

An Oct4-Sall4-Nanog network controls developmental progression in the preimplantation mouse embryo

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Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision

04 November 2011

Thank you again for submitting your work to Molecular Systems Biology. We have now heard back from the three referees who agreed to evaluate your manuscript. As you will see from the reports below, the referees raise substantial concerns on your work, which, I am afraid to say, preclude its publication in Molecular Systems Biology.

While the reviewers appreciated the topic and goals of this work, they all felt that the main conclusions remained rather weakly supported, and that fundamental technical issues cast significant doubt on the biological relevance of these findings. Most importantly, the reviewers raised concerns about potential non-specific or off-targets effects of morpholino treatments, with the first reviewer noting troubling discrepancies with knockout and knockdown phenotypes previously reported in the literature. Additional issues were raised related to the selection of modeling parameters and your ability to rigorously distinguish measurement/experimental error from biological noise.

Given these concerns, and the low level of support from the reviewers, unfortunately, I see no other choice than to return the manuscript with the message that we cannot offer to publish it.

In any case, thank you for the opportunity to examine your work. I hope that the points raised in the reports will prove useful to you and that you will not be discouraged from submitting future work to Molecular Systems Biology.

Sincerely,
 Editor - Molecular Systems Biology
 msb@embo.org

Reviewer #1 (Remarks to the Author):

While the intent of Tan et al in this article, to define the downstream targets of Oct4, Nanog, and Sall4 in mouse pre-implantation development, is certainly an interesting topic worthy of investigation, I am afraid that the quality of the data has something to be desired.

The major issue I have is in the effects of the knock downs, I am unconvinced the phenotypes are a true reflection of loss of these factors but instead are indirect consequences of either microinjection or off-target effects or both. The entire manuscript is based on the subsequent analysis of these knockdowns. There are publications on the zygotic knock outs for all three factors though the authors surprisingly do not even reference these earlier, highly relevant works. In all these knock outs, embryos do progress to the blastocyst stage. The potential advantage of a knockdown approach is the removal/inhibition of maternal mRNA that may be present. It is known that there is indeed maternal Oct4 and Sall4 in the mouse oocyte but there is no maternal Nanog mRNA or protein. Thus the knock down of zygotic Nanog should not present a phenotype earlier than that found in the Nanog null embryos. Nanog null embryos survive to at least the blastocyst stage (Mitsui et al (2003) Cell 113:631; Silva et al (2009) Cell 138:722) yet only <10% of Nanog knockdown embryos in this manuscript make it to the blastocyst. With respect to Sall4, knock downs in the pre-implantation embryo have been well described (Zhang et al (2006) Nat Cell Biol, 8:1114) and these too make it to the blastocyst stage whereas in this manuscript 0% of Sall4 knockdowns make it to the blastocyst. The fact that the authors observed arrested embryos at a wide range of developmental stages in all three knock downs also suggests the non-specific nature of these manipulations. With these contradictions to earlier published work I would have to conclude that any gene expression changes identified as a result of these knock downs are largely not directly a result of the loss of the respective transcription factor thus the networks described within the manuscript are something other than those of Oct4-Sall4-Nanog.

An incomplete list of minor comments:

From Introduction:

1. "and cells at the surface of the ICM becomes the primitive endoderm while cells deeper within the ICM becomes the epiblast (Zernicka-Goetz et al, 2009)."

The authors may want to re-word this as migration, and not just position, plays a role in the epiblast and primitive endoderm differentiation events.

2. "Oct4 is evolutionarily conserved in vertebrates and ..."

This is a bit misleading ... so-called fish Oct4 is really a paralog and not a 1-to-1 ortholog of mammalian Oct4. Mammalian Oct4 actually has quite an interesting evolutionary history and, unlike many other developmental regulators, is in fact not that highly conserved. For instance, see Frankenberg et al (2010) Dev Biol 337:162.

"Other master transcription factors ..."

"Master" is a poor but often used term for such transcription factors. These factors are certainly 'important' and 'key' but not 'master'. MyoD is a master transcription factor as, all by itself, it can drive the muscle program.

4. I do not put much weight on bioinformatic searches of transcription factor binding site motifs nor on microRNA target gene discovery algorithms, both are well known to find many, many more instances of the motif than are actually truly functional. They are useful in a preliminary pre-publication analysis of potential sites but I do not think they warrant presentation in a manuscript as evidence of functional sites.

Reviewer #2 (Remarks to the Author):

In this paper, the authors carried out the experiments for getting the comprehensive views of the regulatory network in preimplantation embryos focusing on Oct4, Sall4, Nanog, including: 1) morpholino-mediated gene knockdowns of these TFs at the different stages of embryonic development, 2) microarray analysis of and comparison of gene expressions between the cells developed into 4 cells or multi-cells even with TFs-knock down treatment and the cells of the same cell-types without injection, and between the cells arrested at the 2-cell or 3-cell-stages and those reached at the 4-cell or multicell-stages, 3) stage-dependent expression analysis of miRNA in the preimplantation embryo 4) deterministic and dynamic simulation for confirming the switch-like property of feed-forward loop inferred between TFs (putting into one group) and Dnmt3b, Rbl2, miR, 5) single cell gene expression analysis of 84 genes including Dnmt3b and house-keeping genes of control, MO-control, Oct4-MO, and Sall4-MO. The concept and approach of doing this work in preimplantation embryos is exciting and should be lauded, however many of the conclusions drawn are not supported by the data presented and the computational approach the authors have taken is quite limited. The authors should address / consider the below issues:

1: The authors have been reported many potentially interesting results, but the chosen discussion points and the analysis and of the data, and in-depth validation of the key novel findings is incomplete. The paper feels somewhat superficial.

2: In the studies where TF-knockdown results in developmental arrest, gene expression of many of the known pluripotency genes (such as Klf2, Klf5, Tbx3, Tcfap2c, Dppa5a) are strongly unregulated in the early-stage-arrested cells rather than in the cells which can reach further stages. There is no discussion on this point and it looks important to be taken into account for the following simulation analysis.

3: They simulated the dynamics in the feed-forward loop between TFs (put into one group) and Dnmt3b, Rbl2, miR, but there is no parameter sensitivity analysis and no reasonable explanation as to why specific parameter values were selected (only the note on this issue is that: "Importantly, we note that, over a wide range of parameters, the switch between an "off" state and an "on" state occurs when the expression level of the pluripotency factors is low." (P.22 line4)). Without these analyses, the deterministic simulations undertaken are meaningless, especially in the situation where big assumptions were made in the model molecular network itself and the actual levels of each of the components is unknown. The simulation certainly cannot be used to quantify differences like "20% and 60%" (P.22 line9).

4: For the noise analysis, they calculated SD values of expression levels of 84 picked-up genes in single cells. However, it is unclear many of the 84 genes are different in the pooled analysis. Moreover, the TFs-knockdown efficiency might be variable among each cells, confusing the analysis. Without this basic information, the difference between the "noise" and the stochasticity of the varied genes can not be distinguished.

Reviewer #3 (Remarks to the Author):

Tan et al. profiled pre-implantation embryos using microarrays, single cell expression using the Fluidigm platform, and genome-wide microRNA profiling, after morpholino-mediated knock-downs of Oct4, Sall4 and Nanog. They applied various bioinformatics analyses of these data and identified some interesting patterns including a role for Dnmt3b and involvement of miR-290-295 in regulation of progression of cell divisions.

While this paper presents a large body of work with important implications and novel insights, the paper is poorly written and there are many conclusions that could be misleading and lack of details about the experiments that raises questions. The following are examples of issues that need to be addressed as reading the text:

- It is never stated in the text what organism was used
- No inclusion of p-values and statistical test provided throughout the text
- The abstract is missing many important details such as the organism, cell type, experiments, when in development, what conditions
- In page 3, the fact that Oct4 binds to thousand of loci does not mean it is playing an important role. Many factors bind to many places.
- Sox2 was never mentioned.
- Early mammalian embryo is poorly defined.
- Page 4, "form critical nodes"... based on what?
- MOs need to be described
- The authors never discuss the differences between the knockdowns of the different three factors
- Was the arrest more specific for some factors?
- Not clear that the MOs were specific
- For the 634 genes identified, how these were determined? What test? How many up/down?
- How the genes from the microarrays compare to genes differentially expression in loss-of-function followed by expression in ESCs?
- Why the GO analysis was done only on the genes with binding sites and changes in expression?
- The different motifs for Nanog in page 8 appear interesting. More details and follow ups needed
- Page 9, how the 63% agreement was determined
- Page 9, direction of change as compared to what?
- The single cell expression is variable across single cells. Was the expression averaged? What was the variability? What was the distribution of expression in single cells for individual genes?
- Page 10, was the perturbation with the MOs done all together or for each factor separately
- Page 11, sentence that starts with "Clearly" no stat are provided
- Page 11, since only few genes are discuss, what are they? What is it that they do?
- MOs for Dnmt3b. How do you know they are specific? How do you know there are no off-target effects?
- Page 12, "large number of miRNA" as compared to what?
- Page 12, why expression was assessed after 46 hours?
- Page 12, again, not clear if MOs to the factors were applied together or individually



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December 5, 2011

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Thank you for your email on 4th November 2011 regarding our manuscript MSB-11-3112R, which is titled "An Oct4-Sall4-Nanog network controls developmental progression in the preimplantation mouse embryo." The reviewers' comments have been very helpful and we appreciate your taking the time to consider our work.

While the reviewers have provided valuable feedback on how we can improve our manuscript, we disagree with a few of the issues that have been raised. The most significant concern pertains to the specificity of our morpholino treatments. This is fundamental to our work and throughout the project, we have taken great pains to ensure that our observations were not mere artifacts. We argue that our knockdowns are on-target and specific for the following reasons:

1) We were able to partially rescue the phenotype for all the three transcription factors (Supplemental Figure S3). In these rescue experiments, when the morpholino was co-injected with the corresponding mRNA, a smaller percentage of embryos arrested by the 4-cell stage compared to embryos injected with the morpholino only. The mRNAs used in the rescue experiments contained several mismatches at the 3rd basepair position, so that they cannot be targeted by the morpholinos while preserving the same amino acid sequence.

2) We have additional evidence (not presented in our submitted manuscript) that the knockdowns are likely to be specific.

(A) For Oct4, we found that two different morpholinos, one of which blocks translation while the other blocks splicing, were able to produce similar phenotypes. This result was presented in one of our previous publications (Foygel et al, 2008).

(B) For *Sall4*, we were able to partially rescue the developmental arrest with both *Sall4* mRNA and *Sall4*-GFP mRNA but not with CAT-GFP mRNA.

