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Integrative multi-omics analysis of intestinal organoid differentiation

Rik G.H. Lindeboom, Lisa van Voorthuijsen, Koen C. Oost, Maria J. Rodríguez-Colman, Maria V. Luna-Velez, Cristina Furlan, Floriane Baraille, Pascal W.T.C. Jansen, Agnès Ribeiro, Boudewijn M.T. Burgering, Hugo J. Snippert and Michiel Vermeulen.

Editor: Maria Polychronidou

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   12th Febuary 2018

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 study. As you will see below, the reviewers appreciate that the study seems potentially interesting and that the presented datasets might be useful for the field. They raise however a series of concerns, which we would ask you to address in a major revision.

Without repeating all the points listed below one of the more fundamental issues refers to the need to perform further analyses in order to better support the role of Hnf4g as a key regulator of enterocyte differentiation. Of course all other issues raised by the referees would need to be thoroughly addressed.

REFEREE REPORTS

Reviewer #1:

In the manuscript entitled "System-wide rewiring of gene expression during intestinal organoid differentiation" perform extensive and thorough dissection of molecular mechanisms that drive differential gene expression during adult intestinal stem cell differentiation. They apply a number technologies, including RNA seq, genome wide accessibility studies using ATAC-seq and transcription factor binding approaches using ChIPmentation. Moreover they also apply proteomics approaches to provide an accurate understanding of a post-transcriptional regulatory network during enterocyte lineage specification. Finally the use of metabolomics and lipidomics enables a more

complete overview of the major changes at functional level. In addition, the authors also make use of publicly available datasets to extend the scope of their work, and to strengthen their hypotheses. Through this holistic approach, they reach the conclusion that during intestinal differentiation a global rewiring of the epigenome, transcriptome and the proteome takes place much faster than what was thought, and that the majority of the changes are driven by transcription. This process facilitated by the fact that the stem cells have their epigenome in a permissive state (Kim, 2014). They also propose that this global change can be, in fact, attributed to a single transcription factor, Hnf4g, that operates as the main driver of enterocyte specification. I found the work compelling. The datasets generated are important resources for the field. However, there are some parts that must be improved:

1. Authors do not present enough evidence to support the claim that Hnf4g is the key TF driving enterocyte differentiation. The authors predict Hnf4g binding motifs in differentiation genes, yet they should show Hnf4g binding to the regulatory regions of these genes in organoids under the distinct culturing conditions.

2. Likewise, overexpression experiments have been performed in cell lines rather than in organoids. It would also be important to include loss of Hnf4g function experiments (shRNA or CRISPR) in organoids to demonstrated altered differentiation.

3. The main driver of the stem cell program is WNT signaling. Is Hnf4g expression negatively regulated by WNT signaling? Does over expression of Hnf4g trigger downregulation of beta-catenin/TCF4 transcriptional activity?

4. In figure 2 authors show markers of intestinal homeostasis enriched in CV (stem cell genes) and in EN (enterocyte lineage specific genes). However, other well-known markers of enterocyte differentiation such as KRT20, ANPEP, CA1/2 are not shown. I would like to see if these genes are expressed in EN organoids. Moreover, authors could leverage their datasets to define novel markers of intestinal differentiation and link them to particular functions in enterocytes.

Minor points:

- Authors do not specify the time points needed to achieve CV nor EN enriched cultures. In Figure 1B, time must be indicated, as well as in all other experiments where CV or EN organoids were used.
- In figure 2 it is somewhat confusing where panel A ends, and panel B starts, panel B must be moved to the right, since there is space.
- In figure 3, panel A is too densely packed, should be broken down into simpler panels, easier to interpret. I cannot understand how the far most right column labeled HNF4g motif should be interpreted.

Reviewer #2:

The mammalian intestinal epithelium became a classical model system to study dynamics of chromatin states during adult stem cell maintenance and differentiation. Several studies mapped the distribution of histone marks associated with either transcriptional activity or repression in adult intestinal stem cells (ISCs) and their differentiated progenies, for example enterocytes, in vivo. Using genome-wide ChIP and ATAC-sequencing, potential cell type specific regulatory elements were identified. The distribution of histone modifications and chromatin accessibility were further associated with transcriptional activity of the nearest genes in both ISCs and their differentiated progenies. In summary, differentiation of the adult ISCs is accompanied by strong changes in the distribution of modified histones in genic and intergenic regions, which leads to transcriptional changes (thousands of genes get up- or downregulated). Moreover, the binding sites for a few transcription factors, including Hnf4a, Cdx2 and Gata4, were mapped by ChIP-sequencing in both ISCs and enterocytes. Functional study in mice showed that Hnf4a in concert with Cdx2 play an important role during ISC differentiation. Finally, the comparative proteomic analysis of ISCs and their differentiated progenies was also performed.
In the present study the authors used intestinal organoids to study transcriptional, chromatin,

proteome and metabolic changes accompanying ISC differentiation. The intestinal organoids were cultured in different conditions to either enrich or deplete for adult ISCs. The authors conclude that strong changes at the transcriptional and protein levels take place during ISC differentiation. Consistent with previous studies, they find that a few ISC signature genes acquire tri-methylation of K27 at histone H3 (H3K27me3) during differentiation. Using ATAC- and ChIP-sequencing they see a strong correlation between open chromatin sites and the presence of H3K27Ac and H3K4me3 histone marks. The genome-wide distribution of open chromatin sites as well as H3K27Ac strongly changes during differentiation. They further searched for potential transcription factor binding sites within dynamic regulatory elements and found Hnf4g motif. An overexpression of Hnf4g in LS174T cells results in up-regulation of a differentiation marker Alpi and downregulation of ISC and proliferation markers, Lgr5 and Mki67. The authors thus conclude that Hnf4g is a major driver of enterocyte specific transcriptional program. Finally, the authors performed profiling of metabolites in ISC enriched and depleted organoids. Yet, the functional studies were not performed to address whether the changes in metabolites have any importance.

