Mechanism of p38 MAPK induced EGFR endocytosis and its crosstalk with ligand-induced pathways

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Review Timeline:

Submission Date:	2021-02-01
Editorial Decision:	2021-03-10
Revision Received:	2021-04-21
Editorial Decision:	2021-04-22
Revision Received:	2021-04-23

Monitoring Editor: Pier Paolo Di Fiore

Scientific Editor: Melina Casadio

Transaction Report:

March 10, 2021

Re: JCB manuscript #202102005

Dr. Alexander Sorkin
University of Pittsburgh School of Medicine
3500 Terrace Str
Pittsburgh, PA 15261

Dear Dr. Sorkin,

Thank you for submitting your manuscript entitled "Mechanism of p38 MAPK induced EGFR endocytosis and its crosstalk with ligand-induced pathways". The manuscript was assessed by expert reviewers, whose comments are appended to this letter. We invite you to submit a revision if you can address the reviewers' key concerns, as outlined here.

You will see that the reviewers felt that the work is interesting and of high technical quality. They provided suggestions for controls and additional analyses to strengthen the conclusions. From their comments, we invite you to focus the revision efforts on the following points that are in our view directly relevant and most important to the main points and conclusions:

Reviewer 1

1) The competition assay on Fig. 2 A-D is not very convincing....
- Please, address experimentally

2) In Fig. 3B, EGFR mutants are overexpressed in PAE cells...
- Please, address experimentally

3) In the IP in Fig. 3D, it would be important to perform an IP of EGFR-WT...
- Dispensable, not a prerequisite for acceptance

4) In Fig. 5B it would be important to measure EGFR endocytosis upon Grb2 KD...
- Dispensable, not a prerequisite for acceptance

5) In Fig. 6A, a sample of +TNFalpha...
- Dispensable, not a prerequisite for acceptance

Reviewer 2

1. Experimental design is a pulse-chase, which tend to bias dose-response curves...
- Dispensable, not a prerequisite for acceptance

2. Basal internalization of EGFR is subtracted to yield the reported values...
- Please, add the requested info

3. The mixing of experiments using TNFa and Anisomycin was confusing...

- Please, address experimentally

4. The statement "phosphorylation of S1006 has not been, to our knowledge, reported previously…" is not correct…
- Please, change the text accordingly

5. Figure 4 shows that mutations on S1006 increases early endosome co-localization, not internalization as described in the text. They need to show an increase in internalization rate as well.
- Please, address experimentally

6. The level of pSer phosphorylation (seen in Fig 3D) is quite high in the constitutive…
- Please address the issue in the text (no experiments needed)

7. The estimate of 50% of all EGFR internalization being p38-mediated (Fig. 5B)…
- Please, add the requested info

8. The comment "these data prove (emphasis mine) that the LL1010/11 motif is crucial for p38-induced EGFR endocytosis because it binds to AP2" is an overstatement…
- Please, rephrase

Reviewer 3

1. The authors propose a CBL-dependent and CBL-independent pathway…
- This would add value, but it is not a prerequisite for acceptance

2. p38 has been implicated before in EGFR signaling via EGFR transactivation…
- Perhaps this can be addressed in the discussion. We do not see compelling reasons for experiments.

3. In Fig. 3, some of the experiments should be repeated with TNFA stimulation for broader applicability.
- Dispensable, not a prerequisite for acceptance

4. The reciprocal effect of EGFR activation on p38 activity needs to be discussed for a potential feedback loop (PMID: 16632517), especially at later time points…
- Please, address this point with text edits

5. Archetypal not archetypical (introduction first line).
- Please correct

Please let us know whether you anticipate any issues addressing the reviews or would like to discuss them further. We would be happy to discuss if you have any questions.

While you are revising your manuscript, please also attend to the following editorial points to help expedite the publication of your manuscript. Please direct any editorial questions to the journal office.

GENERAL GUIDELINES:

Text limits: Character count for an Article is < 40,000, not including spaces. Count includes title page, abstract, introduction, results, discussion, acknowledgments, and figure legends. Count does not include materials and methods, references, tables, or supplemental legends.

Figures: Articles may have up to 10 main text figures. Figures must be prepared according to the policies outlined in our Instructions to Authors, under Data Presentation, https://jcb.rupress.org/site/misc/ifora.xhtml. All figures in accepted manuscripts will be screened prior to publication.

IMPORTANT: It is JCB policy that if requested, original data images must be made available. Failure to provide original images upon request will result in unavoidable delays in publication. Please ensure that you have access to all original microscopy and blot data images before submitting your revision.

