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OCT4/SOX2-independent Nanog autorepression modulates heterogeneous Nanog gene expression in mouse ES cells

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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 13 August 2012

Thank you for submitting your research paper describing an auto-repressive function of Nanog for consideration to The EMBO Journal editorial office.

I did receive comments that obviously reveal an interest in the subject matter, but outline necessary further experimentation before possible further proceedings.

As their reports are constructive and explicit, I do refrain from repeating them here. For guidance on your revisions however, I would like to emphasize that stronger direct support for the intriguing model of auto-inhibition versus -activation would be required. Ref#1 suggests minimally a series of reporter assays (as I do recognize that an ideal knock-in approach seems much too demanding for a single round of revisions). Critically however would be the inclusion of Nanog protein level, preferably throughout the results section. Together with addressing the point of ref #2 on auto-repression under 2i-conditions, we would be happy to re-assess a thoroughly modified and in terms of strength of the conclusions, adequately adjusted manuscript.

Please do note that The EMBO Journal considers only one round of major revisions with the ultimate decision solely depending on the quality and strength of the final dataset that will have to be reassessed by some of the original referees.

Please do not hesitate to get in touch in case of further questions or with an outline of timeline and feasibility of some of the requested experiments (preferably via E-mail).

I am very much looking forward to your revisions and remain with best regards.

Yours sincerely,

Editor
The EMBO Journal

REFEREE REPORTS

Referee #1:

The manuscript by Navarro and colleagues entitled "A Nanog autorepressive loop at the heart of the network controlling ES cell self-renewal" documents a number of observations that are aimed to dissect the function of NANOG within the pluripotency transcription factor circuitry. The authors document that pre-mRNA production from the Nanog locus anticorrelates with the levels of Nanog mRNA; that there is increased NANOG binding on its distal enhancer in cells with low pre-mRNA expression and decreased NANOG binding in cells with high pre-mRNA Nanog expression. The authors interpret these as NANOG functioning as an autorepressor independently of Oct4/Sox2 function.

The work of the authors is very important and of high significance because it provides experimental evidence for the predicted negative feedback necessary to maintain the fluctuations of Nanog expression in the presence of constant high oct4/sox2 levels, which is not compatible with the current assumed exclusively positive feedback. However, I find the model of the authors oversimplified and not consistent with all their data or the data available in the litterature. Also, some of their conclusions are overstated. The manuscript should be substantially rewritten (the authors should tune their conclusions down) and some experimental controls added before publication. My comments follow below, most of them can be addressed by changing the text and/or reinterpreting the data.

The authors conclude categorically that Nanog functions exclusively as an autorepressor. However, there is an alternative, equally plausible and perhaps more realistic interpretation of their data. If NANOG would behave solely as an autorepressor, how is the model of the authors compatible with ES cells grown in 2i medium, which have higher levels of Nanog both at the mRNA level in single cells and at the protein level, and are a much more homogeneous and estabilised ES cell population? In this context, their conclusion that Nanog 'maximises heterogeneity' in ES cells is contradictory. Their model is also not compatible with the observations that high levels of Nanog protect cells from differentiation. Only the variation in protein levels together with a feedback (both positive and negative) mechanism would be consistent with the data presented by the authors and the other data available in the litterature. In other words, at low cellular doses NANOG might be functioning as an activator whereas beyond a certain threshold it might function as a repressor. Mechanisms to explain such behaviour include that the binding kinetics of NANOG to its targets might be different depending on their dose and/or that it associates with different complexes depending on its concentration. The authors present some novel evidence for Nanog behaving as a repressor, and there is evidence of Nanog functioning as an activator (Pan et al, JBC 2005). Indeed, it seems more likely that the variability generated through allele switching that generates an intracellular range of doses of NANOG protein generates heterogeneity.

The authors rule out this latter possibility but their approaches do not allow them to address this, nor to rule out this directly since all the reporters they use behave as heterozygous Nanog cells and therefore without the possibility of the allelic variability to which Nanog expression is subject.

The authors state (Page 10) that the observed differences in Nanog transcription are due to changes in the number of cells transcribing Nanog and not to a variation in the expression level of cells already expressing Nanog nor to allelic switch. This statement is wrong for the following reasons
i) Their analysis of mono- bi-alleic expression is a 'snapshot' (RNA-FISH) and is not dynamic; their reporters, because of what I stated above and because they encode estabilised GFP will not reveal dynamics. Therefore the authors cannot conclude that allele switching is not playing a role in the autoregulation of Nanog.
ii) their conclusion cannot be inferred from their data since RNA-FISH is not quantitative and their RT-PCR only measured bulk cell population steady-state RNA levels. For the authors to make such an assumption, single cell quantitative RT-PCR analysis must be performed.

While the authors show a strong correlation of NANOG binding and presence on the distal enhancer and its repression, their results are only correlative and cannot formally rule out that Nanog can also and additionally behave as an activator of its own promoter. The only direct way of proving this would be to mutate the Nanog binding site within its enhancer either using a knock-in strategy (ideally) or alternatively (less time consuming) by using a series of Luciferase reporter assays and

probing Nanog activity in wt versus mutant enhancer at different doses.

How specific would the effect of Nanog on its own promoter be? Can the authors probe pre-mRNA production of Oct4 and Sox2 similarly to what they did for Nanog?
This will be also important to probe to control that the reduced transcriptional output from Nanog promoter is not due to a general effect of loss of pluripotency and/or precocious differentiation.

The authors assume that the mRNA analysis of Nanog reflects changes in the protein levels of NANOG. However, apart from figure 1 there is not a single indication of the levels of the actual NANOG protein. This is an essential information that needs to be added throughout the results.

In particular, in Figure 2, what are the kinetics of NANOG protein relative to those of pre-Nanog mRNA? The kinetics might help the authors make a stronger, causative argument if lower NANOG protein levels precede increased pre-Nanog mRNA levels. Protein levels of NANOG should be probed for Figure 2 at 0h, 12h, 24h and 48h.

The results shown on Figure 5C suggest that reduced NANOG protein levels (if assumed that they reflect Nanog mRNA levels) are not enough to 'de-repress' Nanog expression (as judged from the reduced pre-Nanog levels in the absence of Dox) in the absence of Sox2 and Oct4 and therefore the autorepression by Nanog is not totallly independent of Sox2/Oct4 as the authors conclude. This part should be rediscussed accordingly.

On Figure 9, it is not surprising that the sorted NG cells with tamoxifene do not 'shift' if according to the authors NANOG can repress the activity of its own promoter. While this can be interpreted as a repressive outcome, it cannot be interpreted as heterogeneity as the authors conclude, so please rephrase. Finally, in the same figure, the 44NERT cells that are treated with tamoxifen for 20 days are presumably undergoing differentiation? Can the authors rule out non-specific effects? In other words, how much of the effects on Nanog expression that the authors observe are due to loss of stemness rather than to an actual direct effect of NANOG?

