Dear Editor,

Please find enclosed the revised version of the manuscript and the point–by–point answers to the questions raised by the three reviewers. We are very grateful to the reviewers for their valuable comments, which have helped us to strongly improve the quality of the manuscript.

Since members of our group that were not previously in the author list performed the additional experiments requested by the reviewers, we inserted them into the author list (i.e. Gloria Ros and Rossella Zanin).

In the revised version, by means of the "Word track changes" and "Comments" options we indicated the changes made.

We feel confident to have addressed all the points raised by the reviewer and we hope that the revised version will be suitable for publication in CANCERS - Special Issue "Epigenetic Dysregulation in Cancer: From Mechanism to Therapy"

We look forward to hearing from you regarding our submission and we would be glad to respond to any further questions and/or comments that you might have.

Sincerely

Riccardo Sgarra

Assistant Professor
Dept. Life Sciences
University of Trieste
Via. L. Giorgieri, 5
Bldg. G, II floor, rooms 210 and 212
34127 - Trieste (TS), Italy
Phone: +39 040 558-8721 (office)
e-mail: rsgarra@units.it
Point–by–point answers

Reviewer #1

Open Review
(x) I would not like to sign my review report
( ) I would like to sign my review report

English language and style
(x) Extensive editing of English language and style required
( ) Moderate English changes required
( ) English language and style are fine/minor spell check required
( ) I don't feel qualified to judge about the English language and style

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We improved the quality of our introduction by including all relevant references.
We improved the research design by adding new experiments, in particular those requested by reviewer #2 and reviewer #3
We improved the materials and methods section by adding more detailed explanation of the protocols used.
We revisited part of the results and conclusions in order to make them clearer.

Comments and Suggestions for Authors

Carlotta Penzo and colleagues reports the role of HMGA1 protein in modulating gene transcription by epigenetics of histone codes.

Minor:

1) Authors have cited multiple review articles. Some of the literature is not specific and citations need to some redoing on first paragraph of introduction sections.

We rewrote the first paragraph trying to refer general concepts with few and very relevant bibliographic references and putting more attention to the specificity of their use. In particular we introduced the review by Hanahan e Weinberg regarding the cancer hallmarks. The reason why we made use of reviews in this paragraph is because the text
illustrates general concepts that, in our opinion, are better and in a more complete manner referenced in a review with respect than a research article. We hope that reviewer #1 will agree with our opinion.

Moreover, in agreement with one of the concerns of Reviewer #3, we explained in a better way the first sentence of the paragraph.

2) Additionally, paragraph 2 lines 51-57 need specific citations

The reviewer is right and we added specific citations regarding HMGA1 and HMGA2 genes, as well as their causal role in cancer.

3) Line 61 needs rephrasing in context of disease specificity

The reviewer is right and we added some sentences in order to better explain the role of HMGA1 and HMGA2 towards p53 and pRB in the context of cancer cells.

4) Line 70 needs specific examples of recent work and cited literature.

The reviewer is right and we added some relevant examples of HMGA1 activity with respect to its involvement in breast cancer.

5) Fig.1 Figure can be divided in sub-pars A and B. Legends need modifications

Accordingly with the reviewer request, we divided figure 1 in panel A and B and, as a consequence, we modified the figure legend and the text.

6) Reference 31 should be replaced with original work

In accordance with the reviewer request we replaced reference 31 with the two original works.

7) Lines 139, 140 need reference

The reviewer raised this request and we realised not to have properly explained this point: the text "Since interphasic H3S10ph and H3S28ph are mediated by the RAS/RAF/MEKK/ERK pathway in MDA-MB-231 cells" refers to our own data" (see figure 2). Therefore we changed this sentence in order to make this point more clear.

Major:

1) It is noted that authors have not cited the original work from multiple laboratories. Authors have relied on review articles heavily. I would love to re-review this manuscript after authors cite and read original literature that has been used for forming hypothesis.

Accordingly with the reviewer request we replaced most of the review with the original articles.

In particular:
We simplified the initial part of the introduction and in this part of the text we used 4 reviews since general cancer and chromatin concepts were discussed.

We introduced the original works regarding HMGA genes and proteins and their role in cancer.

We introduced the original works regarding the impact of HMGA proteins in breast cancer.

