EMBO reports

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# Silencing of Proviruses in Embryonic Cells: Efficiency, Stability, and Chromatin Modifications

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**Review timeline:**

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| Submission date: | 10 May 2012 |
| Editorial Decision: | 04 June 2012 |
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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.)

1st Editorial Decision 04 June 2012

Thank you for the submission of your research manuscript to EMBO reports. We have now received reports from the three referees that were asked to evaluate your study, which can be found at the end of this email. As you will see, although all the referees find the topic of interest and referees 1 and 3 are rather positive about the study, referee 2 raises a number of concerns, requests various technical improvements of the data, and considers that further experiments are needed to provide convincing support for your claims. In addition, referees 2 and 3 note the limited novelty of the study. Upon further consultation with the referees, both 1 and 3 independently considered that the requests of referee 2 should be addressed.

On balance, I would like to give you the opportunity to revise your manuscript. If all the referee concerns can be adequately addressed, we would be happy to consider your manuscript for publication. Please note that it is EMBO reports policy to undergo one round of revision only and thus, acceptance of your study will depend on the outcome of the next, final round of peer-review. A successful revision would need to address or include the scrambled RNA and rescue controls for knockdown studies; the concerns regarding copy number; several inconsistencies noted between different results and/or time-points analyzed in the different experiments; strengthening the results presented in figure 3, as well as their presentation and explanation (as all referees agree this is the most novel part of the study); and examining H3K9me2 in figures 4 and 5.

In addition, please address all concerns regarding the statistical analysis of the data. Referee 2 mentions the issues with Fig 1C and Fig 3. I have also noted that error bars are provided in Fig 1C

when only one representative experiment is shown, and in Fig. 4a when only two experiments have been performed. Please note that such analysis can only be performed from at least three independent experiments (for guidance, please refer to: Cumming et al. JCB 2007). During revision, please ensure that all quantitative experiments are performed at least three independent times in order to provide an adequate statistical analysis and that the figure legends contain information regarding the number of independent experiments and the identity of the error bars.

The length of revised manuscripts must be a maximum of 30,000 characters (including spaces, figure legends and references), and thus you will need to shorten the main text while incorporating new discussion, as requested by the referees. Shortening may be made easier by combining the Results and Discussion into a single section, which we require, and which will help eliminate the redundancy that is inevitable when discussing the same experiments twice. In addition, although basic materials and methods essential to the understanding of the experiments must be described in the main body of the manuscript, more detailed explanations necessary to reproduce them may be presented as supplementary information. Please note that all information pertaining statistics must be retained in the figure legends. We can accommodate a maximum of 5 figures in the main text; to this effect, I would suggest to combine figures 1 and 2 into one, and perhaps also figures 4 and 5.

I look forward to seeing a revised form of your manuscript when it is ready. In the meantime, do not hesitate to get in touch with me if I can be of any assistance.

Yours sincerely,

Editor
EMBO Reports

REFEREE REPORTS:

Referee #1:

Review of Schlesinger et al.

This paper represents a very thorough study that suggests two distinct types of retroviral silencing in mouse embryonic cells. The first response, which is well documented, specifically and rapidly silences viruses that contain a proline tRNA binding site on the murine leukemia virus genome. The second response is less efficient and appears to act independently of the proline tRNA binding site.

Overall, the data strongly support the conclusions; the paper is very suitable for EMBO Reports. I only have some minor suggestions for the authors to consider when preparing a revised manuscript.

1) Pg. 9: I understand what the authors are trying to convey; however, I had to read the following sentence a number of times: "...only partially restrict the incoming GFP PBSproB2 virus (with 30% or 45% remaining GFP-negative...." Perhaps the authors can consider simplifying/editing this sentence to improve the clarity of the passage.

2) Pg. 10: It would be helpful if the authors refer to the color of the bars in Figure 4A and 4B directly in the text. It would make the text a bit more reader friendly. Also, the authors should comment on the controls (Aprt and Polrmt) directly in the text.

3) Pg. 11: The authors should comment further on the differences in the expression and ChIP results for MERV-L class III elements. The H3K9me3 and H3K27me3 results correlate nicely with class I/class II endogenous retroelement expression, but are not as strong for the tested class III retroelement. A few sentences elaborating on this result (in the Results and/or Discussion) would be a welcome addition to the paper.

