molecular systems biology

Regulation of yeast central metabolism by enzyme phosphorylation

Ana Paula Oliveira, Christina Ludwig, Paola Picotti, Maria Kogadeeva, Ruedi Aebersold, Uwe Sauer

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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 06 June 2012

Thank you again for submitting your work to Molecular Systems Biology. We have now heard back from the three referees who agreed to evaluate your manuscript. As you will see from the reports below, the opinions of the referees are somewhat divergent. While reviewer #2 finds that the advance made by this study remains limited, reviewers #1 and #3 are more positive. Given this balance of opinions, we fee that we can consider a major revision of the study, provided the major points raised by the reviewers are convincingly addressed. In particular, the use of the reported correlations to infer causal links is seen as problematic. Furthermore, the follow up functional analysis of the selected phosphosites should be strengthened with more direct evidence. The recommendations provided by the reviewers are clear in this regard.

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.

Yours sincerely,

Editor
Molecular Systems Biology

http://www.nature.com/msb

Referee reports:

Reviewer #1 (Remarks to the Author):

The present paper represents a major advance by integrating enzyme phosphorylation and metabolic flux data to infer potential physiologically important enzyme phosphorylation events. The quality of the data and flux analyses is high. The approach is novel and highly promising.
The present version of the paper, however makes conclusions beyond those supported by the data. Specifically,

1. The current analyses confound correlation and causation. The correlation of enzyme phosphorylation with flux is insufficient to infer a functional role for the phosphorylation event in flux control (although it is certainly suggestive, and a useful tool for identifying phosphorylation events to explore further). This issue is particularly serious in the present paper because
A. The authors arbitrarily use a correlation cut-off of 0.8 to infer a causative relationship. This is particularly problematic when there is almost the same correlation for the total protein abundance and flux (e.g., for Gpm1, R = 0.79 for total protein and 0.86 for phosphoprotein), and there is no test for statistical superiority of the correlation with phosphoprotein level, and no error estimates provided for either the flux or phosphoprotein measurements.
B. The correlation is across only 4 points.
C. There is no attempt to correct for role of substrate or product levels, which also interact with protein and phosphoprotein concentration to exert flux control (as do allosteric effectors).
D. Phosphoprotein levels may correlation with flux through a third variable, such as glucose availability or growth rate, rather than due to a causative relationship, e.g., availability of glucose could lead to both high flux (by increasing substrate levels) and high phosphorylation (by activating PKA), without any causative role of the phosphorylation in flux control.
Rewording the paper may be sufficient to resolve this issue.

2. The studies using phosphosite mutants to show functional relevance studies are insufficient (Pda1, Pfk2) or likely misinterpreted (Gpd1).
A. While Pda1 is a famous example of inhibitory phosphorylation, the authors, with their expertise in isotope tracer studies and flux analysis, should validate that removal of this phosphosite actually increases PDH flux in glucose-fed cells. This is a very important experiment for the field (and if it has a negative outcome, would call into question major assumptions, repeated in this paper, about the importance of enzyme phosphorylation).
B. The Pfk2 phosphorylation event is proposed also to be inhibitory. However, in glucose, substrate levels are lowered, not raised. Also, the growth curve experiment is not very direct proof of the physiological role. Instead, the authors should test directly what happens under conditions where phosphorylation was found (e.g., glycerol-ethanol) and measure reduced growth yield, slower growth, and/or (ideally) the occurrence of futile cycling at the PFK/FBPase node.
C. The Gpd1 phosphorylation event is inferred to be activating. However, removal of all phosphosites results in elevated, not lowered, product levels. Isn't this opposite of the prediction?

3. The apparently better correlation between phosphoprotein levels and flux, than for non-phosphorylated protein levels, and much better than for the non-phosphorylated forms of phosphoproteins, is very striking. It is important, however, to clarify the N for each group in this analysis (it seems to be insufficent for phosphoprotein group?) and associated statistical significance of the differences. Also, why is $R > 0.85$ claimed to be good in this figure, when $R > 0.8$ is used in next figure?

