molecular systems biology

Modularity and hormone sensitivity of the *Drosophila melanogaster* insulin receptor/target of rapamycin interaction proteome

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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 15 July 2011

Thank you again for submitting your work to Molecular Systems Biology. We have now heard back from the three referees whom we asked to evaluate your manuscript. As you will see from the reports below, while two of the three referees are supportive, referee #2 is more reserved. Thus, this reviewer raises several concerns on your work, which should be convincingly addressed in a revision of this study.

One of the major concerns refers to the need to consolidate the main quantitative dataset by providing more convincing evidence for the reliability of your quantitative approach. Reviewer #2 suggests to perform a third replicate to allow statistical analysis of the insulin-dependency of the reported interactions. Since we realize that this recommendation was provided by only one reviewer and may involve time-consuming experimentation, we briefly consulted with an additional independent proteomic expert ('Reviewer #4' below) to ask for advice on this specific point. As you will see, this reviewer agrees that the validity of the 1.5x cutoff should be better justified but makes constructive suggestions for an alternative solution that would not require performing an entire third replicate (see comments by reviewer #4 below). With regard to the follow up experimentations, while some stronger evidence for the existence of the dTTT complex would be highly desirable, the exact determination of its stoichiometry may not represent an essential aspect of the study and toning down the conclusions in this regard would be sufficient, in our opinion.

With regard to the proteomics data we would also kindly remind you of our editorial policies (http://www.nature.com/msb/authors): "mass spectrometry datasets should be deposited in a

machine-readable format (eg mzML if possible) in one of the major public database, for example Pride (http://www.ebi.ac.uk/pride/), PeptideAtlas (http://www.peptideatlas.org), or the Proteome Commons Tranche repository (http://tranche.proteomecommons.org/)" and "molecular interaction data should be deposited with a member of the International Molecular Exchange Consortium (IMEx, http://www.imexconsortium.org)".

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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 favourable.

Yours sincerely,

Editor
Molecular Systems Biology

Referee reports:

Reviewer #1 (Remarks to the Author):

"Modularity and hormone sensitivity of the Drosophila melanogaster insulin receptor/target of rapamycin (InR/TOR) interaction proteome"
Glatter et al.

This is a very nice study looking at interactions between proteins in the insulin/TOR signaling pathway. For the most part, it is largely confirmatory of what is known in the pathway, however there are several new findings which will be of interest to the insulin/TOR community:
-Chico was found to bind three proteins forming an SCF E3 ubiquitin ligase complex, suggesting a novel layer of regulation.
-The protein Unkempt was found to be an integral part of the TOR complex and to regulate its activity. Virtually nothing is currently known about Unkempt function.
-An additional TOR complex, which the authors name dTTT, formed of proteins not observed in TORC1 and TORC2, was identified.

In addition, there is a wealth of other details having to do with single protein-protein interactions, or changes in protein-protein bindings upon stimulation of the pathway with insulin.

On the whole, I think this work will elicit interest in the community. The data quality also seems to be very high from the results obtained, although I cannot judge the mass spec. technical aspects.

Reviewer #2 (Remarks to the Author):

The manuscript by Glatter and colleagues describes and integrated analysis of the D. melanogaster insulin receptor and target of rapamycin (TOR) interaction proteome. The manuscript contains quantitative proteomics analyses of protein interactions, protein complex analysis, RNAi analysis of phosphorylation changes, and D. melanogaster phenotype analysis. This is a good biological system and the study of TOR signaling is important. This research is potentially appropriate for Molecular

Systems Biology but it is in need of very major revisions prior to publication.

Major Issues
1. The authors need to provide supplemental tables documenting the data that they used to make the quantitative proteomic claims. There currently is no mass spectrometry data available showing the peptides, peptide scores, and protein scores used to make the conclusions in the manuscript. Also, the authors need to provide the quantitative data in the form of a supplemental table.

2. This is a quantitative proteomics manuscript but the authors only carried out two replicates of their data on dynamic bait-prey interactions. A third replicate is required and a statistical analysis of the data is required. The authors must provide statistical validation of their claims in the manuscript. Currently, the authors are using a 1.5 fold cutoff to justify insulin sensitive interactions. This is not acceptable practice in modern quantitative proteomics. A third replicate is required and a statistical justification is required for the claim of 22 insulin regulated interactions.