Minor points:
1. The manuscript is not easy to follow. The description of the results is superficial. The authors should describe their findings better instead of only the conclusions they draw from them, that will make the manuscript easier to follow.

2. When the authors refer to Nanog protein, it should be in capitals (NANOG) as per official mouse nomenclature rules.

3. Page 29, figure legend 7, 3rd line, Nanog 'transcription' should be more accurately replaced by Nanog 'RNA-FISH probe'

4. The figures need statistical analysis (p- values for significance) throughout, especially in figures 3, 6, 7.

5. What are the Oct4 and Sox2 protein levels in the 44NRT and the RCNbetaH cells in the presence of tamoxifen?

6. On page 5, the statement 'this suggests that Nanog protein negatively affects transcription of the Nanog gene' is not sustained by their data. This sentence is unnecessary and should therefore be removed.

7. On page 6 the last two lines, the statement related to Figure 3E is not sustained since blocking all protein syntehsis does not tell if other proteins are involved in the repression mediated by Nanog.

8. The transcript levels of 44NERTc3 are almost double to those of clones c1 and c2. Why is the degree of repression equal in Figure 3E? Wouldn't one expect, according to the authors model, to have higher repression if higher mRNA (and presumably protein) levels?

9. Page 6, first line of the last paragraph, 'autorepression' should be more accurately repressed by 'autoregulation'

10. On page 10, first line, the authors conclude that data shown on figure 3 'indicate that Nanog acts as a direct repressor of its own transcription'. This should be removed as there is no such a proof for direct action in Figure 3.

11. First line of page 8 is overstated: their ChIP results are correlative, not causative. This should be rephrased or removed.

12. On page 12, the authors note that "44NERT cells represent the first undifferentiated, yet Nanog:GFP-negative ES cells colonies so far reported". This is a big assumption and I don't understand why the authors conclude this without the proper controls (e.g. Nanog protein levels, differentiation markers etc...). This phrase is unnecessary.

13. On Figure 7, the panel A shows a representative RNA-FISH data that is not in agreement with the percentage shown on graph B (11 full nuclei are visible out of which 72% (8 nuclei) express Nanog, 7 mono- and 1-biallelic). Please correct. Also, why are only 20% of their EF4 (wt ES cells) expressing Nanog? This is not in agreement with previous data (Chambers Nature 2007, Kalmar Plos Genetics 2007, Sing Stem Cells 2007). The authors should specify how many cells were analysed throughout for each condition.

Referee #2:

In this manuscript, the authors reported the mode of the transcriptional regulation of Nanog in mouse embryonic stem (ES) cells. Nanog is regarded as one of the key transcription factors governing pluripotency and many reports proposed its cooperation with Oct4 and Sox2 to activate the transcription each other. However, here the authors demonstrated that Nanog has minimal impact to the expression of Oct4 and Sox2 and vice versa. In contrast, Nanog negatively regulates its own expression directly and this auto-repression is a main cause to generate heterogeneous expression pattern of Nanog in ES cells.
It was recently reported that mono-allelic expression of Nanog causes its heterogeneous expression pattern (Miyanari and Torres-Padia, Nature, 2012). The authors confirmed the mono-allelic expression of Nanog and distinguish its contribution to generate heterogeneous expression from that of the auto-repression. All experiments are well designed and the results look very clear. I think this manuscript is suitable for publication in EMBO Journal after minor revision.

1. The authors used various ES cell lines in which Nanog allele was modified or exogeneous Nanog transgene was introduced or both. The relative expression levels of total Nanog protein were shown for three cell lines (E14tg2a, EF4 and RCNbH-B(t)) but not clear for others. For example, the magnitude of the up-regulation of pre-Nanog in RCNbH ES cells after extinction of Nanog was higher than that of the difference between E14tg2a and RCNbH-B(t), which might due to the different expression level of Nanog in RCNbH and E14tg2a but unclear in the data. Please make sure the relative Nanog expression levels for all ES cell lines appeared in this manuscript to allow clear comparison of the data come from different cell lines.
2. In Figure 7, the authors showed RNA-FISH data to show mono-and bi-allelic expression of Nanog. However, it is better to highlight clear examples of mono- and bi-allelic expression patterns.
3. Why did EF4 ES cells carrying the Nanog transgene still show heterogeneous expression of the endogenous Nanog? If the transgene expresses homogeneously in the population and the auto-repression loop works autonomously, these ES cells would show no expression of the endogenous Nanog. Please show the expression pattern of the Nanog transgene and give clear answer to this comment.
4. Miyanari et al reported that Nanog expresses homogeneously in bi-allelic manner when ES cells are cultured in 2i. Why does the auto-repression stop working in this culture condition? Some additional data for the function of auto-repression in 2i culture will be preferred.

Referee #3

Navarro et al. use gain- and loss-of-function approaches to demonstrate that Nanog negatively regulates its expression in mouse ESCs (Fig 1-3). Nanog autorepression persists in the absence of Oct4 (Fig. 6). Nanog-GFP reporter cells in which the remaining Nanog allele is intact harbour a larger fraction of GFP-negative cells, suggesting that Nanog autorepression contributes to the

generation of cells in which Nanog is silenced (Fig. 8). In addition, forced expression of Nanog suppresses the reactivation of a Nanog-GFP allele in sorted GFP-negative ESCs, indicating that Nanog blocks the exit from the Nanog-inactive state (Fig. 9).

The origin of fluctuating Nanog expression in ESCs has been the subject of considerable interest to systems biologists (Kalmar et al., 2008; Glauche et al., 2010) and to stem cell biologists. The models proposed thus far have incorporated a Nanog-centered positive feedback loop. Thus, the finding that Nanog represses its own expression warrants a revised model, which may provide a better fit with the observed Nanog expression patterns. Thus, the present study should be of interest to stem cell field and suitable for EMBO journal.

However, the following issues need to be resolved.

General points:

1. The current manuscript gives no insight into the mechanism of Nanog autorepression. It is not surprising that Nanog occupancy at the Nanog locus should be abolished when the ectopic Nanog transgene is removed (Fig. 4A-D). Furthermore, the observation that RNA Pol II, TFIIB and active histone marks are depleted when Nanog transcription is reduced (Fig. 4E) gives little mechanistic insight, other than confirming that the Nanog locus is transcriptionally silent. The important question is what mechanism(s) underlie the capacity of Nanog to serve as a repressor of its own locus, but as activator of many other genes in ESCs (Loh et al 2006; Chen et al. 2008; Marson et al. 2008; Kim et al 2008). For instance, could this be explained by partnering with a transcriptional co-repressor vs. co-activator?

