

Phosphoproteome dynamics reveal novel ERK1/2 MAP kinase substrates with broad spectrum of functions

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1st Editorial Decision

08 February 2013

Thank you again for submitting your work to Molecular Systems Biology. We have now heard back from the two referees who agreed to evaluate your manuscript. As you will see from the reports below, the referees find the topic of your study of potential interest. They raise, however, several concerns on your work, which should be convincingly addressed in a major revision of the work. The recommendations provided by the reviewers are very clear in this regard and refer to the need of further verifications.

Molecular Systems Biology strongly encourages authors to upload the 'source data' that were used to generate figures--for example, uncropped gels and blots, tables of individual numerical values and measurements. These files are separate from the traditional supplementary information files and are submitted using the "figure source data" option in the tracking system. Source data are directly linked to specific figure panels so that interested readers can directly download the associated 'source data' (see, for example, <<http://tinyurl.com/365zpej>>), for the purpose of alternative visualization, re-analysis or integration with other data. In the case of this study, this could concern the phosphorylation kinetics shown in Fig 5A, the gels/blots shown in Fig 5B, 6A, the three replicates of the EMSA Fig 6C and quantification Fig 6D.

If you feel you can satisfactorily deal with these points and those listed by the referees, you may wish to submit a revised version of your manuscript. Please attach a covering letter giving details of the way in which you have handled each of the points raised by the referees. A revised manuscript will be once again subject to review and you probably understand that we can give you no guarantee at this stage that the eventual outcome will be favorable.
<http://www.nature.com/msb>

Referee reports:

Reviewer #1 (Remarks to the Author):

Courchelle et al use a quantitative phosphoproteomic strategy to identify ERK substrates. Among 8000 phosphorylation sites they list 155 as putative direct ERK targets according to the following criteria.

- They are up-regulated after stimulation with serum
- They are down-regulated when the cells are incubated in the presence of a MEK inhibitor
- They contain a ERK consensus motif S/T-P. 128 of the identified targets were never reported before

Although earlier manuscripts have reported the identification of ERK substrates by similar strategies, the scale of this experiment and the number of newly identified substrates make the results presented in this report a new valuable resource. I must say, I am surprised of the low overlap with previously published substrate lists. Nevertheless I believe that this study, using more up-to-date phospho-proteomic technics and a carefully controlled approach, offers solid data that are much welcome by scientists interested in characterizing signal propagation started by receptor tyrosine kinases.

I have some comments that the authors should address.

The specificity of the MEK inhibitor PD184352 is an important point for the validation of the whole strategy. I must admit I did not go through all the literature, but the authors should discuss the available evidence. Was the inhibitor ever tested by different techniques with a large panel of kinases in vitro and/or in vivo?

Figure 2 panel A. It looks to me that some peptides with substantial changes under stimulation and inhibition conditions and containing an ERK consensus (P-X-(S/T)-P) are not identified as candidate ERK substrates. It is not clear why.

If many of these putative targets contain ERK docking sites, why have these proteins never been found associated with ERK in PPI networks. How frequent are these docking sites in random proteins.

In Figure 2, reporting the analysis of GO terms enrichment (panel B and C), the authors should present p-values corrected for multiple testing and not just the number of substrates per each category.

The method used to assemble the STRING PPI network is not clearly described neither in the text nor in the legend to Figure 3. How was the interactome assembled? What was the confidence threshold? Why was the rat interactome, combined with the human while the mouse interactome was not considered. It is not clear whether high confidence interactions were considered only for the rat dataset or both rat and human datasets. Are all the interactions involving at least one ERK target shown in Figure 3? If "YES" why are there so many membrane signaling ERK targets that do not show additional interactions aside from the one among themselves.

What is the "new" point that the authors want to make with Fig 3? The concentration of ERK targets in specific functional categories was already shown with the GO enrichment analysis.

Figure 4. It is unclear why do nodes have different shapes. Why RAF and MEK have colored sectors. In general the authors should put more care in the Figure legends.

The EMSA experiment in Figure 6 C/D should be also performed with extracts prepared for cells treated with the MEK inhibitor. Replica experiments should be performed and error bars shown.

By using a fuzzy c-means clustering algorithm the authors propose six different classes of kinetic

profiles (Supplementary Figure S4). I am wondering how different are the six profiles (they do not look very different to me) and how robust is the classification.

