A NANO-G-pERK reciprocal regulatory circuit regulates Nanog autoregulation and ERK signaling dynamics

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Dear Dr. Shekar,

Thank you for the submission of your research manuscript to EMBO reports. We have now received the full set of referee reports that are copied below.

As you will see, the referees feel that the study is of interest (although referee #3 indicates novelty issues). However, all three referees have also several major concerns, indicating that the many effects reported are rather subtle and that the mechanism proposed has not been rigorously established. Moreover, they note technical and experimental shortcomings and also pointed to missing controls, discrepancies between datasets or doubts about reagent/assay specificity that question further the validity the data. As the reports are below, I will not further detail them here.

Given these comments and considering the amount of work required to address them, we cannot offer to publish your manuscript. However, in case you feel that you can address the referee concerns in a timely and thorough manner and can obtain data that would considerably strengthen the study (as outlined in the reports), we would have no objections to consider a new manuscript on the same topic in the future.

Please note that if you were to send a new manuscript this would be assessed again with respect to the literature and the novelty of your findings at the time of resubmission.

Yours sincerely,

Achim Breiling
Senior Editor
EMBO Reports

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Referee #1:

Kale and colleagues address the question how negative autoregulation of Nanog expression pluripotent cells is mediated mechanistically. They show that Fgf4, Fgfbp1 and Fgfr2 expression is positively regulated by NANOG. Furthermore, they provide evidence that negative autoregulation of Nanog expression in inducible cell lines carrying Nanog-reporter constructs is dependent on intact Fgf4, Fgfbp1 and Fgfr2 genes. Together, these findings lead them to propose the existence of a cell-extrinsic negative feedback circuit for Nanog autoregulation. The authors provide further evidence for this loop by substantiating a previously identified mechanism of Nanog repression by ERK through NONO, and by following the dynamics of ERK and NANOG expression in sorted cell populations. In addition, the paper reports some interesting observations on ERK activation by the GSK3 inhibitor Chiron (Fig. 1), which in my view however do not contribute significantly to the main message of the paper.

The authors use an extensive set of appropriately validated transgenic and mutant cell lines to come up with a novel mechanism for an important question in the stem cell field - how the expression of pluripotency factors is controlled to enable both maintenance of pluripotency as well as differentiation. While these ingredients make the paper a potential candidate for publication, I note that the magnitude of some of the effects reported here is comparatively low, raising the question how relevant the author's new mechanism is in comparison to previously proposed mechanisms. I also have issues with some of the experimental modalities that the authors use. These shortcomings could be addressed by a set of additional experiments, as well as by toning down some of the conclusions.

Major points
1. Fig. 2B: The magnitude of the effects reported here is very small. This experiment needs to be repeated to ascertain reproducibility and convey a clearer message about this magnitude.
2. Fig. 2E and following: The authors need to state more clearly in which medium these experiments were carried out. This is not obvious from the current description in the text or figure legends, and impinges on the interpretation of the results.
3. Fig. 3B, Fig. EV1A: The positive regulation of Fgf4, Fgfbp1 and Fgfr2 and the resulting upregulation of pERK is one of the most important parts of the new mechanism proposed by the authors. However, I feel that to establish this link beyond doubt, the paper would benefit from additional data that can be generated with existing reagents, as well as from presenting the current data differently.
   For example, why is the qPCR data in EV1A? I think this should be in the main figure. The same is true for the pERK blot in Fig. EV1E - the increase in pERK upon induction of NANOG provides good evidence for the author's model and should also be included in the main figure. Furthermore, a strong prediction is that this increase in pERK should be absent in Fgf4, Fgfbp1 and Fgfr2 mutant lines, something that the authors can easily test with the existing cell lines.
   In contrast, I am skeptical of the correlation between NANOG and FGFR2 expression inferred from the FACS analysis in Fig. 3B. This kind of assay is prone to artifacts. For example, can the axes be compared between the four panels? If yes, why does
this analysis not show the increase in NANOG expression in SLPD compared to SL? For the analysis shown in Fig. 3C, have the authors controlled for confounding variables, such as cell size?

A more convincing way to establish the dependence between NANOG, FGFR and pERK expression would be to use the NANOG inducible cell lines that the authors have, and to blot for FGFR and pERK after different induction times (I notice that the authors have working antibodies for both FGF receptors). Adding these additional experiments would put more emphasis on the papers main message and significantly strengthen the work.

4. Page 4: "... and discounts that direct role of NANOG..." This statement is too strong and should be reworded. The cell-extrinsic mechanism could well work in addition to the previously described cell-intrinsic ones.

5. Page 4: "...suggests that ZFP281 is dispensable..." I disagree with this statement, particularly because EV1K shows higher NANOG reporter expression in Zfp281 mutant cells compared to the wild type. If I a missing something here please clarify.

6. Fig. 5A, B: This data should be shown at higher resolution, using a smaller field of view. As it is presented right now, it does not support the authors point. Furthermore, the authors need to describe how the data in 5B have been generated from the data in 5A. Have they segmented the cells, and if so, how? How did they assign cytoplasmic pERK to nuclear NANOG?

7. Fig. 5D: Are these measurements derived from FACS data? As pointed out above, using flow cytometry to make these measurements is prone to artifacts. I recommend the authors repeat this experiment, but use western blotting to measure NANOG and pERK levels. This is an doable experiment that could substantiate the link between NANOG and pERK dynamics.

Minor points
1. Fig. 1G: ppERK levels in SL2i seem to be similar to those in SLPD, in contrast to the proposed role of the GSK3 inhibitor in activating MEK. Can the authors comment on this observation?

2. Fig. EV1L: The different conditions are difficult to distinguish in this plot. Consider a different representation.

3. Discussion section: "...specify primitive endoderm by cell-autonomous mechanisms ..." should read. "...non-cell-autonomous..."

4. Discussion section: The authors could more clearly spell out the possibility that NANOG heterogeneity is a consequence of de-synchronized cycling of cells through the NANOG-pERK feedback loop. They can also cite experiments with sorted Nanog-reporter cells (e.g. PMID: 19582141).

5. Molotkov et al. (2017, PMID: 28552557) have demonstrated a central role for FGFR1 and not FGFR2 for pluripotentcy and differentiation. The authors need to discuss their results in the light of these previous findings.

6. Fig. S6: The NANOG staining looks blurred. Please provide higher resolution / smaller field of view representations of this data.

Overall I found the paper hard to review. Page and line numbers as well as a clearer labelling of the figures would have been helpful here. It would also have been helpful to include journal names in the reference list.

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Referee #2:

In this manuscript, Kale and colleagues present evidence of a Nanog-pERK reciprocal regulatory circuit. The focus of the paper is on Nanog autorepression, Nanog induction of components of the FGF/MEK/ERK pathway, MEK/ERK regulation of Nanog transcript and really nice analysis of Nanog/pERK dynamics. The manuscript presents some interesting findings and would be of interest to the community. The main issue in my view is the focus on the MEK/ERK having a role in Nanog AUTOrepression rather than just 'repression'. There are a number of observations that I struggle to see how they fit the narrative presented in the paper, but that hopefully the authors can clarify.

Major points:
- "[...] (Figure 2A, Appendix Fig S2C and D). The data from distinct induction systems conclusively establish and essential role of MEK1/2 in Nanog autoregulation*. I struggle to interpret these results because the phenotypes associated with Nanog expression on a null-background are very subtle compared to the effect of adding PD. To put it in another way, if we look at figure 2A, the median GFP level in conditions that contain PD ~3x higher than SL alone in the absence of OHT. Since TNERT cells -OHT are Nanog-null, it suggests whatever PD is doing to the Nanog locus is independent of Nanog. Addition of Nanog seems to have a very minor effect. Similar to above, deleting elements NBR1 and NBR2 also changes the expression of Nanog/GFP independently of Nanog expression (Figure 3F).

- "Nanog induces and enhances FGF autocrine signalling through FGFR2 to execute Nanog autoregulation" Can the authors
also confirm that mutants of Figure 2D-E show altered levels of pERK? Do these mutants in -OHT conditions also show elevated Nanog::GFP expression compared to control lines, similar to addition of PD? Similarly, to justify the previous statements, the authors would need to show that Nanog induction in the mutant lines does not alter ERK levels.

- Could the authors please explain some inconsistency of results? In figure EV1E, +OHT addition causes very high levels of pERK. However, in figure 4C, +OHT has no effect of pERK levels. The ability of Nanog expression to induce pERK is a central aspect to its a bit worrying to see this discrepancy.

- On a related note, it is also unclear what is happening in the Nono-null cells. These cells overall show much lower levels of Nanog::GFP compared to untargeted controls (Figure Appendix 5C). Since Figure 4C shows pERK is nearly absent in Nono-null cells, I would expect these cells to phenocopy to some extent what happens in PD conditions and show much higher Nanog::GFP.

- Nono and pERK occupancy on Nanog locus. How does FGF and PD treatment affect Nono expression? The changes in pERK binding at Nanog locus are unsurprising given that pERK levels are dictated by the culture treatment. Is the same for Nono? This is important since the authors claim that "pERK is essential for the recruitment of NONO to the Nanog locus".

- All chip-seq and chip-PCR data of the paper. Can the authors please also include a control region for a known/validated target of pERK and Nono to show that the levels of binding at the Nanog locus are biologically relevant? Since pERK levels vary greatly with the treatment, and the binding does not appear enriched in any particular region, it would help to show that the chip signal observed are not just the result of higher background.

- Interpretation of the Nono/pERK/Pol2 binding results. Also here I find the interpretation a bit confusing. The authors say "pERK-NONO affects POL2 loading onto the Nanog locus preventing the initiation of transcription" but there is no evidence that the process is at all dependent on Nono. If this was the case, the Nono-null cells would have higher Nanog::GFP expression, but the opposite appears to be the case.

Other points:
- Introduction: The introduction is very short, and readers would benefit from key topics being included. For example, there is no mention of the literature on the role of ERK; 2iLIF culture is not introduced but CH and PD inhibitors are used regularly; and no description of provided as to what NONO is. The literature is also not always accurately cited (e.g. {Faddah:2013ed, Filipczyk:2013kp} convincingly disproved the earlier report of {Miyanari:2012ef} about mono-allelic regulation of Nanog).

