THE
EMBO
JOURNAL

Manuscript EMBO-2012-84118

Intragenic epigenetic changes modulate NCAM alternative splicing in neuronal differentiation

Ignacio E Schor, Ana Fiszbein, Ezequiel Petrillo and Alberto R Kornblihtt

Corresponding author: Alberto Kornblihtt, University of Buenos Aires

Review timeline:

Submission date:	04 December 2012
Preliminary Decision:	05 February 2013
Editorial Decision:	06 February 2013
Revision received:	27 May 2013
Editorial Decision:	18 June 2013
Revision received:	26 June 2013
Accepted:	28 June 2013

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: Anne Nielsen

1st Editorial Decision   05 February 2013

Thank you for submitting your manuscript for consideration by the EMBO Journal and let me once again apologize for the unusually long reviewing period. We unfortunately experienced difficulties in securing suitable and available referees.

However, I would ask you to now please find enclosed the comments of two of the three reviewers whom we had asked to evaluate the manuscript. We are still waiting for a third report but given the present majority recommendation and to avoid any further delays, I will let you have a preliminary decision now. This decision is still subject to change though, should the third referee offer strong and convincing reasons for doing so.
As you will see, the two present respondents express great interest in your findings, and they would therefore ultimately support publication of the study in the EMBO Journal if you are willing and able to address their multiple criticisms in a revised version of the present manuscript. In particular, we would need you to extend the analysis of splicing pattern and histone methylation status to additional regions of the NCAM gene to support the specific effect of an altered chromatin structure on the inclusion of exon 18 as outlined by the referees.

I would therefore invite you to start thinking about making the requested changes and additions to the manuscript that would render the paper suitable for publication in the view of these two reviewers. We will forward the comments of the third referee to you as soon as we receive them, together with our final editorial decision.

Thank you for submitting your manuscript for consideration by the EMBO Journal. We have now received reports from all three referees as shown below.

As you will see, referee #3 expresses interest in your findings but also raises a number of points that you will need to address in the revised manuscript, especially regarding the direct causality between chromatin status and RNA polymerase speed. In addition, and in line with the other two referees, referee #3 asks you to provide additional statistical analysis and improve the data presentation to support the significance of the reported findings.

Given the referees' positive recommendations, I would like to officially invite you to submit a revised version of the manuscript, addressing the comments of all three reviewers. I should add that it is EMBO Journal policy to allow only a single round of revision, and acceptance or rejection of your manuscript will therefore depend on the completeness of your responses to the satisfaction of all three referees in this revised version.

When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process, please visit our website: http://www.nature.com/emboj/about/process.html

We generally allow three months as standard revision time. As a matter of policy, competing manuscripts published during this period will not negatively impact on our assessment of the conceptual advance presented by your study. However, we request that you contact the editor as soon as possible upon publication of any related work, to discuss how to proceed. Should you foresee a problem in meeting this three-month deadline, please let us know in advance and we may be able to grant an extension.

Thank you for the opportunity to consider your work for publication. I look forward to your revision and would like to to take this opportunity to once more apologize for the unusually long reviewing period for your manuscript.

REFEREE REPORTS

Referee #1

Alternative splicing is regulated by the action of a range of activator and repressor RNA binding proteins. However, in vivo RNA splicing takes place co-transcriptionally in a chromatin context. An increasing body of evidence, much of it emanating from the Kornblihtt lab, has indicated that transcriptional kinetics can influence alternative splicing outcomes and moreover, that chromatin states can also influence splicing by affecting Pol II kinetics. Exon 18 of the NCAM1 gene was previously shown by Kornblihtt's group to be skipped in response to K+ mediated membrane depolarization by a pathway involving histone acetylation and increased Pol II kinetics.
This manuscript addresses the mechanisms underlying increased exon 18 inclusion during neuronal differentiation. The key starting observations are that small molecule effectors of chromatin relaxation (TSA, 5-aza-C) are shown to decrease exon 18 inclusion, particularly in more differentiated cells, and that levels of H3K9 dimethylation throughout the NCAM1 gene body increase during differentiation. In parallel, the Pol II elongation rate decreases in the region of exon 18. Given concerns about multiple possible modes of action of 5-aza-C, a second inhibitor of the H3K9 methyltransferase (BIX) is used to show similar effects. Finally, siRNA-targeted chromatin modification in intron 18, previously shown by to induce repressive chromatin modifications when targeted internally, produced increased exon inclusion in undifferentiated cells, and this effect was dependent upon AGO1, HP1 and H3K9me2. This is an important observation, since it indicates that local modifications are probably sufficient to explain the change in exon 18 inclusion during differentiation.
Overall, this is an interesting piece of work providing significant new insights into the regulation of alternative splicing in a chromatin/co-transcriptional context, that should be of interest to the broad readership of EMBO Journal.

