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Spatio-temporally precise activation of engineered RTKs by light

Michael Grusch, Karin Schlech, Robert Riedler, Eva Reichhart, Christopher Differ, Walter Berger, Álvaro Inglés Prieto and Harald Janovjak

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(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

Editor: David del Alamo

1st Editorial Decision 31 January 2014

Thank you once more for the submission of your manuscript to The EMBO Journal and please accept my apologies for the delay in responding due to the recent holiday break. Your study has been sent to two referees, and we have so far received reports from one of them, which I copy below. As this trusted referee is convinced about the interest, novelty and quality of your study, I would like to ask you to begin revising your manuscript according to the his/her comments. Please note that this decision is made in the interest of time, and I will forward you the second report, very likely including further requests, as soon as I receive it.

Without going into all the details that you will find below, the referee is rather positive as I already mentioned. In general, his/her comments can be summarized in two words: more controls. The referee insists on the need to ascertain subcellular localization of the RTKs (at least of FGFR1), determine whether the pattern of phosphorylation of the opto-RTKs resembles that of wild-type receptors, demonstration of actual dimer formation and assessment of potential cross-interactions with wild-type receptors or other signalling pathways, and a better determination of the spatial resolution that can be achieved by light activation.

Keep in mind that, although the referee suggests specific experiments in each case, alternative approaches may be also acceptable. Given the extensive knowledge available on the mechanisms of RTK activation and the tools that have been developed by the community over the years, we

consider that the assays required to deal with these concerns are rather standard in the field. In any case, do not hesitate to contact me by e-mail or on the phone if you have any questions, you need further input or you anticipate any problems during the revision process.

Thank you very much again for the opportunity to consider your work for publication. I look forward to your revision.

REFEREE REPORTS:

Referee #2:

Here the authors have developed light-regulated derivatives of the FGFR1 receptor tyrosine kinase, by fusing a light-oxygen-voltage (LOV) domain to the C-terminus of FGFR1, and anchoring the expressed cytoplasmic domain-LOV chimera to the membrane with an N-terminal myristoylation signal. Light-induced LOV domain dimerization is required for their biological function, and in principle this dimerization response can be used to drive light-induced dimerization of an RTK such as FGFR1. By surveying various LOV domains, they identified the V. frigida LOV domain as a suitable partner for fusion to the C-terminus of the murine FGFR1 cytoplasmic domain. Using an Elk1-luciferase reporter system to assay MAPK activation, they showed that transfected HEK293 cells expressing mFGFR1-VfAU1-LOV (Opto-mFGFR1) had basal MAPK activity similar to control cells, and that MAPK activity was strongly stimulated by exposure to blue light, but not red or green light. By introducing an R195E mutation in the LOV domain to prevent dimerization, they showed that dimerization of Opto-mFGFR1 was required for MAPK activation. They also demonstrated that a long-lived photoadduct between a Cys and the flavin prosthetic group in the LOV domain is required for Opto-mFGFR1 activation by light. They went on to show that the hEGFR and hRET RTKs could be activated by light using a similar LOVE domain fusion strategy, thus demonstrating the generality of this approach. Next, they stably expressed Opto-mFGFR1 in M38K and SPC212 pleural mesothelioma cells, and showed that 1 min blue light exposure led to increased Tyr phosphorylation of Opto-mFGFR1 and stimulation of ERK, AKT, and PLCg1 phosphorylation, as measured by blotting with phosphospecific antibodies. Similar results were obtained in hBE endothelial cells. Blue light exposure of small areas of SPC212 or hBE cells in a monolayer of cells led to local activation of ERK as determined by IF with phosphospecific ERK antibodies. Next, they showed that 1 hr light activation of Opto-mFGFR1 in MK38 cells caused a small increase in DNA synthesis rate 24 hr later, as measured by EdU incorporation, and also a change in cell morphology. Finally, they showed that stimulation of Opto-mFGFR1-hBE cells caused increased sprouting in a manner similar to VEGFA.

The use of light-induced LOV domain dimerization of RTK-LOV chimeras to activate RTK signalling is a clever new approach to temporal and spatial activation of RTK signalling in cells. The evidence that blue light-activated FGFR1 signalling through induced dimerization leads to both immediate and delayed downstream responses is quire convincing. However, there are a number of major issues.

1. Where is Opto-mFGFR1 located in the expressing cells? The myristoylation signal can localize proteins to internal cytoplasmic membranes as well as to the plasma membrane. The authors need to carry IF studies to determine where Opto-mFGFR1 is localized (i.e. what fraction of the population is on internal membranes?); this analysis could include staining with antibodies against FGFR1 pY sites to determine whether the internal pool of Opto-mFGFR1 is stimulated upon light treatment, and could therefore play a role in MAPK pathway stimulation. Such studies would also determine whether Opto-mFGFR1 moves upon light stimulation, e.g. is there internalization in response to activation. In this connection, they should also determine whether Opto-mFGFR1 is downregulated in response to activation. This analysis could also be extended to assess whether Opto-mFGFR1 is ubiquitylated upon light activation.

2. They need to characterize which Tyr residues are phosphorylated in Opto-mFGFR1 in response to light, and also determine whether there are Tyr residues in the LOV domain itself that are phosphorylated in response to light activation. In this regard, they ought to make and test Tyr to Phe mutants of known FGFR1 autophosphorylation site residues that participate in signalling to establish

that downstream signalling to ERK and AKT occurs in the same way as with WT FGFR1 when stimulated with FGF. They should also test whether there is light-induced association of signalling proteins, such as PLCg1, with Opto-mFGFR1, and it would also be important to check tyrosine phosphorylation (and association) of FRS2.

