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Interplay between Homeobox proteins and Polycomb repressive complexes in p16INK4a regulation

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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.)

Editor: Thomas Schwarz-Romond

1st Editorial Decision 11 April 2012

I now received scientific input on your manuscript, also in light of the associated communication from a distinct title. As you will recognize, both scientists find the conceptual thrust of the paper of potential interest for The EMBO Journal. However, there are currently strong technical concerns with regard to the appropriateness and presentation of some techniques employed. Specifically, the majority of ChIP experiments rely on overexpressed factors instead of analyzing endogenous components. Similarly, the PLA-assays would need strong endorsement by (preferably endogenous) IP's for the key interactions of the paper. Efficiency of knockdowns would have to be controlled again at the level of endogenous factors and experimental replicates clearly labeled (technical versus biological) with the need to provide sufficient numbers of biological ones to bolster the conclusions. Though some of these might be addressed by reorganization and re-write of the existing data, there also appears the demand for significant further experimental work before the paper would be ready to pass the quality- and robustness check for The EMBO Journal.

If you were willing and able to expense the necessary time and experimental resources, I would be ready to present a thoroughly re-drafted manuscript for a second assessment to our referees.
Please do not hesitate to get in touch in case of further questions (preferably via E-mail). Please also inform me on the experimental progress OR in case you decide to seek more rapid publication in a less demanding title.

I hope that specifying our expectations might facilitate efficient proceedings and look forward to hearing from you in the near future.

I do have to formerly remind you that The EMBO Journal considers only one round of major revisions with the ultimate decision solely depend on content and strength of the final manuscript.

REFEREE REPORTS:

Referee #1:

Popov et al. "Interplay between Homeobox proteins and Polycomb repressive complexes in p16INK4a regulation".

This is a potentially interesting story about transcriptional control of very important genes from the CDKN2A and CDKN2B loci. It appears that the authors have been putting more and more data into this manuscript in an attempt to appease reviewers. In order for this to be considered at the EMBO Journal I would encourage the authors to take a step back from whatever trauma they've suffered at G&D and think about this story anew. They need to re-write this manuscript, thinking carefully about what their key points are and to provide robust, well-controlled data that support their arguments.

For example I notice that in Figure 1, F and H they show approximately 50% of IMR-90 cells show BrdU incorporation, whereas in panel G the vector only shows about 18%, and in panel E it's 30%. Most of these data are presented without error bars. Given this degree of variation in cells transfected with vector, how significant are any of the changes seen when overexpressing or knocking things down? These kinds of experiments need to be carefully controlled and carefully presented.

Further, in response to a reviewer's request to show efficiency of knockdown by western blot, they overexpress HLX1 in 293 cells, then knock it down. The point of this control is to see what happens to the endogenous HLX1 levels in the cells being tested here, but this is not what the authors achieve with this rather bizarre choice of control.

I am skeptical that much of this data represents multiple biological replicates. Indeed, in response to a reviewer's comment the authors state that they'd performed biological duplicates, but chose to present technical reps in the figure. I would agree with the previous reviewer, that the data and error bars should represent biological replicates and the variation present therein. This is particularly important for those ChIP experiments in which the recovery is very low: 0.05% of Input can still be

important if the IgG is well below this, however this must include data from at least 3 biological replicates (and triplicate PCR). Finally, I also agree with the reviewer that displaying ChIP-PCR relative to H3 ChIP is not appropriate for transcription factors. I can see the reasoning for expressing histone mod ChIP this way, but it is not appropriate to express PRC2 or HLX1 ChIP this way. I would prefer % input for both sample and IgG, although in some cases displaying it as fold enrichment over IgG can also be acceptable.

Referee #2:

I have now read carefully both Gil's manuscript and the corresponding correspondence.

The topic is surely within the scope of the Journal (i.e. how PRC1 and PRC2 are recruited to target genes in mammalian cells using as model system the INK/ARF locus, with its implication in senescence and proliferation), as it is the approach of this study.

I - in part - agree with some of the "negative" comments of the referee from Gen&Dev. And thus before publication I would request to include the following experiments:

1. I would like to see ChIP assays in HLX1 knock-down cells. This would support also the specificity of the antibody.

2. I would also include the endogenous CoIP between HLX1 and PRC2/HDAC1 in the main manuscript.

3. Add western blot controls for fig. 4C

4. Would HoxA9 knock-down also affect HLX1 occupancy at INK4a locus?

1st Revision - authors' response 08 August 2012

Point by Point rebuttal to the Editor and Reviewer's comments.

Editor comments

I now received scientific input on your manuscript also in light of the associated communication from distinct title. As you will recognize, both scientists find the conceptual thrust of the paper of potential interest for The EMBO Journal.

We are really pleased that the merits and relevance of the manuscript for *The EMBO Journal* are recognized.

However, there are currently strong technical concerns with regard to the appropriateness and presentation of some techniques employed.