Comments

1. Fig 1A is the only place where the raw splicing data is actually shown. In all subsequent panels it is represented as a ratio of E18+/E18-, with the ratio in the control condition normalized to 1.0. This means that in most experiments while we can safely conclude that there are changes in splicing pattern, we have no idea over what range of exon inclusion these occur. It would be useful to provide additional data on the percent spliced in (PSI), at least for the reference condition, for all splicing experiments.

2. Figure 3 should ideally show data from more locations along the NCAM1 gene (minimally the two additional locations mentioned on page 11 as "data not shown").

3. Why does the exon 2 signal in Fig3a not recover to 100%? Minor point: y-axes in A and B should use fraction or %, but not a mixture of both.

4. Figure 4. Should show the effect of BIX on H3K9me2 in the NCAM gene (as in Fig 2B). Minor point: problem with labeling of inset in panel C "siH..."

5. Figure 5. Data should be shown for the effect of siRNAs against AGO1 and HP1alpha on their targets' expression.

6. Figure 5. Additional data should be provided indicating the changes in H3K9me2 at exon 18 and other locations.

7. Figure 5. Ideally, additional data should be provided for siRNAs targeted at other regions of the NCAM1 gene where no effect on exon 18 would be expected.

8. Supplementary Figure 4A. Why are data for probes A and H not shown?

Minor comments

9. p4. In addition to Carillo Oesterreich et al (2010), should probably also cite Alexander et al (2010) for connection between splicing and transcriptional pausing in budding yeast.

10. P5 - should also refer to the more recent paper on CD44 showing the involvement of argonaute proteins as well as HP1gamma in alternative splicing (Ameyar-Zazoua et al, Nat Struct Mol Biol 19, 998-1005, 2012).

11. P8. The screen of potential RNA binding protein regulators of exon 18 appears to be rather limited. Were only four tested?

12. P19 nCHiP methods: would be better to provide primer sequences and PCR conditions as supplementary rather than upon request.

13. There are numerous minor typographical and grammatical errors, as well as labeling errors in some of the figures (although some of these may have arisen during the pdf conversion process e.g. Fig 2B).

Referee #2

In the manuscript entitled "Intragenic epigenetic changes modulate NCAM alternative splicing upon neuronal differentiation", Schor and collaborators show that:

- neuron differentiation of the N2a neuroblastoma cells and differentiation of the embryonic P19 cells induce NCAM exon 18 inclusion.

- NCAM exon inclusion during differentiation (but not in undifferentiated cells) is partially reverted by 5-azacytidine treatment and by TSA treatment expected to induce histone acetylation and therefore chromatin relaxation. This suggests a role of chromatin environment in NCAM splicing regulation during differentiation.

- Further supporting this model, H3K9me2 in NCAM gene body increases during differentiation and this was inhibited by 5-azacytidine treatment. Similar results were obtained using the BIX01294 molecule, a specific inhibitor of EHMT2, the methyltransferase responsible for euchromatic H3K9me2.

- Similarly, H3K27me3 increases in NCAM gene body during differentiation. As H3K9me2 and H3K27me3 are repressive mark, this suggested that NCAM exon 18 inclusion result from slowing down RNA polymerase II, which was indeed observed during cell differentiation.

-Finally, transfection of cells with siRNAs targeting NCAM intron 18 and that were shown to induce H3K9me2 and H3K27me3 induces NCAM exon 18 inclusion.