Supplemental information: There are strict limits on the allowable amount of supplemental data. Articles may have up to 5 supplemental figures. Up to 10 supplemental videos or flash animations are allowed. A summary of all supplemental material should appear at the end of the Materials and methods section.

As you may know, the typical timeframe for revisions is three to four months. However, we at JCB realize that the implementation of social distancing and shelter in place measures that limit spread of COVID-19 also pose challenges to scientific researchers. Lab closures especially are preventing scientists from conducting experiments to further their research. Therefore, JCB has waived the revision time limit. We recommend that you reach out to the editors once your lab has reopened to decide on an appropriate time frame for resubmission. Please note that papers are generally considered through only one revision cycle, so any revised manuscript will likely be either accepted or rejected.

When submitting the revision, please include a cover letter addressing the reviewers' comments point by point. Please also highlight all changes in the text of the manuscript.

We hope that the comments below will prove constructive as your work progresses. We would be happy to discuss them further once you've had a chance to consider the points raised in this letter.

Thank you for this interesting contribution to Journal of Cell Biology. You can contact us at the journal office with any questions, cellbio@rockefeller.edu or call (212) 327-8588.

Sincerely,

Pier Paolo Di Fiore, MD, PhD
Editor, Journal of Cell Biology

Melina Casadio, PhD
Senior Scientific Editor, Journal of Cell Biology

Reviewer #1 (Comments to the Authors (Required)):

This study investigated the p38-dependent clathrin-mediated endocytosis of the EGFR induced by

stress signals (TNFalpha or anisomycin), and its interplay with EGF-induced endocytic pathways. The authors showed that this mechanism of endocytosis is active at low picomolar EGF concentrations and internalizes those EGFRs that are not engaged by the clathrin-dependent pathway (which is Grb2-dependent, at variance with the p38-mediated mechanism). At nanomolar EGF concentrations, clathrin-independent pathways get activated, primarily by subtracting receptors from p38-mediated endocytosis. The authors also identified a previously unknown p38-dependent phosphorylation locus in the EGFR tail that, together with the dileucine motif, contributes to the efficient recruitment of AP2 sigma subunit to drive clathrin-mediated endocytosis of the EGFR.

This study addresses a critical issue in the field of EGFR endocytosis, i.e., how the p38-dependent pathway of EGFR endocytosis is regulated and how it is integrated with ligand-dependent endocytic mechanisms. Indeed, there are two parallel literatures investigating these different modalities of endocytosis and this is the first systematic study where EGF-dependent and p38/stress-dependent mechanisms are investigated in an integrated fashion. In addition, from a technical point of view, the work is of great interest due to the use of FAP-EGFR construct that allow to quantitatively measure ligand-independent endocytosis of the EGFR, which was till now mostly analyzed in qualitative terms and, for this reason, its relevance respect to other endocytic pathways was not investigated.

Below, I listed some issues that need to be addressed prior publication.
1) The competition assay on Fig. 2 A-D is not very convincing. Control for effective competition with AP2-mu and -sigma upon the overexpression of the two constructs (Tac-Y and Tac-LL, respectively) is missing. For this reason, the conclusion that there is a main role for LL motifs as opposed to the Yxxf motif is not fully supported by this data. It would important to show, instead, that the EGFR mutated in the Yxxf and expressed in PAE cells is still internalized via p38-dependent mechanism (to be added in Fig. 3 A-B and compared to the EGFR-LL1010/11A mutant).
2) In Fig. 3B, EGFR mutants are overexpressed in PAE cells. It is important to show that these mutants are equally transported and localized to the PM (in basal condition) by performing either a saturation binding with 125I-EGF or a FACS analysis on the different cell populations using anti-EGFR antibodies recognizing the extracellular domain.
3) In the IP in Fig. 3D, it would be important to perform an IP of EGFR-WT (-/+ anisomycin) in AP2 KD cells, to control for the specificity of the AP2 band that is co-immunoprecipitated with the EGFR (given that there are multiple bands in the WB against AP2alpha).
4) In Fig. 5B it would be important to measure EGFR endocytosis upon Grb2 KD, a condition where only p38-dependent endocytosis should remain active (therefore corresponding to the curve obtained by the subtraction between vehicle and BIRB796-treated curves).
5) In Fig. 6A, a sample of +TNFalpha in Grb2KD cells is critical to effectively measure the TNF alpha-dependent endocytic component.

