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Chk1 phosphorylates the tumor suppressor Mig-6, regulating the activation of EGF signaling

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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.)

1st Editorial Decision 18 July 2011

Thank you very much for submitting your research paper on potential Chk1-signal intersection with the EGFR pathway for consideration to The EMBO Journal editorial office. I received comments from three expert scientists that all appreciate the potential novelty of your study, though at the same time outline major shortcomings when it comes to presenting a conclusive and thoroughly corroborated study. As such, the paper has to be judged as very preliminary indeed. Despite their assessments that request:
-necessary confirmation of RNAi-experiments by additional oligos to rule-out off-target effects
-substantiation of implied functional effects, preferably coupled to rather biological readouts and employing appropriate mutant constructs
-follow-up on further molecular details such as possible phosphorylation-dependent ubiquitination and direct evidence for PI3K-regulation. In fact ref# 3 recommends reconstituting the system to thoroughly address the role of phosphorylated Mig6 on EGFR-interaction and function.
As such, the paper would need a thorough rework and significant (functional) expansion to convince all three of them from its proposal and thus enable necessary, enthusiastic support for publication. This does demand complex and challenging further experimentation and we would thus understand of you might find it easier to seek more rapid publication elsewhere.
However, and seeing the potential of the work we would be prepared to offer more time than the limited amount of three month for revision if you were willing to invest the time and efforts to develop the study (and upon explicit request). I do urge you to take the referee demands serious and carefully consider this option, also to avoid disappointments much later in the process.
Please do not hesitate to contact me in case of further questions or indeed outlining possible experiments and timeline in case you plan to revise (preferably via E-mail).

Finally, I do have to formerly remind you that it is EMBO_J policy to allow one round of revisions only and that the final decision on acceptance or rejection depends on the content and strength of the finally submitted version of your study.

Yours sincerely,

Editor
The EMBO Journal

REFEREE REPORTS:

Referee #1:

Liu and colleagues show that EGF stimulates endogenous Chk1 to phosphorylate and suppress the activity of the tumor suppressor protein Mig6. The overall process is to some degree dependent upon PI-3-kinase and reduces Mig6's modest attenuation of cell proliferation.

The paper is potentially interesting, but is rather superficial and requires substantial additional work to develop the findings adequately. The following concerns need to be addressed.

1) RNAi studies need to be repeated using more than one oligo, in order to rule out off-target effects.

2) A rescue experiment is needed in which Mig6 wt or phosphoacceptor mutant is tested for reversing the effect of Mig6 RNAi on cell proliferation.

3) The smeared bands on the phos-tag gels suggest additional modifications of Mig6 consequent to phosphorylation. Is EGFR-associated Mig6 ubiquitinated? This can be tested using anti Ub and proteasome inhibitors.

4) Does Chk1-dependent phosphorylation of Mig6 affect Mig6 association with the EGFR? What about other members of the EGFR family.

5) In Fig. 4, and elsewhere, the effects of Mig6 overexpression or RNAi on cell proliferation are small, as are (in Fig. 4B) the effects on EGFR phosphorylation. While statistically significant, it is unclear that these effects are that important, at least in the system being used.

6) The only link to PI-3-kinase is a pharmacological study. Inasmuch as many PI-3-kinase inhibitors also block ATM and other related kinases (many of which can regulate Chk1), it is important to use other methods to implicate PI-3-kinase in Chk1 regulation.

7) In addition, there is not any information as to how PI-3-kinase regulates Chk1. Does Akt phosphorylate it directly?

8) A minor point, the upper panel in Fig. 1B is dispensable since most of the drugs tested are not specific, and the lower panel is supported by RNAi data. This upper panel can be deleted.

Referee #2:

This is an interesting manuscript that identifies Mig6, an EGFR feedback inhibitor as a putative target for Chk1 in the control of EGFR activation. Genetic evidence indeed supports a role for Chk1 in the regulation of cell proliferation in addition to its well established role during DNA damage signalling, although the underlying mechanism/s remain virtually unknown. To my knowledge this study provides the first explanation for the mechanism by which Chk1 can regulate growth factor signalling and is therefore of considerable novelty. Although the identification of Mig6 as a Chk1 substrate is convincingly demonstrated, evidence supporting the claim that Chk1 phosphorylation inhibits the ability of Mig6 to inhibit EGFR is weak and not entirely convincing. Extensive revisions

will therefore be needed to substantiate this claim.

Comments:

1) The authors are performing siRNA mediated depletion of Chk1 using only 1 siRNA oligo and need to rescue the key phenotypes (Figure 2D and 6A) by reintroducing Chk1 or at least use an independent siRNA oligo.

2) Fig.4) Ectopic expression of the wt and the S251A/E mutant forms of Mig6 show a very modest (barely significant) difference in the ability of the mutated forms of Mig6 to inhibit EGFR phosphorylation. Since this is a key result the authors should attempt to achieve a more clearcut results, possibly by improving transfection efficiency by stable transfection or lentiviral mediated delivery. Furthermore the authors should ectopically express active Chk1 which would be predicted to increase EGFR activation.

3) Mig6 is known to bind active EGFR and the authors should address whether the S251A/E mutants exhibit different binding affinity for EGFR for example by co-immunoprecipitation.

4) The Mig6S251 site is a predicted 14-3-3 binding site and the authors should show whether Mig6 binding to 14-3-3 is affected by the S251A/E mutations and whether gain and loss of Chk1 activity alters 14-3-3 binding to Mig6.

5) Figure 6 shows that siRNA knockdown of Chk1 results in decreased phosphorylation of EGFR. This is a key result and needs to be repeated multiple times and statistical significance provided in figure 6B.

Referee #3:

Liu et al: Chk1 phosphorylates a tumor suppressor Mig-6, regulating the activation of EGF signaling

This paper investigates the Chk1-dependent phosphorylation of Mig-6 at S251. Although interesting, this reviewer does not think that the current manuscript is qualified for publication in EMBO, at least in its current form. This is because some key experiments have not been performed. It is regrettable that the authors do not have a phospho-specific antibody to Mig-6 residue S251. This makes the analyses and their interpretations more difficult, particularly when conclusions are drawn from studies with proteins expressed from transfected plasmids (i.e. not endogenous material). Although fine for determining the posttranslational status of a protein, it is difficult to make further conclusions. As such it is hard for this reviewer to take Fig. 4A seriously. A way around this and the lack of phospho-antibody is suggested below.
To determine the role of S251-P without a phospho-specific antibody, the authors should knock down Mig-6 with siRNA and reconstitute the system with a RNA-resistant form of the protein that has a mutation at S281. The alanine and the glutamate mutations should both be made in parallel with the wild-type protein. Once reconstituted, the authors should determine the role of S251-P in EGF signaling.
By only eliminating Chk1, one cannot conclude what is happening with respect to only the S251 site as Chk1 has multiple substrate targets. The reconstitution experiments allow the experimentalist to focus on only the site that is deemed to be important. This will greatly strengthen the arguments about S251-P and is a way to circumvent the absence of the phospho-antibody. Additionally, the authors should widen the scope and include the additional phosphorylation sites that were identified by mass spec.
The data regarding EGF stimulation of phosphorylation of Chk1 at 280 does not appear to add anything new to the paper as this has been previously reported (Puc et al., 2005 which is referenced within the paper). As it stands, it is actually a distraction and should be moved to the supplemental data.

1st Revision - Authors' Response 14 January 2012

Response to the reviewers (point by point)

Referee #1

MAJOR COMMENTS

(1) RNAi studies need to be repeated using more than one oligo, in order to rule out off-target effects.

As the reviewer suggests, we designed other siRNA oligos such as *Chk1*(04) and *Mig-6*(05). Using these siRNAs, we performed the same experiments and obtained almost the same data as with the original siRNAs. As shown in Figure 2D and the new Supplementary Figure S2A, depletion of Chk1 by both siRNA(01) and (04) attenuated phosphorylation of Mig-6. As shown in Figure 6 and the new Supplementary Figures S9 and S11, depletion of Chk1 by both siRNA(01) and (04) attenuated auto-phosphorylation of EGFR. Moreover, depletion of Mig-6 by both siRNA *Mig-6* (01) and (05) facilitated auto-phosphorylation of EGFR (new Supplementary Figures S9 and S11). We have not shown the data, but another siRNA oligo *Mig-6*(03), showed the same results. These results strongly suggest that Chk1 is involved in phosphorylation of Mig-6 and the regulation of EGF signaling.

