molecular systems biology

Proteomic and protein interaction network analysis of human T lymphocytes during cell-cycle entry

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1st Editorial Decision 30 August 2011

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 referees are supportive, but raised a series of substantial concerns, which, I am afraid to say, preclude the publication of this work in its present form.

Most importantly, the reviewers raise two important issues regarding potential confounders in the data. Addressing these issues convincingly may require new statistical analyses and/or supporting experiments.

1. The first reviewer is concerned that changes in cellular localization could appear as changes in protein levels, especially if an important part of the protein pool resides in a cellular location that is not well-represented in the chromatin-associated or free protein fractions.

2. The second reviewer notes the mixture of cell-cycle stages present in the 40hr samples. This seems to suggest that the observed protein abundance changes may represent a broader transition from quiescence to cycling & proliferation, rather than focusing specifically on the G0->G1 transition.

In addition, all three reviewers had clear concerns regarding the clarity of presentation, and each noted a series of specific instances where more methodological detail is needed or where existing explanations or figure labels are potentially confusing. Please note that Articles may include up to 8

figures in the main manuscript.

Before submitting any revised work, we ask that all proteomic data is submitted to a public repository (e.g. PRIDE). Please include the resulting accession number (or confidential reviewer login) in the methods section of the revised manuscript.

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.

PLEASE NOTE As part of the EMBO Publications transparent editorial process initiative (see http://www.nature.com/msb/journal/v6/n1/full/msb201072.html), Molecular Systems Biology now publishes online a Review Process File with each accepted manuscript. Please be aware that in the event of acceptance, your cover letter/point-by-point document will be included as part of this file, which will be available to the scientific community. Authors may opt out of the transparent process at any stage prior to publication (contact us at msb@embo.org). More information about this initiative is available in our Instructions to Authors.

Yours sincerely,

Editor - Molecular Systems Biology
msb@embo.org

Referee reports:

Reviewer #1 (Remarks to the Author):

In this manuscript, Orr and colleagues study the human T lymphocyte activation during the G0 to G1 transition. They use label free spectral counting based proteomics analysis of chromatin/nuclear bound proteins versus free proteins from T cells in quiescent and activated states. Notably, theses T cells were from the peripheral blood of normal donors. The authors map the data they obtain onto a modeled protein interaction network in order to interpret their data and suggest follow up experiments. The authors primarily carry out extensive western blotting validation and flow cytometry to validate their results. In general, this is an appropriate manuscript for Molecular Systems Biology, but it is in need of revision prior to publication.

One issue that is not addressed and is difficult to ascertain what impact it has on the interpretation is that of changing localization. Since the authors are looking at different cellular fractions in this study, one possibility is proteins change localization upon activation which then results in the up and down regulation in the C/NM and Free populations. The authors need to determine a way to remove this as a confounding issue in their data analysis. It is possible that proteins that are increased in a fraction come from some other part of the cell that is not covered well by a C/NM and Free fractionation.

A next issue is the issue of statistical significance. Here the authors use the z-score as the method for analyzing data. Since the z-score is not widely used in quantitative proteomics analysis, more justification for its use needs to be given (either in the manuscript or in the supplement). How should readers not familiar with the z-score interpret this information? What z-scores demonstrate statistical significance? The authors state that they use the t-test for statistical analysis, but these values are not clearly provided in the supplemental tables. Also, the t-test is likely not appropriate for use with spectral counting based datasets and other approaches should be used (Choi Het al. 2008 Dec;7(12):2373-85 and Pavelka et al. Mol Cell Proteomics. 2008 Apr;7(4):631-44.)

Some of the figure legends are problematic. Fig. 2 needs more detail for the description of panels D and E in particular which are 6 and 5 panels each. Also, the G1, S, G2/M and Apo values are given in E but not D. These values should be given for all flow cytometry data in all figures (including the supplementary figures). Figure 3B, for example, particularly needs this and additional explanation.

In Figure 3B it appears as if the S +G2/M section is changing from the control siRNA to the eIF6 siRNA since there is a downshift in the location on the y axis in this box. This needs clarification and inclusion of G1, S, G2/M and Apo values. Figure S3 is an important figure for validation, but the legend is insufficient. What is the rational for being included in A, B, C, and D panels in this figure? It would also be valuable if this figure were broken up and the mass spectrometry data included on the side for each of the proteins. It would be much easier to interpret instead of having to go back and forth from the figure to the supp datasets.

Finally, the authors need to carefully spell out in the methods exactly how many biological replicates were used. The supplemental tables suggest that the answer is four, which is commendable.

Reviewer #2 (Remarks to the Author):

In the current manuscript Orr et al. describe a proteomic strategy to identify differentially expressed proteins associated with the nuclear/chromatin matrix (or free "unbound") using label-free quantification. Proteins were isolated from primary human T lymphocytes as they progress from G0 G1. Differentially expressed proteins were than systematically analyzed using gene ontology annotation and protein interaction networks.