Collectively these results support a model where an increase of repressive histone marks within the NCAM gene body during differentiation inhibits RNA pol II elongation speed, inducing exon 18 inclusion.

Major concerns:

1. Does TSA treatment affect histone acetylation and/or Pol II elongation? As the interplay between histone modifications is well documented, does TSA treatment affect H3K9me2 and/or H3K27me3?

2. Do similar histone modification changes occur during P19 cell differentiation?

3. As histone modifications occur from exon 2 and are minor around exon 18, the effect on splicing is not likely due to a local change of RNA pol II velocity. RNA pol II progression is more likely slowed down over the entire NCAM gene, and this globally slower RNA Pol II may induces exon 18 inclusion. Are other NCAM exons (upstream exon 18) alternatively spliced during differentiation? Is there any change during cell differentiation in RNA pol II density/pattern within NCAM gene body, in particular when comparing 5' end of NCAM gene to exon 18? What about RNA pol II phosphorylation status?

4. It would be nice to show that NCAM gene is within a more compacted chromatin environment in differentiated cells than in undifferentiated cells.

5. It must be shown that siI18as indeed affects H3K9me2 and/or H3K27me3 around NCAM exon 18.

6. Statistical analyses should be performed for all histograms.

Minor:
1. Fig1A, 1B, 1C, 4A, 4C and 5C histograms represent the "relative E18+/E18- ratio" (set up as 1 in control cells) rather than the "E18+/E18- ratio".

2. There is a problem with NCAM gene representation in Fig2B.

3. Figures 3 A and B do not have the same axis.

4. page 12: H3K9e2 should be replaced by H3K9me2

Referee #3

The manuscript authored by Schor et al describes experiments aimed at elucidating the connection between histone modification and alternative splicing at the NCAM gene in mouse neuroblastoma cells that can be induced to undergo differentiation. It is a follow-up to a previous study on the effects of induced neuron depolarization published a couple of years ago in PNAS (Schor et al., 2009). It follows a number of other publications aimed at establishing a cause-and-effect relationship between histone modification and alternative splicing, in this case via changes in RNA pol II transcription elongation speeds. The authors are right in their assertion that going beyond mere correlations is extremely important, but direct connections between, for example, histone methylation and slow elongation are clearly extremely hard to establish, and the present paper leaves this reviewer with the feeling that it has, yet again, not quite been achieved. Much of the problem lies in the lack of clear, strong effects. Often, the effects are two-fold or less, and the histone modification is not restricted to the area that is affected; it is a 'gene-global effect' with no real punch.

Specific comments:

1. Throughout the paper, the differences between undifferentiated and differentiated cells are fairly modest. It is therefore somewhat confusing when, for example, the authors claim that there is not significant difference between the results in Figure 2D, while the differences are deemed to be significant in Figure 4A.
2. The results in Figure 3 are crucial for the point the authors are trying to make, but the description and presentation is not ideal. First of all, the authors need to better describe why an intron-exon junction 200 kb into the gene was used as control. In the Singh and Padget paper, they always used

an exon-intron (or intron-exon) junction close to the promoter as control. However, in the present paper, the point is that there is little or no change at 200 kb (exon 2) but a big change at exon 18 (where is that, in kilobases?). This is not obvious from the description. Secondly, I think it would actually be helpful to show another 'unaffected' gene as control, just to show that cell differentiation in itself does not generally affect transcription speed at the end of genes.

3. I know the authors have previously described the phenomenon that DNA becomes 'heterochromatinized' in the DNA region corresponding to an mRNA recognition site for a siRNA. The precise mechanism remains to be resolved, but the results from the use of it here is arguably one of the most important argument for the authors model. Again, the effects are small (less than 2-fold). I do not think the characterization is sufficient. The model in A shows the heterochromatin as a 'road-block to Pol II', but no date to support this is provided. It must be possible to do nascent RNA analysis (BrU incorporation) to show polymerase speed over the regions in question, for example. This is generally true for many of the treatments used.