3. While the evidence that LOV domain dimerization is required for Opto-mFGFR1 stimulation is reasonable, some physical evidence that Opto-mFGFR1 molecules dimerize upon blue light treatment would be reassuring, e.g. through the use of chemical crosslinking. In this regard, they need to test whether Opto-mFGFR1 interacts with endogenous FGFRs (HEK293 and CHO cells express FGFR1 and other FGFRs), in order to test whether some of the signalling responses that are observed might be due to transactivation of endogenous FGFRs (do endogenous FGFR molecules become tyrosine phosphorylated in response to light in Opto-mFGFR1-expressing cells?

4. In terms of how efficient of Opto-mFGFR1 induced signalling is compared to signalling through native FGFR1, the authors need to estimate how many copies of Opto-FGFR1 are expressed in the stably transfected cell lines and compare this to the typical number of endogenous FGFRs that can induce cellular responses.

5. The Elk1/luciferase reporter system can respond to activation of MAPKs other than ERK, e.g. JNK, and they need to check whether JNK and p38 are activated with blue light in Opto-FGFR1-expressing cells to establish whether what they are monitoring with the PathDetect Elk1 trans-Reporting System is activation of ERK or of other MAPKs. In this regard the authors should establish that the blue light treatment is not inducing a DNA damage response, which can activate p38 MAPK.

6. The authors describe their method as providing spatial precision, but in signalling circles this would be taken to mean spatial resolution at the single cell level, and ultimately it will be important to test whether the Opto-mFGFR1 system can be used to activate responses in single cells, and whether exposure of specific regions of a single cell can activate responses.

Points: 1. Page 5 and Figure 2: The authors need to state at the outset how they measured MAPK activation in these cells, because otherwise the reader is left mystified why MAPK activation is measured in RLU! In these initial experiments the authors could strengthen their case for light-induced activation of Opto-mFGFR1 by showing parallel immunoblotting with anti-pFGFR1 and anti-pERK antibodies. The use of a FRET-based ERK activity biosensor could also be considered (c.f. Albeck et al. Mol Cell 49:249).

2. In general, the figure legends need significantly more detail. In particular, the legends (and text) need to indicate how long light treatment was for in different experiments, and when the MAPK activity reporter was assayed.

3. Figure 4A and B: The phospho-Opto-mFGFR1 signal induced by 1 min light treatment in M38K cells was weaker than that in SPC212 cells, and yet the pERK signal was stronger in M38K cells. This deserves discussion. PLCg1 phosphorylation and pERK/pAKT activation in SPC212 cells was extremely transient when the light signal was turned off, whereas in M38K cells pERK was sustained for longer. What does this say about the wiring of FGFR signalling pathways in these two cell types? Is there a similar difference when these cells are stimulated with FGF2?

4. Figure 4D-F: A better description of the light patterns used to stimulate these monolayers is required.

5. With regard to dynamics of ERK activation and downstream responses, the authors could refer to Albeck et al. (op. cit.), and also a very recent paper by Toettcher et al. (Cell 155:1422).

1st Revision - authors' response 03 May 2014

Referee #2:

Here the authors have developed light-regulated derivatives of the FGFR1 receptor tyrosine kinase, by fusing a light-oxygen-voltage (LOV) domain to the C-terminus of FGFR1, and anchoring the expressed cytoplasmic domain-LOV chimera to the membrane with an N-terminal myristoylation signal. Light-induced LOV domain dimerization is required for their biological function, and in principle this dimerization response can be used to drive light-induced dimerization of an RTK such as FGFR1. By surveying various LOV domains, they identified the V. frigida LOV domain as a suitable partner for fusion to the C-terminus of the murine FGFR1 cytoplasmic domain. Using an Elk1-luciferase reporter system to assay MAPK activation, they showed that transfected HEK293 cells expressing mFGFR1-VfAU1-LOV (Opto-mFGFR1) had basal MAPK activity similar to control cells, and that MAPK activity was strongly stimulated by exposure to blue light, but not red or green light.

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Next, they stably expressed Opto-mFGFR1 in M38K and SPC212 pleural mesothelioma cells, and showed that 1 min blue light exposure led to increased Tyr phosphorylation of Opto-mFGFR1 and stimulation of ERK, AKT, and PLCg1 phosphorylation, as measured by blotting with phosphospecific antibodies. Similar results were obtained in hBE endothelial cells. Blue light exposure of small areas of SPC212 or hBE cells in a monolayer of cells led to local activation of ERK as determined by IF with phosphospecific ERK antibodies. Next, they showed that 1 hr light activation of Opto-mFGFR1 in MK38 cells caused a small increase in DNA synthesis rate 24 hr later, as measured by EdU incorporation, and also a change in cell morphology. Finally, they showed that stimulation of Opto-mFGFR1-hBE cells caused increased sprouting in a manner similar to VEGFA.

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Authors:

We thank the reviewer for her/his excellent and constructive comments.

The manuscript was revised significantly following the suggestions of the reviewer. All suggestions were included in the manuscript along with new data in seven new supplementary figures and four additional figures.

Below, we have reproduced the comments of the reviewer and our specific responses follow.

Referee #2:

1. Where is Opto-mFGFR1 located in the expressing cells? The myristoylation signal can localize proteins to internal cytoplasmic membranes as well as to the plasma membrane. The authors need to carry IF studies to determine where Opto-mFGFR1 is localized (i.e. what fraction of the population is on internal membranes?); this analysis could include staining with antibodies against FGFR1 pY sites to determine whether the internal pool of Opto-mFGFR1 is stimulated upon light treatment, and could therefore play a role in MAPK pathway stimulation. Such studies would also determine whether Opto-mFGFR1 moves upon light stimulation, e.g. is there internalization in response to activation. In this connection, they should also determine whether Opto-mFGFR1 is

downregulated in response to activation. This analysis could also be extended to assess whether Opto-mFGFR1 is ubiquitylated upon light activation.