(C) For *Nanog*, we were able to partially rescue the phenotype not only with mouse *Nanog* mRNA but also with human *Nanog* mRNA. This further suggests some degree of functional conservation between human *Nanog* and murine *Nanog*.

The first reviewer believes that our knockdown phenotypes are "indirect consequences of either microinjection or off-target effects or both." However, we firmly disagree with this. Not only were we able to rescue the phenotypes as described above, we also performed control experiments where we injected embryos with a morpholino targeting a human beta-globin intron mutation, whose sequence is not present in the mouse genome. The majority of the control-injected embryos progressed to the morula and blastocyst stages like the uninjected embryos and our gene expression analysis showed that the transcript profile of control-injected embryos was very similar to that of uninjected (normal) embryos (Figure 1).

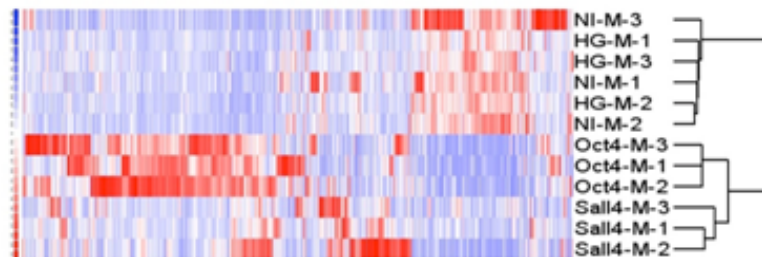


Figure 1. Cluster analysis of the results from our Affymetrix microarray experiments. NI: not injected or uninjected. HG: control morpholino targeting the human globin gene.

Another issue raised by the first reviewer was that our knockdown phenotypes appear to contradict existing knockout mouse models. While knockout mouse models have been and will continue to be indispensable for studying gene functions in mammals, we strongly believe that they are inadequate for studying the earliest stages of development, when the presence of parental transcripts or proteins may confound observations. In other words, when a homozygous null (-/-) embryo is generated from a heterozygous mother (+/-), the maternal transcripts or proteins may delay phenotype onset. Furthermore, if a homozygous null embryo is generated from a mother who is also homozygous null in the germline, any observed defects may reflect oocyte defects from the mother rather than specific gene requirement in the preimplantation embryo itself. Hence, knockout mouse models do not accurately capture molecular events occurring during preimplantation development.

Discrepancies between knockdown phenotypes and knockout mouse models is not without precedence. Our laboratory together with the laboratory of Magdalena Zernicka-Goetz at the Gurdon Institute in the University of Cambridge discovered that the maternal pool of *Cdx2* plays an unexpected role during preimplantation development that was not revealed in studies of a knockout

mouse model (Jedrusik et al, 2010). Specifically, even though the zygotic *Cdx2* knockout (*Cdx2*^{-/-}) embryos appear to progress normally until the blastocyst stage, depletion of both maternal and zygotic *Cdx2* from immediately after fertilization by multiple approaches results in developmental arrest at much earlier stages. In this collaborative work, we further demonstrated that maternal *Cdx2* is essential for normal polarization and compaction of the blastomeres at the 8-cell and 16-cell stages, but the early polarization defects are absent in the *Cdx2* knockout mouse model.

The first reviewer also stated that "there is no maternal *Nanog* mRNA or protein." His or her statement is based on published *in situ* hybridization, immunostaining, or X-gal staining experiments, which failed to detect any *Nanog* in the earliest stages. However, such staining procedures are not sensitive to low levels of gene products. Indeed, we discovered from our single-embryo quantitative real-time PCR experiments that maternal *Nanog* mRNA is present in 1-cell fertilized zygotes (Supplemental Figure S1). In addition, the laboratory of Paul Robson in the Genomics Institute of Singapore has also recently reported the presence of *Nanog* transcripts in 1-cell embryos (Guo et al, 2010).

Besides *Nanog*, the first reviewer pointed out that the laboratory of Bing Lim has knocked down *Sall4* in preimplantation mouse embryos and found that the knockdown embryos apparently developed to the blastocyst stage (Zhang et al, 2006). However, we note that these investigators used *Sall4* siRNA for their experiments, while we injected morpholinos to knockdown *Sall4*. We argue that morpholinos are more effective than siRNAs in embryonic gene knockdown experiments for two reasons. First, morpholinos can act more rapidly than siRNAs. In order for siRNAs to exert their effect, they must first assemble into RNA-induced silencing complexes (RISCs) before guiding the RISC to complementary RNA molecules. Hence, some of the maternal *Sall4* transcripts may be translated before the injected siRNAs are able to bring about their degradation. On the other hand, morpholinos diffuse quickly in embryos and are able to bind directly to mRNAs to block translation or splicing. Second, morpholinos persist for a longer period of time than siRNAs. Morpholinos are more stable and nuclease-resistant compared to siRNAs and can provide sustained gene knockdown for up to four days (or more). In figure 5a of Zhang et al (2006), we note that the *Sall4* transcript level actually increases by the morula stage even in embryos injected with *Sall4* siRNA, which clearly indicates that their siRNA is no longer working by then. In summary, we believe that the siRNA-mediated knockdown of *Sall4* in Zhang et al (2006) is incomplete or only partial.

We wish to highlight that morpholinos have been extensively used in the zebrafish and *Xenopus* communities for more than a decade and have played vital roles in many seminal discoveries in developmental biology. For example, in zebrafish, morpholinos were used to demonstrate that the secreted protein, Twisted gastrulation (Tsg), functions as a BMP antagonist together with Chordin and Short gastrulation (SOG) (Ross et al, 2001). In *Xenopus*, morpholinos were used to dissect the functions of Wnt signaling in embryonic patterning (Heasman et al, 2000). Furthermore, in order to prove the morpholino's specificity, the widely-accepted standard in the zebrafish and *Xenopus*

communities is to show that the phenotype can be rescued or to show that two different morpholinos can give a similar phenotype. In our research, we have adhered by the same standard to convince not only others but also ourselves that what we are observing is not simply due to off-target effects.

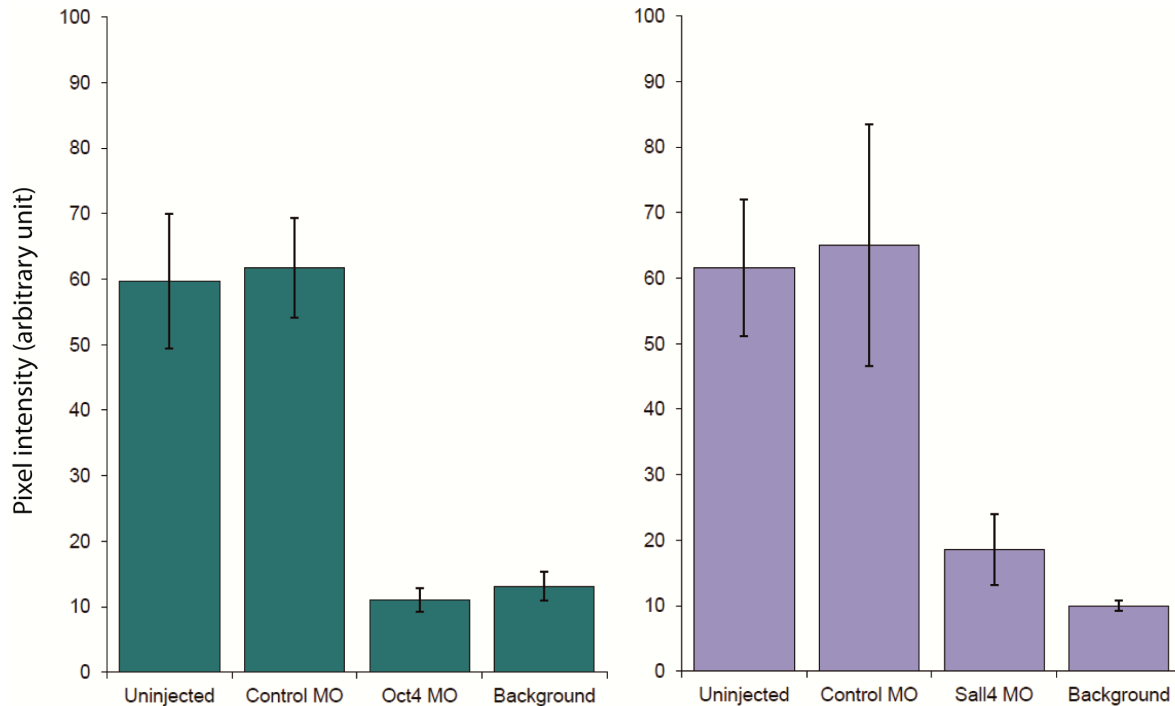


Figure 2. Staining intensities from immunocytochemical experiments. Left panel: α -Oct4 antibody; right panel: α -Sall4 antibody. Background corresponds to signal in the cytoplasm.

There are two other issues that you have displayed concern about in your email. The first issue regards our ability to distinguish experimental error from true biological noise. According to the second reviewer, our knockdown efficiency might be variable among individual cells, thereby confusing our analysis. To address this issue, we have carefully inspected our confocal microscopy images, as our immunostaining results allow us to measure the protein level in each embryo. We quantified the staining intensity in uninjected embryos, embryos injected with a control morpholino, and all embryos injected with an experimental morpholino (Figure 2):

(A) For Oct4, we found that the extent of knockdown is 100% in all the embryos we examined (N = 19), i.e. we did not detect any protein in embryos injected with a morpholino targeting Oct4. Hence, our knockdown of Oct4 is very consistent and does not appear to be variable among individual embryos or cells.

(B) For Sall4, we found that the extent of knockdown did vary from 60% to 97% among the embryos injected with Sall4 morpholinos (N = 20), which suggests that the increase in gene expression noise might be attributed to a certain degree to variability in knockdown efficiency. However, this is unlikely to be the major reason. We calculated the variability in staining intensity (ratio of standard deviation to

mean) for all the control embryos and the knockdown embryos (Table 1) and discovered that while the ratio for control morpholino-injected embryos and the ratio for Sall4 morpholino-injected embryos are similar (0.284 vs. 0.292), the variability in gene expression for Sall4 knockdown embryos is 2.9 times higher than that for control-injected embryos (Table 2). In other words, although the Sall4 protein level is not significantly more variable among knockdown embryos compared to control-injected embryos, gene expression is still noisier in the knockdown embryos.

In summary, we do not believe that the increase in gene expression noise that we observed during knockdown of a pluripotency factor is merely due a variable degree of knockdown among the different embryos.

Table 1. Variability in protein level (standard deviation/ mean) based on quantification of pixel intensities.

Antibody	Uninjected	Control Injected	Knockdown	Background
α -Oct4	0.172	0.123	0.166	0.167
α -Sall4	0.170	0.284	0.292	0.082

Table 2. Variability in gene expression based on single-cell real-time PCR experiments.

