Reviewer 2

We thank the reviewer for the through revision of our manuscript. Thanks to the helpful suggestions of both reviewers, we believe that the manuscript is much improved.

Modifications made to the main text are highlighted in red.

The manuscript presesented by Dr. Noberini et al. makes use of label-free mass spectrometry to quantify histone H1 variants. The method was developed on cell lines and murine tissue samples, and later applied to breast cancer patient samples. The method was capable of identifying unique peptide sequences among the different variants. The authors performed a thorough validation of their method by analyzing wild type mouse embyronic fibroblasts (MEF), MEF with histone H1.0 knock-down, and MEF treated with Quisinostat to induce H1.0 production, and comparing their MS results with immunofluorescence quantitation. Their results showed an acceptable correlation between the two orthogonal techniques. Linearity of the MS method was also established. The data was presented clearly.

The following are some questions or comments that I hope could improve the presentation of the study.

1. The authors mention that PTMs can modulate histone functions. Were any post-translational modifications such as acetylation, methylation, or phosphorylation considered in this analysis? Does the sample preparation workflow eliminate or preserve these modifications? Were the peptide sequences chosen with possible modifications (or lack thereof) in mind?
   The protocols we employed in this study preserve histone PTMs. As a matter of fact, when we ran a search looking for modifications in the initial MEF dataset, we found various acetylations, methylations and phosphorylations occurring on the different H1 variants. However, when we estimated their abundance and found that in the vast majority of cases was below 1% and only in two peptides slightly higher but in any case below 10%, we reasoned that the presence of these PTM would not interfere with the LFQ quantification of protein levels.
   A further, more in depth analysis of PTMs on histone H1 variants would however be extremely interesting to carry out, since a comprehensive study on this subject has not yet been performed. Although we believe that such an analysis would go beyond the scope of the current study, we mention this aspect in the discussion of the manuscript, on pages 10-11. We thank the reviewer for the comment.

2. In Figures 1C and 4A, do the white spaces indicate peptides that were not found in the respective samples?
   Yes, they do. We have added this information to the figure legends.

3. In Figure 2C, what could account for the deviation from linearity of the fold change observed in H1.0?
   We agree with the reviewer that there is a mild deviation from linearity in the graph shown in Figure 2C, however we do have not definitive explanation for this. It is possible that the high levels of histone H1.0 in the HDACi treated sample approached the saturation limit of the LFQ quantification.

4. How much tissue material (in terms of mass, cell counts, or comparable measurement) do the whole slice samples in Figure 4 contain? Can a rough estimate be provided?
   Each of the mouse pancreas sections contains approximately $1.5*10^6$ cells. After protein extraction, we loaded 10 μg of protein extract (corresponding to approximately 1/3 of the total

preparation) on a SDS-PAGE gel for further processing. We added more details about these samples in the Materials and Methods section (pages 11-12), and added the actual protein amount used (10 μg) in figure 4A. We thank the reviewer for the useful comment.

5. In Figure 4A, 2 replicates are shown for each condition (FFPE and OCT). In Figure 4B, correlations are shown for only 1 FFPE. Should there also be 2 replicates here?

   While FFPE whole slices were run in duplicate, the laser-microdissected FFPE samples were run as a single sample, as shown both in figure 4A and 4B.

6. In Line 212, it is mentioned that FFPE tissue samples can have artefactual modifications. What modifications are expected from the FFPE process? Can the modifications be included in the MS search?

   Lysine modifications that may be induced by formalin fixation include methylation, formylation, methylene adducts and methylol adducts (doi: 10.1002/pmic.201400454). Although they could potentially be included in the search, the almost perfect correlation of MaxLFQ values obtained from FFPE and OCT samples (now shown in figure S3B and discussed on page 7) suggests that potential artefacts due to FFPE storage do not influence the quantification of histone H1 isoforms. As such, we believe that for the analysis of linker histones there is no need to include in the search potential artefacts.

7. The authors suggest running a reference sample in parallel for MS/MS identification of peptides. In Figure 4A there seems to be several peptides that could not be quantified in the microdissected samples, even by using the "match between runs" feature. Could quantitation of the lower abundance histones still be done effectively when the amount of sample is low?

   Although some peptides could not be detected in the LMD samples, the detection of at least one peptide for each histone variant allowed the quantification of most variants from all the conditions/cell number tested. The high correlation of histone H1 variant LFQ values shown in panel B demonstrates that the quantitation is not affected by using lower starting amounts of cells. Exceptions are represented by histone H1.1 and H1x, which could not be quantified in a few of the samples containing the lowest amounts of cells. This results is likely due to the particularly low abundance (<0.15%) of these two variants in mouse pancreas. To better convey this message, we have included in the new panel B of Figure 4 a table summarizing which histone H1 variant could be quantified from each of the samples, together with the % abundances of each variant. We also comment on these results on page 7. We thank the reviewer for suggesting this interesting point for discussion.

8. In Figure 4B, why are correlations shown only between microdissected samples, but not with whole slice samples? The LFQ values remain high in Figure 4B, but this does not give the whole picture. In Figure 4A we see that there are some peptides that could not be detected in microdissected samples. If accurate quantitation of H1 variants is the goal, then the caveat would be that less abundant variants would fall below the method detection limit when microdissected samples with less than 20,000 cells are used. Please let me know if I am understanding the results correctly here.

   We chose to show only the correlation among LMD samples because they represent much more homogeneous samples compared with whole slices. While the whole sections were used as a source of information for ID transferring through the “match-between-runs” feature, the true reference for the quantitation of histone H1 levels is represented by the 20000 LMD sample. As also mentioned in the answer to point 7, the very high correlation scores obtained among LMD samples show that an accurate quantification of histone H1 variant can be achieved even from as low as 1000 cells. For completeness, we added in Figure S3 the correlation matrix that also includes the whole sections. Whole sections are slightly more divergent compared with the

LMD samples, likely due to the presence of stroma and other cell types in addition to the acinar cells that were selected by LMD. This is now mentioned on page 7.

9. Please check line 328.

We fixed the issue with this sentence.