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Optogenetic Control of Protein Kinase Activity in Mammalian Cells

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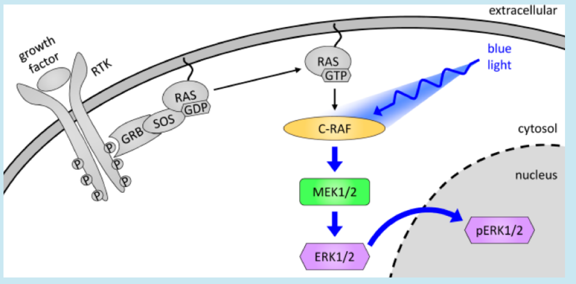
Supporting Information

ABSTRACT: Light-dependent dimerization is the basis for recently developed noninvasive optogenetic tools. Here we present a novel tool combining optogenetics with the control of protein kinase activity to investigate signal transduction pathways. Mediated by *Arabidopsis thaliana* photoreceptor cryptochrome 2, we activated the protein kinase C-RAF by blue light-dependent dimerization, allowing for decoupling from upstream signaling events induced by surface receptors. The activation by light is fast, reversible, and not only time but also dose dependent as monitored by phosphorylation of ERK1/2. Additionally, light-activated C-RAF controls serum response factor-mediated gene expression. Light-induced heterodimerization of C-RAF with a kinase-dead mutant of B-RAF demonstrates the enhancing role of B-RAF as a scaffold for C-RAF activity, which leads to the paradoxical activation of C-RAF found in human cancers. This optogenetic tool enables reversible control of protein kinase activity in signal duration and strength. These properties can help to shed light onto downstream signaling processes of protein kinases in living cells.



KEYWORDS: *optogenetics, RAF, light-controlled protein kinase, cryptochrome 2, dimerization*

Signal transduction pathways are the connection between extracellular stimuli and corresponding cellular responses. Many pathways are intertwined resulting in precise, balanced, dynamic, and sensitive communication networks. Thus, their investigation remains a highly demanding challenge in research. One extensively studied pathway is the mitogen-activated protein kinase (MAPK) pathway, which is involved in regulation of proliferation, differentiation, and changes in cell motility.[1,2] It is stimulated by mitogens, which bind to membrane receptors, leading to activation of the RAF/MEK/ERK protein kinase cascade.[3] Dysregulation of this signaling cascade can be detected in over 30% of human cancers.[4,5]

The RAF family of serine/threonine kinases consists of three known mammalian isoforms (A-, B-, C-RAF).[3] A complex activation mechanism is described for C-RAF, whereas B-RAF is less tightly regulated and frequently mutated in human tumors.[6] In quiescent cells, RAF exists in an autoinhibitory, inactive state in the cytosol stabilized by bivalent binding to the adaptor protein 14−3−3.[7] Upon stimulation by growth factors, receptor tyrosine kinases activate the small G protein RAS (Figure 1a).[1] Active GTP-bound RAS recruits RAF to the plasma membrane inducing a conformational change.[8] Autophosphorylation of its activation loop primes RAF for dimerization and subsequent phosphorylation events by other kinases stabilize the C-RAF side-to-side dimers resulting in full activity.[9−11] RAS-GTP also induces the formation of C-RAF/B-RAF heterodimers showing an elevated kinase activity compared to the respective homodimers.[12] Once activated, RAF proteins phosphorylate and activate MEK1/2, which in turn phosphorylate and activate ERK1/2 (Figure 1a).[2,3] Current methods for further analysis of the intricate regulation mechanisms and role of RAF in signaling networks are limited in respect to time resolution and secondary effects caused by expression of constitutively active kinases, knock-down, or knockout approaches.