Uninjected	Control Injected	Oct4 knockdown	Sall4 knockdown
1.00	1.21	1.74	3.47

The second issue regards our selection of modeling parameters. We are currently working on improving our theoretical analysis, including a detailed parameter sensitivity analysis. Nonetheless, our main conclusions are unlikely to change, especially since another research group has recently shown analytically and through simulations that an incoherent microRNA-mediated feedforward loop can play an important role in noise buffering (Osella et al, 2011).

We hope that you will re-consider our work and allow us to submit a revised manuscript that seeks to address all the major concerns that the three reviewers have raised. Nevertheless, we will respect your decision and we thank you once again for providing us with such valuable feedback to improve our manuscript.

Yours sincerely,

Wing Hung Wong
 Professor of Statistics and Biostatistics
 Chairman, Department of Statistics

References

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Jedrusik A, Bruce AW, Tan MH, Leong DE, Skamagki M, Yao M, Zernicka-Goetz M (2010) Maternally and zygotically provided *Cdx2* have novel and critical roles for early development of the mouse embryo. *Developmental Biology* **344**:66-78.

Osella M, Bosia C, Cora D, Caselle M (2011) The role of incoherent microRNA-mediated feedforward loops in noise buffering. *PLoS Computational Biology* **7**:e1001101.

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Zhang J, Tam WL, Tong GQ, Wu Q, Chan HY, Soh BS, Lou Y, Yang J, Ma Y, Chai L, Ng HH, Lufkin T, Robson P, Lim B (2006) *Sall4* modulates embryonic stem cell pluripotency and early embryonic development by the transcriptional regulation of *Pou5f1*. *Nature Cell Biology* **8**:1114-1123.

Thank you for sending us your appeal letter regarding our decision on your work, entitled "An Oct4-Sall4-Nanog network controls developmental progression in the preimplantation mouse embryo".

We have now had time to revisit your manuscript and the comments from the reviewers, and to consider the points you raise in your appeal letter. I agree that your letter outlines some additional evidence that is relevant to the reviewers' concerns, and, as such, I would be willing to send a revised work back to the reviewers for a second evaluation. I would like to emphasize, however, that the reviewers' concerns were substantial, and I am not entirely convinced that the revised work you outlined would fully address their concerns.

1. Morpholino specificity. The additional morpholino rescue experiments you outline in point #2 do seem like they will help to address, at least in part, the first reviewer's concerns. However, assuming that these rescue experiments have only been conducted at the embryo development level, it is still possible that portions of the transcriptomic changes are the result of off-target effects. Given the importance of these transcriptomic patterns for this work, it may be valuable to demonstrate that two different morpholinos targeting the same gene produce similar transcriptomic profiles.

2. Reviewer #2 seemed to feel that this work remained less than fully conclusive, writing that the "in-depth validation of the key novel findings is incomplete." The sensitivity analysis was one, important aspect of this concern, and new experimental evidence addressing the contribution of knockdown variability to gene expression noise would also be helpful. It is not clear to me, though, that these points alone would provide the kind of substantial additional evidence this reviewer felt would be required to support the most novel biological findings in this work. It may be useful to test whether mRNA rescue of the morpholino treatments can also rescue the increase in gene expression noise. This would directly demonstrate that the increase in noise is not a function of variable knockdown, as well as further addressing Reviewer #1's specificity concerns. Moreover, the evidence that the proposed feedforward loop is directly involved in controlling endogenous gene expression noise (or creating a more hypersensitive response) appears to rest largely on the modeling analysis. Given reviewer #2's comments, additional experimental evidence that directly supports this model would clearly improve this manuscript (for example microRNA inhibitors that disrupt this feedforward loop should alter Dnmt3 expression noise).

Naturally, it would be important to address the other more specific concerns raised by each reviewer, including requests for additional statistical tests supporting the key observations.

You may use the link below to submit your revised manuscript. Please include a covering letter giving details of the way in which you have handled each of the points raised by the referees. A revised manuscript will be once again subject to review and you probably understand that we can give you no guarantee at this stage that the eventual outcome will be favorable.

PLEASE NOTE As part of the EMBO Publications transparent editorial process initiative (see <http://www.nature.com/msb/journal/v6/n1/full/msb201072.html>), Molecular Systems Biology now publishes online a Review Process File with each accepted manuscript. Please be aware that in the event of acceptance, your cover letter/point-by-point document will be included as part of this file, which will be available to the scientific community. Authors may opt out of the transparent process at any stage prior to publication (contact us at msb@embo.org). More information about this initiative is available in our Instructions to Authors.

Sincerely,

Editor - Molecular Systems Biology
msb@embo.org

Overview

We thank the reviewers for insightful comments that have greatly improved the manuscript. In the revised manuscript, we have taken additional steps to address the specificity of the morpholinos used. First, we performed more rescue experiments and showed again that co-injection of embryos with a morpholino and the corresponding mRNA would improve the percentage of embryos that developed until at least the morula stage. Second, we showed that two different morpholinos that targeted the same transcription factor produced similar transcriptome profiles. Third, we demonstrated that the increase in gene expression noise during knockdown of a pluripotency factor could also be rescued. Besides addressing the specificity of the morpholinos, we also performed parameter sensitivity analysis of the feed-forward loop that comprised the pluripotency factors, the miR-290-295 cluster, the Rbl2 transcriptional repressor, and the DNA methyltransferase Dnmt3b. We experimentally broke the feed-forward loop by examining gene expression noise in Dicer knockout embryonic stem cells (ESCs) and showed that without the feed-forward loop, the expression of Dnmt3b did become more variable. Last but not least, we addressed the potential confounding factor of variability in knockdown efficiency by measuring the protein level of the pluripotency factors in individual embryos and showing that the variability in the protein level could not account for the increased gene expression noise.

We address the reviewer's comments point-by-point below.

Reviewer #1

1. The major issue I have is in the effects of the knock downs, I am unconvinced the phenotypes are a true reflection of loss of these factors but instead are indirect consequences of either microinjection or off-target effects or both. The entire manuscript is based on the subsequent analysis of these knockdowns. There are publications on the zygotic knock outs for all three factors though the authors surprisingly do not even reference these earlier, highly relevant works. In all these knock outs, embryos do progress to the blastocyst stage. The potential advantage of a knockdown approach is the removal/inhibition of maternal mRNA that may be present. It is known that there is indeed maternal Oct4 and Sall4 in the mouse oocyte but there is no maternal Nanog mRNA or protein. Thus the knock down of zygotic Nanog should not present a phenotype earlier than that found in the Nanog null embryos. Nanog null embryos survive to at least the blastocyst stage (Mitsui et al (2003) Cell 113:631; Silva et al (2009) Cell 138:722) yet only <10% of Nanog knockdown embryos in this manuscript make it to the blastocyst. With respect to Sall4, knock downs in the pre-implantation embryo have been well described (Zhang et al (2006) Nat Cell Biol, 8:1114) and these too make it to the blastocyst stage whereas in this manuscript 0% of Sall4 knockdowns make it to the blastocyst. The fact that the authors observed arrested embryos at a wide range of developmental stages in all three knock downs also suggests the non-specific nature of these manipulations. With these contradictions to earlier published work I would have to conclude that any gene expression changes identified as a result of these knock downs are largely not directly a result of the loss of the respective transcription factor thus the networks described within the manuscript are something other than those of Oct4-Sall4-Nanog.

We thank the reviewer for highlighting the critical issue of off-target effects and for pointing out previously published work. We agree with the reviewer that there are possible artifacts that may lead us to draw false conclusions. Hence, we have been systematically examining them throughout our study.

First, to address the possibility that our phenotypes may be an indirect consequence of microinjection, we always include an additional experimental condition whereby we inject embryos with a control morpholino, which targets a human beta-globin intron mutation whose sequence is absent from the mouse genome. We have consistently observed that the majority of embryos injected with the control morpholino progress to morula and blastocyst stages after four days of *in vitro* culture, just like the uninjected (normal) embryos (Page 5 and Figure S10). Our gene expression analysis also showed that the transcriptome profile of control injected embryos was very similar to that of uninjected embryos (Figure S8). Taken together, our data indicate that the phenotypes that we observed when we knocked down Oct4, Sall4, or Nanog are not simply a result of microinjection.

Second, to address the possibility that the MOs may have off-target effects, we have performed extensive rescue experiments. Repeatedly, we have observed that compared to embryos that were injected with a MO alone, co-injection of embryos with both the MO and the corresponding mRNA increased the percentage of embryos that reached the morula stage and reduced the percentage of embryos that arrested by the 4-cell stage (Pages 5-7 and Figure 1). To ensure that the knockdown phenotypes could only be rescued by the mRNA corresponding to the gene targeted by the morpholino and not by any other mRNA, we showed that we could partially rescue the developmental arrest induced by the Sall4 MO with Sall4-GFP mRNA but not with CAT-GFP mRNA (Page 6 and Figure S4A). To further confirm the specificity of Nanog knockdown, we tested whether we could rescue the phenotype with human Nanog and observed rescue in 3 out of 4 independent experiments (Page 6 and Figure S4B). This result also suggests that there is some degree of functional conservation between human Nanog and murine Nanog.

As off-target effects are a serious concern, we wanted to perform yet more experiments to verify that we were not merely observing artifacts. We asked whether we could rescue the increase in gene expression noise observed during knockdown of Oct4 or Sall4. Hence, we performed additional single cell expression profiling experiments for embryos that were injected with a MO alone and for embryos that were injected with the MO and the corresponding mRNA. Strikingly, for both pluripotency factors, co-injection of embryos with MO and mRNA did reduce the variability in expression levels (Page 21 and Figure 8), indicating that the increased noise can be rescued. We also asked whether two different morpholinos targeting the same pluripotency factor can give similar transcriptome profiles. For each transcription factor, we injected embryos with either the original morpholino or a second non-overlapping morpholino and performed microarray analysis. Notably, all but one of the arrays corresponding to the knockdown embryos clustered well together against the controls (Page 9 and Figure S8). We suspect that the outlier (an array corresponding to the first biological replicate for the second morpholino targeting Nanog) might be caused by injection failure because the second replicate did cluster with the other knockdown samples. Taken together, the multiple lines of evidence strongly suggest that our MOs are indeed specific and that our results were not simply consequences of off-target effects.