Reviewer #2 (Remarks to the Author):

Review, Courcelles, et al. "Phosphoproteome dynamics reveal novel ERK1/2 MAP kinase substrates with broad spectrum of functions" *Molecular Systems Biology*,

Comments to the authors:

In this work, Courcelles, et al. sought to augment our current inventory of ERK1/2 substrates by presenting dynamic profiles of new targets as determined by mass spectrometry. The authors took a novel approach to acquisition and validation of potential ERK1/2 targets, and consequently identified over one hundred previously-unidentified substrates. Multiple targets were validated in vitro, and functional analysis of the consequences of ERK1/2 phosphorylation on the AP-1 family transcription factor JunB was carried out.

Phosphorylation of JunB by ERK1/2 is necessary for full interaction of a JunB/c-fos dimer on DNA targets as well as robust transcriptional transactivation of a collagenase reporter plasmid. Phosphorylation at the identified target site, S256, has previously been implicated in priming the degradation of JunB. No evidence of that effect was observed in this work, suggesting that that effect is dependent on a different cellular context and/or phosphorylation by another kinase.

One interesting outcome of this work was the mapping of activating phosphorylation events on signaling modules upstream of ERK1/2 following their pharmacological inactivation, suggesting extensive pathway feedback.

Specific comments:

Validation of the phospho-specific JunB S256 antibody does not rule out phosphorylation/recognition of modification at T252 (Figure S7). Despite the lack of phosphorylation on this residue in vitro, demonstrating that the site is not an in vivo ERK1/2 site is important given that T252 clearly contributes to JunB activity (Figure 6).

Response to reviewer's comments

Reviewer 1:

1. The specificity of the MEK inhibitor PD184352 is an important point for the validation of the whole strategy. I must admit I did not go through all the literature, but the authors should discuss the available evidence. Was the inhibitor ever tested by different techniques with a large panel of kinases in vitro and/or in vivo?

PD184352 is a second-generation ATP non-competitive MEK1/2 inhibitor. It is more potent and selective than the first-generation inhibitors PD98059 and U0126. The selectivity of PD184352 has been profiled on a large panel of more than 70 protein kinases (Bain et al. Biochem J 2007). It was found to inhibit the activity of MEK1 and MEK2, but no other kinase in the panel. The first-generation inhibitors PD98059 and U0126 have been reported to inhibit MKK5, a related member of the MAP kinase kinases (MKKs) family, and as a consequence to prevent the activation of ERK5. However, concentrations of PD184352 that block the activation of ERK1/ERK2 in cells (1–2 μM as used in this study) do not affect the activation of ERK5, and higher concentrations (10–20 μM) are needed to prevent the activation of ERK5 (Mody et al. FEBS Letters 2001). Thus, PD184352 is a potent and extremely selective inhibitor of the MEK1/2-ERK1/2 pathway, which does not interfere with the related MKK5-ERK5 pathway when used at a concentration of 2 μM as in the present study. The Results section has been revised to include this information on PD184352.

2. Figure 2 panel A. It looks to me that some peptides with substantial changes under stimulation and inhibition conditions and containing an ERK consensus (P-X-(S/T)-P) are not identified as candidate ERK substrates. It is not clear why.

Based on the t-test, the abundance changes of those phosphopeptides were not significant and thus were not retained as candidate ERK substrates. The following sentence was added in Figure 2 legend: "Note that a few peptides with [pST]P sites in this quadrant did not have significant abundance change and were not retained as ERK1/2 candidates."

3. If many of these putative targets contain ERK docking sites, why have these proteins never been found associated with ERK in PPI networks. How frequent are these docking sites in random proteins. *Can we perform bioinformatics analyses to identify the frequency of Erk docking sites in random proteins?*

Current available PPI interaction networks are known to be incomplete. Different experimental methods give complementary results and thus capture a subset of the interactome. Incompleteness of kinase-phosphatase-substrate network is also more pronounced since these interactions are transient and take place rapidly in the cell. The following text was added to compare the presence of D-DEF domains in candidates ERK substrates against random phosphoproteins:

We found that 22 of the potential ERK1/2 substrates (14 %) contain a D domain, whereas one contains a DEF domain (0.6%) (Supplementary Table S3). In comparison, 17 % (278) and 0.4% (11) of all the phosphoproteins identified in this study have a D or DEF domain, respectively, within 20 amino acids of the phosphorylated site. This indicates that the putative ERK1/2 substrates are not enriched for these docking domains..