Authors:

As requested by the reviewer, we have performed immunofluorescence microscopy to localize light-activated receptors (Opto-mFGFR1, Opto-hEGFR and Opto-hRET). In several cell types, we found that a majority of receptors localizes to the cell membrane and a smaller fraction to internal membranes with no receptors in the nucleus (Supplementary Figure S10). This result is in agreement with previous work on localization of FGFR1 and other FGFRs/RTKs in mammalian cell lines (Johnston et al, 1995; Persaud et al, 2011; Wheldon et al, 2010).

Due to the transparency of single mammalian cells, we expect that all receptors are activated in these experiments. Activation with light is in these experiments thus similar to activation of engineered RTKs by cell permeable synthetic ligands, which have been applied in systems ranging from cultured cells to animal models (Kwiatkowski et al, 2008; Welm et al, 2002). However, only light offers the possibility to address subcellular compartments, and we already performed experiments where light is directed at selected regions of cells (please see below for subcellular recruitment of PLCγ1).

Sites that were previously implicated in FGFR1 internalization are preserved in Opto-mFGFR1 (e.g. the entire C-terminus, Tyr766 and non-pTyr motifs) (Persaud et al, 2011; Sorokin et al, 1994), and we therefore expect internalization also of Opto-mFGFR1 and likewise the other Opto-RTKs. This point is now discussed in the revised manuscript (please refer to page 6).

Binding of fluorescent, biotinylated or radio-labelled FGFs represent common and very powerful methods for measuring FGFR internalization and degradation (Persaud et al, 2011; Sorokin et al, 1994). As these methods were not available to us because of the absence of ligand binding in light-activated receptors, we established a complementary fluorescence-based assay to measure internalization/degradation. It is not advisable to incorporate fluorescent proteins in proximity to LOV domains because of potential Förster resonance energy transfer (FRET) between these domains (as e.g. utilized in the oxygen sensor developed by Drepper, Jaeger and co-workers) (Potzkei et al, 2012). We therefore first created a modified Opto-mFGFR1 receptor with an extracellular fluorescent protein tag. MAPK/ERK pathway activation measurements in HEK293 cells indicate that this receptor, which incorporates a transmembrane helix rather than a MYR domain, produces similar light activation of cellular signalling compared to the original Opto-mFGFR1 (Reviewer Figure 1). We then performed time course measurements of fluorescence to detect receptor trafficking into compartments with reduced pH and receptor degradation. We observed a reduction in total fluorescence for light stimulated but not for unstimulated cells. This decrease occurred on a time scale that is similar to that published previously for FGFR1 internalization/degradation (e.g. compare Reviewer Figure 1C to Figure 4 and 5 of Sorokin et al. or Figure 6B of Haugsten et al.) (Haugsten et al, 2005; Sorokin et al, 1994). In line with this result, our original immunoblot data also revealed a small but distinct decrease of total Opto-mFGFR1 upon light stimulation (Figure 4A and C). While this new data clearly indicates receptor internalization/degradation, we have chosen to include these findings in a reviewer figure as the further development of the modified receptor and the measurement method should be pursued in future studies.

Referee #2:

2. They need to characterize which Tyr residues are phosphorylated in Opto-mFGFR1 in response to light, and also determine whether there are Tyr residues in the LOV domain itself that are phosphorylated in response to light activation. In this regard, they ought to make and test Tyr to Phe mutants of known FGFR1 autophosphorylation site residues that participate in signalling to establish that downstream signalling to ERK and AKT occurs in the same way as with WT FGFR1 when stimulated with FGF. They should also test whether there is light-induced association of signalling proteins, such as PLCg1, with Opto-mFGFR1, and it would also be important to check tyrosine phosphorylation (and association) of FRS2.

Authors:

As requested by the reviewer, we performed additional experiments to characterize phosphorylation and downstream signalling of Opto-mFGFR1.

Using Tyr-Phe/Ser substitutions at known FGFR1 phosphorylation sites, we found that Y271 & Y272 (Y653 & Y654) are required for activation of the MAPK/ERK pathway with light (Figure 2C), while Y81 (Y463), Y201 (Y583), Y203 (Y585), Y348 (Y730) and Y384 (Y766) are dispensable (Supplementary Figure S6; residue numbers correspond to Opto-mFGFR1 while residues in parentheses indicate the corresponding positions in FGFR1). This result is in agreement with the known phosphorylation of FGFR1 (Mohammadi *et al*, 1996).

Also, as requested by the reviewer, we performed additional experiments to test whether a functionally-relevant phosphorylation site exists in VfAU1-LOV. Y447 is the sole surface accessible Tyr in VfAU1-LOV and substitution does not affect activation of the MAPK/ERK pathway with light (Supplementary Figure S6).

We complement our original data from mesothelioma cells (Figure 4) by demonstrating phosphorylation of PLCγ1 and also FRS2 in HEK293 cells (Supplementary Figure S1).

In the original and new data (Figure 4 and Supplementary Figure 1), we observed phosphorylation of ERK and AKT for which the adapter protein FRS2 is required. As requested, we performed additional experiments to test for association of FRS2 with Opto-mFGFR1. Following the work of Ong and co-workers (Ong *et al*, 2000), we introduced Ala substitutions in the FGFR1 juxtamembrane region, which contains the binding site of FRS2. As expected, these Ala substitutions result in the loss of MAPK/ERK pathway activation with light (Supplementary Figure S6).

