Author’s response to reviews

Title: High class I HDAC activity and expression are associated with RelA/p65 activation in pancreatic cancer in vitro and in vivo.

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Author’s response to reviews: see over
Re: Manuscript MS: 3372166472584175

Dear Prof. Bucceri,

Thank you for reviewing our manuscript “High class I HDAC activity and expression are associated with RelA/p65 activation in pancreatic cancer in vitro and in vivo.” (MS: 3372166472584175). We found the reviewers’ suggestions very helpful to improve our manuscript and have performed an extensive revision of the manuscript according to the reviewers’ suggestions.

However, as an introduction to the comments on the reviewers’ suggestions we want to emphasize that our translational research study was designed and performed to investigate the in vivo correlation between HDAC expression and RelA/p65 location/function in a large cohort of human pancreatic carcinomas. The main purpose of our study was to characterize expression patterns of class I HDACs 1, 2 and 3 and to demonstrate a possible link between aberrant HDAC expression and increased nuclear translocation of RelA/p65 in vivo, which has not been demonstrated thus far. In this context, the cell culture part of the study was designed to gather some supportive pancreatic cancer in vitro data for our in vivo findings. We did not aim to clarify the detailed functional crosstalk between these proteins. This has already been tried with conflicting results in a plethora of studies and is clearly beyond the scope of this translational work. Therefore in our revision we focussed on an extensive rework of the translational findings. We decided not to follow all of the reviewer suggestions on additional in vitro experiments, since we believe that even if we would have done so, we by no means have the chance to finally elucidate all of the functional mechanisms implicated in this very complex interaction.

Keeping this in mind, please find below our reply to the reviewers’ comments point by point.
Reviewer #1:

Reviewer comment 1:
How do the authors explain the relationships of HDACS to NF-kB staining do not translate to the same relationship of HDAC staining to patient survival as observed between NF-kB and survival? In this regard, the authors appear to have used univariate methods for multiple comparisons, and it is not clear if the associations and conclusions drawn would be the same after appropriate use of multivariate methods. Have they done so? If not, inclusion of a statistician in the analysis and authorship could be helpful.

Reply: Since we did not find an impact (not even a trend) of HDAC isoform expression in univariate survival analysis we decided not to try for multivariate comparison. However, following the reviewer’s suggestion we did a probatory multivariate survival analysis (Cox regression) under inclusion of nuclear RelA/p65 positivity and HDAC expression groups as well as clinicopathological factors. In this analysis, only nodal stage and grade proved to be independent prognostic markers. As expected, HDAC expression itself had no impact on patient prognosis (p=0.739). All statistics in this work have been rechecked and approved by an experienced Biostatistician (Dr. rer. nat. Jan Budczies,) who is already one of the authors of this manuscript.

The second question raised by the reviewer is an important one. We believe that the differences in the impact on overall survival for HDAC and RelA/p65 might be due to the fact that obviously, based on our in vivo data, RelA/p65 is only partly regulated/activated by strong HDAC expression. This is also underlined by the fact that although we see a correlation between those parameters the correlation is not extremely strong (see new Table 1). This might explain the different impact on survival for both parameters. This has been added to the discussion section of the revised version of our manuscript.

Reviewer comment 2:
The apparent relationship between HDACS expression and NF-kB activation appears to be opposite the functional role of HDACs found in previous studies by several laboratories (ie Chen et al, Science 2001;293: 1653-7; EMBO J 2002; 21:6539-48). HDACs including HDAC3 found in the authors study have been reported to deacetylate RELA, decreasing its transactivating, DNA binding and nuclear localization. HDACIs have been reported to decrease this, favoring acetylation and increased NF-kB activation (Duan, Mol Cancer Ther, 2007:6:37-50). Is it possible the effects the authors see at relatively late time point of 72 hours reflect effects on histones versus RELA itself, or merely decreased relative protein or degradation of cells undergoing growth arrest or apoptosis after exposure to HDACIs? A time course at 12, 24, 48 and 72 hours with NF-kB luciferase reporter and westerns showing total and acetylation of nuclear p65 and an appropriate histone could elucidate functional and biochemical effects.

