Erk signalling eliminates Nanog and maintains Oct4 to drive the formative pluripotency transition
Carla Mulas, Melanie Stammers, Siiri I. Salomaa, Constanze Heinzen, David M. Suter, Austin Smith and Kevin J. Chalut
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Reviewer 1

Evidence, reproducibility and clarity

Summary:
In the present manuscript, Mulas and colleagues address the question how ERK signaling orchestrates the transition from naïve to formative pluripotency in mouse embryonic stem cells. Combining pharmacological MEK inhibition with siRNA knockdown of candidate transcription factors, they conclude that downregulation of Nanog is an immediate function of ERK signaling that underlies exit from the naïve state. Later on they show that ERK signaling has additional functions beyond downregulating Nanog that are required to make cells competent for formative pluripotency and lineage progression, such that Nanog knockdown cells enter a new indeterminate state in the absence of ERK signaling. Finally, they show that Oct4 is a central mediator of this second function of ERK signaling, since forced Oct4 expression rescues the expression of formative markers even in the presence of MEK inhibitors. They also present time-lapse imaging data of a Spry4- and a Nanog-reporter, based on which they propose that metachronous ERK activity is reflected in metachronous NANOG downregulation.

The experiments dissecting the two different functions of ERK signaling in the pluripotency transition are well performed and provide some new interesting perspectives. Overall, the manuscript is well written. I have major concerns regarding the interpretation of the time-lapse imaging experiments (see major point 2 below) that unfortunately feature very prominently in the title of the manuscript.

Major points:
1. In lines 174 and 175, the authors write that "acute ERK activation ... [reduces] NANOG protein, which in turn diminishes Esrrb transcription". Although I am aware that this picture is supported by previous literature (e.g. PMID: 23040477), the author’s data do not fully support this conclusion. In Fig. 1G for example, Esrrb is downregulated even though Nanog expression is maintained. This discrepancy needs to be discussed.
2. The imaging data and analysis presented in Fig. 2 do not support the conclusion that heterogeneous ERK dynamics underlies metachronous pluripotency exit. There are several problems with this section:
- a. As far as I can see, the reporter line used in this study has not been previously used (at least there is no reference to a previous publication), and it has also not been properly validated in the present manuscript. One would for example like to know if the Spry4-FLuc allele encodes for a fusion protein, or whether it disrupts the Spry4 coding sequence. What is the half-life of the Spry4-FLuc protein? A proper description how the line has been generated, as well as an in-depth characterization are essential to evaluate the data.

- b. It is not clear that dynamic expression of a Spry4 reporter reflects dynamic ERK signaling. It has been shown that cumulative transcription from the Spry4 locus correlates with long-term ERK activity (e.g. PMID: 29964027), but short-term Spry4-FLuc dynamics could well be driven by other mechanisms, such as transcriptional bursting. Co-staining of ppERK and reporter expression in single cells would be required to address this issue.

- c. Why is the Spry4-FLuc signal higher at the start of the recording (when cells come out of MEK inhibition and should not have transcribed the reporter) compared to times > 7.5 h, when continuous ERK signaling in N2B27 should drive reporter expression?

- d. What is the evidence for temporally heterogeneous ERK activation? The authors only show one single trace in Fig. 2B, in which the Spry4-FLuc signal peaks right after release from 2i, as would be expected. Another study using a more direct ERK activity sensor (PMID: 31064783) indicated that this initial ERK activity peak after release from 2i is synchronous in all cells in a population. The authors would need to show several or all Spry4-FLuc traces from their experiment to demonstrate the opposite, otherwise one needs to assume synchronous ERK activation upon release from 2i in the author’s experiments as well.

- e. Why does the cross-correlation of the Spry-FLuc promoter activity (which should go up upon ERK signaling) with the NANOG-NLuc signal (which should go down upon ERK signaling) give positive values? Does this positive correlation reflect the transient nature of Spry4-FLuc expression, thus giving a positive value when Spry4-FLuc promoter activity decays? In this case, what is the meaning of the delay? Overall I found the explanation of this cross-correlation analysis very confusing. Given these problems, I recommend the authors to strongly tone down their conclusions or remove this section altogether, since addressing this multitude of problems might be out of scope for the present manuscript.