- First results section (Figure 1). The authors refer to any changes in Nanog in terms of the hypothesised autorepression ("Nanog expression was derepressed above basal levels", "Nanog expression in SPDL indicates insufficient repression", etc). This is not a very helpful description, since the authors present very little evidence at this point that autorepression is involved. I suggest describing the results without assigning a mechanism (e.g. "results in increased expression").

- Please provide some information on the levels, heterogeneity and timing of induction for both TNERT and inducible Nanog lines.

- Figure 2D-E. Can this be confirmed through an alternative method? Maybe RT-qPCR on Nanog UTR, etc? again, the phenotypes are very subtle.

- Please indicate clearly for all figures what the statistics are.

- Beginning of result section 2: lots of acronyms are given to different lines, and the inserts within lines (e.g. NANOGER vs TNERT, etc). This creates more confusion than necessary. I suggest consolidating the nomenclature.

- Please provide some info on the figure as to what Figure 2C shows

- In the literature it's more common to find non cell-autonomous than "cell non-autonomous" as the authors describe.

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Referee #3:

Kale et al explore mechanisms underpinning Nanog autoregulation in ES cells using a series of inducible reporters and cell lines. They conclude that Nanog regulates expression of FGF signalling components such as FGFBP1 and FGFR2 to control FGF-mediated ERK1/2 activation, which in turn leads to high levels of ERK activity in cells with high Nanog expression. This leads to recruitment of NONO to the Nanog locus, which suppresses PolII loading and Nanog transcription to establish autoregulation.

In this area, it is well established that ERK1/2 signalling drives Nanog heterogeneity in ES cells, although this is not discussed or referenced at all in the introduction. Therefore, it does not take much of a leap to consider that because ERK1/2 signalling drives Nanog heterogeneity, it might also underpin Nanog autoregulation. As a result, in my view the conceptual advance presented
here is somewhat limited.

Furthermore, Nanog has been previously proposed to modulate FGF signalling, but by a different mechanism in which Nanog drives expression of FGF4 and suppression of FGFR2 in Nanog high cells. This enables segregation of Nanog high and low populations based on distinct FGF-ERK1/2 signalling responses (reviewed in Lanner and Rossant 2010). However, this study proposes the opposite, that Nanog expression leads to ERK1/2 activation in Nanog high cells. However, from what I can see, the data presented do not fully support this - in Fig5D Nanog and pERK1/2 staining show an inverse correlation, which is opposite to what the authors are proposing. My concern is that in such a dynamic system, it is difficult to confidently distinguish signalling and transcriptional responses in distinct populations of Nanog low and Nanog high cells. Thus, there is a risk that what is being reported here simply reflects what is already known - i.e. that Nanog is regulated by and regulates ERK1/2 signalling.

The area that may be of most interest and novelty is the mechanistic interplay between ERK1/2 signalling and Nanog at the transcriptional level. Is pERK1/2 staining also heterogeneous in regular mouse ES cells cultured in LIF/FCS, which exhibit dynamic Nanog heterogeneity? This would provide evidence that ERK1/2 signalling is not simply influenced by the distinct domed morphology observed in Nanog high EDIN colonies. And is pERK1/2 staining in Fig 5A specific? This can be controlled using MEK1/2 inhibitor PD0325901. Finally, is pERK1/2 recruitment measured by ChIP in Fig 4G specific and inducible - what are the controls for non-specific background signal?

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Referee #1:

Kale and colleagues address the question how negative autoregulation of Nanog expression pluripotent cells is mediated mechanistically. They show that Fgf4, Fgfbp1 and Fgfr2 expression is positively regulated by NANOG. Furthermore, they provide evidence that negative autoregulation of Nanog expression in inducible cell lines carrying Nanog-reporter constructs is dependent on intact Fgf4, Fgfbp1 and Fgfr2 genes. Together, these findings lead them to propose the existence of a cell-extrinsic negative feedback circuit for Nanog autoregulation. The authors provide further evidence for this loop by substantiating a previously identified mechanism of Nanog repression by ERK through NONO, and by following the dynamics of ERK and NANOG expression in sorted cell populations. In addition, the paper reports some interesting observations on ERK activation by the GSK3 inhibitor Chiron (Fig. 1), which in my view however do not contribute significantly to the main message of the paper.

The authors use an extensive set of appropriately validated transgenic and mutant cell lines to come up with a novel mechanism for an important question in the stem cell field - how the expression of pluripotency factors is controlled to enable both maintenance of pluripotency as well as differentiation. While these ingredients make the paper a potential candidate for publication, I note that the magnitude of some of the effects reported here is comparatively low, raising the question how relevant the author’s new mechanism is in comparison to previously proposed mechanisms. I also have issues with some of the experimental modalities that the authors use. These shortcomings could be addressed by a set of additional experiments, as well as by toning down some of the conclusions.

We thank the referee for reading our manuscript thoroughly and appreciating the novel mechanisms for an important question elucidated in the manuscript. We have improved the manuscript by performing multiple experiments based on the constructive comments of the referees.

We thank the referee for pointing out our interesting observations on ERK activation by the GSK3 inhibitor Chiron (Fig 1). We do realize, that we have not been able to convey clearly, how the observations in Fig 1 led to the main work of this manuscript? We apologize for an inadvertent editing error. We had deleted introductory sentences describing, how Fig 1 and other published data prompted us to investigate a possible role of FGF autocrine signaling in Nanog autorepression.

Navarro et. al. had shown that the Nanog autorepression is functional in 2iL (Navarro et al., 2012). Also suggested that Nanog autoregulation is independent of MEK/GSK signaling. In Fig1 we show that MEK activity is not completely and continuously inhibited in 2iL (in presence of CHIR) unlike in SLPD. The presence of residual MEK activity in 2iL from Fig1 and the functioning of Nanog autorepression in 2iL together opened a possibility
for the role of MEK signaling in Nanog autorepression in contrast to a previous report (Navarro et al., 2012).

We have added these introductory sentences in line # 98 “ Nanog autorepression was shown to function in 2iL (Navarro et al., 2012). We show residual MEK activity persist in 2iL and Nanog expression is lower in 2iL than SLPD.” This will put the observations in Fig 1 in the context of the main work and suggest these observations as the basis for investigating the possible role of FGF autocrine signaling in Nanog autorepression.

Major points
1. Fig. 2B: The magnitude of the effects reported here is very small. This experiment needs to be repeated to ascertain reproducibility and convey a clearer message about this magnitude.

We have again repeated the experiments at least 4 times. We find the results are consistently reproducible. Please find the data from multiple experiments for your consideration (given below). Nanog:GFP is repressed in TNERT+OHT, addition of FGFR inhibitor - SU5402 inhibits Nanog autoregulation, suggesting FGFRs are essential for Nanog autoregulation. We would like to bring forth an important observation about the magnitude of the effects of our old and new experiments. We have found that the magnitude of the effect is more pronounced when early passage cells of TNERT or TDiN clones are used. The magnitude of the effects tends to become smaller beyond 15 passages. The older data was obtained from late passage cells. The new experiments were carried out using early passage cells of the same clones. We have replaced the previous representative images of Fig. 2B with representative images from the new experiments. The corresponding quantification data in Fig EV2E is also replaced.
2. Fig. 2E and the following: The authors need to state more clearly in which medium these experiments were carried out. This is not obvious from the current description in the text or figure legends, and impinges on the interpretation of the results.

Thanks for pointing out this oversight. The experiments in Fig. 2E were carried out in SL media. We have added the media conditions into the panels of Fig. 2E to facilitate the interpretation of the results. The culture condition is now described in the figure legend as well.

3. (i) Fig. 3B, Fig. EV1A: The positive regulation of Fgf4, Fgfbp1 and Fgfr2 and the resulting upregulation of pERK is one of the most important parts of the new mechanism proposed by the authors. However, I feel that to establish this link beyond doubt, the paper would benefit from additional data that can be generated with existing reagents, as well as from presenting the current data differently.

We agree additional data and a better representation of the current data can strengthen the proposed mechanism. We have performed additional experiments and also improved the representation of the data to strengthen this part of the manuscript. Since multiple issues are raised in this part of the comments. We have split this part of referee comments into subsections to address all the issues comprehensively.

(ii) For example, why is the qPCR data in EV1A? I think this should be in the main figure. The same is true for the pERK blot in Fig. EV1E - the increase in pERK upon induction of NANOG provides good evidence for the author's model and should also be included in the main figure.

Thanks for this suggestion, now we have included Fig EV1 among the main figures. It is now included as main Fig 3 in the revised manuscript. Fig 3, 4, and 5 are henceforth referred to as Fig 4, 5, and 6 respectively in the revised manuscript.

(iii) Furthermore, a strong prediction is that this increase in pERK should be absent in Fgf4, Fgfbp1 and Fgfr2 mutant lines, something that the authors can easily test with the existing cell lines.

We have performed western blot analysis of pERK and ERK in the mutant lines. We could still detect pERK in all the mutant cell lines. However, the pERK levels were significantly lower in all mutant lines relative to the TNERT line. Further, the treatment with OHT did not significantly induce pERK levels in these mutant cell lines. We did not observe significant changes in pERK levels in the Fgfr1-/- mutant line relative to TNERT.
(iv) In contrast, I am skeptical of the correlation between NANOG and FGFR2 expression inferred from the FACS analysis in Fig. 3B. This kind of assay is prone to artifacts. For example, can the axes be compared between the four panels? If yes, why does this analysis not show the increase in NANOG expression in SLPD compared to SL?

We acknowledge the concerns and skepticism of the referees. We have performed experiments suggested by the referees and repeated the FACS analysis experiments with a new set of antibodies and acquisition voltage settings to strengthen the claims.

The axes of the four panels cannot be compared. Although, the NANOG antibody is common for all four panels. The FGFR1 and FGFR2 antibodies can have different affinities for their antigens. Hence, the fluorescence intensities cannot be compared between FGFR1 and FGFR2 samples. Further, the acquisition voltages for FACS were set based on control cells, stained with primary antibody isotype control and secondary fluorescent antibody. The background fluorescence for the FGFR1 and FGFR2 will be different for these as well. However, within FGFR1 or FGFR2 stained samples, the axes can be compared for SL and SLPD samples.

We agree with the referee that the NANOG expression is expected to show an increase in SLPD. We performed the quantifications for the median fluorescence of NANOG in SL and SLPD samples of FGFR1 and FGFR2 panels. We find a significant increase in NANOG expression in SLPD samples relative to SL in both FGFR1 and FGFR2 stained samples (on the right). However, the difference is not as drastic as seen in the TbC44Cre6 or TNERT cell lines. This could be due to two reasons.