How do we reconcile our results with previous published work? As mentioned by the reviewer, it is known that there are maternal Oct4 and Sall4 products in the 1-cell fertilized zygote. Since maternal transcripts are not eliminated in knockout mouse models, they may drive the beginning developmental stages even in homozygous null embryos. However, the reviewer also stated that there is no maternal Nanog mRNA or protein. This statement is based on published in situ hybridization, immunostaining, or X-gal staining experiments, which failed to detect any Nanog in the earliest stages. However, such staining procedures are not sensitive to low levels of gene products. We discovered from our single-embryo quantitative real-time PCR experiments that maternal Nanog mRNA is in fact present in 1-cell fertilized zygotes (Figure S1). In addition, this result has been independently verified by the laboratory of Paul Robson, who recently reported the presence of Nanog transcripts in 1-cell embryos (Guo *et al.*, 2010).

We also examined the work by Zhang *et al.* (2006), where they showed that Sall4 knockdown embryos apparently developed to the blastocyst stage. We note that these investigators used Sall4 siRNA for their experiments, while we injected morpholinos to knockdown Sall4. We argue that morpholinos are more effective than siRNAs in embryonic gene knockdown experiments for two reasons. First, morpholinos can act more rapidly than siRNAs. In order for siRNAs to exert their effect, they must first assemble into RNA-induced silencing complexes (RISCs) before guiding the RISC to complementary RNA molecules. Hence, some of the maternal Sall4 transcripts may be translated before the injected siRNAs are able to bring about their degradation. On the other hand, morpholinos diffuse quickly in embryos and are able to bind directly to mRNAs to block translation or splicing. Second, morpholinos persist for a longer period of time than siRNAs. Morpholinos are more stable and nuclease-resistant compared to siRNAs and can provide sustained gene knockdown for up to four days (or more). In figure 5a of Zhang *et al.* (2006), we note that the Sall4 transcript level actually increases by the morula stage even in embryos injected with Sall4 siRNA, which clearly indicates that their siRNA is no longer working by then. Hence, we believe that the siRNA-mediated knockdown of Sall4 in Zhang *et al.* (2006) is less complete or only partial.

2. "and cells at the surface of the ICM becomes the primitive endoderm while cells deeper within the ICM becomes the epiblast (Zernicka-Goetz *et al.*, 2009)." The authors may want to re-word this as migration, and not just position, plays a role in the epiblast and primitive endoderm differentiation events.

We thank the reviewer for pointing out the cell sorting model and we have incorporated it into the introduction (Page 3).

3. *"Oct4 is evolutionarily conserved in vertebrates and ..." This is a bit misleading ... so-called fish Oct4 is really a paralog and not a 1-to-1 ortholog of mammalian Oct4. Mammalian Oct4 actually has quite an interesting evolutionary history and, unlike many other developmental regulators, is in fact not that highly conserved. For instance, see Frankenberg et al (2010) Dev Biol 337:162.*

We thank the reviewer for pointing out the evolutionary history of Oct4. In the revised manuscript, we have updated the sentence to “Oct4 is evolutionarily conserved in mammals, lizards, and urodeles” (Page 3).

4. *"Other master transcription factors ..." "Master" is a poor but often used term for such transcription factors. These factors are certainly 'important' and 'key' but not 'master'. MyoD is a master transcription factor as, all by itself, it can drive the muscle program.*

As suggested, we have replaced the word “master” with the word “key” (Page 4).

5. *I do not put much weight on bioinformatic searches of transcription factor binding site motifs nor on microRNA target gene discovery algorithms, both are well known to find many, many more instances of the motif than are actually truly functional. They are useful in a preliminary pre-publication analysis of potential sites but I do not think they warrant presentation in a manuscript as evidence of functional sites.*

We agree with the reviewer that bioinformatic searches typically uncover more motifs or target genes than are actually truly functional. However, they are still valuable for generating hypothesis that can be subsequently followed up. We present the motifs and the microRNA targets as candidates that require further detailed studies and hope that our data and results can serve as a useful resource for developmental biologists and for the general genomics community.

Reviewer #2

In this paper, the authors carried out the experiments for getting the comprehensive views of the regulatory network in preimplantation embryos focusing on Oct4, Sall4, Nanog, including: 1) morpholino-mediated gene knockdowns of these TFs at the different stages of embryonic development, 2) microarray analysis of and comparison of gene expressions between the cells developed into 4 cells or multi-cells even with TFs-knock down treatment and the cells of the same cell-types without injection, and between the cells arrested at the 2-cell or 3-cell-stages and those reached at the 4-cell or multicell-stages, 3) stage-dependent expression analysis of miRNA in the preimplantation embryo 4) deterministic and dynamic simulation for confirming the switch-like property of feed-forward loop inferred between TFs (putting into one group) and Dnmt3b, Rbl2, miR, 5) single cell gene expression analysis of 84 genes including Dnmt3b and house-keeping genes of control, MO-control, Oct4-MO, and Sall4-MO. The concept and approach of doing this work in preimplantation embryos is exciting and should be lauded, however many of the conclusions drawn are not supported by the data presented and the computational approach the authors have taken is quite limited.

We thank the reviewer for highlighting the interest in this work. As described below, we have performed additional computational analysis and experiments to support the conclusions in our manuscript.

1. The authors have been reported many potentially interesting results, but the chosen discussion points and the analysis and of the data, and in-depth validation of the key novel findings is incomplete. The paper feels somewhat superficial.

In our revised manuscript, we have probed deeper into some of our results. Specifically, we have performed parameter sensitivity analysis of the feed-forward loop and have also broken the loop experimentally to show that Dnmt3b expression did become more variable (Pages 16-18, Figure 6, Figure S12, and Figure S13). In addition, we have examined our microscopy images and found that variability in knockdown efficiency cannot account for the increase in gene expression noise during knockdown of Oct4 or Sall4 (Pages 20-21, Figure S16, and Table 2). Furthermore, we also demonstrated that the increased noise could be rescued (Page 21 and Figure 8).

2. In the studies where TF-knockdown results in developmental arrest, gene expression of many of the known pluripotency genes (such as Klf2, Klf5, Tbx3, Tcfap2c, Dppa5a) are strongly unregulated in the early-stage-arrested cells rather than in the cells which can reach further stages.

There is no discussion on this point and it looks important to be taken into account for the following simulation analysis.

We agree with the reviewer that there are potentially many other interesting pluripotency genes that we could examine further. However, we aimed to keep our manuscript focused. We could have included additional genes into our mathematical model, but the simulation analysis will generate more hypotheses that require further experimental validations, which are beyond the scope of the present study.

3. They simulated the dynamics in the feed-forward loop between TFs (put into one group) and Dnmt3b, Rbl2, miR, but there is no parameter sensitivity analysis and no reasonable explanation as to why specific parameter values were selected (only the note on this issue is that: "Importantly, we note that, over a wide range of parameters, the switch between an "off" state and an "on" state occurs when the expression level of the pluripotency factors is low."(P.22 line4)). Without these analyses, the deterministic simulations undertaken are meaningless, especially in the situation where big assumptions were made in the model molecular network itself and the actual levels of each of the components is unknown. The simulation certainly cannot be used to quantify differences like "20% and 60%"(P.22 line9).

We thank the reviewer for bringing this point out. As stated in the revised manuscript (Page 15), we chose the parameters so that it would reproduce known experimental data. Subsequently, we performed parameter sensitivity analysis to determine how the behavior of the system changes with different parameter values (Pages 16-17, Figure 6, Figure S12, and Figure S13). First, we found that the hypersensitive response is robust with respect to the synthesis rate of the Rbl2 transcriptional repressor. A 100-fold change in the synthesis rate did not appear to affect how the system output (Dnmt3b transcript level) responds to changes in the input (expression level of the pluripotency factors). Second, we found that the switch-like behavior does depend on the relative influences of the pluripotency factors and Rbl2 on the transcription of Dnmt3b. When the expression of Dnmt3b is more dependent on the regulatory activity of Rbl2, the switch-like response becomes even more pronounced. We also wish to emphasize that hard numbers are provided simply to help deliver a message. The message that we are trying to deliver is that at high levels of pluripotency factors, variability in their expression will not affect the transcript level of Dnmt3b much, but at low levels of pluripotency factors, variability in their expression can have a big impact on the transcript level of Dnmt3b. The precise numbers, such as 20% or 60%, are less important. They are included only to help readers understand the message.

To obtain experimental evidence to support our model, we examined the variability of Dnmt3b expression in single embryonic stem cells (ESCs). In particular, we studied its variability in wildtype ESCs (feed-forward loop present) and Dicer knockout (Dcr KO) ESCs (feed-forward loop absent). We also performed single cell gene expression profiling experiments on Dcr KO ESCs transfected with a control microRNA (feed-forward loop still absent) and Dcr KO ESCs transfected with miR-295 (feed-forward loop restored). As reported in the revised manuscript (Pages 17-18, Figure 6, Figure S14, and Figure S15), we found that the expression of Dnmt3b was indeed more noisy in Dcr KO ESCs and in control-transfected Dcr KO ESCs than in rescued Dcr KO ESCs and in wildtype ESCs. Hence, our data demonstrated that the feed-forward loop helped to buffer the expression of Dnmt3b against intrinsic noise in the levels of the pluripotency factors.

4. For the noise analysis, they calculated SD values of expression levels of 84 picked-up genes in single cells. However, it is unclear many of the 84 genes are different in the pooled analysis.

According to our microarray analysis of pooled embryos, 68 out of the 84 genes were differentially expressed during knockdown of a pluripotency factor. The remaining 16 genes were related family members or performed similar functions (Page 19).

Moreover, the TFs-knockdown efficiency might be variable among each cells, confusing the analysis. Without this basic information, the difference between the "noise" and the stochasticity of the varied genes can not be distinguished.

We thank the reviewer for the insightful comment. Since MOs work by blocking the translation of their target transcripts, we sought to examine the protein levels of the pluripotency factors in individual embryos. To do so, we examined our confocal microscopy images, as our immunostaining results allowed us to measure the protein level in each embryo. We quantified the staining intensity in uninjected embryos, embryos injected with a control morpholino, and embryos

injected with an experimental morpholino (Figure S16). For Oct4, we found that the extent of knockdown is 100% in all the embryos we examined (N=19) i.e. we did not detect any protein in embryos injected with a morpholino targeting Oct4. Hence, our knockdown of Oct4 is very consistent and does not appear to be variable among individual embryos or cells. For Sall4, we found that the extent of knockdown did vary from 60% to 97% among the embryos injected with a Sall4 morpholino (N = 20), which suggests that the increase in gene expression noise might be attributed to a certain degree to variability in knockdown efficiency. However, this is unlikely to be the major reason. We calculated the variability in staining intensity (ratio of standard deviation to mean) for all the control embryos and the knockdown embryos (Table 2) and discovered that while the ratio for control morpholino-injected embryos and the ratio for Sall4 morpholino-injected embryos were similar (0.284 vs. 0.292), the variability in gene expression for Sall4 knockdown embryos was 2.9 times higher than that for control-injected embryos. In other words, although the Sall4 protein level was not significantly more variable among knockdown embryos compared to control-injected embryos, gene expression was still noisier in the knockdown embryos.