Finally, we performed live cell fluorescence microscopy following Wang and co-workers (Wang *et al*, 2001) to demonstrate membrane recruitment of PLCγ1 upon light activation of Opto-mFGFR1 (Supplementary Figure S7).

Referee #2:

3. While the evidence that LOV domain dimerization is required for Opto-mFGFR1 stimulation is reasonable, some physical evidence that Opto-mFGFR1 molecules dimerize upon blue light treatment would be reassuring, e.g. through the use of chemical crosslinking. In this regard, they need to test whether Opto-mFGFR1 interacts with endogenous FGFRs (HEK293 and CHO cells express FGFR1 and other FGFRs), in order to test whether some of the signalling responses that are observed might be due to transactivation of endogenous FGFRs (do endogenous FGFR molecules become tyrosine phosphorylated in response to light in Opto-mFGFR1-expressing cells?

Authors:

As requested by the reviewer, we performed chemical crosslinking experiments to demonstrate light-induced association of Opto-mFGFR (Supplementary Figure S3). This new data supports our original data that was based on a charge inversion substitution in the Opto-mFGFR1 kinase domain.

Also as requested by the reviewer, we performed additional experiments to test for *trans*-activation of endogenous receptors. Immunoblot data of HEK293 cells that exhibit significant expression of FGFR1 showed no *trans*-activation of the endogenous receptor in response to light stimulation of Opto-mFGFR1 (Reviewer Figure 2). In addition, our original data on cell behaviour (Figure 6) indicate that Opto-mFGFR1 expression does not modulate responses of several cell types to growth factors.

Referee #2:

4. In terms of how efficient of Opto-mFGFR1 induced signalling is compared to signalling through native FGFR1, the authors need to estimate how many copies of Opto-FGFR1 are expressed in the stably transfected cell lines and compare this to the typical number of endogenous FGFRs that can induce cellular responses.

Authors:

As requested by the reviewer, we performed additional experiments to quantify expression levels in stably transfected cells.

Using immunoblotting, we detected similar amounts of Opto-mFGFR1 and endogenous FGFR1 in stably transfected HEK293 cells (Reviewer Figure 2).

Using qPCR, we found Opto-mFGFR1 transcripts at levels of 21- to 47-fold compared to endogenous receptor in stably transfected M38K, SPC212 and hBE cells (Supplementary Figure S9).

Thus, in these cellular models, which are the first ever to have been generated with light-activated RTKs, expression levels of the light-activated receptors are comparable to those of endogenous receptors.

In addition, our original data on cell behaviour (Figure 6) and our data on receptor *trans*-activation (Reviewer Figure 2) indicate that Opto-mFGFR1 expression does not modulate responses of these cells to growth factors.

Referee #2:

5. The Elk1/luciferase reporter system can respond to activation of MAPKs other than ERK, e.g. JNK, and they need to check whether JNK and p38 are activated with blue light in Opto-FGFR1-expressing cells to establish whether what they are monitoring with the PathDetect Elk1 trans-Reporting System is activation of ERK or of other MAPKs. In this regard the authors should establish that the blue light treatment is not inducing a DNA damage response, which can activate p38 MAPK.

Authors:

As requested by the reviewer, we performed additional experiments to test if the commercially-available luciferase reporter system monitors activation of ERK or other MAPKs.

Using an inhibitor of MEK, we demonstrate that activation of the MAPK/ERK pathway is required for reporter activation by Opto-mFGFR1 (Supplementary Figure S5).

Using immunoblotting, we show light-induced phosphorylation of ERK in experiments in the same cellular system that we used for the reporter assay (please see below and Supplementary Figure 1).

Finally, also using immunoblotting, we did not detect light-induced phosphorylation of p38 (Reviewer Figure 3).

As requested by the reviewer, we now also highlight the original data that allow to exclude the potential activation of the reporter as a result of light-induced toxicity (e.g. *via* DNA damage or any other pathway). These experiments show that blue light alone is not sufficient for reporter activation in control cells and that expression of a functional Opto-mFGFR1 is required for reporter activation with light (Figure 2C).

Collectively, we have identified the pathway responsible for reporter activation and excluded unspecific or toxic effects of light stimulation. This is now discussed in the revised manuscript (please refer to pages 5 and 6 of the revised manuscript).

Referee #2:

6. The authors describe their method as providing spatial precision, but in signalling circles this would be taken to mean spatial resolution at the single cell level, and ultimately it will be important to test whether the Opto-mFGFR1 system can be used to activate responses in single cells, and whether exposure of specific regions of a single cell can activate responses.

Authors:

We have performed the experiment suggested by the reviewer and tested whether activation can be confined to single cells or even subcellular regions.

Using confocal microscopy, we achieved activation of Opto-mFGFR1 in subcellular regions of HEK293 cells as detected using PLCγ1 recruitment (Reviewer Figure 4 and also please see above).

While this result demonstrates a level of spatial resolution of receptor activation that can likely only be achieved using light stimulation, this exciting new type of experiment suggested by the reviewer calls for additional work that should be pursued in future studies.

Referee #2:

Points:

1. Page 5 and Figure 2: The authors need to state at the outset how they measured MAPK activation in these cells, because otherwise the reader is left mystified why MAPK activation is measured in RLU! In these initial experiments the authors could strengthen their case for light-induced activation of Opto-mFGFR1 by showing parallel immunoblotting with anti-pFGFR1 and anti-pERK antibodies. The use of a FRET-based ERK activity biosensor could also be considered (c.f. Albeck et al. Mol Cell 49:249).