Advanced methods have been developed for the control and analysis of protein kinase activities. Biosensors for quantitative investigation of RAF interactions in living cells have offered deeper insights into, for instance, the influences of kinase inhibitors.[13] Moreover, distinct concepts for the realization of chemically inducible kinases have been developed. GyrB domains fused to C-RAF allowed coumermycin mediated homodimerization and induction of the kinase activity.[14] In another approach, various kinases were equipped with an allosteric switch by the insertion of a FKBP12 derivative (iFKBP) into the catalytic domain. Upon rapamycin-induced interaction with FRB (FKBP-rapamycin binding−binding domain), those enzymes could be activated.[15] However, the application of chemical inducers may still cause undesired side effects and is limited in temporal resolution by diffusion and stability of the compound. Recent studies demonstrated that

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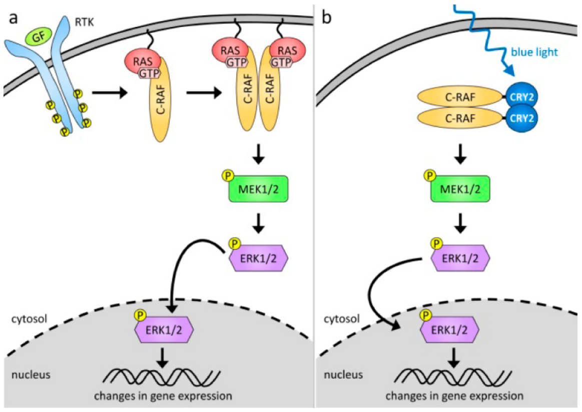

Figure 1. Principle of RAF activation. (a) Growth factor (GF) binding to receptor tyrosine kinase results in GTP-RAS mediated membrane recruitment and dimerization of RAF, thereby inducing its kinase activity. RAF phosphorylates and activates MEK1/2, which in turn phosphorylate and activate ERK1/2. Phosphorylated ERK1/2 (pERK1/2) are able to enter the nucleus, where they phosphorylate several substrates leading to changes in gene expression. (b) Scheme of optogenetically controlled RAF activity. Light-responsive modules such as cryptochtome 2 (CRY2) can be used to induce RAF dimerization and activation on command.

such hurdles can be overcome by the use of light-controlled systems.[16−18] Consequently, we aimed for an optogenetic tool that allows for precise temporal control of protein kinase activity. For this purpose, we capitalized on the photolyase homology domain of *Arabidopsis thaliana* cryptochrome 2 (amino acid 1−498, CRY2). This blue light receptor can either homooligomerize or heterodimerize with the cryptochrome-interacting basic-helix−loop−helix protein CIB1 in its photo-excited state in a reversible manner without the need of exogenous chromophores.[19−21] Both light-induced interaction and dissociation in darkness happen within minutes, making CRY2 a suitable tool for achieving fast and reversible activation of RAF proteins by, for example, mimicking their natural occurring plasma membrane recruitment or dimer formation (Figure 1).

■ RESULTS AND DISCUSSION

Utilizing the photochemical interaction characteristics of CRY2, we designed two general types of RAF-CRY2 fusion constructs. Both approaches are based on known major mechanisms of RAF activation: while one set of constructs was devised to result in blue light-dependent dimerization of RAF monomers, the other set was designed to induce plasma membrane recruitment of RAF.

To achieve CRY2-mediated dimerization of C-RAF, we considered both published principles, namely oligomerization of CRY2 and heterodimerization of CRY2 with the N-terminal part of CIB1 (amino acids 1−170, CIBN).[19,20] As CIBN is a nuclear protein, a version with a mutated, nonfunctional, nuclear localization signal was used (S.W., unpublished data).[22] Upon illumination with 460 nm light, both proteins interact rapidly within seconds and dissociate again in darkness within minutes. Both the CRY2/CRY2 and the CRY2/CIBN interactions were successfully applied in different optogenetic approaches, for example, to photoactivate the *β*-catenin pathway,[20] to regulate transcription,[19] or to facilitate inositol 5-phosphatase membrane recruitment.[23] To determine the most suitable light-responsive system for C-RAF, we initially followed both principles and designed two principal fusion proteins either consisting of C-RAF fused to the N-terminus of CRY2 (C-RAF-CRY2) or of C-RAF-fused to CIBN (C-RAF-CIBN) (Figure 2a).