3. In line 217 (section title), the authors write that "failure to transition is not due to genome-wide chromatin dysregulation". It is true that the changes upon MEK inhibition reported in this section are small, but there are some changes, and it is ultimately difficult to know which ones are essential. I suggest to rephrase this section title.

4. The main finding of Fig. 4 - that Oct4 expression enables formative capacity - is very interesting. One problem throughout this figure is that the authors contrast the control case (N2B27/DMSO + siNeg) with a double perturbation (MEKI + siNanog), making it difficult to demonstrate whether it is the loss of Nanog or the loss of ERK signaling (or both) that results in loss of Oct4 expression. If I have missed something here please clarify.

5. Can the authors speculate, or perhaps even experimentally explore, why Oct4 re-expression enables formative capacity? Oct4 positively regulates Fgf4 expression (PMID: 9814708), raising the possibility that the indeterminate state is caused by insufficient paracrine FGF4 signaling once cells have reached this indeterminate state. Alternatively, Oct4-mediated regulation of a broader set of lineage specifiers might be required to establish formative pluripotency. The authors could explore these possibilities by supplementing cultures with recombinant FGF ligands. While these experiments are not essential for to corroborate conclusions in the present manuscript, could allow the authors to follow up upon what I think is their most interesting finding, and thereby give the manuscript a lift.

Minor points:

6. Please explain in the methods how gates for identifying RGd2-positive and -negative cells in Fig. 1B, E have been determined from the FACS plots in Fig. S1C/1B.

7. For the categorization of marker-positive and -negative cells in immunofluorescence images, the authors should explain in more detail according to which criterion a threshold was determined by ROC analysis. Which positive and negative controls were used in each case?

8. Does a statistical test on the data in Fig. S1A,B reveal significant differences?

9. Please give units on the x-axis in Fig. 2C?

10. Fig. 3E: Consider re-arranging. It is not immediately clear that all five bar charts belong to this panel.

11. There is a typo in Fig. S4A - maintained
12. Methods, lines 408 - 410: Please state units of the parameters used to estimate promoter activity.

Referee Cross-Commenting

I agree with reviewer #1's assessment of significance and their reservation regarding the conclusion "ERK activity is required to maintain Oct4 expression in the naïve to formative pluripotency transition". The experiment that the reviewer suggests is reasonable and doable (see also my major point 4.). Even though reviewer #1 has not explicitly commented on the conclusions drawn from Fig. 2, I disagree with their assessment that these conclusions are convincing (see my major point 2.).

Significance

The control of pluripotency transitions by signaling mechanisms as well as transcription factor circuits have been mapped in quite some detail over the last decade. The main advance of this manuscript is that it looks at the interaction between these two levels and thereby provides some new and interesting links. These results will mainly be of interest to a large community of researchers working with pluripotent stem cells.

To me, the most intriguing finding of the paper is the indeterminate cell state that the authors detect upon combined Nanog knockdown and MEK inhibition. To my knowledge, such a dead end of differentiation has not been reported before, at least not with pluripotent cells. This result could be a starting point for further investigation, and is of potential interest to a broader stem cell community.

Expertise: As a stem cell biologist I have the expertise to evaluate all parts of the paper.

Reviewer 2

Evidence, reproducibility and clarity

Summary:

In this manuscript, the authors investigated the role of Erk signaling in the transition from naïve to formative pluripotency. They found that Erk activation eliminates Nanog to allow naïve state exit. However, when Nanog is knocked down in the absence of Erk activation, ESCs exit the naïve state, and enter an indetermined state, unable to proceed to the formative state. The authors further claimed that the failure to the formative state is due to lack of Oct4 expression. In conclusion, Erk signaling is required for the exit from the naïve state and the entry to the formative state.

Major comments:

- Are the key conclusions convincing?

Most of the key conclusions are convincing, except for the conclusion "ERK activity is required to maintain Oct4 expression in the naïve to formative pluripotency transition". The authors showed that Oct4 expression is diminished under the MEK(i)+siNanog condition, while Oct4 is expressed in N2B27+siNeg (Figure 4C). With these experimental setting, the conclusion that ERK activity is required to maintain Oct4 expression in the naïve to formative pluripotency transition, cannot be reached, because two variations, MEK(i) and siNanog, rather than one variation MEK(i), are there. The experiment should be designed as adding MEK(i) into N2B27+siNeg at various time points, to test whether MEK(i) is able to down-regulate Oct4 expression in the naïve to formative pluripotency transition.

