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CHIP-dependent termination of MEKK2 regulates temporal ERK activation required for proper hyperosmotic response

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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 31 March 2010

Thank you very much for submitting your research manuscript for consideration to The EMBO Journal editorial office.
The enclosed comments reveal that the referee's appreciate the identification of CHIP in terminating MEKK2 signaling to ERK during osmo-stress. However, they also raise important points on the completeness and conclusiveness of the presented dataset that would require significant additional work. Ref#1 raises a number of points for instance phosphorylation versus catalytic-activity dependent binding, quantification of MEKK2-degradation by CHIP and importantly, the possibility that CHIP might regulate ERK-targets not exclusively via the proposed MEKK2-degradation (otherwise, the distinct effects on ERK-targets could hardly be reconciled). Related concerns are raised from refs#2 and #3 that altogether still find the study interesting enough to at least offer you the chance to address their concerns during major revisions. I do have to ask you to take their comments and concerns really serious and address them concisely as it is EMBO_J policy to allow a single round of major revisions only. Also, the final decision on acceptance or rejection of a thoroughly revised paper will depend on a critical assessment of some of these scientists!

Thank you for the opportunity to consider your work for publication.

Yours sincerely,

Editor
The EMBO Journal

REFEREE COMMENTS

Referee #1 (Remarks to the Author):

In this manuscript Maruyama et al. characterize the activation of ERK in response to hyperosmotic stress. The activation of this MAPK is observed at really high osmolarity together with the activation of p38 and JNK MAPKs. However, it is only ERK activation that is affected by the down-regulation of the MAP3K MEKK2. In addition, the authors show that the MAP3K interacts with CHIP, define the interaction domains and show that CHIP is responsible for the ubiquitination and degradation of MEKK2, especially in response to sorbitol. The manuscript also demonstrates that the down-regulation of this ubiquitin-ligase affects ERK activation. This is a very well executed piece of work with really nice biochemical approaches that lead to solid conclusions.

A major concern of this reviewer is related with the role of CHIP in regulation of ERK signaling and the differential expression of ELK-responsive genes and AQP1/5. It seems from experiments presented in figure 4C that binding of CHIP occurs when sorbitol is removed (which does not fit with the fact that binding of CHIP and MEKK2 occurs in the presence of sorbitol (figure 3C)) however, it would suggest that the binding might serve to down-regulate ERK signaling. This is nicely confirmed by data in Figures 5A and B in ELK dependent expression and ERK phosphorylation. However, then data on expression of MEKK2-dependent TonEBP, AQP1 and AQP5 is presented. Here, down-regulation of gene expression, AQP1 and 5 but not TonEBP is shown to be dependent on CHIP. This seems to be contradictory with all the data presented before and suggested an alternative scenario where CHIP might be doing something different than proposed. If this part is to be presented, then more studies are needed such as transcriptional analysis, recruitment of PolII and ERK to those promoters and the characterization of the kinetics of induction in response to sorbitol. Otherwise, I suggest removing this part since it could represent an additional role for CHIP in transcriptional regulation rather than controlling ERK signaling by MEKK2 ubiquitination.

The biological relevance of ERK activation is questionable since the concentration of osmolites used for the experiments results in cell death after a while, similar studies using a lower concentration of osmolites should be performed. At least those related to activation of ERK and interaction of MEKK2 with CHIP.

The authors claim that binding of endogenous MEKK2 to CHIP (Figure 3C) is susceptible of degradation. However, the intensity of the bands is too weak and they do not present what happens in the absence of MG132 at 4 hours. Therefore, other possibilities exist to explain this result such as that in the presence of MG132 more MEKK2 is present (or ubiquinated). Thus, it would be interesting to see whether binding of MEKK2 to CHIP in transfected cells increases in the presence of stress.

The authors conclude from the analysis of binding between CHIP and MEKK2 wild type and catalytically inactive that CHIP interacts depending on a kinase activity-dependent manner. This might not be true, other scenarios are possible such as that phosphorylation is important rather than activity. Actually, the molecular weight of the KN and wild type are very different on the blots presented on figure 2C and later acknowledged that this might correspond to different degree of phosphorylation. It is proposed that the binding of CHIP and MEKK2 occurs with dephosphorylated MEKK2. However, figure 2C shows that it is possibly the phosphorylated MEKK2 that is able to interact with CHIP. Similarly, it is in response to sorbitol that the binding of MEKK2 and CHIP is stronger, and it is under stress-treatment that the MEKK2 is activated and trans-phosphorylated. Therefore, it seems that there is a contradictory scenario here that is not completely understood. May be a more profound analysis of the phosphorylation sites important for activation and binding could help to resolve this point.