(a) The TbC44Cre6 or TNERT have internal fluorescence of GFP from the Nanog locus, whereas in this experiment the fluorescence measures the endogenous NANOG protein by immunofluorescence.

(b) Further, most of the data points in the FACS data are seen between $10^5$ and $10^6$ decades, which is at the end of the scale in the graph. This was due to the high voltage setting during
FACS data acquisition. Although, this would not significantly affect the calculation of the correlation coefficient. It does not represent the data points beyond 10^6 decades, hence, can impact the estimation of expression levels. So, we have repeated the experiments with lower voltage settings to reliably estimate both the correlation coefficient and the expression levels.

The antibodies and double staining can sometimes be a source of artifacts in FACS. To minimize the possibilities of artifacts, we have performed additional FACS experiments using a different set of primary antibodies against the antigens. We have acquired the data by setting the acquisition voltages with cells stained with primary antibody isotype control and secondary fluorescent antibody. All the data points in the FACS are within the 10^5 decade scale. We have analyzed the correlation coefficient as well as the expression levels of NANOG, FGFR1, and FGFR2 (on the right). The expression is significantly increased in SLPD. The data has been replaced with the new data from these experiments in Fig. 4B (Fig. 3B of the previous version of the manuscript).

For the analysis shown in Fig. 3C, have the authors controlled for confounding variables, such as cell size?

Yes, the confounding variable like cell size had been controlled by plotting FSC/SSC and gating a narrow range of FSC. Even the cell doublets had been excluded by plotting the FSC height vs FSC area.

A more convincing way to establish the dependence between NANOG, FGFR and pERK expression would be to use the NANOG inducible cell lines that the authors have, and to blot for FGFR and pERK after different induction times (I notice that the authors have working antibodies for both FGF receptors). Adding these additional experiments would put more emphasis on the paper's main message and significantly strengthen the work.

We cannot agree more with the referees. We have performed western blot of FGFRs and pERK in the TNERT cell line. The OHT treatment can induce translocation of NANOG into the nucleus in 5-10 minutes. We have analyzed the expression of FGFRs and pERK at one-hour time intervals after OHT treatment. FGFRs were induced in OHT treated samples which increased gradually over time. The induction was more pronounced in FGFR2. The pERK levels also increase gradually over time, suggesting NANOG induces FGFRs and
pERK in ESCs. The data is now added to the main figure as Fig 3E. We have also made appropriate edits to the text of the manuscript.

4. Page 4: "... and discounts that direct role of NANOG..." This statement is too strong and should be reworded. The cell-extrinsic mechanism could well work in addition to the previously described cell-intrinsic ones.

   We have reworded the statement to “suggesting that autoregulation predominately operates via non-cell autonomous mechanisms besides the mechanism proposed earlier (Fidalgo et al., 2012).”

5. Page 4: "...suggests that ZFP281 is dispensable..." I disagree with this statement, particularly because EV1K shows higher NANOG reporter expression in Zfp281 mutant cells compared to the wild type. If I a missing something here please clarify.

   We apologize for creating confusion. This could be due to improper wording used by us to describe this result. Now we have reworded the statement to convey the meaning of the result.

   We confer with observations of the referees that Nanog reporter expression in the TNERT Zfp281 mutant line is higher than in TNERT cells. This suggests that Zfp281 is essential for the repression of expression in agreement with a previous report (Fidalgo et al, 2011).

   The induction of NANOG in the TNERTZfp281 mutant line with OHT was able to repress the Nanog:GFP (similar to TNERT + OHT) suggesting that the Nanog autorepression is still functional in TNERTZfp281-/- cells. If ZFP281 was essential for autoregulation; OHT induction in TNERTZfp281-/- should not have repressed Nanog:GFP. The results should have resembled the TNERT FGF4-/- or FGFR2-/- cells. So our results show that ZFP281 is essential for Nanog repression, but does not contribute to Nanog autorepression.

6. Fig. 5A, B: This data should be shown at higher resolution, using a smaller field of view. As it is presented right now, it does not support the authors point. Furthermore, the authors need to describe how the data in 5B have been generated from the data in 5A. Have they segmented the cells, and if so, how? How did they assign cytoplasmic pERK to nuclear NANOG?

   We have repeated the experiments and acquired high-resolution images by confocal microscopy. We have replaced the low-resolution images with higher resolution images.

   For immunostaining, along with NANOG and pERK antibodies, we have utilized the E-Cadherin antibody to mark the boundaries of the cells. The cells in the colonies were segmented manually using E-Cadherin or phalloidin staining in imageJ software. The Mean
fluorescence intensity (MFI) was estimated within the marked boundary in imageJ software. MFI from around 200 cells and correlation plots were plotted.

7. Fig. 5D: Are these measurements derived from FACS data? As pointed out above, using flow cytometry to make these measurements is prone to artifacts. I recommend the authors repeat this experiment, but use western blotting to measure NANOG and pERK levels. This is an doable experiment that could substantiate the link between NANOG and pERK dynamics.

Allaying the skepticism of the referees with possible artifacts from FACS measurements for this experiment, we have repeated the experiments. We have analyzed the NANOG and pERK expression and correlation using confocal microscopy and western blots. In previous FACS experiments, we had cultured the cells in fresh media after sorting. This would remove the impact of the factors (FGF4 and FGFBP1) secreted by the cells into the media. This would affect the pERK levels in the sorted cells. Hence, an inverse correlation was observed between NANOG and pERK in previous FACS experiments in Fig. 5C, D, as pointed out by referee #3. To circumvent this possibility, in the new experiments, we collected the media from the cells before cell sorting and filtered it through 0.22 um filters. The same media was used for culturing the sorted cells. Microscopy and western blot analyses were performed on cells at 4 hr time intervals till 16 hrs. we observe a correlation between the pERK and NANOG western blot and immunostaining. The previous FACS has been replaced with the new one in Fig. 6C, D and the results section has been modified accordingly.

Minor points
1. Fig. 1G: ppERK levels in SL2i seem to be similar to those in SLPD, in contrast to the proposed role of the GSK3 inhibitor in activating MEK. Can the authors comment on this observation?

This is an interesting and keen observation made by the referee. Multiple experiments in our lab which do not directly pertain to the main work of this manuscript have shown that dynamics and levels of pERK in SL2i are intermediate to SLPD and 2iL.

The MEK activity in 2iL increase over a period of time and by 8 hours a significant increase in MEK activity can be observed. In SL2i the MEK activity increase is not as much as 2iL after 24 hrs (Fig EV1C).

The serum contains growth factors, cytokines, and hormones like BMP4, FGF ligands, Insulin, etc. these factors may activate MEK and enhance pERK. In serum-free media like N2B27 (used in 2iL) these factors are missing, hence we expect consistent repression of MEK activity. However, our observations of an increase in MEK activity in 2iL are paradoxical to this logic. We do not have a definitive answer to this paradox. We think some confounding factors present in the serum might be counteracting the role of the GSK3 inhibitor role in activating MEK. These factors are absent in serum-free (2iL) media resulting in a significant increase in MEK activity in 2iL.
2. Fig. EV1L: The different conditions are difficult to distinguish in this plot. Consider a different representation.

We have changed the colors of the plots. All conditioned media where Nanog:GFP is not repressed (TNERT+OHT 0hrs, TNERTFgf4/-, and TbC44Cre6) are plotted in shades of blue. The conditioned media where Nanog:GFP is repressed (TNERT+OHT 48hrs, E14Tg2aFgf4 OE, and 50ngFgf4) are plotted in shades of maroon. Changes have been made in the description of the results to facilitate the interpretation of the data. We hope it would improve the clarity of these results. Now, this is Fig 3L in the revised manuscript.

3. Discussion section: "...specify primitive endoderm by cell-autonomous mechanisms ..." should read. "...non-cell-autonomous..."

Thank you very much for pointing out this error, we have edited the text to insert the correction.

4. Discussion section: The authors could more clearly spell out the possibility that NANOG heterogeneity is a consequence of de-synchronized cycling of cells through the NANOG-pERK feedback loop. They can also cite experiments with sorted Nanog-reporter cells (e.g. PMID: 19582141).

Thanks for the suggestion, we added this possibility into the discussion section.

5. Molotkov et al. (2017, PMID: 28552557) have demonstrated a central role for FGFR1 and not FGFR2 for pluripotency and differentiation. The authors need to discuss their results in the light of these previous findings.

We have addressed this aspect in the discussion section.

6. Fig. S6: The NANOG staining looks blurred. Please provide higher resolution / smaller field of view representations of this data.

We have replaced the images with higher resolution and a larger field of view in Appendix Figure S1. We have added high-resolution smaller field of view images to Fig 6E.

Overall I found the paper hard to review. Page and line numbers as well as a clearer labelling of the figures would have been helpful here. It would also have been helpful to include journal names in the reference list.

We apologize for the inconvenience. We have added the page numbers, and line numbers improved the labelling of the figures and reformatted the reference list to include the journal names. We hope the revised manuscript reads better and clearer.
Referee #2:

In this manuscript, Kale and colleagues present evidence of a Nanog-pERK reciprocal regulatory circuit. The focus of the paper is on Nanog autorepression, Nanog induction of components of the FGF/MEK/ERK pathway, MEK/ERK regulation of Nanog transcript and really nice analysis of Nanog/pERK dynamics. The manuscript presents some interesting findings and would be of interest to the community. The main issue in my view is the focus on the MEK/ERK having a role in Nanog AUTOrepression rather than just ‘repression’. There are a number of observations that I struggle to see how they fit the narrative presented in the paper, but that hopefully the authors can clarify.

We thank the referee for considering the findings interesting and of interest to the community. We relate to the importance of the issue raised by the referees about “the focus on MEK/ERK having a role in Nanog AUTOrepression rather than just ‘repression’”. Thanks for highlighting the clear distinction between the Nanog AUTOrepression and just “repression”.