The conclusions of the study are consistent with previously published literature. The study, however, does not bring significant new knowledge. The transcriptome and chromatin data (and conclusions) are already available from in vivo studies. The same is true for proteome. Although, in the present study 15% more proteins were identified compared to the study by Munoz et al., 2012, ISC markers Lgr5 and Ascl2 were not found by Lindeboom and colleagues.

Major points
1. To conclude whether Hnf4g is a major driver of enterocyte specific gene expression program, loss-of function study using CRISPR/Cas9 in organoids is required.
2. The metabolomics analysis table lacks P values. Therefore, it is not clear, which changes are meaningful.

Minor points:
The authors describe changes in metabolomics or H3K27me3, or TADs in the results part. Yet, it is not clear why. They do not refer to these data even in Discussion part.

Reviewer #3:

The manuscript by Lindeboom et al. describes an intestinal organoid culturing modification that generates most stem cell-enriched small intestinal organoids versus differentiated organoids enriched with enterocytes. Then, a system-wide "multi-omic" approach was used to full characterize the proteome, transcriptome, and epigenome of these different organoid states. From this systems level, analysis it is a little surprising that only Hnf4g transcription factor is identified to be a master regulator of differentiation. While this multi-omic dataset is potentially useful to the community, the biological insights derived from data modeling can be better well-supported by validation experiments.

Major points
1. Kunihiro et al., 2017, AJP. has already worked out the conditions for deriving enriched differentiated cell populations in organoids. Please compare and contrast the phenotypes obtained with the current ones.
2. It was never shown by the authors that the organoid culture derived from the EN condition is actually a pure enterocyte population. Since enterocytes still represent ~90% of the cells, you will get an enterocyte signature with bulk approaches. Immuno-staining of these EN organoids for canonical markers of other differentiate cell types will resolve this issue.
3. As above, better characterization of EN organoids. For instance, if these are differentiated, they would all be "differentiated out" and not be maintainable for more than a week after exposure to EN.
4. Likewise, without stem cell function, the organoids should not be able to be passaged, since there would be no renewal activity.
5. Some of the features hypothesized such as mitochondrial morphological and functional differences between CV and EN can be tested experimentally.
6. Stem cell organoids usually are represented by spherical cyst like structures due to lack of heterogeneous proliferation rates (for bud forming). Here, the stem cell organoids in CV still contains a lot of buds, which seems to contradict the canonical view such as the stem organoids

from the Rodriguez-Colman paper in 2017 (one of the co-authors here). Perhaps, the CV conditions only induces Lgr5 expression but not enriches stem cells. Please compare the gene expression profile of these organoids to that of Lgr5 flow-sorted cells in an analysis similar to 1C.

7. Validation of hypotheses. For instance, using a protein to mRNA ratio, the authors hypothesized that there could a translational / stability differences in important regulators. This is an area that can use some experimental validation such as ribosomal profiling/tagging, etc.

8. Similar to above, the authors predicted increased in a large number of binding sites for Hnf4g in EN organoids. This should be testable with an Hnf4g CHIP experiment.

9. A better validation experiment as opposed to the Hnf4g oe experiment in a cancer cell line is to demonstrate perturbation of Hnf4g by crispr/cas9 in organoids, specifically showing that Hnf4g KO organoids are impaired towards enterocyte differentiation in EN.

10. A missed opportunity is to compare epigenetic data in organoids to in vivo data generated by the Shivdasani lab (quoted by the author). Specifically, the comment on whether the open nature of chromatin in the intestinal system exists in organoids not clarified.

Minor points

1. I am not sure if the small fonts in many of the figures will translate well in print.

1st Revision - authors' response   **23rd May 2018**

12th Feb 2018

RE: MSB-18-8227 System-wide rewiring of gene expression during
intestinal organoid differentiation

Reviewer #1:

In the manuscript entitled "System-wide rewiring of gene expression
during intestinal organoid differentiation" perform extensive and
thorough dissection of molecular mechanisms that drive differential gene
expression during adult intestinal stem cell differentiation. They apply
a number technologies, including RNA seq, genome wide accessibility
studies using ATAC-seq and transcription factor binding approaches using
ChiPmentation. Moreover they also apply proteomics approaches to provide
an accurate understanding of a post-transcriptional regulatory network
during enterocyte lineage specification. Finally the use of metabolomics
and lipidomics enables a more complete overview of the major changes at
functional level. In addition, the authors also make use of publicly
available datasets to extend the scope of their work, and to strengthen
their hypotheses. Through this holistic approach, they reach the
conclusion that during intestinal differentiation a global rewiring of
the epigenome, transcriptome and the proteome takes place much faster
than what was thought, and that the majority of the changes are driven
by transcription. This process facilitated by the fact that the stem
cells have their epigenome in a permissive state (Kim, 2014). They also
propose that this global change can be, in fact, attributed to a single
transcription factor, Hnf4g, that operates as the main driver of
enterocyte specification. I found the work compelling. The datasets
generated are important resources for the field. However, there are some
parts that must be improved:
*We thank the reviewer for his or her positive and constructive feedback on
our manuscript.*

1. Authors do not present enough evidence to support the claim that
Hnf4g is the key TF driving enterocyte differentiation. The authors
predict Hnf4g binding motifs in differentiation genes, yet they should
show Hnf4g binding to the regulatory regions of these genes in organoids
under the distinct culturing conditions.
*This is a valid point. To address this, we performed ChIP-sequencing for
Hnf4g in the three different organoid cultures (CV / ENR / EN) (Widespread
epigenetic modulation facilitates transcription factor driven
differentiation, page 6, paragraph 6). In agreement with our proteomics
and genomics data, we observe a near absence of Hnf4g binding in stem cell
enriched organoids (87 binding sites), whereas many (12357) Hnf4g binding
sites are observed in differentiated (EN) organoid cultures (Figure EV3D).*

Importantly, the observed binding of Hnf4g in EN organoids overlaps with the HNF4 motif and the open and active chromatin regions in EN (Figure 3B).

2. Likewise, overexpression experiments have been performed in cell lines rather than in organoids. It would also be important to include loss of Hnf4g function experiments (shRNA or CRISPR) in organoids to demonstrated altered differentiation.