Reviewer #2 (Remarks to the Author):

Comments to the authors
1. The paper is merely an extension of the manuscript by Costenoble published in Mol Syst Biol last year in which protein level changes were evaluated in relation to alterations in metabolite concentrations under influence of 5 different growth conditions. In my view the additional value presented here is not of general interest.
2. The authors make very general, often bold, statements about the importance of phosphorylation in the regulation of the metabolic pathway (680 proteins), however towards the end of the paper, only

8 protein phosphorylation events are quantifiable events. In my view this is not a systems biology outcome. If only 1.5% (8 out of 680) of proteins show significantly changed phosphorylation sites under these 5 conditions I would not argue that phosphorylation is a major posttranslational modification in these processes, or that perhaps the method used is not sufficient to provide these events.
3. The authors advocate a strong link between metabolic protein phosphorylation and activity. This is too simplistic. As indicated in the introduction the authors state that phosphorlyation can have several other functions besides increasing/decreasing enzyme activity. These are not considered in the remainder of the paper.
4. At several occasions, the authors claim increased correlation of enzyme phosphorylation with flux data as compared to protein data, yet their best examples outlined in figure 3D show increases in Pearson correlation coefficients from for instance 0.8 to 0.86 (Lys20). Given the fact that these linear fits are only based on 4 data points, and log transformed, this seems insignificant to sustain this claim. The same in figure 3E where the correlations at protein level and phosphosite level are not that different.
5. Similarly statistics of figure 3C are insignificant. It is unclear from the figure how many r-values were evaluated here, but based on the text it seems only the 29 quantified phosphorylation events were used.
6. This brings also the unclarity of the numbers throughout the papers to light. In the first part quantified phosphopeptides are mentioned, which in the end shows 49 sites being quantified. However, later when protein levels are included, another number is used. This part reads very confusing and seems to make the whole discussion about the quantified sites in absence of protein levels irrelevant. If the manuscript is considered for revision by the editor, this part needs severe editing and simplification to ease readability.
7. P9 the authors state that their data generally fits the literature. Some positive examples are given. Are there also cases where it did not fit?
8. The follow-up with mutant versions seems thoroughly done, however it is unclear to me how the measurements here compare to the earlier work of Costenoble. Why are there no flux graphs here as in figure 3? That would bridge the different results and ease understanding.
Other points:
1. Citations are often incorrectly used. The authors cite their own lab more than is appropriate. Such as for the use of TiO2 by Bodenmiller, the importance of phosphorylation for yeast metabolic protein activity (Oliveira, is a review, rather cite some crucial original findings). Target decoy approaches by Bodenmiller.
2. P.4 last sentence, point i). How does SRM measure function of a protein?
3. Is a Peptide prophet score of 0.80 sufficient for accurate phosphopeptide identification and site localization. I doubt that.
4. P5. How was the quantitation done. It seems label free methods were used. A sentence or two to explain this would be good. Now the reader is left guessing.
5. P6. The part about proximity of phosphate transferring domains and phosphosites is completely speculative. Why would the phosphosite have to be within 30 amino acids distance in the context of the 3D structure?
6. Several figures seem unnecessary/redundant. For instance 1B, which is trivial. Figure 2 is large and not very informative if not further explained in the text. Figure 3A and C seem not relevant.
7. Supplemental Table 1 would be much more comprehensive if the function would also be mentioned in an extra column. Now it is merely a citation list.
8. P11. How would miss-assigning a phosphosite lead to poor non-correlation? This needs explanation or rephrasing.

Reviewer #3 (Remarks to the Author):

This is an outstanding paper describing the application of proteomics and phosphoproteomics to the study of yeast physiology, in order to identify enzyme phosphorylation events and sites that are important in regulating cellular function. The authors have identified 35 enzymes that are phosphorylated differentially under different growth conditions, and assigned a total of 8 previously unknown phosphorylation events in metabolic enzymes.

I must commend the authors on the high quality of the data presented, as well as the clarity of exposition and presentation of the data. The Supplementary Information contains all the details should any reader want to analyse the data further. In particular I was impressed by Figure 1 and

Supplementary Figure 3 in outlining the experimental approach and clarifying it for the non-expert reader.