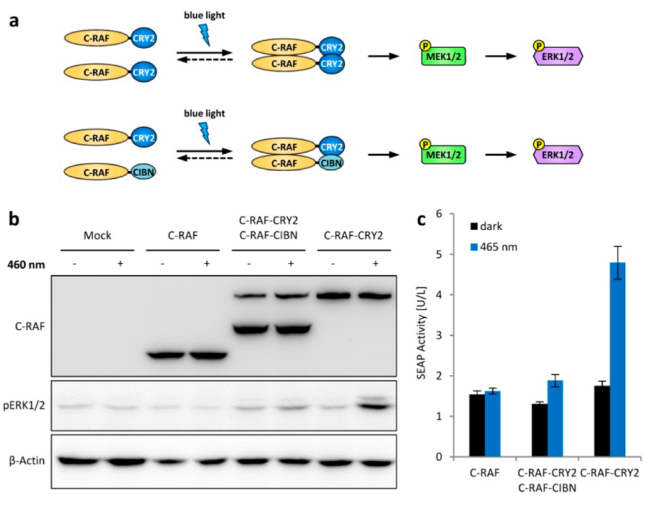

Figure 2. Light-induced activation of C-RAF in mammalian cells. (a) Scheme of the constructs for blue light-dependent activation of C-RAF. (b) ERK1/2 phosphorylation induced by light-activated C-RAF. HEK-293T cells expressing C-RAF, C-RAF-CRY2 alone, or in combination with C-RAF-CIBN, were illuminated with 460 nm light for 5 min (+) or kept in darkness (−). Total cell lysates were analyzed by Western blotting for C-RAF protein expression as well as phosphorylation of ERK1/2 and *β*-actin as loading control. Mock transfected cells served as negative control. (c) Gene expression controlled by light-activated C-RAF. Quantification of long-term, light-induced C-RAF activity. The indicated constructs were cotransfected with the SEAP reporter plasmid pSRE-SEAP into HEK-293T cells. At 24 h post transfection, the culture medium was exchanged with starving medium and the cells were illuminated constantly with 460 nm light (460 nm, blue bars) or were kept in darkness (dark, black bars). Then, 12 h later the SEAP activities were determined.

For evaluation of light-induced C-RAF activation, C-RAF-CRY2 alone or in combination with C-RAF-CIBN was transiently transfected into HEK-239T cells and both approaches were compared in terms of their effects on the downstream MAPK cascade. We assessed the kinase activity by Western blot analysis of phosphorylated ERK1/2 (pERK1/2) under different illumination conditions. Cells expressing the C-RAF-CRY2 construct showed a strong phosphorylation of ERK1/2 when subjected to blue light, whereas in cells coexpressing C-RAF-CRY2 and C-RAF-CIBN only a slight increase in the pERK1/2 signals was detectable (Figure 2b). A possible explanation for the lack of C-RAF activation by the C-RAF-CRY2/C-RAF-CIBN pairing may be deduced from the C-RAF homodimer crystal structure. These C-RAF homodimers show a symmetric side-to-side interaction via the N-lobe of the kinase domains,[24] suggesting that an interaction mediated by two similar-sized CRY2 may work better than dimer formation via CRY2 binding to the much smaller CIBN molecule.

Furthermore, we assessed the effect of the light-regulated C-RAF on downstream gene expression. For this, we monitored serum response element (SRE)-dependent secreted alkaline phosphatase (SEAP) reporter gene expression. SRE-driven transcriptional activation is mediated by the serum response factor, which is activated by stimulated ERK2 phosphorylating the associated ternary complex factor Elk-1.[25] Cells expressing C-RAF-CRY2 showed upon illumination a 2.7-fold increase in the reporter activity compared to a 1.4-fold increase in cells expressing C-RAF-CRY2 and C-RAF-CIBN (Figure 2c).