Specifically,

1. The majority of ChIP experiments rely on overexpressed factors instead of analyzing endogenous components.
We have revamped the ChIP experiments presented in the paper as requested by the Editor and the Reviewers. Although data relying on overexpression of HLX1 and identification of overexpressed HLX1 is presented in Fig 4D, these data is backed up with ChIP of endogenous HLX1 (Fig 4E), which is technically more challenging, due to the amount of primary cells needed. In addition, an improved ChIP experiment analyzing the effect of HLX1 knock down is also presented in Fig 7E.
To analyze the effect of HLX1 in senescence by ChIP, we just have two options: overexpress it or knock it down. It is technically easier to detect it once overexpressed of course. Nevertheless we have ChIP experiments in which we overexpress HLX1 (Fig 4D and 5E-F), where we look to endogenous HLX1 (Fig 4E), and in which we knockdown endogenous HLX1 (Fig 7F). Basically all the experiments concur in the same direction: HLX1 can be located at the *INK4/ARF* locus and recruit Polycomb (and HDAC1s).
As part of this revision we have re-plotted the ChIP as percentage bound versus input or percentage bound vs total H3 for Histone marks. We have added extra biological replicas to the experiments in which we did not have 3 replicas (Fig 4D, E and 5F), and added statistical analysis to the key ChIP experiments shown in Fig 4D, E and 5F. We have added the controls for IgG on the ChIP on Fig 4D,E, 5E,F and 7F.
Regarding the HOXA9 ChIP experiment (Fig 9C,E), these are performed with FLAG antibody in FLAG-HOXA9 cells (Fig 9C), what argues for the specificity even if HOXA9 is overexpressed. Statistical analysis has also been added to the HOXA9 ChIP experiment presented in Fig 9C.

2. Similarly, the PLA-assays would need strong endorsement by (preferably endogenous) IP's for the key interactions of the paper.
We have reorganized the interaction data and presented it in a new Fig 6. In addition to PLA data in different cell types either expressing one or both of the interaction partners (Fig S7), we are presenting PLA assays with **endogenous** proteins (Fig 6A) showing the interaction between HLX1 and PRC2 members and HLX1 and HDAC1. All the PLA experiments include quantification and multiple controls, such as no antibody or isotype control antibodies, or unspecific antibody controls, showing the specificity of the data.
In addition, in Fig 6B, C and S8, we are presenting co-IP data upon overexpression of

HLX1 and tagged versions of HDAC1 and PRC1 and PRC2 components, showing again that HLX1 associates with PRC2 and HDAC1 but not with PRC1.
Finally, we have added in Fig 6D co-IP between endogenous HLX1 and SUZ12. We are aware that the SUZ12 western is not ideal as there is some leakage in the lanes, but it is the best we have and we believe that it adds convincing and important information by showing the association of HLX1 and SUZ12 at the endogenous level. Unfortunately, the experiment is technically very challenging to perform due to expression levels and antibody quality.
We believe that overall, the data presented in Fig 6 and Sup Fig S7 and S8, convincingly demonstrate the interaction between HLX1 and PRC2 and HDAC1. In addition, these data is also consistent with our functional analysis and transcriptome analysis.

3. Efficiency of knockdowns would have to be controlled again at the level of endogenous factors.
We are now presenting data of knockdown experiments for HLX1 using 4 different siRNAs or shRNAs constructs. After a lot of technical difficulties due to the levels of HLX1 expression and absence of decent antibodies recognizing HLX1, we have found antibodies and conditions that work. We are now providing in Fig 2A, 2D and S3B with immunoblot evidence of HLX1 protein depletion with the hairpins used; the data is quantified in the respective figures. These data correlates with our previously presented evidence of KD of HLX1 by qRT-PCR or knockdown of overexpressed HLX1.
We believe that this evidence, together with the use of 4 different siRNAs or shRNAs prove beyond any doubt the specificity of the siRNAs used and the effects assigned to HLX1 depletion.

4. Experimental replicates clearly labeled (technical versus biological) with the need to provide sufficient numbers of biological ones to bolster the conclusions.
We have added a mention in the methods to the statistical analysis, stating that the experiments represent at least 3 biological replicas. As mentioned earlier, we have performed some additional biological replicas for several of the ChIP experiments and added statistical analysis for the key ChIP experiments to confirm the significance of the results.

Referee #1:

This is a potentially interesting story about transcriptional control of very important genes from the CDKN2A and CDKN2B loci. It appears that the authors have been putting more and more data into this manuscript in an attempt to appease reviewers. In order for this to be considered at the EMBO Journal I would encourage the authors to take a step back from whatever trauma they've suffered at G&D and think about this story anew. They need to re-write this manuscript, thinking carefully about what their key points are and to provide robust, well-controlled data that support their arguments.

We appreciate that the reviewer likes the story and we agree that a reorganization of the data it is crucial to present it in a more readable format. He/she should be pleased to learn that we are quickly recovering from the trauma. But I will still appreciate if he/she treats us gently. We have indeed reorganized the manuscript thoroughly and presented the key data to provide a consistent and robust story.

For example I notice that in Figure 1, F and H they show approximately 50% of IMR-90 cells show BrdU incorporation, whereas in panel G the vector only shows about 18%, and in panel E it's 30%. Most of these data are presented without error bars. Given this degree of variation in cells transfected with vector, how significant are any of the changes seen when overexpressing or knocking things down? These kinds of experiments need to be carefully controlled and carefully presented.