To further ensure that the increase in gene expression noise was not simply due to variability in knockdown efficiency, we wondered if we could rescue the noise. We injected embryos with either a morpholino alone or with the morpholino and the corresponding mRNA together and performed the same single cell expression profiling experiments on the Biomark platform (Fluidigm). Strikingly, we found from principal component analysis that gene expression in the rescued cells was less variable than gene expression in the knockdown cells (Page 21 and Figure 8). Taken together, the increase in gene expression noise that we observed during knockdown of Oct4 or Sall4 is unlikely to be caused by variability in transcription factor knockdown efficiency.

Reviewer #3

Tan et al. profiled pre-implantation embryos using microarrays, single cell expression using the Fluidigm platform, and genome-wide microRNA profiling, after morpholino-mediated knock-downs of Oct4, Sall4 and Nanog. They applied various bioinformatics analyses of these data and identified some interesting patterns including a role for Dnmt3b and involvement of miR-290-295 in regulation of progression of cell divisions.

While this paper presents a large body of work with important implications and novel insights, the paper is poorly written and there are many conclusions that could be misleading and lack of details about the experiments that raises questions. The following are examples of issues that need to be addressed as reading the text:

We thank the reviewer for highlighting the interest in this work. We apologize for the lack of clarity in the text and have strived to improve the writing. We have also included more experimental details in the revised manuscript.

1. It is never stated in the text what organism was used

We have now stated in the manuscript title and abstract that the study was performed on preimplantation mouse embryos.

2. No inclusion of p-values and statistical test provided throughout the text

We have included additional p-values and statistical tests and have also tried to make the existing ones more explicit in the text.

3. The abstract is missing many important details such as the organism, cell type, experiments, when in development, what conditions

In the abstract, we have stated that the study was performed on preimplantation mouse embryos (organism and development). We have also stated that we performed morpholino-mediated gene knockdowns of key embryonic stem cell (ESC) factors followed by detailed transcriptome analysis of pooled embryos, single embryos, and individual blastomeres (experiments and conditions).

4. In page 3, the fact that Oct4 binds to thousand of loci does not mean it is playing an important role. Many factors bind to many places.

We agree with the reviewer that many transcription factors bind to numerous genomic loci. Hence, we have weakened the statement to “the protein binds to more than a thousand loci in the genome, which suggests that it may regulate a large population of genes” (Pages 3-4).

5. Sox2 was never mentioned.

There are many interesting pluripotency factors that can be investigated. In order to keep our study within a manageable scope, we have focused only on Oct4, Sall4, and Nanog.

6. *Early mammalian embryo is poorly defined.*

We apologize for the confusion. In the revised manuscript, we have refrained as far as possible from using the term “early embryo” and have instead described our experimental system as “preimplantation embryo.”

7. *Page 4, "form critical nodes"... based on what?*

Genes can be viewed as the nodes in a regulatory network. The pluripotency factors are critical nodes because they appear to control the expression of a large number of genes, based on chromatin immunoprecipitation experiments in ESCs, which we have described in the previous paragraphs.

8. *MOs need to be described*

We have described the MOs in Figure S2 and in the Experimental Procedures (Pages 27-28).

9. *The authors never discuss the differences between the knockdowns of the different three factors*

We have provided a plot summarizing the furthest stages reached by the various knockdown embryos after 4 days of *in vitro* culture (Figure S10) and have discussed the differences in the figure legend.

10. *Was the arrest more specific for some factors?*

As shown in Figure S10, the arrest was not more specific for some factors. Knockdown of any of the pluripotency factor resulted in arrest over a range of developmental stages.

11. *Not clear that the MOs were specific*

We have addressed the specificity of the MOs in different ways. First, we demonstrated that all the phenotypes could be partially rescued (Page 6, Figure 1, and Figure S4). Second, we showed that two different MOs targeting the same pluripotency factor produced comparable transcriptome profiles (Page 9 and Figure S8). Third, we found that the increase in gene expression noise during knockdown of Oct4 or Sall4 could also be rescued (Page 21 and Figure 8). Hence, these different lines of evidence indicate that the MOs were specific.

12. *For the 634 genes identified, how these were determined? What test? How many up/down?*

The microarray data were analyzed using a methodology that we developed (<http://www.stanford.edu/group/wonglab/doc/NewGeneScoreBiostatDraft.pdf>). In comparison with the traditional moderated t-statistic, this methodology takes into account both the fold change and the expression magnitude. The numbers of genes that were down-regulated or up-regulated are provided in Figure 2.

13. *How the genes from the microarrays compare to genes differentially expression in loss-of-function followed by expression in ESCs?*

We have performed the requested comparison and the results are shown in Figure S5.

14. *Why the GO analysis was done only on the genes with binding sites and changes in expression?*

The differentially expressed genes that have promoters or enhancers bound by Oct4, Sall4, or Nanog in ESCs are likely to be directly controlled by these pluripotency factors in the preimplantation embryos, as opposed to genes that are further downstream of the regulatory pathways. In any case, we have also performed GO analysis on differentially expressed genes that are not known to be regulated by the pluripotency factors in ESCs (Figure 2C) in order to gain insights into the embryo-specific genes.

15. *The different motifs for Nanog in page 8 appear interesting. More details and follow ups needed*

We agree with the reviewer that the different motifs for Nanog are interesting. However, a systematic investigation of the motifs would require luciferase assays, mutation analysis, footprinting experiments, gel shift assays, and co-immunoprecipitation experiments, which are beyond the scope of the present study.

16. Page 9, how the 63% agreement was determined

We apologize for the lack of clarity and have revised the text to “On average, 63.9% of the genes that were differentially expressed under a particular knockdown condition in our microarray analysis were also differentially expressed in the same directions under the same knockdown condition in our BioMark analysis” (Page 11).

17. Page 9, direction of change as compared to what?

We meant that if a gene was up-regulated in our microarray analysis, it was also up-regulated in our Biomark analysis and if it was down-regulated in our microarray analysis, it was also down-regulated in our Biomark analysis.

18. The single cell expression is variable across single cells. Was the expression averaged? What was the variability? What was the distribution of expression in single cells for individual genes?

No, we did not average the expression levels across single cells. We have plotted the distributions of the standard deviations for each experimental condition in Figure 7A and have also provided the raw Ct values for all the cells in Supplemental File S16.

19. Page 10, was the perturbation with the MOs done all together or for each factor separately

As stated on Page 11, the perturbation with the MOs was done separately for each factor.

20. Page 11, sentence that starts with "Clearly" no stat are provided

The p-values for all the genes are provided in Figure 4.

21. Page 11, since only few genes are discuss, what are they? What is it that they do?

As requested, we have included in the text that “most of them encode DNA-binding proteins or transcriptional regulators, such as the T-box transcription factor Tbx3, which shares many common targets with Oct4 and Nanog” (Page 12).

22. MOs for Dnmt3b. How do you know they are specific? How do you know there are no off-target effects?

We performed rescue experiments and found that, compared to embryos that were injected with the MO alone, co-injection of embryos with both the MO and *in vitro* transcribed Dnmt3b mRNA increased the percentage of embryos that reached the morula stage (Pages 13-14 and Figure 5).

23. Page 12, "large number of miRNA" as compared to what?

We have replaced the phrase “large number of miRNAs” with “297 distinct miRNAs” (Page 9).

24. Page 12, why expression was assessed after 46 hours?

Expression was assessed after 46 hours because the knockdown phenotypes were readily observable by then (Page 10).

25. Page 12, again, not clear if MOs to the factors were applied together or individually

We apologize for the confusion. We have now stated in the text that we knocked down each transcription factor separately (Page 9).

Thank you again for submitting your work to Molecular Systems Biology. We have now heard back from the three referees who agreed to evaluate your revised manuscript. As you will see from the reports below, the referees find the topic of your study of potential interest. They raise, however, substantial concerns on your work, which, I am afraid to say, must preclude its publication in its present form.

Unfortunately, none of the original reviewers of this work were available to evaluate this revised work. As such, we asked three new reviewers to evaluate this work, and the last two reviewers (#6,

#7) also evaluated the previous reviewers' comments and your responses in detail. These two reviewers found this work of interest, and clearly felt that the new experimental data presented in this work had addressed the fundamental technical raises regarding morpholino specificity raised during the first round of review.

Nonetheless, these reviewers raised a series of important concerns with this work, which they felt were sufficient to prevent publication of this work in its present form. In general, journal policy only allows a single round of major revision. Given, however, the substantial amount of new experimental support presented in this version, and the supportive comments from the last two reviewers, we would like to offer you the exceptional opportunity to prepare another revision of this work. The editor feel that the main issues fall into two main categories:

1. The potential relevance of additional genes not studied in this work. The reviewers note in particular Sox2 and Dnmt3A. Some additional, brief justification of the decision to exclude these genes may be needed. Additional analysis of Dnmt3A in particular was requested by two of the reviewers, and this could potentially require additional experimental work to test or exclude its role in the described feedback mechanism.
2. Issues of presentation. The reviewers indicated that this work required a dramatic restructuring to make the manuscript more concise and to bring out the most important biological results. The editor notes that the lengthy passages confirming MO specificity, while very important for addressing the reviewers concerns, could probably be dramatically shortened and/or moved to the Methods section. The reviewers also felt that additional effort should be made to put this work in the context of other works, and they list a series of previous works that they feel should be mentioned. The Reviewer #6 recommends reducing the Dnmt3b results, but the editor feels that this a crucial aspect of the manuscript (in agreement with Reviewer #7), which should instead be presented more concisely and more clearly. We encourage you to check that the revised manuscript is easily understandable to an outside reader not already familiar with the work.

In addition, when preparing your revised manuscript please see the Revision Checklist (below).

If you feel you can satisfactorily deal with these points and those listed by the referees, you may wish to submit a revised version of your manuscript. Please attach a covering letter giving details of the way in which you have handled each of the points raised by the referees. We reserve the right send any revised work back to some or all of the reviewers, and you probably understand that we can give you no guarantee at this stage that the eventual outcome will be favorable.

In addition, I would like to acknowledge that this review process took somewhat longer than usual due to unfortunate delays while soliciting reviewers. My apologies.

Yours sincerely,

Editor - Molecular Systems Biology
msb@embo.org

Referee reports:

Reviewer #5 (Remarks to the Author):

In this report the authors analyze the affects of MO KD of Oct4/Nanog/Sall4 and Dnmt 3b on gene expression networks during early development of the pre-implantation development and propose a feed forward regulatory network involving these factors and mir290.

major comments:

1. It is not clear why Sox2 was left out of the study when others have shown it is an important regulator of early development and is a key component in the Oct4 and Nanog regulatory

complexes.