Referee #2:

In this manuscript, retroviral restriction in ES cells is claimed to have two distinct types that are targeted quickly to the PBSpro or more slowly to other sites when PBS is mutated. When rare expressing cells are selected, they are silenced over time. Superinfection with PBSpro virus leads to silencing of a different reporter, but only partial silencing is seen with the mutant PBS. ChIP and DNA methylation studies show the expected epigenetic marks with no obvious distinctions between the two pathways. Overall, this work has limited novelty as it has been known for a very long time that PBSmutant MLV is silenced in ES cells, and the epigenetic marks examined here have already been associated with retroviral silencing. There is no new insight into the nonPBS sequences that are recognized nor the specific factors involved. In addition, there are concerns about controls/interpretations of some experiments and inconsistencies between experiments. The claim that the PBS pathway is fast and the nonPBS is slow, does not take into account that the PBS virus contains sites to recruit both pathways, while the nonPBS virus can only use the nonPBS pathway. To claim that the PBS pathway is fast, one would have to test it on its own in the absence of the other pathway, which is not possible at the present time. The data presented could be consistent with slow silencing by each pathway, but when present together they can interact to establish a more rapid silencing.

1. In Figure 1, the two vectors are tested in ES, F9 and NIH3T3 cells. There are several aspects of this experiment that are of concern. If the infectivity of this virus is 80% in NIH3T3 cells, why is the copy number only 0.25-0.55 in ES/F9 cells? This throws considerable doubt on all their copy number data, which should be normalized to a cell line that has been shown to contain a single copy provirus by Southern blot analysis. Since the proB2 virus is silenced after selection in later experiments and in F9 cells in Fig 1B, why isn't it silenced over time in ES cells in Fig1A? In the ZFP809 KD studies, there needs to be evidence presented about what level of knockdown has been attained and its specificity shown for this one factor, including a scrambled RNA control and a rescue experiment performed by expressing a KD resistant version of ZFP809. Why does the KD relieve silencing of the WT virus, but enhances silencing of ProB2? Since the methods say the KD shRNAi construct is delivered in a retrovirus vector, why isn't it silenced in ES cells? What ES cell line is used? Some flow data should be shown as examples for the GFP intensity. Although multiple experiments are mentioned in the figure legends, there are no error bars, except for Fig 1C but this one panel is claimed to be a representative experiment and not a summary of all 5 performed. Overall, these experiments are poorly executed. The interpretation that there is a PBS independent silencing mechanism is also not novel, as MSCV and other vectors with PBS mutations are already reported to be silenced in ES cells.

2. In Figure 2, sorting is performed prior to silencing. Silencing occurs by d4 for both WT and ProB2 in Fig 2A, but in Fig 2B WT is silenced by d4 and B2 is silenced by d10, so only B2 has delayed silencing. Again, the KD data is not shown and there is no rescue with a transgene so this data is incomplete. It can't be claimed that the KD is further delayed to d13, because no earlier silencing time points are shown. These experiments are not conclusive as presented, and again are not novel as extinction of virus expression over time is a well documented.

3. In Figure 3, the attempt to select for cells that escape silencing to superinfect with a virus expressing a different reporter is a novel approach. However, this experiment is poorly explained, especially Fig 3C where the mCherry virus is suddenly introduced with no preamble. The text claims that there are no differences in copy number, but Fig S5A shows that there is a 2 fold change. What does normalization to NIH3T3=100% mean? It is unclear why they examine silencing at d2 post superinfection. No data is shown in the previous figures for d2, and therefore we do not know whether silencing should be expected by this timepoint as d4 is the earliest used otherwise. Of most concern though is that 14d of neo selection led to loss of silencing but sorting for mCherry at d6 did not reproduce this effect. While the authors conclude that long term selection is required, there is no strong rationale to support this contention. To this reviewer, it appears to be a non-reproducible effect as there is no mention of the experimental n and a lack of error bars.

4. The epigenetic studies are of limited novelty. Given their previous focus on H3K9me2/HP1, why do they not examine these effects in Figs 4 and 5? The mCpG data in Fig 5 should be shown with dots illustrating each CpG and not just the %. Finally the experiments on the endogenous retrovirus

suggest that a small subset of selected cells may have relaxed retrovirus silencing to some degree, but the experiments provide no new mechanistic insights into what sequences are being recognized outside the PBS in the exogenous and endogenous retrovirus examined, nor about the distinct factors involved in these processes.