3. The authors need to define their use of the term of modularity early in the manuscript. What exactly do they mean here? It seems as if they are using modularity to in fact describe protein complexes. Therefore modularity in this case would be modules in a network. This also leads to confusion in the section on the pathway interactome where the data that is specifically used to support this section is not listed in the text. For example, on pages 8-9 on the Drosophila TOR modules, where is the data used to support this paragraph? Is it Supplemental Table 3? This section needs more detailed referencing of where the data used to make the claims originates.

4. The data to support the presence of the dTTT complex in their study is not strong. It appears as if the authors are drawing all these conclusions from the use of one bait, that of CG16908 from supplemental table 3. In this table, this is the only protein used to potentially identify the dTTT complex. In the text of the manuscript on page 17 the authors are claiming that dTTT contains CG16908, LqfR, Pontin, Teptin, and Spaghetti. However, supplemental table 3 lists nine total proteins listed as having SAINT scores of 0.99 or 1.0. What is the justification for the inclusion of only 5 proteins in dTTT? What about the other four? The authors need to conduct APMS studies with at least three of the other baits to provide better data to support the presence of the dTTT complex to determine exactly what it contains. This is a major point in the manuscript and needs to be strengthened with more data.

5. This leads to the data regarding the complex abundance ratios calculated from Figure 4. The authors need to provide much more detail on how the 13:1:2 ratio was calculated. What were the exact entry data points and why does this not have a standard deviation associated with it since they are in the data in Figure 4? A more detailed methodological documentation of this is needed here. Also, the dTTT abundance is only estimated from two data points, those of CG16908 and LqfR in the dTOR pulldown, while the other data has many more datapoints to draw from. The authors need to carry out experiments with CG16908, LqfR, and one more dTTT subunit to provide the data necessary to make this conclusion. Also in this figure the authors need to explain why they needed to normalize the data and why there are no error bars on the bait protein column?

6. Finally, the description of the fly genetics and phenotype analysis carried out in Figure 6 is lacking. The authors are to be commended for carrying out this experiment, but they need to better describe this figure, what is being seen, and what is different in all the panels. This is necessary for this manuscript to be approachable by a wide audience. Arrows in the figure pointing to key anatomical features would greatly enhance this figure, for example.

Reviewer #3:

This is an excellent manuscript describing the use of high-end proteomics for the systematic analysis of the InR/TOR interaction proteome in Drosophila. The authors performed a highly comprehensive analysis that mapped the InR/TOR interaction proteome in its basal state, revealed its regulation upon insulin and, by means of targeted MS, determined the relative distribution of network components among different dTOR complexes. This study further includes follow-up experiments that validate the functional significance of network components by RNAi depletion experiments in cell culture as well as genetic studies in flies. This manuscript covers all of its areas in an excellent and thorough manner both experimentally and in interpretation. The paper is well written and the

figures are clear and descriptive. I know this paper will find wide readership, due its highly relevant findings on the evolutionary conserved InR/TOR pathway and due to its well-conceived experimental concepts. This is one of the few papers that I see no fault in and would recommend acceptance outright.

Additional advice by Reviewer #4:

I don't think that three replicates are generally necessary. However, my question would also be (similar to reviewer 2) how reliable their label-free quantification method really is. It boils down to the question how they can justify their 1.5 fold cutoff. Rather than doing a third replicate they could simply do a mixing experiment. For example, they could mix yeast lysate with E. coli lysate in two different ratios (1:1 and 1:1.5). Can their approach then confidently separate E.coli and yeast proteins based on the 1.5 fold change? This is probably less laborious than doing an entire third replicate.

1st Revision - authors' response 09 September 2011

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Responses to the comments of referee two and four:

1. The authors need to provide supplemental tables documenting the data that they used to make the quantitative proteomic claims. There currently is no mass spectrometry data available showing the peptides, peptide scores, and protein scores used to make the conclusions in the manuscript. Also, the authors need to provide the quantitative data in the form of a supplemental table.