The most exiting results obtained are that entry into the cell cycle and entry into the growth cycle can be dissociated from each other. Two proteins were validated through siRNA knock-down and limited functional analyses.

Overall this is an interesting paper that utilizes MS-based proteomics analysis to investigate protein networks of nuclear/chromatin associated proteins in primary T lymphocytes as they progress from G0 to G1.

Comments:

1) It seems the manuscript was previously submitted to a different journal with specific limitation on the number of figures. Several of the supplemental figures need to be moved into the main manuscript. In its current form the manuscript was hard to read and was completely broken up. The authors need to spend some time reformate to turn this into a concise, readable manuscript.
2) It was not clear why the "free" fraction was really analyzed. It seems the authors are mainly interested in nuclear/chromatin/transcription-type networks. Most effort in the analyses are spent on these fractions?
3) Page 4: 2D MudPIT: There is no such thing as 2D MudPIT. It's either 2D-LC or MudPIT.
4) Page 15: please add the references for protein/peptide prophet.
5) Page 6: ...reading primary papers. What papers? Remove or cite.
6) It seems the protein network algorithm HumanNet was recently published in Genome Research. A bit more information in the methods would have been useful.
7) Page 7: The authors choose 2 key proteins for siRNA knock-down and functional analyses. How were these two proteins chosen from the several hundred differentially expressed proteins?
8) Page 5 second paragraph: The authors use spectral counting, just write it. No reason to justify it over an entire paragraph. Any other quantitative proteomics approach also has pros and cons.

There is no doubt that the current manuscript combines careful MS-based proteomics with some strong biochemistry and cell biology. This resource could potentially be interesting to the community. The major problem this reviewer has is that most of the data is jammed into the supplements which make reading the manuscript less than enjoyable. Asides from a couple of minor points, the authors need to change this prior to resubmission.

Reviewer #3 (Remarks to the Author):

Proteomic and protein interaction network analysis of human T lymphocyte activation during G0->G1 transiton by Orr et al.
Orr et al. did proteomic analysis of protein interaction networks activated during the G0/G1 transition of primary human T cells, which then directed detailed molecular studies of two important proteins, SF3B and eIF-6, in coordinating the entry into the cell cycle and growth cycle of the

human T lymphocytes. I like the paper overall. Especially, the conclusion that cell proliferation and cell growth (blastogenesis) in human T cells are not necessarily coupled is of significant interest to cell biology. The approach and comprehensiveness of their work would appeal to the general readers of MSB.
I do, however, have a few questions for the authors to address first, which I hope would help improve the paper:

1. The two parts of the study, proteomic analysis of protein interaction networks and the detailed studies on SF3B and eIF-6, are somewhat disconnected. I did not get how the authors specifically selected SF3B and eIF-6 for detailed study after their proteomic analysis. From the abstract, these two proteins seem to be "two proteins in the most interconnected C/NM-bound sub-networks". However, neither of them appears in the three most highly interconnected sub-networks (Figure 1B and Table S3). In fact, SF3B (SF3B2 and SF3B4) does not even appear in the list of 307 proteins that increase in the C/NM-bound fraction in G1 (Table S2)?

2. The major focus of the study was to compare the protein interaction networks activated at G1 vs. G0. The authors analyzed the proteomic data collected from cells 40h post-stimulation with PMA/ionomycin vs. that from quiescent cells. Cells 40h with PMA/ionomycin, however, were a mixture of cells at G0, G1, S, G2/M, and apoptosis. My question is, why not first sort out G1 cells from the mixture and use that for subsequent proteomic analysis? At least, I would think that the authors should exclude G0 cells from the 40h PMA/ionomycin cell mixture.

3. Data, depending on the assays in individual figures, were collected at different time, from 40h to 3 day to 5 day, after either PMA/ionomycin or +CD3/CD28 stimulation. A general description of expected cell cycle progression (in terms of timing) of human T cells under these conditions would help readers interpret the results.

4. Figure 1B legend states that the three most highly interconnected sub-networks are: (1) RNA splicing, transport, structure, (2) ribosomal proteins, and (3) nucleolar proteins. From a quick look of the list of proteins in Table S3, however, I do not feel that the Sub-network 1 (with the major components associated with Ribosome biogenesis and other functions) is enriched for "RNA splicing, transport, structure".