- Should the authors qualify some of their claims as preliminary or speculative, or remove them altogether?

No.
Would additional experiments be essential to support the claims of the paper? Request additional experiments only where necessary for the paper as it is, and do not ask authors to open new lines of experimentation.

The suggested experiment was described above.

- Are the suggested experiments realistic in terms of time and resources? It would help if you could add an estimated cost and time investment for substantial experiments.

It should not cost too much in terms of funding and time.

- Are the data and the methods presented in such a way that they can be reproduced?

Yes.

- Are the experiments adequately replicated and statistical analysis adequate?

Statistical analysis is lacking for Figure 3E and Figure 4.

Minor comments:

- Specific experimental issues that are easily addressable.

No.

- Are prior studies referenced appropriately?

Yes.

- Are the text and figures clear and accurate?

Yes.

- Do you have suggestions that would help the authors improve the presentation of their data and conclusions?

No.

Significance

This work characterized the role of ERK signaling in the transition between naïve and formative pluripotency. The function of ERK in ESC self-renewal and differentiation has been well recognized. Thus, this work provides new discoveries, but no conceptual advances. It should be of interest to a specialized audience in the pluripotency field, which is my expertise.

Author response to reviewers' comments

Reviewer #1 (Evidence, reproducibility and clarity (Required)):

Summary:

In this manuscript, the authors investigated the role of Erk signaling in the transition from naïve to formative pluripotency. They found that Erk activation eliminates Nanog to allow naïve state exit. However, when Nanog is knocked down in the absence of Erk activation, ESCs exit the naïve state, and enter an indetermined state, unable to proceed to the formative state. The authors further claimed that the failure to the formative state is due to lack of Oct4 expression. In conclusion, Erk signaling is required for the exit from the naïve state and the entry to the formative state.

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required to maintain Oct4 expression in the naïve to formative pluripotency transition, cannot be reached, because two variations, MEK(i) and siNanog, rather than one variation MEK(i), are there. The experiment should be designed as adding MEK(i) into N2B27+siNeg at various time points, to test whether MEK(i) is able to down-regulate Oct4 expression in the naïve to formative pluripotency transition.

We appreciate this point. We have now included data (figure 4C, 4E and S5B) to address this issue. As suggested, we performed exit experiments in MEK(i) only, and found that by 36hrs, a substantial proportion of cells have lost Oct4, unlike cells in N2B27 only. Down-regulation of Oct4 is later than in cells treated with MEK(i) + siNanog because of the delayed exit from the naïve state (in which Oct4 expression is independent of ERK). These data support the proposition that ERK activity is required to maintain Oct4 expression in the formative transition. We previously tried adding MEK(i) at various points in N2B27+siNeg conditions but the lack of synchrony made results impossible to interpret. As long as some Nanog positive cells remained, cells would re-activate the naïve network in the presence of MEK(i) and therefore maintain Oct4.

- Should the authors qualify some of their claims as preliminary or speculative, or remove them altogether?
  No.

- Would additional experiments be essential to support the claims of the paper? Request additional experiments only where necessary for the paper as it is, and do not ask authors to open new lines of experimentation.
  The suggested experiment was described above.

- Are the suggested experiments realistic in terms of time and resources? It would help if you could add an estimated cost and time investment for substantial experiments.
  It should not cost too much in terms of funding and time.

- Are the data and the methods presented in such a way that they can be reproduced?
  Yes.

- Are the experiments adequately replicated and statistical analysis adequate?
  Statistical analysis is lacking for Figure 3E and Figure 4.
  We performed statistical analyses between key comparisons and have added details to the figures and captions.

Minor comments:
- Specific experimental issues that are easily addressable.
  No.

- Are prior studies referenced appropriately?
  Yes.

- Are the text and figures clear and accurate?
  Yes.

- Do you have suggestions that would help the authors improve the presentation of their data and conclusions?
  No.