Reviewer #2 (Comments to the Authors (Required)):

This is a very elegant paper that makes a significant contribution to the field. Using a powerful genetic construct that allows them to follow the endocytosis of empty EGFR, they have studied the specific mechanisms that couple p38 activation to EGFR endocytosis. EGFR endocytosis is very complex, consisting of the overlapping activities of multiple mechanisms and cellular pathways. This complexity has stymied many studies on regulatory mechanisms. The beauty of this paper is that they have found a way to selectively visualize a single endocytic mechanism within the context of

the totality of the pathways, allowing the dissection of specific mechanisms.

The hypotheses they test are well conceived and is based on a particularly large prior body of information. Their experimental results, for the most part, are discriminatory between alternative hypotheses, giving them a solid foundation for their overall conclusions. These data suggest that p38 modulates the engagement of ligand-free EGFR to clathrin-dependent endocytosis through a specific di-leucine motif, probably through a phosphorylation-mediated conformational change in the EGFR. The result is increased endocytosis of the unoccupied EGFR. The paper is very well written, which is important for such a complex subject. The discussion is generally good but seems a bit too descriptive and overly focused on mechanisms rather than discussing the regulatory significance of their findings. Total EGFR at the cell surface is a crucial determinant of a cell's ability to respond to EGF and is regulated by both ligand-induced and constitutive turnover of the receptor. Their studies show that p38 regulates the constitutive turnover of the EGFR, and thus surface receptor levels, through a mechanism that does not compete with ligand-induced endocytosis. Thus, the sensitivity of cells to EGF could be directly controlled by p38 activity without such activity altering the dynamics of EGF-induced signaling. Pathway cross-talk at the level of regulated endocytosis is an important aspect of signal processing by cells. Their studies are very important in establishing the important properties and parameters in this cross-talk mechanism, which is critical in building models of the process. They briefly touched on this aspect of their work at the end of the discussion, but I think that is deserves more emphasis.

There were some concerns and caveats in their experimental design and data presentation, but overall these were relatively minor and do not significantly impact the overall conclusions of their study. Overall, this is a very strong, interesting study that provides important new information on how the dynamics of the EGFR trafficking pathway is regulated, particularly under physiological conditions. I thought the experiments were well designed and produced convincing evidence for a relatively modest set of conclusions.

Minor points:
1. Experimental design is a pulse-chase, which tend to bias dose-response curves. This could be assessed by comparing their results with radiolabeled EGF data. Have they assessed the degree of the bias and the degree to which is would bias the comparison between TNFa and EGF?
2. Basal internalization of EGFR is subtracted to yield the reported values. How big was the basal relative to the induced? They provided one example. Was the basal value consistent between cell type and experiment?
3. The mixing of experiments using TNFa and Anisomycin was confusing, potentially giving rise to secondary effects from the different treatments. Going to the use of a different cell type halfway through the study was poortly justified. Were the inhibitors used in HeLa cells validated to produce the same results in the PAE cells?
4. The statement "phosphorylation of S1006 has not been, to our knowledge, reported previously…" is not correct. The PhosphoSite Plus database list detection of S1006 (listed as S1030-p using the more common +24 proteomics nomenclature) as documented by Zhang G, et al. (2011) J Proteome Res 10, 305-19.
5. Figure 4 shows that mutations on S1006 increases early endosome co-localization, not internalization as described in the text. They need to show an increase in internalization rate as well.
6. The level of pSer phosphorylation (seen in Fig 3D) is quite high in the constitutive (non-p38 activated) state and is abolished by conditions that reduce S1015 phosphorylation. This doesn't make sense because there are dozens of pSer sites on the EGFR. This is more suggestive of an antibody specificity problem than a specific response. A good mass spectrometry EGFR site survey

would be very informative in this regard. They should indicate this potential issue even though it doesn't impact the major conclusions of this study.

7. The estimate of 50% of all EGFR internalization being p38-mediated (Fig. 5B) seems to be an exaggeration. Visually it looks like no more than ~30%. The author should provide specific numbers.

8. The comment "these data prove (emphasis mine) that the LL1010/11 motif is crucial for p38-induced EGFR endocytosis because it binds to AP2" is an overstatement. It is certainly suggestive of such a mechanism, but the complexities of the pool dynamics of different forms of the EGFR and other potentially competing receptor pools complicate a straight-forward interpretation of the data. I would rephrase as "these data suggests..."