Referee #2 (Remarks to the Author):

The manuscript *CHIP-dependent termination of MEKK2 regulates
temporal ERK activation required for proper hyperosmotic response* reports that MEKK2, a member of the MAP3K family, specifically regulates activation of ERK, but not JNK or p38 in response to sorbitol. A novel role was investigated for the E3 ubiquitin ligase, CHIP, in degradation and ultimately regulation of MEKK2 and ERK in response to sorbitol.
This is an interesting story and there is a large amount of data shown in support of the conclusions. Some data are convincing, but the pieces do not link together tightly. Several important issues that are not discussed or that depend on overexpression that keep this manuscript from telling a strong story.

1) MEKK1 has been reported by Tony Hunter*s group to be involved in ERK regulation in response to sorbitol. Where does MEKK1 fit in this picture?

2) All of the experiments involve overexpressed proteins. Some of the proteins in the pathway are not abundant. Overexpression is likely to skew the findings. The authors claim that MEKK2 binds to and is ubiquitinated by CHIP in response to hyperosmotic stress.
Co-immunoprecipitation of endogenous proteins or some equivalent
demonstration in response to sorbitol or any other inducer of osmotic stress seems like a minimal requirement to substantiate this story.

3) The CHIP rescue experiment is not convincing. Will another ligase rescue as well as CHIP?

4) The steps implied by the proposed mechanism have not been
sufficiently delineated. The authors state that dephosphorylation is required for ubiquitination and degradation of MEKK2. Labeling of the protein is a generic way to demonstrate a phosphorylation/dephosphorylation mechanism, but is not directly linked to the subsequent events. Is dephosphorylation causing inactivation of MEKK2? If so, degradation of MEKK2 may not be the essential event. The connections are tenuous.

5) Is there evidence that this event occurs in response to sorbitol treatment?

Referee #3 (Remarks to the Author):

Maruyama and colleagues show that the MAP3K MEKK2 is necessary for ERK activation by hyperosmotic stress (at least at 1h stimulation). They also show that during the activation cycle of MEKK2 by hyperosmolarity, MEKK2 undergoes hyperphosphorylation, then dephosphorylation, at which time MEKK2 associates with the E3 ubiquitin (Ub) ligase CHIP. CHIP does not, however, associate with an inherently kinase-inactive form of MEKK2, nor does it associate with inactive, dephospho-MEKK2 in resting cells. CHIP is required to attenuate a prolonged (12 h) ERK activation seen after withdrawal of a 1h hyperosmolarity stimulus. This ERK deactivation appears necessary for induction of aquaporins (Aqp)-1 and -5.

These results are interesting and potentially quite significant. However, there are several missing mechanistic considerations that need to be addressed.

1) The MEKK2 RNAi data are for only 1 hour of hyperosmotic treatment, and MEKK3 has been implicated in hyperosmolar activation of ERK. The effect of MEKK2 RNAi should encompass the whole time course (5 min-12 h) and be compared with MEKK3 RNAi. Thus it will be clear if MEKK2 is required only for the prolonged phase of ERK activation. This is especially an issue given the data in Fig. 1B showing that MEKK2's kinase activity is activated well after ERK activation goes up (30 min vs. 15 min).

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nonspecific, really does preferentially inhibit phosphatase-2A. Does PP2A catalytic subunit associate with active-phosphorylated MEKK2 before the interaction with CHIP? In a transfection setting, does coexpression of PP2A-C subunit (or A subunit) enhance the binding of CHIP to MEKK2?

3) Fig. 5B needs an MEKK2 RNAi study as described in point "1" above (the experiment can go here or with Fig. 1).

4) Of particular importance, it would be ideal if the authors could provide some insight into how ERK deactivation is translated into an induction of Aqp1/5. The complete picture is not needed, but something should be included to address this issue.