5. Many careless/mistaken statements and typos exist in the current submission (a quick but not comprehensive list is given below). The authors should thoroughly proofread the manuscript and correct the errors.

a. All three pairs of "C/NM" and "Free" labels in Figure S2 were wrong, and should be swapped according to the description in the text.

b. Figure 2D does not seem to match the text on page 7 (percentage of cells that entered S phase, 17.5{plus minus}1.5% with control siRNA vs 7.9{plus minus}1.8% with SF3B2 siRNA). If the percentage of S-phase cells in the control was 17.5%, that with SF3B2 siRNA would look much smaller than 7.9% from the figure. Also, quantitative labeling of cell phases in Figure 2D (like that in Figure 2E) is needed.

c. Again on page 7, it states that "This is not due to a delay in cell cycle entry as there was no increase in the percentage of cells in S phase up to 5 days post stimulation." However, when I compare the "SF3B2, day 5" with "SF3B2" in Figure 2D, I saw a visible increase in the S-phase cells at day 5.

d. Based on the similar gating and population characteristics between the "SF3B2, day 5" in Figure 2D and the knockdowns shown in Figure 2E, I would assume these are all day 5 experiments. However, the legend makes it seem as if Figure 2E corresponds to day 3 experiments. If they are not all day 5, then what is the explanation for their different characteristics compared to the other day 3 populations shown in Figure 2D.

e. On page 8, "e.g. mTOR are phosphorylated in SF3B2/4-depleted cells" - mTOR is shown in SF3B2-depleted cells (Figure 2B), but not shown in SF3B4-depleted cells (Figure 2C).

f. On page 9, "the variability in the protein content of cells in S and G2/M was much reduced when eIF6 was depleted (range for cells in G1: 638.5{plus minus}12 with control siRNA vs 454{plus minus}33 with eIF6 siRNA)" - so the variability seems to increase but not reduce ({plus minus}33 vs. {plus minus}12) with eIF6 siRNA?

g. On page 12, "the cells progress more slowly through the growth cycle but appear to enter the cell cycle as normal." Since there is not really time-course data in the paper, I don't think "slowly" is the appropriate conclusion to make.

h. Figure 2 legend, "A & B, cells transfected with control or SF3B4 siRNA" - "SF3B4" should be "SF3B2".

i. Figure S7 A-E are missing.

1st Revision - authors' response 05 December 2011

We are pleased that the referees are supportive of publication and we are grateful for their helpful comments to improve the paper. We have amended our manuscript in light of their comments and our responses are detailed below, following this letter.

We have addressed the two issues which you highlighted in your letter as follows:

1. The first reviewer is concerned that changes in cellular localization could appear as changes in protein levels, especially if an important part of the protein pool resides in a cellular location that is not well-represented in the chromatin-associated or free protein fractions.
In our study, we have not analysed changes in protein levels between the two cellular fractions and so our analyses are not affected by a protein pool that may not be well represented by the chromatin and nuclear matrix-bound or -free fractions. Rather, we focus our analyses to identify changes in the levels of proteins specifically *within* each of these two fractions as T cells enter the cell cycle from G_0. We have added this clarification to the text on page 6 (line 147-150). Such changes could be due to changes in the overall abundance of individual proteins or changes in their sub-cellular localisation. Our western blotting analyses (were Figures S3-S5, now Figure 2 and Figure 5) showed examples of each of these two possibilities, which was described in the Figure legend to Figure S3 (now Figure 2). To clarify the point, we have now explained this in the text of the revised manuscript on page 6 (line 154-159).

2. The third reviewer (point 2) notes the mixture of cell-cycle stages present in the 40hr samples. This seems to suggest that the observed protein abundance changes may represent a broader transition from quiescence to cycling & proliferation, rather than focusing specifically on the G0->G1 transition.
Our study is indeed focussed on analysing the changes that occur as quiescent T cells are stimulated to enter the cell cycle. As a short-hand, we referred to this as the $G_0 \rightarrow G_1$ transition. We considered sorting cells in G_1, but as we wished to quantify *changes* in C/NM-bound and –free proteins during cell cycle entry we reasoned that any cells remaining in G_0 40h post stimulation would only reduce our signal strength and not significantly affect the data we obtained. Furthermore, we considered that the technical issues of sorting either permeable, fragile fixed cells or of protein losses during sorting nuclei were significant and we decided against it. As we have not focussed on G_0 and G_1 cells exclusively, we have now changed references to the $G_0 \rightarrow G_1$ transition in the title as well as in the text throughout so that it is clear that we are studying entry into the first cell cycle from a quiescent state rather than differences specifically between G_0 and G_1.

As requested, we have posted all our proteomic data to PeptideAtlas (http://www.peptideatlas.org/PASS/PASS00016) and the gene expression array data have been deposited at http://www.ncbi.nlm.nih.gov/geo/. These details have been added just before the Acknowledgements section of the revised manuscript on page 22, lines 628-631.

As also requested: 1. The manuscript text is in MS Word format. 2. A detailed description of the changes made in response to the referees follows this letter, specifying the exact places in the text where each change has been made. 3. Four 'bullet points' highlighting the main findings of our study have been added on the second page of the text (lines 25-32). 4. A 'standfirst text' summarizing the study (~250 characters) has also been added on the second page (lines 35-38). 5. We have also uploaded a "thumbnail image" (width=211 x height=157 pixels, jpeg format), which can be used to highlight our paper on your homepage. 6. An author contributions statement is after the Acknowledgements section (page 22, lines 641-647).