4. In Figure 2, reporting the analysis of GO terms enrichment (panel B and C), the authors should present p-values corrected for multiple testing and not just the number of substrates per each category.

We now included in Figure 2, the p-values for two-sided Fisher test performed on each GO term. Only GO terms with E-values greater than 1 are reported. However, correcting for multiple testing present certain limitations. The high level of redundancy induced by having several genes included in large numbers of functional categories induces a strong positive dependency between p-values. This drastically reduce the power for the FDR procedure {Benjamini, Y., & Hochberg, Y. (1995). Controlling the False Discovery Rate: A Practical and Powerful Approach to Multiple Testing. *Journal of the Royal Statistical Society Series B ...*, 57(1), 289–300}, a well know fact to the authors of the procedure but often ignored by its users. Specifically, in the context of functional category enrichment analysis, using the FDR procedure severely increases the number of type-2 errors or false negatives. More recent procedures developed by Y. Benjamini (e.g. {Reiner, A., Yekutieli, D., & Benjamini, Y. (2003). Identifying differentially expressed genes using false discovery rate controlling procedures. *Bioinformatics*, 19(3), 368–375. doi:10.1093/bioinformatics/btf877}) attempt to address this issue in the context of differential gene expression by using resampling techniques. Unfortunately, such approach requires a high number of replicates per condition (Reiner *et al.* used 8 replicates), which is unrealistic in the context of the studies presented in our paper. Furthermore, these procedures have never been applied in the context of functional category enrichment.

5. A) The method used to assemble the STRING PPI network is not clearly described neither in the text nor in the legend to Figure 3. How was the interactome assembled? What was the confidence threshold?

This point is addressed in point D below.

B) Why was the rat interactome, combined with the human while the mouse interactome was not considered.

We chose the human because its known interactome is larger than mouse.

C) It is not clear whether high confidence interactions were considered only for the rat dataset or both rat and human datasets.

Binary interactions were removed to generate Figure 3.

D) Are all the interactions involving at least one ERK target shown in Figure 3? If "YES" why are there so many membrane signaling ERK targets that do not show additional interactions aside from the one among themselves.

We replaced the following text in the method section: "Protein interaction network of the ERK1/2 pathway was generated from ProteoConnections using STRING interaction dataset (high confidence interactions with score > 0.9 from database and experiments)" with "To generate protein interactions network, we first used ProteoConnections to map the list of candidate ERK1/2 substrates to the STRING interactions database. We retained only the highest confidence (> 0.9) interactions extracted from experiments and databases found in rat. For all candidates found, we gathered extra interactors one level deeper (white node). Since the rat interactome is not well studied, we chose to expand our network by including interactions from human orthologs. After converting rat gene identifiers to human, we extracted mapped interaction from STRING as above. We manually edited the human network to keep interactions that extended our rat network (connectable components) and removed white node (unless needed to connect two subnetworks). Finally, fusion of the rat and the remaining human network was made using Cytoscape. This software was also used to organize spatially the network for Figure 3. Binary interactions were removed to generate the figure."

6. What is the "new" point that the authors want to make with Fig 3? The concentration of ERK

targets in specific functional categories was already shown with the GO enrichment analysis. *Explain that this was necessary for the selection of candidates for further validation.*

This figure shows an important connectivity between the candidates ERK substrates. Out of 155 substrates, 34 are directly interconnected in the STRING network. The text related to figure 3 was modified to read as follows: " One notable feature of this network is that out of 155 candidate ERK1/2 substrates, 34 (22%) are directly interconnected in the STRING network. This high degree of connectivity suggests that ERK1/2 are not phosphorylating proteins randomly in the cell but often regulate members of the same functional pathway or protein complexes." We also modified the legend to Figure 3 which now indicate: "Candidate ERK1/2 substrates show a high degree of connectivity (34/155 are interconnected)."