Reviewer #1 (Significance (Required)):

This work characterized the role of ERK signaling in the transition between naïve and formative pluripotency. The function of ERK in ESC self-renewal and differentiation has been well recognized. Thus, this work provides new discoveries, but no conceptual advances. It should be of interest to a specialized audience in the pluripotency field, which is my expertise.

Reviewer #2 (Evidence, reproducibility and clarity (Required)):
Summary:
In the present manuscript, Mulas and colleagues address the question how ERK signaling orchestrates the transition from naïve to formative pluripotency in mouse embryonic stem cells. Combining pharmacological MEK inhibition with siRNA knockdown of candidate transcription factors, they conclude that downregulation of Nanog is an immediate function of ERK signaling that underlies exit from the naïve state. Later on they show that ERK signaling has additional functions beyond downregulating Nanog that are required to make cells competent for formative pluripotency and lineage progression, such that Nanog knockdown cells enter a new indeterminate state in the absence of ERK signaling. Finally, they show that Oct4 is a central mediator of this second function of ERK signaling, since forced Oct4 expression rescues the expression of formative markers even in the presence of MEK inhibitors. They also present time-lapse imaging data of a Spry4- and a Nanog-reporter, based on which they propose that metachronous ERK activity is reflected in metachronous NANOG downregulation. The experiments dissecting the two different functions of ERK signaling in the pluripotency transition are well performed and provide some new interesting perspectives. Overall, the manuscript is well written. I have major concerns regarding the interpretation of the time-lapse imaging experiments (see major point 2 below) that unfortunately feature very prominently in the title of the manuscript.

Major points:
1. In lines 174 and 175, the authors write that "acute ERK activation … [reduces] NANOG protein, which in turn diminishes Esrrb transcription”. Although I am aware that this picture is supported by previous literature (e.g. PMID: 23040477), the author’s data do not fully support this conclusion. In Fig. 1G for example, Esrrb is downregulated even though Nanog expression is maintained. This discrepancy needs to be discussed.
   There is no real discrepancy because we are not claiming that Nanog is the only factor that regulates Erk or Nanog.”. This does not invalidate the summary conclusion that “the proximal effect of acute ERK activation on the naïve transcription factor network is to reduce Nanog protein, which in turn diminishes Esrrb transcription.”

2. The imaging data and analysis presented in Fig. 2 do not support the conclusion that heterogeneous ERK dynamics underlies metachronous pluripotency exit. There are several problems with this section:
   a. As far as I can see, the reporter line used in this study has not been previously used (at least there is no reference to a previous publication), and it has also not been properly validated in the present manuscript. One would for example like to know if the Spry4-FLuc allele encodes for a fusion protein, or whether it disrupts the Spry4 coding sequence. What is the half-life of the Spry4-Fluc protein? A proper description how the line has been generated, as well as an in-depth characterization are essential to evaluate the data.
   We apologise for not providing full information. Both reporters have been previously published and validated. The Spry4 reporter is not a fusion protein. The Fluc is translated from an IRES. We have amended the text to include details of the construction of all the reporters, references and half-life measurements in the methods section that now reads: “Calibration cells (PGK-Nluc-Fluc - (Mandic et al., 2017) and cells carrying Spry4-Fluc transcriptional reporter (Phillips et al., 2019) and Nanog::Nluc fusion were routinely cultured in 2i/LIF as described above. The Spry4-FLuc construct contains a splice acceptor site, followed by an IRES and an Bsd/F2A/NLSluc cassette and has a half-life of 1.5hrs (Phillips et al., 2019). The Nanog::Nluc targeting construct was generating using the nanotemplated targeting construct for Sox2 (Strebinger et al., 2019) in which 5’ and 3’ homology arms flank a Nluc-loxP-P2A-Puro-sFgfp-loxP cassette. Integration of Nanog::Nluc was initially verified by GFP and Nluc expression, and finally by PCR following excision of the loxP cassette. Using cycloheximide treatment we determined that the reporter had a half-life of 3.0hrs.”
   b. It is not clear that dynamic expression of a Spry4 reporter reflects dynamic ERK signaling. It has been shown that cumulative transcription from the Spry4 locus correlates with long-term ERK activity (e.g. PMID: 29964027), but short-term Spry4-FLuc dynamics could well be driven by other
mechanisms, such as transcriptional bursting. Co-staining of ppERK and reporter expression in single cells would be required to address this issue.

