molecular systems biology

Extensive quantitative remodeling of the proteome between normal colon tissue and adenocarcinoma

Jacek R Wisniewski, Pawel Ostasiewicz, Kamilla Dus, Dorota F Zielinska, Florian Gnad and Matthias Mann

Corresponding author: Matthias Mann, Max-Planck Institute for Biochemistry

Review timeline:

Submission date:	12 March 2012
Editorial Decision:	24 April 2012
Resubmission:	25 May 2012
Editorial Decision:	13 July 2012
Revision received:	02 August 2012
Accepted:	08 August 2012

Transaction Report:

(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 24 April 2012

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, the referees raise substantial concerns on your work, which, I am afraid to say, preclude its publication.

While the reviewers each acknowledge the depth of the dataset provided in this work exceeded previous reports, they felt that the novel biological insights provided in this work remained modest. The second reviewer wrote, that given other previous proteomic analyses of colorectal cancer, "the burden is on the authors ... to do a more detailed analysis and comparisons, to identify novel findings leading to improved understanding of the colon cancer progression." This sentiment was echoed by the first reviewer's third point. Moreover, these reviewers felt that some of the most potentially novel observations made in this work, such as the broad increase in nuclear protein abundance or the change in core/linker histone ratio, required more in depth investigation and analysis. The reviewers also felt that the total protein abundance (TPA) method required more direct validation. With these concerns in mind, the second reviewer rated the suitability of this work for Molecular Systems Biology as "low", and the third reviewer indicated to the editor that they felt this work at present would be better suited for a journal more specialized on proteomics.

Given these concerns, I see no choice but to return this work with message that we cannot offer to publish it.

Nevertheless, the reviewers did express interest in this work, and acknowledged the depth of the underlying datset. For this reason, we would like to suggest that we would be willing to reconsider a

new submission based on this work that includes additional work both validating the existing dataset and the TPA method, and investigating in more depth some of the most novel observations. We acknowledge that this would involve substantial additional experimentation and analysis.

A resubmitted manuscript would have a new number and receipt date, and, as you probably understand, we can give no guarantee about its eventual acceptability. If you do decide to follow this course, then please enclose with your re-submission an account of how the work has been altered in response to the points raised in the present review.

I am sorry that the review of your work did not result in a more favorable outcome on this occasion, but I hope that you will not be discouraged from sending your work to Molecular Systems Biology in the future.

Thank you for the opportunity to examine this work.

Yours Sincerely,

Editor - Molecular Systems Biology
msb@embo.org

REFEREE REPORTS

Reviewer #1

This is a well written and quite interesting paper from a proteomics point of view. Remarkable depth of analysis was achieved based on microdissected primary colon cancer cells, metastasis and normal mucosa.
The authors comment about the limitations of prior proteomic work on lung cancer and indeed the data generated in this study is quite remarkable. However some caveats should be noted by the authors:
1- Analysis of microdissected cells does not favor detection of secreted proteins which are relased into the extra-cellular environment.
2- The microenvironment itself is of substantial interest and it would have been useful if the authors compared microdissected cells with non-microdissected tumor tissue.
3- It is not clear from the authors description what fundamental new findings were obtained that could not be inferred from say gene expression analysis.
Overall the manuscript is well written, although the english could be improved, and would recomment publication.

Reviewer #2

The manuscript by Wisniewski et al. describes the results of an in-depth analysis of tissues from colorectal cancer patients. The dataset was generated using a very small amount of material obtained using LCM, and proteins were quantified using label-free quantification. The resulting dataset is among the best out there in terms of the depth of the coverage. However, it comes from a lab that recently published many similar papers showing similar numbers in various cell lines and tissues (including quantitative studies using Super-SILAC), so that these numbers are no longer that impressive.

The main problem with the manuscript is that it is highly descriptive and does not provide much new insight. Where it attempts to do so, the details of the analysis are lacking. Overall, the manuscript appears to be rushed, as it contains so many obvious typos that they really distract from reading it. The novelty should also be clarified since there were a number of recent studies on colorectal cancer, only some of which are cited.

More specific comments:

1. There were recent papers, e.g.
Besson D et al. A quantitative proteomic approach of the different stages of colorectal cancer establishes OLFM4 as a new nonmetastatic tumor marker. Mol Cell Proteomics. 2011 Dec;10(12):M111.009712.

This manuscript (and there were others that I recall) have identified 3-4000 proteins. Although not as high as in the submitted manuscript, it is still significant. So the burden is on the authors of the manuscript under review now to do a more detailed analysis and comparisons, and to identify novel findings leading to improved understanding of the colon cancer progression.

2. The manuscript provides a standard discussion of GO categories and pathways that are differentially expressed. The manuscript suggests that "the knowledge of the magnitude of protein expression changes ... should provide interesting information for the characterization of cancer". The authors should really expand on this, and give examples of how one can use this "magnitude of changes" (as compared to just calling proteins differentially expressed or not).

3. There are some substantial claims made but no in-depth discussion of what they mean. For example, what does it mean that "nuclear proteins were almost uniformly up-regulated"? How do we know there are no biases in the technology (e.g. in the data normalization procedures, or sample preparation that may introduce some bias toward nuclear proteins when handling cancer tissues, etc.).

4. Comparison with previous transcriptome studies is weak. The comparison is limited to 128 genes identified in at least 3 studies. The transcriptome was profiled using microarrays, and the citation is quite old (2007).

5. The entire section 'Estimation of absolute copy of proteins" is hard to follow. There are theoretical estimates, and indirect comparisons to confirm them. The proposed Total Protein Approach (TPA) should be directly compared with more accurate estimates obtained using spiked labeled synthetic peptides or similar methods. For example, Mann's lab recently published a nice method called PrEST:

Zeiler M et al. Protein Epitope Signature Tag (PrEST) library allows SILAC-based absolute quantification and multiplexed
determination of protein copy numbers in cell lines. Mol Cell Proteomics. 2012 Mar;11(3):O111.009613.

Why not compare TPA with PrEST to validate the claims about its accuracy?

6. The authors apply t-test to identify differentially expressed proteins, but there is confusion about which version of the test. On p. 7 they mention Student's t-test, but in Methods they mention paired t-test.

7. Regardless of the version of t-test, is it even applicable to datasets where it is estimated that half of the proteins show a significant change in the abundance across the samples that are compared?