Reviewer #1:

1. *One issue that is not addressed and is difficult to ascertain what impact it has on the interpretation is that of changing localization. Since the authors are looking at different cellular fractions in this study, one possibility is proteins change localization upon activation which then results in the up and down regulation in the C/NM and Free populations. The authors need to determine a way to remove this as a confounding issue in their data analysis. It is possible that proteins that are increased in a fraction come from some other part of the cell that is not covered well by a C/NM and Free fractionation.*
   In our study, we have not analysed changes in protein levels between the two cellular fractions and so our analyses are not affected by a protein pool that may not be well represented by the chromatin and nuclear matrix-bound or -free fractions. Rather, we focus our analyses to identify changes in the levels of proteins specifically *within* each of these two fractions as T cells enter the cell cycle from G_0. We have added this clarification to the text on page 6 (line 147-150). Such changes could be due to changes in the overall abundance of individual proteins or changes in their sub-cellular localisation. Our western blotting analyses (were Figures S3-S5, now Figure 2 and Figure 5) showed examples of each of these two possibilities, which was described in the Figure legend to Figure S3 (now Figure 2). To clarify the point, we have now explained this in the text of the revised manuscript on page 6 (line 154-159).

2. *A next issue is the issue of statistical significance. Here the authors use the z-score as the method for analyzing data. Since the z-score is not widely used in quantitative proteomics analysis, more justification for its use needs to be given (either in the manuscript or in the supplement). How should readers not familiar with the z-score interpret this information? What z-scores demonstrate statistical significance? The authors state that they use the t-test for statistical analysis, but these values are not clearly provided in the supplemental tables. Also, the t-test is likely not appropriate for use with spectral counting based datasets and other approaches should be used (Choi Het al. 2008 Dec;7(12):2373-85 and Pavelka et al. Mol Cell Proteomics. 2008 Apr;7(4):631-44.)*
   The z-score is commonly used to determine statistical significance of deviation when comparing normal distributions. It is an established method and the test statistic associated with the APEX method of label-free protein quantitation which we have used in our previous publications (see (Lu et al., 2007) and (Vogel and Marcotte, 2008) in the manuscript). The APEX method is also referenced in (Choi et al, 2008). The reviewer is correct that the t-test is inappropriate for use in this context, and in fact we did not use it. We did, however, make reference to the p-value corresponding to the z-score cut-off for significance (a z-score of +/- 1.96 corresponds to a p-value of 0.05 for a normal distribution). We have clarified this point and the use of z-scores more fully, including the z-score value of +/- 1.96 used to determine significance, on page 6, line 140-141 and page 18, line 537-538 of the revised manuscript.

3. *Some of the figure legends are problematic.*
   Fig. 2 needs more detail for the description of panels D and E in particular which are 6 and 5 panels each. Also, the G1, S, G2/M and Apo values are given in E but not D. These

values should be given for all flow cytometry data in all figures (including the supplementary figures).
Figure 2 is now Figure 6 of the revised manuscript. The Figure legend has been amended to include more detail and percentage values have been added for Panel D.
Figure 3B, for example, particularly needs this and additional explanation. In Figure 3B it appears as if the S +G2/M section is changing from the control siRNA to the eIF6 siRNA since there is a downshift in the location on the y axis in this box. This needs clarification and inclusion of G1, S, G2/M and Apo values.
Percentages have been added for Figure 7B (was Figure 3B). The observation that there is a down-shift in the protein content of the S+G2/M cells (y-axis) is described and quantified on page 12, line 329-336 of the revised manuscript.
Figure S3 is an important figure for validation, but the legend is insufficient. What is the rational for being included in A, B, C, and D panels in this figure? It would also be valuable if this figure were broken up and the mass spectrometry data included on the side for each of the proteins. It would be much easier to interpret instead of having to go back and forth from the figure to the supp datasets.
The legend of Figure S3 (now Figure 2) has been expanded to provide more explanation. As requested, the fold-change values identified by mass spectrometry for each of the proteins have been added to the Figure. We have not split the data in panels A-D as the lysates used in each panel are common to the proteins analysed in that panel and so loading and cell cycle controls also apply to that panel.

4. *Finally, the authors need to carefully spell out in the methods exactly how many biological replicates were used. The supplemental tables suggest that the answer is four, which is commendable.*
We did indeed carry out mass spectrometry analyses of 4 independent biological replicates, which is now stated in the text (see page 5, line 97 of the revised manuscript).

Reviewer #2:

1) *It seems the manuscript was previously submitted to a different journal with specific limitation on the number of figures. Several of the supplemental figures need to be moved into the main manuscript. In its current form the manuscript was hard to read and was completely broken up. The authors need to spend some time reformate to turn this into a concise, readable manuscript.*
We have moved Figures S2-S5 into the main paper (now Figures 1,2,5 and 4) and we have amended the text to include these changes. The section on Xirp1/Xin1 has also been moved from Supplementary Results into the main text (page 7, line 187-218 of the revised manuscript). The paper now has 7 figures in the main text and we hope it is now more readable, without recourse to the Supplementary sections.