This is a valid point of discussion. Comparing between reporter systems is difficult since the Spry4 reporter used in PMID: 29964027 is fluorescent protein based and is therefore dependent on the time of protein maturation (although very fast compared to other fluorescent proteins) and a half-life of 9 hrs as reported by the authors. The bioluminescent reporter requires no maturation time and has a half-life of 1.65 hrs (PMID: 28456689).

Below are our considerations for the choice of reporter and the interpretation of results:

1. We previously showed that during exit from the naïve state, at the bulk level, pERK activity and some pERK transcriptional targets show dynamic patterns of activity (PMID: 29895711). Therefore, a dynamic pattern of Spry4 expression is not unexpected.
2. We initially tested different means of measuring ERK activity more directly (ERK-KTR and EKAREV-NLS and EKAREV-NES) but the imaging frequency (<2min) and resolution were incompatible with the time scale of transitions (24+hrs) without compromising cell viability. Moreover, little is known about how specific promoters integrate the activity of peaks of pERK. Therefore, we opted for an approach suitable for long term imaging to measure the transcriptional output of a well validated ERK target gene. Our reporter has a half-life of 1.65 hours (PMID: 30872573), and a negligible maturation time.
3. We cannot know how individual pERK peaks are translated into a transcriptional response, and our ability to measure changes in ERK activity is dependent on the reactor half-life, which is longer than the reported duration of individual peaks of ERK activity in mESCs (mode of 6.33 minutes - PMID: 35175328). Therefore, we limit our conclusion to “the level or dynamics of pERK that activate a Spry4 transcriptional response in individual cells also initiate downregulation of Nanog protein (line 160)”.
4. Regarding the suggestion of “Co-staining of ppERK and reporter expression in single cells” - since pERK activity is more transient compared to reporter activity, we did not expect a clear correlation between the reporter and ppERK immunostaining. Moreover, we did not find antibodies good enough for Luc proteins. However, have performed immunostainings that support the heterogeneity observed (Figure S2A-B, and see point d below).

c. Why is the Spry4-FLuc signal higher at the start of the recording (when cells come out of MEK inhibition and should not have transcribed the reporter) compared to times > 7.5 h, when continuous ERK signaling in N2B27 should drive reporter expression?

After media change, we typically allowed cells to equilibrate for 30 min in the incubator before setting up the imaging. Therefore, there is a ~45 min window in which we lack data. From experience (Nett*, Mulas* et al 2018), we know that pERK activity increases within 5-10 min after MEK(i) withdrawal and that explains why Spry4-FLuc signal is high as soon as we start the recording. We have now included this clarification in the methods (line 392).

d. What is the evidence for temporally heterogeneous ERK activation? The authors only show one single trace in Fig. 2B, in which the Spry4-FLuc signal peaks right after release from 2i, as would be expected. Another study using a more direct ERK activity sensor (PMID: 31064783) indicated that this initial ERK activity peak after release from 2i is synchronous in all cells in a population. The authors would need to show several or all Spry4-FLuc traces from their experiment to demonstrate the opposite, otherwise one needs to assume synchronous ERK activation upon release from 2i in the author's experiments as well.

Following the reviewer’s suggestion, we now provide an additional supplementary figure with all the traces (Supporting Figure 1). This demonstrates asynchrony in the response. Moreover, as per the reviewer’s suggestion, we have now included immunostainings of the first wave of pERK response (<1 hr) showing heterogeneity in nuclear pERK (Figure S2A-B). Indeed, it is possible to reconcile the results of our study with the cited reference. Deathridge et al. (PMID: 31064783) show that there is some asynchrony in the time of the first peak (spread in T50up parameter), as well as a spread in the height of the first peak (Fmax parameter). If Spry4 responds to integrated levels of ERK signalling, cells with a slower and/or lower initial peak of ERK would take longer to reach the cumulative ERK level necessary to activate Spry4 expression, creating asynchrony in the transcriptional response.