Next, we examined whether simultaneous CRY2-mediated dimerization and membrane recruitment can further enhance the pERK1/2 signals. Fusion of C-RAF to the K-RAS derived CaaX sequence leads to prenylation of this motif and plasma membrane recruitment of the whole construct, resulting in a constitutively active C-RAF.[26] To render this mechanism light-dependent, a CRY2-mCherry-CaaX fusion protein was coexpressed with C-RAF-CIBN or C-RAF-CRY2 (Supporting Information Figure S1). Simultaneous light-dependent membrane recruitment and homodimerization of C-RAF-CRY2 did not elevate the pERK1/2 levels compared to C-RAF-CRY2 expression alone, with both remaining in the range of the signal resulting from constitutively active C-RAF-CaaX. Blue-light mediated recruitment of C-RAF-CIBN to the membrane did not result in detectable kinase activation, indicating that CRY2/CIBN mediated membrane recruitment of C-RAF was not sufficient to induce C-RAF activity under these conditions.

We then continued with a more detailed characterization of the C-RAF-CRY2 homodimer alone, which not only provided clear activation properties on the MAPK cascade but also offered the advantage of working with a single construct, thereby enabling simple application of our system. The characterization focused on the time and dose responses of C-RAF-CRY2. In a first step, C-RAF-CRY2 expressing cells were illuminated for increasing periods of time ranging from 10 s to 2 h (on kinetics). As presented in Figure 3a, the pERK1/2 signals increased to a maximum after 5 min of illumination.

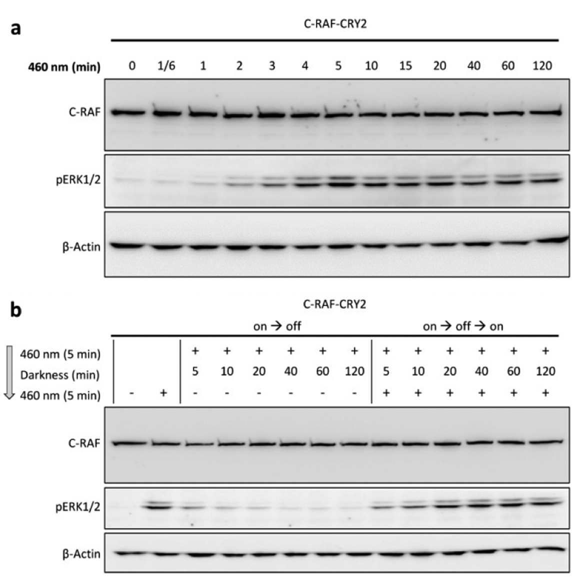

Figure 3. Kinetic analysis of light-controlled C-RAF-CRY2. (a) On kinetics. HEK-293T cells expressing C-RAF-CRY2 were illuminated with 460 nm light for the indicated periods of time. C-RAF-CRY2 protein expression and phosphorylation of ERK1/2 were determined by Western blot analysis of total cell lysates. (b) On−off and on−off−on kinetics. After illuminating HEK-293T cells expressing C-RAF-CRY2 with 460 nm light for 5 min (+) they were incubated in darkness for the indicated periods of time. Afterward, the samples were directly lysed (on−off) or subjected to an additional 5 min illumination step directly after the denoted dark incubation periods (on−off−on) before lysis.

Longer illumination periods did not significantly influence the level of activation and resulted in a sustained signal.

Using the 5-min illumination regime as a basis, we next determined the time needed for inactivation of the light-activated C-RAF (on−off kinetics). Already after 5 min of incubation in the dark, the pERK1/2 signals were strongly reduced and, after 20 to 40 min, completely returned to background levels (Figure 3b). To evaluate the restimulation of the kinase activity, cells were illuminated a second time after dark incubation (on−off−on kinetics; Figure 3b). Phosphorylation of ERK1/2 indicated that C-RAF could be fully reactivated although with a delay of about 20 min. Such a retention of reactivation may be due to pathway inherent inhibitory feedbacks.[27] The restimulation of C-RAF activity after a dark period of up to 2 h indicates that the cells are not impaired by the previous light exposure. Furthermore, the strength of activation could also be controlled by variation of the light intensity used for illumination of the cells (Supporting Information Figure S2). These results suggest that light-dependent C-RAF can be rapidly and strongly activated in a repeated manner and used for short and long-term signaling studies. Moreover, the kinase activity can be fine-tuned by adjusting the light intensity. This flexibility makes this light-controlled protein kinase C-RAF a valuable tool allowing for a detailed investigation of signal transduction dynamics of this pathway and cross-talk within the signaling network.