MEK/ERK and FGF4’s role in Nanog repression is well established. Similarly, TCF3, GATA6, GATA4, TCF15, OTX2, ZFP281, SNAI1, and AMPK also have been reported to repress Nanog expression (Chae & Broxmeyer, 2009; Davies et al, 2013; Di Giovannantonio et al, 2021; Fidalgo et al., 2011; Galvagni & Neri, 2015; Pereira et al, 2006; Schröter et al, 2015; Singh et al, 2007). These molecules may be induced in different contexts and by different mechanisms leading to the repression of Nanog. Whereas, Nanog autorepression is a distinct context where increased levels of NANOG, induce a negative feedback regulatory mechanism to limit Nanog expression levels within those cells. It seemed logical to us, to the hypothesis that Nanog might activate and recruit one or more of the repressive mechanisms for its feedback autorepression, rather than evolve a distinct repressive mechanism for autorepression. Among these repressive mechanisms, ERK signaling was found to be essential for autorepression in our initial experiments. We investigated the possible role of other repressive mechanisms in autorepression -TCF15 (Fig EV5A), ZFP281 (Fig 3K), and TCF3 (given below). Although these factors are essential for Nanog repression they do not seem to be critical for Nanog autorepression. Along with the essential role of ERK signaling in Nanog repression, our work suggests that ERK signaling is activated by Nanog to execute its own feedback autorepression.
The essential role of ERK signaling in Nanog repression is demonstrated by earlier reports, here we have focused predominately on the role of ERK signaling in Nanog AUTorepression. We have performed additional experiments and analyses to clarify all the comments of the referees.

Major points:
- "[...] (Figure 2A, Appendix Fig S2C and D). The data from distinct induction systems conclusively establish and essential role of MEK1/2 in Nanog autoregulation". I struggle to interpret these results because the phenotypes associated with Nanog expression on a null-background are very subtle compared to the effect of adding PD. To put it in another way, if we look at figure 2A, the median GFP level in conditions that contain PD ~3x higher than SL alone in the absence of OHT. Since TNERT cells -OHT are Nanog-null, it suggests whatever PD is doing to the Nanog locus is independent of Nanog. Addition of Nanog seems to have a very minor effect. Similar to above, deleting elements NBR1 and NBR2 also changes the expression of Nanog:GFP independently of Nanog expression (Figure 3F).

We agree with the observation of the referees that the median GFP level in SLPD is multiple folds higher relative to SL in TNERT in Fig. 2A. We also agree with the conclusion drawn by the referees that “it suggests whatever PD is doing to the Nanog locus is independent of Nanog”. This conclusion is true when comparing partial data, about only -OHT samples of SL and SLPD. This conclusion is incomplete and pertains to repression (just repression) of Nanog, but not autorepression. FGF, INSULIN, and other ligands present in the serum can activate basal levels of ERK signaling in TNERT cultured in SL. These moderate ERK levels can induce repression of the Nanog locus as seen by the lower median GFP levels in TNERT in SL relative to SLPD. This suggests that Nanog repression (just repression) is independent of NANOG but dependent on ERK activation mediated by factors. Hence the median GFP levels in SL are multiple folds less than SLPD where ERK signaling is completely abrogated. When NANOG is induced in TNERT by adding OHT (TNERT+OHT) the Nanog locus is further repressed. This repression is due to induction of Nanog in the cells and hence this decrease in median GFP levels relative to -OHT represents Nanog Autorepression. However, in the presence of PD where pERK is absent, OHT induction of NANOG fails to repress the Nanog locus and the median GFP levels remain unchanged. Repression is dependent on ERK signaling, Autorepression is dependent on both Nanog and ERK signaling. These data suggest that ERK is essential for just repression and as well as autorepression of Nanog.

Addition of Nanog seems to have a very minor effect.

We also agree that the addition of Nanog seems to have a minor effect on Nanog repression compared to the difference seen between the SL and SLPD. However, this difference is significant and reproducible. Multiple serum factors in SL, feed into ERK signaling continuously to repress Nanog. This results in a consistent and high magnitude of
repression of Nanog in SL relative to SLPD. The addition of Nanog in SL enhances ERK signaling to repress Nanog further. Nanog autorepression is one more layer of repression that acts in addition to the regular repression. This notion is also shared by referee #1. The reasons for the minor effect could be (a) The base level of Nanog expression used for comparison of Nanog autorepression is already too low in TNERT-OHT cultured in SL. It might not be appropriate to compare the difference between SL and SLPD using SLPD as the base level; with +OHT and -OHT in SL using -OHT as the base level. These two contexts are different - one is repression the other is autorepression. (b) Nanog autorepression is a feedback repression mechanism to reduce the expression levels of Nanog in the cell when it exceeds a threshold limit. Logically it should limit the expression of Nanog only to the extent of the excessive expression beyond the threshold limit. This is also clear from our data (figure EV4 A-C), where mere expression of Nanog does not induce autorepression, but it is induced only when Nanog is overexpressed. The magnitude of the difference in repression also appears to be dependent on the over-expression levels of Nanog. (c) Further, the TNERT cell line is similar to the Nanog autorepression experimental system (NERTC3) used by Navarro et al. The magnitude of Nanog autorepression observed by us is similar to and consistent with the data of Navarro et al. 2012.

Similar to above, deleting elements NBR1 and NBR2 also changes the expression of Nanog:GFP independently of Nanog expression (Figure 3F).

The NBR1 and NBR2 regions encompass consensus sequences for NANOG binding as well as for multiple other factors (Browser track of CHIP data given below). NANOG is one of the many factors that binds to these regions and activate FGFR2. Deletion of NBR1 and NBR2 is likely to change the expression of Nanog:GFP independent of Nanog. Our data do not suggest/claim that NBR1 and NBR2 are exclusively dependent on Nanog expression to change Nanog:GFP. Instead, our data suggest that NBR2 is essential for NANOG to repress Nanog:GFP. The possibility of NBR1 and NBR2 regions being regulated by other factors leading to Nanog repression in a different context cannot be ruled out.

- "Nanog induces and enhances FGF autocrine signaling through FGFR2 to execute Nanog autoregulation" Can the authors also confirm that mutants of Figure 2D-E show altered levels of pERK? Do these mutants in -OHT conditions also show elevated Nanog:GFP expression compared to control lines, similar to addition of PD? Similarly, to justify the previous statements, the authors would need to show that Nanog induction in the mutant lines does not alter ERK levels.

We have analyzed the pERK levels in all mutant lines- Fgfr2/-/-, Fgfbp1/-/-, Fgf4/-/-, and Fgfr1/-/-. We observed an overall reduction in the pERK levels in all mutants relative to TNERT. Fgfr1/-/- did not show very significant changes in the pERK levels. Further, the
induction of Nanog by the addition of OHT did not significantly enhance the pERK activity in these mutants. The data is provided in part 3 (iii) of the response to Referee #1.

We have performed experiments to compare the expression of Nanog:GFP in -OHT condition of all the mutants with the control TNERT line. We do observe an increase in the Nanog:GFP expression in all the mutant lines. The magnitude of increase in Nanog:GFP is different in all mutant lines. The highest increase was seen in the ligand mutant (Fgf4-/-) which was relatively close to PD. None of the mutants showed Nanog:GFP expression equivalent to PD treatment (on the right). This suggests pERK might also be activated by other growth factor pathways to some extent in ESCs.

- Could the authors please explain some inconsistency of results? In figure EV1E, +OHT addition causes very high levels of pERK. However, in figure 4C, +OHT has no effect of pERK levels. The ability of Nanog expression to induce pERK is a central aspect so it’s a bit worrying to see this discrepancy.

We thank the referees for pointing out seemingly inconsistent results. The increase in pERK levels in +OHT is not obvious in the representative western blot images due to a smudge in the TNERT+OHT samples in Fig 4C (now Fig. 5C). However, The western blot quantification data corresponding to Fig 4C which was provided in Fig. S5D clearly showed a significant increase in pERK levels from four independent experiments. We are sorry for the confusion arising due to poor quality representative blots in Fig 4C. We have replaced the blots with another set of representative blots without smudges to represent the results clearly in the revised manuscript in Fig 5C (previously Fig 4C). The quantification data remains the same in Fig EV5D (previous Fig. S5D)

- on a related note, it is also unclear what is happening in the Nono-null cells. These cells overall show much lower levels of Nanog:GFP compared to untargeted controls (Figure Appendix 5C). Since Figure 4C shows pERK is nearly absent in Nono-null cells, I would expect these cells phenocopy to some extent what happens in PD conditions and show much higher Nanog:GFP.

We were also puzzled by the lower levels of Nanog:GFP in TNERTNono-/- cells relative to TNERT. We have analyzed multiple independent clones of TNERTNono-/-.. The Nanog:GFP level was found to be low in all the clones. We do not understand, why the Nanog:GFP levels are low in TNERTNono-/- cells contrary to the expected increase in GFP expression? At this juncture, we can only speculate on possible reasons. TNERTNono-/- lines carry null mutations of both Nanog and Nono genes. The anomalous behavior of Nanog:GFP could be a result of epistatic interactions between these two mutations. We do not have any evidence to confirm this. Alternately, we have deleted the Nono gene in E14Tg2a ESCs to understand its
effect on Nanog expression without interference with any other mutations. We have performed western blot and immunostaining to assess the impact of the loss of NONO on pERK and NANOG. The Nono-/- cells showed low levels of PERK and enhanced expression of Nanog as expected, suggesting that Nono is essential for sustaining pERK levels and repression of Nanog (data given below). The anomalous behavior of Nanog:GFP in TNERTNono-/- cells is interesting but does not in any way impact the central findings and conclusions of the manuscript. We have not included any discussion about this aspect in the manuscript.

- Nono and pERK occupancy on Nanog locus. How does FGF and PD treatment affect Nono expression? The changes in pERK binding at Nanog locus are unsurprising given that pERK levels are dictated by the culture treatment. Is the same for Nono? This is important since the authors claim that "pERK is essential
for the recruitment of NONO to the Nanog locus“.

- We have performed additional western blot analysis for NONO expression in FGF4 and PD treatment. The result shows that the expression of NONO correlates with pERK expression. The NONO expression was increased in FGF4 and reduced in PD treatment. The related data is added to Fig 5E.
- Yes, the changes in NONO binding on the Nanog locus are mostly similar to pERK. The occupancy of NONO was generally increased in FGF4 and reduced in PD treatment. This data was already provided in Fig 4H (Fig 5H in the revised manuscript) of the manuscript.