In addition, Deathridge et al. included serum in all ESC culture conditions (according to their Methods) which creates a more complex signalling environment.
e. Why does the cross-correlation of the Spry-FLuc promoter activity (which should go up upon ERK signaling) with the NANOG-NLuc signal (which should go down upon ERK signaling) give positive values? Does this positive correlation reflect the transient nature of Spry4-FLuc expression, thus giving a positive value when Spry4-FLuc promoter activity decays? In this case, what is the meaning of the delay? Overall I found the explanation of this cross-correlation analysis very confusing. Given these problems, I recommend the authors to strongly tone down their conclusions or remove this section altogether, since addressing this multitude of problems might be out of scope for the present manuscript.

Cross-correlation explicitly includes an analysis of the changes in correlation when a lag (meaning a shift in time) is applied to one of the signals. Therefore, there is no “positive correlation” but rather “positive correlation with a given lag applied”. An example is cross-correlation between a sine wave and a cosine wave (which are going in opposite directions for half the points in any given period and so show a positive correlation with a time lag). In our case, if we time shift the Spry4 signal, it will cross-correlate with the Nanog signal. It is possible we are misunderstanding the point of confusion, but we have reviewed our analysis of the data and believe it to be sound. Moreover, in our opinion the findings represent an important component of our paper and add weight to the conclusions drawn.

However, we agree that the analysis could be better explained. We have substantially re-written the section with this aim. The main text now reads:

“We examined the relationship between Spry4 activation and Nanog protein downregulation. After smoothing to remove noise, we used a simple set of ordinary differential equations to calculate the Spry4 promoter activity for each Spry4-Fluc trace (Figure S2C, see methods for details). We created continuous traces by adding the measurements made from each cell end-to-end (Figure S2D). We then measured the cross-correlation between activity of the Spry4 promoter and Nanog protein level. As controls we randomised the Spry4 signal in two ways: first, we randomised the Spry4 signals to measure correlations between Spry4 promoter activity and Nanog downregulation that could be attributable to noise; second, we assigned random time shifts to the Spry4 traces recorded (schematic diagrams shown in Figure S2D). Cross-correlation for the real data is higher than for either of the controls, meaning that the measured Spry4 and Nanog signals are correlated above noise levels and there is a consistent time delay between the two signals. We repeated the analysis for individual traces and observed the same trend (Figure S2F-G, 2D-E). The average lag time is ~80min, indicating that activation of the Spry4 promoter precedes Nanog downregulation. We repeated the analysis for RSK(i) treated cells and observed a stronger correlation at the level of the combined dataset (Figure 2F) as well as in individual cells (Figure 2G, S2H). Interestingly, RSK(i) treatment, which leads to a more sustained peak of pERK1/2 activity (Figure S2B), decreased the average delay (lag) between Spry4 promoter activation and Nanog downregulation to 20 min (Figure 2H, S2I). The fact that the lag is short, and not evident in all cells, suggests that Nanog downregulation might not require transcriptional activation.”

Overall we observe a significant cross-correlation between the rise in Spry-Fluc promoter activity (indicating active ERK-signalling) and the fall in Nanog-Nluc signal. However, we agree that this is not the most decisive result in the study and have changed the title of the paper to “Erk signalling eliminates Nanog and maintains Oct4 to drive the formative pluripotency transition”.

3. In line 217 (section title), the authors write that “failure to transition is not due to genome-wide chromatin dysregulation”. It is true that the changes upon MEK inhibition reported in this section are small, but there are some changes, and it is ultimately difficult to know which ones are essential. I suggest to rephrase this section title.

We have adjusted the section title.

4. The main finding of Fig. 4 - that Oct4 expression enables formative capacity - is very interesting. One problem throughout this figure is that the authors contrast the control case (N2B27/DMSO + siNeg) with a double perturbation (MEKi + siNanog), making it difficult to demonstrate whether it is the loss of Nanog or the loss of ERK signaling (or both) that results in loss of Oct4 expression. If I have missed something here please clarify.