I agree with the reviewer that the data can lead to confusion, and may seems contradictory. However, regarding that specific example, the apparent contradictions can be mitigated with some explanations:

1. All the experiments are internally consistent and show differences between vector and shHLX1 in the same direction.
2. We perform a full set of controls that typically include Ras overexpression, shp16, shp53 and others to assess the quality of the experiments; all of these were not displayed due to presentation purposes and space limitation.
3. Importantly, the overall BrdU incorporation (between experiment and experiment) varies depending of the passage of the cells, and other technical issues, what can confound the comparison of the data directly from different experiments.

Although all the above explain the apparent contradictions, we agree with the reviewer that the reason for the contradiction are not obvious and can lead to confusion. To avoid this we have taken different actions:

- We have included the passage numbers in which the experiments were performed.
- For similar experiments, we present the data on equivalent passages so that it can be more easily cross-compared between figures. In the particular case that the reviewer mentioned, BrdU data on equivalent shRNA experiments is for example now shown in Fig 2C (left) and 2D right and S3B (centre right) and controls show a similar 40 % level. We hope that the improvements on the presentation and the reference to the passage numbers help to clarify this point.

Further, in response to a reviewer's request to show efficiency of knockdown by western blot, they overexpress HLX1 in 293 cells, then knock it down. The point of this control is to see what happens to the endogenous HLX1 levels in the cells being tested here, but this is not what the authors achieve with this rather bizarre

choice of control.
As suggested by the reviewer, immunoblots showing the effect of siRNA and shRNA vectors over knockdown of HLX1 are shown now in Fig 2A, 2D and S3B. This is consistent with the previous data showing the effect by qRT-PCR or over overexpressed HLX1.

I am skeptical that much of this data represents multiple biological replicates. Indeed, in response to a reviewer's comment the authors state that they'd performed biological duplicates, but chose to present technical reps in the figure. I would agree with the previous reviewer, that the data and error bars should represent biological replicates and the variation present therein. This is particularly important for those ChIP experiments in which the recovery is very low: 0.05% of Input can still be important if the IgG is well below this, however this must include data from at least 3 biological replicates (and triplicate PCR).
The data represents biological replicas. We have added a mention to this in the methods. We have performed additional biological replicas to some of the ChIP experiments in which there were not enough replicas on all of the points. Particularly, we have performed and added additional replicas for the ChIP experiments shown in Fig 4D, E and 7F, to which we have also added statistical analysis.

Finally, I also agree with the reviewer that displaying ChIP-PCR relative to H3 ChIP is not appropriate for transcription factors. I can see the reasoning for expressing histone mod ChIP this way, but it is not appropriate to express PRC2 or HLX1 ChIP this way. I would prefer % input for both sample and IgG, although in some cases displaying it as fold enrichment over IgG can also be acceptable.
As part of this revision we have re-plotted the ChIP as percentage bound versus input or percentage bound vs total H3 for Histone marks. We have added extra biological replicas to the experiments in which we did not have 3 replicas (Fig 4D, E and 5F), and added statistical analysis to the key ChIP experiments shown in Fig 4D, E and 5F. We have added the controls for IgG on the ChIP on Fig 4D,E, 5E,F and 7F. For the ChIP experiments with HOXA9 (Fig 9C,E), these are performed with FLAG antibody in FLAG-HOXA9 cells (Fig 9C), what argues for the specificity of the ChIP (as the signal is significant over the background observed in vector cells). Statistical analysis has been added to the experiment presented in Fig 9C. We thank the reviewer for his/her comments and hope that he/she agrees that this improves the presentation of the ChIP data.

Referee #2:

The topic is surely within the scope of the Journal (i.e. how PRC1 and PRC2 are recruited to target genes in mammalian cells using as model system the INK/ARF locus, with its implication in senescence and proliferation), as it is the approach of this study.
We appreciate the positive comments of the reviewer.

I - in part - agree with some of the "negative" comments of the referee from Gen&Dev. And thus before publication I would request to include the following experiments:

1. I would like to see ChIP assays in HLX1 knock-down cells. This would support also the specificity of the antibody.
Regarding the specificity of the HLX1 antibody and the hairpins, we have added data in Fig 2A, 2D and S3B showing by western blot that the antibodies specifically recognize HLX1, and that the hairpins are able to ablate the signal. As shown also in Fig 1A, overexpression of HLX1 increases the HLX1 signal. We have included attached as **Fig just for the reviewer**, a summary of the evidence showing the specificity of the antibody. Although we tried to show evidence of the decrease of the endogenous HLX1 ChIP signal upon shHLX1 knockdown, the experiment is very challenging as we need to accumulate huge numbers of cells to obtain enough chromatin to detect the endogenous HLX1. We have done that in Fig 4E, but given that cells infected or transfected with shHLX1 are arrested, the amount of cells that we would need to use to detect HLX1 in those conditions make the experiment extremely difficult and we have failed to accumulate enough chromatin to perform such a experiment.
However, we believe that the new evidence provided in Fig 2A, 2D and S3B showing the specificity of the shHLX1 hairpins and the antibody used, together with the accompanying data regarding the specificity of the antibody settles this beyond any doubt.

2. I would also include the endogenous CoIP between HLX1 and PRC2/HDAC1 in the main manuscript.
As mentioned in the comments to the Editor, we have revamped the data in Fig 6 and Fig S7 and S8 to strengthen the evidence for the interaction between HLX1 and PRC2/HDAC1. As part of this, we have included in Fig 6D evidence for the co-IP of endogenous HLX1 and Suz12.