1st Revision - authors' response 15 May 2010

Reviewer #1:

In this manuscript Maruyama et al. characterize the activation of ERK in response to hyperosmotic stress. The activation of this MAPK is observed at really high osmolarity together with the activation of p38 and JNK MAPKs. However, it is only ERK activation that is affected by the down-regulation of the MAP3K MEKK2. In addition, the authors show that the MAP3K interacts with CHIP, define the interaction domains and show that CHIP is responsible for the ubiquitination and degradation of MEKK2, especially in response to sorbitol. The manuscript also demonstrates that the down-regulation of this ubiquitin-ligase affects ERK activation. This is a very well executed piece of work with really nice biochemical approaches that lead to solid conclusions.

A major concern of this reviewer is related with the role of CHIP in regulation of ERK signaling and the differential expression of ELK-responsive genes and AQP1/5. It seems from experiments presented in figure 4C that binding of CHIP occurs when sorbitol is removed (which does not fit with the fact that binding of CHIP and MEKK2 occurs in the presence of sorbitol (figure 3C)) however, it would suggest that the binding might serve to down-regulate ERK signaling. This is nicely confirmed by data in Figures 5A and B in ELK dependent expression and ERK phosphorylation. However, then data on expression of MEKK2-dependent TonEBP, AQP1 and AQP5 is presented. Here, down-regulation of gene expression, AQP1 and 5 but not TonEBP is shown to be dependent on CHIP. This seems to be contradictory with all the data presented before and suggested an alternative scenario where CHIP might be doing something different than proposed. If this part is to be presented, then more studies are needed such as transcriptional analysis, recruitment of PolII and ERK to those promoters and the characterization of the kinetics of induction in response to sorbitol. Otherwise, I suggest removing this part since it could represent an additional role for CHIP in transcriptional regulation rather than controlling ERK signaling by MEKK2 ubiquitination.

Re: We appreciate the reviewer's suggestion. The data on the sorbitol-induced expression of TonEBP and AQPs is an important point to assess the physiological role of CHIP-dependent down-regulation of MEKK2-ERK pathway. To address the reviewer's concern, we have examined the kinetics of the gene expression and the requirement of ERK activity for the induction of these genes. We confirmed that MEK-ERK activity was required for the inductions of mRNAs of TonEBP, AQP1, and AQP5 in response to sorbitol (Figure 6A). Interestingly, whereas TonEBP mRNA was rapidly induced within 1h after stimulation, AQP1 and AQP5 were relatively slowly induced after 4 h (Figure 6A). These results suggest, as this reviewer pointed-out, that different mechanisms may be involved in the inductions of AQP1/AQP5 and TonEBP. The reason why CHIP knockdown-dependent prolonged ERK activity did not affect the induction of TonEBP (Fig. 5F, 6C) may be because the ERK activation at an early phase may not only be necessary but also sufficient for TonEBP induction. In contrast, AQP1/AQP5 may not be simply induced by the activation of ERK (Figure 6D), and AQP1/AQP5 may require CHIP-dependent down-regulation of ERK activity at the prolonged phase (Figure 6F, G). Since the time course of CHIP-dependent ERK down-regulation which starts after 4h (Figure 5B, C) correlated well with the onset of induction of AQP1/AQP5 (Figure 6A), these new data further strengthens our model that not only the early phase activation but also the late phase inhibition of ERK is required for the induction of AQP1/AQP5. Indeed,

U0126-mediated artificial transient ERK activation reversed the down-regulation of AQP1/AQP5 expression in CHIP knockdown cells (Figure 6F, G). Although still we cannot rule out the possibility that CHIP contributes not only to MEKK2 degradation but also to other unknown functions, we may at least conclude that CHIP-dependent down-regulation of MEKK2-ERK pathway contributes to the expression of AQP1 and AQP5 in response to hyperosmotic stress. We included this new data (Figure 6A) and related discussion (p15 9-p17 13) in the revised manuscript.

The biological relevance of ERK activation is questionable since the concentration of osmolites used for the experiments results in cell death after a while, similar studies using a lower concentration of osmolites should be performed. At least those related to activation of ERK and interaction of MEKK2 with CHIP.

Re: We understand the reviewer's concern. Since the transient treatment with 500 mM sorbitol for 1 h in HEK293 cells does not induce cell death, we believe this concentration of sorbitol represented the physiological condition to some extent. However, in order to examine the physiological relevance of this interaction in more detail, we performed the experiments using lower concentrations of sorbitol. The treatment with 100 mM and 300 mM sorbitol, which induced slight and moderate activations of ERK, respectively (Figure 1A), also induced the interaction between MEKK2 and CHIP (Figure S4A, please see long exposure panel, lanes 5, 6, 8, 9). We included this new supplemental data (Figure S4A) and related discussion (p11 22-p12 3) in the revised manuscript.

