄₇.
Experimental validation of dark-state Jα helix stabilization by NMR spectroscopy

We used solution nuclear magnetic resonance (NMR) spectroscopy to investigate the structural and thermodynamic effects of the four LOV core and three Jα mutations in dark-state AsLOV2. The $^{15}\text{N}$-$^1\text{H}$ heteronuclear single quantum coherence (HSQC) spectra of all mutants in the isolated AsLOV2 are similar to that of the WT protein (data not shown), suggesting they maintain the same overall structure. (HSQC spectra of F429K and I510K suggested that these mutations undock the helix, therefore these two mutations were not pursued further.)

To evaluate how these mutations affect the docking affinity between the LOV core and Jα in AsLOV2, we performed NMR relaxation dispersion (RD) measurements. Conformational exchange processes occurring on the micro- to to millisecond time scale contribute to NMR transverse relaxation rates ($R_2$) of the nuclei experiencing these dynamic processes. This exchange-originated contribution to $R_2$ is termed $R_{\text{ex}}$ and can be suppressed by a Carr-Purcell-Meiboom-Gill (CPMG) spin echo pulse train sequence, provided that the exchange rate is similar to the CPMG pulsing frequency ($\nu_{\text{CPMG}}$ typically ranges from 100 s$^{-1}$ to 1000 s$^{-1}$). Changes in the effective relaxation rate ($R_{2\text{eff}}$) as a function of $\nu_{\text{CPMG}}$ give rise to a RD curve, where $R_{\text{ex}}$ is defined as following:

$$R_{\text{ex}} = R_{\nu_{\text{CPMG}} \to 0} - R_{\nu_{\text{CPMG}} \to \infty}$$  \hspace{1cm} \text{Eq. 8}$$

For two-state exchange under conditions where $R_{\text{ex}}$ can be completely suppressed at higher $\nu_{\text{CPMG}}$, the fitting of the RD curves provides thermodynamic, kinetic and structural information of the process$^{21,22}$. 
Backbone $^{15}$N RD measurements indicate that the G528A, I532A, and N538E mutations on the Jα helix indeed stabilize the docked conformation. Of the two LOV core mutations predicted by HyPARE, only D419K stabilizes the docked conformation of the helix while Y508K mutation increases the Jα undocked population by ~ 1.6-fold in the dark. Similar to WT AsLOV2, the residues that exhibit significant $R_{ex}$ are clustered at the LOV–Jα interface (Supplementary Fig. 3). The RD curves of one of these residues, His517, indicates that $R_{ex}$ values decrease for the G528A and N538E mutations and the D419K-I532A double mutation (Supplementary Fig. 2a). Consistent with the decreased $R_{ex}$ values, fit of the RD data indicate that those mutations indeed reduce the Jα undocked population in the dark (Table 1). In the WT, G528A and I532A mutants, the percentage of molecules with the helix undocked is 5.98 ± 0.08%, 4.5 ± 0.9% and 1.76 ± 0.03%, respectively (note that our previously reported value of 1.7 % for the WT protein$^{17}$ was measured for a construct containing a longer C-terminus). More generally, substantial variations were found in the equilibrium populations with the length of Jα (data not shown). More dramatic effects were observed for the D419K-I532A double mutant and the N538E mutant, where the Jα undocked populations are reduced ~ 8 fold, to 0.74 ± 0.02% for N538E and 0.73 ± 0.04% for D419K-I532A. These results demonstrate that the mutations stabilize interactions between the LOV core and Jα in the dark.

NMR experiments were performed at 25ºC on Varian Inova 600 MHz spectrometers. All NMR data were processed using NMRDraw and NMRview software$^{23,24}$. Relaxation compensated constant time CPMG pulse sequences were used to acquire $^{15}$N relaxation dispersion data$^{25,26}$. A 40 ms constant time period was used for all experiments. For each CPMG series, repeats of one or two data points were performed and the intensity of the repeats were used to calculate standard deviation of the series. In cases where the standard deviation was less then 2%, 2% was
used in data fitting. For data points with repetition, the average of the two repeats was used for data fitting. Data were fit using software kindly provided by Dr. Lewis Kay. Our previous NMR analysis of AsLOV2–Ja established that residues showing relaxation dispersion experience the same two-state exchange process. Therefore relaxation dispersion curves were fit by a two-state model, using the same kinetic and thermodynamic values for all dynamic residues within the same protein. This global fit improves the accuracy of the fitting.

Backbone resonance assignments of the mutant D419K-I532A were carried out by HNCACB and CBCA(CO)NH experiments. The spectra of lit state AsLOV2 proteins were acquired as described. Briefly, the output from a 5 W Coherent Inova 90-C argon ion laser (472.7 nm) was delivered to the NMR tube (Wilmad, 5 mm) through a quartz fiber optic. The amount of light delivered to the NMR sample is directly measured before each experiment. All lit state $^{15}$N-$^1$H HSQC spectra were acquired using 50 mW laser pulses with duration of 200 ms that are controlled by a shutter.