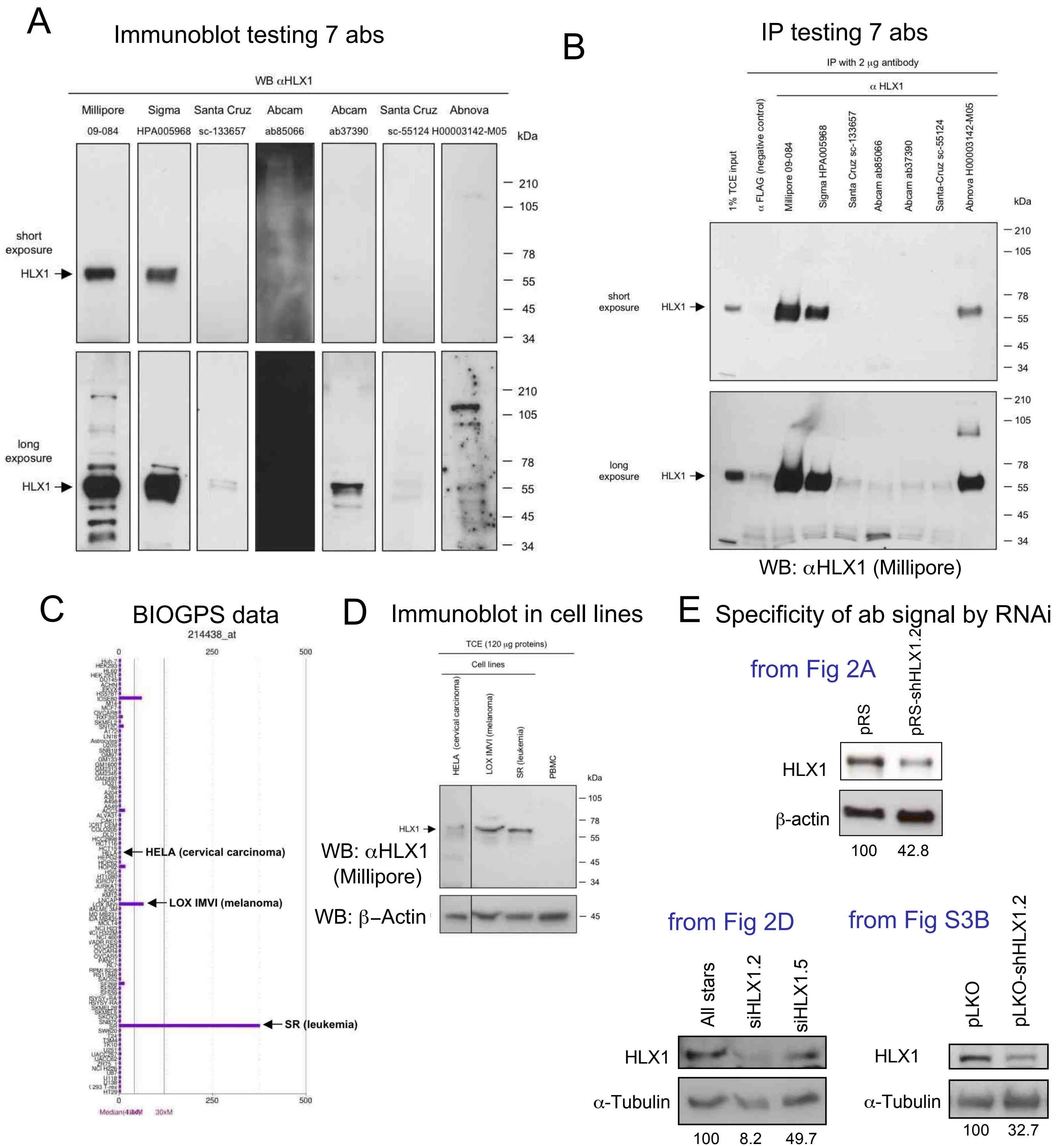
3. Add western blot controls for fig. 4C
The old Figure 4C is now presented in Fig 7A-D. I assume that the reviewer ask for western controls for the KD of Suz12 and HDAC1. We now present the evidence for the KD by quantitative immunofluorescence in Fig 7B and C. We include representative pictures and the quantification of positive cells. We believe that this should satisfy the request of the reviewer and contributes to more clearly and convincingly present the experiment described in Fig 7A-D.

4. Would HoxA9 knock-down also affect HLX1 occupancy at INK4a locus?
This is a very interesting question but think that it should be part of another study and it is beyond the scope of this manuscript. A major drawback to perform this experiment is again technical; to collect the amount of cells necessary to perform ChIP with endogenous HLX1 in conditions in which the cells would arrest (such as HOXA9

knockdown) is not a trivial matter.

Overall I hope that the revamped organization and the new data added satisfy to the reviewer.