8. p. 6. Estimation of the abundances of the linker and the core histones, is questionable. How reliable is label-free quantification here to make statements about the ratio of linker vs. core histones? The error bars in the Figure 3 are huge, especially for the linker. The way it is plotted, again especially for the linker, is misleading because of the scale on the y-axis. Furthermore, histones have a high degree of sequence homology. Are there enough unique peptides to accurately quantify them?

9. Hierarchical clustering figure raises additional questions. For example, what was the run order of those samples on the instrument? Was it random? It appears that it was not, and the clustering reflects that. For example, samples 1,2, and 3 are next to each other, then 6,7, and 8 are next to each other (looking at the clustering of the normal samples). The order, from right to left, is (1,3,2)-(5,4)-(8,7,6).

10. Figure 2 legends mentions HSA protein levels, but I do not see it in the Figure.

11. Figure 2, panel 'extracellular'. Why is there two sets of boxes? Same for Figure 3. This is not explained in the Figure legend.

12. Data description is incomplete. LC MS/MS only mentions HCD MS/MS data, but the analysis part mentions both HCD and CID.

13. Statistical analysis needs more details, including normalization steps and missing value imputation.

14. Has the data been uploaded to any public repositories?

15. So many typos (and mislabeled Figures), I am not even going to list examples.

Reviewer #3

In-depth proteomics of patient-matched series of normal, primary CRC, and metastases (N= 7-8) with over 7000 proteins identified from FFPE tissue. Gene ontology mining is performed for global analysis of (changes in) protein functions (nothing special here). The strong point is the large dataset (yet that awaits validation). A bit a disconnected point is the method to estimate protein copy numbers.

Major comment:
#The total protein approach is an important message in this manuscript. Why do the protein estimation exercise on these ill-defined patient samples and not on controlled cell lysates +/- gold standard spike-in at different copy-number levels?

Other comments:
One of the differences between normal colon mucosa and CRC is the increase in amount of stroma. Does stroma content explain a significant part of the difference between normal and CRC? Or, was laser capture microdissection used to predominantly isolate epithelial cells?
Images of tissue (preferably before and after laser capture microdissection) should be provided. Some discussion on the potential influence of stroma content should be added.
What is surprising is that no significant changes in protein abundance were found between primary and the nodal metastasis. This is not expected since the tumors need to acquire certain features to leave the primary tumor site and the tumor environment is different. Some discussion should be added.
Abstract first sentence. The authors claim they report the first in-depth proteomics analysis of colorectal cancer. This not correct. In-depth analyses of colorectal cancer by nanoLC-MS/MS-based proteomics with several thousands of proteins identified have been reported before for colon cancer subproteomes such as the cell surface (Conrotto et al., Int J Cancer. 2008; De Wit et al., Gut 2011) and the nuclear matrix (Albrethsen et al., 2010) or a colon cancer stem cell enriched culture model (Van Houdt et al., MCP 2011). What is true that this is the largest dataset to date. Please modify text.
Introduction, sentence stating that proteomics In particular, proteomics so far did not allow systematic insights into changes in signaling pathway, alterations on the cell surface proteome, or variations in processing of genetic information in CRC. This is not completely true, see previous point and references. Please modify text and include references.
Figure 2F shows a putative decrease in mitochondrial protein content in metastases compared to primary CRC. While resemblance between primary CRC and metastases is recognized throughout the paper, this putatively interesting difference should be mentioned as well.
Table 3, note 3 in legend refers to de Wit et al for HeLa cell protein info while this reference does not contain HeLa info; correct reference needs to be added.
Check text for reference to figures (e.g. Pages 5- 7, references to figures seem incorrect and have shifted (1C should be 1B, etc).
page 10 first sentences. The authors play the number game where they compare their own large dataset to the numbers of identified proteins in previous studies. This is a bit an apple-orange comparison since most previous studies focused on subproteomes and this study on total tissue lysates. Please modify text.

The total protein approach for copy number estimation relies on correct estimation of the cellular volume and protein content. What is the spread in the numbers used for cell volume and protein content?
At page 10 the enterocyte volume is quoted to be 1400 µm3 quoting references (Buschmann and Manke 1981; MacLeod, Hamilton et al. 1991;Crowe and Marsh 1993) while in Table 3 1200 µm3 is mentioned for the enterocyte volume quoting the same references. Please clarify.
in Table 3 the values presented should be in scientific notation with the appropriate number of decimals reflecting the precision of the determination. Additionally, single values are presented for Normal mucosa and Carcinoma. However, multiple individuals have been analyzed, therefore the median +/- stdev or the individual values should be presented. The variation also illustrates the level of precision for copy-number estimates in these LCM-captured cell populations. Please modify table content.
What is the rationale for using the summed peptide intensities for absolute protein quantitation? Within the MaxQuant software other -possibly better suited- label-free protein quantification options are available such as LFQ intensity (label-free quantitation) or IBAQ (intensity-based absolute quantification). The authors should argue why summed intensity was chosen as the most appropriate quantitation method. Please modify text.
pg 15 typo: 'The analysis was performed as described previously. Briefly, SAXfractionated peptides were separated on a 15 cm ReproSil-Pur C18-AQ, 3 µm resin (Dr. Maisch,Ammerbuch-Entringen, Germany) column using a 230 min acetonitrile gradient and were analyzed with an LTQ-Orbitrap Velos mass spectrometer using a 'high-high' strategy with HigherEnergy Collisional Dissociation (HCD) (Olsen, Macek et al. 2007)'. Please modify text.
Discussion page 12. The strength and feasibility of label-free proteomics especially for the analysis of clinical samples has been recognized by several others in the last few years, eg., all the above mentioned CRC proteomics studies were label-free. It would be nice if the authors acknowledge this.
#Please resolve duplicates references: Luber, C. A., J. Cox, et al; Ostasiewicz, P., D. F. Zielinska, et al.; Wisniewski, J. R., P. Ostasiewicz, et al.

Resubmission 25 May 2012

Reviewer #1

This is a well written and quite interesting paper from a proteomics point of view. Remarkable depth of analysis was achieved based on microdissected primary colon cancer cells, metastasis and normal mucosa.
The authors comment about the limitations of prior proteomic work on lung cancer and indeed the data generated in this study is quite remarkable. However some caveats should be noted by the authors:

We thank the reviewer for the positive assessment of our work. In the revised manuscript we have further clarified potential caveat as described below.
1.Analysis of microdissected cells does not favor detection of secreted proteins which are relased into the extra-cellular environment.