2) *It was not clear why the "free" fraction was really analyzed. It seems the authors are mainly interested in nuclear/chromatin/transcription-type networks. Most effort in the analyses are spent on these fractions?*
We have indeed concentrated our analyses on the C/NM-bound proteins. However, we have included mass spectrometry and western blot analyses of both fractions so that others interested in T cell biology can make full use of the data we have generated.

3) *Page 4: 2D MudPIT: There is no such thing as 2D MudPIT. It's either 2D-LC or MudPIT.*
This has been corrected (page 5, line 112 of the revised manuscript).

4) *Page 15: please add the references for protein/peptide prophet.*
This reference has been added to the text (page 17, line 516-517 of the revised manuscript).

5) *Page 6: ...reading primary papers. What papers? Remove or cite.*
Our reading of the primary literature is reflected in the Supplementary Review of T Cell Proteomics, which contains many of the references. A sentence has been added to the

legend of Figure 3 (page 30, line 954-955) and in the Methods section page 18 (lines 545-548) to explain this.

6) *It seems the protein network algorithm HumanNet was recently published in Genome Research. A bit more information in the methods would have been useful.*
A brief explanation has been added to the text on page 21 (line 617-619) and we have updated the reference.

7) *Page 7: The authors choose 2 key proteins for siRNA knock-down and functional analyses. How were these two proteins chosen from the several hundred differentially expressed proteins?*
We chose proteins involved in two of the main functional categories that were C/NM-bound and upregulated during cell cycle entry, namely hnRNA splicing and ribosome biogenesis (described on page 6, line 175-178). SF3B2 was identified in our initial mass spectrometry analyses and increases in C/NM-binding in stimulated cells (verified by western blotting). We chose this, as well as its binding partner, SF3B4, as they are involved early in 3'-splice recognition by the U2 major spliceosome. eIF-6 was chosen as it is involved in biogenesis of the 60S ribosomal subunit and it is highly upregulated during cell cycle entry. An explanation has been added to the text (page 9, line 231-239).

8) *Page 5 second paragraph: The authors use spectral counting, just write it. No reason to justify it over an entire paragraph. Any other quantitative proteomics approach also has pros and cons.*
This explanation has been removed.

Reviewer #3:

I do, however, have a few questions for the authors to address first, which I hope would help improve the paper:

1. *The two parts of the study, proteomic analysis of protein interaction networks and the detailed studies on SF3B and eIF-6, are somewhat disconnected. I did not get how the authors specifically selected SF3B and eIF-6 for detailed study after their proteomic analysis. From the abstract, these two proteins seem to be "two proteins in the most interconnected C/NM-bound sub-networks". However, neither of them appears in the three most highly interconnected sub-networks (Figure 1B and Table S3). In fact, SF3B (SF3B2 and SF3B4) does not even appear in the list of 307 proteins that increase in the C/NM-bound fraction in G1 (Table S2)?*
See response to Reviewer #2, point 7 and we have added an explanation to the text (page 9, line 231-239). Reference to the "most interconnected sub-networks" in the abstract has also been amended (line 51).

2. *The major focus of the study was to compare the protein interaction networks activated at G1 vs. G0. The authors analyzed the proteomic data collected from cells 40h post-stimulation with PMA/ionomycin vs. that from quiescent cells. Cells 40h with PMA/ionomycin, however, were a mixture of cells at G0, G1, S, G2/M, and apoptosis. My question is, why not first sort out G1 cells from the mixture and use that for subsequent proteomic analysis? At least, I would think that the authors should exclude G0 cells from the 40h PMA/ionomycin cell mixture.*
As explained the covering letter to the Editor, our study is focussed on analysing the changes that occur in C/NM-bound and –free proteins as quiescent T cells are stimulated to enter the cell cycle. We discussed sorting and analysing cells in G_1, but we considered that the technical issues of sorting either permeable, fragile fixed cells or of protein losses during sorting nuclei would be significant and we decided against it. As we wished to quantify *changes* in C/NM-bound and –free proteins during cell cycle entry we considered that any cells remaining in G_0 40h post stimulation would only reduce the changes quantified and would not significantly affect the identification of proteins that changed. Since we have not focussed on G_0 and G_1 cells exclusively, we have now changed

references to the $G_0 \rightarrow G_1$ transition in the title as well as in the text throughout to be clear that we are studying entry into the first cell cycle from a quiescent state rather than differences specifically between G_0 and G_1.

3. *Data, depending on the assays in individual figures, were collected at different time, from 40h to 3 day to 5 day, after either PMA/ionomycin or +CD3/CD28 stimulation. A general description of expected cell cycle progression (in terms of timing) of human T cells under these conditions would help readers interpret the results.*
   An explanation has been added to the text on page 4, line 59-61.