We introduced the original works regarding histone H3 serine 10 phosphorylation by MSK1, RSK-2, IKK-alpha, and AURKB.

We introduced the original works regarding the context-dependent functions of CBP/p300.

We introduced the original works regarding the reduction of HMGA DNA-binding affinity by phosphorylation.

In summary, now we have used 4 reviews for general concepts in the very first part of the introduction, 1 review on the global network of HMGA proteins, 1 on the role of HMGA proteins in breast cancers, 1 on CBP/p300, 1 on the post-translational modifications of HMGA proteins, and 1 on the general role of HMGA in cancer.

2) Additionally, authors have relied heavily on si-RNA technique. Can some experiments be reproduced on stable HMGA1 knockout cells?

We have not developed a stable HMGA1 knockout cellular model for HMGA1 and therefore these experiments cannot be performed in a time frame compatible with the revision of this manuscript. In the past we used a MDA-MB-231 cellular model in which HMGA1 expression was lowered by means of the inducible expression of shRNA molecules, but this cellular model was very close to the silencing of HMGA1 by siRNA (shRNA and siRNA we used were based on the same functional sequences) and the results that we generally obtained using these two slightly different cellular models were almost equivalent (Pegoraro et al 2013, Pegoraro et al 2015, Resmini et al, 2017).

In the discussion, we added a sentence recognizing the limitation of our approach.
Reviewer #2

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Does the introduction provide sufficient background and include all relevant references? (x) ( ) ( ) ( )
Is the research design appropriate? ( ) (x) ( ) ( )
Are the methods adequately described? (x) ( ) ( ) ( )
Are the results clearly presented? ( ) (x) ( ) ( )
Are the conclusions supported by the results? ( ) ( ) (x) ( )

We improved the quality of our introduction by including all relevant references.
We improved the research design by adding new experiments, in particular those requested by reviewer #2 and reviewer #3
We improved the materials and methods section by adding more detailed explanation of the protocols used.
We revisited part of the results and conclusions in order to make them clearer.

Comments and Suggestions for Authors

The manuscript by Penzo et al represents a well-composed and well-presented manuscript exploring the role of HMG1A as modifier of chromatin accessibility and gene expression particularly in the previously reported HMG1A subset signature (Hsss) of genes downstream of RAS signalling.

Strengths of this paper include the diversity of targets, assay and approaches to illustrate the importance of several genes (HMG1A, RSK2) in altering either gene expression or overall histone PTM (serine phosphorylation and methylation) in the MDA231 breast cancer cell line.

Major weakness in this paper are the use of only one cell line, however this is acceptable for this particular journal. The authors convincingly illustrate the importance of each of their key molecules (RSK2 pathway and HMG1A) in modifying gene expression of similar genes (HSSS) attempting to establish the flow of the signalling network (as diagrammed in Figure 9), which would establish novel understandings previously unappreciated about these two molecules. However, the reviewer was confused in reconciling contradictions in
interpretation of the data that has confused the accuracy of the model they have proposed.

1) Of specific concern is in consistently and fairly interpreting the data. Figure 2C, they state that the impacts of siRNA against HMG1A DOES NOT significantly impact RSK2 (line 147). Yet in line 255 it is argue that HMG1A is necessary for RSK2 activities and that knockdown of HMG1A does impact RSK2 expression (line 272).

The reviewer is right in evidencing these discrepancies. We missed to discriminate in a better way between protein (western blot data) and mRNA (RT-PCR data). Indeed, what we found was that at the protein level RSK2 was not significantly downregulated (Fig. 2C and Fig. 8B), while at the mRNA level it turned out to be significantly down regulated (Fig. 8A). As a matter of fact, the protein expression level of RSK2 and its phosphorylated form did not change upon HMGA1 silencing and this data is the most important one considering the activity of RSK2 toward histone H3 serine 10. We changed the text in order to be clearer about this point.

2) While knocking down CBP phenocopies RSK2 and HMG1A, it is unclear, what impact if any knockdown of CBP/P300 have on RSK2 or HMG1A expression or if HMG1A or RSK2 knockdown or inhibition impact CBP activity/binding (authors do show HMG1A knockdown does not impact CBP expression - what about localization).