4. Figure 2B has not come through (the PDF conversion) well. It is impossible to read. There are no DNA size-indications in this figure (or Figure S4A, which looks much better). These are important to judge Figure 3.

5. The writing needs to be improved. There are several examples of poor editing and sub-optimal English.

6. The introduction is unnecessarily long. It can easily be cut to about 3 pages.

7. At the end of page 8, the authors conclude that "....upon differentiation the chromatin environment is changed in the NCAM gene, affecting E18 inclusion." However, at this point in the paper, the reviewers have not shown any data to support this assertion.

8. At the beginning of page 11, the authors write "The direct relationship between repressive marks and higher E18 inclusion...". Which 'direct relationship' is that?

1st Revision - authors' response   27 May 2013

Referee #1

Major comments
1. Fig 1A is the only place where the raw splicing data is actually shown. In all subsequent panels it is represented as a ratio of E18+/E18-, with the ratio in the control condition normalized to 1.0.
We have now added raw splicing data (native gels of radioactive RT-PCRs) to Figs. 1B and 1D.

It would be useful to provide additional data on the percent spliced in (PSI), at least for the reference condition, for all splicing experiments.
Due to big size of E18 (801 bp), the intensity of the inclusion band observed by radioactive RT-PCR with a single pair of primers that amplifies both isoforms, underestimates the real percentage of E18 inclusion. Except for Fig. 1 in which we used radioactive RT-PCR, in the rest of the figures we used Real time RT-PCR to assess the abundance of each isoform with different pairs of primers. Due to the nature of this procedure, we are certain about the fold change in splicing index in each treatment but we cannot assess the absolute abundance of each isoform. However, as we discussed in our previous paper (Schor et al., 2009), approximate absolute percentages can be estimated from independent determinations: using RNase protection assays (RPA) we estimated the basal inclusion percentage of E18 in undifferentiated N2a cells to be about 30%, and it increases to nearly 60% in differentiated cells. This is consistent with published results (Tacke and Goridis, Genes & Dev., 1991). So, this can give a hallmark to assess the magnitude of isoform changes.

2. Figure 3 should ideally show data from more locations along the NCAM1 gene (minimally the two additional locations mentioned on page 11 as "data not shown").
We have now included 8 amplicons in the elongation analysis by the Singh and Padgett method in Figure 3C.

3. Why does the exon 2 signal in Fig3a not recover to 100%?

As explained in the original Singh and Padgett, since we are analyzing amplicons spanning splice junctions, the pre-mRNA levels measured are the consequence of both their recovery due to transcription and their disappearance due to splicing and/or RNA degradation. This is now explained in the text.

Minor point: y-axes in A and B should use fraction or %, but not a mixture of both.

Thanks for pointing this out. The y-axes have been corrected to make them uniform.

4. Figure 4. Should show the effect of BIX on H3K9me2 in the NCAM gene (as in Fig 2B).

Experiments to satisfy this request were performed together with the effects of TSA on H3K9me2, requested by another Reviewer. The TSA experiment worked very well and is now shown in new Fig. S4. Unfortunately, while the ChIP in BIX-treated cells suggested that H3K9me2 was indeed reduced in the NCAM gene, the low DNA yields from the immunoprecipitates was insufficient to have accuracy about this result, mainly because no amplification was observed for the control amplicons. On the one hand we interpret that the BIX treatment is affecting the DNA yield, but we must also bear in mind that the epitope recognized by the antibody is a translational modification performed by the actual enzyme inhibited by BIX. In any case, although we failed with the ChIP of BIX-treated cells, we hope that the Reviewer will be in part satisfied by the fact that TSA downregulates the H3K9me2 mark (new Fig. S4), something that points at the same mechanism and that was not present in the first version of this manuscript. Also, the BIX specificity and effectiveness in reducing H3K9 methylation in vitro and in cells is well documented (for example Kubicek et al, Mol. Cell, 2007; Chang et al, Nat Struct Mol Biol., 2009).

Minor point: problem with labeling of inset in panel C "siH..."

Problem solved.