2. One of the more important observations related to stem cell heterogeneity in recent years has been that transcriptional fluctuations are highly dependent on the culture environment. In particular, the expression of Nanog, Rex1 and other markers was found to become uniform upon switching from serum to serum-free media containing dual inhibition of MAP kinase and GSK3 signaling (2i) (Wray et al., 2010; Marks et al., 2012). In addition, Nanog expression was reported to switch from a mono- to biallelic mode in 2i conditions (Miyanari and Torres-Padilla, 2012). The authors propose that Nanog autorepression is a critical mechanism for regulating heterogeneity, but should address whether this negative feedback loop persists under conditions where Nanog heterogeneity is abolished (i.e. 2i). Without such experiments it seems premature to conclude, as the authors do in their Discussion, 'that heterogeneity is not exclusively driven by extrinsic cellular signalling, but that inherent dynamism arises from the activity of the network'.

3. CHX is applied in Fig 3E to assess whether Nanog autorepression is dependent on protein synthesis of a secondary regulator. While there is a slight reduction in Nanog pre-mRNA expression at 2.5h, the pulse of combined CHX/Tam treatment is too short to conclude that Nanog autorepression is direct. Do the authors observe significant Nanog pre-mRNA downregulation at 6h, as in Fig. 3D, in the presence of CHX?

Specific points:
4. Figure 2: The increase in pre-Nanog in D is rather subtle: is this due to heterogeneity in the cell population? Using Authors FACS sorted GFP+ and GFP- populations at 12 hr, 24 hr, 48 hrs may make these data more convincing.

5. Figure 7: How many Nanog negative cells are in each cell line and do these Nanog negative cells express Sox2 and Oct4? What is the false negative rate of this RNA- FISH? This could skew the quantification of monoallelic cells.

1st Revision - authors' response 07 November 2012

Reviewer #1:

The manuscript by Navarro and colleagues entitled "A Nanog autorepressive loop at the heart of the network controlling ES cell self-renewal" documents a number of observations that are aimed to dissect the function of NANOG within the pluripotency transcription factor circuitry. The authors document that pre-mRNA production from the Nanog locus anticorrelates with the levels of Nanog mRNA; that there is increased NANOG binding on its distal enhancer in cells with low pre-mRNA expression and decreased NANOG binding in cells with high pre-mRNA Nanog expression. The authors interpret these as NANOG functioning as an autorepressor independently of Oct4/Sox2 function.

The work of the authors is very important and of high significance because it provides experimental evidence for the predicted negative feedback necessary to maintain the fluctuations of Nanog expression in the presence of constant high oct4/sox2 levels, which is not compatible with the current assumed exclusively positive feedback. However, I find the model of the authors oversimplified and not consistent with all their data or the data available in the litterature. Also, some of their conclusions are overstated. The manuscript should be substantially rewritten (the authors should tune their conclusions down) and some experimental controls added before publication. My comments follow below, most of them can be addressed by changing the text and/or reinterpreting the data.

If NANOG would behave solely as an autorepressor, how is the model of the authors compatible with ES cells grown in 2i medium, which have higher levels of Nanog both at the mRNA level in single cells and at the protein level, and are a much more homogeneous and estabilised ES cell population? In this context, their conclusion that Nanog 'maximises heterogeneity' in ES cells is contradictory.

We show now in Figure 9 that in 2i conditions Nanog is still able to repress *Nanog*. However, this does not lead to generation of Nanog negative cells, presumably because in the absence of Erk/GSK3-signalling the expression of one or several activators of *Nanog* transcription is too strong and permanently keeps high levels of *Nanog* transcription.

Their model is also not compatible with the observations that high levels of Nanog protect cells from differentiation.

It sounds like the reviewer is referring to our own data (Chambers et al., Cell, 2003), in which we show that high levels of Nanog protect cells from differentiation. In this case enforced Nanog expression is derived from a transgene and uncoupled from the regulation at the *Nanog* locus, so there is no issue here. Moreover, it may be possible that *Nanog* autorepression has evolved as a mechanism allowing the cells to express levels of Nanog that are compatible with their inherent ability to undergo differentiation. This has been included in the discussion (page 20 lines 15-18: ***"it is known that ectopically enforced NANOG expression captures ES cells in a self-renewal state (Chambers et al, 2003). Therefore, Nanog autorepression may be an important component that restrains NANOG from reaching a level which completely blocks exit from the undifferentiated state."***).

Only the variation in protein levels together with a feedback (both positive and negative) mechanism would be consistent with the data presented by the authors and the other data available in the litterature. In other words, at low cellular doses NANOG might be functioning as an activator whereas beyond a certain threshold it might function as a

repressor. Mechanisms to explain such behaviour include that the binding kinetics of NANOG to its targets might be different depending on their dose and/or that it associates with different complexes depending on its concentration. The authors present some novel evidence for Nanog behaving as a repressor, and there is evidence of Nanog functioning as an activator (Pan et al, JBC 2005).

We can agree with the referee when he/she claims that a positive feedback may be required. This notion was in fact already introduced in our previous version of the discussion which has now been extended by suggesting that *Klf4* and *Esrrb* may establish positive feedback loops with *Nanog*. See page 21 lines 21-25: ***"NANOG-dependent feedback loops have recently been suggested to contribute to ES cell heterogeneity (MacArthur et al, 2012). The NANOG targets Esrrb and Klf4 (Festuccia et al, 2012) have been suggested to act as transcriptional activators of Nanog (Niwa et al, 2009; Van der Berg et al, 2008). Esrrb and Klf4 are therefore likely to establish positive feedback loops"***

However, the referee is suggesting that the positive feedback may be directly mediated by Nanog itself, which would act as an auto-activator at low cellular doses. We would like to mention that there is nothing in the literature suggesting a bimodal function of Nanogmediated transcriptional control, neither at *Nanog* nor at any other locus. Although the work cited by the referee (Pan et al, JBC 2005) examines the function of artificial fusions of Nanog in heterologous molecular settings to reach the conclusion that Nanog can act as an activator, this is something we do not dispute. In fact we find that Nanog can act as a direct activator of a range of target genes (Festuccia et al. Cell Stem Cell 2012), and we provide some evidences in the new version of our manuscript too: although Nanog protein acts repressively at *Nanog*, it does activate other genes such as *Rex1*, *Klf4* and *Esrrb*.