Popov et al Figure for reviewers only



Specificity of the αHLX1 antibodies tested for these study. (A) Immunoblots showing the ability of 7 different commercially available antibodies to recognize HLX1 on extracts of HEK293T cells transfected with a HLX1 plasmid. The arrow indicates the expected HLX1 size. (B) 2 μgs of the indicated antibodies were used to IP HLX1 from 4.5 mg total cell extract prepared in RIPA buffer from HEK293T cells expressing HLX1. Membranes were blotted using the αHLX1 Millipore antibody. (C) The expression of HLX1 mRNA transcript in the NCI60 panel of cell lines was analyzed using data available at BIOGPS (www.biogps.org). (D) Based on this data, HeLa cells (cervical carcinoma, low expressers), LOX IMVI (melanoma, high expressers) and SR (leukemia, high expressers) cell extracts were prepared, together with that of peripheral blood mononuclear cells (PMBCs). The extracts were blotted with the αHLX1 Millipore antibody and a specific band of the expected size for HLX1 was observed and the level of this protein was higher in LOX IMVI and SR cells than in HeLa cells as suggested from the BIOGPS mRNA data. (E) Confirmation of the specificity of the Millipore antibody using RNAi. Independent experiments, using 4 different RNAi sequences show the specificity of the HLX1 Millipore antibody as the signal corresponding to the presumed HLX1 band decreases or disappears in the presence of RNAi targeting HLX1 (these data is extracted from the paper).

2nd Editorial Decision 26 August 2012

Thank you once more for submitting a revised version of your paper that has now been assessed by the two original referees. You will recognize that at least one of them appreciates significant further efforts and new experimentation you provided.

However, and I am really sorry to have to be the bearer of bad news here, as both remain unconvinced that in the absence of definitive formal proof at the endogenous ChIP-level the proposed role of Hlx1, (respective other homeobox factors) as mediator of PRC/HDAC function at the p16 locus has sufficiently been established.

We discussed their remarks and indeed the full paper once more in the context of the entire editorial team. One suggestion that came up was indeed to possibly shift to stronger functional support to make these points, for instance exploring genetically deficient material from flies that do also present with reduced Hox-factor complexity/less redundancy?

Please also accept my sincere apologies for some rather harsh remarks from ref#1.

I still hope that the requested revisions at least improved quality and clarity of the dataset and might thus ease rapid proceedings elsewhere.

Once more, I am sorry that I have no other choice than to transmit a negative decision at this stage in the process.

REFEREE REPORTS:

Referee #1:

Identifying proteins that help control senescence, and understanding how they work, is important. These authors have shown that HLX1 overexpression results in an extended lifespan of human cell lines. To work out the molecular mechanism behind this, the authors perform SILAC to identify differentially expressed proteins. At this point the authors find one protein that looks interesting: p16INK4a, and decide to construct an argument that HLX1 directly regulates the expression of this protein by recruitment of PRC2 and perhaps HDAC1. There is no attempt made to show to what extent control of p16INK4a expression is responsible for the phenotypes observed.

If this were a paper exploring the mechanisms of how HLX1 regulates senescence in general, with the identification of interesting targets and pathways, and a clear demonstration that it is the misregulation of these targets and/or pathways by HLX1 that results in the phenotype, then I would find it very interesting. But that's not what this manuscript is about.

There are plenty of different factors which have been shown to regulate the INK4A locus (including HOXA9, which this group showed previously), and here they find some more proteins that appear to bind and repress this locus in a few cancer cell lines. There is no attempt to show any in vivo relevance, or relevance in primary tumour lines. This doesn't feel like it's telling me some new insight into how PRCs function, but rather about how one gene might be under the influence of a number of different factors, most of which are already known, without much evidence about how regulation by these different factors is correlated.

As in the previous version I find the flow of the paper to be very confusing as I'm constantly having to flick back and forth between the main manuscript and the supp figs. For example the validation of altered expression of INK4a is referred to in the middle of page 6. They show one very minimal western in Figure 3B, but then also refer to S3C and S4B-C. They then go on to present some other experiments shown in Figures 3, S5 and S2. It would be much easier to read if they had constructed one Figure that shows, convincingly, that the INK4a protein is very responsive to the levels of

HLX1 so that the reader can look at it and be convinced, and not have to flick back and forth.

Figure 5: they do gene expression analysis on cells in which HLX1 is knocked down. The finding that HLX1 depletion caused upregulation of HDAC1 and PRC targets is really the same as saying that some genes went up. HDAC1 and PRC are used by the cell to keep genes down or off. so of course if some genes go up they'll be HDAC1 or PRC targets. While I don't doubt that these upregulated genes are normally associated with proteins that keep genes off, was this analysis done using PRC and HDAC1 targets in this same cell line? This would be the only way such a comparison would be legitimate.

If overexpression of HLX1 interferes with senescence, then it is reasonable to do ChIP for HLX1 in those overexpressing cells to find out where it's binding. If they want to argue that endogenous HLX1 is normally sitting on INK4a keeping it off, then they have to show this by ChIP for endogenous protein. In the end they show both, but both to the same locus. What is the point of showing the ChIP from overexpressed protein if they also show ChIP of the endogenous protein to the same locus (Figure 4D and E)? These two figures don't appear to be making distinct points. Similarly, the relevance of doing IPs between overexpressed proteins is questionable (Figure 6). If they want to see what overexpressed HLX is binding, then fair enough to use OE HLX1, but then probe for endogenous proteins. I don't find it helpful to then go and perform an IP in a completely different cell line (HeLa cells, Figure 6D).

p.9: "These results suggest that HLX1 recruits PRCs and HDAC1 to repress INK4a expression." I disagree that there is any demonstration of a direct link shown in this paper. They show that genes showing increased expression are associated with increased active marks and decreased silent marks. It is really not clear that these things are directly related to HLX1 or HOXA9. How soon after HLX1 binding does the INK4A show reduced expression? Can they link loss of HLX1 temporally with increased INK4A expression? Does PRC recruitment to INK4a require HLX1, or can they show that PRC or HDAC1 is recruited to the locus upon overexpression of HLX1 with the same kinetics?