As such I only have one comment and a minor technical point:

1. I like the idea of first obtaining a global picture of phosphorylation envents and then delving further into a few specific details. As such the identification of functional phosphorylation sites for Pfk2 and Gpd1 using site-directed mutagenesis (p. 13 ff.) provides important novel biochemical insights. However, the evidence presented (Fig. 4) is rather of an indirect nature, consisting of determination of growth curves as well as in vivo substrate and product metabolite concentrations. Both enzymes (phosphofructokinase and glycerol phosphate dehydrogenase) are in or closely linked to the glycolytic pathway and enzyme activity assays have been available for decades. The data and conclusions would have been much stronger if directed enzyme activity measurements had been performed on the various site-directed mutants.

2. A number of the references are incomplete and are lacking volume and page numbers.

1st Revision - authors' response 05 September 2012

Reviewer #1 (Remarks to the Author):

The present paper represents a major advance by integrating enzyme phosphorylation and metabolic flux data to infer potential physiologically important enzyme phosphorylation events. The quality of the data and flux analyses is high. The approach is novel and highly promising.

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B. The correlation is across only 4 points.

C. There is no attempt to correct for role of substrate or product levels, which also interact with protein and phosphoprotein concentration to exert flux control (as do allosteric effectors).

D. Phosphoprotein levels may correlation with flux through a third variable, such as glucose availability or growth rate, rather than due to a causative relationship, e.g., availability of glucose could lead to both high flux (by increasing substrate levels) and high phosphorylation (by activating PKA), without any causative role of the phosphorylation in flux control.

Rewording the paper may be sufficient to resolve this issue.

The point is well taken! We have reworded the entire document to make it very clear – as it was our intention from the beginning on – that correlations generate hypotheses for candidate phospho-regulated enzymes. In particular, we specify on page 9 the assumptions under which the dependency model is valid (e.g., no other allosteric effectors or post-translational modifications regulate the enzyme, and the reaction rate is determined by the amount of active enzyme rather than by the concentration of the limiting substrate). To identify which candidate enzymes are regulated by phosphorylation, we have now calculated the p-value of the correlation coefficient and selected only the most significant cases, which resulted in a shorter list of candidates to be phospho-regulated.

2. The studies using phosphosite mutants to show functional relevance studies are insufficient (Pda1, Pfk2) or likely misinterpreted (Gpd1).

A. While Pda1 is a famous example of inhibitory phosphorylation, the authors, with their expertise in isotope tracer studies and flux analysis, should validate that removal of this phosphosite actually increases PDH flux in glucose-fed cells. This is a very important experiment for the field (and if it has a negative outcome, would call into question major assumptions, repeated in this paper, about the importance of enzyme phosphorylation).

B. The Pfk2 phosphorylation event is proposed also to be inhibitory. However, in glucose, substrate levels are lowered, not raised. Also, the growth curve experiment is not very direct proof of the physiological role. Instead, the authors should test directly what happens under conditions where phosphorylation was found (e.g., glycerol-ethanol) and measure reduced growth yield, slower growth, and/or (ideally) the occurrence of futile cycling at the PFK/FBPase node.

C. The Gpd1 phosphorylation event is inferred to be activating. However, removal of all phosphosites results in elevated, not lowered, product levels. Isn't this opposite of the prediction?

We took these comments very serious and performed therefore additional experiments with the phosphosite mutants. We now show phosphorylation to control the in vivo activity of these three enzymes through physiological data that back the metabolomics data up. Briefly, Pda1 [S313A] increased the flux to the TCA cycle with increased glutamine and alpha-ketoglutarate secretion, Pfk2 [S163A] increased probably a futile cycle that leads to 20% decreased biomass yield, and the quadruple Gpd1 mutant increased the flux to glycerol (compare the entirely new Figure 4). The final section of the results and the discussion were entirely rewritten.