We show these data in the new Supplementary Figures S2A and S11. Below is the text that we have added to the Results section (page 8, line 1 and page 13, line 16) and the Methods section (page 21, line 23).

Page 8, line 1: "To confirm the Chk1-mediated Mig-6 phosphorylation, we designed and used another siRNA oligo, *Chk1*(04). As shown in Figure 2D and Supplementary Figure S2A, depletion of Chk1 by both siRNA(01) and (04) attenuated phosphorylation of Mig-6."

Page 13, line 16: "The data shown in Figure 6A and Supplementary Figure S9 were analyzed statistically and the results are presented in Figure 6B. Moreover, another *Chk1* siRNA oligo (04) showed the same result as did *Chk1* siRNA oligo (01) (Supplementary Figure S11). These data indicate that depletion of Chk1 results in decreased phosphorylation of EGFR."

Page 21, line 23: "The nucleotide sequence of the *Chk1*(01) siRNA was 5′-gcgugccguagacugucca-3′, *Chk1*(04) siRNA was 5′-gaaguugggcuaucaaugg-3′ with a 3′dTdT overhang. The nucleotide sequence of the human *Mig-6*(01) siRNA was 5′-cuacacuuucugauuucaa-3′, *Mig-6*(05) siRNA was 5′-gcaggguauccauucuuua-3′ with a 3′dTdT overhang. The nucleotide sequence of the human *ATR* siRNA was 5′-cctccgtgatgttgcttga-3′, *S6K1* siRNA was 5′-ggacatggcaggagtgttt-3′ with a 3′dTdT overhang. *ATM* siRNA used in this study was purchased from Ambion."

2) A rescue experiment is needed in which Mig6 wt or phosphoacceptor mutant is tested for reversing the effect of Mig6 RNAi on cell proliferation.

Thank you very much for the comment. We performed rescue experiments using siRNA-resistant Mig-6 wild type, S251A, or S251E. MDA-MB-231 cells were infected with retrovirally encoded Mig-6 wild type, S251A, or S251E, and then treated with an siRNA targeting *Mig-6*. After EGF stimulation, auto-phosphorylation of EGFR was analyzed by immunoblotting. As shown in the new Figure 4, A and B, treatment with *Mig-6* siRNA increased the auto-phosphorylation of EGFR to approximately 120% of control levels. Introduction of wild-type Mig-6 inhibited the auto-phosphorylation of EGFR to 66% of that for the empty vector control. The S251A mutant suppressed the auto-phosphorylation of EGFR significantly more strongly than did wild-type Mig-6. Because Mig-6 is highly phosphorylated in EGF-stimulated MDA-MB-231 cells, the effect of S251E on auto-phosphorylation was almost the same as that of wild-type Mig-6. Next, we performed rescue experiments on cell proliferation using siRNA-resistant Mig-6 wild type, S251A, or S251E. As described above, MDA-MB-231 cells were infected with retrovirally encoded Mig-6 wild type, S251A, or S251E, and then treated with siRNA for *Mig-6*. Then, cell proliferation assays

were performed. As shown in the new Figure 4C, the doubling time of cells expressing S251A was significantly prolonged compared with that of both wild-type and the S251E mutant. Because Mig-6 is highly phosphorylated in MDA-MB-231 cells in normal culture conditions, but not in serum-starved conditions, it is consistent that the effect of S251E on cell proliferation was almost the same as that of wild-type Mig-6. Moreover, we obtained almost the same data using HEK293 cells that were transfected with Mig-6 plasmids (formerly Figure 4, now changed to Supplementary Figure S7). These results were reproducible in three independent experiments, and the effect of the S251A mutant was always moderate. We conclude that phosphorylation of S251 in Mig-6 is involved in the regulation of EGF signaling.

We have revised Figure 4 and added the text below to the Results section (page 10, line 21).

Page 10, line 21: "For further information, we performed rescue experiments using siRNA-resistant Mig-6 wild type, S251A, or S251E. MDA-MB-231 cells were infected with retrovirally encoded Mig-6 wild type, S251A, or S251E, and then treated with an siRNA targeting *Mig-6*. After EGF stimulation, auto-phosphorylation of EGFR was analyzed by immunoblotting. As shown in Figure 4, A and B, treatment with a *Mig-6* siRNA increased the auto-phosphorylation of EGFR to approximately 120% of control levels. Introduction of wild-type Mig-6 inhibited the auto-phosphorylation of EGFR to 66% of that for the empty vector control. The S251A mutant suppressed the auto-phosphorylation of EGFR significantly more strongly than did wild-type Mig-6. Because Mig-6 is highly phosphorylated in EGF-stimulated MDA-MB-231 cells, the effect of S251E on auto-phosphorylation was almost the same as that of wild-type Mig-6. Next, we performed rescue experiments on cell proliferation using siRNA-resistant Mig-6 wild type, S251A, or S251E. As described above, MDA-MB-231 cells were infected with retrovirally encoded Mig-6 wild type, S251A, or S251E, and then treated with siRNA for *Mig-6*. Then, cell proliferation assays were performed. As shown in Figure 4C, the doubling time of cells expressing S251A was significantly prolonged compared with that of the wild-type and the S251E mutant. Because Mig-6 is highly phosphorylated in MDA-MB-231 cells in normal culture conditions, it is consistent that the effect of S251E on cell proliferation was almost the same as that of wild-type Mig-6. Moreover, we obtained almost the same data using HEK293 cells that were transfected with Mig-6 plasmids (Supplementary Figure S5). These results were reproducible in three independent experiments, and the effect of the S251A mutant was always moderate. Therefore, we conclude that phosphorylation of Mig-6 S251 is involved in the regulation of EGF signaling."

3) The smeared bands on the phos-tag gels suggest additional modifications of Mig6 consequent to phosphorylation. Is EGFR-associated Mig6 ubiquitinated? This can be tested using anti Ub and proteasome inhibitors.

As the reviewer suggests, we examined whether EGF promotes ubiquitylation of Mig-6. MDA-MB-231 cells were treated with or without EGF stimulation and/or the proteasome inhibitor MG132. Mig-6 was immunoprecipitated with anti-Mig-6 and then immunoblotted with anti-ubiquitin to detect ubiquitylation of Mig-6. As shown in the new Supplementary Figure S2B, EGF did not promote ubiquitylation of Mig-6 (lanes 7 *vs*. 8), whereas ubiquitylation of Mig-6 was observed *in vivo* in the presence of MG132 (lanes 5 *vs.* 7). The smearing band of Mig-6 prepared from MDA-MB-231 cells with EGF in a phos-tag gel did not indicate ubiquitylation. Therefore, we believe that EGF promotes the phosphorylation of Mig-6 but not its ubiquitylation.

We have added Supplementary Figure S2B and the text below to the Results section (page 7, line 23).

Page 7, line 23: "Furthermore, we proved the smearing band of Mig-6 prepared from EGF-stimulated MDA-MB-231 cells in a phos-tag gel did not indicate ubiquitylation (Supplementary Figure S2B)."

4) Does Chk1-dependent phosphorylation of Mig6 affect Mig6 association with the EGFR? What about other members of the EGFR family.

As the reviewer points out, we examined whether Chk1-dependent phosphorylation of Mig-6 affects its association with EGFR. Wild-type FLAG-Mig-6 or its mutants (such as S251A, S251E, or S302A) were transfected into HEK293 cells and immunoprecipitated with anti-FLAG antibody. Then, the immunoprecipitates were analyzed by immunoblotting with anti-EGFR antibody. Phosphorylation of Mig-6 may not affect its association with EGFR (attached Figure A1A). Next, we evaluated whether phosphorylation of Mig-6 affects its association with ERBB2, another member of the EGFR family. As shown in the attached Figure A1B, phosphorylation of Mig-6 may not affect its association with ERBB2. In contrast, we found that depletion of Chk1 inhibited not only phosphorylation of EGFR but also of ERBB2 and ERBB3 (new Figure 7A, lanes 2 *vs*. 4). Depletion of Mig-6 facilitated the phosphorylation of not only EGFR but also of ERBB2 and ERBB3 (new Figure 7A, lanes 2 *vs*. 6). These results suggest that Chk1 increases the activation of EGFR, ERBB2, and ERBB3 *via* inhibition of Mig-6, although the effects of Mig-6 phosphorylation on its association with EGFR family members could not be detected in this co-immunoprecipitation assay. Chk1-mediated phosphorylation of Mig-6 may modulate activation of EGFR family members *via* some conformational change in the EGFR-Mig-6 complex.