- all chip-seq and chip-PCR data of the paper. Can the authors please also include a control region for a known/validated target of pERK and Nono to show that the levels of binding at the Nanog locus are biologically relevant? Since pERK levels vary greatly with the treatment, and the binding does not appear enriched in any particular region, it would help to show that the chip signal observed are not just the result of higher background.

We agree with the referees. We had already analyzed the enrichment of pERK, NONO, H3K27, H3K4, and Pol2 in known validated targets (positive control) and a non-genic region (negative control). Now, we provided this data in Fig EV5G.

- Interpretation of the Nono/pERK/Pol2 binding results. Also here I find the interpretation a bit confusing. The authors say "pERK-NONO affects POL2 loading onto the Nanog locus preventing the initiation of transcription" but there is no evidence that the process is at all dependent on Nono. If this was the case, the Nono-null cells would have higher Nanog:GFP expression, but the opposite appears to be the case.

We had provided evidence for the dependence of this process on pERK in Fig 4I (Now Fig 5I). We have carried out ChIP-qPCR to evaluate POL2 occupancy on the Nanog locus in Nono-/- ESCs (the Nono-/- cell line is described in the above section on the previous page). The occupancy of POL2 on the Nanog locus was significantly increased in the Nono-/- cell relative to wildtype. The data is now added in Fig EV5F. Our data shows that either loss of pERK or NONO can affect the other, eventually affecting POL2 loading onto the Nanog locus.

Other points:
- introduction: The introduction is very short, and readers would benefit from key topics being included. For example, there is no mention of the literature on the role of ERK; 2iLIF culture is not introduced but CH and PD inhibitors are used regularly; and no description of provided as to what NONO is. The literature is
also not always accurately cited (e.g. {Faddah:2013ed, Filipczyk:2013kp} convincingly disproved the earlier report of {Miyanari:2012ef} about moni-allelic regulation of Nanog).

Thanks for the suggestions, we have made changes in the introduction section to address these points.

- First results section (Figure 1). The authors refer to any changes in Nanog in terms of the hypothesised autorepression ("Nanog expression was derepressed above basal levels", "Nanog expression in SPDL indicates inefficient repression", etc). This is not a very helpful description, since the authors present very little evidence at this point that autorepression is involved. I suggest describing the results without assigning a mechanism (e.g. "results in increased expression").

Our intention of using the terms “derepressed” and “repression” in the first results section was in the context of the well-known Nanog repression phenomenon and not the hypothesized autorepression. We agree with the referee that describing the result without ascribing to any mechanisms would be appropriate at this stage of the manuscript. We have made necessary edits accordingly in the manuscript text.

- Please provide some information on the levels, heterogeneity, and timing of induction for both TNERT and inducible Nanog lines.

  - The TNERT and TDiN cell lines clones with a similar or higher expression level of Nanog compared to the WT ESC were used in this study. The clones which expressed lower levels of Nanog did not show Nanog autorepression and they were not used for any experiments.
  - None of these clones showed heterogeneity of NANOG expression.
  - The timing of induction for TNERT and inducible Nanog lines was already described in the Methods section – “The cells were cultured as described earlier (Festuccia et al, 2012). 4-Hydroxytamoxifen (4-OHT), Doxycycline, and Cycloheximide were used at a concentration of 1 μg/ml, 1 μg/ml, and 100 μg/ml respectively. The TNERT and its derivative cell lines were treated with 4-OHT for 18 hrs except when indicated. TDiN and EDiN were treated with Doxycycline for 48 hrs unless indicated.”

- Figure 2D-E. Can this be confirmed through an alternative method? Maybe RT-qPCR on Nanog UTR, etc? again, the phenotypes are very subtle.
We have performed RT-qPCR analysis to assess Nanog:GFP transcript using a primer set corresponding to the 5’UTR region of Nanog mRNA. A significant reduction was observed in transcript levels after OHT treatment in TNERT and TNERTFgfr1-/- cells. No significant change was observed in Fgfr2, Fgf4, and Fgfbp1 mutant lines upon OHT treatment. It is tempting to cross-compare the magnitude of differences between the FACS and qPCR data. However, in FACS, we are analyzing the GFP levels which will depend on the protein stability, and in qPCR, we are comparing the transcript which would follow entirely different dynamics. However, both the data suggest that the Nanog locus is repressed in TNERT and TNERTFgfr1-/- cells upon induction of NANOG by OHT. The data is now presented in Fig 2F. We have also analyzed the Nanog:GFP in all the mutants relative to TNERT, we observe that the transcript levels are increased in all the mutants relative to TNERT (data given on the right).

- please indicate clearly for all figures what the statistics are.

The detailed statistics information was provided in the methods section. Now we have also included it in all the figure legends.

- Beginning of result section 2: lots of acronyms are given to different lines, and the inserts within lines (e.g. NANOGER vs TNERT, etc). This creates more confusion than necessary. I suggest consolidating the nomenclature.

We have cut down on the acronyms for the inserts within cell lines to reduce the acronyms and limited the nomenclature to the names of different cell lines.

- please provide some info on the figure as to what Figure 2C shows

We have described more detailed information about Figure 2C in the result section.

- in the literature it’s more common to find non cell-autonomous than "cell non-autonomous" as the authors describe.

Thanks for pointing out this error. We have edited the text appropriately as suggested.
Kale et al explore mechanisms underpinning Nanog autoregulation in ES cells using a series of inducible reporters and cell lines. They conclude that Nanog regulates expression of FGF signalling components such as FGFBP1 and FGFR2 to control FGF-mediated ERK1/2 activation, which in turn leads to high levels of ERK activity in cells with high Nanog expression. This leads to recruitment of NONO to the Nanog locus, which suppresses PolII loading and Nanog transcription to establish autoregulation.

In this area, it is well established that ERK1/2 signalling drives Nanog heterogeneity in ES cells, although this is not discussed or referenced at all in the introduction. Therefore, it does not take much of a leap to consider that because ERK1/2 signalling drives Nanog heterogeneity, it might also underpin Nanog autoregulation. As a result, in my view the conceptual advance presented here is somewhat limited.

We thank the referee for the critical review and suggestions which helped us to improve the quality of the manuscript. We have addressed all the comments of the referee. We beg to differ with the view of the referee about the conceptual advance presented in this manuscript. We would like to present our case with more information and our point of view to persuade the referee to reconsider their opinion on the conceptual advances presented in this manuscript.

We have discussed and referenced the essential function of ERK1/2 signalling in Nanog heterogeneity in the relevant context in the results and discussion section. We agree it would have been more appropriate to discuss and reference it in the introduction. Now, we have included the discussion of the function of ERK1/2 signalling in Nanog heterogeneity in the introduction of the revised manuscript.

The referee’s suggestion “that because ERK1/2 signalling drives Nanog heterogeneity, it might also underpin Nanog autoregulation” sounds like an obvious possibility. However, there is no literature that suggests anywhere in this direction. The available literature indicates the contrary.

(a) Among the two studies which reported Nanog autorepression (Fidalgo et al. 2012 and Navarro et al. 2012), Navarro et al. suggested that ERK signalling is dispensable for Nanog autorepression. Navarro et al. state “Overall, these results suggest that Nanog autorepression persists in ‘2i + LIF’ and is therefore independent of the MEK/GSK3 signaling pathways.”

(b) ERK signalling is essential for heterogeneous expression of pluripotency factors Rex1, KLF4, and Nanog (Marks et al., 2012; Wray et al., 2010). If ERK signalling can underpin Nanog autoregulation then it might also underpin autorepression of Rex1 and KLF4. However, there is no report of the autorepression phenomenon of Rex1 or KLF4 in ESCs. KLF4 autorepression is reported in breast cancer cells by an entirely different mechanism involving miRNA (Lin et al., 2011).

Furthermore, Nanog has been previously proposed to modulate FGF signalling, but by a different mechanism in which Nanog drives expression of FGF4 and
suppression of FGFR2 in Nanog high cells. This enables segregation of Nanog high and low populations based on distinct FGF-ERK1/2 signalling responses (reviewed in Lanner and Rossant 2010). However, this study proposes the opposite, that Nanog expression leads to ERK1/2 activation in Nanog high cells.

We have carefully studied Lanner and Rossant’s review of 2010. We did not find any information suggesting Nanog drives expression of FGF4 and suppression of FGFR2. On the contrary, they have proposed a model (Fig 3, Lanner and Rossant 2010) where FGF/ERK is suggested to function upstream of Nanog and Gata6. They cite Guo et al. 2010 -“detected a stronger and earlier inverse correlation between Fgf4 and Fgfr2 transcripts in ICM cells than between Nanog and Gata6, supporting a model in which FGF/Erk signaling variation is upstream of Nanog and Gata6 (Fig. 3)” - to support their model. This review does not describe Nanog high and Nanog low cells, they describe Nanog and Gata6 expressing cells in the context of segregation of ICM cells into primitive endoderm and epiblast. We do not think Nanog low cells can be equated to Gata6 positive cells in ICM. Cells expressing Gata6 would have mostly committed to primitive endoderm, whereas most of the Nanog low cells can rise to Nanog high cells (Chambers et al., 2007).

We agree with the referee that Nanog was shown to function upstream of FGF4. But, this was reported later by Frankenberg et al. in 2011(Frankenberg et al., 2011). We have not been able to find any report suggesting Nanog suppresses Fgfr2, so we would like to politely disagree with the referee on this part of the comment. Our data shows that Fgfr2 is induced by Nanog and Fgfr2 is essential for autorepression. Although Nanog is shown to be essential for Fgf4 induction, it is a secreted protein. It cannot distinguish between Nanog high and low cells. Whereas FGFR2 is induced by Nanog and retained on the membrane of Nanog high cells. This can help the FGF4 ligand to distinguish between the Nanog high and low cells and induce stronger ERK signalling in Nanog high cells. This can lead to induction of Nanog autorepression predominately in the Nanog high cells. Our data (Fig 4A) and the logic of Nanog autorepression proposed by Fidalgo et al. and Navarro et al.(Fidalgo et al., 2012; Navarro et al., 2012) suggest that Nanog autorepression operates in Nanog high cells to prevent Nanog levels from crossing levels which can inhibit differentiation of ESC.

However, from what I can see, the data presented do not fully support this - in Fig5D Nanog and pERK1/2 staining show an inverse correlation, which is opposite to what the authors are proposing. My concern is that in such a dynamic system, it is difficult to confidently distinguish signalling and transcriptional responses in distinct populations of Nanog low and Nanog high cells. Thus, there is a risk that what is being reported here simply reflects what is already known - i.e. that Nanog is regulated by and regulates ERK1/2 signalling.