We agree with this comment (also pointed out by the other reviewer). We have now included a new figure, showing that treatment with MEK(i) alone leads to loss of Oct4 expression after naïve state exit (updated figure 4E and S5B).
5. Can the authors speculate, or perhaps even experimentally explore, why Oct4 re-expression enables formative capacity? Oct4 positively regulates Fgf4 expression (PMID: 9814708), raising the possibility that the indeterminate state is caused by insufficient paracrine FGF4 signaling once cells have reached this indeterminate state. Alternatively, Oct4-mediated regulation of a broader set of lineage specifiers might be required to establish formative pluripotency. The authors could explore these possibilities by supplementing cultures with recombinant FGF ligands. While these experiments are not essential for to corroborate conclusions in the present manuscript, could allow the authors to follow up upon what I think is their most interesting finding, and thereby give the manuscript a lift.

The reviewer raises an interesting point of discussion. In our study transgene driven Oct4 expression was able to induce formative gene expression in MEK(i) conditions, which block FGF/ERK signalling (Figure 4F). Previous studies have shown that relocation of Oct4 to multiple gene loci is instrumental in the formative transition (PMID: 24905168 and PMID: 23271975) and it is known that Oct4 is an essential factor for formative stem cells (PMID: 33271069) and primed EpiSCs (PMID: 29915126).

Nonetheless we performed experiments to test whether addition of FGF could help rescue expression of formative genes after MEK(i) withdrawal (not shown). However, addition of FGF reduced neural differentiation in control cells and further reduced Sox1 expression in MEK(i)/siNanog treated cells (not shown). Moreover, we saw no significant upregulation of formative genes with addition of FGF (not shown). We decided not to include these results since the literature on the essential role of Oct4 throughout pluripotency is extensive.

Minor points:
6. Please explain in the methods how gates for identifying RGd2-positive and -negative cells in Fig. 1 B, E have been determined from the FACS plots in Fig. S1C/1B.
We have added a section in the methods to explain how this is done (Methods section “Flow Cytometry”), and we have now included a representative example in Figure S1C.

7. For the categorization of marker-positive and -negative cells in immunofluorescence images, the authors should explain in more detail according to which criterion a threshold was determined by ROC analysis. Which positive and negative controls were used in each case?
We have added the information to the methods section (Immunostaining and quantification).

8. Does a statistical test on the data in Fig. S1A,B reveal significant differences?
We have now performed appropriate statistical tests and have added them to both plots to show that there is indeed a significant difference.

9. Please give units on the x-axis in Fig. 2C?
Amended.

10. Fig. 3E: Consider re-arranging. It is not immediately clear that all five bar charts belong to this panel.
The experiments were carried out in parallel so we feel that the best way to present them is as currently shown.

11. There is a typo in Fig. S4A - maintained
Amended.

12. Methods, lines 408 - 410: Please state units of the parameters used to estimate promoter activity.
Amended.

**Referee Cross-Commenting**

I agree with reviewer #1's assessment of significance and their reservation regarding the conclusion "ERK activity is required to maintain Oct4 expression in the naïve to formative pluripotency transition". The experiment that the reviewer suggests is reasonable and doable (see also my major point 4.). Even though reviewer #1 has not explicitly commented on the conclusions drawn from Fig. 2, I disagree with their assessment that these conclusions are convincing (see my major point 2.).

Reviewer #2 (Significance (Required)):
The control of pluripotency transitions by signaling mechanisms as well as transcription factor circuits have been mapped in quite some detail over the last decade. The main advance of this
manuscript is that it looks at the interaction between these two levels and thereby provides some new and interesting links. These results will mainly be of interest to a large community of researchers working with pluripotent stem cells.

To me, the most intriguing finding of the paper is the indeterminate cell state that the authors detect upon combined Nanog knockdown and MEK inhibition. To my knowledge, such a dead end of differentiation has not been reported before, at least not with pluripotent cells. This result could be a starting point for further investigation, and is of potential interest to a broader stem cell community.

Expertise: As a stem cell biologist I have the expertise to evaluate all parts of the paper.

Original submission

First decision letter

MS ID#: DEVELOP/2024/203106

MS TITLE: Erk signalling eliminates Nanog and maintains Oct4 to drive the formative pluripotency transition

AUTHORS: Carla Mulas, Melanie Stammers, Siiri I Salomaa, Constanze Heinzen, David Suter, Austin G Smith, and Kevin J Chalut

Thank you for submitting your paper via Review Commons, I have now received the referees reports on the manuscript, and have reached a decision. The referees' comments are appended below, or you can access them online: please go to BenchPress and click on the 'Manuscripts with Decisions' queue in the Author Area.