Authors:

We have revised the manuscript following the suggestion of the reviewer.

We now clearly state the reasons for our choice of the multi-well plate luciferase assay and we explain that results are obtained as RLU (please refer to pages 4 and 5 of the revised manuscript).

We also performed additional experiments and provide immunoblot data conducted in the same cell model as the luciferase reporter assay. As expected, this data reveals light-induced phosphorylation of Opto-FGFR1 and of the key intracellular signalling molecules mentioned by the reviewer (Supplementary Figure 1 and Reviewer Figure 2; also please see above).

Collectively, the revised manuscript now contains independent measurements of receptor activation and pathway activation with light in several cell types.

The application of the suggested recent and very exciting FRET-sensors will be pursued in future studies.

Referee #2:

2. In general, the figure legends need significantly more detail. In particular, the legends (and text) need to indicate how long light treatment was for in different experiments, and when the MAPK activity reporter was assayed.

Authors:

We have revised the figure legends following the suggestion of the reviewer. We added incubation times and light stimulation times to all legends that did not already contain this information (legend to Figure 2B and C, Figure 3A to D, Figure 4D to F, Figure 5C, Figure 6A, B and D; please refer to pages 24 to 26 of the revised manuscript).

3. Figure 4A and B: The phospho-Opto-mFGFR1 signal induced by 1 min light treatment in M38K cells was weaker than that in SPC212 cells, and yet the pERK signal was stronger in M38K cells. This deserves discussion. PLCg1 phosphorylation and pERK/pAKT activation in SPC212 cells was extremely transient when the light signal was turned off, whereas in M38K cells pERK was sustained for longer. What does this say about the wiring of FGFR signalling pathways in these two cell types? Is there a similar difference when these cells are stimulated with FGF2?

Authors:

We are currently investigating cellular responses to FGF2 and to FGFR inhibitors in a panel of mesothelioma cell lines. Indeed responses to both stimulation and inhibition of FGFR1 can be quite diverse in mesothelioma cells and likely reflect different expression and/or activity levels of adaptor proteins or feedback mechanisms. The systematic evaluation that is required to understand these effects can best be pursued in future studies. To revise the manuscript following the suggestion of the reviewer, this point is now mentioned on page 8 of the revised manuscript.

Referee #2:

4. Figure 4D-F: A better description of the light patterns used to stimulate these monolayers is required.

Authors:

We have revised Figure 4D and F following the suggestion of the reviewer. We added visual cues to the figure and extended the figure legend. We also moved this data to a separate figure (Figure 5) so that it may appear bigger in the final manuscript.

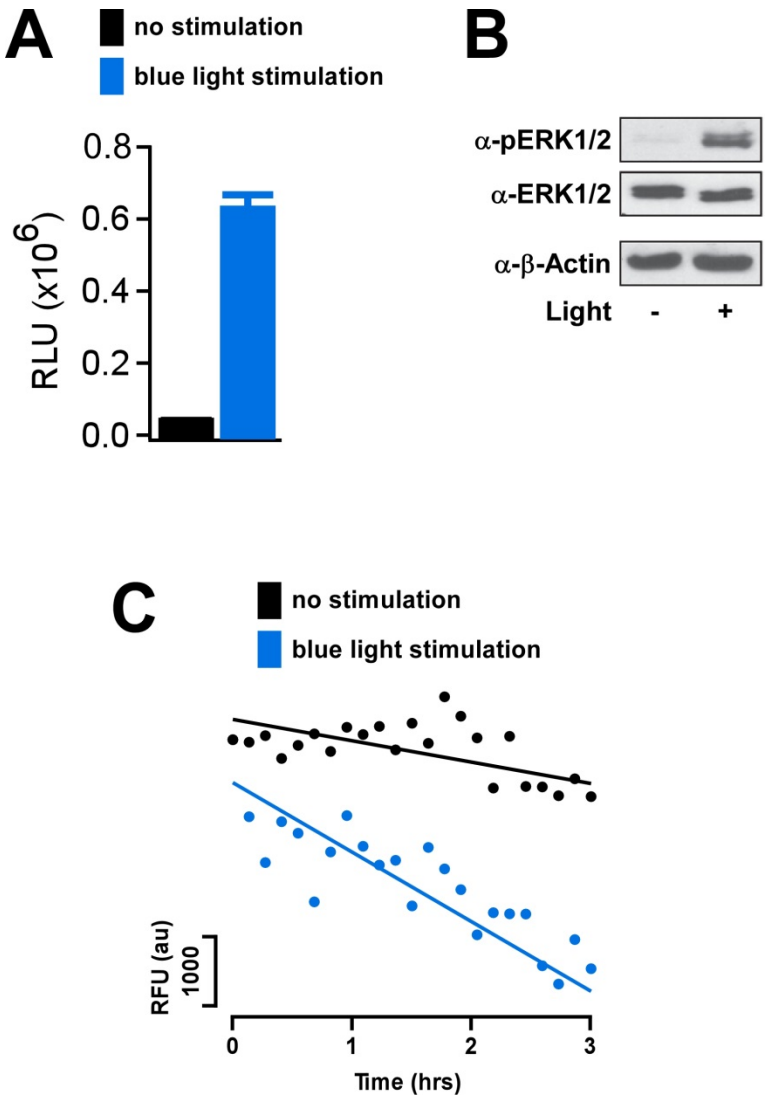
Referee #2:

5. With regard to dynamics of ERK activation and downstream responses, the authors could refer to Albeck et al. (op. cit.), and also a very recent paper by Toettcher et al. (Cell 155:1422).