4. *Figure 1B legend states that the three most highly interconnected sub-networks are: (1) RNA splicing, transport, structure, (2) ribosomal proteins, and (3) nucleolar proteins. From a quick look of the list of proteins in Table S3, however, I do not feel that the Sub-network 1 (with the major components associated with Ribosome biogenesis and other functions) is enriched for "RNA splicing, transport, structure".*
   The legend (now Figure 3B) has been corrected (page 32-33, line 950-963).

5. *Many careless/mistaken statements and typos exist in the current submission (a quick but not comprehensive list is given below). The authors should thoroughly proofread the manuscript and correct the errors.*

a. *All three pairs of "C/NM" and "Free" labels in Figure S2 were wrong, and should be swapped according to the description in the text.*
   These have been changed (note this Figure is now Figure 1).

b. *Figure 2D does not seem to match the text on page 7 (percentage of cells that entered S phase, 17.5{plus minus}1.5% with control siRNA vs 7.9{plus minus}1.8% with SF3B2 siRNA). If the percentage of S-phase cells in the control was 17.5%, that with SF3B2 siRNA would look much smaller than 7.9% from the figure.*
   There is biological variability between experiments involving different T cell isolates. We note that the percentage of cells in S-phase when transfected with SF3B2 siRNA varied from 0.95%-20.53% and we did not exclude any of the experiments from our statistical analyses. We have now updated the statistics in the text for n=15 experiments (page 9, lines 252-253).
   Also, quantitative labeling of cell phases in Figure 2D (like that in Figure 2E) is needed.
   The percentages have been added to the Figure (now Figure 6).

c. *Again on page 7, it states that "This is not due to a delay in cell cycle entry as there was no increase in the percentage of cells in S phase up to 5 days post stimulation." However, when I compare the "SF3B2, day 5" with "SF3B2" in Figure 2D, I saw a visible increase in the S-phase cells at day 5.*
   The rationale for doing the experiment and analysing the sample at day 5 was to be sure that the effects of depleting SF3B2 persisted and so quantifying at day 3 was valid. An explanation has been added to the text on page 9, lines 252-255.

d. *Based on the similar gating and population characteristics between the "SF3B2, day 5" in Figure 2D and the knockdowns shown in Figure 2E, I would assume these are all day 5 experiments. However, the legend makes it seem as if Figure 2E corresponds to day 3 experiments. If they are not all day 5, then what is the explanation for their different characteristics compared to the other day 3 populations shown in Figure 2D.*
   Samples were normally taken on day 3 post stimulation. The panel shown in Figure 2D (now Figure 6D) taken on day 5 was a specific experiment to test whether the effect of the siRNA persisted, as described in the text on page 9, line 252-255. The samples in Figure 2E (now Figure 6E) were of cells transfected with each of the individual SF3B2 siRNA and all samples were taken on day 3. As the effects on cell cycle progression were observed with 3 of the 4 individual siRNA duplexes, we considered it unlikely that the results are due to off-target effects (described on page 9, line 256-258). We did not increase the concentrations of the individual siRNA used to determine whether we could attain the same level of inhibition as we obtained with the siRNA pool.

e. *On page 8, "e.g. mTOR are phosphorylated in SF3B2/4-depleted cells" - mTOR is shown in SF3B2-depleted cells (Figure 2B), but not shown in SF3B4-depleted cells (Figure 2C).*
   The text on page 11, line 297-298 has been amended to reflect what is shown in the Figure (now Figure 6).

f. *On page 9, "the variability in the protein content of cells in S and G2/M was much reduced when eIF6 was depleted (range for cells in G1: 638.5{plus minus}12 with control siRNA vs 454{plus minus}33 with eIF6 siRNA)" - so the variability seems to increase but not reduce ({plus minus}33 vs. {plus minus}12) with eIF6 siRNA?*
The point we wanted to make is that the mean protein content of cells in $S+G_2/M$ is smaller in cells transfected with eIF6 siRNA *vs* control siRNA (454 *vs.* 638). The range is also less in cells transfected with eIF6 siRNA, which we describe in the text on page 12, line 329-332. However, we cited the effects of eIF6 *vs* control siRNA for the mean but the effects of control *vs* eIF6 siRNA for the range. As this could be confusing for the reader, it has now been amended to be consistent.

g. *On page 12, "the cells progress more slowly through the growth cycle but appear to enter the cell cycle as normal." Since there is not really time-course data in the paper, I don't think "slowly" is the appropriate conclusion to make.*
The text has been changed (page 14, line 414-415).

h. *Figure 2 legend, "A & B, cells transfected with control or SF3B4 siRNA" - "SF3B4" should be "SF3B2".*
The legend has been changed (note Figure 2 is now Figure 6).

i. *Figure S7 A-E are missing.*
We thought that these had been uploaded and we apologise if not. These have now been uploaded (now Figure S4 A-E).