We have added three additional Supplementary Tables to better support the claims (Supplementary Table 4, 6 and 7). Supplementary Table 4 lists all identified network components with the corresponding protein and peptide scores. Supplementary Table 6 and 7 list all quantitative data that has been used to measure changes in protein abundances between insulin stimulated and non-stimulated AP-MS experiments.

2. This is a quantitative proteomics manuscript but the authors only carried out two replicates of their data on dynamic bait-prey interactions. A third replicate is required and a statistical analysis of the data is required. The authors must provide statistical validation of their claims in the manuscript. Currently, the authors are using a 1.5 fold cutoff to justify insulin sensitive interactions. This is not acceptable practice in modern quantitative proteomics. A third replicate is required and a statistical justification is required for the claim of 22 insulin regulated interactions.

Following the comments of referee two and the suggestions by referee four we performed additional experiments to demonstrate the validity of 1.5 fold change cut off used in our study for the identifcation insulin regulated protein interactions. To mimic a 1.5 fold change in abundance we prepared two different dilution samples in triplicates where we added 1x and 1.5x of purified Chico complexes purified from Kc167 cells to constant amounts of a sample prepared from GFP purifications. We could demonstrate by applying a Student's t-test that the measured changes in the abundance of Chico and its interacting proteins are highly significant and match the values expected from the 1.5 fold dilution.

We have added the results from this experiment and the primary data as an additional Supplementary Figure 1 and Supplementary Table 9 to our manuscript and referenced the new data in the main text at page 6 “The validity of a 1.5-fold cut off was carefully evaluated by statistical analysis of controlled dilution experiments (Supplementary Figure 1 and Supplementary Table 9).”

3. The authors need to define their use of the term of modularity early in the manuscript. What exactly do they mean here? It seems as if they are using modularity to in fact describe protein complexes. Therefore modularity in this case would be modules in a network. This also leads to confusion in the section on the pathway interactome where the data that is specifically used to support this section is not listed in the text. For example, on pages 8-9 on the Drosophila TOR modules, where is the data used to support this paragraph? Is it Supplemental Table 3? This section needs more detailed referencing of where the data used to make the claims originates.

The term modules may indeed be misleading without definition. We have made the following changes to clarify the use of this term in our manuscript as suggested by referee 2: The term “modules” was replaced in the abstract and on page 6 by the term “complexes” prior to its first use, where we inserted a definition at the end of page 6 to clarify its use in the text: “The term module is defined here as a group of proteins with high connectivity in the interaction network model due to complex formation or sharing of binding partners indicating a related biochemical context.”

The data to support the pathway interactome shown in Figure 3 is now better referenced by changing the first sentence of the section “**Overview on the *Drosophila* InR/TOR pathway interactome”**

at page 6: “We next assembled the interaction data (Supplementary Table 2 and Supplementary Table 6) into a quantitative network model to identify signaling modules and their changes upon insulin treatment (Figure 3)”.

4. The data to support the presence of the dTTT complex in their study is not strong. It

appears as if the authors are drawing all these conclusions from the use of one bait, that of CG16908 from supplemental table 3. In this table, this is the only protein used to potentially identify the dTTT complex. In the text of the manuscript on page 17 the authors are claiming that dTTT contains CG16908, LqfR, Pontin, Teptin, and Spaghetti. However, supplemental table 3 lists nine total proteins listed as having SAINT scores of 0.99 or 1.0. What is the justification for the inclusion of only 5 proteins in dTTT? What about the other four? The authors need to conduct APMS studies with at least three of the other baits to provide better data to support the presence of the dTTT complex to determine exactly what it contains. This is a major point in the manuscript and needs to be strengthened with more data.

We thank the reviewer for his comments on the composition of the dTTT complex. The reason whey we included these five proteins (dTOR, Pontin, Reptin, LqfR, CG16908 and Spaghetti) in our dTTT complex model is based on the detection of these five proteins in both, dTOR and CG16908 purifications. In addition all five proteins have been reported to form orthologous complexes in human cells using the human TTT subunits Tel2 and Tti2 as a bait (Horejsi et al, 2010; Hurov et al, 2010; Kaizuka et al, 2010; Takai et al, 2010). The other four proteins found in CG16908 purifications were not identified in dTOR purifications and were therefore excluded from the dTTT complex model. We have compiled the orthologous evidence in Supplementary Table 8 for clarification of the conserved dTTT complex composition. In addition we performed further validation experiments by reciprocal co-purification to obtain additional interaction information. In agreement with interactions between orthologous TTT proteins from human cells (Horejsi et al, 2010; Takai et al, 2010) we find that purified Spaghetti complexes contain dTOR as well as LqfR. Reciprocal experiments using LqfR as a bait showed that LqfR complexes contain Spaghetti, dTOR and CG16908. We have added the additional experimental data as a novel Supplementary Figure 3 and changed the text on page 18 to account for this.