Response: We agree. In this work we have focused and microdissected samples and tried to avoid contamination by stroma. We now explain this in the revised manuscript on page 4.

2- The microenvironment itself is of substantial interest and it would have been useful if the authors compared microdissected cells with non-microdissected tumor tissue.

Response: We have done such analysis of lysates of non-microdissected tissue but they revealed high content of blood proteins amounting to over 10% of the sample. Moreover other stromal proteins appeared to be highly abundant. Since cancer cells are the focus of our studies, we chose to analyze only microdissected samples to eliminate this variability. This is explained in the manuscript.

3- It is not clear from the authors description what fundamental new findings were obtained that could not be inferred from say gene expression analysis.

Response: The fundamental contribution of this work is that it is the first global proteomic description of protein composition of normal and cancers cells. We show that that the extent of changes involves about half of the proteome, a much larger fraction than commonly supposed. Our findings contrast with transcriptomics analyses, which were only able to identify a limited number of changes during cell transformation from the enterocyte into the cancer cell. Likely reasons for differences between proteome and transcriptome have been widely discussed in the literature. In the revised manuscript, we reconstitute our major findings in a cell model, which further strengthening the main findings of our study (see below).

Overall the manuscript is well written, although the english could be improved, and would recomment publication.

Response: We have tried to improve the English in the revised manuscript.

Reviewer #2

The manuscript by Wisniewski et al. describes the results of an in-depth analysis of tissues from colorectal cancer patients. The dataset was generated using a very small amount of material obtained using LCM, and proteins were quantified using label-free quantification. The resulting dataset is among the best out there in terms of the depth of the coverage. However, it comes from a lab that recently published many similar papers showing similar numbers in various cell lines and tissues (including quantitative studies using Super-SILAC), so that these numbers are no longer that impressive.

Response: We thank the reviewer for his or her kind remarks about the quality of our work but respectfully disagree on the assessment that this depth is not impressive any more. Recent publications from our and other labs were done in cancer cell lines, an incomparably easier system than the one we tackle here.
In any case, the goal of the present study was to provide a picture of changes in the CRC proteomes. We believe that our data are a substantial advance in studying archival clinical material formalin fixed material and in providing information on global changes in the cell architecture during cancer development. Not only did we characterize the proteome in unprecedented depths but we were able to discern 1,800 proteins significantly changing between normal and cancer cells. In comparison to recent proteomic papers on CRC, this study provides a significant advance in the depth of analysis and covers almost all previously found potential markers in comprehensive and easy to compare manner.

The main problem with the manuscript is that it is highly descriptive and does not provide much new insight. Where it attempts to do so, the details of the analysis are lacking.

Response: Our work was not primarily aimed at the identification of novel biomarkers or drug targets or at mechanistic studies of the transformation process on which there are thousands of papers. Using our technological platform allowing profiling proteomes on a scale of close to 10,000 proteins, we wanted to provide a general systems wide picture of changes between normal, cancer, and metastatic cell proteomes. To address the reviewer's concern we have added a cell model. In the revised work and we show that similar changes occur during spontaneous differentiation in that model (see below).
Furthermore, our data includes a large group of proteins of which some are potential biomarkers or drug targets. Cell surface proteins seem to be the most promising group. In the revised manuscript we now list such proteins - up-regulated in cancer (Table 4) - and show an IHC validation for 3 proteins, including two low abundant ones (Figure 4).

Overall, the manuscript appears to be rushed, as it contains so many obvious typos that they really distract from reading it.

Response: We apologize submitting a manuscript with typographical errors (although we and reviewer 1 thought it was well written). We hope that we have caught them in the revised manuscript.

The novelty should also be clarified since there were a number of recent studies on colorectal cancer, only some of which are cited.

Response: In the revised version of the manuscript we have additionally cited and discussed several recent papers (see specific comment 1 below) in CRC area and highlighted the unique contributions of this paper (see also above and below).

More specific comments:

1. There were recent papers, e.g.
Besson D et al. A quantitative proteomic approach of the different stages of colorectal cancer establishes OLFM4 as a new nonmetastatic tumor marker. Mol Cell Proteomics. 2011 Dec;10(12):M111.009712.
This manuscript (and there were others that I recall) have identified 3-4000 proteins. Although not as high as in the submitted manuscript, it is still significant. So the burden is on the authors of the manuscript under review now to do a more detailed analysis and comparisons, and to identify novel findings leading to improved understanding of the colon cancer progression.

Response: We have updated our manuscript for citing recent publications on CRC and have made more comparisons between our and previously published studies on page 13:

"For example, proteins reported to be up-regulated in cancer and discussed as potential biomarkers in very recent studies - STOML2 (Han, Chen et al. 2011), BIRC6 (Van Houdt, Emmink et al. 2011), OLFM4 (Conrotto, Roesli et al. 2008; Besson, Pavageau et al. 2011), NGAL (Conrotto, Roesli et al. 2008), and GLUT1 (de Wit, Jimenez et al. 2011)- have relatively high abundances between 1.6×10^{-2} % and 1.9×10^{-3} % of total protein mass, respectively (Suppl. Table 3). Our data confirm that STOML2, BIRC6, and GLUT1 are significantly (2.4-20-fold) - up-regulated in cancer samples (Suppl. Table 4). In contrast, the extent of up-regulation of OLFM4 and NGAL were extremely variable, with cancer to normal ratios changing from 0.09 to 109 (Suppl. Table 3). This variability in the abundance of OLFM4 was already observed by IHC analysis, likely reflecting its transient rise during pre- and early cancer stages (Besson, Pavageau et al. 2011). The variability in C/N rations of NGAL may reflect the fact that this protein appears abundantly in stroma (Conrotto, Roesli et al. 2008), which can contaminate the microdissected material.

Since our large scale analysis covers the previously identified potential biomarkers to a large extent, we believe that our set of outliers, in particular those up-regulated in cancer, can be useful in future research aimed at the identification of novel biomarkers. In particular, our analysis contains almost all of the proteins identified in studies involving subcellular fractionation of clinical material (Conrotto, Roesli et al. 2008; Albrethsen, Knol et al. 2010; de Wit, Jimenez et al. 2011). Such fractionation strategies typically require relatively large amounts of starting material and are seldom of a purity that can substantially increase the depth of analysis. For example nuclear and cell surface proteomes contain albumin and abundant mitochondrial proteins (Conrotto, Roesli et al. 2008; Albrethsen, Knol et al. 2010). "

2. The manuscript provides a standard discussion of GO categories and pathways that are differentially expressed. The manuscript suggests that "the knowledge of the magnitude of protein expression changes ... should provide interesting information for the characterization of cancer". The authors should really expand on this, and give examples of how one can use this "magnitude of changes" (as compared to just calling proteins differentially expressed or not).