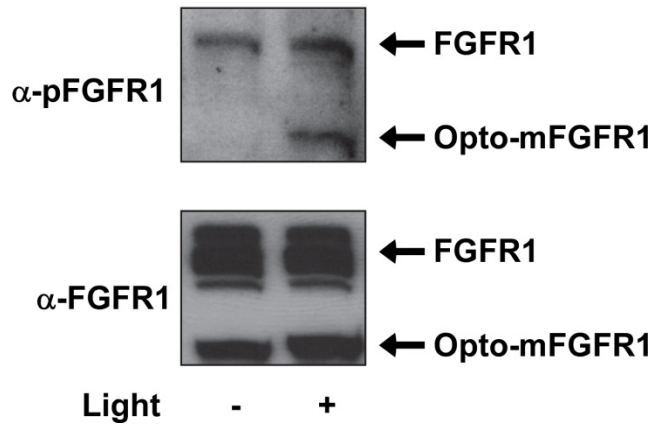
Authors:

Following the suggestion of the reviewer, the recent works of Albeck *et al.* and Toettcher *et al.* are now cited in the manuscript (please refer to page 4 of the revised manuscript).

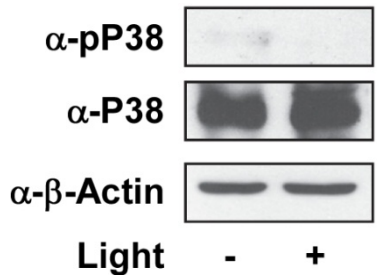
Reviewer Figures



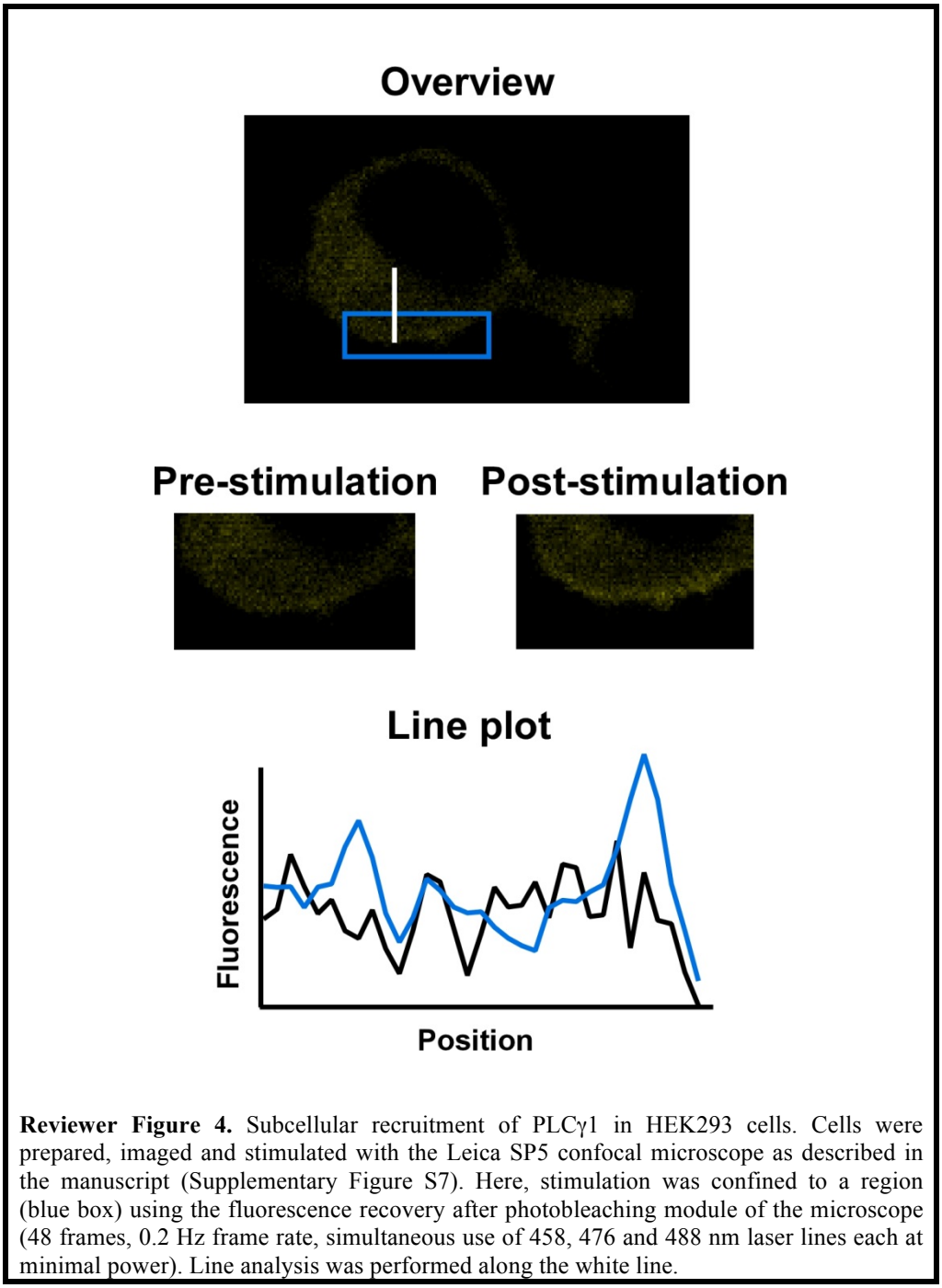
Reviewer Figure 1. Fluorescence measurements of receptor internalization/degradation. (A and B). We created a modified Opto-mFGFR1 receptor with an extracellular fluorescent protein tag (mVenus) by including the transmembrane and extracellular domain of p75. These domains were previously utilized in the creation of engineered receptor tyrosine kinases (Muthuswamy *et al*, 1999). mVenus fluorescence can be detected by excitation with green light (~515 nm) that does not activate LOV domains (compare to Supplementary Figure S8, inset). In HEK293 transfected with this receptor, we observed activation of the MAPK/ERK reporter and phosphorylation of ERK that is comparable to that of Opto-mFGFR1. (C) We measured cellular fluorescence over the period of several hours using a microplate reader at 37°C. Cells that were stimulated with blue light for 15 min prior to imaging show a marked decrease in total fluorescence. This decrease is on a similar time scale as observed previously for FGFR1 degradation in response to FGF stimulation (Sorokin *et al*, 1994). In contrast, unstimulated cells show a minor decrease similar to that observed previously for FGFR1 degradation in unstimulated cells or potentially caused by mVenus bleaching during fluorescence measurements.