Although we note that this point was only raised by Reviewer #1, we have performed a set of experiments to address the possibility that at lower cellular concentrations, Nanog may activate *Nanog* gene transcription. To do this, we introduced an independent inducible system of Nanog function in which a Doxycycline-driven *Nanog* cDNA is integrated into the genome of *Nanog*-null ES cells. We show now that at low concentrations of Doxycycline, which produce levels of Nanog protein far below wild-type levels, endogenous *Nanog* transcription and *Nanog*-driven luciferase activity are downregulated. This rules out the hypothesis submitted by the referee. These results form the basis of a new figure (Figure 4) and are described in a new subsection entitled ***"Dose response of NANOG-mediated repression of Nanog."***

"Indeed, it seems more likely that the variability generated through allele switching that generates an intracellular range of doses of NANOG protein generates heterogeneity. The authors rule out this latter possibility but their approaches do not allow them to address this, nor to rule out this directly since all the reporters they use behave as heterozygous Nanog cells and therefore without the possibility of the allelic variability to which Nanog expression is subject.

The authors state (Page 10) that the observed differences in Nanog transcription are due to changes in the number of cells transcribing Nanog and not to a variation in the expression level of cells already expressing Nanog nor to allelic switch. This statement is wrong for the following reasons: i) Their analysis of mono-bi-alleic expression is a 'snapshot' (RNA-FISH) and is not dynamic; their reporters, because of what I stated above and because they encode estabilised GFP will not reveal dynamics. Therefore the authors cannot conclude that allele switching is not playing a role in the autoregulation of Nanog.

ii) their conclusion cannot be inferred from their data since RNA-FISH is not quantitative and their RT-PCR only measured bulk cell population steady-state RNA levels. For the authors to make such an assumption, single cell quantitative RT-PCR analysis must be performed."

We agree with the referee that our approaches do not reveal dynamics and to clarify this to any reader we have therefore modified the text. Page 13, lines 22-23: ***"Although using RNA-FISH we cannot exclude that Nanog does not control the dynamic properties of mono/biallelic switching ..."***

More importantly, we feel like the referee has overstated our conclusions as we never meant to suggest that "*allele switching is not playing a role in the autoregulation of Nanog*" or in heterogeneity. We believe this misunderstanding to be the cause of the categorical opinion of the reviewer that our "*statement is wrong"*. What we stated in the manuscript and still maintain because we believe it is an objective conclusion (as acknowledged by Reviewer #2) is that *Nanog* autorepression does not influence the proportion of mono-versus biallelically transcribing cells. In no way does this dispute or contradict the potential importance of allelic switching in the generation/control of Nanog heterogeneity.

To clarify this we have added a subsection to our discussion (page 22 lines 3-9, ***"As recently reported, …"***) in which we acknowledge that allelic switching may generate additional variability to the regulatory system controlling *Nanog* heterogeneity. However, and as demonstrated with the Dox-inducible system described in our response to the previous point, we want to stress that the allelic variability cannot influence heterogeneity through the mechanisms hypothesized by Reviewer #1, in which low Nanog levels activate *Nanog* transcription.

Finally, Reviewer #1 asks us to perform single cell PCR to quantify monoallelic vs biallelic transcription as a function of Nanog autorepressive activity. We do agree that this would be ideal and in fact required should the control of allelic switching be the main focus of our manuscript, which is not. Moreover, this would require regenerating all our inducible systems in a hybrid ES line carrying the *Nanog*:GFP reporter, in order to be able to use polymorphisms for allelic PCR. Finally, it is far from guaranteed that single cell PCR strategies will succeed in detecting extremely lowly abundant pre-mRNA species.

While the authors show a strong correlation of NANOG binding and presence on the distal enhancer and its repression, their results are only correlative and cannot formally rule out that Nanog can also and additionally behave as an activator of its own promoter. The only direct way of proving this would be to mutate the Nanog binding site within its enhancer either using a knock-in strategy (ideally) or alternatively (less time consuming) by using a series of Luciferase reporter assays and probing Nanog activity in wt versus mutant enhancer at different doses.

Here again Reviewer #1 suggests that at low concentrations Nanog may act positively on its own promoter. As stated above, we have ruled out this possibility by using a Doxinducible Nanog restoration system in *Nanog*-null cells: at all tested concentrations of exogenous Nanog expression (above and below wild-type levels), endogenous *Nanog* is downregulated, and so is a luciferase reporter of *Nanog*-driven transcription (new Figure 4).
The referee further asks for a mutational approach of the Nanog binding site either at the endogenous locus or using a luciferase reporter. On the face of it, this seems like a reasonable experiment that we agree would be of general interest. However, the Nanog binding motif is not clearly defined. In fact, the in-vitro defined motif (the homeobox TAAT(G/T)(G/T), Jauch et al. J. Mol. Biol. 2008) is different from the motif derived from

ChIP-Seq studies (which corresponds to the Oct4/Sox2 motif due to the high incidence of corecruitment of the 3 factors; Chen et al. Cell 2008). Furthermore, our ChIP analysis shows that the Nanog signal occupies 1.3kb of sequence across the -5kb region, suggesting that more than one DNA site may be important to recruit Nanog, which would excessively complicate a mutational approach. Nevertheless, Wu et al. (Wu et al. JBC 2006) deleted 16 bases from the *Nanog* -5kb region that were shown by EMSA to contain a Nanog binding site. With this mutant construct they observed reduced luciferase activity and concluded that Nanog activates *Nanog*, a result which contradicts our findings. To meet the referee's criticism and address such discrepancy, we have tried to reproduce Wu et al. experiments.

As shown by Wu et al., deletion of the putative Nanog binding site leads to a strong decrease of luciferase activity. However, this loss of activity is observed both in cells expressing Nanog and in *Nanog*-null cells. This conclusively demonstrates that Nanog is not the key factor responsible for the loss of activity observed upon introduction of the Wu et al. mutation. Furthermore, upon restoration of nuclear Nanog to *Nanog*-null cells transfected with the mutated luciferase construct we observe a further reduction in luciferase activity, clearly showing that Nanog represses *Nanog* through sequences independent of those mutated by Wu et al. These experiments are now shown in Figure 3.

How specific would the effect of Nanog on its own promoter be? Can the authors probe premRNA production of Oct4 and Sox2 similarly to what they did for Nanog? This will be also important to probe to control that the reduced transcriptional output from Nanog promoter is not due to a general effect of loss of pluripotency and/or precocious differentiation.

We already showed in the previous version of the manuscript that *Oct4* and *Sox2* mRNA levels are not controlled by Nanog. Therefore, addressing their pre-mRNA levels is unlikely to be informative, particularly given that *Sox2* consists of a single exon.
However, because we think the criticism made by the referee about the specificity of Nanog-mediated control is still valid we have included analysis of *Klf4* and *Esrrb* mRNA and pre-mRNA (two targets of Nanog that we recently identified; Festuccia et al, Cell Stem Cell, 2012). Instead of being repressed by Nanog, these two genes are trans-activated (Figures 2 and 3). Moreover, we also provide luciferase data showing that while the *Nanog*-driven luciferase reporter is downregulated by Nanog, a *Rex1*-driven promoter is activated (data included in Figure 1). Therefore, the variations of *Nanog* transcription caused by our experimental manipulation of Nanog activity do not result from non-specific mechanisms.