5. Figure 5. Data should be shown for the effect of siRNAs against AGO1 and HP1alpha on their targets' expression.

We show now the effects of siRNAs against AGO1 and HP1alpha on their corresponding mRNA levels at the bottom of Fig. 5C.

6. Figure 5. Additional data should be provided indicating the changes in H3K9me2 at exon 18 and other locations.

In order to satisfy this request we performed H3K9me2 ChIP experiments at different regions of the NCAM gene after transfection with control (siLuc) or the intronic siRNA. Result in new Fig. 5D clearly show that the intronic siRNA increases H3K9me2 levels at the E18 region (amplicons H and J) but not at E5 (amplicon C) or at the promoter (amplicon A). To complement the reviewer's request we have also measured Pol II elongation upon transfection of the intronic siRNA. These results are in new Fig. 3C. We thank the reviewer for this request because it allowed us to confirm, in a more robust way, the TGS-AS mechanism, originally described for fibronectin E33, in the NCAM gene.

7. Figure 5. Ideally, additional data should be provided for siRNAs targeted at other regions of the NCAM1 gene where no effect on exon 18 would be expected.

We have now included in Fig. 5C (lane 9) evidence for the lack of effect of siI17as, an intronic siRNA targeting a sequence in intron 17, on E18 alternative splicing. It should be noticed that, since this intronic siRNA will form a repressive chromatin environment upstream the alternative exon, the pol II pausing caused by it would not affect the splicing efficiency of E18.

8. Supplementary Figure 4A. Why are data for probes A and H not shown?

Former Supplementary Figure 4A is now Supplementary Figure 5A (i.e., Fig. S5). We performed a new ChIP to H3K27me3 using 8 amplicons.

Minor comments

6

9. p4. In addition to Carillo Oesterreich et al (2010), should probably also cite Alexander et al (2010) for connection between splicing and transcriptional pausing in budding yeast.
Done.

10. P5 - should also refer to the more recent paper on CD44 showing the involvement of argonaute proteins as well as HP1gamma in alternative splicing (Ameyar-Zazoua et al, Nat Struct Mol Biol 19, 998-1005, 2012).
Done.

11. P8. The screen of potential RNA binding protein regulators of exon 18 appears to be rather limited. Were only four tested?
We tried to focus on true E18 regulators, but clearly it is difficult to cover all of them since they are mostly unknown. On the other hand, we don't discard completely the possibility of a trans regulator-dependent effect, but present evidence that also there is a chromatin effect involved.
Nevertheless, to expand the analysis we test four more SR proteins for their effect on E18 by overexpressing them in N2a cells and co-transfecting with an E18 reporter minigen

As no effect was detected, we conclude that we couldn't find any other E18 splicing regulators than the ones mentioned in the test.

12. P9 nCHiP methods: would be better to provide primer sequences and PCR conditions as supplementary rather than upon request.
A new Supplementary Methods section with the sequences of all primers and siRNAs used in this study was added.

13. There are numerous minor typographical and grammatical errors, as well as labeling errors in some of the figures (although some of these may have arisen during the pdf conversion process e.g. Fig 2B).
We have thoroughly checked the spelling, the grammar and typos.

Referee #2

Major comments

1. Does TSA treatment affect histone acetylation and/or Pol II elongation? As the interplay between histone modifications is well documented, does TSA treatment affect H3K9me2 and/or H3K27me3?
New Figure S4 shows now a ChIP experiment to assess the effects of TSA on H3K9me2 along the NCAM gene. Indeed TSA brings down H3K9me2 levels but only around E18 and not further upstream. These new results turned out to be quite interesting because they match the restricted character (also around E18) of the hyperacetylation of histones upon neuron depolarization previously described by our group (Schor et al., 2009).

2. Do similar histone modification changes occur during P19 cell differentiation?
We apologize for not satisfying this request but the idea to show similar results of differentiation on NCAM E18 in P19 cells (Fig. 1C) was only to demonstrate that the effect was not restricted to N2a cells and not to study the mechanism in depth in P19 cells. We preferred to use the limited time allowed to generate a revised version to perform experiments that were more crucial to strengthen the mechanism in N2a cells.