2. Unclear why Dnmt 3A was not included in the analysis along with Dnmt 3B.
3. The wide variation in gene expression is worrisome and points to stochastic variation in gene expression or the measurement. One would expect that genes repressed by oct4 and Nanog to go up upon KD and genes that are positively regulated by Oct4 etc to go down.
4. The equations defining the feed forward regulatory mechanisms are hard to validate and as a whole are unconvincing in establishing the cause of the noisy gene expression observed in the experiments.
5. An important control the effectiveness of KD of target proteins by the various MOs should not be relegated to the supplemental data. They should be a crucial first figure.
6. The authors do not discuss their results in relation to other published data in the area.
7. The paper can be significantly shortened in every section.

Reviewer #6 (Remarks to the Author):

In the revised paper by Tan et al., the authors have included additional experiments to thoroughly show the specificity of the morpholino targeting to TFs and have addressed many other issues raised by previous reviewers. The work is substantial and provides an excellent resource for future study. However, the revised work is repetitive in many places and needs to be extensively revised to be more concise. The authors need to rigorously evaluate how to re-structure their paper in order to emphasize and highlight the appropriate points. Otherwise, the excessive verbiage distracts readers from the "bottom-line message" and dilutes away main points.

Specific Comments:

1. The authors shall consider to remove the Dnmt3b result/discussion, which was a bit disjointed compared to other data surrounding TFs Oct4, Sall4, and Nanog. Although interesting, the study on Dnmt3b expands to the epigenetic mechanism underlying preimplantation development. One obvious question concerns on how the effect of Dnmt3b is compared to the role of Dnmt3a (another de novo DNA methylase with known overlapping function to Dnmt3b), its functional relevance to shaping the preimplantation methylome (see Smith et al., 2012. Nature PMID:22456710). Thus, revising this paper as a TF-centric study will help shorten the paper, re-focus main points, and afford the authors more space for in-depth commentary.
2. There is a glaring omission of proper references that make the paper appear more novel than it actually is. Morpholino expression has been performed by others (also in mouse preimplantation embryos), yet there is no mention of prior works, nor details on how they carried out morpholino injection. Secondly, some seminal works on gene expression profiling in mouse preimplantation embryos (Wang et al., 2004 and Hamatani et al., 2004 both published in Dev. Cell) as well transcription network analyses (Xie et al., 2010, Genome Res) need to be addressed. Moreover, gene profiling in mouse preimplantation embryos at the single-cell resolution has been performed by Tang et al., 2011, PLoS One. The authors should make conclusion in the context of these works. It would be very helpful to cross-reference their current datasets to these previous studies as a verification of the authors' dataset. For example, the authors argue that the discrepancy between their results and genetic knockout studies is that maternal RNAs, but not the zygotic genome, are capable of driving preimplantation development. As morpholinos targeting splicing event can knock-down newly synthesized RNAs from ZGA, the authors should be able to use this approach to assess the requirement of ZGA for the gene regulatory network of Oct4/Nanog/Sall4 in driving preimplantation development.
3. How does miRNA and pluripotency network identified by Rick Young's group compare with the current findings?
4. The forward-feed loop is not well explained and suffers from lack of citations of previous works

that may be similar or have employed this method. The authors might discuss whether the model will perform in the case of cellular reprogramming, where expressions of Oct4, Sall4, Nanog, Dnmt3b are staggered between partially and fully reprogrammed cells.

5. Statistical significance are still lacking in both texts and in figures, including figures 1b, 6C-D, 7A. As another reviewer mentioned, motif discovery and miRNA target prediction are both algorithms that give more results than are actually true. The authors may perform statistics on the overlap between two algorithms and determine whether the list of intersect is more significant than background. This is needed for data on pages 8, 10, etc.

Reviewer #7 (Remarks to the Author):

In this version, the authors have spent considerable effort to address the potential problem of MOs having off target effects through an approach of co-injection of the MO with the corresponding mRNA for rescue. One of the most interesting results in the manuscript is the demonstration of existence of a feed-forward loop and its importance in controlling variability of the gene expressions. Here are some minor comments.

- 1) Bottom of page 15 - "the same Hill coefficient and Michaelis constant.". What exactly did the authors mean by this? It is important to list H1, H2, ..., H6 used for the simulation in the supplement because those numbers dictate the switch-like behavior.
- 2) Page 18 - "In this case, robustness is lost .. appreciably". The statement is way too strong: The system only becomes a bit more sensitive with minor change in the slope of its response curves with or without the feed-forward loop.
- 3) From the biological point of view, is there an intuitive way to argue that feed-forward loop can usually make an output response less switch-like? From the modeling point of view, as long as the feed-forward term (the 2nd term of the equation 3 in Fig. 6A) dominates, the overall slope of the response curve will be dictated by H3 and the strength of this term relative to the others in the equation.
- 4) What is the importance of the cooperative term (4th term of the equation 3)? How did you choose H5 and H6?
- 5) All legends of the figures can be enlarged to be more readable.
- 6) Bottom of page 4: "unprecedented insight"?

2nd Revision - authors' response

05 November 2012

Overview

We thank the reviewers for helpful suggestions that have improved the manuscript. We have extensively revised the manuscript to make it more concise and to bring out the most important biological results. We have also performed additional analysis to strengthen the manuscript and to put our work in the context of previous related publications.

We address the reviewers' comments point-by-point below.

Reviewer #5

In this report the authors analyze the affects of MO KD of Oct4/Nanog/Sall4 and Dnmt 3b on gene expression networks during early development of the pre-implantation development and propose a feed forward regulatory network involving these factors and mir290.

We thank the reviewer for succinctly summarizing our work.

Major comments:**1. It is not clear why Sox2 was left out of the study when others have shown it is an important regulator of early development and is a key component in the Oct4 and Nanog regulatory complexes.**

We have tried to study Sox2 in preimplantation embryos, as the reviewer has rightly pointed out that it is a well-known component of the Oct4 and Nanog regulatory complexes. Unfortunately, maternal Sox2 protein is difficult to deplete. In embryos injected with a MO targeting the translation start site of Sox2, we could still detect by immunocytochemistry a substantial amount of Sox2 protein 24 hours after injection. Unsurprisingly, these embryos did not show an obvious phenotype until the morula stage. Since we are interested in studying the regulatory network in earlier stages, we did not pursue Sox2 further in the current project. We have also noted the reason why Sox2 was left out of the study in the Supplementary Information (Page 14).

2. Unclear why Dnmt 3A was not included in the analysis along with Dnmt 3B.

There are three reasons why we focused on Dnmt3b in this study. First, we did include both Dnmt3a and Dnmt3b in our BioMark experiments. However, only Dnmt3b allowed us to distinguish early-arrested embryos from late-arrested embryos. The expression of Dnmt3b was 2.1-fold higher in embryos that progressed to the 4-cell and multicell stages than in embryos that arrested at the 2-cell and 3-cell stages ($p=1.29 \times 10^{-20}$), while the expression of Dnmt3a was only 1.1-fold higher ($p=0.00296$). Second, Dnmt3a^{-/-} mice developed to term and appeared grossly normal at birth. In contrast, the Dnmt3b-null allele resulted in embryonic lethality, indicating a more prominent role for Dnmt3b during embryogenesis (Okano et al., *Cell* 1999). Third, there is only one known promoter for the Dnmt3b gene, but there are two separate promoters for the Dnmt3a gene (Chen et al., *J. Biol. Chem.* 2002), which can complicate analysis. For example, the level of the full-length Dnmt3a protein is similar between Dicer^{-/-} embryonic stem cells (ESCs) and wildtype ESCs, while the level of the smaller Dnmt3a protein, which originates from a downstream intronic promoter, is appreciably lower in Dicer^{-/-} ESCs (Sinkkonen et al., *Nat. Struct. Mol. Biol.* 2008). Clearly, the regulatory activities at the two promoters can be different. Further work is needed to tease apart the regulation occurring at the two distinct Dnmt3a promoters, which is beyond the scope of the present study. We have also included a brief note of Dnmt3a in the figure legends of the main text (Page 33) and a more detailed discussion of Dnmt3a vs. Dnmt3b in the Supplementary Information (Pages 10-11).

3. The wide variation in gene expression is worrisome and points to stochastic variation in gene expression or the measurement. One would expect that genes repressed by oct4 and Nanog to go up upon KD and genes that are positively regulated by Oct4 etc to go down.

Transcription factor-target gene relationships are typically studied using experiments that are averaged over cell populations. For example, one may knockdown Oct4 in a plate of ESCs, collect all the cells, and then perform experiments on them to determine the transcript levels of some known target genes. Such measurements represent mean values over a few million cells. Hence, on average, it is true that genes repressed by a transcription factor go up upon knockdown of the regulator and vice versa.

However, gene regulation is often probabilistic in nature, especially since transcription factors may not be present in high copy numbers. In some cells, the transcription factor may be present but by chance, it does not find the correct promoter. Alternatively, the transcription factor may bind the correct promoter, but other essential proteins may not have bound at the same time. Hence, when the regulator is knocked down in these cells, there will be no change in the expression of the target genes and this can only be detected in single cell gene expression profiling experiments.

We are indeed interested in stochastic gene expression. The actual concentration of a transcription factor can vary significantly from cell-to-cell or from embryo-to-embryo. Yet, there are some biological processes that have to be precisely controlled. How does a cell or an embryo take a variable regulatory input and convert it into a precise or reproducible output? There have been multiple studies in the past that investigated this important question (for example, see He et al., *Dev. Cell* 2008). In our study, we showed that a feed-forward network motif helps to buffer the expression of Dnmt3b (output) against fluctuations in the level of the pluripotency factors (input) in wildtype preimplantation embryos in order to achieve a reliable outcome (developmental progression). Furthermore, the differences in gene expression variability between control and

knockdown cells is unlikely to be a result of measurement errors because the measurement error profile should be similar across different cell populations or different experimental conditions.

4. The equations defining the feed forward regulatory mechanisms are hard to validate and as a whole are unconvincing in establishing the cause of the noisy gene expression observed in the experiments.

We disagree with the reviewer on this point. Every mathematical model is an abstraction of some complex underlying biological process and has to be experimentally validated. Our model is no exception and we have validated it in two different ways. First, we broke the feed-forward loop and demonstrated that the expression of *Dnmt3b* did become noisier, as predicted by the model (Pages 14-15, Figure 6E, and Supplementary Figure S17). Second, we knocked down *Oct4* or *Sall4* and showed that the noise buffering mechanism was lost, which was also predicted by the model (Pages 15-17 and Figure 7).

5. An important control the affectiveness of KD of target proteins by the various MOs should not be relegated to the supplemental data. They should be a crucial first figure.