Again, this is a valid point. In the revised manuscript we replaced the HNF4g overexpression experiment in human colorectal cells with Hnf4g KO experiments in mice and organoids (*Hnf4g drives enterocyte differentiation, page 6, paragraph 1*). We obtained Hnf4g KO organoids by isolating small intestines from Hnf4g KO mice (Baraille et al. 2015, Diabetes). Using RNA sequencing, we observed an enrichment of expressed genes that are specific for secretory cells concomitant with a depletion of enterocyte-specific genes in these Hnf4g KO organoids, indicating that Hnf4g is important for driving expression of enterocyte specific genes (Figure 4A). In agreement with these observations, staining of goblet cells in the small intestinal epithelium of Hnf4g KO mice and Hnf4g KO organoids revealed a significant increase in goblet cell numbers compared to WT intestines and organoids (Figure 4B and 4C). Altogether, these experiments revealed disrupted intestinal epithelial homeostasis in the absence of Hnf4g, characterized by skewed differentiation towards secretory cells.

3. The main driver of the stem cell program is WNT signaling. Is Hnf4g expression negatively regulated by WNT signaling? Does over expression of Hnf4g trigger downregulation of beta-catenin/TCF4 transcriptional activity?

Intestinal stem cells located at the crypt base are exposed to a high concentration of Wnt, whereas cells located in the villus, are not. Our cell type enrichment in organoids mimics this Wnt gradient by supplementing the medium with CHIR99021 or by removing R-spondin to generate stem cell enriched or depleted organoids, respectively. In our model, we observe a strong upregulation of Hnf4g when the Wnt signals are removed, suggesting that Wnt signaling is regulating Hnf4g expression, at least indirectly. However, to investigate whether Hnf4g and Wnt signaling are directly regulating each other, we investigated the activation of WNT signaling pathway signature in the transcriptome of Hnf4g KO organoids. In these organoids we did not observe differential expression of Wnt responsive genes compared to WT organoids (Reviewer Figure 1), suggesting that Hnf4g is not a direct regulator of Wnt signaling. However, we believe that further research is needed to decipher the exact molecular interplay between Wnt signaling and enterocyte factors such as Hnf4g, but such experiments are in our opinion beyond the scope of the current manuscript.
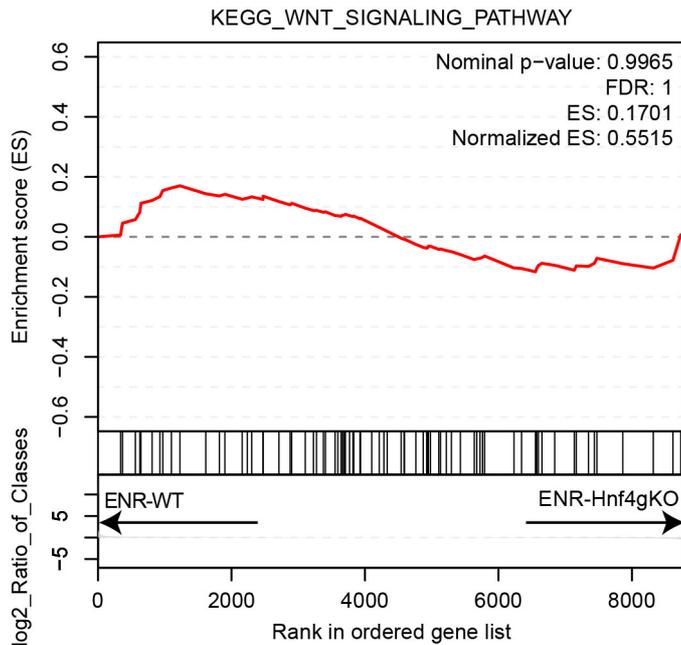
Reviewer Figure 1: Gene Set Enrichment Analysis (GSEA) of Hnf4g KO organoids compared to WT organoids for Wnt responsive genes.

4. In figure 2 authors show markers of intestinal homeostasis enriched in CV (stem cell genes) and in EN (enterocyte lineage specific genes). However, other well-known markers of enterocyte differentiation such as KRT20, ANPEP, CA1/2 are not shown. I would like to see if these genes are expressed in EN organoids. Moreover, authors could leverage their datasets to define novel markers of intestinal differentiation and link them to particular functions in enterocytes.
We thank the reviewer for pointing this out. We mined our transcriptome and proteome data for expression of these marker genes (*Global rewiring of the proteome and transcriptome during adult intestinal stem cell differentiation, page 3, paragraph 1*). Krt20 and Anpep are significantly upregulated in EN organoids, both at the transcript and protein level. Both markers are now highlighted in Figure 2A. We did not detect Ca1/2 expression in our organoids cultures, both at the mRNA and protein level. Besides the identification of Hnf4g as a major driver of differentiation, we indeed detect many more genes and pathways that show specific expression in our cell type enriched organoids. As suggested by the reviewer, we now included a section in the discussion where we highlight the potential of this study as a resource to define enterocyte and stem cell specific gene sets (*Discussion, page 7, paragraph 3*).

Minor points:

- Authors do not specify the time points needed to achieve CV nor EN enriched cultures. In Figure 1B, time must be indicated, as well as in all other experiments where CV or EN organoids were used.
We thank the reviewer for pointing out the missing description of the CV and EN culturing method, this is indeed essential information. We added the culturing conditions and timing to the methods sections (*Organoid culture, page 9, paragraph 1*), and indicated the culturing time points in Figure 1B.

- In figure 2 it is somewhat confusing where panel A ends, and panel B starts, panel B must be moved to the right, since there is space.
We moved panel B from figure 2 to the right, hopefully this makes the separation of the different panels more clear.

- In figure 3, panel A is too densely packed, should be broken down into simpler panels, easier to interpret. I cannot understand how the far most right column labeled HNF4g motif should be interpreted.

In the revised manuscript we split Figure 3A into two separate panels. Furthermore, we moved the legends down to create more space between heatmaps and we added more descriptive labels. Panel A now only shows clustered dynamics in DNA accessibility and histone marks, while panel B now contains the expression dynamics of the closest gene, the HNF4 motif and the newly generated Hnf4g ChIP-seq data. The HNF4 motif and Hnf4g binding data reveals that clusters of accessible and active genomic regions in enterocytes display strong binding of Hnf4g, while the stem cell specific accessible regions are devoid of Hnf4g binding. We hope that these data in panel B are now easier to interpret and link to the observed epigenetic dynamics in panel A.