We have added the data to the new Figures 7A, and have added the text below to the Results section (page 13, line 22) and the Discussion section (page 17, line 6).

Page 13, line 22: “Next, we investigated whether phosphorylation of Mig-6 affects activation of other EGFR family members. As shown in Figure 7A, depletion of Mig-6 facilitated phosphorylation of not only EGFR but also of ERBB2 and ERBB3 (Figure 7A, lanes 2 *vs*. 6). Moreover, we found that depletion of Chk1 inhibited phosphorylation of not only EGFR but also of ERBB2 and ERBB3 (Figure 7A, lanes 2 *vs*. 4). These results suggest that Chk1 also enhances the activation of other EGFR members *via* phosphorylation of Mig-6.”

Page 17, line 6: “We noticed that no effect of Mig-6 phosphorylation on its association with EGFR family members was detected using co-immunoprecipitation (data not shown). We speculate that Chk1-mediated phosphorylation of Mig-6 may increase activation of the EGFR family members *via* some conformational change in the EGFR-Mig-6 complex. Alteration in the binding of Mig-6 to 14-3-3ξ may affect the conformation of Mig-6-EGFR family complex, although further study is required to clarify the mechanism.”

5) In Fig. 4, and elsewhere, the effects of Mig6 overexpression or RNAi on cell proliferation are small, as are (in Fig. 4B) the effects on EGFR phosphorylation. While statistically significant, it is unclear that these effects are that important, at least in the system being used.

As described above, we performed rescue experiments using siRNA-resistant Mig-6 wild type, S251A, or S251E. MDA-MB-231 cells were infected with retrovirally encoded Mig-6 wild type, S251A, or S251E, and then treated with siRNA for *Mig-6*. Cell proliferation assays were then performed. As shown in the new Figure 4, the doubling time of cells expressing S251A was significantly prolonged compared with that for the wild type and S251E mutant. The effect of S251E on auto-phosphorylation was almost the same as that of wild-type Mig-6, because Mig-6 is highly phosphorylated in MDA-MB-231 cells in normal culture conditions. Therefore, it is consistent that the effect of S251E on cell proliferation was almost the same as that of wild-type Mig-6. S251A also decreased cell proliferation in both HEK293 and MDA-MB-231 cells, although its effect was moderate. These results were reproducible in three independent experiments. We conclude that phosphorylation of Mig-6 at S251 is involved in cell proliferation *via* EGFR activation. As the referee pointed out, the contribution of the Chk1-Mig-6 pathway in EGFR activation is significant and reproducible but moderate, because EGF signaling is regulated by multiple mechanisms. In the revised manuscript, we state that the Chk1-Mig-6 pathway promotes EGFR activation as one of the regulatory mechanisms of EGF signaling.

We have revised Figure 4 and added text to the Results section as given in the answer to point 2. We have also added the following text to the Discussion section (page 16, line 10).

Page 16, line 10: “The contribution of the Chk1-Mig-6 pathway to EGFR activation is significant and reproducible but moderate, because EGF signaling is regulated by multiple mechanisms. We

believe that the Chk1-Mig-6 pathway modulates EGFR activation as one of the regulatory mechanisms of EGF signaling."

6) The only link to PI-3-kinase is a pharmacological study. Inasmuch as many PI-3-kinase inhibitors also block ATM and other related kinases (many of which can regulate Chk1), it is important to use other methods to implicate PI-3-kinase in Chk1 regulation.

We apologize for our errors in the units in Supplementary Table S1 and the old Figure 5E (the new Figure 5C); we have revised "mM" to the correct "mM".

To address the reviewer's comment, we examined whether depletion of ATM or ATR attenuates S280 phosphorylation of Chk1. As shown in the new Figure 5B, depletion of neither ATM nor ATR affected EGF-dependent S280 phosphorylation of Chk1. This result is consistent with those shown in Figure 2C, in which the ATM/ATR inhibitor caffeine did not affect the phosphorylation of Mig-6. Next, we investigated the PI3-kinase pathway, as shown in the new Figure 5, C–E. Both a PI3-kinase inhibitor and an Akt inhibitor inhibited S280 phosphorylation of Chk1 in a dose-dependent manner. We conclude that EGF-dependent S280 phosphorylation of Chk1 is mediated by the PI3-kinase/Akt pathway but not by the ATM/ATR pathway.

We have revised Supplementary Table S1 and the old Figure 5E (the new Figure 5C), have added the above data as the new Figure 5B, and have added the following text to the Results section (page 12, line 17).

Page 12, line 17: "As shown in Figure 5B, depletion of neither ATM nor ATR affected EGF-dependent S280 phosphorylation of Chk1. This is consistent with the data shown in Figure 2C, in which the ATM/ATR inhibitor caffeine did not affect the phosphorylation of Mig-6.

Next, we investigated the PI3-kinase pathway (Figure 5, C–E). The PI3K inhibitor LY294002 inhibited phosphorylation of not only Akt-S473 but also Chk1-S280 and Mig-6 (Figure 5C). Similarly Akt inhibitor IV inhibited S280 phosphorylation of Chk1 in a dose dependent manner (Figure 5D)."

7) In addition, there is not any information as to how PI-3-kinase regulates Chk1. Does Akt phosphorylate it directly?

Thank you for the comment. To address this, we performed *in vitro* phosphorylation experiments using recombinant Akt and Chk1. However, it was difficult to evaluate whether Akt directly phosphorylates S280 of Chk1, because recombinant Chk1 is autonomously phosphorylated at S345, S317, and also S280. Therefore, background phosphorylation was strongly detected, as indicated by asterisks in the new Supplementary Figure S8A, whereas anti-phosph-Chk1 (S280) antibody specifically recognized S280 phosphorylation of Chk1 (supplementary Figure S8B). S280 of Chk1 was slightly phosphorylated by Akt. We found that p70S6K, a downstream kinase of Akt, phosphorylated S280 of Chk1. Next, we investigated whether inhibition of Akt or p70S6K attenuated S280 phosphorylation of Chk1. As shown in the new Figure 5D, Akt inhibitor IV inhibited S280 phosphorylation of Chk1 in a dose-dependent manner. Depletion of p70S6K inhibited S280 phosphorylation of Chk1 (as shown in the new Figure 5E). These results suggest that p70S6K, a downstream kinase of Akt, is involved in phosphorylation of Chk1 at S280 *via* the EGFR-PI3K-Akt pathway.

We have added the above results as the new Figure 5, D and E, and Supplementary Figure S8. We have added the following text to the Results section (page 12, line 24).

Page 12, line 24: "To address whether Akt phosphorylates Chk1 at S280 directly, we performed *in vitro* phosphorylation experiments using recombinant Akt and Chk1. As shown in Supplementary Figure S8A, Akt-induced S280 phosphorylation of Chk1 was not observed. Whereas we found that p70S6K, a downstream kinase of Akt, phosphorylated Chk1 at S280. And further experiment confirmed that depletion of p70S6K inhibited EGF-induced S280 phosphorylation of Chk1 (Figure 5E). These results suggest that p70S6K, a downstream kinase of Akt, is involved in phosphorylation of Chk1 at S280 *via* the EGFR-PI3K-Akt pathway."

8) A minor point, the upper panel in Fig. 1B is dispensable since most of the drugs tested are not specific, and the lower panel is supported by RNAi data. This upper panel can be deleted.

As described above, we apologize for our errors in the units in Supplementary Table S1 and the old Figure 5E (the new Figure 5C); we have revised "mM" to the correct "mM". As the reviewer suggested, we have moved the old Figure 1B to the new Supplementary Figure S1.

Referee #2:

1) The authors are performing siRNA mediated depletion of Chk1 using only 1 siRNA oligo and need to rescue the key phenotypes (Figure 2D and 6A) by reintroducing Chk1 or at least use an independent siRNA oligo.