3. The apparently better correlation between phosphoprotein levels and flux, than for non-phosphorylated protein levels, and much better than for the non-phosphorylated forms of phosphoproteins, is very striking. It is important, however, to clarify the N for each group in this analysis (it seems to be insufficent for phosphoprotein group?) and associated statistical significance of the differences. Also, why is R > 0.85 claimed to be good in this figure, when R > 0.8 is used in next figure?

We removed the claim that flux correlates better with phosphorylated protein than with the total protein. Although the trend is clearly present, 4 points and 16 protein/phosphoprotein pairs indeed do not provide a sufficient statistical basis. As outlined in point 1, we use these correlations merely to identify candidates for follow up experiments.

Reviewer #2 (Remarks to the Author):

Comments to the authors
1. The paper is merely an extension of the manuscript by Costenoble published in Mol Syst Biol last year in which protein level changes were evaluated in relation to alterations in metabolite concentrations under influence of 5 different growth conditions. In my view the additional value presented here is not of general interest.

We apologize for apparently not being clear enough in the first version, which has now been reworded completely. The Costenoble paper dealt with protein abundance measurements where we learned that enzymes are often present when there is actually no need for flux through them. The present manuscript has an exclusive focus on post-translational modifications (ie, phosphorylation) of enzymes and their functional consequence. While we do use the Costenoble protein abundance data to relate our here determined phosphoprotein data to the amount of total protein, there is obviously a major difference between protein abundance and protein modification., and thus the scope of the two papers is very different.
Moreover, the phosphodata and their correlation to flux are only one part of the present manuscript, essentially allowing us to generate entirely novel hypotheses on the functional relevance of phosphorylation. At least half of the work deals with verifying these correlations through measuring, for the first time, phosphopeptides and absolute phosphorylation stoichiometry directly from crude cell extracts without enrichment using SRM as well as metabolomics and physiology in phosphosite mutants. In sharp contrast to the Costenoble paper, we present here a novel approach to identify

FUNCTIONALITY of phosphorylation events, essentially increasing the number of functionally characterized phosphoenzymes by more than 50% in central yeast metabolism.

2. The authors make very general, often bold, statements about the importance of phosphorylation in the regulation of the metabolic pathway (680 proteins), however towards the end of the paper, only 8 protein phosphorylation events are quantifiable events. In my view this is not a systems biology outcome. If only 1.5% (8 out of 680) of proteins show significantly changed phosphorylation sites under these 5 conditions I would not argue that phosphorylation is a major posttranslational modification in these processes, or that perhaps the method used is not sufficient to provide these events.

Apparently our first version left room for potential misinterpretations of our goals and achievements. Firstly, we focused on the network of 204 proteins of yeast central carbon and amino acid metabolism, where we found 35 enzymes to be differently phosphorylated, which would be 17%. Second, perhaps somewhat in contrast to this reviewer's preference, we decided to not go for a "measure as much as you can", but focused instead on identifying phosphorylation functionality. We believe this is a dearly missing part in the phosphoproteomics field (and other omics as a matter of fact), as is illustrated by the fact that such functionality was only known for 9 enzymes in our network of 204. By providing evidence for 5 new functions of phosphoproteins (and specific phosphosites), we increased the present functional knowledge by more than 50%.
The text has been reworded extensively to avoid overly general and potentially unclear claims.

3. The authors advocate a strong link between metabolic protein phosphorylation and activity. This is too simplistic. As indicated in the introduction the authors state that phosphorlyation can have several other functions besides increasing/decreasing enzyme activity. These are not considered in the remainder of the paper.

We fully agree with the reviewer. This is why we specify in the Introduction how phosphorylation can potentially regulate an enzyme; i.e, altering of conformation, (un)blocking the access of the substrate, translocation, complex formation or degradation. All of these mechanisms of regulation result in either of two global functional outputs: enzyme activation or inhibition. In our work, we assessed such global functional output, that is, whether an enzyme becomes activated or inhibited by a given phosphorylation event, without detailing the precise mechanism of regulation. We believe we are fully in line with the reviewer's opinion and make no claims to particular mechanisms.