Reviewer #3 (Comments to the Authors (Required)):

This is a focused and detailed examination of p38 MAPK-stimulated EGFR endocytosis by a leader in the field of EGFR function and trafficking. Sorkin and co-workers have previously shown that low concentrations of EGF (20-200 pM) results in clathrin-dependent endocytosis (CME) of EGFR whereas higher concentrations of ligand result in clathrin-independent endocytosis (CIE). It has also been shown that p38-stimulated EGFR endocytosis is CME. The authors utilize cleverly designed endogenous FAP-tagged EGFR in Hela cells that can accept different fluorogenic versions of the non-permeant MG dye to quantitatively monitor the transit of EGFR through different intracellular compartments, as well as separate experiments using EGFR-GFP-tagged constructs in porcine aortic endothelial cells (PAE). They show that p38-stimulated EGFR endocytosis is dependent on a cytosolic dileucine motif that interacts with AP2 clathrin adaptors, which in turn depends on phosphorylation of EGFR serine 1006. They report the cross-talk between p38 and low ligand concentration CME and that high ligand concentration utilizes CIE at least partially through reducing p38-mediated endocytosis. The experiments appear well-performed and shed new mechanistic insights into EGFR endocytosis. I have the following relatively minor concerns and suggestions.

Minor concerns:
• The authors propose a CBL-dependent and CBL-independent pathway. It is further proposed that the CBL-dependent pathway could be via a CBL/EGFR interaction or a CBL/GRB2 interaction. While inability of GRB2 loss to affect EGFR internalization after TNFA/anisomycin stimulation is shown (Fig. 1H), it should be tested if this pathway is completely independent of CBL, perhaps by performing CBL knockdown in a similar assay.
• p38 has been implicated before in EGFR signaling via EGFR transactivation, which typically requires cleavage of EGFR ligands by metalloproteases like TACE (PMID: 26658844). Did the authors consider the possibility of EGFR internalization after endogenous ligand binding induced by the transactivation pathway? Might what is referred to as ligand-free endocytosis (that is, receptors without labelled ligand) actually be occupation by endogenous ligand? Have the authors determined amounts of ligands produced by these Hela and PAE cells?
• In Fig. 3, some of the experiments should be repeated with TNFA stimulation for broader applicability.
• The reciprocal effect of EGFR activation on p38 activity needs to be discussed for a potential feedback loop (PMID: 16632517), especially at later time points. This relates to findings in Figs. 5A, 5C, 6C. Is it possible that p38 is also involved in the recycling after ligand stimulation and BIRB addition modulates that pathway?
• Archetypal not archetypical (introduction first line).

Pier Paolo Di Fiore, MD, PhD
Editor
Melina Casadio, PhD
Senior Scientific Editor
Journal of Cell Biology

Dear Drs. Di Fiore and Casadio,

We would like to submit the revised manuscript "Mechanism of p38 MAPK induced EGFR endocytosis and its crosstalk with ligand-induced pathways". We appreciate encouraging and constructive comments of the reviewers, and your thorough editorial analysis of these comments and the manuscript. We addressed all reviewers' concerns by additional experimentation and modifications of the text in all parts of the manuscript and most figures. While reviewers designated their concerns as "minor", addressing those comments required development of new methodologies, such as, for example, an antibody-uptake internalization assay; hence the delay in the submission of the revision.

Our point-by-point response to reviewers' comments is below. Changes in the main text (except corrected typos and stylistic errors) are marked as red font.

We do appreciate consideration of our work by the Journal of Cell Biology. We believe that new experiments and other changes in the manuscript substantially strengthened this study, and hope it is now suitable for publication.

Best regards,

Alexander Sorkin

Response to comments

Reviewer #1 (Comments to the Authors (Required)):

Below, I listed some issues that need to be addressed prior publication.
1) The competition assay on Fig. 2 A-D is not very convincing. Control for effective competition with AP2-mu and -sigma upon the overexpression of the two constructs (Tac-Y and Tac-LL, respectively) is missing. For this reason, the conclusion that there is a main role for LL motifs as opposed to the Yxxf motif is not fully supported by this data. It would important to show, instead, that the EGFR mutated in the Yxxf and expressed in PAE cells is still internalized via p38-dependent mechanism (to be added in Fig. 3 A-B and compared to the EGFR-LL1010/11A mutant).