The authors claim that binding of endogenous MEKK2 to CHIP (Figure 3C) is susceptible of degradation. However, the intensity of the bands is too weak and they do not present what happens in the absence of MG132 at 4 hours. Therefore, other possibilities exist to explain this result such as that in the presence of MG132 more MEKK2 is present (or ubiquinated). Thus, it would be interesting to see whether binding of MEKK2 to CHIP in transfected cells increases in the presence of stress.

Re: We newly examined whether MEKK2 interacts with CHIP in the absence or presence of MG132 at 4 h and 8 h, and incorporated the new data as Figure 3C. The endogenous MEKK2-CHIP interaction was clearly observed only in the presence of MG132 without apparent changes in the amount of MEKK2 (Figure 3C, lanes 5-8), supporting our original conclusion. The sorbitol-induced increased interaction and ubiquitination in CHIP-transfected cells had been originally shown in Figure 4C and D.

The authors conclude from the analysis of binding between CHIP and MEKK2 wild type and catalytically inactive that CHIP interacts depending on a kinase activity-dependent manner. This might not be true, other scenarios are possible such as that phosphorylation is important rather than activity. Actually, the molecular weight of the KN and wild type are very different on the blots presented on figure 2C and later acknowledged that this might correspond to different degree of phosphorylation. It is proposed that the binding of CHIP and MEKK2 occurs with dephosphorylated MEKK2. However, figure 2C shows that it is possibly the phosphorylated MEKK2 that is able to interact with CHIP. Similarly, it is in response to sorbitol that the binding of MEKK2 and CHIP is stronger, and it is under stress-treatment that the MEKK2 is activated and trans-phosphorylated. Therefore, it seems that there is a contradictory scenario here that is not completely understood. May be a more profound analysis of the phosphorylation sites important for activation and binding could help to resolve this point.

Re: We understand the reviewer's concern that the phosphorylated MEKK2 may interact with CHIP. Since the intensity of the lower (dephosphorylated) band of $MEKK2^{WT}$ was much weaker compared with the upper (phosphorylated) band in previous Figure 2C, the data may have tended to lead to a confusion that CHIP interacts with the phosphorylated MEKK2. Thus, we have examined and improved the experimental conditions to make clear the lower band of $MEKK2^{WT}$ by changing the amount of DNA for the transfection of MEKK2 and CHIP. $MEKK2^{WT}$ was clearly detected as a doublet of bands on SDS-PAGE (new Figure 2C). Moreover, the lower band of $MEKK2^{WT}$ was specifically co-immunoprecipitated with CHIP (Figure 4A, arrow). These data are also confirmed by

using MEKK2-CT^{WT} (Figure S4C). Furthermore, we also found that only dephosphorylated MEKK2, but not phosphorylated MEKK2, interacted with CHIP *in vitro* (Figure 4B). Thus, we believe that we can conclude that the dephosphorylation step allows MEKK2 to interact with CHIP. Moreover, according to the reviewer's suggestion we conducted a more profound analysis of the phosphorylation sites for the binding to reveal the binding mechanisms. We performed the experiments using several mutant forms of MEKK2-CT (Figure S4B). Contrary to the results from the full length MEKK2^{KN}, MEKK2-CT^{KN} clearly interacted with CHIP (Figure S4C), suggesting that dephosphorylated MEKK2-CT may be able to interact with CHIP. However, this interaction was abolished by the phosphorylation mimetic mutation (MEKK2-CT^{EE}), but not by the non-phosphorylation mutation (MEKK2-CT^{AA}), at Ser 519 and Ser 523, which are the essential phosphorylation sites for MEKK2 activation (Figure S4D). Although we cannot rule out the potential involvement of other unknown phosphorylation sites in MEKK2-CHIP interaction, the phosphorylation of Ser 519 and Ser 523 in MEKK2 is likely to prevent the interaction with CHIP. We included these new supplemental data (Figure S4B, C, D) and related discussion (p12 19-p13 15).

