## Online Methods

**DNA cloning** The cDNA encoding the LOV2 domain of *Avena sativa* (oat) Phototropin1 (404-546), including the C-terminal helical extension (Jα), was a gift from Dr. Keith Moffat of the University of Chicago. Chimeric fusion constructs consisting of LOV2-Jα fused to Rac1 or Cdc42 were generated using an overlapping PCR approach so that precise junctional sequences could be engineered without being limited by restriction sites. These included truncations/extensions of the LOV2-Jα C-terminus (539-547), the N-terminus (2-4) of the GTPases, or insertions of designed Schellman caps (KEAGADQI and KELKEAGADQI)[1]. The QuickChange (Stratagene) protocol was used to introduce additional point mutations, including C450A or C450M, and I539E to mimic the dark and lit state of the LOV domain, respectively.

PA-Rac1 was constructed as follows: LOV2-Jα(404-546)-Rac1(4-192)/Q61L/E91H/N92H. These constructs were inserted into a pTriEx (Novagen) vector for transient expression in mammalian cells as well as in bacteria. For crystallization, C-terminally truncated PA-Rac1 (Δ181-192) was subcloned into the pQE-30 vector (Qiagen). Fluorescent proteins mVenus[2], mCherry[3] and mPA-GFP[4] were inserted at the N-terminus of the LOV domain with a short GSGS linker to monitor expression and subcellular localization. After initial characterization, PA-Rac1 with different fluorescent protein tags was subcloned into pBabe-TetCMV vector for retroviral production and establishment of stable MEF Tet-Off cell lines. High fidelity Pfu Turbo DNA polymerase (Stratagene) was used in PCR reactions and all plasmids were verified by DNA sequencing.

**Pull-down assay of effector binding** mVenus-tagged LOV2-Jα and Rac1 fusion constructs were coexpressed with FLAG-tagged PAK1 in HEK 293 (LinXE) cells by transient transfection using Fugene 6 (Roche). The cells were lysed in 50 mM Tris pH 7.5, 150 mM NaCl, and 1% Triton X-100 (lysis buffer) with addition of EDTA-free protease inhibitor cocktail (Roche). After brief centrifugation, the supernatants were incubated with FLAG/M2-agarose (Sigma) followed by washes with lysis buffer, and elution with lysis buffer containing 200 μg/ml 3xFLAG peptide (Sigma). All procedures were done at 4°C under red light, facilitated using Handee spin columns (Pierce). The purified protein complexes as well as cell lysates were fractionated on 4-12% NuPAGE gels (Invitrogen) followed by Western blot analysis using antibodies against fluorescent protein (JL-8, Clontech) and PAK (N-20, Santa Cruz). Myc pull-down experiments were performed similarly using anti-myc agarose and myc peptide (Sigma) to investigate the interaction of PA-Rac constructs with p50RhoGAP.

**Expression, purification and characterization of proteins used for crystallization** C-terminal truncated PA-Rac1 (Δ181-192) was expressed in *E.coli* strain XL-10 Gold (Stratagene) at 30°C overnight. All purification steps were done under yellow light at 4°C. Cells were lysed in 20 mM Tris pH 8.5, 50 mM NaCl, 5 mM $MgCl_2$ and 2 mM 2-mercaptoethanol. Protein was purified with a Ni-NTA-FastFlow column (Qiagen) exploiting the N-terminal 6xHis tag. The elution was dialyzed against 10 mM Tris pH 8.5, 20 mM NaCl, 5 mM $MgCl_2$ and 2 mM 2-mercaptoethanol. The protein was bound to a MonoQ column (GE Healthcare) and eluted with a linear gradient (0-250 mM NaCl in 50 CV). Fractions containing the protein were concentrated (30 kDa cutoff, Millipore) and further purified by Superose 6 gel filtration chromatography (GE Healthcare, 10 mM Tris pH 8.5, 20 mM NaCl, 5 mM $MgCl_2$, and 2 mM DTE). Prior to crystallization the protein was concentrated to 10 mg/ml (30 kDa cutoff, Millipore). All proteins were characterized spectroscopically. PA-Rac1 showed reversible light dark conversion. The dark recovery rate of PA-Rac1 was measured as described previously[5]. The inactive C450A mutation showed no effect upon light illumination whereas truncated C450M (Δ181-192) was prone to aggregation upon light illumination and, therefore, no data for light dark conversion could be measured.