Response: As the reviewer requested, we demonstrate robust p38-dependent endocytosis of the Y974A EGFR mutant but not the LL1010/11A mutant. For these experiments we used our previously published PAE cell lines stably expressing these mutants. Please see new Figures 2C-D and corresponding text on page 8, second paragraph. We kept the competition assay in the manuscript (Figures 2A-B) because we believe it is imperative to show the importance of the dileucine motif as oppose to Tyr-based motif in p38-dependent endocytosis of endogenous EGFR in HeLa/FAP-EGFR cells, the key experimental model in the present study. It should be emphasized that Tac chimeras were originally developed and used in similar experiments to demonstrate competition for endocytosis with endogenous cargo containing Tyr- and LL-based AP2 binding motifs (cited as Marks et al.). Due to space limitation, the bar graph comparing transient expression levels of Tac-Y and Tac-LL chimeras in our experiments (former panel 2B) was omitted in the revised manuscript. The information about relative expression levels of Tac chimeras is instead presented in the Figure 2 legend.

2) In Fig. 3B, EGFR mutants are overexpressed in PAE cells. It is important to show that these mutants are equally transported and localized to the PM (in basal condition) by performing either a saturation binding with 125I-EGF or a FACS analysis on the different cell populations using anti-EGFR antibodies recognizing the extracellular domain.

Response: To address this concern, we performed a series of experiments to examine whether EGFR mutants are efficiently transported to and localized at the cell surface. Because 125I-EGF binding or FACS could not be used to measure the fraction of total cellular EGFR located at the cell surface – a measure of the efficiency of the delivery of EGFR mutants to the PM - we used single-cell analysis to measure the fraction of extracellularly exposed EGFR-GFP. These experiments demonstrated that 70-80% of total cellular WT or mutant EGFR-GFPs were present at the cell surface. The data are presented in new Figure S3C (Supplemental Materials).

3) In the IP in Fig. 3D, it would be important to perform an IP of EGFR-WT (-/+ anisomycin) in AP2 KD cells, to control for the specificity of the AP2 band that is co-immunoprecipitated with the EGFR (given that there are multiple bands in the WB against AP2alpha).

Response: Western blot image of α-adaptin immunoreactivity in PAE cells transfected with non-targeting or $\mu2$ siRNA (to deplete AP2) is presented in new Figure S2F (Supplemental Materials). The position of α-adaptin band (~102-103 kDa) that is depleted in $\mu2$ siRNA transfected cells, precisely corresponds to the position of the α-adaptin band detected with the same antibody in EGFR-GFP immunoprecipitates in Figure 3D.

4) In Fig. 5B it would be important to measure EGFR endocytosis upon Grb2 KD, a condition where only p38-dependent endocytosis should remain active (therefore corresponding to the curve obtained by the subtraction between vehicle and BIRB796-treated curves). 5) In Fig. 6A, a sample of +TNFalpha in Grb2KD cells is critical to effectively measure the TNF alpha-dependent endocytic component.

Response: Comments #4 and #5 are largely addressed in Figure 7C, where the effects of Grb2 knockdown on FAP-EGFR endocytosis in the presence of low and high EGF concentrations, and with or without TNFα, are shown. In essence, <u>Grb2-independent</u> component of EGFR internalization is slightly larger than <u>p38-dependent component</u> at low EGF concentrations. Although Grb2 is the major player during endocytosis of activated EGFR, knockdown of Grb2 only partially inhibits internalization of ligand-bound receptors due to compensatory clathrin-mediated endocytosis (CME) through other redundant and cryptic mechanisms, as well as a small contribution of clathrin-independent endocytosis (CIE) (*see page 16, second paragraph*). This is illustrated in Fig 7C (data with 1 ng/ml EGF; <u>Grb2-independent</u> component ~60% of NT; compare with <u>p38-dependent</u> component of 40-50% in Fig 5B). At high EGF concentrations, <u>Grb2-independent</u> component is dramatically larger than <u>p38-dependent</u> component of endocytosis because Grb2 knockdown does not significantly inhibit CIE in our cells (commented in Discussion on *page 22, end of the first paragraph*). This is illustrated in Fig 7C (data with 10 ng/ml EGF; <u>Grb2-independent</u> component ~90% of NT; compare with <u>p38-dependent component</u> of ~10% in Fig 5B).