Reply: We agree with the reviewer, that our data is apparently in contrast to the results of some preceding functional studies. However, the effect of HDAC inhibition on RelA/p65 DNA binding activity is controversially discussed in the current literature. Chen et al. showed that deacetylation of RelA/p65 promotes binding between NF-xB and IkBα which leads to decreased nuclear import of the protein (Science 2001, 293:pp.1653-1657). In contrast, Kiernan et al. showed that class I HDACs, in particular HDAC3, are able to deacetylate RelA/p65 in cervical carcinoma cells (JBC 2003, 278:pp.2758-2766). Acetylation of RelA/p65 decreases IkBα mediated nuclear import of RelA/p65 by reducing its ability to bind κB-DNA in this study, in turn deacetylation increases nuclear RelA/p65 accumulation which is in line with our findings. The effects of HDAC inhibition
on RelA/p65 activity in pancreatic carcinoma cells, to our knowledge, has not been characterised so far. Thus, the discrepancy between the observations might be explained by the fact, that different cell culture models of various tumor entities and different HDAC inhibitors were used. We extended the discussion on this point on pages 15-16 of our manuscript.

To further elucidate the effects of HDIs on RelA/p65 activity in the course of time we performed our NF-κB Luciferase reporter assay after 12h, 24h, 48h and 72h of HDI treatment. We only observed minor effects at early time points concluding that HDI treatment periods shorter than 72h are not sufficient to decrease RelA/p65 DNA binding activity. In contrast, increased histone H3 acetylation is already present after 12h of HDI exposure (see Western Blot at new Figure 3). Based on these results we can’t make a statement whether the effect on RelA/p65 and phosphorylation of IκBα is a direct one or whether it is mediated by histone modifications. In our opinion, the characterisation of the underlying mechanism which leads to the observed effects is beyond the scope of this translational work and needs to be addressed in future functional studies. We discussed this in the revised version of our manuscript.

Furthermore, we followed the reviewers’ suggestion and added a Western Blot against β-Actin in figure 3 showing a constant total protein level over the time course to exclude that increased degradation of cells is responsible for the observed decrease in RelA/p65 activity.

**Reviewer comment 3:**
*Along this line, IHC of serial sections of same area within tumors with weak, partial or strong staining with HDACS and relationship to cytoplasmic versus nuclear RELA and a regression analysis of these from the series could help show such a relationship is dominant in tumors in situ.*

Reply: This is an important point. All staining were done on serial sections in the first place. We apologize for not mentioning this in the text and added this information in the revised version of our manuscript. In addition, to properly illustrate this, we exchanged figure 1 which now depicts two examples of the same tumor areas for HDAC1, HDAC2, HDAC3 and RelA/p65. In the new figure 1 the overlap between strong HDAC positivity and nuclear RelA/p65 staining can bee easily seen. In addition, we added a new Table 1 which represents, as suggested by the reviewer, a more in depth statistical analysis of the correlation of HDAC and RelA/p65 expression. In this Table we show the distribution of the raw RelA/p65 cytoplasmic and nuclear expression scores in dependence of HDAC1, HDAC2 and HDAC3 expression scores as well as in dependence of the respective grouped HDAC expression scores. Our new analysis shows that nuclear RelA/p65 as well as cytoplasmic (borderline significant) RelA/p65 positivity is linked to increased HDAC expression, which suggests at least in part a transcriptional regulation and not only a post transcriptional modification of RelA/p65 activity by HDACs. We added this information in the revised version of our manuscript.
**Reviewer #2:**

**Reviewer comment 1:**
*In Figure 1, the evidence for the correlated staining of HDACs and RelA was not convincing. In the present format, the staining is not good and so the evidence is very fragmented. The authors should provide double staining to indicate their coexistence in the same cell.*