**Crystallization** Crystallization was carried out under dimmed red light at 20°C. PA-Rac1 and its C450A and C450M mutants were crystallized using the vapor diffusion method by mixing equal volumes of protein (10 mg/ml) and precipitant solution (100 mM calcium acetate / 100 mM sodium cacodylate / 12% (w/v) PEG 8000 or 4% (w/v) PEG 4000 / 100 mM potassium chloride). Yellow pencil shaped crystals appeared overnight and grew to a final size of 50 x 50 x

1000 $\mu m^3$ in a week. To avoid photoactivation, crystal handling was done by shielding the microscope light bulb with a 2 mm thick RG630 filter (ITOS, Mainz, Germany). Prior to cooling the crystals in liquid nitrogen they were transferred stepwise to precipitant solution supplemented with 20% (v/v) ethylene glycol for cryoprotection.

**Data collection and structure determination** Diffraction data were collected at the X10SA beamline (Swiss Light Source, Villigen, Switzerland) under standard cryogenic settings. Data was reduced using the XDS suite[6] (Table S2) and the structure was solved by molecular replacement[7] using subsequentially 2VOU[8] and 1MH1[9] as the initial models. During several rounds of refinement with PHENIX[10] and manual model building in COOT[11], FMN, GTP, $Mg^{2+}$, $Ca^{2+}$ and solvent molecules were included in the model. Structures were validated using MOLPROBITY[12] and PROCHECK[13] (see Table S2 for final statistics).

**Structural modelling for linker optimization** The Rosetta program[14,15] was used to predict the dark state structure of LOV2-Rac1 based on the solved crystal structures of dark state LOV2[8] (PBD code 2V0U) and Rac1[9] (PBD code 1MH1). Structure prediction simulations were performed on LOV2-Rac1 545-4, 546-4, and 547-4 constructs. In these simulations, the torsion angles of the residues connecting the two proteins were optimized with Monte Carlo sampling. Using the Rosetta domain assembly protocol[16], we first applied 1000 $\Phi$ and $\Psi$ backbone torsion angle movements of up to 180° each to three residues connecting LOV2 to Rac1 in a low resolution representation. Small backbone torsion angle moves of up to 4° were then performed on a high-resolution representation of LOV2-Rac1, followed by a global repacking of all sidechain rotamers. After every 15 cycles of small moves and repacking, further repacking was

restricted to the rotamers at the interface and next to the LOV2-Rac1 linkers. This sequence of refinement was repeated for a total of 150 cycles. Next, we adopted a series of small moves, global rotamer repacking, as well as backbone minimization within 5 residues of the LOV2-Rac1 linker for high-resolution optimization cycles. After every ten cycles, only rotamers at the interface and next to the LOV2-Rac1 linkers were repacked. A total of 100 such high resolution optimizations were used to generate models, which were further scored using Rosetta's energy function. One thousand models, each representing a different folding trajectory, were generated per construct from simulations using the domain assembly protocol.

The complex structure of Rac3 and the CRIB domain of PAK4 (PBD code 2OV2) was used to model the interaction of CRIB-containing effectors with LOV2-Rac1 constructs. The crystal structure of Rac1[9] (1MH1) was superimposed onto the complex structure by mapping the Cα atoms of Rac1 onto those of Rac3. This derived complex structure was then superimposed onto the LOV2-Rac1 models to create model-CRIB complexes. Side chain rotamers at the interface of each complex were optimized using rotamer repacking[17]. These complexes were scored using the Rosetta energy function. A low-scoring model-CRIB complex indicated the model could bind CRIB, while a high-scoring model-CRIB complex indicated clashes between atoms of the model and the CRIB domain, resulting in reduced binding.

Models generated in a simulation were grouped into clusters according to their pair-wise root mean square deviation (RMSD). The RMSD (in Å) of the Cα atom positions of each model from all other models in the simulation was calculated. Those models falling within a radius of 3 Å RMSD from each other were grouped into a cluster. A cluster member representing the center of each cluster was chosen.

**Isothermal titration calorimetry** Dark and lit state mimetics of PA-Rac1, C450A and I539E, were cloned into a pTriEx vector with an N terminal six Histidine tag. Residues 65-150 of PAK1, comprising the extended CRIB domain, were cloned into a pET23 vector, with a C-terminal 6xHistidine tag. All proteins were expressed in *E.coli* strain BL21(DE3) cells (Stratagene) at 16°C overnight in the dark. Cells were lysed in 50 mM sodium phosphate pH 7.0, 300 mM NaCl, and 5 mM $MgCl_2$. Proteins were purified under yellow light using TALON Metal Affinity Resin (Clontech) and eluted with 150 mM Imidazole at pH 7.0. The proteins were dialyzed against 50 mM sodium phosphate, 150 mM NaCl, 7.15 mM 2-mercaptoethanol, 5 mM $MgCl_2$, and 1% glycerol. Protein concentrations were quantified based on the estimated molar extinction coefficients (280 nm) of the corresponding polypeptides or the reported molar extinction coefficient (447 nm) of LOV2-FMN, and were confirmed with SDS-PAGE followed by Coommassie staining.