2nd Editorial Decision 02 January 2012

Thank you again for submitting your work to Molecular Systems Biology. We have now heard back from the three referees who evaluated your revised study. As you will see, the reviewers feel that the revisions made to this work have satisfied their main concerns. They raise, however, a series of minor concerns and make suggestions for modifications. In addition, certain issues have arisen during our editorial evaluation of this work, which we would ask you to carefully address in a revision of the present work.

Most importantly, while checking the figure files supplied with this work, the editor noticed that the PDF images of the gels presented in Figures 2 and 6, in some cases, appear to be compositions of different image layers. Please note that our data presentation policies require that "images gathered at different times or from different locations should not be combined into a single image ... If juxtaposing images is essential, the borders should be clearly demarcated in the figure and described in the legend." Please see our Instructions for Authors for more details (http://www.nature.com/msb/authors/index.html#a3.4.3).

Separate image layers are apparent, for example, in Figure 2A in the STAT3 lane, Figure 2D for all of the left panels, and in Figure 6A in the SF3B2 lane. We ask that you supply the unprocessed image files for all gels presented in this work (including the supp. figures). They can be supplied in a single zip file, with descriptive filenames for each image. We encourage you to investigate the origin of this image layering, and to provide a brief explanation in a reply email.

In addition to the important issue above and the minor changes suggested by the reviewers, we have some more minor points regarding formatting and organization, which we ask you to take into consideration when preparing your revised work (detailed at the end of this email).

Thank you for submitting this paper to Molecular Systems Biology.

Sincerely,

Editor - Molecular Systems Biology
msb@embo.org

Referee reports:

Reviewer #1 (Remarks to the Author):

The revised manuscript addresses most of my concerns regarding the original manuscript. It is a nice combination of approaches and fits very well in Molecular Systems Biology. I think that it will make an excellent contribution to the literature. There are a couple of minor 'clerical' issues that need to be addressed prior to final publication. One issue is on page 8 lines 195-200, there are references to figures in the text that do not match. It seems likely that these references are to figure 2 and not 4. Figure 4C is not a western, for example. The second issue is there is a great deal of important information in the supplemental information that is available in the zipped SI file. The authors provide info for both a Mac and PC. Inside of each of these folders, for the sake of ease of use, the authors should provide a word document or text document describing what is provided in the tables and the figures. This will make this information more accessible to readers who would have to go back to the main document to figure out what is where.

Reviewer #2 (Remarks to the Author):

The authors have addressed all my previous concerns.

The paper is suitable for publication in MSB

Reviewer #3 (Remarks to the Author):

The authors have addressed all my major questions.

Minor comments:
1. Figure 6D: the S(%) of SF3B2 and SF3B4 panels are labeled as 8.3 and 3.87, respectively. However, it looks that the number/percentage of S-phase cells in SF3B2 panel is smaller than that in SF3B4?

2. line 37: " ... show roof of principle", "roof" should be "proof".

Formatting & Organization Points for Revision
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1. The manuscript title is currently longer than our 100 character limit (including spaces). Moreover, the editor feels that this work may benefit from a simpler, more declarative title that highlights this work's most novel findings, for example "Proteomic analysis of T cell activation reveals separable control of cell growth and the cell cycle." This is only a suggestion; please select a title, within the length limits, that you feel best represents your manuscript.

2. The resolution of the existing figure images is low in some cases, particularly for the network diagrams and some of the gel images. Please provide higher resolution images, if possible. You may also wish to present the tabular data in Fig. 2 as an independent manuscript Table, to ensure that this figure remains easily readable when fit to a single page by the typesetters. For the same reason you may want to increase the font size, and decrease the white space in Fig. 3A. Please supply each main manuscript figure as an individual image file, preferably in the EPS format (although PDF may also be acceptable).

3. Please provide a single Supplementary Information pdf that begins with a Table of Contents explaining all of the supplementary materials and files provided with this work. This PDF should also include all of the Supplementary Figures (with legends immediately below), as well as the Supplementary Results and Discussion. Supplementary Tables and other materials can then be provided as additional files. Please merge all of the Supplementary high-resolution figure panels and Table 6A-G into single zip folders to help reduce the total number of supplementary files.

23rd January, 2012

Manuscript Number: MSB-11-3098R

We are pleased that the referees are happy with the revisions we have made to the manuscript. We have amended the manuscript further to address the additional points you and the reviewers have raised. Our amendments are as follows (corrections to the text are in red):

Editorial comments:

1. *…the editor noticed that the PDF images of the gels presented in Figures 2 and 6, in some cases, appear to be compositions of different image layers.*
   In the course of this study, western blot membranes were either routinely cut into sections and each section probed with a different antibody or the blots were re-probed sequentially with different antibodies, which recognise proteins of different molecular weights (*e.g.* Mcm2 and Mcm7). Sections of the films that showed the bands corresponding to each antibody (determined by size comparison with molecular weight markers) were selected in the scanner software (Epson Scan 3.04), scanned individually as separate image files and saved as JPEGs. In some cases, changes in brightness and/or contrast were applied linearly equally across the entire image.

   This explanation has been added to the Materials and Methods section of the manuscript on page 20 (line 586-594).