Reply: In general, in our opinion, the quality of staining for all four proteins was exceptionally good. We have published several works in high ranked journals including review articles on expression analyses for these proteins in human tumors (Weichert et al. Clin Cancer Res. 2008 14:1669-77; Br J Cancer. 2008 98:604-10; Lancet Oncol. 2008; 9:139-48). The specificity of the antibodies used has been extensively evaluated in the mentioned publications. Therefore, we believe that our results on expression are in fact very reliable. However, we agree with the reviewer that the illustration of the staining and the presentation of the data were not optimal. Therefore, we followed the reviewer’s suggestion and exchanged the entire Fig.1. On the new figure corresponding areas on serial section of two tumors stained for HDAC1, HDAC2 and HDAC3 as well as for RelA/p65 are shown. We decided not to perform double staining due to the fact that we are comparing expression patterns of 4 proteins and not only two. Since we see the best correlations for grouped HDAC staining and RelA/p65 positivity, this means we would have to stain all 4 proteins at once, which is not possible by IHC but only by immunofluorescence. Immunofluorescence staining on paraffin embedded tissue is very unreliable. Therefore, we decided to stain serial sections, since by this method different tumor areas could be easily compared with respect to expression, as well (see new Figure 1).

**Reviewer comment 2:**
*For figure 2E, would the treatment of the cells with VPA or SAHA cause cell death? If it is the case, the decrease of p65 activity may due to the "side effect".*

Reply: We followed the reviewer’s suggestion and included a western blot against β-Actin for the cell suspensions used for the RelA/p65 activity assay at the time points 12h, 24h, 48h and 72 h in figure 3, showing that increased cell death is not responsible for the decrease in RelA/p65 activity.

**Reviewer comment 3:**
*In figure 2F, the authors should show the Western blot data of IκB and the acetylated NFκB.*

Reply: We followed the reviewer’s suggestion and included a western blot showing IκBα for time points 12h, 24h, 48h and 72 h in figure 3. We do not see any effects on total IκBα protein level after exposure to HDIs. Interestingly we could observe an effect of HDIs on IκBα phosphorylation, which depicts one possible mechanism by which RelA/p65 is sequestered in the cytoplasm. We also discussed this point in the revised version of our manuscript. Since, to our knowledge, functioning antibodies against acetylated NFκB are not commercially available, we were not able to perform this analysis. An immunoprecipitation with antibodies against acetylated lysine as used in other studies on this topic (Dai et al.; Mol. Cell. Biol. 25: 5429; 2005) is, in our opinion, beyond the scope of this translational research paper.
Reviewer comment 4:
The authors illustrated that there was a positive correlation between HDAC and p65 stainings; but what are the underlying regulatory mechanisms in linking up these two activity? Other studies reported that HDACi treatment increases the translocation and the activity of p65, by the process of acetylation. Would there be any conflicts between these observations?

Reply: As mentioned in the introductory section of this reply letter, our work focuses on the translational link between aberrant HDAC expression and increased RelA/p65 activity *in vivo*. In our study the cell culture part merely acts as a model to additionally confirm the relationship of HDACs and RelA/p65 *in vitro*. We clarified this in the revised version of our manuscript. The detailed process involved in activation and deactivation of these proteins can’t be elucidated completely within the scope of this work and remains to be done in future functional studies. In fact, some authors reported an increase in nuclear translocation and activation of RelA/p65 in response to HDI treatment (i.e. Chen et al). However, some HDAC isoforms, i.e. HDAC3, are known to deacetylate RelA/p65 promoting its ability to bind kB-DNA and decreasing nuclear export of RelA/p65 (Kiernan et al. JBC 2003). The underlying mechanism leading to this discrepancy is not clarified so far. Obviously, HDIs like SAHA, VPA and TSA may have different impact on RelA/p65 in different tumor entities (see also discussion part on pages 15-16 of the manuscript). We reworked the respective part in the discussion.

Reviewer comment 5:
The conclusion (page 14, line 8-9), the statement is in conflict with what the authors claimed in the abstract.

Reply: We followed the Reviewers suggestion and changed the statement in our manuscript.

Reviewer #3:

Reviewer comment 1:
Previous studies by Miyake et al. (Pancreas 2008) has shown HDAC1 as a prognostic marker in pancreatic cancer, but the authors fail to observe similar effect in their tissues.