Minor points:

They argue in response to a reviewer's comment that they cannot do ChIP in the HLX1 knockdown cells because they cells stop proliferating once HLX1 is knocked down. But this simply means that they should start with more cells.

Some explanation or at the very least a citation of 'senescence-associated fl-gal activity' should be given in the text, not just left in the methods.

p. 8, end of first paragraph, and Figure 5 legend: I have no idea what "anti-regulated" genes might be.

Figure 5D: the y-axis is labelled "mRNA Expression" but there is no indication in the text as to what is actually being displayed here.

Figure 6B. The EED IP blots are very poor. Surely FLAG IPs and westerns should be better than this. All of the IPs showing things that don't interact are in the supplement, but I would prefer to see a figure made which clearly shows the relevant proteins that do and do not interact here.

Strange to switch to the epithelial cell line at the end. Does this mean that the proteins they find in this section have no effect in the other cell line? Is this why they didn't find HOXA9 in this screen?

Figure 1C, 5D, 6A, 7B, 7C, 9D, S7, S9, S10: some of the data plotted as graphs are missing error bars.

Referee #2:

Although the topic of this manuscript is potentially interesting for the EMBO readers, the authors failed to provide key evidences to support their conclusions.
Without providing the proper controls (i.e. ChIPs in knocked-down cells), it is not clear that the signal obtained in ChIP assays is specific. There are plenty of cases when an antibody works in western-blots but not in ChIPs.

Also the quality of the endogenous CoIP is unacceptable for a journal such as EMBO J.
Moreover, the fact that several experiments cannot be performed "do to large amount of required cells" make me wonder not only on the relevance of these findings, but also whether other groups will be able to reproduce the experiments presented here.
As I mentioned in my previous email, this work was borderline for EMBO J few months ago, and after the revision, it remains at this level.

Appeal 29 August 2012

I have now shared the comments with the main authors in my lab and our key collaborators, Eric So included, and everyone agrees in a few key points that I will summarise here:

- Although nobody doubts the scientific stand of reviewer 1, his/her review is extremely unfair, and not adequate; he/she is not judging the rebuttal and the revised experiments but throwing in a rant that is inappropriate and should grant the dismissal of his/her opinion in this particular review as he is coming with novel demands in this review (e.g. asking for the extent to which p16 is responsible of the phenotype; we have that data and we could have added that if asked in a first instance), missing some key points on the paper (e.g. we do not identify 'some more proteins that appear to bind and repress this locus in a few cancer cell lines' but a role for HLX1 and other homeobox in repression of p16 in PRIMARY NORMAL cell lines, which is very different). In addition he/she makes some assumptions that are just not true (e.g. 'the finding that HLX1 depletion caused upregulation of HDAC1 and PRC targets is really the same as saying that some genes went up. HDAC1 and PRC are used by the cell to keep genes down or off. so of course if some genes go up they'll be HDAC1 or PRC targets'. This is not true, and indeed we compare the expression with the 'Bracken signature' that we extracted from primary fibroblasts, and compared with siCbx7, on the SAME CELL LINES). I juts mentioned three examples, I could go on and on rebutting many of the points that this reviewer raises and I am happy to have this conversation on the phone and summarize the rebuttal in written later.

-One of the key things that in general the authors appreciate from EMBO J, and that EMBO J itself is proud of, is the interaction and feedback with the editors to guide the revision process. In that spirit we have a very clear communication on what we could do and for example we got asked for you 'Similarly, the PLA-assays would need strong endorsement by (preferably endogenous) IP's for the key interactions of the paper'. In the spirit of the comment we presented the data that we have for the co-IPs and also for the endogenous co-IP giving you and the reviewers' choice to show it or not. We believe that the data all together proves more than convincingly the interactions and relation between HLX1 and PRC2.

-We believe that the other points made by reviewer 2 are arguable, and in fact we can provided now the ChIP for HLX1 in the shHLX1 cells that seem to be a major bone of contention.

3rd Editorial Decision 19 September 2012

Thank you for your recent correspondence. I understand your disappointment and, as indicated before, have been similarly surprised by the turns the comments on your revised manuscript have taken.

With the regard to the editorial process however, please allow me to remind you that these scientists were asked initially to more generally assess potential suitability of your results, at that time based on a still preliminary version and existing comments from earlier peer-review at an alternative title. Originally devised to accelerate further proceedings, I am not surprised that a new version is been considered much more critically, being closer to potential publication.

To solve the current situation and recognizing from your letter that you do have already substantial more data at hand that could be easily incorporated, I did consult first within the editorial team and solicited a third, independent opinion from an external scientist solely on the grounds of the scientific paper.

As you will see below, the general message of the paper is once again appreciated and judged to be of interest to The EMBO Journal. Furthermore, this expert suggests very sensible modifications that appear feasible within a decent timeframe and would, if successful, also overcome the reservations expressed from the initial referees.
Conditioned on addressing these points (some of them you already offered in your communication) as well as constructive integration of the recent Cancer Cell paper, I would be willing to expose a further improved version for ultimate assessment by this scientist.