In order to clarify the interdependence of RSK2 and CBP we silenced both CBP and RSK2 evaluating the effects towards the gene expression of HMGA1, CBP, and RSK2 (Figure 7C). The silencing of both CBP and RSK2 did not affect HMGA1 gene expression level, and the silencing of RSK2 did not affect CBP gene expression. On the contrary, CBP silencing causes a decrease in RSK2 gene expression level. We inserted these data in Figure 7C within the paragraph "2.5. Histone H3S10ph and Histone H2BK5ac are Interdependent PTMs" and discussed them in the Discussion.

3) These stories require some connecting or clarifying thoughts or connecting data to validate the model they propose. As they authors have done an excellent job in eliminating concerns of MSK1/2 and P38MAPK, additional information needs to be provided other than phenocopying of gene expression in a limited set of genes to validate their model as the exclusive mechanism of action. While subject to the strength of interpretation of the observations depicted, their model mostly fits the data displayed, however are other models possible that also explain the data. If the proposed model is the strongest explain why, provide additional and clear details.

The reviewer is right in evidencing this point. We demonstrated that the silencing of HMGA1 causes a reduction of histone H3S10ph and histone H2BK5ac and proved that these two modifications are dependent on the activity of RSK2 and CBP/p300. It was previously demonstrated that both ERK1/2 and RSK2 were promoter-bound kinases and suggested that the positioning of kinases in such locations could allow them to target substrates not otherwise accessible (Lawrence et al 2008). CBP/p300 is a well known transcriptional coregulators, it is recruited onto chromatin through the interaction with transcription factors and is generally involved in the organization of a bridge between the promoter/enhancer bound factors and the general transcription factors (reviewed in Wang et al. 2013). We provided a model in which HMGA1 binds to nucleosomes and therein organizes the assembly of a complex that in turn could recruit RSK2 and CBP onto
chromatin. However, HMGA1 has been also demonstrated to be able to enhance the binding of transcription factor through direct DNA-binding mechanisms or via direct protein-protein contacts, such as for example NF-kB (ref) or SRF (ref). Therefore it could be possible that the mechanism that we depicted is not correct, or at least could only partially explain one HMGA1 mechanism of action. It is obvious that HMGA1 influences histone post-translational modifications, and this imply that the effect of HMGA1 is towards chromatin and therefore onto nucleosomes, but this does not imply necessary that HMGA1 acts exclusively via a mechanism involving its interaction with nucleosomes as depicted in the model we proposed. HMGA1 could simply allow the binding of transcription factor onto their own consensus sequence and thereafter they could be responsible for the recruitment of RSK2 and CBP/p300. Moreover, it was also demonstrated that HMGA1 is involved in perturbing DNA topology and influencing long-range enhancer transcriptional activity transcription by organizing DNA loops (Bagga 2000). It is therefore possible that HMGA1 could act directly onto nucleosomes, indirectly by facilitating the binding of other transcription factors, or through long-range topological mechanisms and that these three different modes of action occur concomitantly.

For these reasons we included in our model (Figure 9) other possibilities; all of them compatible with our data. We hope to have interpreted the reviewer concerns in the proper way.

Minor Comments:

1) Unlike other figures - figure 1 does not report how statistical evaluations were performed (what test to acquire p values). The reviewer prefers to see individual dots of experimental repeats rather than only bar graphs but present form is acceptable.

The reviewer was right in evidencing this point. Now the figure legend correctly reports the method for the evaluation of the statistical significance.

2) The strength of figure 8 at this position of the manuscript is not apparent to the reviewer. This is another methodology that draws a connection between RSK2 and the phosphorylation of histones. Might serve better occurring before CBP story.

As suggested by the reviewer, we moved figure 8 at the end of the RSK-2 story.

Overall a strong presentation of data from strong experiments. Some additional care in composition of the manuscript will more effectively and consistently communicate how the data fit their model, or what other models may explain their data or the gaps in their model not well explained by the presented data. I look forward to reading the revised manuscript and approving for publication.
Reviewer #3

Open Review
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Does the introduction provide sufficient background and include all relevant references?
( ) ( ) ( ) ( )

Is the research design appropriate?
( ) ( ) (x) ( )

Are the methods adequately described?
( ) ( ) (x) ( )

Are the results clearly presented?
( ) ( ) (x) ( )

Are the conclusions supported by the results?
( ) ( ) (x) ( )

We improved the quality of our introduction by including all relevant references.
We improved the research design by adding new experiments, in particular those requested by reviewer #2 and reviewer #3
We improved the materials and methods section by adding more detailed explanation of the protocols used.
We revisited part of the results and conclusions in order to make them clearer.