Reviewer #2:

*The manuscript *CHIP-dependent termination of MEKK2 regulates temporal ERK activation required for proper hyperosmotic response* reports that MEKK2, a member of the MAP3K family, specifically regulates activation of ERK, but not JNK or p38 in response to sorbitol. A novel role was investigated for the E3 ubiquitin ligase, CHIP, in degradation and ultimately regulation of MEKK2 and ERK in response to sorbitol.*
This is an interesting story and there is a large amount of data shown in support of the conclusions. Some data are convincing, but the pieces do not link together tightly. Several important issues that are not discussed or that depend on overexpression that keep this manuscript from telling a strong story.

1) MEKK1 has been reported by Tony Hunter's group to be involved in ERK regulation in response to sorbitol. Where does MEKK1 fit in this picture?

Re: We appreciate the reviewer's comment. As the reviewer's point, Lu et al. have shown that MEKK1 mediates the degradation of ERK1/2 in response to sorbitol (Lu et al. Mol. Cell 2002). The concentration of sorbitol used for their experiments was 500 mM which eventually induced cell death after the prolonged treatment of NIH3T3 cells for 10 h because of the down-regulation of MEK1-ERK1/2 survival signals. In our experimental settings, the treatment of MEFs with 300 mM sorbitol for 8 h or the transient treatment of HEK293 cells with 500 mM sorbitol for 1 h induced certain genes expression but not cell death. Thus, it is not easy to integrate both studies in a single picture. However, we agree that we should carefully discuss this issue with appropriate citation. We newly incorporated the paper in the reference and discussed in our revised manuscript (p20 18-p21 5).

2) All of the experiments involve overexpressed proteins. Some of the proteins in the pathway are not abundant. Overexpression is likely to skew the findings. The authors claim that MEKK2 binds to and is ubiquitinated by CHIP in response to hyperosmotic stress.
Co-immunoprecipitation of endogenous proteins or some equivalent demonstration in response to sorbitol or any other inducer of osmotic stress seems like a minimal requirement to substantiate this story.

Re: Endogenous MEKK2 interaction with CHIP and ubiquitination and degradation of endogenous MEKK2 had been shown in previous Figure 3C, 3D, 3E (in the revised manuscript, Figure 3C, 3D, 3F), respectively. In addition to the endogenous data, we have newly confirmed the ubiquitination of MEKK2 in response to not only sorbitol but also NaCl (Figure S2B). These data strengthen our conclusion that hyperosmotic stress induces the MEKK2 degradation by CHIP. We included this new supplemental data (Figure S2B) and related discussion (p9 11-13).

3) The CHIP rescue experiment is not convincing. Will another ligase rescue as well as CHIP?

Re: We do not understand why this reviewer was not convinced with this solid data. As shown in Figure 3E and 3F, the inhibition of MEKK2 degradation in *CHIP*$^{-/-}$ MEFs was almost completely recovered by the lentivirus-mediated expression of CHIP. To avoid the misinterpretation of data, we improved the detailed indication of these figures (Figure 3E, F) and the manuscript (p9 17-19).

4) The steps implied by the proposed mechanism have not been sufficiently delineated. The authors state that dephosphorylation is required for ubiquitination and degradation of MEKK2. Labeling of the protein is a generic way to demonstrate a phosphorylation/dephosphorylation mechanism, but is not directly linked to the subsequent events. Is dephosphorylation causing inactivation of MEKK2? If so, degradation of MEKK2 may not be the essential event. The connections are tenuous.

Re: We agree with the reviewer's point. To examine whether dephosphorylated MEKK2 maintains its inactive state or not, we have newly tested the phosphorylation and activation status of MEKK2 by using *in vitro* kinase assay (Figure S8A and B). MEKK2 dephosphorylated by cell lysate was re-autophosphorylated and re-activated by the preincubation with ATP *in vitro* in a time-dependent manner (Figure S8B). Although we cannot rule out the possibility that dephosphorylation contributes to the inactivation of MEKK2 *in cells*, we may conclude that the dephosphorylation step is not sufficient for the complete shutdown of the MEKK2-ERK pathway. We included this new supplemental data (Figure S8A and B) and related discussion (p19 13-15).

5) Is there evidence that this event occurs in response to sorbitol treatment?

Re: We might not completely understand this comment. If "this event" means the dephosphorylation-dependent interaction of MEKK2 with CHIP, as shown in figure 4C, the MEKK2-CHIP interaction (lane 7, time point 75 min) was observed after the peak of phosphorylation of MEKK2 (lane 6, time point 60 min). Since MEKK2 is unphosphorylated in the non-stimulated condition (lane 1) and is phosphorylated by the treatment with sorbitol, all the events including interaction with CHIP, ubiquitination, and degradation are the dephosphorylation-dependent events initiated by sorbitol.