Reviewer #2:

The mammalian intestinal epithelium became a classical model system to study dynamics of chromatin states during adult stem cell maintenance and differentiation. Several studies mapped the distribution of histone marks associated with either transcriptional activity or repression in adult intestinal stem cells (ISCs) and their differentiated progenies, for example enterocytes, in vivo. Using genome-wide ChIP and ATAC-sequencing, potential cell type specific regulatory elements were identified. The distribution of histone modifications and chromatin accessibility were further associated with transcriptional activity of the nearest genes in both ISCs and their differentiated progenies. In summary, differentiation of the adult ISCs is accompanied by strong changes in the distribution of modified histones in genic and intergenic regions, which leads to transcriptional changes (thousands of genes get up- or downregulated). Moreover, the binding sites for a few transcription factors, including Hnf4a, Cdx2 and Gata4, were mapped by ChIP-sequencing in both ISCs and enterocytes. Functional study in mice showed that Hnf4a in concert with Cdx2 play an important role during ISC differentiation. Finally, the comparative proteomic analysis of ISCs and their differentiated progenies was also performed.
In the present study the authors used intestinal organoids to study transcriptional, chromatin, proteome and metabolic changes accompanying ISC differentiation. The intestinal organoids were cultured in different conditions to either enrich or deplete for adult ISCs. The authors conclude that strong changes at the transcriptional and protein levels take place during ISC differentiation. Consistent with previous studies, they find that a few ISC signature genes acquire tri-methylation of K27 at histone H3 (H3K27me3) during differentiation. Using ATAC- and ChIP-sequencing they see a strong correlation between open chromatin sites and the presence of H3K27Ac and H3K4me3 histone marks. The genome-wide distribution of open chromatin sites as well as H3K27Ac strongly changes during differentiation. They further searched for potential transcription factor binding sites within dynamic regulatory elements and found Hnf4g motif. An overexpression of Hnf4g in LS174T cells results in up-regulation of a differentiation marker Alpi and downregulation of ISC and proliferation markers, Lgr5 and Mki67. The authors thus conclude that Hnf4g is a major driver of enterocyte specific transcriptional program. Finally, the authors performed profiling of metabolites in ISC enriched and depleted organoids. Yet, the functional studies were not performed to address whether the changes in metabolites have any importance.
The conclusions of the study are consistent with previously published literature. The study, however, does not bring significant new knowledge. The transcriptome and chromatin data (and conclusions) are already available from in vivo studies. The same is true for proteome. Although, in the present study 15% more proteins were identified compared to the study by Munoz et al., 2012, ISC markers Lgr5 and Ascl2 were not found by Lindeboom and colleagues.
We thank the reviewer for his or her constructive feedback. Indeed, the intestinal epithelium is a well-studied model for
adult stem cell maintenance and differentiation and many seminal studies have been published in recent years. In our current manuscript, we, for the first time, used a holistic approach to interrogate many aspects of intestinal stem cell differentiation in an integrative manner. We respectfully disagree with the point raised by the reviewer regarding the

lack of significant new knowledge brought by our study. While some of our findings indeed nicely agree with literature, our approach allowed us to answer fundamental questions related to intestinal stem cell differentiation. In addition to uncovering previously underappreciated global changes at the epigenome, transcriptome and proteome level during lineage commitment in the intestinal epithelium, we are for example, to the best of our knowledge, the first to report a transcription factor (Hnf4g) that specifically drives enterocyte differentiation. In our revised manuscript, we further strengthened this observation by performing experiments on Hnf4g KO mice and organoids (*Hnf4g drives enterocyte differentiation, page 6, paragraph 1*), and profiled the Hnf4g binding in organoid cultures using ChIP-seq (*Widespread epigenetic modulation facilitates transcription factor driven differentiation, page 6, paragraph 6*).

Moreover, moving beyond mouse models, the established systems biology platform that we present in this manuscript has the potential to be applicable to all types of organoid cultures, which include human organoid cultures of normal and diseased origin. To illustrate the relevance of our findings for human biology and disease, we analyzed human colorectal cancer stem cell (CSC) transcriptomes for evidence of regulation by Hnf4g (*Hnf4g drives enterocyte differentiation, page 6, paragraph 3*). Strikingly, Hnf4g is one of the most downregulated transcription factors in CSCs (Figure EV4A). Consequently, the binding motif of Hnf4g is the most significantly enriched transcription factor motif in the promoters of genes that are specifically upregulated in tumor progression organoid cells that lack a CSC phenotype (Figure EV4B). This suggests that Hnf4g is playing a role in gene expression regulation in cancer cells, which is in line with recent reports linking perturbed Hnf4g function to pancreatic (Klein et al., Nature Communications, 2018), prostate (Shukla et al, Cancer Cell, 2018) and lung cancer (Wang et al., Oncotarget, 2017).
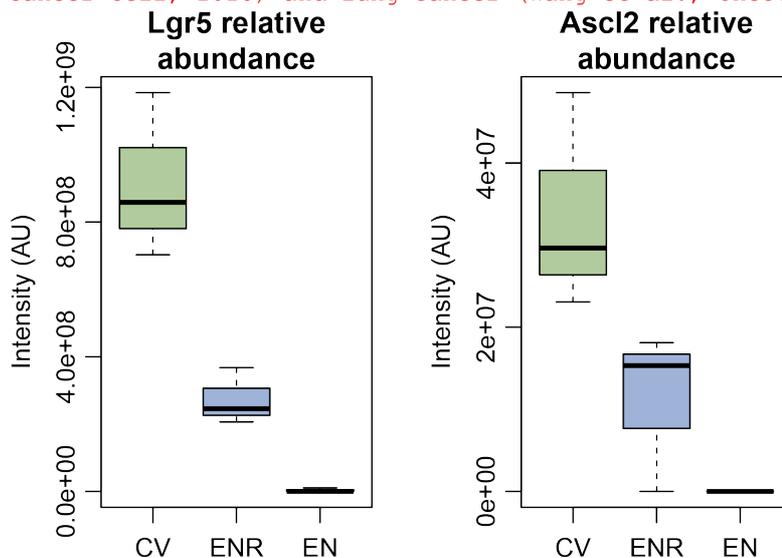
Reviewer Figure 2: relative abundance Lgr5 and Ascl2 in the different culture conditions. Mass spectrometry intensities of Lgr5 and Ascl2 in three replicates per organoid condition are shown.