ITC experiments were performed by injecting the dark state mutant C450A of PA-Rac1 (0.14 mM) or the lit state mutant I539E (0.13 mM) into the CRIB domain of PAK1 (10 μM) using a Microcal VP-ITC calorimeter at 25$^{o}$C. Each titration consisted of 29 injections of 10 μL of mutants of PA-Rac1. The baseline of each titration was determined and subtracted from all of the data points. Titration data for the heat change per injection were fitted to a one-site binding model using Origin software (OriginLab).

**Cell Culture** HeLa, HEK293 (ATCC) and parental MEF/3T3 Tet-Off cells (Clontech) were maintained in DMEM containing 10% FBS following the supplier's culturing instructions. Stable MEF lines were passaged with addition of 1 ng/ml Doxycyclin, sufficient to suppress protein expression under the Tet-CMV promoter. Doxycyclin was removed 24 hours before live cell

imaging. It was important to control expression level because small amounts of Rac activity from PA-Rac were apparently present prior to irradiation, as evidenced by increased ruffling at high expression levels. This was likely due to the equilibrium amount of active Rac present in the dark state. For constructs tagged with fluorescent proteins, expression level could be roughly approximated as proportional to brightness/unit area, enabling use of cells with similar expression.

**Live cell microscopy** Cells for live cell imaging were seeded on coverslips coated with 5 µg/ml fibronectin in Ham's F-12K medium free of Phenol Red and containing 2% fetal bovine serum (FBS). Coverslips were mounted in an Attofluor live cell chamber (Invitrogen) placed in a microscope stage with a heated stage adapter (Warner). Initial characterization and photoactivation of PA-Rac1, diffusion studies by FRAP and PA-GFP, and protrusion/retraction analyses were carried out using an Olympus FluoView 1000 confocal scanning microscope system equipped with a 60x 1.42 NA oil objective and lasers at 405, 458, 488, 515, 568 and 633 nm. Fluorescence images were acquired using 0.1% power from a 30mW multi-line Ar ion laser (Olympus, minimum power possible without introducing a neutral density filter) and scanned at 2µs/pixel. The illumination used for photoactivation of PA-Rac1 was between 0.1% power for 10 µs and 1% power for 1 ms in a 10-µm spot, at 458 nm. A more precise measure of the light dose used for activation was obtained by measuring the power after the objective using a power meter (Thorlabs), as described below (Fig. S7).

Biosensor images were acquired using an Olympus IX81-ZDC microscope equipped with a CoolSNAP HQ2 14-bit camera (Photometrics) and ET-CFP/YFP filters (Chroma) as described previously[18,19]. Bandpass and neutral density filters were switched using motorized filter wheels

under computer control (Ludl). CFP, FRET and YFP images were acquired using a 100W Hg arc lamp with a 3% ND filter for 500, 250 and 250 ms, respectively. FRET/CFP ratio images were calculated after shading correction, background subtraction, binary masking, and image registration using MetaMorph and MatLab software as described previously[18-20].

Our imaging conditions, exposure times and filters resulted in a donor (ECFP) bleedthrough factor (into the FRET channel) of 0.25 and an acceptor (Citrine) bleedthrough factor of 0.07, using the previously described approach[18-20]. The E-FRET calculation was used as a measure of apparent FRET efficiency of the RhoA biosensor, producing a G factor of 1.57. Photobleaching-corrected FRET efficiency ($E_{corr}$) images were processed based on acceptor photobleaching as previously described[21]. Imaging YFP acceptor fluorescence of the RhoA biosensor was carried out using bandpass filters 500/20 (excitation) and 545/30 (emission).

Simultaneous photoactivation and biosensor imaging was achieved using the FRAP-3D instrument (MAG Systems), an illumination system with galvanometer-driven laser positioning. A laser source at 473 nm was incorporated into the illumination pathway using beam combining mirrors (Chroma, or a 94%/6% Magic Mirror from Olympus).