Reviewer #3:

Maruyama and colleagues show that the MAP3K MEKK2 is necessary for ERK activation by hyperosmotic stress (at least at 1h stimulation). They also show that during the activation cycle of MEKK2 by hyperosmolarity, MEKK2 undergoes hyperphosphorylation, then dephosphorylation, at which time MEKK2 associates with the E3 ubiquitin (Ub) ligase CHIP. CHIP does not, however, associate with an inherently kinase-inactive form of MEKK2, nor does it associate with inactive, dephospho-MEKK2 in resting cells. CHIP is required to attenuate a prolonged (12 h) ERK activation seen after withdrawal of a 1h hyperosmolarity stimulus. This ERK deactivation appears necessary for induction of aquaporins (Aqp)-1 and -5.

These results are interesting and potentially quite significant. However, there are several missing mechanistic considerations that need to be addressed.

1) The MEKK2 RNAi data are for only 1 hour of hyperosmotic treatment, and MEKK3 has been implicated in hyperosmolar activation of ERK. The effect of MEKK2 RNAi should encompass the whole time course (5 min-12 h) and be compared with MEKK3 RNAi. Thus it will be clear if MEKK2 is required only for the prolonged phase of ERK activation. This is especially an issue given the data in Fig. 1B showing that MEKK2's kinase activity is activated well after ERK activation goes up (30 min vs. 15 min).

Re: As far as we know, there is no report that MEKK3 is implicated in the hyperosmotic stress-induced ERK activation. It has been shown that the hyperosmotic stress-induced activation of p38, but not ERK, is mediated by MEKK3 by Uhlik et al. (Nat. Cell Biol. 2003). However, to explore the possibility that MEKK3 is involved in the ERK activation in response to sorbitol, we have compared the effects of siRNAs for MEKK3 and MEKK2 on the sorbitol-induced ERK activation in the whole time course [new Figure S1A and B (5 min-30 min), S1C, D, E and F (1 h-12 h)]. MEKK2

knockdown, but not MEKK3 knockdown, significantly inhibited not only the transient ERK activation but also the prolonged ERK activation. We included these new data (Figure S1A-F) and related discussion (p6 23-24 and p14 9-13).

2) The nature of the MEKK2-CHIP interaction is still unclear. Why doesn't MEKK2, which is dephosphorylated and inactive in "resting" cells, not interact with CHIP in resting cells? The authors correctly suggest that a phosphatase is recruited to the active MEKK2 and that this might stabilize or enhance the CHIP interaction. This should be demonstrated. Okadaic acid, while somewhat nonspecific, really does preferentially inhibit phosphatase-2A. Does PP2A catalytic subunit associate with active-phosphorylated MEKK2 before the interaction with CHIP? In a transfection setting, does coexpression of PP2A-C subunit (or A subunit) enhance the binding of CHIP to MEKK2?

Re: Although we used high dose (100 nM) of okadaic acid (OA), which nonspecifically inhibits Ser/Thr phosphatase with a wide spectrum, it is possible that PP2A, which is preferentially inhibited by OA, fascilitates the MEKK2-CHIP interaction. According to the reviewer's suggestion, we examined the effect of PP2A-A subunit on the interaction between MEKK2 and CHIP, and the phosphorylation of MEKK2. Inhibition of the MEKK2-CHIP interaction and the dephosphorylation of MEKK2 were not observed in the PP2A-A co-expressed cells (Figure S7). We included this new supplemental data (Figure S7) and related discussion (p18 20-p19 2).

3) Fig. 5B needs an MEKK2 RNAi study as described in point "1" above (the experiment can go here or with Fig. 1).

Re: We thank the reviewer's suggestion. We included the new supplemental data (Figure S1C-F) and related discussion (p14 9-13). Please see also our response to the point 1.

4) Of particular importance, it would be ideal if the authors could provide some insight into how ERK deactivation is translated into an induction of Aqp1/5. The complete picture is not needed, but something should be included to address this issue.

Re: We agree with the reviewer's point. Accordingly, we included the new supplemental data (Figure S9).

Editorial Decision 31 May 2010

The paper has been re-reviewed by two original referees with no further comments.