As the reviewer suggests, we designed other siRNA oligos such as *Chk1*(04) and *Mig-6*(05). Using these siRNAs, we repeated the experiments and obtained almost the same data as with the original siRNAs. As shown in Figure 2D and the new Supplementary Figure S2A, depletion of Chk1 by both siRNA(01) and (04) attenuated phosphorylation of Mig-6. As shown in Figure 6 and the new Supplementary Figures S9 and S11, depletion of Chk1 by both siRNA(01) and (04) attenuated auto-phosphorylation of EGFR. Moreover, depletion of Mig-6 by both siRNA(01) and (05) facilitated auto-phosphorylation of EGFR (new Supplementary Figures S9 and S11). We have not shown the data, but another anti-*Mig-6* oligo, siRNA (03), showed the same results. These results strongly suggest that Chk1 is involved in phosphorylation of Mig-6 and the regulation of EGFR activation.

We have added the data to the new Supplementary Figures S2A and S11. We have added the text below to the Results section (page 8, line 1, and page 13, line 16) and Methods section (page 21, line 23).

Page 8, line 1: "To confirm the Chk1-mediated Mig-6 phosphorylation, we designed and used another siRNA oligo, *Chk1*(04). As shown in Figure 2D and Supplementary Figure S2A, depletion of Chk1 by both siRNA(01) and (04) attenuated phosphorylation of Mig-6."

Page 13, line 16: "The data shown in Figure 6A and Supplementary Figure S9 were analyzed statistically and the results are presented in Figure 6B. Moreover, another *Chk1* siRNA oligo (04) showed the same result as did *Chk1* siRNA oligo (01) (Supplementary Figure S11). These data indicate that depletion of Chk1 results in decreased phosphorylation of EGFR."

Page 21, line 23: "The nucleotide sequence of the *Chk1*(01) siRNA was 5′-gcgugccguagacugucca-3′, *Chk1*(04) siRNA was 5′-gaaguugggcuaucaaugg-3′ with a 3′dTdT overhang. The nucleotide sequence of the human *Mig-6*(01) siRNA was 5′-cuacacuuucugauuucaa-3′, *Mig-6*(05) siRNA was 5′-gcaggguauccauucuuua-3′ with a 3′dTdT overhang. The nucleotide sequence of the human *ATR* siRNA was 5′-cctccgtgatgttgcttga-3′, *S6K1* siRNA was 5′-ggacatggcaggagtgttt-3′ with a 3′dTdT overhang. *ATM* siRNA used in this study was purchased from Ambion."

2) Fig.4) Ectopic expression of the wt and the S251A/E mutant forms of Mig6 show a very modest (barely significant) difference in the ability of the mutated forms of Mig6 to inhibit EGFR phosphorylation. Since this is a key result the authors should attempt to achieve a more clearcut results, possibly by improving transfection efficiency by stable transfection or lentiviral mediated delivery. Furthermore the authors should ectopically express active Chk1 which would be predicted to increase EGFR activation.

Thank you very much for the useful suggestion. We performed rescue experiments using siRNA-resistant Mig-6 wild type, S251A, or S251E. MDA-MB-231 cells were infected with retrovirally encoded Mig-6 wild type, S251A, or S251E, and then treated with an siRNA targeting

Mig-6. After EGF stimulation, auto-phosphorylation of EGFR was analyzed by immunoblotting. As shown in the new Figure 4, treatment with *Mig-6* siRNA increased the auto-phosphorylation of EGFR to 120% of control levels. Introduction of wild-type Mig-6 inhibited auto-phosphorylation of EGFR to 66% of that for the empty vector control. The S251A mutant suppressed the auto-phosphorylation of EGFR significantly more strongly than did wild-type Mig-6. Because Mig-6 is highly phosphorylated in EGF-stimulated MDA-MB-231 cells, the effect of S251E on auto-phosphorylation was almost the same as that of wild-type Mig-6. Next, we performed rescue experiments on cell proliferation using siRNA-resistant Mig-6 wild type, S251A, or S251E. As described above, MDA-MB-231 cells were infected with retrovirally encoded Mig-6 wild type, S251A, or S251E, followed by treatment with siRNA for *Mig-6*. Cell proliferation assays were performed. As shown in the new Figure 4, the doubling time of cells expressing S251A was significantly prolonged compared with that of wild type and the S251E mutant. Because Mig-6 is highly phosphorylated in MDA-MB-231 cells in normal culture conditions, it is consistent that the effect of S251E on cell proliferation was almost the same as that of wild-type Mig-6. These results were reproducible in three independent experiments.

As the reviewer suggested, we evaluated whether EGFR activation was promoted by over-expressed Chk1. We found that over-expression of Chk1 did not affect EGFR activation.

We have revised Figure 4 and added new text to the Results section (page 10, line 21, as detailed in the response to Reviewer 1, point 2.

Page 10, line 21: “For further information, we performed rescue experiments using siRNA-resistant Mig-6 wild type, S251A, or S251E. MDA-MB-231 cells were infected with retrovirally encoded Mig-6 wild type, S251A, or S251E, and then treated with an siRNA targeting *Mig-6*. After EGF stimulation, auto-phosphorylation of EGFR was analyzed by immunoblotting. As shown in Figure 4, A and B, treatment with a *Mig-6* siRNA increased the auto-phosphorylation of EGFR to approximately 120% of control levels. Introduction of wild-type Mig-6 inhibited the auto-phosphorylation of EGFR to 66% of that for the empty vector control. The S251A mutant suppressed the auto-phosphorylation of EGFR significantly more strongly than did wild-type Mig-6. Because Mig-6 is highly phosphorylated in EGF-stimulated MDA-MB-231 cells, the effect of S251E on auto-phosphorylation was almost the same as that of wild-type Mig-6. Next, we performed rescue experiments on cell proliferation using siRNA-resistant Mig-6 wild type, S251A, or S251E. As described above, MDA-MB-231 cells were infected with retrovirally encoded Mig-6 wild type, S251A, or S251E, and then treated with siRNA for *Mig-6*. Then, cell proliferation assays were performed. As shown in Figure 4C, the doubling time of cells expressing S251A was significantly prolonged compared with that of the wild-type and the S251E mutant. Because Mig-6 is highly phosphorylated in MDA-MB-231 cells in normal culture conditions, it is consistent that the effect of S251E on cell proliferation was almost the same as that of wild-type Mig-6. Moreover, we obtained almost the same data using HEK293 cells that were transfected with Mig-6 plasmids (Supplementary Figure S5). These results were reproducible in three independent experiments, and the effect of the S251A mutant was always moderate. Therefore, we conclude that phosphorylation of Mig-6 S251 is involved in the regulation of EGF signaling.”

3) Mig6 is known to bind active EGFR and the authors should address whether the S251A/E mutants exhibit different binding affinity for EGFR for example by co-immunoprecipitation.

As the reviewer suggests, we examined whether Chk1-dependent phosphorylation of Mig-6 affects its association with EGFR. Wild-type FLAG-Mig-6 or its mutants (such as S251A, S251E, or S302A) were transfected into HEK293 cells and were immunoprecipitated with anti-FLAG antibody. Then the immunoprecipitates were analyzed by immunoblotting with anti-EGFR antibody. It seemed that phosphorylation of Mig-6 did not affect its association with EGFR (attached Figure A1A). Next, we evaluated whether phosphorylation of Mig-6 affects its association with ERBB2, another member of the EGFR family. As shown in the attached Figure A1B, phosphorylation of Mig-6 did not affect its association with ERBB2. In contrast, we found that depletion of Chk1 inhibited phosphorylation of not only EGFR but also of ERBB2 and ERBB3 (new Figure 7A). Depletion of Mig-6 facilitated phosphorylation of not only EGFR but also of ERBB2 and ERBB3. These results suggest that Chk1 regulates the activation of EGFR, ERBB2, and ERBB3 *via* inhibition of Mig-6. Phosphorylation of Mig-6 may effect a conformational change in the Mig-6-

EGFR family complex, even though its effect was undetectable in a conventional *in vivo* binding assay.

We have added the new Figure 7A and have added new text to the Results section (page 13, line 22) and the Discussion section (page 17, line 6), as detailed in the response to Reviewer 1, point 4.