7. Figure 4. It is unclear why do nodes have different shapes. Why RAF and MEK have colored sectors. In general the authors should put more care in the Figure legends. *Clarify in figure legend that each protein is represented as a circle divided according to the number of individual psites, each with a color gradient showing the extent of regulation.*

The legend of the Figure 4 was changed accordingly: "**Figure 4.** Phosphorylation changes in the upstream regulators of ERK1/2 MAP kinases. Identified phosphoproteins (round circles) were mapped on the interaction network from the STRING database. A color gradient is used to represent the modulation (summed log fold change) of each individual phosphorylation site following treatment with PD184352. Protein nodes with multiple phosphorylation sites are represented using a pie chart where each slice correspond to the abundance change of a unique phosphorylated site."

8. The EMSA experiment in Figure 6 C/D should be also performed with extracts prepared for cells treated with the MEK inhibitor. Replica experiments should be performed and error bars shown.

As requested, we have treated 293T cells expressing Flag-c-Fos and HA-JunB with 2 μ M of PD184352, for either 12 h or 24 h, and performed EMSA experiments on corresponding nuclear extracts. In agreement with results of mutagenesis experiments, MEK1/2 inhibition affected the binding of AP-1 complexes to target DNA (new Figure 7A). The assay was performed on three replicates and the results quantified in new Figure 7B.

9. By using a fuzzy c-means clustering algorithm the authors propose six different classes of kinetic profiles (Supplementary Figure S4). I am wondering how different are the six profiles (they do not look very different to me) and how robust is the classification. *The number of profiles is somewhat subjective. Can we provide a rationale for the selection of six, and how to evaluate robustness of classification?*

As indicated in the Supplementary Figure S4 legend but not in the text, these 6 classes were arbitrary chosen to display the general stimulation/inhibition trends of selected candidates. It would have been impossible to distinguish all profiles in one graph. Number of clusters were not optimized and don't represent any biological functional classes. Those are representative trends so that the readers can understand the type of profiles selected and are provided as a supplementary figure for convenience. We modified the text as follow: "Representative stimulation and inhibition temporal profiles of regulated phosphopeptides were grouped using fuzzy c-means clustering into 6 groups (arbitrary chosen number) to graphically report the general trends of the selected candidates (Supplementary Figure S4). "

Reviewer #2 (Remarks to the Author):

Phosphorylation at the identified target site, S256, has previously been implicated in priming the degradation of JunB. No evidence of that effect was observed in this work, suggesting that that effect is dependent on a different cellular context and/or phosphorylation by another kinase.

1. Validation of the phospho-specific JunB S256 antibody does not rule out phosphorylation/recognition of modification at T252 (Figure S7). Despite the lack of phosphorylation on this residue *in vitro*, demonstrating that the site is not an *in vivo* ERK1/2 site is important given that T252 clearly contributes to JunB activity (Figure 6).

Several arguments strongly suggest that T252 is phosphorylated by another kinase than ERK1/2. First, *in vitro* phosphorylation assays clearly demonstrate that T252 is not directly phosphorylated by ERK1 (Figure 5B). However, the lack of a commercially available phospho-T252 specific antibody did not allow the confirmation of these results *in vivo*. Second, single phosphorylation at T252 is not modulated by either ERK1/2 and/or inhibition, consistent with a possible lack of involvement of ERK1/2 *in vivo*. Third, data from the literature suggest that T252 is phosphorylated by GSK3 β (Perez-Benavente et al. Oncogene 2012). Treatment with the GSK3 β inhibitor VIII decreases the phosphorylation of T252A but not that of S256. It was proposed that the phosphorylation of S256 serves as a priming event for GSK3 β -mediated phosphorylation of T252, and consecutive Fbw7-mediated degradation. Similarly, it has been previously reported that the JunB homologue c-Jun is phosphorylated on T239 (equivalent to T252 of JunB) by GSK3 β , while the S243 (equivalent to S256 of JunB) kinase has remained unknown (Wei et al. Cancer Cell 2005). All together, these data strongly suggest that T252 is phosphorylated by GSK3 β *in vivo*. However, the identification of the kinase that specifically phosphorylates T252 falls outside the scope of this paper.

Availability of metadata:

We include an Excel file “Figure 5A_source_data” describing the phosphorylation kinetics shown in Figure 5A. We also provided source data (gels and blots) as “figure source data” in the tracking system for Figures 5B, 6A, 7B and 7D.