We agree with the referee, that the data presented by us in Fig 5D showed an inverse correlation between Nanog and pERK. This may not necessarily mean the opposite of what we are proposing. Since Nanog induces the Fgfr, Fgf4, and Fgfbp1 to activate pERK. There will be a time lag between the highest level of Nanog and the highest levels of pERK, with intermediate levels. However, this may not generate a complete inverse correlation as observed in Fig 5D.
After careful examination of the data and the experimental design, we recognized a flaw in our experimental design. We apologize for this mistake. Nanog autorepression functions only in Nanog high population. So, we sorted the top 10% Nanog high cells and cultured them to follow the dynamics of NANOG and pERK during the course of culture. In our experiments, we used fresh media to culture the cells. This was a mistake, by using fresh media we have effectively removed the impact of the factors secreted from the cells on the dynamics of NANOG and pERK. The right experiment would have been to culture the cells in the media collected from the cells before sorting Nanog high cells. This would retain all the secreted factors and allow further progression of the trajectory of NANOG and pERK. We think the culture of cells in fresh media removed the induction of FGF signaling by the secreted proteins and led to reduction in pERK resulting in an inverse correlation with NANOG.

We have repeated the experiments considering all these factors with changes in the experimental design. The media from the cells was collected before cell sorting and filtered through a 0.22 μM filter. This media was used for culturing the cells to ensure the autocrine signaling factors secreted by the cells were retained. We analyzed NANOG and pERK expression every 4 hr intervals. Referee #1 had expressed reservation about FACS artifacts. Hence, we performed immunofluorescence and western blot analysis of these cells. Both data show a positive correlation between NANOG and pERK. They follow a similar pattern over a period of 16 hrs, with high expression at 8 hrs and low expression by 16 hrs. This behavior of NANOG and pERK expression is consistent with our suggestion that NANOG and pERK feed into each other and cycle through different intermediate levels of NANOG and pERK in culture.

The area that may be of most interest and novelty is the mechanistic interplay between ERK1/2 signalling and Nanog at the transcriptional level. Is pERK1/2 staining also heterogeneous in regular mouse ES cells cultured in LIF/FCS, which exhibit dynamic Nanog heterogeneity? This would provide evidence that ERK1/2 signalling is not simply influenced by the distinct domed morphology observed in Nanog high EDiN colonies. And is pERK1/2 staining in Fig 5A specific? This can be controlled using MEK1/2 inhibitor PD0325901. Finally, is pERK1/2 recruitment measured by ChIP in Fig 4G specific and inducible - what are the controls for non-specific background signals?

We have performed additional experiments to address all the questions.

- Yes, pERK1/2 was also heterogeneous in regular ESC cultured in SL, which exhibits dynamic Nanog heterogeneity. This data was already provided in Fig. 5 A. The results might not have been very clear due to the low resolution of the images. Now we have provided high resolution and high magnification images in Fig 6A, which clearly show heterogeneous expression of pERK.
- Yes, pERK staining is very specific. pERK can be seen as two puncta in mitotic cells (pERK has been shown to localize to spindle poles in mitosis (Shapiro et al, 1998). As suggested, we have also used PD treatment where pERK staining is completely lost (Fig 6A).
- Yes, the pERK1/2 recruitment measured by ChIP in Fig 4G (revised as Fig 5G) is specific and inducible. This is true for NONO as well. The inducibility can be seen in
the samples treated with FGF4 and PD in all the ChIP-qPCR s. We have analyzed the recruitment of pERK and other proteins shown in Fig 4G-K (revised a Fig 5G-K) to a non-genic region (negative control) and known validated target locus for each of these proteins. We do not observe significant enrichment of these proteins in the non-genic region but see significant enrichment in the known validated targets in the genome. The data is provided in Fig EV5G.

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Dear Dr. Shekar

Thank you for the re-submission of your revised manuscript to our editorial offices. I have now received the reports from three of the referees that have re-evaluated, you will find below. As you will see, the two referees now fully support publication of your study in EMBO reports. Both have some final points and suggestions to improve the manuscript.

Referee #3 was more critical, indicating novelty issues and conflicts to previously published data. During cross-commenting with the other referees indicated that they disagree with this assessment. They state:

- ‘Nanog-lo ESCs will almost never give rise to primitive endoderm in culture, in contrast to the situation in the ICM. I therefore think the authors are right to state that their mechanism operates in a different context, a context that could still be developmentally highly relevant.’

- ‘The comparison between PE/EPI segregation is, in my opinion, not particularly helpful since a whole genetic program (that of the PE cells) that is involved in establishing the reciprocal relationship between FGF4 and FGFR2 observed in the embryo.’

- ‘Moreover, the literature has proven that ESCs recapitulate a stage in development equivalent to the peri-implantation development, not the ICM. The Nanog low cells are not turning towards a primitive endoderm lineage. All evidence suggests these cells are differentiating further towards a post-implantation-like stage (pre-gastrulation). Therefore, it’s more likely that the autoregulation described here is part of the circuit that allows the naïve pluripotency to collapse as the embryo implants.’

Considering these comments, I decided to proceed with the manuscript and to ask you to address the remaining points of referees #1 and #2 in a final revised manuscript. Please also provide a final point-by-point-response to the referee concerns, including your specific responses to referee #3.

Moreover, the manuscript needs formatting according to our journal style. Please carefully review the instructions that follow below.

PLEASE NOTE THAT upon resubmission revised manuscripts are subjected to an initial quality control prior to exposition to re-review. Upon failure in the initial quality control, the manuscripts are sent back to the authors, which may lead to delays. Frequent reasons for such a failure are the lack of the data availability section (please see below) and the presence of statistics based on n=2 (the authors are then asked to present scatter plots or provide more data points).

When submitting your final revised manuscript, we will require:

1) a .docx formatted version of the final manuscript text (including legends for main figures, EV figures and tables), but without the figures included. Figure legends should be compiled at the end of the manuscript text.

2) individual production quality figure files as .eps, .tif, .jpg (one file per figure), of main figures and EV figures. Please upload these as separate, individual files upon re-submission.

The Expanded View format, which will be displayed in the main HTML of the paper in a collapsible format, has replaced the Supplementary information. You can submit up to 5 images as Expanded View. Please follow the nomenclature Figure EV1, Figure EV2 etc. The figure legend for these should be included in the main manuscript document file in a section called Expanded View Figure Legends after the main Figure Legends section. Additional Supplementary material should be supplied as a single pdf file labeled Appendix. The Appendix should have page numbers and needs to include a table of content on the first page (with page numbers) and legends for all content. Please follow the nomenclature Appendix Figure Sx, Appendix Table Sx etc. throughout the text, and also label the figures and tables according to this nomenclature.

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4) that primary datasets produced in this study (e.g. RNA-seq, ChIP-seq, structural and array data) are deposited in an appropriate public database. If no primary datasets have been deposited, please also state this in a dedicated section (e.g. 'No primary datasets have been generated and deposited'), see below.

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The accession numbers and database should be listed in a formal "Data Availability" section (placed after Materials & Methods) that follows the model below. This is now mandatory (like the COI statement). Please note that the Data Availability Section is restricted to new primary data that are part of this study. This section is mandatory. As indicated above, if no primary datasets have been deposited, please state this in this section

# Data availability

The datasets produced in this study are available in the following databases:

- RNA-Seq data: Gene Expression Omnibus GSE46843 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE46843)
- [data type]: [name of the resource] [accession number/identifier/doi] ([URL or identifiers.org/DATABASE:ACCESSION])

*** Note - All links should resolve to a page where the data can be accessed. ***

Moreover, I have these editorial requests:

6) As they are significantly cropped, please provide the source data for the Western blots shown in the manuscript (including the EV figures). The source data will be published in separate source data files online along with the accepted manuscript and will be linked to the relevant figures. Please submit scans of entire gels or blots together with the final revised manuscript. Please include size markers for scans of entire gels, label the scans with figure and panel number, and send one PDF file per figure (main and EV figures).

7) Our journal encourages inclusion of *data citations in the reference list* to directly cite datasets that were re-used and obtained from public databases. Data citations in the article text are distinct from normal bibliographical citations and should directly link to the database records from which the data can be accessed. In the main text, data citations are formatted as follows: "Data ref: Smith et al, 2001" or "Data ref: NCBI Sequence Read Archive PRJNA342805, 2017". In the Reference list, data citations must be labeled with "[DATASET]". A data reference must provide the database name, accession number/identifiers and a resolvable link to the landing page from which the data can be accessed at the end of the reference. Further instructions are available at: http://www.embopress.org/page/journal/14693178/authorguide#referencesformat

8) Regarding data quantification and statistics, please make sure that the number "n" for how many independent experiments were performed, their nature (biological versus technical replicates), the bars and error bars (e.g. SEM, SD) and the test used to calculate p-values is indicated in the respective figure legends (also for potential EV figures and all those in the final Appendix). Please also check that all the p-values are explained in the legend, and that these fit to those shown in the figure. Please provide statistical testing where applicable. Please add complete statistical testing to all diagrams (for main, EV figures). Please also indicate (e.g. with n.s.) if testing was performed, but the differences are not significant. Please avoid the phrase 'independent experiment', but clearly state if these were biological or technical replicates. See also: http://www.embopress.org/page/journal/14693178/authorguide#statisticalanalysis

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11) We updated our journal's competing interests policy in January 2022 and request authors to consider both actual and perceived competing interests. Please review the policy https://www.embopress.org/competing-interests and update your competing interests if necessary. Please name this section 'Disclosure and Competing Interests Statement' and put it after the Acknowledgements section.

12) Please make sure that all the funding information is entered into the online submission system and is complete and similar to the one in the acknowledgements section of the manuscript text file.
13) The colour coding of panels A and B of Fig. 3 is not ideal, as it is difficult to distinguish adjacent bars. Please try to depict this differently.

14) Please make sure that the manuscript sections are ordered like this:
Title page - Abstract - Introduction - Results - Discussion - Materials and Methods - Data availability section (DAS) -
Acknowledgements - Author contributions - Disclosure and Competing Interests Statement - References - Figure legends -
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In addition, I would need from you:
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- three to four short one sentence bullet points highlighting the key findings of your study.
- a schematic summary figure (synopsis image) in jpeg or tiff format with the exact width of 550 pixels and a height of not more than 400 pixels that can be used as a visual synopsis on our website.