Referee #3:

Schlesinger and Goff (EMBOR-2012-36138-T)
In this manuscript, the authors describe two distinct types of ES/EC specific retrovirus silencing, one is against PBSpro-MLV and other is to non-PBSpro (variant PBSproB2)-MLV. PBSpro-MLV is quite efficiently silenced and non-PBSpro-MLV is less efficiently silenced. For silencing of PBSpro-MLV, PBSpro sequence is targeted by the specific zinc finger DNA binding protein, ZFP809 and ZFP809 recruits TRIM28/Kap-1/Tif1b. Then, TRIM28 recruits multiple transcriptional silencing molecules including ESET, HP1, NuRD complex and Suz12, some of which are probably redundantly involved in this silencing. For silencing of non-PBSpro-MLV, how this MLV genome is targeted for silencing is not known, but eventually similar silencing machineries are involved in. Using some clever experimental assay systems, the authors challenged to clarify the nature of two different MLV silencing mechanisms and regulation. Although the obtained results are not surprising much, some of the results are novel and additional piece of data for further understanding of regulation mechanism(s) of retrovirus/ERV silencing. Therefore, I'm supportive for publication of this manuscript in EMBO report. However, this reviewer had strong concern about the "two silencing mechanisms". The authors frequently use the term"PBS-independent". But, the reviewer guesses this should be "PBSpro-independent". Or, for the variant PBSproB2-MLV silencing, the variant PBS sequence is completely dispensable? If not, "PSB-independent" is not appropriate. In the same line of concern, the author's group has proposed that different (KRAB) zinc finger molecules recognize different types of PBS for the TRIM28 recruitment (for exp, Wolf et al, PNAS 2008). In the case of variant PBSproB2-MLV silencing, different ZFP other than ZFP809 is still possible for this recognition and recruitment of TRIM28. If so, above two silencing mechanisms are fundamentally not different, just utilizing different DNA recognition module of KRAB ZFPs. Therefore, the authors should include the different ZFP possibility and more carefully discuss about the multiple silencing mechanisms in ES/EC.

One minor point,
Fig, 3(c) figure legend should be explained more details. Although it is stated in the text that the mCherry-positive population is selected and then subjected for second infection, it is better to explain same thing in the legend.

1st Revision - authors' response 01 August 2012

Response to reviewers' comments:

Referee #1:

We appreciate the referee's enthusiastic support.
In response to his minor suggestions:

*1) Pg. 9: I understand what the authors are trying to convey; however, I had to read the following sentence a number of times: "...only partially restrict the incoming GFP PBSproB2 virus (with 30% or 45% remaining GFP-negative...." Perhaps the authors can consider simplifying/editing this sentence to improve the clarity of the passage.*

1) p. 9 (previous p. 9): Reworded for clarity.

*2) Pg. 10: It would be helpful if the authors refer to the color of the bars in Figure 4A and 4B directly in the text. It would make the text a bit more reader friendly. Also, the authors should comment on the controls (Aprt and Polrmt) directly in the text.*

2) p. 10: Bars in previous Figures 4A and B (now 3A and B) are now referenced directly in the text. We left mention of the ChIP control DNAs in the legend to save space.

*3) Pg. 11: The authors should comment further on the differences in the expression and ChIP results for MERV-L class III elements. The H3K9me3 and H3K27me3 results correlate nicely with class I/class II endogenous retroelement expression, but are not as strong for the tested class III retroelement. A few sentences elaborating on this result (in the Results and/or Discussion) would be a welcome addition to the paper.*

3) p. 11: We now say a little more about the data for the class III retroelements, which are indeed distinctive, though we are constrained by the character count.

Referee #2:

*Overall, this work has limited novelty as it has been known for a very long time that PBSmutant MLV is silenced in ES cells, and the epigenetic marks examined here have already been associated with retroviral silencing. There is no new insight into the nonPBS sequences that are recognized nor the specific factors involved.*

This reviewer worries about lack of novelty of our findings, arguing that the epigenetic marks examined here have already been associated with retroviral silencing. In fact chromatin marks have been studied mainly for the endogenous proviruses but not for newly introduced exogenous wild-type MuLV DNAs. Further, the main point is that we report here for the first time the timing of establishment and the stability of the silent state, and the consequences of the silent or active state on the response to superinfecting genomes introduced by a second infection.

*In addition, there are concerns about controls/interpretations of some experiments and inconsistencies between experiments. The claim that the PBS pathway is fast and the nonPBS is slow, does not take into account that the PBS virus contains sites to recruit both pathways, while the nonPBS virus can only use the nonPBS pathway. To claim that the PBS pathway is fast, one would have to test it on its own in the absence of the other pathway, which is not possible at the present time. The data presented could be consistent with slow silencing by each pathway, but when present together they can interact to establish a more rapid silencing.*

We agree that it is not possible to test the PBS pathway in the absence of the nonPBS pathway, and that the characterization of the PBS pathway is necessarily done in the presence of the background nonPBS pathway. Thus it is formally possible that the fast PBS pathway is only fast in concert with the slow pathway, but this has no bearing on the characterization of that pathway as fast, nor that it is a PBS-dependent pathway. We now make it clear in the text that it is acting in the context of the nonPBS pathway.