As B-RAF plays an important role in tumorigenesis, this isoform has become the predominant target of current RAF research. B-RAF has an increased basal kinase activity compared to C-RAF and heterodimerization of B-RAF and C-RAF leads to higher activity compared to the respective homodimers.[12] Even B-RAF mutants impaired in their kinase activity are able to activate the MAPK pathway comparable to wild-type B-RAF by forming heterodimers with C-RAF.[12,28] To address this intriguing aspect of paradoxical C-RAF activation, we adapted our tool to induce a light-dependent heteromerization of C-RAF with a kinase-dead B-RAF mutant (Figure 4a). For this purpose, we fused the kinase-dead mutant B-RAF(K483M) to CRY2 and cotransfected it with C-RAF-CRY2 or C-RAF-CIBN into HEK-293T cells. Coexpression of C-RAF-CIBN and B-RAF(K483M)-CRY2 led to a strong phosphorylation of ERK1/2 upon illumination (Figur 4c). This demonstrates the functionality of the C-RAF-CIBN construct and also indicates another underlying activation mechanism: kinase-dead B-RAF may act as a scaffold that upon illumination facilitates activation of bound C-RAF-CIBN. The light-induced pERK1/2 levels were even higher when coexpressing C-RAF-CRY2 with B-RAF(K483M)-CRY2 (Figures 4b and 4c). The ability to form heterodimers with B-RAF(K483M) clearly elevated the C-RAF activity compared to C-RAF-CRY2 homodimers but also enhanced the background of RAF activity in darkness. We further quantified the pERK2 dependent SEAP reporter expression upon blue light illumination (Figure 4d). Light stimulation of cells coexpressing C-RAF-CIBN and B-RAF(K483M)-CRY2 led to a 2.9 fold increase in reporter activity which was comparable to the results obtained with the C-RAF-CRY2 homodimer. In contrast, blue light mediated C-RAF-CRY2 heterodimerization with B-RAF(K483M)-CRY2 led to a 14.7 fold increase in reporter activity.

To assess if the formation of C-RAF/B-RAF heterodimers showed different activation kinetics compared to those obtained with C-RAF-CRY2 homodimers (Figure 3), we analyzed the C-RAF activity upon increasing times of