4. At several occasions, the authors claim increased correlation of enzyme phosphorylation with flux data as compared to protein data, yet their best examples outlined in figure 3D show increases in Pearson correlation coefficients from for instance 0.8 to 0.86 (Lys20). Given the fact that these linear fits are only based on 4 data points, and log transformed, this seems insignificant to sustain this claim. The same in figure 3E where the correlations at protein level and phosphosite level are not that different.

We removed the claim that flux correlates better with phosphorylated protein than with the total protein, since we cannot defend it statistically, although the general trend is there (please see also response 3 to reviewer 1). We have also re-done the linear fits in a more direct manner, by correlating absolute fluxes directly with the abundances relative to glucose (direct fold-changes, not transformed). Furthermore, we now assess the statistical significance of the correlations and use them only to identify CANDIDATE enzymes to be regulated by phosphorylation. Regarding Figure 3D (former Figure 3E): while both correlations with phosphoprotein and total protein are significant, the correlation coefficient is much higher in the phosphoprotein case, suggesting that phosphorylation here fine-tunes the catalytic activity of the enzyme. Therefore, we did not change the figure and text, except by adding the p-value of the correlation coefficient.

5. Similarly statistics of figure 3C are insignificant. It is unclear from the figure how many r-values were evaluated here, but based on the text it seems only the 29 quantified phosphorylation events were used.

We have removed the former Figure 3C and the claim that flux correlates better with phosphorylated protein than with the total protein.

6. This brings also the unclarity of the numbers throughout the papers to light. In the first part quantified phosphopeptides are mentioned, which in the end shows 49 sites being quantified. However, later when protein levels are included, another number is used. This part reads very confusing and seems to make the whole discussion about the quantified sites in absence of protein levels irrelevant. If the manuscript is considered for revision by the editor, this part needs severe editing and simplification to ease readability.

Based on this suggestion, we rephrased the text to clarify the numbers. Though we focus most of the work on the set of 204 enzymes for which we previously quantified total protein abundance, for the sake of completeness we still start the results with a reference to the total number of phosphoproteins (556) and of phosphoenzymes (70) detected. We then focus most of our analysis on the subset of 35 detected phosphoenzymes with central carbon and amino acid metabolism for which we previously quantified total protein abundance.

7. P9 the authors state that their data generally fits the literature. Some positive examples are given. Are there also cases where it did not fit?

Whenever the comparison was possible, our data was actually consistent with the literature. Sometimes, published studies report the role of phosphorylation without assigning it to a particular condition(s), and in such cases we cannot say whether our data fits or not the literature reports.

8. The follow-up with mutant versions seems thoroughly done, however it is unclear to me how the measurements here compare to the earlier work of Costenoble. Why are there no flux graphs here as in figure 3? That would bridge the different results and ease understanding.
Costenoble et al. estimated the flux through the reactions, which is the RATE of converting one metabolite into another and we used these data for our correlations. These rates, however, do not tell anything about the metabolite CONCENTRATIONS per se. Hence the metabolite concentrations that we report here cannot be compared in any way to the reported fluxes.
Based on the reviewers suggestions, for the revised manuscript we now report additional experiments that measured flux directly (through physiological data) and thereby provide functional evidence of phosphorylation in regulating Pda1, Pfk2 and Gpd1 activity.

Other points:
1. Citations are often incorrectly used. The authors cite their own lab more than is appropriate. Such as for the use of TiO2 by Bodenmiller, the importance of phosphorylation for yeast metabolic protein activity (Oliveira, is a review, rather cite some crucial original findings). Target decoy approaches by Bodenmiller.

We apologize for leaving the impression of overly extensive self-citations, but these citations report the precise protocols that we used. We have now added further original citations.

2. P.4 last sentence, point i). How does SRM measure function of a protein?
The sentence was removed.

3. Is a Peptide prophet score of 0.80 sufficient for accurate phosphopeptide identification and site localization. I doubt that.

A Peptide-prophet score of 0.80 was selected because it accounts for a false discovery rate of 2.5% for peptide identification. We now mention the false discovery rate instead of the peptide-prophet score in the Materials and Methods. We agree that this score does not address the issue of site localization, and that was the reason why we investigated the phosphosite localization in detail for the followed-up cases using selected reaction monitoring. In particular, we have introduced a new Supplementary Figure 4 to show how we validated the phosphosite positions by SRM.