On the specific issues raised:

*If the infectivity of this virus is 80% in NIH3T3 cells, why is the copy number only 0.25-0.55 in ES/F9 cells? This throws*

*considerable doubt on all their copy number data, which should be normalized to a cell line that has been shown to contain a single copy provirus by Southern blot analysis.*

1. When performing infections in different cell lines, it is important to know that there are no major differences in the copy number, and that the differences in reporter readout are therefore correctly attributable to gene expression. Retroviral infections are performed at multiplicities of approximately 1 (meaning in the range of 0.5-2 or so) so as to introduce one provirus into as many cells as possible without generating many cells with multiple integrations. All the results presented here are not sensitive to minor changes in the multiplicity of less than two-fold. The key feature of the system is that these various cells all acquire remarkably similar DNA copy numbers when treated similarly, and yet can exhibit effects on expression that can be ten-fold or even hundred-fold. The values for the multiplicity and resulting DNA copy numbers being discussed by the reviewer here are not significant. We present all the DNA copy number data in supplementary Figures S2 and S5A, which shows that all the cells being tested have approximately equivalent DNA numbers. The values are normalized to a cloned cell line with one copy, so represent true copy numbers per cell genome.

*Since the proB2 virus is silenced after selection in later experiments and in F9 cells in Fig 1B, why isn't it silenced over time in ES cells in Fig1A?*

With respect to proB2 expression in Figures 1B and A, the proB2 virus is never totally silenced (this is the main point we would like to make) and the time course of its expression is always slowly declining; we don't consider the small variations in this timing to be significant. The error bars now help make this apparent.

*In the ZFP809 KD studies, there needs to be evidence presented about what level of knockdown has been attained and its specificity shown for this one factor, including a scrambled RNA control and a rescue experiment performed by expressing a KD resistant version of ZFP809.*

In the ZFP809 knockdown studies, we are repeating published knockdown work (Wolf D, Goff SP (2009) *Nature* **458:** 1201-1204) and to make our point we only required a level of RNAi function that is adequate to relieve repression. The level of knockdown of the mRNA as judged by QPCR is in fact about five fold (see supplementary Figure S1C). We now include the presentation of the scrambled RNAi as in Figure 1B. The result does not depend on the absolute specificity of the RNAi and so does not justify the engineering of an RNAi-resistant expression construct of ZFP809 to establish absolute specificity.

*Why does the KD relieve silencing of the WT virus, but enhances silencing of ProB2?*

The knockdown of ZFP809 clearly shows major relief of silencing of the WT virus, while the slight apparent increase of silencing of the ProB2 virus here is just noise in the system and not significant. The standard deviations shown in the error bars now make this clear.

*Since the methods say the KD shRNAi construct is delivered in a retrovirus vector, why isn't it silenced in ES cells?*

With respect to delivery of shRNA genes on retroviral vectors that could in principle silence their own expression, it is an interesting (and useful) aspect of the system that there is sufficient expression of the shRNAs from these vectors that we can successfully select for the expressing lines. We can be confident that the effects seen are correctly attributable to the specific shRNA and not to the selection for expression of the vector alone because the scrambled control shRNAs act like the uninfected control cells.

*What ES cell line is used?*

We use both E14 ES cell line and primary ES cells taken directly from the embryo ICM.

*Some flow data should be shown as examples for the GFP intensity.*

We initially left the flow plots of the GFP intensity out because of space constraints, but now include examples of these data in supplementary Figure S3C and S4B.

*Although multiple experiments are mentioned in the figure legends, there are no error bars, except for Fig 1C but this one panel is claimed to be a representative experiment and not a summary of all 5 performed.*

With respect to the statistics, we have performed independent repeats of all experiments three or more times and now include the error bars on the figures.

*Overall, these experiments are poorly executed.*

Obviously we disagree with the reviewer's thought that the experiments are poorly executed.