I look forward to seeing a revised version of your manuscript when it is ready. Please let me know if you have questions or comments regarding the revision.

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Best,

Achim Breiling
Senior Editor
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Referee #1:

In the revised version of their manuscript, the authors have added an extensive set of additional experiments that address a large number of my concerns raised in the first round of review. They have also re-organized some of the figures, and re-written the manuscript accordingly. Overall, the new analyses largely support their original claims. One exception is the data in the new Figure 6D, where, in contrast to the previous version where pERK and NANOG were anticorrelated, the authors now find a positive correlation. This discrepancy however does not necessarily invalidate the author's model, it rather indicates that the timescales over which the proposed regulatory circuit operates are still not fully understood.

I notice that the ChIP-qPCR data in Fig. 3C,D and 5 G-K is still lacking negative controls. These had been requested by reviewers #2 and #3, and I agree that these controls are important to distinguish specific binding to the respective loci from a mere increase in background due to higher levels of the respective proteins. If I've been missing something here please clarify.

Overall, the manuscript now makes a convincing case for the existence of a new type of negative feedback loop acting on Nanog expression that relies on the FGF signaling system, and that might complement previously proposed mechanisms for Nanog autorepression. In my opinion, the work now merits publication in EMBO Reports.

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Referee #2:

In this revised manuscript the authors have answered some of the key concerns raised in the first round of reviewing. However, I would suggest that the western blot data of the ERK levels in the Fgf4, Fgfbp1 and Fgfr2 mutant lines {plus minus} OHT is an essential control and should be included in the paper. Similarly, the flow plots of the Nanog:GFP levels of the mutant lines in S/L should be included. This data is fundamental for showing that ERK activity is altered in the mutants.

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Referee #3:

In the revised version, the authors have done some control experiments to improve the technical quality of the paper. My main concern remains that the overall conceptual advance is limited, although the authors provide several arguments as to why this work goes beyond the large amount of literature on interplay between Nanog and FGF-ERK1/2 signalling in both ES cells and the early embryo.

Most of the work in this area supports the notion that FGF-ERK1/2 signalling suppresses Nanog expression, and that Nanog promotes FGF4 expression to establish an amplification loop. Indeed, the authors concede that Nanog knockout embryos fail to
express FGFR4 (Frankenberg et al, 2011), but suggest that Fgfr2 regulation has not been studied. However, Fgf4 and Fgfr2 exhibit reciprocal expression in the early embryo (Guo et al, 2010), reflecting Nanog hi/lo states. Therefore, the link between Nanog and FGF4/FGFR2 in the early embryo has been clearly established, and does not support the authors conclusions.

Another argument the authors present is that the context is different - either that this is specific to Nanog auto-regulation, or that Nanog hi/lo ES cells are not equivalent to the process of ICM segregating to Nanog hi EPI and Nanog lo PE in the embryo. Although strictly speaking Nanog heterogeneity represents a short window in embryonic development, ICM segregation to Nanog hi/lo cells nevertheless represents the developmental process most analagous to Nanog heterogeneity observed in ES cells. This raises a further concern that what is being reported here is specific to ES cells and is not relevant for development in vivo.
Referee #1:

In the revised version of their manuscript, the authors have added an extensive set of additional experiments that address a large number of my concerns raised in the first round of review. They have also re-organized some of the figures, and re-written the manuscript accordingly. Overall, the new analyses largely support their original claims. One exception is the data in the new Figure 6D, where, in contrast to the previous version where pERK and NANOG were anticorrelated, the authors now find a positive correlation. This discrepancy however does not necessarily invalidate the author's model, it rather indicates that the timescales over which the proposed regulatory circuit operates are still not fully understood.

Thanks for pointing out the discrepancy between the NANOG and pERK correlation between the previous version and the current version of Figure 6D. This difference is a result of the changes in the experimental design in the previous and current versions. We sorted the Nanog-high cells and analyzed the dynamics and correlation of NANOG and pERK over 16 hrs.

In the previous version after cell sorting, we cultured the cells in fresh media which eliminated the autocrine components secreted by the cells, which resulted in an anticorrelation between NANOG and pERK. We realized that to maintain the natural progression of NANOG and pERK dynamics, we should have retained the autocrine components secreted by the cells. To ensure this, in the current version, we cultured the cells in the same media collected from the cells before sorting. This ensured a natural progression of the NANOG and pERK dynamics, where we found a positive correlation between NANOG and pERK. In the light of the change in experimental design, both the results support the proposed model. As pointed out by the referee, these experiments are correlative and suggest that the timescales of operation of the regulatory circuit operate is still unknown.

I notice that the ChIP-qPCR data in Fig. 3C,D and 5G-K is still lacking negative controls. These had been requested by reviewers #2 and #3, and I agree that these controls are important to distinguish specific binding to the respective loci from a mere increase in background due to higher levels of the respective proteins. If I've been missing something here please clarify.

We have added the ChIP-qPCR data of the non-genic region (negative control) in Fig. 3C, D. The ChIP-qPCR data of a known validated target locus (positive control) and non-genic region (negative control) for Fig. 5G-K have already been provided in Fig. EV5G.

Overall, the manuscript now makes a convincing case for the existence of a new type of negative feedback loop acting on Nanog expression that relies on the FGF signaling system, and that might complement previously proposed mechanisms.
for Nanog autorepression. In my opinion, the work now merits publication in EMBO Reports.

Thank you very much for your time and constructive criticism for helping us to improve the quality of our work and the manuscript.

Referee #2:

In this revised manuscript the authors have answered some of the key concerns raised in the first round of reviewing. However, I would suggest that the western blot data of the ERK levels in the Fgf4, Fgfbp1 and Fgfr2 mutant lines (plus minus) OHT is an essential control and should be included in the paper. Similarly, the flow plots of the Nanog:GFP levels of the mutant lines in S/L should be included. This data is fundamental for showing that ERK activity is altered in the mutants.

We have included the flow plots and western data in Fig. 2 G & H respectively. Thank you very much for your time and constructive criticism to help us improve the quality of our work and manuscript.

Referee #3:

In the revised version, the authors have done some control experiments to improve the technical quality of the paper. My main concern remains that the overall conceptual advance is limited, although the authors provide several arguments as to why this work goes beyond the large amount of literature on interplay between Nanog and FGF-ERK1/2 signalling in both ES cells and the early embryo.

We thank the referee for their time and constructive criticism to help us improve the quality of our work and manuscript. We would like to clarify that we have performed all the experiments suggested by all the referees (including referee #3). We have generated new resources and performed additional experiments to further strengthen the findings presented in the manuscript.

We are sorry if the conceptual advances did not come through clearly in our manuscript or our response to referee #3’s previous comments. Here, we again attempt to provide the salient conceptual advances of the manuscript.

- The major work in this manuscript is focused on delineating the mechanism of Nanog autoregulation. Contrary to the current understanding that ERK signaling is dispensable for Nanog autorepression, our data demonstrate that Nanog autoregulation operates through ERK signaling.
• The existing literature on Nanog autorepression provides some molecular mechanisms but still cannot explain the basic logic of autorepression—how autorepression is triggered and operated only in the Nanog-high cells in the ESC population. Here we show a new mechanism for Nanog autorepression, which elucidates how NANOG concentration-dependent induction of FGFR2 in Nanog-high cells sensitizes the cells for FGF signaling and induces Nanog autorepression exclusively in Nanog-high cells.

• Dynamic and heterogeneous expression of pERK has been reported in ESCs and embryos. The mechanisms that induce pERK heterogeneity in ESCs and embryos are not understood. Our data show that Nanog heterogeneity can feed into pERK heterogeneity and vice versa through the Nanog autoregulation mechanism.

Most of the work in this area supports the notion that FGF-ERK1/2 signalling suppresses Nanog expression, and that Nanog promotes FGF4 expression to establish an amplification loop. Indeed, the authors concede that Nanog knockout embryos fail to express FGF4 (Frankenberg et al, 2011), but suggest that Fgfr2 regulation has not been studied.

The fact that Nanog function is essential for FGF4 secretion was discussed and cited from our first version of the manuscript in the results and discussion sections as given below. We have also explained - why the Fgf4 induction by Nanog cannot explain the logic of Nanog autoregulation, which prompted us to investigate FGFR further.

• “Cell non-autonomous function of Nanog in the induction of primitive endoderm (Frankenberg et al, 2011; Messerschmidt & Kemler, 2010) and essentiality of secreted proteins FGF4 and FGFBP1 in autoregulation prompted us to investigate cell non-autonomous mechanisms.”

• “Nanog is considered to induce FGF paracrine signaling through FGF4 secretion and specify primitive endoderm by non-cell-autonomous mechanisms (Frankenberg et al, 2011; Messerschmidt & Kemler, 2010). Although FGF4 is essential for Nanog autoregulation, it is a secreted protein. Its induction by NANOG can neither explain the functioning of autoregulation exclusively in Nanog-high cells nor the heterogenous pERK activation…….”.

However, Fgf4 and Fgfr2 exhibit reciprocal expression in the early embryo (Guo et al, 2010), reflecting Nanog hi/lo states. Therefore, the link between Nanog and FGF4/FGFR2 in the early embryo has been established, and does not support the authors conclusions.

We disagree with the comments of the referee. In our opinion, the Fgf4 and Fgfr2 inverse correlation reflecting Nanog hi/lo state in Guo et. al., 2010 may not be comparable to Nanog hi/lo ESCs. The link between Nanog and FGF4/FGFR2 established in early embryos may not be extrapolated to ESC for the following reasons.

• Guo et al. analyzed gene expression in single cells of 8C, 16C, 32C, and 64C stage embryos. The Fgf4 and Fgfr2 show inverse correlation in 32-cell and 64-cell ICM
in Fig. 5. In the same figure (Fig 5C &D) they clearly show that Fgf4 correlates with Nanog; Fgfr2 correlates with Gata4 and Gata6. This is due to the segregation of the EPI and the PE. The Nanog-low cells at this stage express Gata6 & Gata4 which have acquired PE fate, this fate choice is driven actively by a PE transcription network involving Gata6, Gata4, and Sox17. On the contrary, the Nanog-low cells in ESC culture, do not express Gata6 or Gata4 nor they are differentiated to PE. They retain pluripotency and can revert to Nanog-high states as shown by Chambers et al., 2007 and Kalmar et al., 2009.