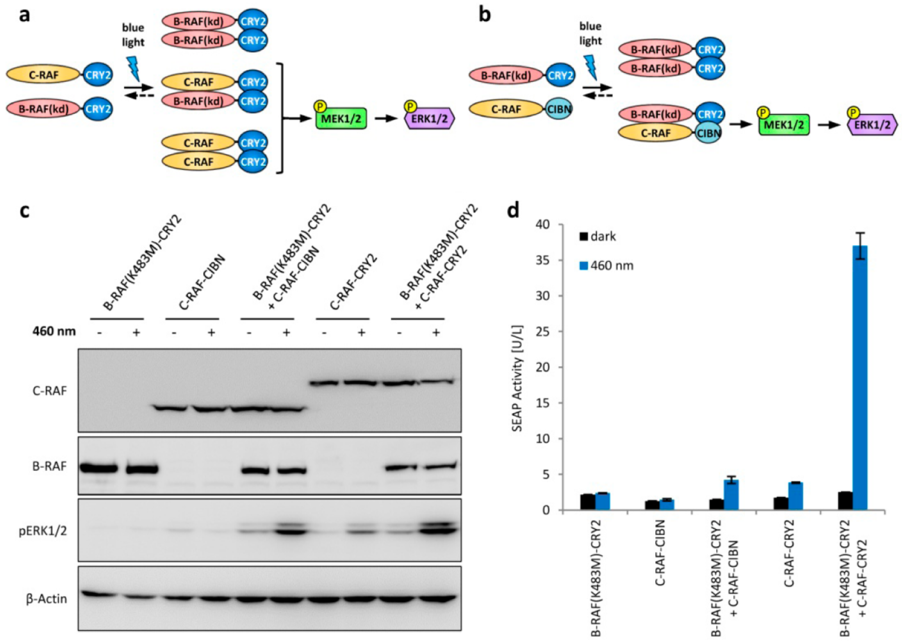

Figure 4. Light-triggered heterodimerization of C-RAF with the kinase-dead B-RAF mutant B-RAF(K483M). (a, b) Overview showing possible combinations of C-RAF and kinase-dead B-RAF(kd) constructs and their stimulation of MEK/ERK. (c) HEK-293T cells expressing the indicated C- and/or kinase-dead B-RAF constructs were illuminated with 460 nm light for 5 min (+) or kept in darkness (−). Total cell lysates were analyzed by Western blotting for C-RAF or B-RAF expression as well as for phosphorylation of ERK1/2. *β*-Actin served as a loading control. (d) SEAP quantification of light-induced B-/C-RAF heterodimer activity. HEK-293T cells were transfected with the SEAP reporter plasmid, pSRE-SEAP, and the indicated B- and C-RAF constructs. Twenty-four hours later, the culture medium was exchanged with starving medium and the cells were illuminated with 460 nm constant light (460 nm, blue bars) or were kept in darkness (dark, black bars) for 12 h, followed by determination SEAP activity.

illumination (Figure 5a). Similar to the C-RAF-CRY2 homodimer, the maximal pERK1/2 signals of both the B-RAF(K483M)-CRY2 heterodimer with C-RAF-CIBN as well as with C-RAF-CRY2 could already be detected after 5 min. Longer illumination led to sustained pERK1/2 signals. Having selected the 5 min illumination regime, we determined the time needed for inactivation (on−off; Figure 5b). Both kinase-dead B-RAF heterodimers showed a decrease in pERK1/2 signals already after 5 min of incubation in the dark. This effect was less pronounced in cells expressing B-RAF(K483M)-CRY2 and C-RAF-CRY2, most likely due to the high signal strength. In both cases, a reduction of pERK1/2 signals to basal levels could be observed after 20 min of dark incubation. To estimate the restimulation of the kinase activity, cells were illuminated a second time after 5 or 60 min of dark incubation (on−off−on). C-RAF activity of cells expressing B-RAF(K483M)-CRY2 and C-RAF-CIBN could be reactivated after 5 as well as after 60 min of dark incubation but seemingly not to full extent compared to initial pERK1/2 signal strength. The restimulation of B-RAF(K483M)-CRY2/C-RAF-CRY2 dimers led to maximal activity regardless of the time incubated in the dark. Thus, this light-controlled system allows for studying the mechanism of the paradoxical C-RAF activation by kinase dead B-RAF that contributes to higher kinase activity of RAF heterodimers, playing an important role in cancer.

In conclusion, we engineered the first optogenetically controlled eukaryotic protein kinase, which can be rapidly and reversibly activated by blue-light illumination and fine-tuned by the applied light intensity. This tool will be of value to study how differences in ERK dynamics decide between cell proliferation and differentiation.[29] Furthermore, this tool may be used to contribute to unraveling the riddle of paradoxical C-RAF activation by impaired or kinase-dead B-RAF. It may also be useful for studying the efficiency of RAF inhibitors for cancer therapy, especially such molecules impeding dimerization. Since this light-induced C-RAF is uncoupled of upstream pathway components, a more precise investigation of C-RAF regulation may be possible. Optogenetic approaches, as shown here for the control of C-RAF, could be applied to other protein kinases, thus providing additional tools for analyzing the complexity of signaling networks.