The referee also thinks that it would be important to show that reduced *Nanog* transcription does not result from a general loss of pluripotency and/or precocious differentiation. Since we observe *Nanog* gene downregulation upon ectopic overexpression of Nanog, a condition which leads to a clear resistance to differentiation (Chambers et al., 2003), we do not think that the possibility suggested by the referee is pertinent. Moreover, we show *Oct4* and *Sox2* mRNA and protein to remain expressed at normal levels upon Nanog overexpression (data included in Figure 2), further showing that under these conditions pluripotency is preserved.

The authors assume that the mRNA analysis of Nanog reflects changes in the protein levels of NANOG. However, apart from figure 1 there is not a single indication of the levels of the actual NANOG protein. This is an essential information that needs to be added throughout the results. In particular, in Figure 2, what are the kinetics of NANOG protein relative to those of pre-Nanog mRNA? The kinetics might help the authors make a stronger, causative argument if

lower NANOG protein levels precede increased pre-Nanog mRNA levels. Protein levels of NANOG should be probed for Figure 2 at 0h, 12h, 24h and 48h.

We have now included Western-Blot analyses of Nanog in the most important experimental conditions that we have analysed, in particular those concerning the experiments described in Figure 2 and highlighted by the referee.

The results shown on Figure 5C suggest that reduced NANOG protein levels (if assumed that they reflect Nanog mRNA levels) are not enough to 'de-repress' Nanog expression (as judged from the reduced pre-Nanog levels in the absence of Dox) in the absence of Sox2 and Oct4 and therefore the autorepression by Nanog is not totally independent of Sox2/Oct4 as the authors conclude. This part should be rediscussed accordingly.

In the cell line used to perform this experiment, ZHBTc4, only the *Nanog* loci produce Nanog. Therefore, the reduction of *Nanog* pre-mRNA that we observe is the cause of the reduced *Nanog* mRNA and binding of Nanog at *Nanog*, and not the opposite as shown in all other experiments in which Nanog is expressed from additional transgenes and the endogenous *Nanog* loci do not produce *Nanog* transcripts or proteins.

Thus, upon loss of Oct4/Sox2 binding in ZHBTc4, *Nanog* transcription is downregulated, leading to reduced pre-mRNA levels which cause a reduction of *Nanog* mRNA. Does this reduction of endogenous mRNA (and in fact of binding of Nanog at *Nanog* as we show in Figure 6) lead to a derepression of endogenous *Nanog* transcription? This is hard to know because it is impossible to establish what level of *Nanog* pre-mRNA would have been observed upon loss of Oct4/Sox2 binding in a non-autorepressive context.

On Figure 9, it is not surprising that the sorted NG cells with tamoxifene do not 'shift' if according to the authors NANOG can repress the activity of its own promoter. While this can be interpreted as a repressive outcome, it cannot be interpreted as heterogeneity as the authors conclude, so please rephrase.

We disagree with the referee. Nanog transcription heterogeneity results from the dynamic transition between states of activity and silencing of *Nanog* (Chambers et al., 2007; Singh et al., 2007; Kalmar et al., 2009). Therefore, finding a regulator which affects the ability of cells in a given state to reach the other (as we show for Nanog in our manuscript) is a clear proof of altered dynamic transitions and, thus, of altered heterogeneity.

Finally, in the same figure, the 44NERT cells that are treated with tamoxifen for 20 days are presumably undergoing differentiation? Can the authors rule out non-specific effects? In other words, how much of the effects on Nanog expression that the authors observe are due to loss of stemness rather than to an actual direct effect of NANOG?

Treatment of 44NERT cells with Tamoxifen causes a shift in Nanog protein into the nucleus (Figure 3). As we have shown previously (and the referee acknowledges in a previous comment), enforced expression of Nanog prevents differentiation (Chambers et al. 2003). This can bee seen in the brightfield images in Figure 9 where the colonies formed in the presence of Tamoxifen (bottom panels) are uniformly undifferentiated, while untreated cells do exhibit significant levels of differentiation (upper panels) as expected for cells lacking Nanog.

Minor points:

1. The manuscript is not easy to follow. The description of the results is superficial. The authors should describe their findings better instead of only the conclusions they draw from them, that will make the manuscript easier to follow.

None of the other two referees have commented on this particular point. However, in this new version we have tried to make it easier for the reader to follow our experiments clearly and endeavour to describe our findings more fully rather than just our conclusions.

2. When the authors refer to Nanog protein, it should be in capitals (NANOG) as per official mouse nomenclature rules.

This has been changed.

3. Page 29, figure legend 7, 3rd line, Nanog 'transcription' should be more accurately replaced by Nanog 'RNA-FISH probe'

This has been corrected.

4. The figures need statistical analysis (p-values for significance) throughout, especially in figures 3, 6, 7.

We agree that there is a general tendency to provide such p-values, even though in most cases the number of experimental replicates should not rigorously allow the use of statistical analysis. The global strategy of our study was to provide several independent lines of evidence for what we believe is an important phenomenon (*Nanog* autorepression) to support our conclusion. Indeed, we have used: (1) stable *Nanog*-null cells, (2) Nanog overexpressing cells, (3) three independent clones in which Nanog activity can be restored by tamoxifen, (4) an independent line in which Nanog can be restored by doxycycline treatment and (5) an inducible knock-out system. These different genetic contexts have been explored by different and complementary analytical techniques such as RT-(Q)PCR, FACS, RNAFISH, Luciferase activity and ChIP. The outcome is a highly robust and consistent data set that, in our opinion, does not require further support from what to our minds would be weak statistical analyses. However, should Reviewer #1 and/or the editorial team persist in this request, we will incorporate it.

5. What are the Oct4 and Sox2 protein levels in the 44NRT and the RCNbetaH cells in the presence of tamoxifen?

We have included Western-Blot analyses of Oct4 and Sox2 expression in both 44NERT and RCNβH cells (Figures 2 and 3)

6. On page 5, the statement 'this suggests that Nanog protein negatively affects transcription of the Nanog gene' is not sustained by their data. This sentence is unnecessary and should therefore be removed.

It is unclear what the reviewer is objecting to here. Because this sentence is located at an early stage of our demonstration of *Nanog* autorepression we have further toned down this early interpretation. The current sentence (page 5, lines 17-18) now reads: ***"This may suggest that NANOG negatively affects transcription of the Nanog gene"***.