- As suggested by other referees, it’s well established in the literature that ESCs are equivalent to the pre-implantation epiblast cells of the embryos and not the ICM (Nichols et al., 2009). We think, comparing the links of Nanog-Fgf4-Fgfr2 in 32-cell and 64-cell stage ICM to ESCs may not be relevant. In our opinion, it may be appropriate to compare ESCs to epiblast of later embryonic stages as described in the response to the below comments.

Another argument the authors present is that the context is different - either that this is specific to Nanog auto-regulation, or that Nanog hi/lo ES cells are not equivalent to the process of ICM segregating to Nanog hi EPI and Nanog lo PE in the embryo. Although strictly speaking Nanog heterogeneity represents a short window in embryonic development, ICM segregation to Nanog hi/lo cells nevertheless represents the developmental process most analogous to Nanog heterogeneity observed in ES cells. This raises a further concern that what is being reported here is specific to ES cells and is not relevant for development in vivo.

While we agree that Nanog heterogeneity represents a short window in embryonic development, we disagree with “ICM segregation to Nanog hi/lo cells nevertheless represents the developmental process most analogous to Nanog heterogeneity observed in ES cells”.

ESCs are shown to be the cell culture counterparts of the pre-implantation epiblast (Nichols et al., 2009). We suggest ESCs may be comparable to the Nanog hi/lo cells of the epiblast rather than ICM. Although Nanog expression is mostly homogenous in epiblast from E3.5 to E4.5, some of the cells lose Nanog expression and re-establish Nanog heterogeneity in the epiblast from E4.5-E4.7 as shown in Fig.1 of Acampora et al., 2016. Acampora et al., suggest that during the naïve-to-prime pluripotency transition, Nanog expression is heterogeneous in the epiblast and ESCs (Acampora et al, 2016, Acampora et al, 2017). The Nanog hi/lo cells in ESCs may be more analogous to the peri-implantation stage epiblast. Other referees have suggested that the Nanog autoregulation might be part of the circuit that allows exit from naïve pluripotency in the peri-implantation stages. In the light of this information, We suggest that the autoregulation mechanism and the reciprocal circuit being reported by us in ESCs is not an in vitro artifact, but a highly relevant mechanism for embryonic development, which opens up possibilities for further investigations.
References:

Frankenberg S, Gerbe F, Bessonnard S, Belville C, Pouchin P, Bardot O, Chazaud C (2011) Primitive endoderm differentiates via a three-step mechanism involving Nanog and RTK signaling. Developmental cell 21: 1005-1013

Messerschmidt DM, Kemler R (2010) Nanog is required for primitive endoderm formation through a non-self-autonomous mechanism. Developmental biology 344: 129-137

Nichols J, Silva J, Roode M, Smith A (2009) Suppression of Erk signaling marks ground state pluripotency in the mouse embryo. Development 136: 3215-3222

Acampora D, Omodei D, Petrosino G, Garofalo A, Savarese M, Nigro V, et al. (2016) Loss of the Otx2-binding site in the Nanog promoter affects the integrity of embryonic stem cell subtypes and specification of inner cell mass-derived epiblast. Cell reports 15: 2651-2664

D. Acampora, L. G. Di Giovannantonio, A. Garofalo, V. Nigro, D. Omodei, A. Lombardi, et al. (2017) Functional antagonism between OTX2 and NANOG specifies a spectrum of heterogeneous identities in embryonic stem cells. Stem cell reports 9: 1642-1659
Dear Dr. Shekar,

Thank you for the submission of your revised manuscript to our editorial offices. I went through this, and we are nearly done.

I have these final editorial requests I ask you to address in a further revised manuscript:

- Please reduce the number keywords on the title page to five.

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- The dotted line in the FACS profiles shown (unstained control -?) is still not uniformly labelled. There should be a legend for this line in all the FACS profiles shown using the same symbol and name. Please define the name ('-ve c') in the respective figure legends. There are still profiles where the dotted line is not defined in the legend in the figure (2B, 3J, 4A, EV2C/D and EV4B), or where in the legend a full line is shown (2H and 3F). Moreover, in the legend other profiles shown a clear dotted line should be shown (resembling the line in the profile - 1A/B, 2A/E, 3K/L, 4C/F, 5B, EV3B and EV5A). Please check.

- Moreover, for some FAX profiles shown, this unstained control peak looks identical (e.g. Fig. 2B second and third panel, or 2B second and third panel - there are more examples in 2E, 4F and EV4B). Why is that? Aren't these different experiments and even the control should look individual for each experiment?

- Regarding data quantification and statistics, please add complete statistical testing to all diagrams (for main, EV figures). Please also indicate (e.g. with n.s.) if testing was performed, but the differences are not significant. There are still many diagrams with only partial testing. Please check.

- There are figures in the Appendix and Tables in the Appendix file with no name ('Mycoplasma Test', 'PCR control for genomic DNA', 'STR Analysis Report' and 'PSet- Primer set used for STR analysis'). Please name these as the other Appendix items (Appendix Figure Sx; Appendix Table Sx), add legends, mention these with these names in the TOC and add callouts for these in the manuscript text.

- Appendix Figure 1 has only one panel, thus it does not need the 'A' label. Please remove this and correct the callouts.

I look forward to seeing the final revised version of your manuscript when it is ready. Please let me know if you have questions regarding the revision.

Please use this link to submit your revision: https://embor.msubmit.net/cgi-bin/main.plex

Best,

Achim Breiling
Senior Editor
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Dear Dr Breiling,

Thanks for your final editorial suggestions. We sincerely appreciate the due diligence and suggestion made to improve the quality of the manuscript. We have addressed all the points raised by you. We have also carefully looked at the manuscript and figures, made small changes to keep up the consistency and remove inadvertent errors, where-ever we could notice. Please find below details of the changes made and actions taken by us to address the comments.

Yours sincerely

Chandra

Response to the final editorial suggestions:

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  > We have reduced the number of key words to five in the title page.

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- The synopsis image provided has too small fonts. Please provide this with bigger fonts but keeping the size (exact width of 550 pixels and a height of not more than 400 pixels).

  > We changed the text to bigger and bold fonts keeping the size of the synopsis image same.

- Please provide as separate related manuscript text file a short, two-sentence summary of the manuscript (not more than 40 words) and three to four short bullet points highlighting the key findings of your study (not more than 2 lines
We have provided a separate manuscript related file with two sentence summary and four bullet points highlighting key findings of our study.

- The dotted line in the FACS profiles shown (unstained control -?) is still not uniformly labelled. There should be a legend for this line in all the FACS profiles shown using the same symbol and name. Please define the name ('-ve c') in the respective figure legends. There are still profiles where the dotted line is not defined in the legend in the figure (2B, 3J, 4A, EV2C/D and EV4B), or where in the legend a full line is shown (2H and 3F). Moreover, in the legend other profiles shown a clear dotted line should be shown (resembling the line in the profile - 1A/B, 2A/E, 3K/L, 4C/F, 5B, EV3B and EV5A). Please check.

- We have added dotted lines in the FACS profiles figure panels of Figure 2B, 3J, 4A, EV2C, Ev2D and EV4B.
- We have described the ‘-ve c’ in the all the respective figure legends. The name ‘-ve c’ is described as unstained control in the legends of Fig1A,B; Fig 2A,B,E,H; Fig 3F,J,K,L; Fig 4A,C,F and Fig 5B. Also in the legends of EV figures - EV2C,D; Ev3B, Ev4B and EV5A.
- The full line for ‘-ve c’ in 2H and 3F has been changed to dotted line consistent with other figures.
- We have labelled all the dotted lines in the FACS profile uniformly in all figure panels of the main figures and the EV figures.

- Moreover, for some FAX profiles shown, this unstained control peak looks identical (e.g. Fig. 2B second and third panel, or 2B second and third panel - there are more examples in 2E, 4F and EV4B). Why is that? Aren’t these different experiments and even the control should look individual for each experiment?

- Yes, these are different experiments. We agree that the controls should look individual for each experiment.
- Although superficially, the FACS profiles of unstained control peaks in Fig2B (2nd and 3rd panel), Fig2E (3rd and 4th panel), Fig4F (1st and 3rd panel) and also Fig2A (2nd and 3rd panel) might appear identical, but they are not identical. The differences among these profiles can be noticed under higher magnification. Since these profiles were acquired around same time for each experiment, and the unstained controls only have autofluorescence, their FACS profile will appear nearly similar (they may not be identical). We understand that having similar FACS profiles of the unstained controls can lead to confusion about the experimental design.
Keeping this mind, we have replaced panels in Fig.2A (1st and 2nd panel), Fig.2B (2nd panel), Fig.2E (3rd panel) and Fig. 4F (3rd panel) with different set of representative images from other sets of replicate data.

> In case of Fig.EV4B, there was inadvertent mistake. We had used same control in 1st and 2nd panel. We regret this oversight. We have now replaced the second panel in EV4B with FACS profile of a different replicate data.

- Regarding data quantification and statistics, please add complete statistical testing to all diagrams (for main, EV figures). Please also indicate (e.g. with n.s.) if testing was performed, but the differences are not significant. There are still many diagrams with only partial testing. Please check.

> We have added complete statistical testing to Fig. 1A,B,E & I; Fig. 3A-D,G & H; Fig. 4G-K; Fig. EV1H; Fig. EV3D-F & H-K; Fig. EV5 F & H. We have indicated n.s in the figure panels, where ever test was done and no difference were found.

- There are figures in the Appendix and Tables in the Appendix file with no name (‘Mycoplasma Test’, ‘PCR control for genomic DNA’, ‘STR Analysis Report’ and ‘PSet- Primer set used for STR analysis’). Please name these as the other Appendix items (Appendix Figure Sx; Appendix Table Sx), add legends, mention these with these names in the TOC and add callouts for these in the manuscript text.

> The appendix and tables in the Appendix file are given names, the legends for each figure is provided. These are now indexed in the TOC with page numbers. Call outs for these are given in the manuscript text in the materials and method section in line number 412.

- Appendix Figure 1 has only one panel, thus it does not need the ‘A’ label. Please remove this and correct the callouts.

> We have removed ‘A’ label in the Appendix Figure 1 and also changed the call out in the manuscript text.
Dear Dr. Shekar,

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