**CD spectroscopy**

We examined the effects of the G528A, I532A, N538E, G528A-N538E and D419K-I532A mutations on the lit state AsLOV2 protien. We previously reported a measurement of lit-state docking in the wild-type protein determined by NMR chemical shift analysis. However, the lit state $^{15}$N-$^1$H HSQC spectra of proteins containing the D419K, G528A and N538E mutations show varying degrees of line broadening in the J$\alpha$ region (Supplementary Fig. 4), indicating the presence of intermediate timescale exchange and thus preventing the use of chemical shift analyses to estimate the J$\alpha$ docked population.
Instead, far-UV circular dichroism (CD) was used to measure changes in the $J\alpha$ docking equilibrium from the changes in $\Theta_{222\text{ nm}}$ values accompanying the unfolding of this helix. Given our signal-to-noise ratio, $K_{\text{helix}}$ values in the range of 0.1–10 can be reliably measured. Because $K_{\text{helix}}^{\text{lit}} \approx 10$ for WT AsLOV2, docking stabilization down to $K_{\text{helix}}^{\text{lit}} \approx 0.1$ is readily measurable.

Circular dichroism measurements were performed by using a Jasco J-715 spectropolarimeter equipped with a temperature-controlled cuvette holder and a 0.1-cm path-length cuvette. Illumination was from a single blue LED connected to an external switch. The sample (1-7 µM AsLOV2) was illuminated from above for 60 s to saturated photoexcitation, and then the LED was switched off and CD at 222 nm was recorded for 250 s. The data were recorded in triplicate, averaged, converted to mean residue ellipticity, and fit to a single-exponential function by using IGOR Pro (Wavemetrics). The fitted parameters (amplitude, dark-state ellipticity) were normalized by the dark-state ellipticity.

After steady-state photoexcitation, we monitored $\Theta_{222\text{ nm}}$ until the sample recovered to the ground state (Supplementary Fig. 2b). We fit the data to a single exponential model and used the CD values at $t = 0$ and $\infty$ as the lit- and dark-state values, respectively. We incorporated the NMR-derived dark- and lit-state helix docking data for the WT protein to determine the conversion between CD amplitude and helix docking. We then normalized the CD amplitude for the mutants by their individual dark-state docking, and calculated $x_{\text{mut}}^{\text{lit}}$ from their normalized lit state CD amplitudes. We propagated errors from the NMR analysis through the conversion, although we feel that this error is systematic and its inclusion leads to an overestimation of the statistical error in helix docking. Nevertheless, we would prefer to overestimate the error than to underestimate it. We also note that this analysis assumes that the helicity in the undocked state is negligible. To
the extent that mutations change this helicity, CD may underestimate the fraction of undocked helix.

We were unable to independently determine $x_{\text{dark mut}}$ for the G528A-N538E double mutant, and we estimated this as the product of the values from the individual mutants. The error in the determination of $x_{\text{dark mut}}$ for G528A is large compared to that of other mutants (Table 1) and this error is propagated to $x_{\text{dark mut}}$ for the G528A-N538E double mutant. Thus, the relative lack of agreement between $x_{\text{dark mut}}$ and $x_{\text{lit mut}}$ at these two points likely stems from the same source.

In order to determine $x_{\text{eff}}$ for the various mutations, we used a reference value of $x_{\text{fus}}$ calculated from LovTAP DNA binding data. We used the same value, $x_{\text{fus}} = 0.28$, in all calculations. We are interested in the stabilization of helix docking relative to the wild-type protein. Therefore, we did not propagate the error on this value through subsequent calculations because it would be highly correlated among the individual mutants and thus overstate the statistical error.

**Illumination**

Samples were illuminate using blue AlGaInP LEDs (thefledlight.com, 20° viewing angle, 8,000 mcd, 468 nm $\lambda_{\text{max}}$ at 3.4 V) powered by a 3.4-V power source.

**RsaI protection assays**

The plasmid pUC19-KpnIΔ-trpRs(3A)$_2$ was digested with PvuI and the resulting linear fragment containing only a single RsaI restriction site buried in the trp operator was gel purified. This 1680 bp reactant is digested to 375 and 1305 bp products by RsaI. Protection assays were performed with 19 nM pUC19- KpnIΔ-trpRs(3A), 1X NEB Buffer 2 (10 mM Tris·HCl, pH 7.9,
10 mM MgCl₂, 50 mM NaCl, 1 mM DTT), 0.1 mg/mL BSA (Promega), 0.1 mM L-tryptophan (Sigma). Protein in G-buffer was diluted 10-fold into this mixture and incubated for 1 minute under ambient conditions. RsaI was diluted to a concentration of 0.5-2 U/μL in dilution buffer (50% v/v glycerol, 1X NEB Buffer 2, 0.2 mg/mL BSA) and then diluted 20-fold into the reaction mixture. Reactions were performed in 0.5 mL thin walled tubes placed in the block of a thermocycler set at 25° C. The reaction was quenched at the desired time points by adding loading buffer (Promega) to 1X and EDTA to 15mM. The samples were run on a 1.5% agarose gel in 1X TAE, stained with ethidium bromide and imaged. The amount of digestion was determined as the ratio of the intensity of the product bands to that of the product bands plus the reactant band. The bands were quantified using ImageJ (http://rsb.info.nih.gov/ij/), and the data was analyzed using IGOR Pro (WaveMetrics, Lake Oswego, OR). For all fits, the amplitude was constrained to unity and only the rate was allowed to vary. The observed association constant \( K_{\text{obs}} \) in the lit and dark states was calculated using the equation: \( K_{\text{obs}} = \frac{R - 1}{L} \) where \( L \) is the concentration of LovTAP and \( R \) is the ratio of the intrinsic rate of RsaI digestion \( (k_0) \) and the rate in the presence of LovTAP \( (k_{\text{obs}}) \).

We were unable to carry out DNA cleavage assays on Lov-TAP containing the D419K mutation due to poor behavior of the protein although potentially this mutation can still be used to improve the photoswitchability of other artificial systems.