4. P5. How was the quantitation done. It seems label free methods were used. A sentence or two to explain this would be good. Now the reader is left guessing.

A label-free quantitative proteomic approach was used, as mentioned in the main text and described in the Materials and Methods. A detailed description of the data analysis and post-processing steps applied for label-free quantification is given in the Supplementary Materials and Methods. A comment to this is now added to the main text.

5. P6. The part about proximity of phosphate transferring domains and phosphosites is completely speculative. Why would the phosphosite have to be within 30 amino acids distance in the context of the 3D structure?

We deleted this part.

6. Several figures seem unnecessary/redundant. For instance 1B, which is trivial. Figure 2 is large and not very informative if not further explained in the text. Figure 3A and C seem not relevant.

We removed Figure 3C but decided to leave the Figures 1B, 1C, 3A and 3B. As pointed out by reviewer 3, we think that Figure 1 (including panel B) makes it easier for the non-expert to get familiarized with the different quantities mentioned in the paper. Similarly, we think that Figure 3A draws the attention to an aspect that is not trivial, in particular for non-experts. Figure 2 summarizes the entire phosphoproteomics results in the network context, and we think this figure is particularly relevant, at least for the metabolic community.

7. Supplemental Table 1 would be much more comprehensive if the function would also be mentioned in an extra column. Now it is merely a citation list.
Thanks for the comment. We have included the function in one extra column.

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We deleted this part.

Reviewer #3 (Remarks to the Author):

This is an outstanding paper describing the application of proteomics and phosphoproteomics to the study of yeast physiology, in order to identify enzyme phosphorylation events and sites that are important in regulating cellular function. The authors have identified 35 enzymes that are phosphorylated differentially under different growth conditions, and assigned a total of 8 previously unknown phosphorylation events in metabolic enzymes.
I must commend the authors on the high quality of the data presented, as well as the clarity of exposition and presentation of the data. The Supplementary Information contains all the details should any reader want to analyse the data further. In particular I was impressed by Figure 1 and Supplementary Figure 3 in outlining the experimental approach and clarifying it for the non-expert reader.
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As with reviewer 1, we fully agree on this matter and therefore performed several additional experiments with the phospholoss Pda1, Gpd1 and Pfk2 mutants to provide more direct evidence. These new experiments included determining in vivo activity through physiological flux analysis and, for Gpd1, in vitro enzyme activity. For Pfk2 we did not pursue the in vitro enzymatic assays due to the unavailability in the market (for a couple of years now) of the required allosteric effector

fructose 2,6-bisphosphate, but we believe that the demonstrated 20% reduction in biomass yield, presumably as a consequence of the Pfk2 driven futile cycle, provides sufficient evidence.

2. A number of the references are incomplete and are lacking volume and page numbers.

We have corrected this.

Acceptance letter 05 October 2012

Thank you again for sending us your revised manuscript. We are now satisfied with the modifications made and I am pleased to inform you that your paper has been accepted for publication.

Thank you very much for submitting your work to Molecular Systems Biology.

Your sincerely,
Editor
Molecular Systems Biology

http://www.nature.com/msb

Reviewer #1 (Remarks to the Author):

The paper is an ambitious and important attempt to relate enzyme phosphorylation to metabolic flux. While the yield is only a few examples, the paper will still interest a wide audience. The major inaccuracies of the initial submission have now been resolved.

To maximize impact/timeliness of the paper, a brief discussion of a recent paper on the same topic (reaching orthogonal conclusions) is warranted: Regulation of Yeast Pyruvate Kinase by Ultrasensitive Allostery Independent of Phosphorylation. Xu YF, Zhao X, Glass DS, Absalan F, Perlman DH, Broach JR, Rabinowitz JD. Mol. Cell.

Also, I would encourage the authors to use both enzyme names and gene abbreviations throughout the paper, including in the abstract. For readers working on other organisms than yeast, enzyme names are much more informative than 3-letter gene codes.