Finally, our proteomics dataset exceeds any currently available intestine proteome datasets in depth, and it does actually cover the stem cell markers (Ascl2 and Lgr5) mentioned by the reviewer. As seen in Reviewer Figure 2, both Ascl2 and Lgr5 display strong upregulation in stem cell enriched organoids. The reason that these proteins are not included in Figure 2 is that we filter for proteins which are identified with at least 2 unique peptides and three independent identifications by mass spec in at least three replicates to ensure high confidence quantification of protein abundance. Lgr5 and Ascl2 did not meet these criteria, likely because even in stem cells they have low expression (Lgr5) or because they are very small proteins with few tryptic peptides (Ascl2). Nevertheless, we increased the confidence and depth of proteome datasets by repeating the mass spectrometry experiments (*Generation of stem cell enriched and - depleted mouse small intestinal organoid cultures, page 3, paragraph 1*).

Importantly, we observe high correlations and reproducible dynamics between the cell type enriched organoid cultures (Figure EV1C).

Major points
1. To conclude whether Hnf4g is a major driver of enterocyte specific gene expression program, loss-of function study using CRISPR/Cas9 in organoids is required.
This is a valid point, see our response to reviewer 1, point 2.

2. The metabolomics analysis table lacks P values. Therefore, it is not clear, which changes are meaningful.
We thank the reviewer for pointing this out. We now visualized and included false discovery rates in both Figure EV1B and Dataset EV2.

Minor points:
The authors describe changes in metabolomics or H3K27me3, or TADs in the results part. Yet, it is not clear why. They do not refer to these data even in Discussion part.
We agree with the reviewer that these datasets are underused and not thoroughly discussed. In the revised manuscript we now included functional follow-up experiments based on observations from the lipidomics experiments (*Global rewiring of the proteome and transcriptome during adult intestinal stem cell differentiation, page 4, paragraph 2*). Based on the global upregulation of glycero(phospho)lipids in our stem cell enriched organoids, we hypothesized that the rapidly dividing stem cells require more organelles such as mitochondria. We experimentally tested this hypothesis with live-cell metabolic assays (Seahorse technology, Bioscience), by quantifying metabolic complexes with western-blot, and by qPCR on mitochondrial DNA. Indeed, we found that stem cell enriched organoids have a higher mitochondria load, revealing cell type specific differences in metabolic activity and energy use in the intestine (Fig EV1D-G), thus illustrating the usefulness of our metabolomics data as a resource. In addition, we now included a discussion section about H3K27me3 domains and changing TAD activity (*Discussion, page 7, paragraph 3*).
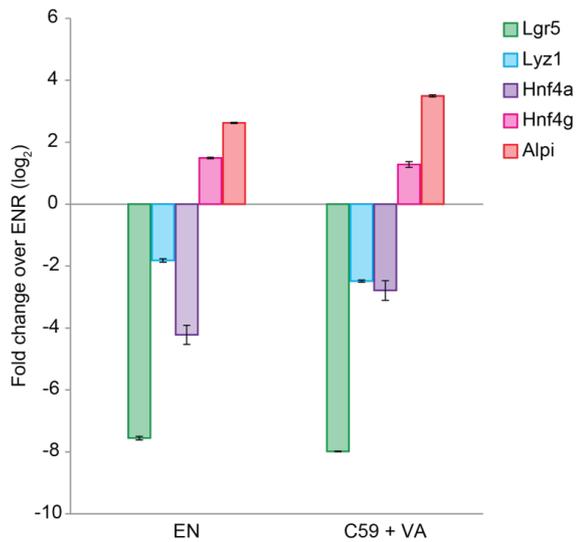
Reviewer #3:

The manuscript by Lindeboom et al. describes an intestinal organoid culturing modification that generates most stem cell-enriched small intestinal organoids versus differentiated organoids enriched with enterocytes. Then, a system-wide "multi-omic" approach was used to full characterize the proteome, transcriptome, and epigenome of these different organoid states. From this systems level, analysis it is a little surprising that only Hnf4g transcription factor is identified to be a master regulator of differentiation. While this multi-omic dataset is potentially useful to the community, the biological insights derived from data modeling can be better well-supported by validation experiments.
We thank the reviewer for his or her constructive feedback on our manuscript.
Major points
1. Kunihiro et al., 2017, AJP. has already worked out the conditions for deriving enriched differentiated cell populations in organoids. Please compare and contrast the phenotypes obtained with the current ones.

Reviewer Figure 3: gene expression of enterocyte enrichment cultures in small intestinal organoids. Relative expression of cell type specific markers in enterocyte enriched cultures compared to ENR gene expression. Gene expression is normalized over Actb expression and fold changes are shown in log2. Error bars show 1x the standard deviation.

We thank the reviewer for pointing out this paper which describes different culture conditions to achieve cell type enrichments in mouse small intestinal organoids. In the revised manuscript we cite this paper in the introduction section. We compared the enterocyte enrichment culture method (C59 + VA) of Kishida et al. (2017) with our enterocyte enrichment method (EN) (Reviewer Figure 3), since the stem cell enrichment method used in the paper and our manuscript are similar (CV). Both cultures were harvested after a differentiation period of 2,5 days. The enrichment methods both show a decrease of the stem cell marker Lgr5 and the Paneth cell marker Lyz1 compared to a typical ENR culture, which is also shown in Figure 1b of Kishida et al. (2017). As discussed in the manuscript, Hnf4a has an alternative transcript, which is downregulated during enterocyte differentiation. Both culture conditions show a reduction of this Hnf4a transcript. The enterocyte differentiation markers Hnf4g and Alpi are increased in both culture conditions, which confirms that both culture methods enrich for enterocytes. We conclude that both the EN and the C59 + VA method can be used to enrich for enterocytes in intestinal organoid cultures.