We thank the reviewer for the suggestion. As requested, we have moved our immunostaining images to the main manuscript (Figure 1A).

6. The authors do not discuss their results in relation to other published data in the area.

We apologize for missing out some related publications. In our revised manuscript, we have now cited previous works on gene expression profiling in mouse preimplantation embryos, including the first genome-wide transcriptome profiling efforts by Wang et al. (2004) and Hamatani et al. (2004) as well as the first comprehensive transcriptome study at the single-cell resolution by Tang et al. (2011). We also compared our results with some of these previously published datasets. First, we found that 376 genes that were up-regulated during the maternal-embryonic transition in our microarray data for uninjected embryos had previously been shown to be activated between the 2-cell stage and the 8-cell stage in wildtype mouse embryos (see Supplementary Information Page 3 and Figure S5A). Second, we found that the gene expression variability of our uninjected cells is comparable to the variability observed in the real-time PCR dataset from Tang et al. (2011) (the standard deviations of Ct values are 1.00 and 1.19 respectively). Interestingly, out of all the genes that are common between the two datasets, the gene that showed the largest variability in its expression level is the same in both cases. We speculate that this gene, *Fgf4*, may play a role in biasing blastomeres from the same embryo towards different cell fates (see Supplementary Information Page 5).

7. The paper can be significantly shortened in every section.

We thank the reviewer for the comment. We have made every effort to write the manuscript as concisely as possible without sacrificing clarity. The character count of the manuscript (including spaces) is now ~60,000, which meets the requirement of the journal.

Reviewer #6

In the revised paper by Tan et al., the authors have included additional experiments to thoroughly show the specificity of the morpholino targeting to TFs and have addressed many other issues raised by previous reviewers. The work is substantial and provides an excellent resource for future study.

We thank the reviewer for highlighting the interest in this work.

However, the revised work is repetitive in many places and needs to be extensively revised to be more concise. The authors need to rigorously evaluate how to re-structure their paper in order to emphasize and highlight the appropriate points. Otherwise, the excessive verbiage distracts readers from the "bottom-line message" and dilutes away main points.

We thank the reviewer for the comment. We have tried our best to re-structure the paper, so as to bring out the key biological results. We have also moved all supporting material to the Supplementary Information.

Specific Comments:

1. The authors shall consider to remove the Dnmt3b result/discussion, which was a bit disjointed compared to other data surrounding TFs Oct4, Sall4, and Nanog. Although interesting, the study on Dnmt3b expands to the epigenetic mechanism underlying preimplantation development. One obvious question concerns on how the effect of Dnmt3b is compared to the role of Dnmt3a (another de novo DNA methylase with known overlapping function to Dnmt3b). , its functional relevance to shaping the preimplantation methylome (see Smith et al., 2012. Nature PMID:22456710). Thus, revising this paper as a TF-centric study will help shorten the paper, re-focus main points, and afford the authors more space for in-depth commentary.

We thank the reviewer for the suggestion. However, we feel that the Dnmt3b results form an important component of the manuscript. Hence, we have tried to present these results more concisely and clearly instead.

Dnmt3b appears to perform a more important role than Dnmt3a during embryogenesis. In our BioMark experiments on single embryos, we analyzed the expression of both Dnmt3a and Dnmt3b. However, only Dnmt3b allowed us to distinguish early-arrested embryos from late-arrested embryos. The expression of Dnmt3b was 2.1-fold higher in embryos that progressed further to the 4-cell and multicell stages than in embryos that arrested at the 2-cell and 3-cell stages ($p=1.29 \times 10^{-20}$), while the expression of Dnmt3a was only 1.1-fold higher ($p=0.00296$). This result is in agreement with existing knockout mouse models. Dnmt3a^{-/-} mice develops to term and appears grossly normal at birth. In contrast, the Dnmt3b-null allele results in embryonic lethality, which supports a more prominent role for Dnmt3b during development (Okano et al., *Cell* 1999).

Understanding how Dnmt3a and Dnmt3b contribute to shaping the preimplantation methylome is both an interesting and also an important question. Two sets of experiments are necessary to address the question. First, chromatin immunoprecipitation coupled with high throughput DNA sequencing (chIP-seq) using anti-Dnmt3a and anti-Dnmt3b antibodies will allow us to map the genome-wide locations of the enzymes. Second, depletion or knockdown of Dnmt3a/Dnmt3b followed by bisulfite sequencing will allow us to determine which DNA methylation marks are dependent on Dnmt3a and which marks are dependent on Dnmt3b. However, these experiments are quite involved and such a systematic study is beyond the scope of the current project. Nevertheless, we have included a brief note of Dnmt3a in the figure legends of the main text (Page 33) and a more detailed discussion of Dnmt3a vs. Dnmt3b in the Supplementary Information (Pages 10-11).

2. There is a glaring omission of proper references that make the paper appear more novel than it actually is. Morphino expression has been performed by others (also in mouse preimplantation embryos), yet there is no mention of prior works, nor details on how they carried out morpholino injection. Secondly, some seminal works on gene expression profiling in mouse preimplantation embryos (Wang et al., 2004 and Hamatani et al., 2004 both published in Dev. Cell) as well transcription network analyses (Xie et al., 2010, Genome Res) need to be addressed. Moreover, gene profiling in mouse preimplantation embryos at the single-cell resolution has been performed by Tang et al., 2011, PLoS One. The authors should make conclusion in the context of these works. It would be very helpful to cross-reference their current datasets to these previous studies as a verification of the authors' dataset. For example, the authors argue that the discrepancy between their results and genetic knockout studies is that maternal RNAs, but not the zygotic genome, are capable of driving preimplantation development. As morpholinos targeting splicing event can knock-down newly synthesized RNAs from ZGA, the authors should be able to use this approach to assess the requirement of ZGA for the gene regulatory network of Oct4/Nanog/Sall4 in driving preimplantation development.

We apologize for missing out some relevant publications and have included them in the revised manuscript.

(i) Morpholinos. In our paper, we did not claim any novelty on the usage of morpholinos in mouse preimplantation embryos. Nevertheless, we have now mentioned some prior works that reported the application of morpholinos to mouse embryos (see Supplementary Information Page 14). Details on how we carried out the microinjections are present in the Materials and Methods section (Page 22).

(ii) Expression profiling in mouse preimplantation embryos. We have highlighted the major publications that profiled the transcriptome during preimplantation developmental stages in the Introduction section of our manuscript (Page 4). In addition, we compared our microarray data for uninjected embryos with one of the previously published datasets. Specifically, we found that 376 genes that were up-regulated during the maternal-embryonic transition in our data for uninjected embryos had previously been shown to be activated between the 2-cell stage and the 8-cell stage in wildtype mouse embryos (see Supplementary Information Page 3).

(iii) Requirement of Oct4/Sall4/Nanog in zygotic genome activation or ZGA. We analyzed how the transcriptome changes in the embryos between our 20 hour timepoint (before ZGA) and our 44 hour timepoint (after ZGA). Interestingly, we found that depletion of Oct4, Sall4, or Nanog reduced the extent to which the embryonic genome was activated. The number of up-regulated genes in knockdown embryos was 21.7-31.8% less than that in uninjected embryos (Supplementary Figure S5B). In contrast, the number of activated genes in control-injected embryos was nearly identical to that in uninjected embryos. Hence, our results suggest that the maternally-deposited pluripotency factors may play an important role in ensuring the full activation of the zygotic genome. We have included this analysis in the Supplementary Information (Page 3).

(iv) Transcriptional network analysis. In the paper pointed out by the reviewer (Xie et al., *Genome Res.* 2010), the authors first performed microarray analysis of the transcriptome in wildtype embryos of three different species and then intersected the zygotic activated genes with the genes whose promoters were bound by Oct4 and Nanog in ESCs. In contrast, we intersected genes that were differentially expressed upon knockdown of Oct4/Nanog with genes whose promoters were bound by Oct4/Nanog. Conceptually, the two approaches are different and it would be informative to compare them. Unfortunately, the only relevant data that are publicly available for the Xie et al. (2010) paper are the microarray datasets. We further requested for the analysis files from the authors but were informed that they no longer have those files. Hence, we were unable to cross-reference our work with their network analysis. In any case, the network deconstructed by Xie et al. (2010) was based purely on association with no evidence of causality. Furthermore, the authors only identified the activated genes but not the repressed genes. Hence, there are likely to be some differences between our network and the network presented by Xie et al. (2010).

(v) Single-cell profiling. We have highlighted the paper that reported gene expression profiling at single-cell resolution (Tang et al., *PLoS One* 2011) in the Introduction section of our manuscript (Page 4). In addition, we have compared our single-cell data with the authors' dataset and reported the results in the Supplementary Information (Page 5). Briefly, the gene expression variability of our uninjected cells is comparable to the variability observed in the authors' real-time PCR dataset (the standard deviations of Ct values are 1.00 and 1.19 respectively). Interestingly, out of all the genes that are common between the two datasets, the gene that showed the largest variability in its expression level is the same in both cases. We speculate that this gene, *Fgf4*, may play a role in biasing blastomeres from the same embryo towards different cell fates.

3. How does miRNA and pluripotency network identified by Rick Young's group compare with the current findings?

We have compared our findings with publicly available chIP datasets in ESCs, which included results from Richard Young's laboratory (see Pages 7-9, Figure 3C, Supplementary Figures S6 and S7, as well as Supplementary Files S6, S13, and S15). Briefly, the overlaps between our microarray results and the chIP results were 20.4% for down-regulated genes and 18.8% for up-regulated genes. In addition, 33.3% of the miRNAs that were differentially expressed during knockdown of a pluripotency factor in embryos also had regulatory regions that were bound by the corresponding factor in ESCs.

4. The forward-feed loop is not well explained and suffers from lack of citations of previous works that may be similar or have employed this method. The authors might discuss whether the model will perform in the case of cellular reprogramming, where expressions of Oct4, Sall4, Nanog, Dnmt3b are staggered between partially and fully reprogrammed cells.

We apologize for the lack of clarity. We have included a short description in the figure legends and cited three publications from Uri Alon's laboratory that studied network motifs and the

feed-forward loop in great detail as well as other relevant publications that may enhance the reader's understanding of transcriptional networks (Page 35).