Comments and Suggestions for Authors

Title: HMGA1 Protein Affects Breast Cancer Cells Gene Transcription by Modulating the Histone Epigenetic Code

Manuscript ID: cancers-521015

Reviewer comments: The article describes a potential additional mechanism exploited by the High Mobility Group A1 (HMGA1) non-histone protein to exert its oncogenic effect in a breast cancer cell model.

The article is interesting but a little ambiguous. Is this HMGA1 effect a general mechanism in all cells, in all cancer cells, specific sub-sets of cancer cells, exclusive to TNBC, or all breast cancer cells. Recommend major revision if it is to be accepted for publication.

Comment 1: The title is ambiguous, ‘Affects’ should be specified, i.e. promote BC aggressiveness??? Or promote tumor-signalling pathways???
We agree with the comment regarding the title of our manuscript. We changed the title as follow: "HMGA1 Modulates Gene Transcription Sustaining a Tumor Signalling Pathway Acting on the Epigenetic Status of Triple Negative Breast Cancer Cells"

**Comment 2:** Abstract is too waffly, should be more succinct, direct and the point of the article.

We agree with the comment regarding the abstract of our manuscript. We provided a new version of the abstract in which we go straightforward to the results and the message provided by the article.

**Comment 3a:** Was the MDA-MB-468 a convenient cell line to use to demonstrate the mechanism of HMGA1 action because the idea of this being a mechanism of 'oncogenic effect' in TNBC seems to be secondary to the paper's overall study. The study is more focused on the mechanism of HMGA1 in one cell line (MDA-MB-231), which happens to be a TNBC cell line.

This manuscript is the last of a series of articles in which our laboratory investigated the role of HMGA in MDA-MB-231 cells and other TNBC cell lines (MDA-MB-157 and MDA-MB-468). By using this cellular model we were able to demonstrate that HMGA1 is involved in maintaining a mesenchymal phenotype and to confer aggressive and stemness traits to TNBC cells (Pegoraro et al 2013) and that part of this activity is performed by modulating a downstream mediator of the Hippo pathway, i.e. YAP (Pegoraro 2015). We were able to demonstrated that a set of HMGA1-dependent genes constitute a molecular signature with prognostic value in breast cancers (Maurizio et al. 2016) and HMGA1 expressing cells secreted glycosylated factors involved in modulating cell motility and invasiveness (Resmini et al. 2017). Very recently we demonstrated that HMGA1 is involved in modulating breast cancer cells nuclear stiffness trough a mechanism involving histone H1 (Senigagliesi et al. 2019) and that it drives also angiogenic properties acting in cooperation with FOXM1 (Zanin et al. 2019). Overall, HMGA1 exerts pleiotropic oncogenic effects in MDA-MB-231 cells, which have been often confirmed in other TNBC cells. In this work we continue this characterization of the oncogenic role of HMGA1 exploring an additional mechanism, i.e. the contribution of HMGA1 to the epigenetic status of cancer cells. We focused on MDA-MB-231 cells because they are triple negative and basal-like breast cancer cells and we previously showed, by performing a bioinformatic analysis of a primary breast cancer public microarray data collection (1881 different samples), that HMGA1 mRNA levels were higher in the basal-like than the HER2+, the luminal A and B, and normal-like subtypes (Pegoraro et al 2013). We are aware of the limitations of studies based mainly on cellular models and therefore we have added in the discussion a part of text in which we explain in a better way the context of this work and highlights its pro but also its limitations.

**Comment 3b:** MDA-MB-468 is mentioned in the methods and the discussion but there are no results using these cell lines?