I do kindly ask you to take this rare exception to further work on an already re-reviewed paper very seriously. I would thus appreciate a short message stating whether and in which timeframe you see yourself in a position to meet this demands.

REFEREE REPORTS:

Referee #3:

The authors describe an interesting set of data showing that the homeodomain protein HLX binds to the INK4A promoter and regulates the repression of this gene. Knockdown of HLX or expression of dominant negative forms of HLX, with mutants in the DNA binding homeodomain, increase INK4A expression and induce cellular senescence. Proximity ligation and CoIP experiments reveal an interaction between HLX and PRC2 (EED and SUZ12) and HDAC1 proteins (and possibly not RNF2). ChIP experiments indicate occupancy of these proteins as well as of H3K27me3 at promoter of INK4A, suggesting that HLX may be directly involved in targeting of PRC2 and HDAC1 complexes to the INK4A locus to mediate transcriptional repression.

To strengthen the later conclusion, the following experiments are proposed:
1: To show that HLX knockdown leads to reduced occupancy of this protein at the INK4A locus, ChIPs are needed in knockdown and control conditions (Figure 7E and 7F).

2: As a second, independent approach to show an involvement of HLX in targeting PRC2 to INK4A, the authors are recommended to use the homeodomain mutants (see Figure 1D) to show, upon overexpression, reduced occupancy of H3K27me3 and PcG proteins and supposedly a reduced HLX occupancy.
CoIP experiments would be needed to show that the homeodomain mutants still interact with PRC2 and HDAC1 protein (but would not be targeted to the INK4A domain).

3: To be able to assess the extent by which HLX would contribute to targeting of PRC2 complexes to the INK4A locus, the authors need to show (as a positive control) ChIP data for H3K27me3, SUZ12 and RING1B in knock down for another regulator, e.g. CBX7. Knockdown of CBX7 should lead to a clear reduction of RING1B (directly) as well as H3K27me3 (indirectly). These control experiments would demonstrate the sensitivity of the assay.

4: To assess the relative contribution of INK4A reactivation to the cellular senescence upon HLX knockdown, rescue experiments with INK4A need to be performed.

5: To test the hypothesis that various homeodomain proteins repress INK4A expression by targeting PRC2 complexes, it would be revealing to show that HOXA9 overexpression in HLX knockdown cells would rescue INK4A repression and H3K27me3 enrichment at the INK4A promoter.

2nd Revision - authors' response 15 January 2013

Point by Point answer to comments:

Referee #3

The authors describe ... ChIP experiments indicate occupancy of these proteins as well as of H3K27me3 at promoter of INK4A, suggesting that HLX may be directly involved in targeting of PRC2 and HDAC1 complexes to the INK4A locus to mediate transcriptional repression. To strengthen the later conclusion, the following experiments are proposed:

1: To show that HLX knockdown leads to reduced occupancy of this protein at the INK4A locus, ChIPs are needed in knockdown and control conditions (Figure 7E and 7F).

A new Figure 7E has been included showing the HLX1 occupancy of the *INK4a* promoter in this experiment. As expected, HLX1 is lost upon knockdown of HLX1 and this correlates with the reduced occupancy by PRC1 and PRC2 members and decreased levels of H3K27me3 as previously shown (Fig 7F).

2: As a second, independent approach to show an involvement of HLX in targeting PRC2 to INK4A, the authors are recommended to use the homeodomain mutants (see Figure 1D) to show, upon overexpression, reduced occupancy of H3K27me3 and PcG proteins and supposedly a reduced HLX occupancy. CoIP experiments would be needed to show that the homeodomain mutants still interact with PRC2 and HDAC1 protein (but would not be targeted to the INK4A domain).

This is a constructive and clever suggestion although to perform this experiment was not trivial and took us a long time. We have presented the results in Sup Fig S12. The HLX1 mutants retain their ability to interact with SUZ12 and HDAC1 as proven by co-IP (Fig S12A,B). We picked the T328M mutant for further experiments as it caused the strongest effects. The T328M mutant shows reduced binding *in vitro* to homeodomain binding sequences present in the INK4a promoter (Fig S12C). Expression of the T328M mutant results in induction of $p16^{INK4a}$ (Fig S12D), however the derepression is more modest than that observed upon HLX1 knockdown. Compared to HLX1 wt, overexpression of HLX1 T328M homeodomain mutant results in a reduced occupancy of the INK4a promoter by HLX1, H3K27me3 and PRC proteins (Fig S12E).

3: To be able to assess the extent by which HLX would contribute to targeting of PRC2 complexes to the INK4A locus, the authors need to show (as a positive control) ChIP data for H3K27me3, SUZ12 and RING1B in knock down for another regulator, e.g. CBX7. Knockdown of CBX7 should lead to a clear reduction of RING1B (directly) as well as H3K27me3 (indirectly). These control experiments would demonstrate the sensitivity of the assay.

We presented the experiment suggested by the reviewer in Fig S11. We knocked down the

expression of CBX7 in IMR90 cells, what resulted in INK4a induction (Fig S11A). We then observed by ChIP that levels of CBX7, other PRC proteins and H3K27me3 on the *INK4a* promoter were reduced upon CBX7 knockdown, proving the sensitivity of the assay (Fig S11B). We have previously published a similar experiment in Maertens et al. PLoS ONE 2009 vol. 4 (7) pp. e6380.