Page 13, line 22: "Next, we investigated whether phosphorylation of Mig-6 affects activation of other EGFR family members. As shown in Figure 7A, depletion of Mig-6 facilitated phosphorylation of not only EGFR but also of ERBB2 and ERBB3 (Figure 7A, lanes 2 *vs*. 6). Moreover, we found that depletion of Chk1 inhibited phosphorylation of not only EGFR but also of ERBB2 and ERBB3 (Figure 7A, lanes 2 *vs*. 4). These results suggest that Chk1 also enhances the activation of other EGFR members *via* phosphorylation of Mig-6."

Page 17, line 6: "We noticed that no effect of Mig-6 phosphorylation on its association with EGFR family members was detected using co-immunoprecipitation (data not shown). We speculate that Chk1-mediated phosphorylation of Mig-6 may increase activation of the EGFR family members *via* some conformational change in the EGFR-Mig-6 complex. Alteration in the binding of Mig-6 to 14-3-3ξ may affect the conformation of Mig-6-EGFR family complex, although further study is required to clarify the mechanism."

4) The Mig6S251 site is a predicted 14-3-3 binding site and the authors should show whether Mig6 binding to 14-3-3 is affected by the S251A/E mutations and whether gain and loss of Chk1 activity alters 14-3-3 binding to Mig6.

Thank you for your insightful suggestion. We examined whether phosphorylation of Mig-6 at S251 affects the binding of Mig-6 to 14-3-3. As shown in the new Supplementary Figure S12A, wild-type Mig-6 bound to 14-3-3b, d and ξ. Whereas S251A mutant showed low binding affinity to 14-3-3b and ξ but not d. Moreover, depletion of Chk1 attenuated the binding of Mig-6 to 14-3-3ξ but not to 14-3-3b or d (Supplementary Figure S12B). Phosphorylation of S251, which is located in the 14-3-3-binding domain of Mig-6, may be involved in the binding of Mig-6 to 14-3-3ξ. Alteration in the binding of Mig-6 to 14-3-3ξ may affect the conformation of the Mig-6-EGFR family complex.

We have added the data to the new Supplementary Figure S12 and the text below to the Discussion section (page 16, line 24).

Page 16, line 24: "Our data indicated wild-type Mig-6 bound to 14-3-3b, d and ξ (Supplementary Figure S12A). Whereas S251A mutant showed low binding affinity to 14-3-3b and ξ but not d. Moreover, Depletion of Chk1 attenuated binding of Mig-6 to 14-3-3ξ but not to 14-3-3b and d (Supplementary Figure S12B). Therefore, phosphorylation of S251, which is located in the putative 14-3-3 binding sequence in Mig-6, may be involved in binding of Mig-6 to 14-3-3ξ.

We noticed that no effect of Mig-6 phosphorylation on its association with EGFR family members was detected using co-immunoprecipitation (data not shown). We speculate that Chk1-mediated phosphorylation of Mig-6 may increase activation of the EGFR family members *via* some conformational change in the EGFR-Mig-6 complex. Alteration in the binding of Mig-6 to 14-3-3ξ may affect the conformation of Mig-6-EGFR family complex, although further study is required to clarify the mechanism."

5) Figure 6 shows that siRNA knockdown of Chk1 results in decreased phosphorylation of EGFR. This is a key result and needs to be repeated multiple times and statistical significance provided in figure 6B.

As the reviewer suggests, we repeated the experiment three times. As shown in the new Supplementary Figure S9, the results were almost the same as those shown in Figure 6A. The data were analyzed statistically and are presented in the new Figure 6B. Moreover, another *Chk1* siRNA

oligo (04) showed the same result (shown in the new Supplementary Figure S11). These data indicate that depletion of Chk1 results in decreased phosphorylation of EGFR.

We have added the data to the new Supplementary Figures S9 and S11, have revised the graph in Figure 6B and added the text below to the Results section (page 13, line 16).

Page 13, line 16: "The data shown in Figure 6A and Supplementary Figure S9 were analyzed statistically and the results are presented in Figure 6B. Moreover, another *Chk1* siRNA oligo (04) showed the same result as did *Chk1* siRNA oligo (01) (Supplementary Figure S11). These data indicate that depletion of Chk1 results in decreased phosphorylation of EGFR."

Referee #3:

1) It is regrettable that the authors do not have a phospho-specific antibody to Mig-6 residue S251. This makes the analyses and their interpretations more difficult, particularly when conclusions are drawn from studies with proteins expressed from transfected plasmids (i.e. not endogenous material).

As the reviewer suggests, we tried to raise phospho-specific polyclonal and monoclonal antibodies against p-S251 of Mig-6 using an extended phospho-peptide as the new antigen. However, the new antibodies could not detect a specific endogenous p-S251 Mig-6 signal, even though they could recognize S251 phosphorylation of Mig-6 by Chk1 using recombinant proteins (data not shown). We speculate that the neighboring sequence is not suitable for a high potential phospho-specific antibody.

2) To determine the role of S251-P without a phospho-specific antibody, the authors should knock down Mig-6 with siRNA and reconstitute the system with a RNA-resistant form of the protein that has a mutation at S281. The alanine and the glutamate mutations should both be made in parallel with the wild-type protein. Once reconstituted, the authors should determine the role of S251-P in EGF signaling. By only eliminating Chk1, one cannot conclude what is happening with respect to only the S251 site as Chk1 has multiple substrate targets. The reconstitution experiments allow the experimentalist to focus on only the site that is deemed to be important. This will greatly strengthen the arguments about S251-P and is a way to circumvent the absence of the phospho-antibody.

Thank you very much for the comment. We performed rescue experiments using siRNA-resistant Mig-6 wild type, S251A, or S251E. MDA-MB-231 cells were infected with retrovirally encoded Mig-6 wild type, S251A, or S251E, and then treated with an siRNA target for *Mig-6*. After EGF stimulation, auto-phosphorylation of EGFR was analyzed by immunoblotting. As shown in the new Figure 4, A and B, treatment with *Mig-6* siRNA increased auto-phosphorylation of EGFR to approximately 120% of control levels. Introduction of wild-type Mig-6 inhibited auto-phosphorylation of EGFR to 66% of that induced by the empty vector control. The S251A mutant suppressed the auto-phosphorylation of EGFR significantly more strongly than did wild-type Mig-6. Because Mig-6 is highly phosphorylated in EGF-stimulated MDA-MB-231 cells, the effect of S251E on auto-phosphorylation was almost the same as that of wild-type Mig-6. Next, we performed rescue experiments on cell proliferation using siRNA-resistant Mig-6 wild type, S251A, or S251E. As described above, MDA-MB-231 cells were infected with retrovirally encoded Mig-6 wild type, S251A, or S251E, and then treated with siRNA for *Mig-6*. Then, cell proliferation assays were performed. As shown in the new Figure 4C, the doubling time of cells expressing S251A was significantly prolonged compared with that of the wild-type and S251E mutant. Because Mig-6 is highly phosphorylated in MDA-MB-231 cells in normal culture conditions, it is consistent that the effect of S251E on cell proliferation was almost the same as that of wild-type Mig-6. Moreover, we obtained almost the same data using HEK293 cells that were transfected with Mig-6 plasmids (the old Figure 4, now moved to Supplementary Figure S5). These results were reproducible in three independent experiments, and the effect of the S251A mutant was always moderate. We conclude that phosphorylation of S251 in Mig-6 is involved in the regulation of EGF signaling.

In addition, because S251 is located in a putative 14-3-3-binding sequence in Mig-6, in the revised manuscript we examined whether phosphorylation of Mig-6 at S251 affects its binding

to 14-3-3. As shown in Supplementary Figure S12A, wild-type Mig-6 bound to 14-3-3b, d and ξ. Whereas S251A mutant showed low binding affinity to 14-3-3b and ξ but not d. Moreover, depletion of Chk1 attenuated the binding of Mig-6 to 14-3-3ξ but not to 14-3-3b or d. This suggests that phosphorylation of S251, which is located in the putative 14-3-3-binding sequence in Mig-6 may be involved in the binding of Mig-6 to 14-3-3ξ. Alteration in the binding of Mig-6 to 14-3-3ξ may affect the conformation of the Mig-6-EGFR family complex.