7. On page 6 the last two lines, the statement related to Figure 3E is not sustained since blocking all protein syntehsis does not tell if other proteins are involved in the repression mediated by Nanog.

The purpose of this experiment is to indicate whether a secondary gene activated by Nanog is involved in the repression, rather than Nanog itself. It is not meant to test if Nanog **alone** is responsible for the repression.

8. The transcript levels of 44NERTc3 are almost double to those of clones c1 and c2. Why is the degree of repression equal in Figure 3E? Wouldn't one expect, according to the authors model, to have higher repression if higher mRNA (and presumably protein) levels?

Although we agree with the referee that the difference in the magnitude of the repression observed in 44NERT#3 is not much greater than in the other two clones, there is still a difference that we have now confirmed using FACS analysis (Figure 4). As this suggests that the Nanog-mediated repression of *Nanog* is dose-responsive, we aimed at clarifying this using the doxycycline-dependent system of Nanog restoration described above and shown in Figure 4 of the new version of our manuscript. The conclusion is clear: the more Nanog is expressed, the higher the level of repression is.

9. Page 6, first line of the last paragraph, 'autorepression' should be more accurately repressed by 'autoregulation'.

We have changed the whole sentence to: "***Our results show that the Nanog gene responds rapidly to the inducible depletion and restoration of Nanog.***" (Page 7, lines 13-14)

10. On page 10, first line, the authors conclude that data shown on figure 3 'indicate that Nanog acts as a direct repressor of its own transcription'. This should be removed as there is no such a proof for direct action in Figure 3.

We disagree with Reviewer #1, in particular because the fact that the repression of *Nanog* by Nanog does not require protein synthesis (Figure 3) warrants a sentence including the word "***indicate***".

11. First line of page 8 is overstated: their ChIP results are correlative, not causative. This should be rephrased or removed.

The conclusion of the ChIP section is now as follows: "***Altogether, our results suggest that Nanog acts as a direct transcriptional repressor of Nanog gene transcription.***" (page 11, lines 4-5)

12. On page 12, the authors note that "44NERT cells represent the first undifferentiated, yet Nanog:GFP-negative ES cells colonies so far reported". This is a big assumption and I don't understand why the authors conclude this without the proper controls (e.g. Nanog protein levels, differentiation markers etc...). This phrase is unnecessary.

This sentence has now been deleted.

13. On Figure 7, the panel A shows a representative RNA-FISH data that is not in agreement

with the percentage shown on graph B (11 full nuclei are visible out of which 72% (8 nuclei) express Nanog, 7 mono- and 1-biallelic). Please correct. Also, why are only 20% of their EF4 (wt ES cells) expressing Nanog? This is not in agreement with previous data (Chambers Nature 2007, Kalmar Plos Genetics 2007, Sing Stem Cells 2007). The authors should specify how many cells were analysed throughout for each condition.

The figure has been modified accordingly.
EF4 are not wild-type but Nanog overexpressing cells, and were not used in the papers cited by the reviewer. We therefore fail to understand the referee's concern.

The number of counted nuclei has been included in the legend to the RNA-FISH data.

Reviewer #2:

In this manuscript, the authors reported the mode of the transcriptional regulation of Nanog in mouse embryonic stem (ES) cells. Nanog is regarded as one of the key transcription factors governing pluripotency and many reports proposed its cooperation with Oct4 and Sox2 to activate the transcription each other. However, here the authors demonstrated that Nanog has minimal impact to the expression of Oct4 and Sox2 and vice versa. In contrast, Nanog negatively regulates its own expression directly and this auto-repression is a main cause to generate heterogeneous expression pattern of Nanog in ES cells. It was recently reported that mono-allelic expression of Nanog causes its heterogeneous expression pattern (Miyanari and Torres-Padia, Nature, 2012). The authors confirmed the mono-allelic expression of Nanog and distinguish its contribution to generate heterogeneous expression from that of the auto-repression. All experiments are well designed and the results look very clear. I think this manuscript is suitable for publication in EMBO Journal after minor revision.

1. The authors used various ES cell lines in which Nanog allele was modified or exogeneous Nanog transgene was introduced or both. The relative expression levels of total Nanog protein were shown for three cell lines (E14tg2a, EF4 and RCNbH-B(t)) but not clear for others. For example, the magnitude of the up-regulation of pre-Nanog in RCNbH ES cells after extinction of Nanog was higher than that of the difference between E14tg2a and RCNbH-B(t), which might due to the different expression level of Nanog in RCNbH and E14tg2a but unclear in the data. Please make sure the relative Nanog expression levels for all ES cell lines appeared in this manuscript to allow clear comparison of the data come from different cell lines.

We have now included Western-Blot analyses of Nanog expression for all *Nanog*mutant lines used in this manuscript. As expected by the referee, RCNβH ES cells express higher levels of Nanog than wild-type cells (Figure 2), and this is likely to explain why upon removal of the transgene to generate *Nanog*-null cells the relative fold induction is higher than when wild-type cells are directly compared to *Nanog*-null RCNβH-B(t) cells.

2. In Figure 7, the authors showed RNA-FISH data to show mono-and bi-allelic expression of Nanog. However, it is better to highlight clear examples of mono-and bi-allelic expression patterns.

We have now modified the RNA-FISH picture such that mono and biallelically transcribing cells are clearly indicated.

3. Why did EF4 ES cells carrying the Nanog transgene still show heterogeneous expression of the endogenous Nanog? If the transgene expresses homogeneously in the population and the auto-repression loop works autonomously, these ES cells would show no expression of the endogenous Nanog. Please show the expression pattern of the Nanog transgene and give clear answer to this comment.

We agree that it would have been preferable to see no cells transcribing *Nanog* in EF4. Similarly, and although this is not mentioned by Reviewer #2, the same is also valid for untreated RCNβH cells and Tamoxifen-treated 44NERT cells. Also, it may appear surprising that not all *Nanog*-null cells actively transcribe *Nanog*.