The situation in the presence of TNFα is more complex. As shown in Fig 7C (data with 1 ng/ml EGF; supported in Fig 7D), strong activation of p38 by TNFα in Grb2-depleted cells results in re-routing of ligand-bound active EGFRs to the p38-dependent pathway of CME, which leads to compensatory endocytosis. Under these conditions, all endocytosis is virtually TNFα-induced and Grb2-independent. This is illustrated in Fig 7C (data with 1 ng/ml plus TNFα; <u>Grb2-independent component</u> is ~100% of NT; compare with <u>TNFα–induced</u> component of ~40% at 1 ng/ml EGF in Fig 6A). At high EGF concentration (10 ng/ml) plus TNFα, Grb2-independent component of endocytosis is much larger than TNFα-dependent endocytosis (~10% under the same conditions in Fig 6A) because Grb2 depletion does not significantly inhibit CIE. This is illustrated in Fig 7C (data with 10 ng/ml EGF plus TNFα; <u>Grb2-independent</u> component is ~95% of NT; compare with Fig 6A with <u>TNFα-induced component</u> at 10 ng/ml EGF is ~15%). Given the complexity of the data described in Figures 5-9, we feel that including additional EGF-dose-dependence curves in Figures 5B and 6A will be more confusing than helpful to the reader. At the same time, we believe that the data in Figures 7C-D are sufficient to illustrate the interplay between Grb2- and p38-dependent pathways of EGFR endocytosis. However, we would be happy to include an additional figure directly comparing contributions of Grb2-independent, p38-dependent and TNFα-induced endocytosis in the presence of low and high EGF concentrations if further requested.

Reviewer #2:
Minor points:
1. Experimental design is a pulse-chase, which tend to bias dose-response curves. This could be assessed by comparing their results with radiolabeled EGF data. Have they assessed the degree of the bias and the degree to which is would bias the comparison between TNFa and EGF?

Response: We apologize but we did not understand this comment. We are not sure how 125I-EGF can be used to assess endocytosis of ligand-free EGFR, and for comparison of the data

between EGF and TNFα. The FERI assay is not a pulse-chase experiment, such as for example experiments in which cells are first pre-labeled with the radioactive ligand at 4oC, washed and then chased at 37oC. The FERI assay does involve a quick "pulse" step of labeling cell-surface FAP-EGFR with MG dyes at 37oC; however, endocytosis is then stimulated by ligand binding or p38 activation, much like as in a conventional time-course experiment with the radioactive ligand.

2. Basal internalization of EGFR is subtracted to yield the reported values. How big was the basal relative to the induced? They provided one example. Was the basal value consistent between cell type and experiment?

Response: FAP-bound MG-Bis-SA displays a "basal" level of FRET (excitation at 561 nm; emission at 700 nm) at high pH. As a consequence, the 561/640 ratio obtained from untreated HeLa/FAP-EGFR cells labeled with MG-Bis-SA ("basal" 561/640 ratio) typically varied between 0.3-0.4 as shown in our preceding method paper (cited as Larsen et al. 2018). In the FERI assay, the values of this basal ratio obtained in untreated cells are subtracted from raw 561/640 ratio values obtained in wells treated with ligands and/or inhibitors. Therefore, the basal 561/640 ratio is the sum of the basal (minimal) FRET signal and FRET signal resulting from the constitutive FAP-EGFR internalization that occurs during the incubation time (typically 15 min). To estimate this constitutive internalization component of the basal 561/640 ratio, we used time-course experiments presented in Figure S1A. Constitutive internalization data are now shown in the revised Figure S1A ("Untreated" data points) and additional methodological details are provided in the figure legend.

3. The mixing of experiments using TNFa and Anisomycin was confusing, potentially giving rise to secondary effects from the different treatments. Going to the use of a different cell type halfway through the study was poorly justified. Were the inhibitors used in HeLa cells validated to produce the same results in the PAE cells?

Response: We must admit we did struggle with the organization of the manuscript but could not come up with the logical outline without switching between two cell models. Both TNFα and anisomycin treatments produced essentially similar results in experiments with HeLa cells. We have chosen to focus on the TNFa data because they are more physiologically relevant. We did not include the parallel anisomycin data, except in Figure 1, due to the space limitation. PAE cells were necessary for the analysis of EGFR mutants. However, in these cells, TNFα did not significantly activate p38, and we therefore exploited the anisomycin treatment (see Figure S3A-B). As the reviewer suggested, in the revised version of Figure S3A-B, we demonstrate that anisomycin effects were inhibited by BIRB976 in PAE cells, much like in HeLa cells.

4. The statement "phosphorylation of S1006 has not been, to our knowledge, reported previously..." is not correct. The PhosphoSite Plus database list detection of S1006 (listed as S1030-p using the more common +24 proteomics nomenclature) as documented by Zhang G, et al. (2011) J Proteome Res 10, 305-19.

Response: We thank the reviewer for this information. We missed this paper. This study is now cited, and the text is modified on page 11, second paragraph.

5. Figure 4 shows that mutations on S1006 increases early endosome co-localization, not internalization as described in the text. They need to show an increase in internalization rate as well.