Moreover, in the revised manuscript we investigated whether phosphorylation of Mig-6 affects activation of other EGFR family members. As shown in the new Figure 7A, depletion of Mig-6 facilitated phosphorylation of not only EGFR but also of ERBB2 and ERBB3. Moreover, we found that depletion of Chk1 inhibited phosphorylation of not only EGFR but also of ERBB2 and ERBB3. These results suggest that Chk1 also modulates the activation of ERBB2 and ERBB3 *via* Mig-6.

Taken together, these results indicate that Chk1 regulates activation of the EGFR family proteins *via* S251 phosphorylation of Mig-6.

We have revised Figure 4 and added a new Figure 7A and Supplementary Figure S12. We have added the text below to the Results section (page 10, line 21, page 13, line 22) and the Discussion section (page 16, line 24)

Page 10, line 21: “For further information, we performed rescue experiments using siRNA-resistant Mig-6 wild type, S251A, or S251E. MDA-MB-231 cells were infected with retrovirally encoded Mig-6 wild type, S251A, or S251E, and then treated with an siRNA targeting *Mig-6*. After EGF stimulation, auto-phosphorylation of EGFR was analyzed by immunoblotting. As shown in Figure 4, A and B, treatment with a *Mig-6* siRNA increased the auto-phosphorylation of EGFR to approximately 120% of control levels. Introduction of wild-type Mig-6 inhibited the auto-phosphorylation of EGFR to 66% of that for the empty vector control. The S251A mutant suppressed the auto-phosphorylation of EGFR significantly more strongly than did wild-type Mig-6. Because Mig-6 is highly phosphorylated in EGF-stimulated MDA-MB-231 cells, the effect of S251E on auto-phosphorylation was almost the same as that of wild-type Mig-6. Next, we performed rescue experiments on cell proliferation using siRNA-resistant Mig-6 wild type, S251A, or S251E. As described above, MDA-MB-231 cells were infected with retrovirally encoded Mig-6 wild type, S251A, or S251E, and then treated with siRNA for *Mig-6*. Then, cell proliferation assays were performed. As shown in Figure 4C, the doubling time of cells expressing S251A was significantly prolonged compared with that of the wild-type and the S251E mutant. Because Mig-6 is highly phosphorylated in MDA-MB-231 cells in normal culture conditions, it is consistent that the effect of S251E on cell proliferation was almost the same as that of wild-type Mig-6. Moreover, we obtained almost the same data using HEK293 cells that were transfected with Mig-6 plasmids (Supplementary Figure S5). These results were reproducible in three independent experiments, and the effect of the S251A mutant was always moderate. Therefore, we conclude that phosphorylation of Mig-6 S251 is involved in the regulation of EGF signaling.”

Page 13, line 22: “Next, we investigated whether phosphorylation of Mig-6 affects activation of other EGFR family members. As shown in Figure 7A, depletion of Mig-6 facilitated phosphorylation of not only EGFR but also of ERBB2 and ERBB3 (Figure 7A, lanes 2 *vs*. 6). Moreover, we found that depletion of Chk1 inhibited phosphorylation of not only EGFR but also of ERBB2 and ERBB3 (Figure 7A, lanes 2 *vs*. 4). These results suggest that Chk1 also enhances the activation of other EGFR members *via* phosphorylation of Mig-6.”

Page 16, line 24: “Our data indicated wild-type Mig-6 bound to 14-3-3b, d and ξ (Supplementary Figure S12A). Whereas S251A mutant showed low binding affinity to 14-3-3b and ξ but not d. Moreover, Depletion of Chk1 attenuated binding of Mig-6 to 14-3-3ξ but not to 14-3-3b and d (Supplementary Figure S12B). Therefore, phosphorylation of S251, which is located in the putative 14-3-3 binding sequence in Mig-6, may be involved in binding of Mig-6 to 14-3-3ξ.

We noticed that no effect of Mig-6 phosphorylation on its association with EGFR family members was detected using co-immunoprecipitation (data not shown). We speculate that Chk1-mediated phosphorylation of Mig-6 may increase activation of the EGFR family members *via* some

conformational change in the EGFR-Mig-6 complex. Alteration in the binding of Mig-6 to 14-3-3ξ may affect the conformation of Mig-6-EGFR family complex, although further study is required to clarify the mechanism."

3) Additionally, the authors should widen the scope and include the additional phosphorylation sites that were identified by mass spec.

As the reviewer points out, we tested additional phosphorylation site such as S302 in which phosphorylation as well as S251 was identified by mass spec in *in vitro*, *in vivo* and endogenous status (Figure 5F). As shown in the new Supplementary Figure S6A, S302A/S334A/S369A mutant showed same inhibitory effect on auto-phosphorylation of EGFR as wild-type Mig-6 in HEK293 cells. Further more, retrovirally expression of Mig-6 S302A had no effect on both auto-phosphorylation of EGFR and proliferation in MDA-MB-231 cells (new Supplementary Figure S6, B-D). All together, we think that S251 but no S302 in Mig-6 is a main phosphorylation site which is involved in regulation of EGFR-activation.

We added the new supplementary Figure S6 and the results to the Result section (Page 11 line 18).

Page 11 line 18: "We tested additional phosphorylation site such as S302 in which phosphorylation as well as S251 was identified by mass spec in *in vitro*, *in vivo* and endogenous status (Figure 5F). As shown in the Supplementary Figure S6A, S302A/S334A/S369A mutant showed same inhibitory effect on auto-phosphorylation of EGFR as wild-type Mig-6 in HEK293 cells. Further more, retrovirally expression of Mig-6 S302A had no effect on both auto-phosphorylation of EGFR and proliferation in MDA-MB-231 cells (Supplementary Figure S6, B-D)."

4)*The data regarding EGF stimulation of phosphorylation of Chk1 at 280 does not appear to add anything new to the paper as this has been previously reported (Puc et al., 2005 which is referenced within the paper). As it stands, it is actually a distraction and should be moved to the supplemental data.*

As per the reviewer's comment, we have moved the old Figure 5, A–C to the new Supplementary Figure S7.

In addition, in the revised manuscript we found that p70S6K, a downstream kinase of Akt, phosphorylated S280 of Chk1 (Supplementary Figure S8). Next, we investigated whether inhibition of Akt or p70S6K attenuated S280 phosphorylation of Chk1. As shown in the new Figure 5D, Akt inhibitor IV inhibited S280 phosphorylation of Chk1 in a dose-dependent manner. Depletion of p70S6K inhibited S280 phosphorylation of Chk1 (as shown in the new Figure 5E). We found that p70S6K, a downstream kinase of Akt, phosphorylated S280 of Chk1, whereas S280 of Chk1 was slightly phosphorylated by Akt (Supplementary Figure S8). In contrast, depletion of neither ATM nor ATR affected EGF-dependent S280 phosphorylation of Chk1, as shown in the new Figure 5B. This is consistent with the data shown in Figure 2C, in which the ATM/ATR inhibitor caffeine did not affect the phosphorylation of Mig-6. These results suggest that p70S6K, a downstream kinase of Akt, is involved in the phosphorylation of Chk1 at S280 *via* the EGFR-PI3K-Akt pathway but not the ATM/ATR pathway.

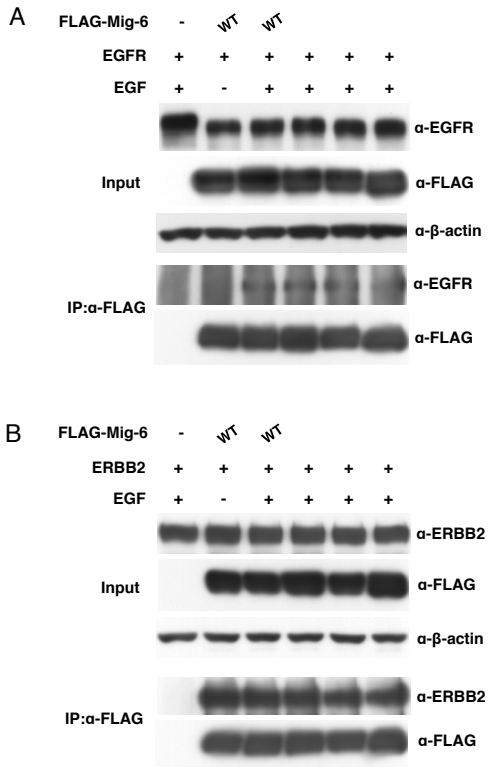
We have added the results to the new Figure 5, B, D, and E, and to Supplementary Figure S8. We have added the text below to the Results section (page 12, line 17).