*The interpretation that there is a PBS independent silencing mechanism is also not novel, as MSCV and other vectors with PBS mutations are already reported to be silenced in ES cells.*

Our major point of the paper is not that there is a PBS-independent silencing mechanism. Rather, we report the distinctive kinetics of the silencing of the PBS-dependent and the PBS-independent silencing, the distinctive efficiencies of the two mechanisms, and the instability of the rare expression of cells derived after selection. We further report the linkage between exogenous and endogenous retroviral DNA silencing, and the presence of the epigenetic marks on these DNAs with and without selection for the expression. These are the novel findings.

*2. In Figure 2, sorting is performed prior to silencing. Silencing occurs by d4 for both WT and ProB2 in Fig 2A, but in Fig 2B WT is silenced by d4 and B2 is silenced by d10, so only B2 has delayed silencing. Again, the KD data is not shown and there is no rescue with a transgene so this data is incomplete. It can't be claimed that the KD is further delayed to d13, because no earlier silencing time points are shown. These experiments are not conclusive as presented, and again are not novel as extinction of virus expression over time is a well documented.*

2. Figure 2: The initial sorting is performed at a time postinfection (6 d) when the vast majority of the cells are silenced. In this experiment we are now following the kinetics of the silencing in the rare subpopulation that were selected as initially expressing – a novel set of data to our knowledge. The ZFP809 knockdown is as done previously, and is clearly successful since there is a large effect; we now include the level of knockdown as judged by QPCR in the supplementary Figure S1C. The major finding, not addressed by the reviewer, is that the knockdown profoundly affects the final extent of the silencing. With respect to the time course, we now have looked at earlier time points and show SDs on the graphs.

*3. In Figure 3, the attempt to select for cells that escape silencing to superinfect with a virus expressing a different reporter is a novel approach.*

3. We are pleased that the reviewer appreciates the novel aspect of these superinfection experiments.

*However, this experiment is poorly explained, especially Fig 3C where the mCherry virus is suddenly introduced with no preamble.*

We now try to explain these superinfection experiments more clearly and introduce the rationale for the mCherry virus.

*The text claims that there are no differences in copy number, but Fig S5A shows that there is a 2 fold change.*

Since the multiplicity of infection is approximately ~1, the copy number will be slightly less than 1, and then after selection the copy number will be greater than 1. But again, the point is that these are not significant changes in copy number, and the changes in expression are much larger than this.

*What does normalization to NIH3T3=100% mean?*

Normalization to NIH3T3 means that we give the numbers as GFP-positive F9 cells/GFP-positive NIH3T3 cells x100; this is to correct for variation in multiplicity with different viruses. The copy numbers are presented in Supplementary Figure S5A.

*It is unclear why they examine silencing at d2 post superinfection. No data is shown in the previous figures for d2, and therefore we do not know whether silencing should be expected by this timepoint as d4 is the earliest used otherwise.*

With respect to the choice of examining silencing at d2, we did previously show data for d2 in Figure 1A. Here we chose to show the data 2d after the mCherry infection. We have looked at later time points, but there was no significant difference.

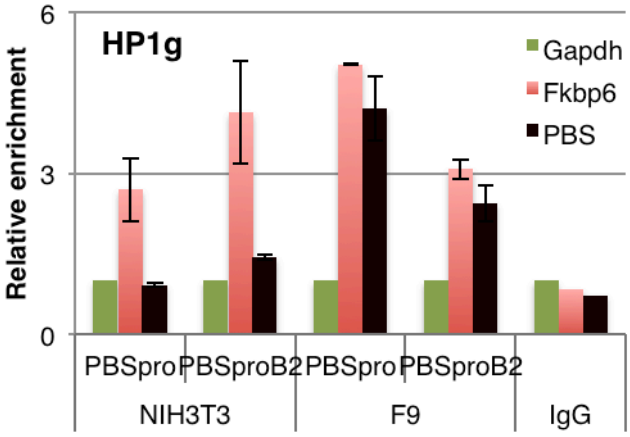
*Of most concern though is that 14d of neo selection led to loss of silencing but sorting for mCherry at d6 did not reproduce this effect. While the authors conclude that long term selection is required, there is no strong rationale to support this contention. To this reviewer, it appears to be a non-reproducible effect as there is no mention of the experimental n and a lack of error bars.*

Concerning the distinction between short term selection for expression (via sorting) vs. long term selection (via drug selection) we performed three independent repeats of these experiments and now include the error bars. The results are reproducible.