We think that the referee has highlighted an important observation that suggests that additional activities are important to control *Nanog* heterogeneity. We now acknowledge this in the results (page 13, lines 12-16: "***Interestingly, in populations of cells permanently expressing exogenous NANOG (EF4, Tamoxifen-untreated RCNβH and Tamoxifentreated 44NERT), Nanog is not homogenously silent. Conversely, in cells lacking NANOG activity (RCNβH-B(t), Tamoxifen-treated RCNβH and Tamoxifen-untreated 44NERT), NANOG is not homogenously active.***"), and in the discussion (page 21, lines 11-13: "***Interestingly, not all the cells constituvely expressing exogenous NANOG display silent Nanog genes and, conversely, not all cells lacking NANOG permanently transcribe Nanog***"). We propose that such activities may activate *Nanog* and be themselves activated by Nanog. It is indeed to be expected that genes upregulated by Nanog and which in turn trans-activate *Nanog*, may overcome in some cells the effects of direct *Nanog* autorepression, upon modulation of Nanog activity (page 21, lines 13-15: "***This suggests that other activities are likely to buffer the efficiency of Nanog autorepression in NANOG-overexpressing cells or to restrain full activation of Nanog.***")

We already commented in the previous discussion on the possibility that indirect *Nanog* auto-activation through the establishment of positive feed-back loops with other pluripotency genes may contribute to Nanog heterogeneity. Starting from the reviewer's question we have now further developed this idea in the discussion, particularly proposing *Esrrb* and *Klf4* as interesting candidate factors (page 21 line 22 to page 23 line 2: "***The NANOG targets Esrrb and Klf4 (Festuccia et al, 2012) have been suggested to act as transcriptional activators of Nanog (Niwa et al, 2009; Van der Berg et al, 2008). Esrrb and Klf4 are therefore likely to establish positive feedback loops that may explain the fact that Nanog is neither homogenously silent in cells overexpressing NANOG from a transgene, nor homogeneously active in cells lacking NANOG.***").

The referee also asks for the expression pattern of the *Nanog* transgene in EF4. However, we cannot specifically show exogenous Nanog expression in this line because it was unfortunately not tagged and the endogenous alleles are wild-type such that we cannot distinguish transgenic from endogenous Nanog expression.

4. Miyanari et al reported that Nanog expresses homogeneously in bi-allelic manner when ES cells are cultured in 2i. Why does the auto-repression stop working in this culture condition? Some additional data for the function of auto-repression in 2i culture will be preferred.

Reviewer #2 assumes that the auto-repression stops working in 2i. However, we now show in Figure 9 that, even in 2i, Nanog autorepression occurs though not to a sufficient extent to drive heterogeneity. We show indeed that *Nanog*-null;*Nanog*:GFP cells grown in 2i

express higher levels of *Nanog*:GFP than *Nanog*:GFP cells expressing Nanog from the second allele. Also, we provide both FACS and RT-(Q)PCR data showing that restoration of nuclear Nanog to *Nanog*-null;*Nanog*:GFP cells downregulates endogenous *Nanog* expression, although this does not lead to *Nanog*:GFP-negative cells.

We speculate at the end of the discussion that in wild-type cells grown under 2i conditions additional regulations impede Nanog autorepression to generate Nanog-inactive cells (page 22 line 20 to page 23 line 7: "***the repression of Nanog by exogenous NANOG persists in "2i+LIF", yet without giving rise to Nanog-negative cells. This suggests that, although the MEK/GSK3 signalling pathways are not required for Nanog autorepression to occur, they do promote the ability of Nanog autorepression to generate cells in which Nanog is not transcribed. A multitude of transcription factors, including KLF4 and ESRRB, are upregulated in "2i+LIF" (Marks et al, 2012), suggesting that the global level of Nanog activators might be too high to allow Nanog autorepression to generate cells expressing no NANOG. Conversely, the transcriptional repressor TCF3 is not functional in "2i+LIF" (Wray et al, 2011), releasing the repression it normally exerts on Nanog and on several other components of the pluripotency network. In this regard, the MEK/GSK3 signalling pathways should not be viewed as specific drivers of Nanog heterogeneity, but rather as the inducers of a regulatory landscape in which the consequences of Nanog autorepression can be fully unfolded to give rise to heterogeneous and fluctuating Nanog transcription.")***

Reviewer #3:

Navarro et al. use gain-and loss-of-function approaches to demonstrate that Nanog negatively regulates its expression in mouse ESCs (Fig 1-3). Nanog autorepression persists in the absence of Oct4 (Fig. 6). Nanog-GFP reporter cells in which the remaining Nanog allele is intact harbour a larger fraction of GFP-negative cells, suggesting that Nanog autorepression contributes to the generation of cells in which Nanog is silenced (Fig. 8). In addition, forced expression of Nanog suppresses the reactivation of a Nanog-GFP allele in sorted GFP-negative ESCs, indicating that Nanog blocks the exit from the Nanog-inactive state (Fig. 9). The origin of fluctuating Nanog expression in ESCs has been the subject of considerable interest to systems biologists (Kalmar et al., 2008; Glauche et al.,2010) and to stem cell biologists. The models proposed thus far have incorporated a Nanog-centered positive feedback loop. Thus, the finding that Nanog represses its own expression warrants a revised model, which may provide a better fit with the observed Nanog expression patterns. Thus, the present study should be of interest to stem cell field and suitable for EMBO journal.

However, the following issues need to be resolved.

General points:

1. The current manuscript gives no insight into the mechanism of Nanog autorepression. It is not surprising that Nanog occupancy at the Nanog locus should be abolished when the ectopic Nanog transgene is removed (Fig. 4A-D). Furthermore, the observation that RNA Pol II, TFIIB and active histone marks are depleted when Nanog transcription is reduced (Fig. 4E) gives little mechanistic insight, other than confirming that the Nanog locus is transcriptionally silent. The important question is what mechanism(s) underlie the capacity of Nanog to serve as

a repressor of its own locus, but as activator of many other genes in ESCs (Loh et al 2006; Chen et al. 2008; Marson et al. 2008; Kim et al 2008). For instance, could this be explained by partnering with a transcriptional co-repressor vs. co-activator?

It is important to stress that, in fact, it is not infrequent that Nanog acts as a repressor, as the referee seems to suggest by claiming that it activates many other genes while it represses its own promoter. In fact, around half of Nanog-responsiveness genes are repressed by Nanog (Festuccia et al; Cell Stem Cells 2012). However, we agree with the referee that we do not provide significant molecular details on how *Nanog* autorepression operates, although we do show that it is Oct4/Sox2-independent.

Given that an independent manuscript (Fidalgo et al; PNAS 2012) has confirmed Reviewer #3's hypothesis that Nanog partners with a transcriptional co-repressor complex to repress Nanog, we now cite this in the discussion page 20, lines 5-7: ***"Nanog autorepression occurs through interaction between NANOG and the transcriptional repressors ZFP281 and NURD (Fidalgo et al, 2012)"***

2. One of the more important observations related to stem cell heterogeneity in recent years has been that transcriptional fluctuations are highly dependent on the culture environment. In particular, the expression of Nanog, Rex1 and other markers was found to become uniform upon switching from serum to serum-free media containing dual inhibition of MAP kinase and GSK3 signaling (2i) (Wray et al., 2010; Marks et al., 2012). In addition, Nanog expression was reported to switch from a mono-to biallelic mode in 2i conditions (Miyanari and Torres-Padilla, 2012). The authors propose that Nanog autorepression is a critical mechanism for regulating heterogeneity, but should address whether this negative feedback loop persists under conditions where Nanog heterogeneity is abolished (i.e. 2i). Without such experiments it seems premature to conclude, as the authors do in their Discussion, 'that heterogeneity is not exclusively driven by extrinsic cellular signalling, but that inherent dynamism arises from the activity of the network'.