Page 12, line 17: "As shown in Figure 5B, depletion of neither ATM nor ATR affected EGF-dependent S280 phosphorylation of Chk1. This is consistent with the data shown in Figure 2C, in which the ATM/ATR inhibitor caffeine did not affect the phosphorylation of Mig-6.

Next, we investigated the PI3-kinase pathway (Figure 5, C–E). The PI3K inhibitor LY294002 inhibited phosphorylation of not only Akt-S473 but also Chk1-S280 and Mig-6 (Figure 5C). Similarly Akt inhibitor IV inhibited S280 phosphorylation of Chk1 in a dose dependent manner (Figure 5D). To address whether Akt phosphorylates Chk1 at S280 directly, we performed *in vitro* phosphorylation experiments using recombinant Akt and Chk1. As shown in Supplementary Figure S8A, Akt-induced S280 phosphorylation of Chk1 was not observed. Whereas we found that p70S6K,

a downstream kinase of Akt, phosphorylated Chk1 at S280. And further experiment confirmed that depletion of p70S6K inhibited EGF-induced S280 phosphorylation of Chk1 (Figure 5E). These results suggest that p70S6K, a downstream kinase of Akt, is involved in phosphorylation of Chk1 at S280 *via* the EGFR-PI3K-Akt pathway."

Liu *et al.* Attached Figure A1



Attached FigureA. Effect of Mig-6 phosphorylation on its association with EGFR family members.

A, B. Plasmids encoding human EGFR (A), or ERBB2 (B) were co-expressed with wild type (WT), or the indicated mutant FLAG-Mig-6 into HEK293 cells. Quiescence was induced by 16 h serum starvation, then the cells were stimulated with 20 ng/ml EGF for 15 min. The cell lysates were subjected to immunoprecipitation with anti-FLAG antibody, followed by immunoblotting with the indicated antibodies.

2nd Editorial Decision 03 February 2012

Thank you very much for submission of your revised paper that has now been seen by two of the original referees. Although one of them raises no further concerns, the second brings up the issue of standard errors/error bars. As we take this rather seriously since it impinges on data quality and

reproducibility, this certainly needs to be thoroughly and satisfactorily addressed, similar to the remaining questions from this referee.

Please also notice that The EMBO Journal (in the very likely event of eventual publication) encourages the provision of source data, particularly for electrophoretic gels and blots, with the aim of making primary data more accessible and transparent to the reader. This is for the moment a voluntary policy to present un-cropped/unprocessed scans minimally for data central to the published work. We would thus be grateful for such files that should each combine un-cropped blots presented in an individual figure(s).
Please let me know if you have any questions about this AND please check the below URL for a recent example:
http://www.nature.com/emboj/journal/v30/n20/suppinfo/emboj2011298as1.html

I am very much looking forward to your final revisions.

Yours sincerely,

Editor
The EMBO Journal

REFEREE REPORTS:

Referee #2:

Revisions are satisfactory and my concerns have now been addressed.

Referee #3:

Overall I think that the paper has improved, but there are still a few issues:

1. . Fig. 6 D, E. There is a big problem with the error bar presentation. In most of the data points, only one side of the error bar is shown, but if both sides (the full error bar) were shown it clearly seems that the data wouln not be statistically significant as the bars would overlap. In Fig. 6D, the 8 hour time point has a "full" error bar, while the other data points do not. Why is this the case? Please put the full error bars in all data points to show how significant the overlap is. And then in other figures (7C) there are no error bars....

2. Figure 2 phosphorylation and other phorphorylation figures. In some cases, the phosphorylation is a large smear (Fig. 2C) and in others (Fig 3C0, it appears to migrate as two distinct bands). I do not recall seeing any phosphatase treatment to collapse the band and to control for the phos-tag. This would be comforting. This will also address the question regarding the nature of posttranslational events outside of phosphorylation that might affect band migration.

3. What is the IEC50 of SB218078?

4. Figure 3 A. There is no loading control for the retrovirally expressed mutants to make sure that they actually expressed protein.

Point-by-point response to reviewer #3

Referee #3:

1. Fig. 6 D, E. There is a big problem with the error bar presentation. In most of the data points, only one side of the error bar is shown, but if both sides (the full error bar) were shown it clearly seems that the data wouln not be statistically significant as the bars would overlap. In Fig. 6D, the 8 hour time point has a "full" error bar, while the other data points do not. Why is this the case? Please put the full error bars in all data points to show how significant the overlap is. And then in other figures (7C) there are no error bars....

As the reviewer suggested, we have revised Figure 6 to show full error bars, as shown in Attached Figure A1. We believe that it is difficult to avoid some unevenness of the data in this type of time-course experiment. What is important, however, is whether the conclusions drawn from these results are reproducible. Therefore, we have indicated each of three independent sets of data. As shown in the new Figure 6 (expt. 1) and Supplementary Figure S8 (expts. 2 and 3), depletion of Chk1 attenuated EGFR phosphorylation and ERK phosphorylation at 0.25–0.50 h after EGF treatment; in contrast, depletion of Mig-6 enhanced EGFR phosphorylation and ERK phosphorylation in the same period. These tendencies were reproducible in the three independent experiments. As we mention in the Discussion, attenuation of Mig-6 activity by Chk1 may be required for efficient activation of EGFR in the early stages of EGF signaling.

Additionally, as the reviewer suggested, we have performed the three independent experiments of Figure 7C to show error bar, and indicated in new Figure 7C and D.

We have revised Figure 6 and Supplementary Figure S8 and revised the text below to the Result section (page 14, line 14-18). Moreover, we have revised to Figure 7C and D with error bars.

Page 14, line 14-18: “Depletion of Chk1 attenuated EGFR phosphorylation and ERK phosphorylation at 5-30 min after EGF treatment. In contrast, depletion of Mig-6 enhanced EGFR phosphorylation and ERK phosphorylation in the same period (Figure

6, Supplementary Figure S9). These tendencies were reproducible in three independent experiments (Figure 6 and Supplementary Figure S8)."

2. Figure 2 phosphorylation and other phorphorylation figures. In some cases, the phosphorylation is a large smear (Fig. 2C) and in others (Fig 3C, it appears to migrate as two distinct bands). I do not recall seeing any phosphatase treatment to collapse the band and to control for the phos-tag. This would be comforting. This will also address the question regarding the nature of posttranslational events outside of phosphorylation that might affect band migration.

As the reviewer suggested, we have performed a phosphatase-treatment experiment to prove the EGF-promoted phosphorylation of Mig-6 by Phos-tag gel analysis. As shown in the new Supplementary Figure S2B, the smeared Mig-6 band was apparently shifted down by Alkaline Phosphatase treatment. Therefore, the smeared Mig-6 band in the Phos-tag gel is because of phosphorylation via EGF treatment. Because the lot number of the Phos-tag solution has changed, we repeated the experiments in Figure 2C and D and used the new data in the figure.

We have added Supplementary Figure S2B, revised Figure 2C and D, and added the text below to the Results section (page 8, line 23 - page 9, line 3).

Page 8, line 23 - page 9, line 3: "Furthermore, we performed a phosphatase-treatment experiment to prove that the smeared Mig-6 band on the Phos-tag gel was because of phosphorylation (Supplementary Figure S2B). We also demonstrated that the smeared Mig-6 band prepared from EGF-stimulated MDA-MB-231 cells on a Phos-tag gel did not indicate ubiquitylation (Supplementary Figure S2C)."

3. What is the IEC50 of SB218078?

First, we noticed an error in the units of the old Figure 2B, and have revised "mM" to the correct "μM".