Response: What we meant to say is that one can think of this data analysis as a two step process: First one performs the standard GO category analysis for enriched functions. In a second step, one can look into the proteomic data for each of the members of the category and discover the extent of the quantitative change. Depending on the abundance of the protein, statistically significant upregulation can involve a percent change or it can be several fold. We believe this is a particular

strength of proteomics as opposed to transcriptomics, for instance. We have added text explaining what we mean at the end of discussion in the revised manuscript.

3. There are some substantial claims made but no in-depth discussion of what they mean. For example, what does it mean that "nuclear proteins were almost uniformly up-regulated"? How do we know there are no biases in the technology (e.g. in the data normalization procedures, or sample preparation that may introduce some bias toward nuclear proteins when handling cancer tissues, etc.).

Response: No method is perfect and we agree that we cannot rule out minor biases, however, the main trends that we describe are crystal clear. In response to the reviewer's concerns we changed the sentence to: "In contrast, many nuclear proteins were up-regulated." Furthermore, to mitigate the possibility that our data showing that the overall changes including the up-regulation of nuclear proteins is biased by sample preparation or data normalization procedure we performed an analysis using cultured cells. We measured changes in colon cancer CaCo-2 cells upon spontaneous cell differentiation. The overall changes observed follow those observed in clinical samples of CRC. We have added these results to the revised manuscript as a separate sections entitled "Differentiation related changes in cultured colon cancer cells recapitulate the extensive subcellular and functional differences between the cancer and normal cells" . This section contains a new figure (now Figure 6).

4. Comparison with previous transcriptome studies is weak. The comparison is limited to 128 genes identified in at least 3 studies. The transcriptome was profiled using microarrays, and the citation is quite old (2007).

Response: To strength the comparison we now provide additional data analysis which shows that we identified 80% of proteins identified in array studies as changed in metastasis (citation year 2010):

The absence of significant changes between adenocarcinoma and nodal metastasis was an unexpected observation of this study. One of reason could simply have been the insufficient sensitivity of our analysis which might not have allowed identification of proteins which change specifically in metastasis. We tested this possibility by comparing the list of 41 proteins found to be changed between normal mucosa and cancer and cancer and metastasis (Nambiar et al., 2010) with our data. We found 80% of these proteins in our dataset (Supplemental Table 9). Most of these proteins were abundant.

5. The entire section 'Estimation of absolute copy of proteins" is hard to follow. There are theoretical estimates, and indirect comparisons to confirm them. The proposed Total Protein Approach (TPA) should be directly compared with more accurate estimates obtained using spiked labeled synthetic peptides or similar methods. For example, Mann's lab recently published a nice method called PrEST:

Zeiler M et al. Protein Epitope Signature Tag (PrEST) library allows SILAC-based absolute quantification and multiplexed determination of protein copy numbers in cell lines. Mol Cell Proteomics. 2012 Mar;11(3):O111.009613.

Why not compare TPA with PrEST to validate the claims about its accuracy?

Response: We agree with the reviewer's point. In the revised version we explain the TPA method in more detail and validate it with protein standards and also compare TPA with the PrEST on HeLa cell as suggested. This comparison worked very well and we find a high correlation. Furthermore, we show that TPA is even a valuable tool for analysis of datasets generated with different methods in the past. This section is now extended by added text and two figures (Figure 7 and supp. Figure 2).

6. The authors apply t-test to identify differentially expressed proteins, but there is confusion about which version of the test. On p. 7 they mention Student's t-test, but in Methods they mention paired t-test.
Response: we used paired t-test for clinical samples. Now we mention this on p.7 and in the methods section. Analysis of CaCo-2 data was performed using just the t-test (Methods)

7. Regardless of the version of t-test, is it even applicable to datasets where it is estimated that half of the proteins show a significant change in the abundance across the samples that are compared?

Response: Normalization on total protein amount is not problematic even when this many proteins change. Furthermore, there are no intrinsic problems in applying the t-test in a situation where half the population has significant changes.

8. p. 6. Estimation of the abundances of the linker and the core histones, is questionable. How reliable is label-free quantification here to make statements about the ratio of linker vs. core histones? The error bars in the Figure 3 are huge, especially for the linker. The way it is plotted, again especially for the linker, is misleading because of the scale on the y-axis. Furthermore, histones have a high degree of sequence homology. Are there enough unique peptides to accurately quantify them?

Response: Indeed linker histones have a large extent of sequence homology, but in the quantification of the total abundance of H1 each unique peptide is counted only one time. Furthermore the high sequence conservation of liker histones is limited to their globular domain representing only about 40% of the whole protein sequence.

9. Hierarchical clustering figure raises additional questions. For example, what was the run order of those samples on the instrument? Was it random? It appears that it was not, and the clustering reflects that. For example, samples 1,2, and 3 are next to each other, then 6,7, and 8 are next to each other (looking at the clustering of the normal samples). The order, from right to left, is (1,3,2)-(5,4)-(8,7,6).

Response: The samples were analyzed in the order: 1(C, N, M), 2(C, N, M)...8(C, N, M), but the order of N,C,M in each group was random. This is pointed out in the revised manuscript (on page 19).

10. Figure 2 legends mentions HSA protein levels, but I do not see it in the Figure.

Response: We corrected Figure 2 indicating the boxes corresponding to HSA.

11. Figure 2, panel 'extracellular'. Why is there two sets of boxes? Same for Figure 3. This is not explained in the Figure legend.

Response: The lower set of boxes refers to serum albumin in the samples. Now we mentioned this in the legend. In Figure 2 the histones panel also contains separate boxes for linker and core histones. We marked both on in the figure and explained this in the legend.

12. Data description is incomplete. LC MS/MS only mentions HCD MS/MS data, but the analysis part mentions both HCD and CID.

Response: All MS-analyses were exclusively performed with HCD fragmentation. The text has been corrected accordingly.