*4. The epigenetic studies are of limited novelty. Given their previous focus on H3K9me2/HP1, why do they not examine these effects in Figs 4 and 5? The mCpG data in Fig 5 should be shown with dots illustrating each CpG and not just the %. Finally the experiments on the endogenous retrovirus suggest that a small subset of selected cells may have relaxed retrovirus silencing to some degree, but the experiments provide no new mechanistic insights into what sequences are being recognized outside the PBS in the exogenous and endogenous retrovirus examined, nor about the distinct factors involved in these processes.*

4. Re: H3K9me2, we now add this ChIP for at least some of the experiments. We have in fact performed the HP1 ChIP experiments but simply do not have room to include it. We attach the results here for the reviewer to see – as expected, HP1 is enriched on the PBSpro virus, less so on the B2 virus, and not on the 3T3 cells (see below). We present the mCpG data with dots in supplementary Figure S6 as requested. While we do not know the basis for the shared response to both endogenous and exogenous in our selected cell populations, the link is interesting and is a subject of much study by many labs. Exploring this mechanism is beyond the scope of this paper.




Referee #3:

*Using some clever experimental assay systems, the authors challenged to clarify the nature of two different MLV silencing mechanisms and regulation. Although the obtained results are not surprising much, some of the results are novel an d additional piece of data for further understanding of regulation mechanism(s) of retrovirus/ERV silencing. Therefore, I'm supportive for publication of this manuscript in EMBO report.*

We were happy that this reviewer was supportive of publication.

*However, this reviewer had strong concern about the "two silencing mechanisms". The authors frequently use the term"PBS-independent". But, the reviewer guesses this should be "PBSpro-independent". Or, for the variant PBSproB2-MLV silencing, the variant PBS sequence is completely dispensable? If not, "PSB-independent" is not appropriate.*

He raises an issue of the terminology for the two silencing mechanisms. As noted, we should certainly refer to the PBS-targeted mechanism as “PBSpro-dependent” . We made this change throughout.

*In the same line of concern, the author's group has proposed that different (KRAB) zinc finger molecules recognize different types of PBS for the TRIM28 recruitment (for exp, Wolf et al, PNAS 2008). In the case of variant PBSproB2-MLV silencing, different ZFP other than ZFP809 is still possible for this recognition and recruitment of TRIM28. If so, above two silencing mechanisms are fundamentally not different, just utilizing different DNA recognition module of K RAB ZFPs. Therefore, the authors should include the different ZFP possibility and more carefully discuss about the multiple silencing mechanisms in ES/EC.*

We expect that the mutant PBS sequence in the PBSproB2 silencing is indeed dispensable – since any one of many variant PBSs seem to work this way. We now show both B2 and glutamine PBSs in the supplementary figures. We have also edited so we do not exclude the possibility that for some PBSs there may indeed be new ZFPs that recognize them.

*One minor point, Fig, 3(c) figure legend should be explained more details. Although it is stated in the text that the mCherry-positive population is selected and then subjected for second infection, it is better to explain same thing in the legend.*

With regard to the Figure 3C legend, we now try to explain the design of the experiment more clearly in the text.

In addition to the above changes, we have trimmed the paper to fit within the limits of the Report format. We hope you will consider that the revisions are responsive to the suggestions of the reviewers and that the draft will now be considered suitable for publication.

2nd Editorial Decision 08 October 2012

Thank you for your patience while we have reviewed your revised manuscript and please accept our apologies for the unusual delay in this process. As you will see from the reports below, the referees appreciate the efforts in the improvement of the study and acknowledge that their main technical concerns have been addressed. Although referee #2 still raises concerns about the limited novelty of the study, based on the fact that the other two referees do not agree on this point and are supportive of publication of your manuscript, we would be happy to accept your study in principle, under the condition that the following minor changes are addressed in the text for clarification.

Minor revisions:

1) Referee #1 suggests that you clarify the last paragraph on pg 8.
2) Referee #2 suggests that the abstract is modified to better reflect the novel aspects of the study including the superinfection studies as a novel experimental approach using the F9 cells and the fact that the last sentence should not refer to ES cells but EC cells (since Fig2-5 all use F9 cells). In addition he/she feels that the abstract and text should clearly state that the PBSPro dependent pathway is actually operating within the context of at least 3 other independent pathways.
3) Referee #2 has four other specific concerns, two of which refer to clarifications in the text, inversion of Figure S6E and S6F, and additional labeling of Y axis in the supplementary data.

If all remaining corrections have been attended to, you will then receive an official decision letter from the journal accepting your manuscript for publication in the next available issue of EMBO reports. This letter will also include details of the further steps you need to take for the prompt inclusion of your manuscript in our next available issue.

Thank you for your contribution to EMBO reports.