The cell line MDA-MB-468 is mentioned in the discussion where we were discussing the possibility of using inhibitors against CBP/p300 to fight cancer cells and we mentioned the action of the CBP/p300 inhibitor L002 towards a MDA-MB-468 mouse xenograft model (Yang et al. 2013). The fact that MDA-MB-468 are mentioned in the material and methods section is due to the fact that in supplementary figure 1 we showed an experiment
demonstrating that the silencing of HMGA1 in MDA-MB-468 cells causes a decrease of Histone H3 S10 phosphorylation as well as in MDA-MB-231 and MDA-MB-157 cells.

Comment 3c: Only one experiment shown with MDA-MD-157, the majority of the results only show MDA-MB-468? The authors should provide the results from all three cell lines to be inclusive.

In order to be more inclusive, as suggested by the reviewer, we performed additional experiments using MDA-MB-157 cells. In particular we assessed the effect of the RSK2 inhibitor BI-D1870 with respect to cell proliferation (MTS assay) and invasiveness (transwell assay). Moreover, we assessed the effects of HMGA1, RSK2, and CBP silencing towards the expression of the HSSS by qRT-PCR.

Comment 3d: Following on from the general comment above - Is this a generic mechanism or is this mechanism exclusive to TNBC. It is very unclear if this is a unique mechanism to these cell lines or all breast cancers or even all cancers or normal cell lines. There is no information or comparison - the title is very specific to breast cancer - has the authors looked at any other cell lines. Is there any information in human breast tissue?

In the first part of our answer to comment #3, we introduced in the discussion a paragraph in which we explain in a better way the experimental context of this series of data and highlights their limitations. Unfortunately, there are not dataset containing expression data of histone post-translational modification in breast cancer patients and therefore it is not possible to correlate HMGA1 expression levels with specific epigenetic post-translational modifications affecting histones and/or other chromatin factors. For sure this kind of data would have strongly strengthen the impact of our data. However, we believe that the information provided by this article could constitute a strong base for an immunohistochemistry screening to find out a link/correlation between specific epigenetic post-translational modifications and HMGA1 expression. Given post-translational modification are carried out by enzymes, these represent more valuable targets for the exploration of drug-based therapies. We tried to explain this concept in a better way in the final part of the discussion.

Comment 4: Can the authors comment or discuss the mechanism described - exclusively affecting only one pathway (RAS) or is it a master regulator of other pathways?

The reviewer is right and this comment reconnects to a major concern of reviewer #2. We missed to discuss with a more wide view the results that we obtained. It seems quite obvious that HMGA1 could affect in the same way other aberrantly activated pathways in cancer cells. HMGA1 is usually expressed at very high level in cancer cells (up to a ratio 1/10 with histone H1). They displace histone H1, bind to DNA and chromatin (nucleosomes) and in this way they facilitate the assembly of DNA-bound macromolecular complexes, which are mainly, but not only, involved in transcriptional activation. This chromatin architectural activity could allow HMGA1 to participate in the delivery toward chromatin of signals from other oncogenic pathways. Indeed, RSK2 is not the only kinase able to phosphorylate histone H3 serine 10. MSK1 is known to be a downstream effector of the RAS pathway and to be recruited onto the chromatin as part of macromolecular complexes that, for instance include CBP/p300, NF-kB, and AP1, or ERK and PR (Ndlovu at al. 2009, Vicent et al. 2006). Moreover, MSK1 was shown to be activated also in a PKC dependent way. IKK-alpha is recruited onto chromatin by means of a NF-kb mediated mechanism and therein is involved in the phosphorylation of histone H3 serine 10.
IKK-alpha is known to be activated by a plethora of different stimuli (reviewed in Huang and Hung 2013). Aurora kinase B, is mainly involved in mitosis, but it has a role also in interphasic cells where, for instance it is recruited to promoter by a thyroid hormone (T3)-dependent mechanism (Tardáguila et al. 2011). In addition, it is worthwhile to evidence that RSK2 has a prominent role in the activation of the transcription factor YB-1 which is a main factor leading to the development of basal like cancer cells (Stratford et al. 2008, Stratford et al. 2012, Davies et al. 2014). Albeit in a different cellular model, i.e. in non-small-cell lung cancer (NSCLC) cells A549, it is intriguing that both HMGA1 and YB-1 have been demonstrated to bind to the promoter of cyclin D1 (HMGA1 at 1752 to -1622 and YB-1 at -1539 to -1114 from the transcription start site) and to be responsible for its transcriptional regulation (Zhao et al. 2018 and Harada et al. 2014). In breast cancer cells it was demonstrated that chromatin remodelling is upstream of YB-1 landing onto promoters (Davies et al. 2014). Albeit at a speculative level, HMGA1 could be one of the chromatin factors that cooperating with YB-1 in breast cancer onset and development.