We agree with the referee's opinion that the loss of heterogeneity in 2i is a recent important observation. He/she asks us to assess whether *Nanog* autorepression is operational in 2i to warrant our conclusion "***that heterogeneity is not exclusively driven by extrinsic cellular signalling***". Although we understand the referee's request, we believe that the fact that *Nanog*-null cells are compromised in their ability to generate and maintain *Nanog*-inactive cells in the absence of any artificial manipulation of MAP/GSK3 signalling, as we show in our manuscript, should be sufficient to suggest that "***heterogeneity is not exclusively driven by extrinsic cellular signalling***". This sentence has been nevertheless deleted.

We understand however that, if the autorepression was molecularly dependent upon extrinsic signalling, then our conclusion would turn out to be wrong because *Nanog* autorepression would just be one of the mechanisms stimulated by MAP/GSK3 signalling to downregulate *Nanog* transcription and lead to Nanog-negative cells. However, we now show that *Nanog*-null;*Nanog*:GFP cells grown in 2i express higher levels of *Nanog*:GFP than *Nanog*:GFP cells expressing Nanog from the second allele. Also, we provide both FACS and RT-(Q)PCR data showing that restoration of nuclear Nanog to *Nanog*-null;*Nanog*:GFP cells downregulates endogenous *Nanog* expression, although this does not lead to *Nanog*:GFPnegative cells. These data, included in Figure 9, show that in 2i the negative feedback loop persists.

At the end of the discussion we highlight that *Nanog* autorepression, without being itself dependent on MAP-and GSK3-related regulations, requires these signalling pathways

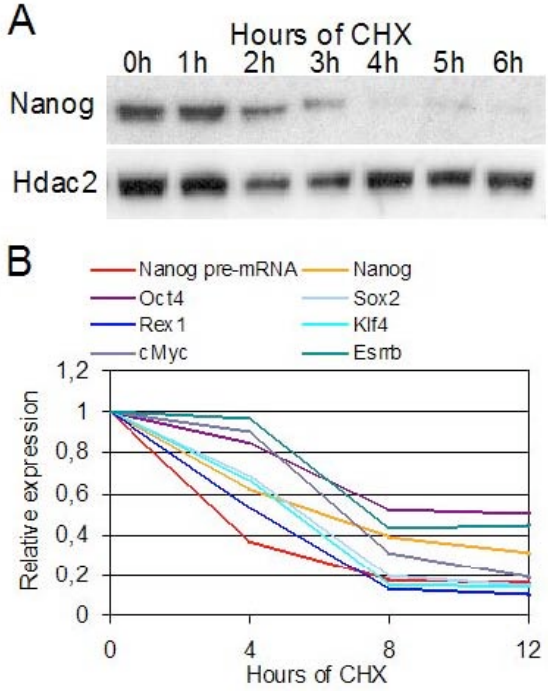
to lead to Nanog-negative cells. More specifically, we speculate that the MAP and GSK3 signalling pathways "***should not be viewed as specific drivers of Nanog heterogeneity, but rather as the inducers of a regulatory landscape in which the consequences of Nanog autorepression can be fully unfolded to give rise to heterogeneous and fluctuating Nanog transcription.***" (page 23, lines 5-7)

3. CHX is applied in Fig 3E to assess whether Nanog autorepression is dependent on protein synthesis of a secondary regulator. While there is a slight reduction in Nanog pre-mRNA expression at 2.5h, the pulse of combined CHX/Tam treatment is too short to conclude that Nanog autorepression is direct. Do the authors observe significant Nanog pre-mRNA downregulation at 6h, as in Fig. 3D, in the presence of CHX?

We agree with the referee that, because at 2.5h of treatment the reduction of pre-mRNA is quite slight, a longer treatment of 6h would have been more appropriate. However, as we show here to illustrate our response (Figure R1A), Nanog half-life is about 2-3 hours. Therefore, longer treatments of CHX would significantly reduce the level of exogenous Nanog and, thus, the extent of the Nanog-mediated repression of *Nanog*.

Moreover, while setting up the CHX experiment we noticed that upon treatments of 4 or more hours, CHX leads to a global downregulation of pluripotency genes, in particular of *Nanog*, an inappropriate molecular context to analyse *Nanog* downregulation (Figure R1B).

Figure R1



Specific points:

4. Figure 2: The increase in pre-Nanog in D is rather subtle: is this due to heterogeneity in the cell population? Using Authors FACS sorted GFP+ and GFP-populations at 12 hr, 24 hr, 48 hrs may make these data more convincing.

The increase in *Nanog* pre-mRNA after 48h of Tamoxifen treatment is 3-fold which does not seem very subtle to us. Moreover, we have monitored the proportion of cells having undergone the deletion at 12, 24 and 48h and found that, even at the first time-point, around 75% of the cells have already undergone the deletion (Figure 2). Therefore, it is hard to believe that better fold-induction values could be observed following a rather time-consuming FACS sorting approach. Moreover, the referee should also take into account that such proposed sort will require the cells to stay in PBS for a long time, raising concerns as to whether unstable pre-mRNA species would still be detectable.

5. Figure 7: How many Nanog negative cells are in each cell line and do these Nanog negative cells express Sox2 and Oct4? What is the false negative rate of this RNAFISH? This could skew the quantification of monoallelic cells.

The number of Nanog negative cells is virtually zero in all our lines expressing Nanog from a transgene. In wild-type cells, around 10-30% display undetectable levels of Nanog, and the large majority express relatively low levels, at least as compared to the highest levels observed in wild-type cells as quantified by Immunofluorescence experiments (Descalzo *et al*, 2012). In *Nanog*-null cells all cells are obviously negative.

All Nanog-negative cells, when undifferentiated, express Oct4 and Sox2.

The negative rate of the RNA-FISH probe is difficult to be rigorously assessed, in particular because it is too short to be used in DNA-FISH experiments that could indicate its hybridisation efficiency. We know however that in '2i' culture conditions the percentage of wild-type cells that we detect as transcribing *Nanog* increases to around 80%. This is a percentage routinely observed with longer probes that detect permanently expressed house keeping genes, indicating that transcription of *Nanog* in our RNA-FISH conditions is equivalent to the maximum routinely detected by this technique.