■ METHODS

Plasmids. All plasmids were generated by introducing PCR amplified DNA fragments into the pEF6/V5-His-TOPO mammalian expression vector (Life Technologies, Carlsbad, CA) using an restriction-enzyme-free isothermal assembly method described elsewhere.[30] All linkers and tags were included into PCR primers (Supporting Information Table S1). C-RAF fragments were amplified from pcDNA3FLAG-Rafwt.[8] The plasmid containing the sequence coding for B-RAF(K483M) was kindly provided by Dr. T. Brummer, University of Freiburg, Germany (unpublished). CRY2 and CIBN were amplified from plasmids of Chandra Tucker's lab (Addgene plasmid no. 26888 and plasmid no. 26889, respectively).[19] CIBN was further mutated (K92,93A) to circumvent accumulation in the nucleus[22] (S.W., unpublished data). The reporter plasmid pGR53 for SRE_6-$pCMV_{min}$ controlled SEAP expression was constructed by annealing the oligonucleotides oGR86 and oGR87 as well as oGR88 and oGR89 and ligating them into pKM006[17] (*Aat*II/*Nhe*I), thereby replacing the $tetO_{13}$-*365bp* sequence.

Antibodies. Polyclonal c-Raf (Cell Signaling Technology, Danvers, MA, cat no. 9422) and Phospho-p44/42 MAPK

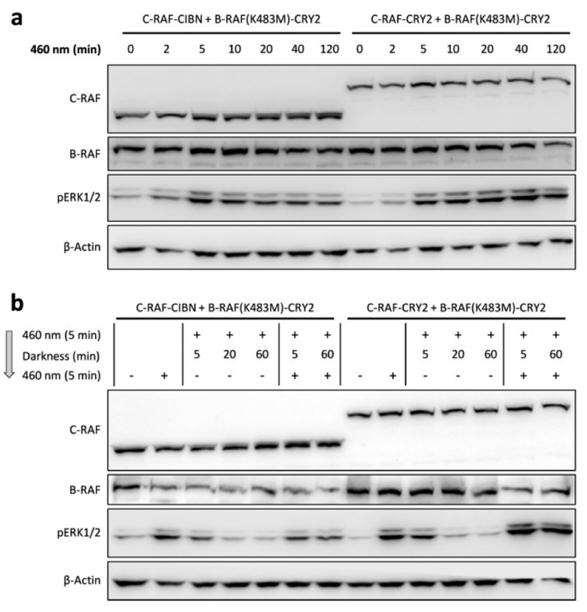

Figure 5. Kinetics of light-induced B-RAF/C-RAF heterodimerization. (a) On kinetics of B-RAF(K483M) cotransfected with C-RAF-CIBN (left) or with C-RAF-CRY2 (right) into HEK-293T cells. Forty-eight hours post transfection, the cells were illuminated with 460 nm light for the indicated time periods. B-RAF and C-RAF expression as well as phosphorylation of ERK1/2 were analyzed by Western blot of total cell lysates. (b) On−off and on−off−on kinetics of light-triggered activation of B-RAF(K483M)/C-RAF-CIBN (left) or B-RAF-(K483M)/C-RAF-CRY2 (right) heterodimers. HEK-293T cells expressing the indicated constructs were illuminated for 5 min (+), followed by an incubation step in darkness for the indicated time periods (5, 20, or 60 min). The samples were either directly lysed (on−off) or illuminated for additional 5 min (on−off−-on) prior to lysis. B-RAF and C-RAF expression as well as pERK1/2 levels were determined by Western blot analysis.

(Erk1/2) (Thr202/Tyr204) (Cell Signaling Technology, cat no. 4370) antibodies were used at a 2500-fold dilution in phosphate buffered saline (PBS) containing 2% (w/v) milk powder. β-Actin antibody (Santa Cruz Biotechnology, Dallas, TX, cat no. sc-47778) was applied as a 1500-fold dilution in 2% milk and monoclonal ANTI-FLAG M2 antibody (Sigma-Aldrich, St. Louis, MO, cat no. F3165) as a 2000-fold dilution in 2% milk.