Reviewer Figure 2. Phosphorylation of endogenous FGFR1 and Opto-mFGFR1 in HEK293 cells stably transfected with Opto-mFGFR1. Cells were stimulated with blue light for 5 min. Light intensity was ~2.5 μW/mm^2. Comparable levels of expression are observed for endogenous FGFR1 and Opto-mFGFR1. Increased phosphorylation of Opto-mFGFR1 but not endogenous FGFR1 is observed in response to light.



Reviewer Figure 3. Absence of phosphorylation of P38 in HEK293 cells (for experimental procedures, please see Supplementary Figure S1). Cells were stimulated with blue light for 5 min. Light intensity was ~2.5 μW/mm^2.



Reviewer Figure 4. Subcellular recruitment of PLCγ1 in HEK293 cells. Cells were prepared, imaged and stimulated with the Leica SP5 confocal microscope as described in the manuscript (Supplementary Figure S7). Here, stimulation was confined to a region (blue box) using the fluorescence recovery after photobleaching module of the microscope (48 frames, 0.2 Hz frame rate, simultaneous use of 458, 476 and 488 nm laser lines each at minimal power). Line analysis was performed along the white line.

References

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2nd Editorial Decision 19 May 2014

Thank you for the submission of your revised manuscript to The EMBO Journal and please accept my apologies again for the unexpected delay in responding. Thank you for your patience.

Your study was sent back to the referee, who now believes that all major concerns have been properly addressed and your manuscript is almost ready for publication (see below). Only a few minor issues will still require your attention, mostly related to the presentation and discussion of your data, but no further experimental evidence is required. I find particularly important the idea of including key 'supplementary' or 'reviewer' experiments into the main text. Keep in mind that supplementary information or the 'Review process file' are not documents accessed as frequently as the main text of the paper, and readers might be missing crucial information.

Browsing through the manuscript myself I have also noticed a few small issues with data presentation. Micrographs throughout the manuscript (Figs. 6, S7, S13) lack scale bars, which we require for clarity. Furthermore, the statistical analysis of the results requires a more detailed description. As a guide, statistical analyses must be described either in the Materials and Methods section or in the legend of the figure to which they apply and will include a definition of the error bars used and the number of independent experiments performed. The statistical significance analysis tool used, if any, must be also clearly stated.

Once these minor issues have been solved, I will be glad to accept your manuscript for publication in The EMBO Journal.

Every paper now includes a 'Synopsis', displayed on the html and freely accessible to all readers. The synopsis includes an image, normally provided as a model/summary figure by the authors or

cropped by us from one of the final figures of the manuscript, as well as 2-5 one-short-sentence bullet points that summarize the article. I would appreciate if you could provide these bullet points. If you are also interested in providing a small figure summarizing your technique, please keep in mind that final dimensions should be 550 pixels wide X 150-400 pixels (variable) tall.

I would also like to mention that we now encourage the publication of source data, particularly for electrophoretic gels and blots but also for numerical data in graphs, with the aim of making primary data more accessible and transparent to the reader. Although optional at the moment, would you be willing to provide a PDF file per figure that contains the original, uncropped and unprocessed scans of all or key gels used in the figures? The PDF files should be labelled with the appropriate figure/panel number, and should have molecular weight markers; further annotation could be useful but is not essential. Raw numerical data for graphs can be provided as Excel (or related) tables. The files will be published online with the article as supplementary "Source Data" files.

If you have any questions regarding this initiative or any other part of the publication process, please let me know.

Thank you very much again for your patience. I am looking forward to seeing the final version of your manuscript.

REFEREE REPORT:

Referee comment:

To address the reviewers' comments, the authors have added a significant number of new experiments confirming the utility and signal pathway specificity of light-induced Opto-mFGFR1 signalling, including the use of Opto-mFGFR1's mutated at individual tyrosines known to be autophosphorylated and involved in signalling downstream of FGFR1. However, the large amount of supplementary data has been only briefly described in the main text. As a consequence it is not always exactly clear what was done, a fuller description of these experiments in the main text and legends is needed.

1. In response to a request that the authors cite the recent Toettcher et al. paper that uses light-activated ERK MAPK pathway signalling, the authors have simply added a sentence on page 4 mentioning these papers in totally inappropriate context. Since the authors' paper will appear more than six months after the Toettcher et al. paper, the authors need to include in the Discussion section a proper discussion that compares and contrasts their method for light activation of FGFR signalling leading to ERK MAP kinase activation with the method described by Toettcher et al., which activates the ERK MAPK pathway at the level of Sos recruitment to the membrane, and indicates the advantages of their approach.