   The individual image scans (JPEGs) are attached in a Zip-file, entitled "Figure source data for Orr et al" and explanations are in "Explanations of image files".

2. *License to publish.*
   We have FAXed the License to Publish form.

Formatting and Organization Points for Revision

1. *The manuscript title is currently longer than our 100 character limit (including spaces).* The title has been amended to: "Proteomic and protein interaction network analysis of human T lymphocytes during cell cycle entry" (97 characters)

2. *The resolution of the existing figure images is low in some cases, particularly for the network diagrams and some of the gel images. Please provide higher resolution images, if possible.*
   High resolution PDF images of the network diagrams were uploaded with our original manuscript and we also uploaded the Cytoscape .cys files so that readers can view the data interactively. We referred to these in the text on page 7, lines 172-175.

   You may also wish to present the tabular data in Fig. 2 as an independent manuscript Table, to ensure that this figure remains easily readable when fit to a single page by the typesetters.
   These data have now been removed from the Figures and inserted as Table S8.

   For the same reason you may want to increase the font size, and decrease the white space in Fig. 3A.
   This Figure has been modified and the percentages have been inserted in Table S3. We have also made similar modifications to Figure S2A and added the percentages to Table S1. The Figure legends on page 34, lines 964-965 and page 36, lines 1052-1053 have been amended.

   Please supply each main manuscript figure as an individual image file, preferably in the EPS format (although PDF may also be acceptable).
   Individual PDFs are now uploaded.

3. *Please provide a single Supplementary Information pdf that begins with a Table of Contents explaining all of the supplementary materials and files provided with this work. This PDF should also include all of the Supplementary Figures (with legends immediately below), as well as the Supplementary Results and Discussion.*
   These changes have been made and a single PDF has been uploaded.

Supplementary Tables and other materials can then be provided as additional files. Please merge all of the Supplementary high-resolution figure panels and Table 6A-G into single zip folders to help reduce the total number of supplementary files.
These changes have been made.

4. *Please provide three to four 'bullet points' highlighting the main findings of your study.*
These bullet points were provided on page 2 of the main text (lines 24-32).

5. *Please provide a 'standfirst text' summarizing your study in one or two sentences (approx. 250 characters).*
The text was provided on page 2 of the main text (lines 34-39).

6. *Please provide a "thumbnail image" (width=211 x height=157 pixels, jpeg format), which can be used to highlight your paper on our homepage.*
This was provided.

Reviewer comments:

Reviewer #1

1. *page 8 lines 195-200, there are references to figures in the text that do not match. It seems likely that these references are to figure 2 and not 4. Figure 4C is not a western, for example.*
The reference is to Figure 5C and this has been corrected in the text on page 8, line 197.

2. *The the authors should provide a word document or text document describing what is provided in the tables and the figures*
This Contents page has been added to the Supplementary Information, as requested by the Editor (see Point 3 above).

Reviewer #2

No changes required.

Reviewer #3

1. *Figure 6D: the S(%) of SF3B2 and SF3B4 panels are labeled as 8.3 and 3.87, respectively. However, it looks that the number/percentage of S-phase cells in SF3B2 panel is smaller than that in SF3B4?*
The percentages indicated in the Figure are correct, but only relate to the data shown in that Figure. These were provided in response to an earlier review comment. Because the percentages in S-phase are low, a visual inspection of the Figure is not always reliable. It is more pertinent to see the mean±SEM values for the 15 experiments, as noted on pages 9-10, lines 250-260 of main text.

2. *line 37: " ... show roof of principle", "roof" should be "proof".*
This has been changed (line 37).

We hope that we have answered the minor queries raised satisfactorily and that the paper is now acceptable for publication.

Acceptance letter 30 January 2012

Thank you again for sending us your revised manuscript. The reviewer that evaluated this revised work, and the editor, are fully satisfied with the changes made, and, as such, I am pleased to inform you that your paper has been accepted, in principle, for publication.

One last issue remains before we can send this work to production. Molecular Systems Biology generally requires that high-throughput proteomic data are deposited into a public repository, such as PRIDE (<http://www.nature.com/msb/authors/index.html#a3.5>). When submission is complete, we ask that send a revised main manuscript document with the accession number in the Methods section. Please contact us if submitting these data to PRIDE, or a similar public repository, will present a problem.

I did note that you indicate that these data will be available via the Marcotte lab website, but this website was apparently not working when I tested it today. Regardless, we would much prefer if these data are also deposited in a public repository to help ensure their long-term availability.

When preparing the adding the accession number to the main manuscript you may also delete the Supp. Figure and Table Legends, since these are already included in the Supp. Information pdf. This revised manuscript file can be sent as an attachment to a reply email.

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

Sincerely,

Editor - Molecular Systems Biology
msb@embo.org

Referee reports:

Reviewer #2 (Remarks to the Author):

Modifications made to the western blot images is adequate.
This reviewer has no issues with this.