Yours sincerely,

Editor
EMBO Reports

REFEREE REPORTS:

Referee #1

The authors have adequately addressed my previous concerns. They may wish to clarify the last paragraph on pg. 8 to improve the text. For example, what does "expressing cells" refer to? Another noun would be great here (as well as a better topic sentence). Otherwise, I am happy with the paper.

Referee #2

The revisions by Schlesinger and Goff and their response to this reviewer's comments require careful consideration. The revised figures incorporating error bars, flow data, knock down controls and H3K9me2 ChIP are appreciated and help to explain some of the authors' rationale about what they consider novel and significant and what they consider to be background noise. It would have been helpful if revisions were highlighted in red text. Overall, this work has modest novelty as the efficiency of exogenous proviral silencing and associated chromatin modifications are well established in the literature. The finding that the stability of silencing in superinfected F9 cells is affected by the selection process is new, but there is no insight into the mechanisms involved and this is only correlated with upregulation of ERVs. To be of wide interest, it is important to determine why only certain types of selection have this effect and what chromatin modifying pathways are required for it to be active on both exogenous and endogenous proviruses. I will focus my comments on novelty and address some of the specific comments.

Comments on Novelty
The authors state in italics that:

In fact chromatin marks have been studied mainly for the endogenous proviruses but not for newly introduced exogenous wild-type MuLV DNAs.
I disagree. Chromatin marks have been extensively studied in ES cells on the silencing of exogenous virus in the papers by the authors (Wolf and Goff), and in papers on MSCV vectors that lack the PBSPro (Refs 12, 18, 19) that have very similar marks as those described here. It is logical that these MSCV modifications are also present on highly silenced wild-type MMLV proviral DNA as well. Figures 1A/B are not novel, as this PBS independent silencing is already known. Figures 1C/D show the ZFP809 KD data which is already published for the PBSPro and B2. The authors' response acknowledges there is no activity on B2 in the current paper. Therefore Figure 1 brings no novelty and should be deleted. The rest of Figures 2-5 in fact use only F9 EC cells and the ChIP data in these F9 cells largely replicates what is already known about MSCV in ES cells. Overall the ChIP data is confirmatory and has limited novelty.

Further, the main point is that we report here for the first time the timing of establishment and the stability of the silent state, and the consequences of the silent or active state on the response to superinfecting genomes introduced by a second infection.
The Abstract does not fully reflect what the authors themselves consider novel. I agree that they have studied "efficiency, stability and associated chromatin modifications" as stated in the title. However, there is no mention of the superinfection studies in the Abstract which I acknowledged as a novel experimental approach using the F9 cells. The efficiency and chromatin work is not novel as explained above. Also I disagree about the emphasis on timing/kinetics. The authors refer to a rapid PBS dependent pathway but further examination of their own data in Fig 1 shows that the PBS independent pathway is also silenced to its maximum level in ES cells by the earliest timepoint. This fits with the older MSCV data in ES cells from Ref 12. There is no difference in kinetics, the difference is in efficiency in ES cells. In F9 cells, there may be some prolonged silencing over time, but this is not seen in the ES data shown. Thus it appears the most accurate conclusion that summarizes both the ES and F9 data is that the PBS pathway is very efficient and the PBS independent pathway(s) is less efficient in ES cells. This is already known from MSCV and other vector studies. Also with respect to the Abstract, the last sentence should not refer to ES cells but rather to EC cells since Fig 2-5 all use F9 cells.

We agree that it is not possible to test the PBS pathway in the absence of the nonPBS pathway, and that the characterization of the PBS pathway is necessarily done in the presence of the background nonPBS pathway. Thus it is formally possible that the fast PBS pathway is only fast in concert with the slow pathway, but this has no bearing on the characterization of that pathway as fast, nor that it is a PBS dependent pathway. We now make it clear in the text that it is acting in the

context of the nonPBS pathway.
Given my above argument about kinetics, this issue should really be about efficiency not speed. The authors agree that the PBS dependent mechanism cannot be studied without the independent mechanisms. Since there is no difference in kinetics in ES cells, the simplest interpretation is that "the presence of two or more mechanisms is more efficient than having just one or more mechanisms". After all, there are at least 3 other silencer elements known to be present in the wild-type LTR (see ref 10 for review) and their activity is probably additive. This must be clearly stated in the abstract and text as the PBSPro dependent pathway is actually operating within the context of at least 3 other independent pathways. Efficiency remains confirmatory and not novel.