We evidenced this aspect in the final part of the discussion.

Comment 5: The authors states ‘Finally, we show that these two effectors are involved in the gene expression regulation of factors involved in cancer aggressiveness.’ The wound healing assays are inconclusive and there is no functional endpoint shown to how this mechanism affects the cancer biology of the cell i.e. proliferation, or migration. Perhaps the authors should do some cell proliferation assays or simple counts or migration assays and some more convincing images of changes in morphology.

We agree with the reviewer comment and, as suggested, we performed additional experiments to demonstrate that BI-D1870 treatment affect cancer cell aggressiveness. We performed both a cell proliferation assay (MTS) and a transwell assay. Moreover, we assessed by qRT-PCR the expression of mesenchymal markers in order to verify the mesenchymal to epithelial transition. In addition, more convincing images of cell morphology were provided. In order to extend these data we provided cell proliferation, transwell migration and morphology data on MDA-MB-157 cells treated in the same way. These data were in accordance with those obtained in MDA-MB-231 cells (Supplementary figure 5).

Comment 6: Clarity of introduction…. The first sentence reads, "Most of the stimuli a cell is subjected to..."? Explain. Also throughout the manuscript past and present tenses are misused (the authors used present and past tenses randomly). For example, line 73 "We investigated...", line 75 "We provided...", line 78 "We show...". Same problem can be seen in the Materials and Methods section. The authors used "we suggested" in many occasions in the paper. A better expression should "The data suggested, or the results suggested".

As concern the clarity of the introduction we modified the first sentence trying to be more explicit.

We are sorry for the misuse of the past and present tenses throughout the manuscript. We carefully checked all the manuscript.

We used "the data suggested" or "the results suggested" in place of "we suggested".
Comment 7: ‘HMGA1 Expression Influences the Histone Code’ explain what you mean in this section. No reference to histone code in this section – need to link the concept to the explanation in the results.

We changed the text introducing a little explanation for the histone code and its reference.

Comment 8: Results are presented in the introduction, repeated in results (suggest remove).

We removed the text regarding the anticipation of results that was included in the introduction.

Comment 9: When abbreviating in the body of the text for the first time (excluding abstract) should be full name, i.e. line 51 HMGA.

We checked throughout the text for this kind of oversights

Comment 10: Figure 4a need clearer images. Wound healing assays are not convincing (4b). The visual results do not match the analyses (4b and c).

We almost remade figure 4, also in accordance with comment 5. We removed from figure 4 the 0.1 and 1.0 µM treatment leaving only the most evident one, i.e. the 10 µM. In figure 4C it is reported the closure percentage after 4 (images not shown) and 8 hours of BI-D1870 incubation. The more effect the inhibitor has towards the migration of cells, the less cells close the wound and therefore the two images shown at 0 and 8 hours should be very similar, on the contrary, control cells migrate more efficiently and therefore the wound should appear smaller after 8 hours. After 4 hours control cells close the 15% of the original wound and after 8 hours about the 30%. On the contrary, cell treated with the inhibitor close the wound more slowly, and indeed after 4 hours the wound is closed for less than 5% while after 8 hours it is close for about the 10%. Therefore, in our opinion the visual results match the analyses and they were supported by statistical evaluation.

Comment 11: Please explain in the figure legend or the methods - nuclear/cytoplasmic mask.

We added the explanation for nuclear/cytoplasmic mask in the methods. Briefly, we used the images obtained by Hoechst staining to identify nuclei. Once identified, this area was subtracted from the whole cell area to identify the cytoplasmic portion of the cell. The nuclear/cytoplasmic mask reported in figure 8C is the graphical evidence of this process. After this operation, it is possible to identify two different cellular regions, i.e. nucleus and cytoplasm and to assign the fluorescence intensity of RSK2 to the nuclear or cytoplasmic compartment.