3. As histone modifications occur from exon 2 and are minor around exon 18, the effect on splicing is not likely due to a local change of RNA pol II velocity. RNA pol II progression is more likely slowed down over the entire NCAM gene, and this globally slower RNA Pol II may induces exon 18 inclusion. Are

other NCAM exons (upstream exon 18) alternatively spliced during
differentiation?
*Following the Reviewer's suggestion we assessed alternative splicing patterns of two
additional NCAM alternative splicing events placed upstream of E18. These events
are named VASE and MSD1. New Fig. S3 shows that while VASE inclusion is not
detected in either undifferentiated or differentiated cells, MSD1 splicing pattern is
modified with differentiation, and the changes observed are reverted by TSA and 5aC.*

4. It would be nice to show that NCAM gene is within a more compacted
chromatin environment in differentiated cells than in undifferentiated cells.
*In view of this comment we now show that differentiation does not affect H3K9me2 in
an intergenic region located approximately 10 kb upstream of the NCAM gene (New
Fig. 2C, center). This indicates that although the increase in the H3K9me2 mark is
observed allover the NCAM, it is not present 10 kb away in the same chromosome nor
in an unrelated gene (HPRT, Fig. 2C, right). However, we detected an increase in
H3K27me3 in the same intergenic region where H3K9me2 does not change (Fig. S5A,
right). This indicates that chromatin changes triggered by differentiation are various
and not restricted to the NCAM gene.*

5. It must be shown that siI18as indeed affects H3K9me2 and/or H3K27me3
around NCAM exon 18.
*This was also asked by Reviewer 1. In order to satisfy this request we performed
H3K9me2 ChIP experiments at different regions of the NCAM gene after transfection
with control (siLuc) or the intronic siRNA. Result in new Fig. 5D clearly show that
the intronic siRNA increases H3K9me2 levels at the E18 region (amplicons H and J)
but not at E5 (amplicon C) or at the promoter (amplicon A). To complement the
reviewer's request we have also measured Pol II elongation upon transfection of the
intronic siRNA. These results are in new Fig. 3C. We thank the reviewer for this
request because it allowed us to confirm, in a more robust way, the TGS-AS
mechanism, originally described for fibronectin E33, in the NCAM gene.*

6. Statistical analyses should be performed for all histograms.
Done for all experiments involving biological replicates.

Minor comments
1. Fig1A, 1B, 1C, 4A, 4C and 5C histograms represent the "relative E18+/E18-
ratio" (set up as 1 in control cells) rather than the "E18+/E18- ratio".
The relative nature of the E18+/E18- ratio was now clarified in all pertinent figures.

2. There is a problem with NCAM gene representation in Fig2B.
*This was due to an error in the pdf conversion upon submission. We have redrawn the
gene representation and hope that the problem is now solved.*

3. Figures 3 A and B do not have the same axis.
The y-axes have been corrected to make them uniform.

4. page 12: H3K9e2 should be replaced by H3K9me2
Sorry for this typo. Corrected.

Referee #3

Comments

1. Throughout the paper, the differences between undifferentiated and
differentiated cells are fairly modest. It is therefore somewhat confusing when,
for example, the authors claim that there is not significant difference between
the results in Figure 2D, while the differences are deemed to be significant in
Figure 4A.
Statistical analysis was performed and p values were added to all experiments

involving biological replicates.

2. The results in Figure 3 are crucial for the point the authors are trying to make, but the description and presentation is not ideal. First of all, the authors need to better describe why an intron-exon junction 200 kb into the gene was used as control. In the Singh and Padget paper, they always used an exonintron (or intron-exon) junction close to the promoter as control. However, in the present paper, the point is that there is little or no change at 200 kb (exon 2) but a big change at exon 18 (where is that, in kilobases?). This is not obvious from the description.