Our major point of the paper is not that there is a PBS-independent silencing mechanism. Rather, we report the distinctive kinetics of the silencing of the PBS-dependent and the PBS-independent silencing, the distinctive efficiencies of the two mechanisms, and the instability of the rare expression of cells derived after selection. We further report the linkage between exogenous and endogenous retroviral DNA silencing, and the presence of the epigenetic marks on these DNAs with and without selection for the expression. These are the novel findings.
I agree that PBS independent silencing is not novel and argue that Figure 1 does not provide new data because the kinetics are actually the same in ES cells, and the only difference is in efficiency which was already known. The instability of rare expression and the superinfection approach in F9 cells is new, but the mechanisms are not explained by the experiments conducted here. The finding that endogenous virus is reactivated after certain types of selection is new, but the mechanism by which only certain types of selection systems reactivate is unknown and should be confirmed in ES cells. The authors associate reactivation with chromatin marks but the causative mechanisms are not actually identified using knockout or knockdown ES cells. Therefore, there is no novel mechanistic insight into the selection effects or ERV reactivation reported here.

Specific comments

1. We use both E14 ES cell line and primary ES cells taken directly from the embryo ICM. Please add this to the text somewhere. This refers only to Fig 1.

2. Normalization to NIH3T3 means that we give the numbers as GFP-positive F9 cells/GFP-positive NIH3T3 cells x100; this is to correct for variation in multiplicity with different viruses. The copy numbers are presented in Supplementary Figure S5A.  Please add this to the text somewhere. Why is this normalization used here when all the other responses suggest that there is no significant difference in copy #/multiplicity of infection? It is still hard to imagine how the NIH3T3 cells have greater than 80% EGFP+ cells but a copy number of about 0.3, demonstrating that the copy number determination used here is very inaccurate.

3. Figures S6E and S6F seem to have been inverted. The closed and open circles should be defined in the figure legend, the lettering in this figure is unnecessary.

4. Y axis labels are missing in many graphs in the supplementary data (figS2B, S4A, S4C etc). In FigS5B, What does "relative expression" mean?

2nd Revision - authors' response 15 October 2012

We were pleased to hear the positive response to the revised draft of our manuscript on retroviral silencing. We have made final revisions to address the last remaining issues raised by the reviewers as detailed below.

1*) Referee #1 suggests that you clarify the last paragraph on pg 8.*

We now explain that the “expressing” cells are mCherry-positive cells in this paragraph.

*2) Referee #2 suggests that the abstract is modified to better reflect the novel aspects of the study including the superinfection studies as a novel experimental approach using the F9 cells and the fact that the last sentence should not refer to ES cells but EC cells (since Fig2-5 all use F9 cells).*

We have added back to the Abstract the description of the superinfection experiments (and deleted some words to fit within the limit) and fixed the last sentence.

*In addition he/she feels that the abstract and text should clearly state that the PBSPro dependent pathway is actually operating within the context of at least 3 other independent pathways.*

When we introduce the PBS-independent silencing in the introduction (pp. 3, 4), we now explain that this may include more than one mechanism.

*3) Referee #2 has four other specific concerns, two of which refer to clarifications in the text, inversion of Figure S6E and S6F, and additional labeling of Y axis in the supplementary data.*

*1. Please add the fact that both E14 ES cell line and primary ES cells were used to the text somewhere. This refers only to Fig 1.*

We have now added this to the Figure legend and the supplementary methods.

*2. Please add comments about… normalization… to the text somewhere. Why is this normalization used here when all the other responses suggest that there is no significant difference in copy #/multiplicity of infection? It is still hard to imagine how the NIH3T3 cells have greater than 80% EGFP+ cells but a copy number of about 0.3, demonstrating that the copy number determination used here is very inaccurate.*

We described this in the supplementary text, and it seems an appropriate place for it. With respect to the copy number, the number he is referring to come from several different experiments – one is an average of several experiments and the % EGFP is just one example. There is no issue about this variation.

*3. Figures S6E and S6F seem to have been inverted. The closed and open circles should be defined in the figure legend, the lettering in this figure is unnecessary.*

Corrected. But we kept the lettering, which we feel is helpful.

*4. Y axis labels are missing in many graphs in the supplementary data (figS2B, S4A, S4C etc).*

Fixed.

*In FigS5B, What does "relative expression" mean?*

This is explained in the legend to Fig. 5 and in the Methods.

We here upload the draft with these revisions. Again, we thank you for your attention to the paper.

3rd Editorial Decision 22 October 2012

I am very pleased to accept your manuscript for publication in the next available issue of EMBO reports. Thank you for your contribution to our journal.

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Yours sincerely,

Editor
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