**Immunocytochemistry** MEF cells expressing mVenus-PA-Rac were plated onto coverslips with etched grids (Bellco) that were coated with fibronectin as described above. The cells were locally irradiated at 473 nm through a 20x phase-contrast objective. Immediately after protrusions were induced, the cells were fixed in 3.7% formalin (Sigma), permeablized in 0.2% Triton X-100, incubated with anti-phospho-PAK antibody (Cell Signaling), and finally incubated with Alexa Fluor 594-conjugated secondary antibody (Molecular Probes). The numbers on the

etched-grid coverslips were used to locate the immunostained cells that had been previously irradiated.

**Measurement of protrusion length** Protrusions were analyzed by drawing up to 8 lines per cell, perpendicular to the cell border and at least 45 degree apart (a few lines were dropped because of cell junctions). More than 14 cells were analyzed for each construct. The line scans were analyzed as kymographs, oriented as shown in Supplementary Fig. S4. The protrusion length was defined as the maximal distance of the membrane from the left hand border of the kymograph after irradiation minus the average distance of the cell border from the left hand edge of the kymograph prior to irradiation.

**FRAP (Fluorescence Recovery After Photobleaching)** MEF cells expressing mVenus-tagged PA-Rac1 were irradiated (515 nm, 10% power for 100 μs) at a 10-μm spot using the Olympus FluoView1000 confocal scanning microscope and laser detailed above. The intensity of mVenus fluorescence was monitored (515 nm, 0.1% power for 2 μs) before and after photobleaching at 2 second intervals. The intensity of fluorescence within the bleached spot was normalized against the integrated intensity of the entire cell. The data were plotted and fitted to a single-exponential decay to obtain t1/2 ($\tau_D$). A t1/2 of 12.1 s and Fi (immobile fraction) of 18.6 ± 0.6% were obtained (n = 26). The diffusion coefficient (D = 0.55 $\mu m^2/s$) was estimated using $D = \omega^2/4\tau_D$ ($\omega$ = the radius of the circular bleached spot), assuming exclusively free lateral diffusion [22,23].

**PA-GFP tracking** MEF cells expressing mPA-GFP-tagged PA-Rac1 were irradiated (405 nm, 6 mW Diode laser, 10% power for 10 μs) in a 10-μm spot to switch on PA-GFP. The fluorescence

of PA-GFP was monitored using a 488 nm laser, acquiring an image every 2 seconds. The intensities of fluorescence within the irradiated spot and an adjacent spot of the same size were quantified and normalized against the entire cell. The decay of activated PA-GFP fluorescence was fitted to a single exponential decay, yielding a t1/2 of 14.6 s.

**Inhibitor studies** MEF cells expressing mVenus-tagged PA-Rac1 were incubated with 1 μM myosin II inhibitor Blebbistatin, 1 μM MLCK inhibitor ML-7, or 10 μM ROCK inhibitor Y-27632 (Calbiochem). Cells underwent the cell shape changes previously described [24] and then reached a stable state within 30 minutes. After this cells were irradiated with the 458 nm laser at a 10-μm spot to induce PA-Rac activation.

**Protrusion/retraction analysis** Fluorescence images of MEF cells expressing mVenus-tagged PA-Rac1 and its mutants were masked based on intensity thresholding to produce binary images. Regions of protrusion were isolated by subtracting the binary image at a given time point from that at time 0. Conversely the binary images of retraction were obtained. Areas not part of protrusions or retractions, those that overlapped the time 0 image, were obtained by subtracting the above two images from that at time 0. Each binary image was assigned a different color: red = protrusion, blue = retraction, green = area overlapping with time 0, and white = background. These operations were carried out using MetaMorph software.

**Polarity index calculation** To obtain the polarity index ($\cos\theta$) of the migrating MEF cells, X and Y coordinates were obtained for the centroid before movement ($x_0$,$y_0$), the centroid after movement ($x_1$,$y_1$), and for the center of the irradiation spot ($x_2$,$y_2$), using MetaMorph software.

The cos and sin values of the angles were obtained using simple triangle calculations. First two angles were defined using an arbitrarily selected horizontal line $\theta_1$ = angle between the arbitrary line and the line from $(x_0,y_0)$ to $(x_1,y_1)$, and $\theta_2$ = angle between the arbitrary line and the line between $(x_0,y_0)$ and $(x_2,y_2)$. The cos used to characterize polarity (see main text and Main Fig 2d) was obtained using the following formula:

$$\cos\theta = \cos(\theta_1-\theta_2) = \cos\theta_1 \cos\theta_2 + \sin\theta_1\sin\theta_2$$