Reply: The reviewer is right that our data are somewhat in contrast to the findings of Miyake et al. In fact, before starting our study we expected to find a correlation between HDAC expression and survival based on our experiences from other tumor entities (Weichert et al Clin Cancer Res. 2008 14:1669-77; Br J Cancer. 2008 98:604-10; Lancet Oncol. 2008; 9:139-48). However, in pancreatic cancer we were not able to show such a correlation. We cannot exclude that in certain cohorts of pancreatic cancer (e.g. Japanese patients) such a correlation might exist, however, in our Western European cohort which is twice as large as Miyake’s cohort, we were not able to confirm his findings and therefore have to conclude that this observed correlation is not universally applicable. Since cutoff values for defining HDAC1 positive/negative cases in Miyake’s and our study were different, we repeated our analysis with the cutoffs used by Miyake. However, this did not result in a significant survival difference, either (p>0.05, data not shown). We discussed this in more detail in the revised version of our manuscript.
**Reviewer comment 2:**
The case for pursuing HDAC inhibition as a strategy because it inhibits NF-kappaB activity is not compelling. First, the authors claim that HDAC in addition to altering histones have been shown to deacetylate other non-histone proteins such as p53, NF-kB. Going by the literature, the acetylation of RelA is an important post-translational modification that marks it for phosphorylation and increased nuclear accumulation. If HDAC deacetylates RelA, the assumption is that it leads to inactivation. Thus treating cells with HDAC inhibitor should augment RelA activity, rather the authors show the opposite. So, the effect that they observe is indirect.

Reply: As mentioned above both, enhancement and inhibition of RelA/p65 activity by HDAC inhibitors has been published (Chen et. al., Science 2001 and Kiernan et. al. JBC 2003). The consequences of enhanced RelA/p65 acetylation might be dependent on the cell culture model and the HDI used in the experiments (see reply to comment 2, reviewer 1). However, the detailed regulatory mechanisms still remain undetermined (see also discussion on pages 15-16 of the revised manuscript).

**Reviewer comment 3:**
VPA or SAHA both inhibit most forms of HDAC, then why is the effect of VPA not statistically significant whereas SAHA did? This clearly implies HDAC inhibition alone is insufficient to reverse RelA activity.

Reply: This is a very important point. Most likely the effects of HDIs on RelA/p65 activity are dependent on the HDI concentration used. Furthermore, previous studies have shown that SAHA is more likely to act as a pan HDAC inhibitor whereas VPA preferentially inhibits class I HDACs (Xu WS et. al. Oncogene 2007; Göttlicher M et. al. Embo J 2001) The differences in the mode of action may have different impact on acetylation and deacetylation of RelA/p65 and may be also responsible for the different effectiveness of the two HDIs in deactivating RelA/p65. We discussed this in more detail in the revised version of our manuscript.

**Reviewer comment 4:**
Page 3, line 6 change "aminoterminal" to "amino terminal".

Reply: We corrected this.

**Reviewer comment 5:**
Figure 2B, a bar graph comparing the %nuclear versus cytoplasmic RelA may be needed. As is, only three cells show cytoplasmic retention of RelA in VPA treated cells compared to majority that are in the nucleus. The authors should also consider adding a DAPI stain to clearly show nuclear staining.

Reply: The reviewer is right, that such an analysis might be useful to underscore the functional relevance of nuclear RelA/p65 translocation. However, in our opinion, the results of the extended transcription factor assay analyses (see above), which proved such a functional relevance, are quantitative and clearly more reliable than a pure phenomenologic observational result obtained by immunofluorescence. The immunofluorescence in our manuscript merely served as an additional tool to depict these changes. We added a sentence in the revised version of our manuscript, that clarifies the pure qualitative nature of this experiment.
Please find enclosed a thoroughly revised version of our manuscript with extensive changes in the Results and the Discussion. In addition, we replaced the original Figures and added several References. The changes made are marked in blue in the revised version of our manuscript. We hope that these changes to the manuscript are satisfactory and render our manuscript acceptable for publication in BMC Cancer.

Sincerely yours,

Annika Lehmann and Wilko Weichert