Response: We developed an approach to monitor EGFR-GFP internalization in PAE cells expressing WT and mutant EGFR-GFP based on antibody uptake in living cells and performed a series of time-course experiments. This "antibody-feeding" method is described in "Materials and Methods". The representative experiment is shown in Figure 4G. The data confirm that constitutive internalization of the S1006E mutant is significantly faster compared to this internalization of the WT receptor. The biggest difference was observed at 5-min time point. It should be noted that while these data are consistent with the results of EEA1-colocalization experiments (Figure 4B-C), the caveat of the antibody-uptake method is that cross-linking of EGFR by antibodies may augment accumulation of both WT and mutant receptor in endosomes at later time points due to reduced recycling of dimerized/oligomerized EGFR as compared to the monomeric receptor.

6. The level of pSer phosphorylation (seen in Fig 3D) is quite high in the constitutive (non-p38 activated) state and is abolished by conditions that reduce S1015 phosphorylation. This doesn't make sense because there are dozens of pSer sites on the EGFR. This is more suggestive of an antibody specificity problem than a specific response. A good mass spectrometry EGFR site survey would be very informative in this regard. They should indicate this potential issue even though it doesn't impact the major conclusions of this study.

Response: The reviewer is right in that this is an interesting and puzzling observation. That R1 cluster is the major p38-dependent Ser/Thr phosphorylation site in EGFR is evident not only from the detection with the pS1015 antibody but also using the pan-phosphoserine antibody (please see Figure 3D). In fact, this finding was made in previous studies by Sakurai (author in the present study) and co-workers (cited as Tanaka et al., 2018). They developed the pS1015 antibody and additionally demonstrated the prominent LL-dependent phosphorylation of the R1 cluster using an antibody-independent technique (gel-shift method). We think that the basal phosphorylation detected by pS1015 and pSer antibodies is higher in PAE cells compared to HeLa cells [see "control" lanes in Figure S4A (PAE) and S4B (HeLa cells)], because, unlike HeLa cells, PAE cells were serum-starved only for 1-2 hs as they do not tolerate longer serum starvation.

7. The estimate of 50% of all EGFR internalization being p38-mediated (Fig. 5B) seems to be an exaggeration. Visually it looks like no more than ~30%. The author should provide specific numbers.

Response: The reviewer is correct – 50% was a rough estimation. We have included specific mean numbers (40-48%) from 3 independent experiments performed as in Figure 5B. See page 13, bottom: "As shown in Figure 5B, the p38-dependent component of FAP-EGFR endocytosis (endocytosis inhibited by BIRB796) peaked at ~1 ng/ml EGF when about 40-50% of internalized FAP-EGFR was dependent on p38 activity [48.4±12.8% (SD) at 0.78 ng/ml EGF and 40.9±7.8% (SD) at 1.56 ng/ml EGF; mean of 3 experiments exemplified in Figure 5B]." Also, data points in the calculated curve representing p38-component of endocytosis are now shown with propagated errors in the revised Figure 5B. Corresponding minor changes were made in the model in Fig 9C.

8. The comment "these data prove (emphasis mine) that the LL1010/11 motif is crucial for p38-induced EGFR endocytosis because it binds to AP2" is an overstatement. It is certainly suggestive of such a mechanism, but the complexities of the pool dynamics of different forms of the EGFR and other potentially competing receptor pools complicate a straight-forward interpretation of the data. I would rephrase as "these data suggests..."
This sentence is rephrased on page 19, end of second paragraph.

Reviewer #3:
Minor concerns:
• The authors propose a CBL-dependent and CBL-independent pathway. It is further proposed that the CBL-dependent pathway could be via a CBL/EGFR interaction or a CBL/GRB2 interaction. While inability of GRB2 loss to affect EGFR internalization after TNFA/anisomycin stimulation is shown (Fig. 1H), it should be tested if this pathway is completely independent of CBL, perhaps by performing CBL knockdown in a similar assay.

Response: The data demonstrating the effect of Cbl siRNA knockdown on p38-dependent endocytosis is included in the revised Figure 1H, and the efficiency of siRNA knockdown is shown in the revised Figure S2A. See page 7, second paragraph.

• p38 has been implicated before in EGFR signaling via EGFR transactivation, which typically requires cleavage of EGFR ligands by metalloproteases like TACE (PMID: 26658844). Did the authors consider the possibility of EGFR internalization after endogenous ligand binding induced by the transactivation pathway? Might what is referred to as ligand-free endocytosis (that is, receptors without labelled ligand) actually be occupation by endogenous ligand? Have the authors determined amounts of ligands produced by these Hela and PAE cells?