Since we do not focus on the development of cell type specific enrichment methods in our manuscript, this figure is not included in the expanded view of the revised manuscript, but we only provide this figure in our rebuttal for the reviewers.

2. It was never shown by the authors that the organoid culture derived from the EN condition is actually a pure enterocyte population. Since enterocytes still represent ~90% of the cells, you will get an enterocyte signature with bulk approaches. Immuno-staining of these EN organoids for canonical markers of other differentiate cell types will resolve this issue.

It is indeed correct that the EN culture does not consist of a pure enterocyte population. The EN culture is used to obtain enterocyte enriched organoids. As depicted in Figure 2A of the manuscript, we observe an upregulation of enterocyte markers like Vil1, Ptk6 and Alpi in the EN condition compared to CV and ENR. However, other differentiated cell types are also present in the EN condition as shown by the expression of Muc2, Dclk1, and Reg4 (for goblet cells, tuft cells, and enteroendocrine cells respectively). Importantly, the expression of enterocyte markers is the strongest in EN organoids compared to the ENR condition, which indicates a strong enrichment of enterocytes compared to secretory cell types.

In addition to the observed dynamics in Lgr5-DTR-GFP+ cells, we set out to visualize differentiated cell types in the CV, ENR and EN organoids, as the reviewer suggested. While we were technically not able to visualize enterocyte cells by microscopy, we used Alcian Blue staining to visualize

goblet cells in CV, ENR and EN (see Reviewer Figure 4), which revealed an absence and enrichment for goblet cells in CV and EN organoids, respectively. However, as pointed out by the reviewer, enterocytes are by far the most dominant cell type in absolute abundance in the intestinal epithelium, which is why the enterocyte signature is dominant in the bulk assays that we performed.
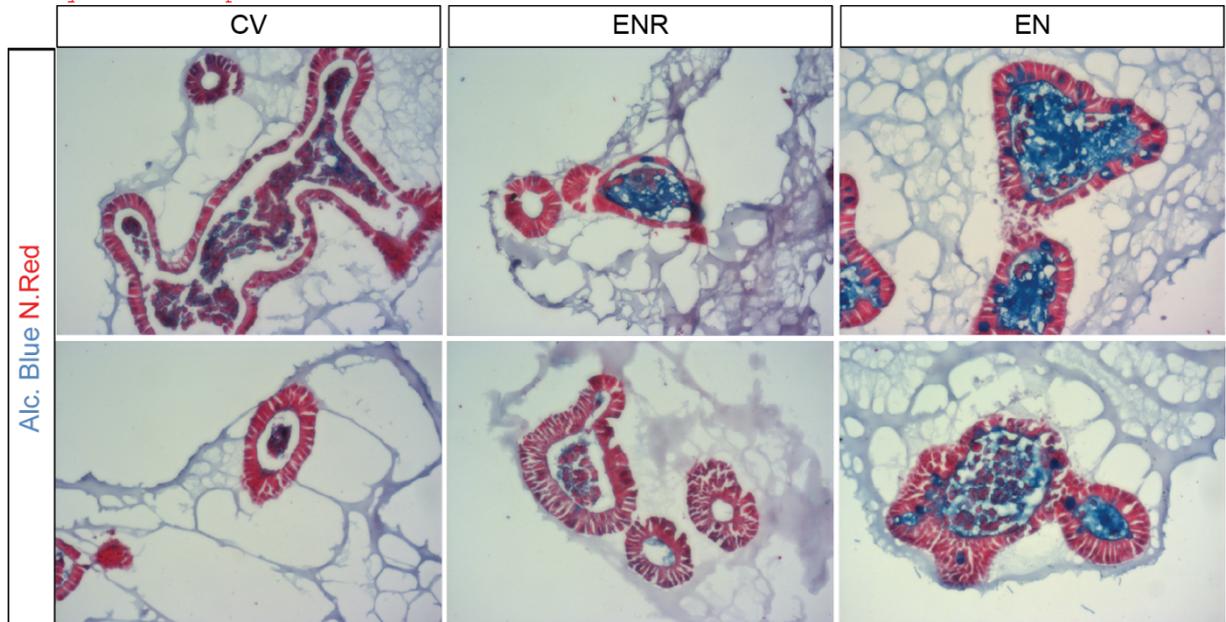
Reviewer Figure 4: Alcian blue and Nuclear Fast Red staining of CV, ENR, and EN small intestinal organoids. Stainings are performed on fixed WT organoids in the different culture conditions. Alcian blue is used to visualize the goblet cells and the cells are visualized using nuclear fast red.

3. As above, better characterization of EN organoids. For instance, if these are differentiated, they would all be "differentiated out" and not be maintainable for more than a week after exposure to EN.
We indeed forgot to mention the duration of differentiation using the EN culture method, we now included this in our methods section (*Organoid culture, page 9, paragraph 1*) and in Figure 1B. It is indeed correct that the organoids stop proliferating and die after continued culturing on EN medium. We passage and expand organoids on ENR medium, followed by induction of differentiation by washing out R-spondin. We cultured these EN organoids for 2,5 days after which we harvested the organoids. Apologies for the confusion this may have caused.

4. Likewise, without stem cell function, the organoids should not be able to be passaged, since there would be no renewal activity.
It is indeed also not possible to passage the EN organoids, as mentioned above, we now included a methods section for this and highlighted the timepoints used in Figure 1B.

5. Some of the features hypothesized such as mitochondrial morphological and functional differences between CV and EN can be tested experimentally.
We now tested the hypothesized differences in mitochondrial load between CV and EN (*Global rewiring of the proteome and transcriptome during adult intestinal stem cell differentiation, page 4, paragraph 2*). Western-blot and qPCR for mitochondria shows an increased presence of mitochondria in the CV organoids (Fig EV1E-F). Reassuringly, when investigating bioenergetics with Seahorse technology (Biosciences) we observe an increase in relative mitochondrial respiration metabolic activity in the CV population (Fig EV1D,G). These results are in agreement with the decrease in glycerolipids observed in the EN population and reveal differences in energy metabolism in different intestinal cell types.