13. Statistical analysis needs more details, including normalization steps and missing value imputation.

Response: this information is already given in the Methods sections:
“Protein abundance was calculated on the basis of the normalized spectral protein intensity”.
“Zero intensities were filled with intensities from the lower part of normal distribution (imputation width=0.3, shift=1.8)”.

14. Has the data been uploaded to any public repositories?

Response: We uploaded the raw ms files to the "Tranche". The files are available: Passphrase: ISIILXKa1ou3gcpFomhP; Hash key: 4J7wto4aNCmjeYCcihnNWakm2M94h8orULE0obgNeg2f/8f1mPMCuTtbGmIoeqRRvCltK4pGmvGWSdC3VFmON9e/X90AAAAAAABiXw==

15. So many typos (and mislabeled Figures), I am not even going to list examples.

Response: We apologize providing a manuscript with typographical errors. We believe that we corrected all of them in the revised version of the manuscript.

Reviewer #3

In-depth proteomics of patient-matched series of normal, primary CRC, and metastases (N= 7-8) with over 7000 proteins identified from FFPE tissue. Gene ontology mining is performed for global analysis of (changes in) protein functions (nothing special here). The strong point is the large dataset (yet that awaits validation). A bit a disconnected point is the method to estimate protein copy numbers.

We thank the reviewer for the appreciation of our data set.

Major comment:
#The total protein approach is an important message in this manuscript. Why do the protein estimation exercise on these ill-defined patient samples and not on controlled cell lysates +/- gold standard spike-in at different copy-number levels?

Response: In the new version of the manuscript we extended the description and validation of the total protein approach for copy number estimation (see also point 5 of reviewer 2). We compared the SILAC - PrEST values measured for HeLa cell lysates by Zeiler et al (2011) with the current values and a previously published HeLa lysate datasets. The results are shown in Figure 7 and show excellent agreement.

Other comments:
One of the differences between normal colon mucosa and CRC is the increase in amount of stroma. Does stroma content explain a significant part of the difference between normal and CRC? Or, was laser capture microdissection used to predominantly isolate epithelial cells?

Response: All clinical samples analyzed in this study were prepared from laser microdissected material. We mentioned this in the Abstract, Results and Methods sections. The main purpose of using laser microdissection was indeed to reduce the chance that observed cellular changes were instead due to differential contribution of stroma.

Images of tissue (preferably before and after laser capture microdissection) should be provided. Some discussion on the potential influence of stroma content should be added.

Response: We have already published pictures showing how the microdissection was performed (Wisniewski, et al., J. Proteome Res. 10(7): 3040-3049, 2012). This is now pointed out more clearly in the revised manuscript on page 4.

What is surprising is that no significant changes in protein abundance were found between primary and the nodal metastasis. This is not expected since the tumors need to acquire certain features to leave the primary tumor site and the tumor environment is different. Some discussion should be added.

Response: We agree with the reviewer and have extended the discussion part on metastasis as follows: "Despite the fact that our analysis is the most comprehensive proteomic study on CRC so far, we cannot exclude the possibility that some proteins which are specifically up-regulated in nodal metastases escaped detection due their lower abundance or due to high variation between samples originating from different donors. It is also possible that proteins enabling the primary tumor to metastasize were already upregulated in the cancer cells in situ (adenocarcinoma) and therefore cannot be detected in our differential analysis."

Abstract first sentence. The authors claim they report the first in-depth proteomics analysis of colorectal cancer. This not correct. In-depth analyses of colorectal cancer by nanoLC-MS/MS-based proteomics with several thousands of proteins identified have been reported before for colon cancer subproteomes such as the cell surface (Conrotto et al., Int J Cancer. 2008; De Wit et al., Gut 2011) and the nuclear matrix (Albrethsen et al., 2010) or a colon cancer stem cell enriched culture model (Van Houdt et al., MCP 2011). What is true that this is the largest dataset to date. Please modify text.

Response: we removed…." the first in-depth" in the abstract

Introduction, sentence stating that proteomics In particular, proteomics so far did not allow systematic insights into changes in signaling pathway, alterations on the cell surface proteome, or variations in processing of genetic information in CRC. This is not completely true, see previous point and references. Please modify text and include references.

Response: We disagree somewhat with the reviewer but agree that it depends somewhat on one's point of view. Conrotto et al., identified only a small number of proteins, namely 367, thus 'per se' it cannot be systematic for the entire proteome. The study of De Wit et al. involves proteomic identification of surface proteins in various cultured cells and IHC analysis with two antibodies on clinical specimens. That work neither maps proteome changes to the cell surface or attempts to provide any general insights. Finally, the focus of Albrethsen et al. was the nuclear matrix (1153 proteins) and for this reason we do not consider this work as providing systematic insights in CRC.

Figure 2F shows a putative decrease in mitochondrial protein content in metastases compared to primary CRC. While resemblance between primary CRC and metastases is recognized throughout the paper, this putatively interesting difference should be mentioned as well.

Response: The decrease of the mitochondrial component is not statistically significant, which is the reason that we did not comment on it.

Table 3, note 3 in legend refers to de Wit et al for HeLa cell protein info while this reference does not contain HeLa info; correct reference needs to be added.

Response: we corrected the reference. The correct one is: Kimura et al., 1999.

Check text for reference to figures (e.g. Pages 5- 7, references to figures seem incorrect and have shifted (1C should be 1B, etc).

Response: we thank the reviewer for picking this up. We have now corrected the references to the figures.

page 10 first sentences. The authors play the number game where they compare their own large dataset to the numbers of identified proteins in previous studies. This is a bit an apple-orange comparison since most previous studies focused on subproteomes and this study on total tissue lysates. Please modify text.

Response: Apparently it looks like we comparing subcellular proteomes with a whole cell proteomes. However, we note that the "subcellular proteomes" contain many proteins that are obviously contaminants, and even more important our global proteome cover more subcellular

specific (e.g. nuclear or cell surface proteins) than the directed analyses. Therefore we believe that we are justified in pointing out the large coverage of our study.

The total protein approach for copy number estimation relies on correct estimation of the cellular volume and protein content. What is the spread in the numbers used for cell volume and protein content?

Response: In contrast to the majority of cultured cells, native cells can be very variable in shape and size. Thus our calculation for the enterocytes and the cancers in tissue provides only an estimate for the average.