From: “This study revealed the existence of a third dTOR complex here referred to as dTTT. Based on orthology information and reciprocal AP-MS data we propose that dTTT contains, besides dTOR, the gene product of CG16908, LqfR, Pontin, Reptin and Spaghetti.”

To “This study revealed the existence of a third dTOR complex here referred to as dTTT. Based on orthology information (see Supplementary Table 8) and reciprocal AP-MS and co-immunoprecipitation data (see Supplementary Figure 3) we propose that dTTT contains, besides dTOR, the gene product of CG16908, LqfR, Pontin, Reptin and Spaghetti”

5. This leads to the data regarding the complex abundance ratios calculated from Figure 4. The authors need to provide much more detail on how the 13:1:2 ratio was calculated. What were the exact entry data points and why does this not have a standard deviation associated with it since they are in the data in Figure 4? A more detailed methodological documentation of this is needed here. Also, the dTTT abundance is only estimated from two data points, those of CG16908 and LqfR in the dTOR pulldown, while the other data has many more datapoints to draw from. The authors need to carry out experiments with CG16908, LqfR, and one more dTTT subunit to provide the data necessary to make this conclusion. Also in this figure the authors need to explain why they needed to normalize the data and why there are no error bars on the bait protein column?

The relative protein abundance has been estimated using the average intensity of the three most intense peptide ions (TOP3) (Silva et al. 2006) relative to the bait dTOR. To estimate the abundance of the three dTOR complexes we used the abundance of CG16908 and LqfR for dTTT, dRaptor for TORC1 and dRictor for dTORC2 from four dTOR purifications. Since we used two proteins to estimate dTTT abundance we cannot exclude that this may affect the accuracy compared to the other two complexes. In order not to overstate our claim on the abundance distribution we followed the editor’s suggestion and tone down our claim in the text as follows (page12): From “Overall, the quantitative data from the dTOR purifications indicate that dTORC1, dTORC2 and dTTT are concurrently present in the cells at estimated abundance ratios of 13:1:2 (for details see Supplementary Table 6).” To: “Overall, the quantitative data from the dTOR purifications indicate that dTORC1 is the most abundant dTOR complex we have identified in Kc167 cells (for details see Supplementary Table 10).

Following the suggestion of referee two we also have modified Figure 4b and the corresponding legend. Figure 4b represents the abundance of proteins in the indicated

purifications relative to the bait. The legends for Figure 4b on page 29 has been changed as follows:

(B) Abundance distribution of proteins identified in dTOR and dGβL (upper panel), dRictor and dRaptor purifications (lower panel) relative to the bait. The average TOP3 signal intensity was used to infer protein abundances within individual AP-MS/MS experiments (data are listed in Supplementary Table 10). The average signal intensities of dTOR core components in each of the indicated purifications were calculated relative to corresponding bait intensity (set to $10E^5$) from four purification experiments and are shown in log scale in the bar chart. Error bars represent standard deviation in log scale.

6. Finally, the description of the fly genetics and phenotype analysis carried out in Figure 6 is lacking. The authors are to be commended for carrying out this experiment, but they need to better describe this figure, what is being seen, and what is different in all the panels. This is necessary for this manuscript to be approachable by a wide audience. Arrows in the figure pointing to key anatomical features would greatly enhance this figure, for example.