The elongation analysis using the Singh and Padgett method was now extended to other amplicons (8 in total), including one closer to the promoter (amplicon α). The complex results are now shown in new Fig. 5 C and discussed in the text. In any case it is now more clear that elongation is inhibited around E18 but not at two regions located further upstream. While exon 1-intron 1 junction is also affected by differentiation, we think that this is most likely due to changes in the initiation or promoter release efficiency, and not elongation across the gene body, since the difference is lost on exon 2 and exon 5 regions.

Secondly, I think it would actually be helpful to show another 'unaffected' gene as control, just to show that cell differentiation in itself does not generally affect transcription speed at the end of genes.

The requested control (HPRT) is now shown in new Fig. 5C right.

3. I know the authors have previously described the phenomenon that DNA becomes 'heterochromatinized' in the DNA region corresponding to an mRNA recognition site for a siRNA. The precise mechanism remains to be resolved, but the results from the use of it here is arguably one of the most important argument for the authors model. Again, the effects are small (less than 2-fold). I do not think the characterization is sufficient. The model in A shows the heterochromatin as a 'road-block to Pol II', but no date to support this is provided.

In order to satisfy this request we have measured Pol II elongation by the method of Singh and Padgett upon transfection of the intronic siRNA. Results showing that inhibition of elongation is restricted to the boundaries of E18 (amplicon ε and ζ) are in new Fig. 5E. Indeed, no changes in elongation were observed downstream (amplicon η) or upstream (amplicons α and δ) of E18. Complementary, as requested by other Reviewers, we performed H3K9me2 ChIP experiments at different regions of the NCAM gene after transfection with control (siLuc) or the intronic siRNA. Result in new Fig. 5D clearly show that the intronic siRNA increases H3K9me2 levels at the E18 region (amplicons H and J) but not at E5 (amplicon C) or at the promoter (amplicon A). These results are in new Fig. 5D. We thank the reviewer for this request because it allowed us to confirm, in a more robust way, the TGS-AS mechanism, originally described for fibronectin E33, in the NCAM gene.

4. Figure 2B has not come through (the PDF conversion) well. It is impossible to read. There are no DNA size-indications in this figure (or Figure S4A, which looks much better). These are important to judge Figure 3.

This was due to an error in the pdf conversion upon submission. We have redrawn the gene representation and hope that the problem is now solved.

5. The writing needs to be improved. There are several examples of poor editing and sub-optimal English.

Done.

6. The introduction is unnecessarily long. It can easily be cut to about 3 pages.

The Introduction was cut down by about 300 words.

7. At the end of page 8, the authors conclude that "....upon differentiation the chromatin environment is changed in the NCAM gene, affecting E18 inclusion." However, at this point in the paper, the reviewers have not shown

any data to support this assertion.
Removed.

8. At the beginning of page 11, the authors write "The direct relationship between repressive marks and higher E18 inclusion...". Which 'direct relationship' is that?
We replaced "direct relationship" by "correlation".

2nd Editorial Decision   18 June 2013

Thank you for submitting your revised manuscript to the EMBO Journal. It has now been seen by two of the original referees who both find that all criticisms originally raised have been adequately addressed and are thus broadly in favour of publication, pending satisfactory minor revision as outlined below.

During our routine check of the manuscript text and figures we noticed that a number of subfigures (1B, 2C, 2D, 3A, 3B, 5D, 5E) include error bars that according to the figure legends are based on duplicate experiments, not on triplicates as we require for reliable statistics. In line with our journal guidelines to authors, I would therefore ask you to alter the data presentation to reflect the statistical basis by either plotting duplicate values separately or by including a third replica in your statistical analysis.

Given the overall positive recommendations by the referees, I would like to invite you to submit a final revised version of the manuscript, where the data presentation has been amended as outline above. Please do not hesitate to contact me if you have further questions concerning this issue.

Thank you for the opportunity to consider your work for publication. I look forward to your revision.

REFEREE REPORTS

Referee #2
The authors answer to all my concerns

Referee #3
The authors did a fine job dealing with my queries

2nd Revision - authors' response   26 June 2013

In the case of Fig. 1B, we have replaced it by a figure in which the mean values were obtained through triplicates. In the cases of Figs. 2C, 2D, 3A, 3B, 5D and 5E, we have included separate duplicates.