2. The new fluorescence data in Reviewer Figure 1 showing that light activation causes slow internalization of plasma membrane associated Opto-mFGFR1 are reasonable, but it is unclear why the authors did not simply use IF imaging to determine whether light activation causes Opto-mFGFR1 to move from a plasma membrane localization, which they demonstrated in Figure S10, to a more intracellular localization. Was this attempted?

3. The requests for a demonstration of which Tyr in Opto-mFGFR1 are phosphorylated and that FRS2 associates with Opto-mFGFR1 in response to light have been addressed with mutation analysis, which demonstrates that specific Tyr are required for ERK activation and that the JMD region required for FRS2 binding is needed. However, this is not exactly the same as blotting with site-specific anti-pTyr antibodies or testing FRS2 binding by coprecipitation. However, new experiments are not needed.

4. In Reviewer Figure 2, the authors have used immunoblotting to show that stable Opto-mFGFR1-expressing 293 cells contained a level of Opto-mFGFR1 protein approximately equal to that of endogenous FGFR1, and in the main text on page 7 they state that the levels of Opto-mFGFR1 were comparable to endogenous FGFR based on the RNA data in Figure S9. However, from the ordinate

scale in that figure it looks as though the level of Opto-mFGFR1 RNA was 30 times higher than that of endogenous FGFR1 RNA, although the Figure S9 legend is so cryptic that it is hard to know exactly what was done and this needs to be explained. Since the issue of level of expression and efficiency of signalling downstream of Opto-mFGFR1 in terms of the level of phosphorylated Opto-mFGFR1 is important, I strongly recommend that the protein data in Reviewer Figure 2 be included in one of the figures in the main paper.

2nd Revision - authors' response 22 May 2014

Referee #2:
To address the reviewers' comments, the authors have added a significant number of new experiments confirming the utility and signal pathway specificity of light-induced Opto-mFGFR1 signalling, including the use of Opto-mFGFR1's mutated at individual tyrosines known to be autophosphorylated and involved in signalling downstream of FGFR1. However, the large amount of supplementary data has been only briefly described in the main text. As a consequence it is not always exactly clear what was done, a fuller description of these experiments in the main text and legends is needed.

Authors:

We thank the reviewer for her/his comment.
The manuscript was revised following the suggestions of the reviewer, and the new data is now discussed in the main text and the legends in more detail.

Referee #2:
1. In response to a request that the authors cite the recent Toettcher et al. paper that uses lightactivated
ERK MAPK pathway signalling, the authors have simply added a sentence on page 4 mentioning these papers in totally inappropriate context. Since the authors' paper will appear more than six months after the Toettcher et al. paper, the authors need to include in the Discussion section a proper discussion that compares and contrasts their method for light activation of FGFR signalling leading to ERK MAP kinase activation with the method described by Toettcher et al., which activates the ERK MAPK pathway at the level of Sos recruitment to the membrane, and indicates the advantages of their approach.

Authors:

We thank the reviewer for pointing this out.
The manuscript was revised following the suggestions of the reviewer (please refer to the Discussion section of the revised manuscript).

Referee #2:
2. The new fluorescence data in Reviewer Figure 1 showing that light activation causes slow internalization of plasma membrane associated Opto-mFGFR1 are reasonable, but it is unclear why the authors did not simply use IF imaging to determine whether light activation causes OptomFGFR1 to move from a plasma membrane localization, which they demonstrated in Figure S10, to a more intracellular localization. Was this attempted?

Authors:

Yes, this was attempted using several anti-pYFGFR1 antibodies. No clear picture emerged from these experiments and we applied the method described previously.

Referee #2:
3. The requests for a demonstration of which Tyr in Opto-mFGFR1 are phosphorylated and that FRS2 associates with Opto-mFGFR1 in response to light have been addressed with mutation

analysis, which demonstrates that specific Tyr are required for ERK activation and that the JMD region required for FRS2 binding is needed. However, this is not exactly the same as blotting with site-specific anti-pTyr antibodies or testing FRS2 binding by coprecipitation. However, new experiments are not needed.

Authors:

We attempted immunoblotting experiments with anti-pYFGFR1 antibodies against all commercially available sites (anti-pY653/654, anti-pY463, anti-pY766). For antibodies other than those directed against pY653/654 (Supplementary Figure S3 and Figure 5), we did not observe technically sound band patterns in the blots irrespective of the many experimental conditions that we tried. The substitution approach we ultimately utilized was originally suggested by the referee in her/his comments to the previous version of the manuscript. This approach may have the additional feature that requirement of the respective sites for signalling is assessed.

Referee #2:
4. In Reviewer Figure 2, the authors have used immunoblotting to show that stable Opto-mFGFR1-expressing 293 cells contained a level of Opto-mFGFR1 protein approximately equal to that of endogenous FGFR1, and in the main text on page 7 they state that the levels of Opto-mFGFR1 were comparable to endogenous FGFR based on the RNA data in Figure S9. However, from the ordinate scale in that figure it looks as though the level of Opto-mFGFR1 RNA was 30 times higher than that of endogenous FGFR1 RNA, although the Figure S9 legend is so cryptic that it is hard to know exactly what was done and this needs to be explained. Since the issue of level of expression and efficiency of signalling downstream of Opto-mFGFR1 in terms of the level of phosphorylated Opto-mFGFR1 is important, I strongly recommend that the protein data in Reviewer Figure 2 be included in one of the figures in the main paper.

Authors:

We thank the reviewer for pointing this out.
Reviewer Figure 2 contained data collected from HEK293 cells, while Supplementary figure S9 contained data collected from mesothelioma cells. These data are now more clearly distinguished in the manuscript, and, as requested by the reviewer, Reviewer Figure 2 has been moved from the reviewer comments to the manuscript (it is now Supplementary Figure S3) and the legend was modified.