At page 10 the enterocyte volume is quoted to be 1400 µm3 quoting references (Buschmann and Manke 1981; MacLeod, Hamilton et al. 1991;Crowe and Marsh 1993) while in Table 3 1200 µm3 is mentioned for the enterocyte volume quoting the same references. Please clarify.

Response: for the calculations we used the 1400 µm3 value. We corrected the value on page 10.

in Table 3 the values presented should be in scientific notation with the appropriate number of decimals reflecting the precision of the determination. Additionally, single values are presented for Normal mucosa and Carcinoma. However, multiple individuals have been analyzed, therefore the median +/- stdev or the individual values should be presented. The variation also illustrates the level of precision for copy-number estimates in these LCM-captured cell populations. Please modify table content.

Response: We have modified the Table 3 according to the reviewer's suggestions. We also added data comparing differentiated and undifferentiated CaCo-2 cells.

What is the rationale for using the summed peptide intensities for absolute protein quantitation? Within the MaxQuant software other -possibly better suited- label-free protein quantification options are available such as LFQ intensity (label-free quantitation) or IBAQ (intensity-based absolute quantification). The authors should argue why summed intensity was chosen as the most appropriate quantitation method. Please modify text.

Response: For protein quantitation only the LFQ intensities were used. Now, we state this in the results and methods sections. Some of our data use two enzymes and the iBAQ method does not currently support this. Furthermore, we do not find large differences between iBAQ or LFQ as we show in Figure 7A.

pg 15 typo: 'The analysis was performed as described previously. Briefly, SAXfractionated peptides were separated on a 15 cm ReproSil-Pur C18-AQ, 3 μm resin (Dr. Maisch,Ammerbuch-Entringen, Germany) column using a 230 min acetonitrile gradient and were analyzed with an LTQ-Orbitrap Velos mass spectrometer using a 'high-high' strategy with Higher Energy Collisional Dissociation (HCD) (Olsen, Macek et al. 2007)'. Please modify text.

Response: we thank the reviewer for picking this up. We changed this section of the manuscript.

Discussion page 12. The strength and feasibility of label-free proteomics especially for the analysis of clinical samples has been recognized by several others in the last few years, eg., all the above mentioned CRC proteomics studies were label-free. It would be nice if the authors acknowledge this.

Response: We agree and now acknowledge this by citing a few recent papers….." and was used in several recent proteomic studies on cancer (Gamez-Pozo, Sanchez-Navarro et al. 2012; Meding, Balluff et al. 2012; Pan, Chen et al. 2012)".

#Please resolve duplicates references: Luber, C. A., J. Cox, et al; Ostasiewicz, P., D. F. Zielinska, et al.; Wisniewski, J. R., P. Ostasiewicz, et al.

Response: the duplicates were resolved.

2nd Editorial Decision 13 July 2012

Thank you again for submitting your work to Molecular Systems Biology. We have now heard back from two referees who agreed to evaluate this resubmitted study. As you will see, the referees felt that the revisions made had improved this manuscript. They raise however a series of concerns and make suggestions for modifications, which we would ask you to carefully address in a revision of the present work.

Reviewer #1 was Reviewer #3 during the review of the previous version of this work, and Reviewer #3 is new reviewer who provided a fresh opinion on this manuscript.

Importantly, both reviewers felt that more detailed clinical information described the samples is needed.

Reviewer #3, in particular, was clearly supportive of this work, although s/he indicated that some important revisions and clarifications were needed before this manuscript would be appropriate for publication. Addressing these concerns will likely require some additional analysis. In particular, Reviewer #3 felt that it would be important to analyze whether greater heterogeneity among the metastatic samples may contribute to the lack of significant protein shifts between these samples and the primary cancers.

Thank you for submitting this paper to Molecular Systems Biology.

Yours sincerely,

Editor - Molecular Systems Biology
msb@embo.org

REFEREE REPORTS

Reviewer #1

The authors greatly improved the manuscript and now have addressed most of my concerns. Of course, it does not change the fact that the paper is mainly about technology, and not about systems biology of CRC.

Two issues remain to be addressed:

1. A crucial point that the authors still need to clear up is the normalization (also comment of reviewer 2). The assumption of their label-free quantitation (LFQ) is that the MAJORITY of proteins do not change in abundance. Here, about 50% are significantly changed using LFQ, which indicates something is not right.

2. The tissue images before and after LCM of the 8 patients samples is still missing. Clinical information per patient is also missing (such as age, sex, location, etc).

Reviewer #3

Jacek R. Wiśniewski et al. "Extensive quantitative remodeling of the proteome between normal colon tissue and adenocarcinoma".

This is a paper by the Mann group that seems to have undergone one round of review already. This "new" reviewer has carefully read all previous assessments and certainly agrees with many of the previous comments. In summary, Wiśniewski and colleagues have used a limited number of FFPE samples and preformed an in-depth proteomics analyses using normal colon tissue, adenocarcinoma and matched metastasis. Using a latest generation mass spectrometer an impressive number of proteins were identified and quantified. The study certainly shows the power of current proteomics technologies. It is a very descriptive study that will unlikely have much clinical impact. It is almost a proteomics equivalent of the cancer genome atlas projects, since most/all of these next-gen sequencing exercises are published in Nature I think it is reasonable to accept this paper in Mol Syst Biol.

The major conclusions of this paper are: 1) proteomics technologies have come a long way; 2) FFPE samples are useful and applicable for these technologies now; 3) label-free quantification can provide useful data in these screening type approaches. The authors have mentioned this in the discussion and this reviewer would strongly agree with these conclusions.

There are some more major comments (#1, 5) this reviewer would like the authors to address and some more philosophical comments (#2-4) one could address to further improve this manuscript.