4: To assess the relative contribution of INK4A reactivation to the cellular senescence upon HLX knockdown, rescue experiments with INK4A need to be performed.

To address this comment of the reviewer we performed two sets of experiments, presented in Fig 3G and Sup Fig S6. In Fig 3G, we infected IMR90 cells with a control vector (pRS) or a vector knocking down $p16^{INK4a}$ expression (shp16) and analyzed the effect that knocking down HLX1 has in both cells. While as we observed previously knocking down HLX1 expression causes a significant growth arrest of IMR90 cells, this arrest is not significant in cells with $p16^{INK4a}$ depleted, suggesting that $p16^{INK4a}$ is a key mediator of HLX1 effects. In addition, in the experiment presented in Sup Fig S6 we took advantage of the oncoviral protein E7, which degrades pocket proteins and therefore inhibits the $p16^{INK4a}$/RB pathway. The effect that knocking down HLX1 has over cell growth is significantly rescued by inhibiting the $p16^{INK4a}$/RB pathway. Although regulation of additional genes or mechanisms by HLX1 can contribute to the senescence phenotype, these results suggest that the $p16^{INK4a}$/RB pathway is a key mediator of the arrest induced by HLX1 loss.

5: To test the hypothesis that various homeodomain proteins repress INK4A expression by targeting PRC2 complexes, it would be revealing to show that HOXA9 overexpression in HLX knockdown cells would rescue INK4A repression and H3K27me3 enrichment at the INK4A promoter.

To address these comments, we have performed two experiments that we present in Sup Fig S15A. First as suggested by the reviewer we analyzed how HOXA9 expression affects to $p16^{INK4a}$ levels upon knockdown of HLX1 (Fig S15A left). While knocking down HLX1 in cells expressing HOXA9 resulted in higher levels of $p16^{INK4a}$ expression, these levels didn't reach those observed in control cells (cells not overexpressing HOXA9). We also performed the converse experiment, analyzing how HLX1 expression affects to $p16^{INK4a}$ levels upon knockdown of HOXA9 (Fig S15A right). We obtained similar results.

To perform the experiments shown in Fig S15A, we had to generate IMR-90 cells expressing HOXA9 (left) or HLX1 (right) and their respective empty vector controls and work with IMR-90 cells at passage 24-27 to observe a difference in $p16^{INK4a}$ expression. This meant that $p16^{INK4a}$ levels in the control cells were already elevated and as a consequence further knocking down HLX1 or HOXA9 does not result in a massive increase in $p16^{INK4a}$ levels as its expression is already high.

The results presented in Fig S15A could be taken as a proof in favor of redundancy in the regulation of INK4a by homeobox proteins: HOXA9 (HLX) overexpression in HLX (HOXA9) knock down cells rescues INK4a repression. However, we preferred not to jump into any conclusions in the paper and leave options open as other interpretations might be possible and further work will be needed to clarify this. In particular we have added data proving that homeobox proteins can heterodimerize (we have evidence that HLX1 and HOXA9 heterodimerize, Fig S15B, C) what could suggest that more complex scenarios than mere redundancy need to be examined.

Editor comments.

The editor also asked for a 'constructive integration of the recent Cancer Cell paper'.

We have integrated our data with the recent Kawahara et al. paper (Cancer Cell, 2012 vol. 22 (2) pp. 194-208). We believe that in view of HLX1 being highlighted as a potent oncogene, our study

supports HLX1 role with mechanistic insights of its ability to interplay with Polycomb complexes and regulate senescence. More specifically, a point that is mentioned in the Sup information of the Cancer Cell paper is that BMI1 and HDAC signatures are associated with HLX1 knockdown. We reanalyzed the microarray data present in the Cancer Cell manuscript (arrays in mouse cells with HLX1 knockdown) and in addition to the mentioned signatures, we observed that the expression of EZH2 and HDAC1 regulated genes is associated to HLX1. We have mentioned this in our manuscript and included a selection of these GSEA signatures in Fig S7E. This provides additional confirmation of our observations of a direct relation between HLX, PRCs and HDAC1 using different cells and datasets. In addition, we have added a paragraph discussing how our study relates to the Kawahara et al paper: 'Interestingly, a recent study identified HLX1 as a gene frequently overexpressed pin patients with acute myeloid leukemia (AML) that correlates with poor survival (Kawahara et al, 2012). Although the authors suggest regulation of PAK1 and BTG1 as mechanisms explaining the prevalence of HLX1 in AML, it is tempting to speculate that the ability of HLX1 to regulate senescence may be an additional trait contributing to its role in AML.'

4th Editorial Decision 01 February 2013

I am pleased to inform you that your paper has been positively evaluated from the critical referee (comments attached below) and the editorial office will soon be in touch regarding formal acceptance.

REFEREE REPORT:

The authors have performed all the experiments suggested by this reviewer. Their new findings support the former claim of HLX1 having a direct role in targeting PRC2 complexes to the INK4a locus and strengthen the manuscript. Concerning point 5, the explanation that the authors provide for their results is acceptable.

The manuscript is now suitable for publication in the EMBO J.