Cell Culture. Human embryonic kidney HEK-293T cells were maintained in Dulbecco's modified Eagle medium (DMEM, PAN Biotech GmbH, Aidenbach, Germany, cat. No. P03-0710) supplemented with 10% fetal calf serum (FCS, PAN Biotech GmbH, cat. no. 1502, batch P123002) and 1% (v/v) penicillin/streptomycin (PAN Biotech GmbH, cat. no. P06-07100) at 37 °C in a humidified atmosphere containing 5% CO_2. Transfections were conducted by seeding 250 000 cells per well in 6-well plates. Twenty-four hours later, 2.5 μg plasmid DNA and 5 μL of polyethylenimine (PEI, linear, MW = 25 kDa, Polyscience, U.S.A.) (1 mg/mL) per well were assembled in 1/10 cell culture volume of OptiMEM (Life technologies, cat. no. 22600-134), incubated at room temperature for 20 min and then added dropwise to the cells. A fluorescent protein construct with pEF6/V5-His-TOPO plasmid backbone was used for mock transfections. Six hours post transfection, the cell culture medium was exchanged with fresh DMEM to remove PEI. In coexpression experiments, single construct controls were cotransfected with mock plasmid and the plasmids were applied in a ratio of 1:1, except for experiments with B-RAF(K483M)-CRY2, which was cotransfected with a mock plasmid or C-RAF constructs in a ratio of 1:7.

Cell Illumination, Lysis, and Western Blotting. Fourteen hours prior to lysis, cell culture medium was exchanged with starving medium (DMEM containing no FCS). If not indicated otherwise, all illumination experiments were performed 48 h post transfection with self-built light boxes containing 460 nm light-emitting diodes with a radiation angle of 120° of which the light intensities were adjusted to 5 μE m^{-2} s^{-1} using a quantum sensor (LI-250A Light Meter, LI-COR, Lincoln, NE). Subsequent cell lysis was conducted by removing the culture medium from the cells and adding 300 μL of ice-cold lysis buffer (20 mM Tris/-HCl, 100 mM NaCl, 1 mM EDTA, 0.5% Triton X-100, 1% (w/v) SDS, pH 7.5) supplemented with protease (complete protease inhibitor cocktail, Roche, Basel, CH, cat. No. 04693116001) and phosphatase inhibitors (1 mM Na_3VO_4, 10 mM sodium pyrophosphate, 50 mM sodium fluoride, 10 mM β-glycerophosphate). After 10 min of incubation on ice, lysates were sonified 4 times 30 s each and centrifuged at 15 000g and 4 °C for 4 min. Proteins were separated on 9% (w/v) sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gels at 90 V following transfer to PVDF membranes at 350 mA per blot. Membranes were blocked in PBS containing 4% (w/v) milk powder for 1 h at room temperature. Primary antibody incubation was conducted at 4 °C overnight. After three washing steps with PBST (PBS containing 0.05% (v/v) Tween-20), blots were incubated with the corresponding secondary antibody coupled to horseradish peroxidase (HRP) at room temperature for 1 h. Following washing steps with PBST, chemiluminescence was detected after incubation in ECL I (1.25 mM luminol, 0.2 mM coumaric acid, 0.1 M Tris/-HCl, pH 8,5) and ECL II (0.1 M Tris/-HCl, 0.01% H_2O_2, pH 8.5) reagents in a 1:1 ratio.

SEAP Reporter Assay. HEK-293T cells were seeded into 24 well plates at a density of 75 000 cells/well. After 24 h, cells were cotransfected with different RAF-constructs and pERK2 inducible SEAP reporter in a 5:1 ratio (w:w). The medium was exchanged with fresh DMEM after 6 and 18 h later with starving medium and cells were directly incubated under blue light (5 μE m^{-2} s^{-1}) or in darkness for 12 h. The activity of SEAP reporter (pGR53) was quantified using a colorimetric assay in cell culture medium described elsewhere.[31]

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Figure S1. Simultaneous light-induced dimerization and membrane recruitment of C-RAF-CRY2. Figure S2. Light dose dependency of C-RAF-CRY2. Table S1. Primers designed and used for PCR amplification or annealing and assembly of resulting DNA fragments in this work. This material is available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions

‖S.W. and H.J.W. contributed equally to this work. S.W. and H.J.W. designed and performed experiments and wrote the manuscript. K.M. and M.D.Z. designed and analyzed experiments. W.W. and G.R. planned the study and analyzed the data and edited the manuscript.

Notes

The authors declare no competing financial interest.

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