The overall evaluation is positive and we would like to publish a revised manuscript in Development, provided that the referees' comments can be satisfactorily addressed. As you will see, the referees have minor comments that should be addressed in your revised manuscript. If you do not agree with these suggestions please explain clearly why this is so.

Reviewer 1

Advance summary and potential significance to field

In this manuscript, the authors investigated the role of Erk signaling in the transition from naive to formative pluripotency. They found that Erk activation eliminates Nanog to allow naive state exit. However, when Nanog is knocked down in the absence of Erk activation, ESCs exit the naive state, and enter an indetermined state, unable to proceed to the formative state. The authors further claimed that the failure to the formative state is due to lack of Oct4 expression. In conclusion, Erk signaling is required for the exit from the naive state and the entry to the formative state. In summary, the discovery is of interest to the field of stem cells.

Comments for the author

I have only one concern. The data presented in Figure 1J is inconsistent with the conclusion that “during pluripotency transition, the proximal effect of acute ERK activation on the naive transcription factor network is to reduce Nanog protein, which in turn diminishes Esrrb transcription”. The regulation of Esrrb is more complexed than what the authors proposed. Particularly, compared to 2i, MEK(i) leads to the activation of Esrrb, while other naive pluripotency genes are down-regulated. The authors should discuss the complexed regulation of Esrrb by Nanog and MEK(i).

Reviewer 2
Advance summary and potential significance to field

See my comments from the first round of review.

Comments for the author

The authors have appropriately addressed all of my comments from the previous round of review. The conclusions are now well supported by the evidence, such that the paper is suitable for publication in Development.

One minor thing that the authors might want to change in the final version is to match the color code of the Nanog::NLuc and Spry4-Fluc intensity traces between Fig. 2B and Supporting Figure 1.

First revision

Author response to reviewers' comments

Reviewer 1 Comments for the author

I have only one concern. The data presented in Figure 1J is inconsistent with the conclusion that “during pluripotency transition, the proximal effect of acute ERK activation on the naive transcription factor network is to reduce Nanog protein, which in turn diminishes Esrrb transcription”. The regulation of Esrrb is more complexed than what the authors proposed. Particularly, compared to 2i, MEK(i) leads to the activation of Esrrb, while other naive pluripotency genes are down-regulated. The authors should discuss the complexed regulation of Esrrb by Nanog and MEK(i).

RESPONSE: We thank the reviewer for their positive outlook on our paper. We have amended the text to include a references to the multiple inputs into Esrrb regulation which we hope addressed the concerns. The full paragraph now reads (line 130+): "These results suggest that Erk signalling leads directly to depletion of Nanog protein. We also analysed mRNA expression of a panel of naive ESC transcription factors (Fig. 1J). These markers are barely expressed after 30h in N2B27. In MEK(i) we saw reduction in mRNA for all except Esrrb, which increased slightly (Fig. 1J). When we combined MEK(i) with siNanog, Esrrb mRNA was lost, in line with the observed exit from the naïve state. Transcriptional regulation of Esrrb is multifaceted, including input from Oct4, Sox2, and Tcf3 (Festuccia et al., 2018a; Martello et al., 2012; Whyte et al., 2013). However, Esrrb has been shown to be a direct transcriptional target of Nanog (Festuccia et al., 2012). Persisting Nanog protein in MEK(i) conditions is therefore likely to be the main driver of maintained Esrrb transcription and delay in naïve state exit."

Reviewer 2:

One minor thing that the authors might want to change in the final version is to match the color code of the Nanog::NLuc and Spry4-Fluc intensity traces between Fig. 2B and Supporting Figure 1.

RESPONSE: many thanks for the positive review. We have amended the colour in figure 2B.

Second decision letter

MS ID#: DEVELOP/2024/203106

MS TITLE: Erk signalling eliminates Nanog and maintains Oct4 to drive the formative pluripotency transition
Thank you for sending your manuscript to Development through Review Commons.

I am happy to tell you that your manuscript has been accepted for publication in Development, pending our standard ethics checks.