Comments:
1) The biggest problem this reviewer has is the sample selection. There is absolutely no information on how these samples were selected, matched, etc. that is a major caveat! Detailed information on the samples and the selection need to be presented. Especially since an incredibly low number of samples were analyzed.
a. What are the normal colon tissue samples? Adjacent, pathologically normal tissue? If so please state this in the text. Many of us use this, but it's certainly not normal!
2) What is the clinical question the authors tried to address? Since the authors mention biomarkers several times, it would have been nice if a more detailed analysis had been performed in this context. Start with cell surface markers (suggested by the authors) perform more comprehensive analyses and validate better (I leave it to the authors, but at least discuss this caveat!)
a. If the authors choose IHC, well-annotated FFPE blocks should be chosen. In this case patient outcome is crucial to demonstrate if marker expression correlates with clinical progression?
b. Alternatively, the authors could tailor their mining strategy towards secreted proteins (there are many options to do this). In this case serum/plasma samples are required and markers could be quantified by ELISA. This would add major clinical context to this paper, especially since all the other analyses are very high-level.
3) It is a bit surprising that no significant changes were identified between primary cancers and mets (at least intuitively to me). Although our lab has performed a similar study in a different cancer using >70 FFPE samples and LC-MS with label-free quantification and similarly few changes were found in cancers with stratified outcome. In this context, these primary cancers and mets seem to be matched samples from the same patient? Could the authors at least perform some type of pair wise comparisons to at least suggest some proteins that change with in a patient pair? Maybe the cancer samples are so heterogeneous and poorly matched that not even the best technology can identify changes within the biological noise?
4) The correlation to the CaCo-2 cells is surprising? Although we all use cell line models they are completely irrelevant and do not provide relevant models to recapitulate human disease.
5) The comparison to the array datasets is very superficial. What is the global correlation of these two different datasets? I would assume poor? Are their functional differences in RNA-protein pairs that correlate vs. don't' correlate. The proteomics data will be way more informative than the array, the authors could make a much stronger case for this.
a. Figure 8A is a bit confusing. It wasn't clear to this reviewer what the 2 different pie charts actually represent? Could this be merged into some type of heat map or even better a scatter plot?

In summary, this is a well-performed proteomics study that impressively showcases how far MS-based proteomics has come. This reviewer is convinced these technologies will make significant contributions in biological sciences and cancer research. A shortcoming of the paper is the limited number of samples and their poor (absent) annotation. If samples are poorly selected without the direct input of a clinician even the most powerful proteomics technology will likely not result in much novel insights let alone clinical impact. The authors also sell themselves a bit short by a

somewhat superficial analysis. Some suggestions were made and it would be appreciated if the authors would be able to address some of these points. Overall, I recommend publication in MSB simply due to potential implication of the presented technology for future translational cancer proteomics studies.

1st Revision - authors' response 02 August 2012

Responses to the reviewers' comments

Reviewer #1

The authors greatly improved the manuscript and now have addressed most of my concerns. Of course, it does not change the fact that the paper is mainly about technology, and not about systems biology of CRC.

We would like to thank the reviewer for the second review and thank him or her for acknowledging the extensive changes that we performed. We do not fully agree with the opinion that our work is not about systems biology, although it is certainly true that we do not include formal modeling of the system (but this goes for basically all work involving patient samples). Rather, our work breaks new group by describing, in unprecedented depth, the proteome changes occurring upon transformation of the enterocyte to the adenocarcinoma cell. The results achieved can not only be used to identify potential biomarkers, but in contrast to other proteomic studies in this field, it offers a high quality catalog of protein changes that is complementary to genomic studies such as recently published in Nature (Cancer Genome Atlas, vol. 487, p.330, 2012) and many other top journals.

Two issues remain to be addressed:

1. A crucial point that the authors still need to clear up is the normalization (also comment of reviewer 2). The assumption of their label-free quantitation (LFQ) is that the MAJORITY of proteins do not change in abundance. Here, about 50% are significantly changed using LFQ, which indicates something is not right.

I this study we identified more than 8,100 proteins in CRC samples and of these about 7,500 were quantified. The proteins abundances were calculated on the basis of the normalized spectral protein intensity (LFQ intensity) as mentioned in the methods section.
Statistical analysis revealed that about 25% of protein levels were significantly changed at $p<0.05$. Using more stringent criteria ($p<0.01$) only levels of 762 (~10%) were significantly changed (Page 7). The estimate that a majority of proteins change is based on extrapolation from more abundant, and more precisely quantified, to less abundant proteins.

Regardless of this the reviewer raises a pertinent point. Given a sufficiently large percentage of the proteome that is changing, the ratios could be skewed. However, fortunately the effect is on the main 'background populations', whose ratio is slightly underestimated. The ratio from background population to the large outlier population is not affected and – most importantly – the t-test is not affected either. These observations are from benchmark tests that we have performed and that we would be happy to provide to the reviewer if requested. We now provide a sentence explaining that this is not an issue in the Methods:
"Although a large proportion of the proteome was changing in the comparison of carcinoma with enterocytes, this does not affect the statistical test (t-test), used to calculate the significance of the change".

2. The tissue images before and after LCM of the 8 patients samples is still missing. Clinical information per patient is also missing (such as age, sex, location, etc).

We have supplemented the manuscript with the requested patient information (Supplementary Table 1) and with tissue images before and after microdissection (Supplementary Figure 1).

Reviewer #3

Jacek R. Wiśniewski et al. "Extensive quantitative remodeling of the proteome between normal colon tissue and adenocarcinoma".

This is a paper by the Mann group that seems to have undergone one round of review already. This "new" reviewer has carefully read all previous assessments and certainly agrees with many of the previous comments. In summary, Wiśniewski and colleagues have used a limited number of FFPE samples and preformed an in-depth proteomics analyses using normal colon tissue, adenocarcinoma and matched metastasis. Using a latest generation mass spectrometer an impressive number of proteins were identified and quantified. The study certainly shows the power of current proteomics technologies. It is a very descriptive study that will unlikely have much clinical impact. It is almost a proteomics equivalent of the cancer genome atlas projects, since most/all of these next-gen sequencing exercises are published in Nature I think it is reasonable to accept this paper in Mol Syst Biol.

We would like to thank the reviewer from positive recommendation for publication of our manuscript, which indeed has already undergone one previous round of reviews. As noted above, the study is indeed descriptive but we do not see this as a disadvantage. Rather it complements the dozens of cancer genome papers that have appeared in high profile journals, which are also mainly of a descriptive nature.

The major conclusions of this paper are: 1) proteomics technologies have come a long way; 2) FFPE samples are useful and applicable for these technologies now; 3) label-free quantification can provide useful data in these screening type approaches. The authors have mentioned this in the discussion and this reviewer would strongly agree with these conclusions.

There are some more major comments (#1, 5) this reviewer would like the authors to address and some more philosophical comments (#2-4) one could address to further improve this manuscript.

Comments:
1) The biggest problem this reviewer has is the sample selection. There is absolutely no information on how these samples were selected, matched, etc. that is a major caveat! Detailed information on the samples and the selection need to be presented. Especially since an incredibly low number of samples were analyzed.