Response: In the above cited studies, TACE activation by p38/MAPK was shown in gastric mucosa cells. In HeLa and PAE cells used in the present study, we did not observe any activation of EGFR by TNFα or anisomycin within the time scale of our experiments, indicating that EGF ligands were not produced in a significant amount in response to p38 activation. Please see immunoblotting with pY1068 antibodies in Figure 8A (lane 4) and S4A (lane 3). Moreover, TNFα− and anisomycin-induced endocytosis was insensitive to EGFR kinase inhibitors (example with erlotinib is illustrated in Figure 7A). Similarly, previous studies in HeLa cells have not reported transactivation of EGFR by TNFα (cited as Singhirunnusorn et al.).

• In Fig. 3, some of the experiments should be repeated with TNFA stimulation for broader applicability.

Response: As shown in Figure S3A, TNFα has a very weak effect on p38 activity in PAE cells, which precluded measurements of TNFα effects on EGFR-GFP endocytosis in these cells due to an extremely low signal-to-noise ratio.

• The reciprocal effect of EGFR activation on p38 activity needs to be discussed for a potential feedback loop (PMID: 16632517), especially at later time points. This relates to findings in Figs. 5A, 5C, 6C. Is it possible that p38 is also involved in the recycling after ligand stimulation and BIRB addition modulates that pathway?

Response: This is indeed an important point. p38 has been shown to phosphorylate components of endosomal sorting machinery such as EEA1. The present study is focused on early steps of endocytosis, and unfortunately, we have not been able to discuss a potential role of p38 signaling in endosomal sorting due to space limitation.

• Archetypal not archetypical (introduction first line).

Corrected.

April 22, 2021

RE: JCB Manuscript #202102005R

Dr. Alexander Sorkin
University of Pittsburgh School of Medicine
3500 Terrace Str
Pittsburgh, PA 15261

Dear Dr. Sorkin,

Thank you for submitting your revised manuscript entitled "Mechanism of p38 MAPK induced EGFR endocytosis and its crosstalk with ligand-induced pathways". We would be happy to publish your paper in JCB pending final revisions necessary to meet our formatting guidelines (see details below).

To avoid unnecessary delays in the acceptance and publication of your paper, please read the following information carefully.

1) eTOC summary: A 40-word summary that describes the context and significance of the findings for a general readership should be included on the title page. The statement should be written in the present tense and refer to the work in the third person.

- Please include a summary statement on the title page of the resubmission. It should start with "First author name(s) et al…" to match our preferred style.

2) Figure formatting: Scale bars must be present on all microscopy images, including inset magnifications. Please add scale bars to 6D magnification

3) Statistical analysis: Error bars on graphic representations of numerical data must be clearly described in the figure legend. The number of independent data points (n) represented in a graph must be indicated in the legend. Statistical methods should be explained in full in the materials and methods. For figures presenting pooled data the statistical measure should be defined in the figure legends.

4) Materials and methods: Should be comprehensive and not simply reference a previous publication for details on how an experiment was performed. Please provide full descriptions in the text for readers who may not have access to referenced manuscripts.
- For all cell lines, vectors, constructs/cDNAs, etc. - all genetic material: please include database / vendor ID (e.g., Addgene, ATCC, etc.) or if unavailable, please briefly describe their basic genetic features *even if described in other published work or gifted to you by other investigators*
- Please include species and source for all antibodies, including secondary, as well as catalog numbers/vendor identifiers if available.
- Sequences should be provided for all oligos: primers, si/shRNA, gRNAs, etc.
- Microscope image acquisition: The following information must be provided about the acquisition and processing of images:
a. Make and model of microscope
b. Type, magnification, and numerical aperture of the objective lenses

c. Temperature
d. imaging medium
e. Fluorochromes
f. Camera make and model
g. Acquisition software
h. Any software used for image processing subsequent to data acquisition. Please include details and types of operations involved (e.g., type of deconvolution, 3D reconstitutions, surface or volume rendering, gamma adjustments, etc.).

5) A summary paragraph of all supplemental material should appear at the end of the Materials and methods section.
- Please include one brief descriptive sentence per item.

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Thank you for this interesting contribution, we look forward to publishing your paper in the Journal of Cell Biology.

Sincerely,

Pier Paolo Di Fiore, MD, PhD
Editor, Journal of Cell Biology

Melina Casadio, PhD
Senior Scientific Editor, Journal of Cell Biology