6. Stem cell organoids usually are represented by spherical cyst like

structures due to lack of heterogeneous proliferation rates (for bud forming). Here, the stem cell organoids in CV still contains a lot of buds, which seems to contradict the canonical view such as the stem organoids from the Rodriguez-Colman paper in 2017 (one of the co-authors here). Perhaps, the CV conditions only induces Lgr5 expression but not enriches stem cells. Please compare the gene expression profile of these organoids to that of Lgr5 flow-sorted cells in an analysis similar to 1C.

Organoids growing in medium containing high Wnt3a concentrations (WENR) are indeed growing in cyst like structures due to homogeneous proliferation, as also shown by Rodríguez-Colman et al. (2017, Nature). These WENR organoids, which resemble embryonic small intestine organoids and have decreased Lgr5 expression, are used by Rodríguez-Colman et al. (2017, Nature) to study the differentiation process before bud formation. Crypt structures are a typical phenotype for CV organoids and have increased expression of Lgr5 (Kishida et al. (2017, AJPGLP), Yin et al. (2014, Nature Methods)). Together, this suggest that CV organoids are enriched for adult intestinal stem cells as shown by increased Lgr5 expression, WENR organoids have a decrease in Lgr5 expression because of their more embryonic state.
As suggested by the reviewer, we compared our gene expression data with Lgr5 flow-sorted cells from Munõz et al. (2012, The EMBO journal) which revealed a strong enrichment of the Lgr5+ gene signature in the CV population (Figure EV1A) (*Global rewiring of the proteome and transcriptome during adult intestinal stem cell differentiation, page 3, paragraph 1*). This again validates the stem cell identity of the CV organoid population.

7. Validation of hypotheses. For instance, using a protein to mRNA ratio, the authors hypothesized that there could a translational / stability differences in important regulators. This is an area that can use some experimental validation such as ribosomal profiling/tagging, etc.

We agree that further research is needed to experimentally validate the changes in proteins / mRNA ratios. The interesting observation of potential post transcriptional regulation of certain transcripts could be of great interest to the field, and the method we use to identify post transcriptional regulated transcripts takes a unique angle to address this problem, which could be explored by others. However, we now emphasize in the revised manuscript that these observations are predictive only, and require further functional validation (*Discussion, page 7, paragraph 3*). Such validations are, however, beyond the scope of the current manuscript.

8. Similar to above, the authors predicted increased in a large number of binding sites for Hnf4g in EN organoids. This should be testable with an Hnf4g CHIP experiment.

To address this valid point, we performed Hnf4g ChIP-sequencing in the three organoid culture conditions (CV / ENR / EN) (*Widespread epigenetic modulation facilitates transcription factor driven differentiation, page 6, paragraph 6*). Strikingly, global Hnf4g binding is observed in EN (12357 binding sites) compared to ENR and CV organoids (1619 and 87 binding sites, respectively; Figure EV3D). Importantly, Hnf4g binding as observed by ChIP-seq overlaps with open and active chromatin regions in the EN culture, which shows that Hnf4g is indeed binding to these enterocyte specific regulatory elements (Figure 3B).

9. A better validation experiment as opposed to the Hnf4g oe experiment in a cancer cell line is to demonstrate perturbation of Hnf4g by crispr/cas9 in organoids, specifically showing that Hnf4g KO organoids are impaired towards enterocyte differentiation in EN.

This is a valid point, see our response to reviewer 1, point 2.

10. A missed opportunity is to compare epigenetic data in organoids to in vivo data generated by the Shivdasani lab (quoted by the author). Specifically, the comment on whether the open nature of chromatin in the intestinal system exists in organoids not clarified.

This is a very good suggestion. We integrated the mentioned datasets with the DNA accessibility and histone mark profiles we generated (*Discussion,*

page 8, paragraph 4). Reassuringly, we see that the enterocyte specific dynamics we observe is most similar to the profiles from enterocyte precursor sorted cells, while our stem cell enriched organoids show dynamics that are more similar to their Lgr5+ and secretory progenitor populations (Figure EV5A).

As already mentioned in the manuscript, our results partially challenge the conclusions made by the Shivdasani lab that the intestinal epithelium is characterized by a globally permissive epigenetic landscape. While we focus on terminally differentiated cells, their study focusses on progenitor cells that are likely in a more permissive state. On the other hand, it is conceivable that global and extensive changes are less pronounced due to technical differences. However, because of the technical and biological differences with our study, we do not wish to make bold statements about this, as it goes beyond the scope of our manuscript. Interestingly, when investigating predicted Hnf4g binding in secretory / enterocyte progenitors in the Shivdasani dataset, a significant enrichment of the Hnf4g motif is observed in regions that are more active / open in enterocyte progenitors compared to secretory progenitors (Figure EV5B) (*Discussion, page 8, paragraph 4*). This strengthens our observation that Hnf4g is an enterocyte specific factor, and a not pan-differentiation factor. Furthermore, this observation indicates that Hnf4g is already of importance during the progenitor stage of differentiation.

Minor points

1. I am not sure if the small fonts in many of the figures will translate well in print.

We thank the reviewer for pointing this out. We enlarged some figures (for example figure 3A) to make sure they will be easy to read. All font sizes are now according the guidelines to authors.

References

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2nd Editorial Decision   23rd May 2018

Thank you for sending us your revised manuscript. We acknowledge the addition of follow up analyses, including experimental analyses better supporting the proposed role of Hnf4g in enterocyte differentiation. We are satisfied with the modifications made and we think that the study is now suitable for publication in Molecular Systems Biology.

Before we formally accept your study for publication, we would ask you to address a few remaining editorial issues listed below.

YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND ⬇

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: Michiel Vermeulen
Journal Submitted to: Molecular Systems Biology
Manuscript Number: MSB-18-8227

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http://www.consort-statement.org
http://www.consort-statement.org/checklists/view/32-consort/66-title

http://www.equator-network.org/reporting-guidelines/reporting-recommendations-for-tum

http://datadryad.org

http://figshare.com

http://www.ncbi.nlm.nih.gov/gap

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Reporting Checklist For Life Sciences Articles (Rev. July 2015)

This checklist is used to ensure good reporting standards and to improve the reproducibility of published results. These guidelines are consistent with the Principles and Guidelines for Reporting Preclinical Research issued by the NIH in 2014. Please follow the journal's authorship guidelines in preparing your manuscript.

A- Figures
1. Data
The data shown in figures should satisfy the following conditions:

➔ the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
➔ figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
➔ graphs include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
➔ if n< 5, the individual data points from each experiment should be plotted and any statistical test employed should be justified
➔ Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the author ship guidelines on Data Presentation.

2. Captions

Each figure caption should contain the following information, for each panel where they are relevant:

➔ a specification of the experimental system investigated (eg cell line, species name).
➔ the assay(s) and method(s) used to carry out the reported observations and measurements
➔ an explicit mention of the biological and chemical entity(ies) that are being measured.
➔ an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.

➔ the exact sample size (n) for each experimental group/condition, given as a number, not a range;
➔ a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
➔ a statement of how many times the experiment shown was independently replicated in the laboratory.
➔ definitions of statistical methods and measures:
  • common tests, such as t-test (please specify whether paired vs. unpaired), simple χ2 tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  • are tests one-sided or two-sided?
  • are there adjustments for multiple comparisons?
  • exact statistical test results, e.g., P values = x but not P values < x;
  • definition of 'center values' as median or average;
  • definition of error bars as s.d. or s.e.m.

Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

Please ensure that the answers to the following questions are reported in the manuscript itself. We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

In the pink boxes below, provide the page number(s) of the manuscript draft or figure legend(s) where the information can be located. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable).

B- Statistics and general methods

Please fill out these boxes ⬇ (Do not worry if you cannot see all your text once you press return)

Question	Answer
1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	No tests were used to estimate sample size to ensure adequate power to detect pre-specified effect size.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	We estimated that three independent experiments with 4 to 5 animals per genotype (WT or Hnf4g KO) were the needed sample size to obtain results with a statistical significance. The mice number is 11 WT mice and 14 Hnf4g KO mice (as specified in figure 4 legend).
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	No exclusion criteria were used to analyze the goblet cell number: Goblet cells (PAS positive cells) were counted in villi on one section of jejunum from WT and Hnf4g KO mice without mouse or tissue section exclusion (In Materials and Methods, Tissue Isolation and histology).
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	The effects of subjective bias have been minimized: mice were matched in age and housed in the same house in a specific pathogen free area (SPF) with a monitored light/dark cycle and fed with a controlled diet (In Materials and Methods, Hnf4g knock out mouse model).
For animal studies, include a statement about randomization even if no randomization was used.	No randomization was used.
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	No steps were taken to minimize the effects of subjective bias during group allocation and when assessing results.
4.b. For animal studies, include a statement about blinding even if no blinding was done.	No blinding was used when assessing results.
5. For every figure, are statistical tests justified as appropriate?	We believe all performed tests were appropriate.
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	Most tests performed are non-parametric. We used a Fisher transformation when comparing pearson correlation coefficients on mRNA/prot ratios between different replicates to ensure a normal distribution for a Student's t-test.
Is there an estimate of variation within each group of data?	We did not explicitly analyse within-group variation.
Is the variance similar between the groups that are being statistically compared?	The employed statistical tests do not depend similar variance between groups.

C- Reagents

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	anti-H3K4me3 (C15410003, Diagenode), anti-H3K27ac (C15410196, Diagenode), anti-H3K27me3 (C15410195, Diagenode), anti-Hnf4g (Sigma, HPA005438), Anti-beta Actin (Abcam,ab16039), Polyclonal Swine Anti-Rabbit Immunoglobins/HRP (Dako, P0399), Total OXPHOS Rodent WB Antibody Cocktail (ab110413, Abcam), anti-vinculin (V9131, Sigma).
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	All organoids cultures were montly tested for mycoplasma contamination where all culteres tested negative. Source of the organoids is described in the material & methods and the acknowledgements.

* for all hyperlinks, please see the table at the top right of the document

D- Animal Models

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	Species, strain, genetic modification status as eel as housing and husbandry conditions and source of animals are described in Materials and Methods, Hnf4g knock out mouse model, page9. Age of animals is specified in figure 4 legend.
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	Compliance with ethical regulations and identification of the committee(s) approving the experiments were described in Materials and Methods, Hnf4g knock out mouse model.
10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance.	We confirm compliance

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	NA
12. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	NA
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	NA
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	NA
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	NA
16. For phase II and III randomized controlled trials, please refer to the CONSORT flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list.	NA
17. For tumor marker prognostic studies, we recommend that you follow the REMARK reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	NA

F- Data Accessibility

18. Provide accession codes for deposited data. See author guidelines, under 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	Next-generation sequencing data has been deposited at Gene Expression Omnibus (GEO) with accession code GSE114113. The mass spectrometry data has been deposited at the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD009700.
19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal's data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under 'Expanded View' or in unstructured repositories such as Dryad (see link list at top right) or Figshare (see link list at top right).	In addition to the deposited data, processed datasets are deposited as expanded view datasets.
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21. As far as possible, primary and referenced data should be formally cited in a Data Availability section. Please state whether you have included this section. Examples: **Primary Data** Wetmore KM, Deutschbauer AM, Price MN, Arkin AP (2012). Comparison of gene expression and mutant fitness in Shewanella oneidensis MR-1. Gene Expression Omnibus GSE39462 **Referenced Data** Huang J, Brown AF, Lei M (2012). Crystal structure of the TRBD domain of TERT and the CR4/5 of TR. Protein Data Bank 4O26 AP-MS analysis of human histone deacetylase interactions in CEM-T cells (2013). PRIDE PXD000208	We included a Data Availablility section.
22. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biomodels (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information.	NA

G- Dual use research of concern

23. Could your study fall under dual use research restrictions? Please check biosecurity documents (see link list at top right) and list of select agents and toxins (APHIS/CDC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could.	NA