We agree with the reviewer and have now supplemented the manuscript with the requested patient information (Supplementary Table 1) and with tissue images before and after microdissection (Supplementary Figure 1). Regarding the low number of samples, we note that the combination of depth of analyzed proteins and number of samples is certainly at the forefront of cancer proteomics. We anticipate that developments by our and other groups will allow analysis of larger numbers in the not too distant future.

a. What are the normal colon tissue samples? Adjacent, pathologically normal tissue? If so please state this in the text. Many of us use this, but it's certainly not normal!

We used the adjacent pathologically normal tissue. This is stated in the legend of the Figure 3. Now we have additionally stated this in the method section and we also refer to “normal appearing” rather than “normal” (Page 20).

2) What is the clinical question the authors tried to address? Since the authors mention biomarkers several times, it would have been nice if a more detailed analysis had been performed in this context. Start with cell surface markers (suggested by the authors) perform more comprehensive analyses and validate better (I leave it to the authors, but at least discuss this caveat!)

The surface markers are described as such in Table III. We now discuss possible follow ups in a new paragraph added to the revised manuscript (see just below).

a. If the authors choose IHC, well-annotated FFPE blocks should be chosen. In this case patient outcome is crucial to demonstrate if marker expression correlates with clinical progression?

b. Alternatively, the authors could tailor their mining strategy towards secreted proteins (there are many options to do this). In this case serum/plasma samples are required and markers could be quantified by ELISA. This would add major clinical context to this paper, especially since all the other analyses are very high-level.

These are valuable suggestions, with which we completely agree in principle. However, the analyses recommended by the reviewer would require a larger number of samples, to which we do not have access from our collaborators. Certainly, this would add many months to our study and would not be a very unique contribution in any case (given the very large number of changing proteins). This study was not aimed to identify such correlations but is rather a baseline study elucidating general proteome changes in CRC for the first time. As such it breaks new ground in cancer proteomics. Nevertheless, the knowledge resulting from this work may contribute to identification of biomarkers. In this regards we added a comment on it on page 8:

"Knowledge on proteins significantly changed between normal tissue and adenocarcinoma can be used for subsequent targeted analysis of larger number of samples, using either IHC or mass spectrometry-based methods. This may identify clinically relevant correlations between protein up-regulation in cancer and the disease outcome. Another attractive follow up to this study would be to search for the proteins identified as outliers as secreted proteins in plasma samples (Hanash et al. 2008). In this way clinically useful biomarkers could be identified and potentially later monitored by conventional ELISA. "

3) It is a bit surprising that no significant changes were identified between primary cancers and mets (at least intuitively to me). Although our lab has performed a similar study in a different cancer using >70 FFPE samples and LC-MS with label-free quantification and similarly few changes were found in cancers with stratified outcome. In this context, these primary cancers and mets seem to be matched samples from the same patient? Could the authors at least perform some type of pair wise comparisons to at least suggest some proteins that change with in a patient pair? Maybe the cancer samples are so heterogeneous and poorly matched that not even the best technology can identify changes within the biological noise?

We indeed used exclusively patient matched samples and accordingly the statistical analysis was conducted using paired t-test. We are glad to know that our findings are of a similar nature as the reviewer's study. (We assume this is unpublished as we are not aware of it – we would of course be happy to cite it if it is published). It would be very interesting to know whether the few proteins found changed between cancer and metastasis are indeed similar between the two datasets (even without individual statistical significance). In response to the reviewer's suggestions, we have repeated analyses of our patient datasets, however, the outcome was as before. Of course this lack of metastatic outliers in the outcome of our study could also be due to limited sensitivity of our platform or due to analysis of too small a number of samples to allow identification of significant changes. In the revised manuscript, we have additionally pointed out these caveats by adding the above sentence to the discussion.

4) The correlation to the CaCo-2 cells is surprising? Although we all use cell line models they are completely irrelevant and do not provide relevant models to recapitulate human disease.

We do not completely subscribe to the point of view that cell lines are completely irrelevant to studying human disease. For instance, the currently most discussed promise of iPS cells is the construction of patient specific disease model cell lines with defined genomic changes. The point is that cell lines have been poor representations of human disease but very good at identifying molecular mechanisms. We use the experiment with the CaCo-2 cells in that functional sense, and such functional experiments were also suggested by previous reviewers.
Thus, the experiment with CaCo-2 cells was performed to demonstrate that overall large extent (an extrapolated half of the proteome) of changes observed between normal colon and adenocarcinoma (clinical samples) can be observed in another system. We found a similar extent of changes that occurred under differentiation (reverse to cancer) process, thus, providing evidence for our main

finding in a completely different and well controlled system.

5) The comparison to the array datasets is very superficial. What is the global correlation of these two different datasets? I would assume poor? Are their functional differences in RNA-protein pairs that correlate vs. don't' correlate. The proteomics data will be way more informative than the array, the authors could make a much stronger case for this.

We agree that it would be interesting to perform a detailed comparison between our proteomic results and the array based studies. However, we believe that this would be beyond the scope of this study. Ideally it should be done on the same sample and using RNA-seq, rather than microarrays. Since such data was not available, we decided to provide at least this simplified comparison of both approaches to stress the potential of proteomics. Given the limitations of the comparison, we think it might be misleading to calculate specific correlation values. Again, we completely agree that the proteomics data is likely to be more 'functionally relevant' and we hope that future studies will bear this out.

Figure 8A is a bit confusing. It wasn't clear to this reviewer what the 2 different pie charts actually represent? Could this be merged into some type of heat map or even better a scatter plot?

We thank the reviewer for the suggestion. In the revised manuscript, we have extended the legend explained the Figure and we have converted the abundance data into a scatter plot.

In summary, this is a well-performed proteomics study that impressively showcases how far MS-based proteomics has come. This reviewer is convinced these technologies will make significant contributions in biological sciences and cancer research. A shortcoming of the paper is the limited number of samples and their poor (absent) annotation. If samples are poorly selected without the direct input of a clinician even the most powerful proteomics technology will likely not result in much novel insights let alone clinical impact. The authors also sell themselves a bit short by a somewhat superficial analysis. Some suggestions were made and it would be appreciated if the authors would be able to address some of these points. Overall, I recommend publication in MSB simply due to potential implication of the presented technology for future translational cancer proteomics studies.

We thank the reviewer for the overall positive evaluation of our paper and hope that we have addressed at least some of the concerns in this revision.