As shown in Figure 2B and Attached Figure A2B, 0.02 μM of SB-218078 inhibited phosphorylation of Mig-6 by Chk1 to about 50% *in vitro*. The data are consistent with a previous report that the IC50 value of SB-218078 against recombinant Chk1 is 0.015 μM (Jackson *et al*, Cancer Res. 60, 566-572, 2000). Moreover, we indicated that 1, 5, and 10 μM of SB-218078 (Figure A2A) inhibited the phosphorylation of endogenous Mig-6 in MDA-MB231 cells. Jackson *et al* also reported that 2.5 μM of SB-218078 inhibited the G2-DNA damage checkpoint mediated by Chk1 (Cancer Res. 60, 566-572, 2000). This report is in accordance with our findings.

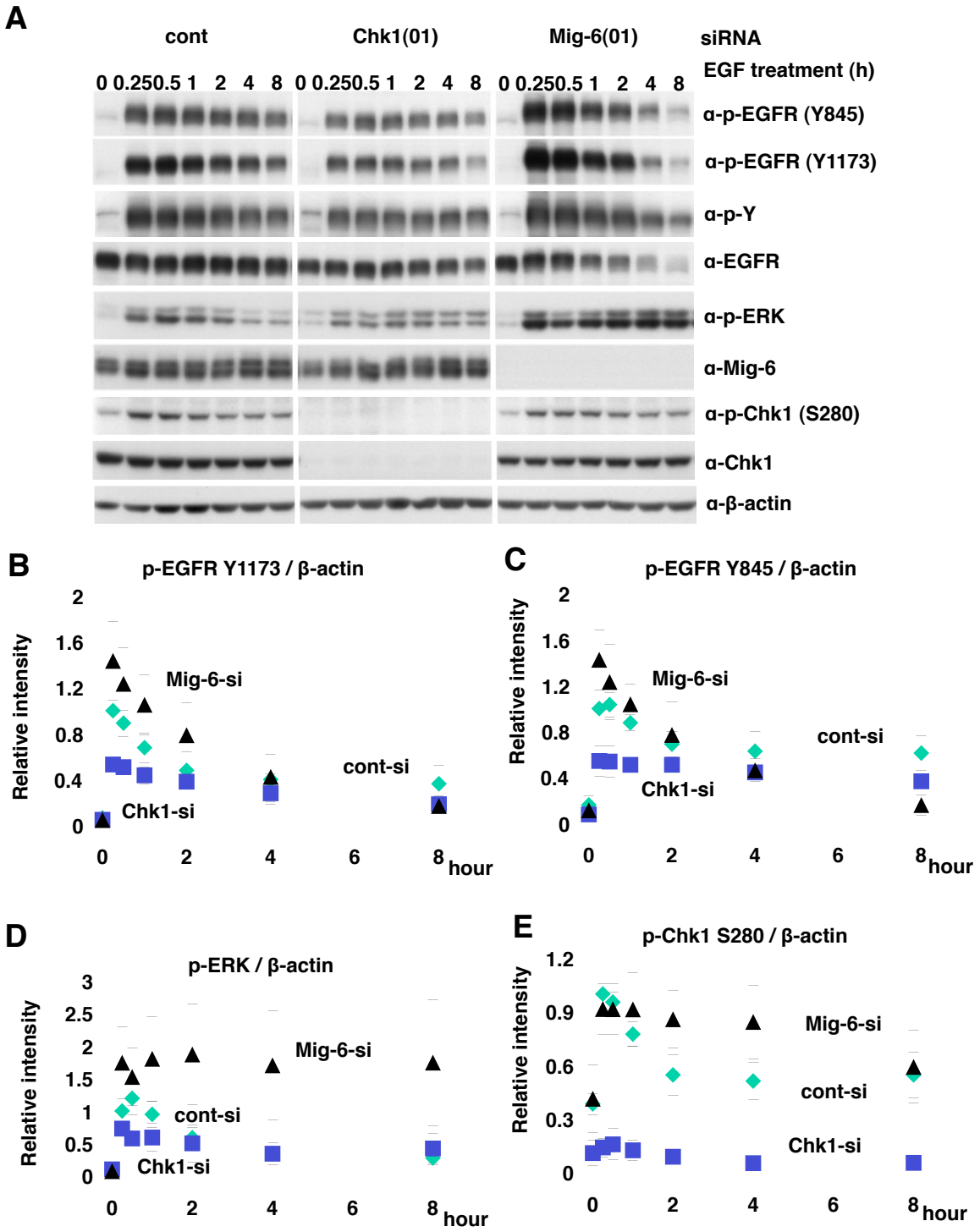
4. Figure 3 A (provably Figure 4A)*. There is no loading control for the retrovirally expressed mutants to make sure that they actually expressed protein.*

As the reviewer suggests, we have reprobed the blot shown in Figure 4A with an anti-FLAG antibody to detect retrovirally expressed FLAG-Mig-6 mutant proteins. The arrow indicates the retrovirally expressed wild-type and mutant full-length FLAG-Mig-6 protein. The asterisk indicates retrovirally expressed Mig-6 protein without the FLAG-tag, which is translated using the original first methionine of the *Mig-6* cDNA.

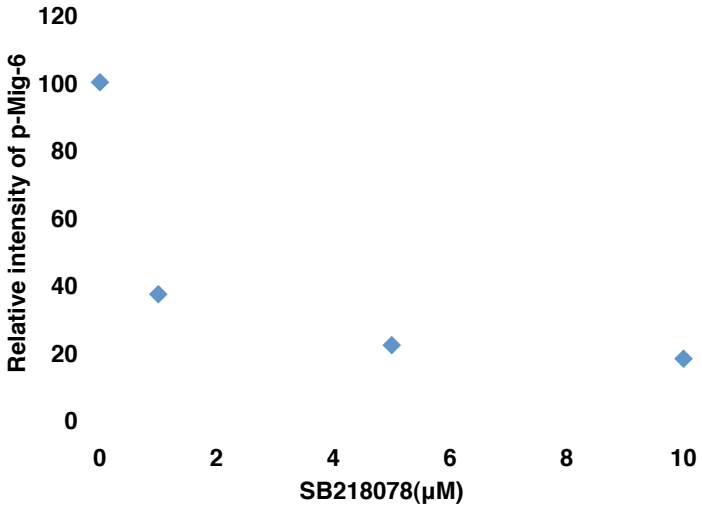
We have added the above data to Figure 4A, and have added the following description to the legend for Figure 4A (page 35, line 24 –page 36, line 1).

P age 35, line 24 –page 36, line 1: “The asterisk indicates retrovirally expressed Mig-6 protein without the FLAG-tag, which is translated using the original first methionine of the *Mig-6* cDNA.”

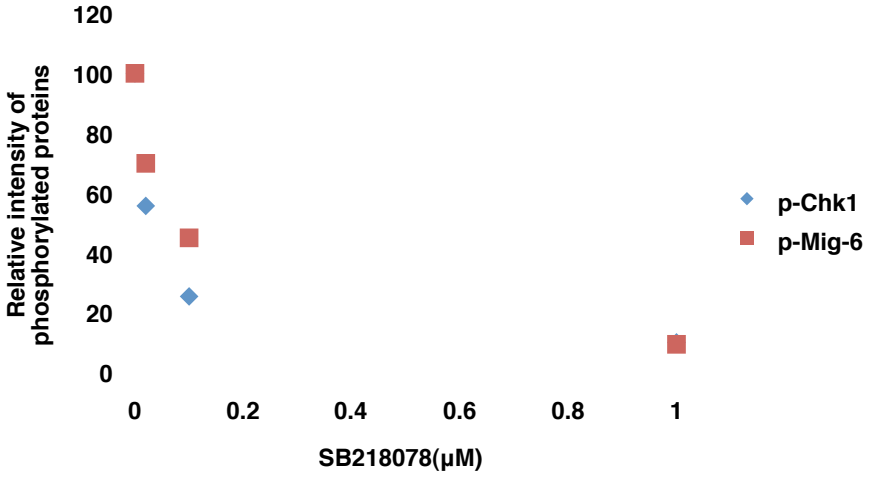
Liu_Attached Figure A1 (Figure 6)



A Graph for Figure 1C



B Graph for Figure 2B



Attached Figure A1. Effects of SB218078 on Mig-6 phosphorylation *in vivo* and *in vitro*. (A) Phosphorylated Mig-6 bands in Figure 1C were scanned and the relative intensities were graphically indicated. (B) Phosphorylated Mig-6 bands and auto-phosphorylated Chk1 bands in Figure 2B were scanned and the relative intensities were graphically indicated.