We have changed the Figure legends and the Materials and Methods section in the revised manuscript to better explain the experiment shown in Figure 6:

Page 30-31

Legend Figure 6: “Regulation of cell growth by LqfR/Tel2 and CG16908/Tti1. (A) Knock down experiments of *lqfR* and *CG16908* in the *Drosophila* eye. *ey-GAL4* was used to drive expression of short hairpin *UAS*-constructs in the *Drosophila* eye. Depletion of *lqfR* (*UAS-lqfR^{RNAi}*) or *CG16908* (*UAS-CG16908^{RNAi}*) resulted in a severe reduction of eye size suggesting that both proteins might act as positive growth regulators. A short hairpin construct against *CG1315*, which does not affect eye size, was used as control (*control*).
(B) Flp-FRT based mutagenesis of *lqfR* and *CG16908.* Flp-FRT mediated recombination was used to create mutant clones of *Tor, lqfR* or *CG16908* (*white tissue*) surrounded by wild-type tissue (*red tissue*) in the *Drosophila* eye. In comparison to control clones (*white*

tissue, first image; the FRT chromosome carries no mutation), clones mutant for *lqfR* (*white tissue, third image*) or *CG16908* (*white tissue, fourth image*) are severely smaller and show a growth disadvantage similar to clones mutant for *Tor* (*white tissue, second image*) suggesting a positive growth regulating function of lqfR and the product of *CG16908*. Exact genotypes are: (1) *y w ey-Flp; FRT82B,* cl^{3R3} w^{+} */ FRT82B,* (2) *y w ey-Flp; FRT40A,* cl^{2L3} w^{+} */ FRT40A,* Tor^{2L1}, (3) *y w ey-Flp; FRT82B,* cl^{3R3} w^{+} */ FRT82B,* $lqfR^{\Delta 117}$, (4) *y w ey-Flp; FRT82B,* cl^{3R3} w^{+} */ FRT82B,* $CG16908^{MB01483}$."

(page 25)

" Fly genetics

The *UAS* hairpin lines 25707 (*UAS-*$lqfR^{RNAi}$), 47096 (*UAS-*$CG1315^{RNAi}$) and 16908R-1 (*UAS-*$CG16908^{RNAi}$) were obtained from the Vienna *Drosophila* RNAi Center and the National Institute of Genetics (Japan), respectively. $CG16908^{MB01483}$ (Metaxakis et al, 2005) and the *GAL4* driver line *ey-GAL4* (Hazelett et al, 1998) were from the Bloomington *Drosophila* Stock Center. The alleles $lqfR^{\Delta 117}$ (Lee et al, 2009) and TOR^{2L1} (Oldham et al, 2000) as well as the FRT insertions *FRT40A* and *FRT82B* (Xu & Rubin, 1993) and the lines *y w eyFLP; FRT40A,* w^{+}*,* cl^{2L3} */ CyO* and *y w eyFLP; FRT82B,* w^{+}*,* cl^{3R3} */ TM6B, Tb, Hu,* y^{+} (Newsome et al, 2000) have been described. Lines carrying mutations on FRT chromosomes were established by meiotic recombination.

For figure 6A, *ey-GAL4* females have been crossed to males carrying the different *UAS* transgene insertions. For figure 6B, *y w eyFLP; FRT40A,* w^{+}*,* cl^{2L3} */ CyO* or *y w eyFLP; FRT82B,* w^{+}*,* cl^{3R3} */ TM6B, Tb, Hu,* y^{+} females have been crossed to males of the following lines: (1) *y w; FRT82B / TM6B, Tb, Hu,* y^{+}, (2) *y w; FRT40A,* Tor^{2L1} */ CyO*, (3) *y w; FRT82B,* $lqfR^{\Delta 117}$ */ TM6B, Tb, Hu,* y^{+}, (4) *y w; FRT82B,* $CG16908^{MB01483}$ */ TM6B, Tb, Hu,* y^{+}."

Acceptance letter 29 September 2011

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.

Sincerely,

Editor
Molecular Systems Biology

Reviewer #2 (Remarks to the Author):

I am satisfied with the authors revisions and now support publication of this strong manuscript in Molecular Systems Biology

Reviewer #3 (Remarks to the Author):

All the points raised in the previous round of review have been satisfactorily addressed.

Reviewer #4 (Remarks to the Author):

Based on the new dilution experiment and the corresponding fig S1 I think the 1.5 cutoff is well justified. A more thorough analysis of the accuracy of label free quantification would require a larger dataset (i.e. mixing experiments with more complex protein samples. However, this is beyond the scope of this paper and therefore *not* required for publication.