We interpret "cellular reprogramming" as nuclear reprogramming of somatic cells to pluripotent stem cells. As the reviewer pointed out, distinct markers are activated or repressed sequentially over the course of reprogramming. After a series of intermediate stages that are still not clearly understood, endogenous pluripotency factors such as Oct4 and Nanog are activated with the concomitant formation of ESC-like colonies. Even though the expression of Dnmt3b rises slightly during the intermediate stages, it increases by more than four-fold after activation of the pluripotency genes (Stadtfeld et al., *Cell Stem Cell* 2008), suggesting that Dnmt3b expression relies predominantly on the endogenous pluripotency factors. Furthermore, introduction of the miR-290 cluster of miRNAs significantly improved reprogramming efficiency (Judson et al., *Nat. Biotechnol.* 2009), while knockdown of the Rbl2 transcription factor also enhanced reprogramming, albeit more modestly (Subramanyam et al., *Nat. Biotechnol.* 2011). Hence, the feed-forward loop may represent a gene module that regulates the transcription of Dnmt3b during the formation of induced pluripotent stem cells. We have included this discussion in the Supplementary Information (Page 12).

5. Statistical significance are still lacking in both texts and in figures, including figures 1b, 6C-D, 7A.

We apologize for the oversight. As suggested, we have included the statistical significance for Figures 1C (previously Figure 1B), 5A, 5C, 5D, 6E, and 7A as well as for Supplementary Figure S3.

As another reviewer mentioned, motif discovery and miRNA target prediction are both algorithms that give more results than are actually true. The authors may perform statistics on the overlap between two algorithms and determine whether the list of intersect is more significant than background. This is needed for data on pages 8, 10, etc.

The Oct4 motif that we presented is almost identical to the well established Oct4-Sox2 binding site, which has already been experimentally validated (Loh et al., *Nat. Genet.* 2006). For Nanog, we asked if we could recover the same (or at least comparable) motifs that were presented in Figure 2E using a different program. To this end, we performed a *de novo* search for *cis*-regulatory elements using SCOPE (Chakravarty et al., *BMC Bioinformatics* 2007; Carlson et al., *Nucleic Acids Res.* 2007) and found that the top motifs resembled the TGACCTT core sequence that was predicted by CisGenome (Ji et al., *Nat. Biotechnol.* 2008) (Supplementary Figure S8).

To increase confidence in the miRNA-target predictions, we asked which of the miRNAs outputted by miRanda (Betel et al., *Nucleic Acids Res.* 2008) were also predicted by at least one other program, including DIANA-microT (Maragkakis et al., *BMC Bioinformatics* 2009; Maragkakis et al., *Nucleic Acids Res.* 2009), miRDB (Wang et al., *Bioinformatics* 2008; Wang, *RNA* 2008), miRWalk (Dweep et al., *J. Biomed. Inform.* 2011), PITA (Kertesz et al., *Nat. Genet.* 2007), RNA22 (Miranda et al., *Cell* 2006), and TargetScan (Lewis et al., *Cell* 2005; Friedman et al., *Genome Res.* 2009) (Page 9). Besides the original files containing all the miRNAs predicted by miRanda only (Supplementary Files S12-S13), we have now provided additional files containing the miRNAs that are predicted by at least two different algorithms (Supplementary Files S14-15). In these additional files, each listed miRNA is followed by [x], where x is the number of programs that predicted a particular miRNA-target pair and ranges from 2 to 7.

Reviewer #7

In this version, the authors have spent considerable effort to address the potential problem of MOs having off target effects through an approach of co-injection of the MO with the corresponding mRNA for rescue. One of the most interesting results in the manuscript is the demonstration of existence of a feed-forward loop and its importance in controlling variability of the gene expressions. Here are some minor comments.

We thank the reviewer for highlighting the interest in this work.

1) Bottom of page 15 - "the same Hill coefficient and Michaelis constant.". What exactly did the authors mean by this? It is important to list H1, H2, ..., H6 used for the simulation in the supplement because those numbers dictate the switch-like behavior.

We apologize for the confusion. We meant that we used the same numbers for all the common parameters between the feed-forward loop and the simple two-component model (pluripotency factors and Dnmt3b only). We have adjusted the text (Page 13 and Supplementary Information Page 37) and provided all parameters used for the simulations in Supplementary Files S17-S18.

2) Page 18 - "In this case, robustness is lost .. appreciably". The statement is way too strong: The system only becomes a bit more sensitive with minor change in the slope of its response curves with or without the feed-forward loop.

The reviewer has misunderstood the statement. In the paragraph that contains the statement, we were not comparing the response curve for the feed-forward loop with the response curve for the simple two-component system without the feed-forward loop. Instead, we were describing how the slope of the same response curve (blue graph in Figure 6C only) changes at different levels of the pluripotency factors. At high levels of the pluripotency factors (i.e. right side of the response curve), the slope is shallow and Dnmt3b expression is robust with respect to intrinsic fluctuations in the levels of the factors. However, at low levels of the factors (i.e. left side of the same response curve), the gradient increases dramatically and Dnmt3b expression becomes much more sensitive to changes in the levels of the factors. We have rewritten the paragraph in order to improve its clarity (Page 15).

3) From the biological point of view, is there an intuitive way to argue that feed-forward loop can usually make an output response less switch-like? From the modeling point of view, as long as the feed-forward term (the 2nd term of the equation 3 in Fig. 6A) dominates, the overall slope of the response curve will be dictated by H3 and the strength of this term relative to the others in the equation.

An intuitive argument from the biological point of view is as follows. The ultrasensitivity in the system originates from the Oct4-miRNA-Rbl2-Dnmt3b pathway. The Rbl2 3'UTR contains multiple binding sites for the miR-290-295 cluster of miRNAs (Sinkkonen et al., *Nat. Struct. Mol. Biol.* 2008). It is possible that the binding of the miRNAs is cooperative. For example, the 3'UTR may be highly structured, such that the miRNAs have limited access to their binding sites. Nevertheless, when the first miRNA manages to bind, it opens up the double-stranded structure to allow other miRNAs to bind more easily. Hence, the concentration of the miRNAs may have to exceed a certain threshold to bring about rapid degradation of the Rbl2 mRNA or efficient translation blockage of the Rbl2 transcripts.

However, when the direct Oct4-Dnmt3b pathway is added on to create the feed-forward loop, it provides a means to bypass the indirect regulatory pathway, from which the ultrasensitivity originates. Thus, even when the miRNAs have not accumulated beyond the requisite threshold to relieve the Dnmt3b promoter from repression by Rbl2, Oct4 itself is already impacting on the transcription of Dnmt3b, thereby short-circuiting the process. As a result, the output response becomes less switch-like. We have included this argument in the Supplementary Information (Pages 9-10).

4) What is the importance of the cooperative term (4th term of the equation 3)? How did you choose H5 and H6?

We investigated the importance of the cooperative term (fourth term of the third equation) by varying the parameter, β_c . We observed that the precise value of β_c does not affect the steepest gradient of the input-output curve appreciably, i.e. the sensitivity of the dynamical system seems to be unaffected. Instead, the curve appears to move to the left with smaller values of β_c or move to the right with larger values of β_c (Supplementary Figure S15). Hence the cooperative term determines where the switch in the response curve occurs.

We chose H5 and H6 to be equal to H3 and H4 respectively. These Hill coefficients were set to either 1 or 2, which are commonly used values (Supplemental File S17).

5) All legends of the figures can be enlarged to be more readable.

We apologize for the small font and have tried to increase the font size in the figures wherever possible to improve readability.

6) Bottom of page 4: "unprecedented insight"?

We apologize for the lack of clarity. We have changed the phrase to “novel and unexpected insights” (Page 4).

4th Editorial Decision

06 November 2012

Thank you again for submitting your revised work to Molecular Systems Biology. We have now had time to read and consider your revised manuscript and your response to the reviewers. While we found your responses satisfactory, and agree that the scientific content of this work is now, in principle, appropriate for Molecular Systems Biology, we feel that some additional work is needed to address the substantial concerns regarding clarity of presentation raised during the previous round of review. We therefore ask that you prepare a final revision of this work to address these remaining textual and content issues.

1. To help expedite this process, I have attached an edited document where I attempted to further improve the conciseness and the clarity of the Results section of this work. I did feel that this section still remained overly long and included unnecessary discussion and repetitive of methods details. I hope these suggested changes are helpful, and apologies if they garble your intended meaning. Similar streamlining of other sections of the manuscript may also be helpful.

2. In addition, I feel that the issues regarding Sox2 and Dnmt3a were important enough to the reviewers that they deserve brief discussion in the main manuscript, rather the supplementary material. Please move your responses on these points to the Introduction, Results, or Discussion, as appropriate.

3. Please submit all new microarray data to public repository, such as GEO or ArrayExpress, and include the resulting accession number(s) in the Methods section of this manuscript.

Please feel free to contact us if any of the above issues will be problematic.

Thank you for submitting this paper to Molecular Systems Biology.

Yours sincerely,
Editor - Molecular Systems Biology

3rd Revision - authors' response

13 November 2012

Overview

We thank the reviewers for helpful suggestions that have improved the manuscript. We have extensively revised the manuscript to make it more concise and to bring out the most important biological results. We have also performed additional analysis to strengthen the manuscript and to put our work in the context of previous related publications. In addition, we have uploaded all our microarray datasets onto the NCBI Gene Expression Omnibus (GEO) under the accession number GSE42135.

We address the reviewers' comments point-by-point below.

Reviewer #5

In this report the authors analyze the affects of MO KD of Oct4/Nanog/Sall4 and Dnmt 3b on gene expression networks during early development of the pre-implantation development and propose a feed forward regulatory network involing these factors and mir290.

We thank the reviewer for succinctly summarizing our work.

Major comments:

1. It is not clear why Sox2 was left out of the study when others have shown it is an important

regulator of early development and is a key component in the Oct4 and Nanog regulatory complexes.

We have tried to study Sox2 in preimplantation embryos, as the reviewer has rightly pointed out that it is a well-known component of the Oct4 and Nanog regulatory complexes. Unfortunately, maternal Sox2 protein is difficult to deplete. In embryos injected with a MO targeting the translation start site of Sox2, we could still detect by immunocytochemistry a substantial amount of Sox2 protein 24 hours after injection. Unsurprisingly, these embryos did not show an obvious phenotype until the morula stage. Since we are interested in studying the regulatory network in earlier stages, we did not pursue Sox2 further in the current project. We have also noted the reason why Sox2 was left out of the study in the discussion section of the main text (Pages 17-18) and in the Supplementary Information (Page 13).

2. Unclear why Dnmt 3A was not included in the analysis along with Dnmt 3B.

There are three reasons why we focused on Dnmt3b in this study. First, we did include both Dnmt3a and Dnmt3b in our BioMark experiments. However, only Dnmt3b allowed us to distinguish early-arrested embryos from late-arrested embryos. The expression of Dnmt3b was 2.1-fold higher in embryos that progressed to the 4-cell and multicell stages than in embryos that arrested at the 2-cell and 3-cell stages ($p=1.29 \times 10^{-20}$), while the expression of Dnmt3a was only 1.1-fold higher ($p=0.00296$). Second, Dnmt3a^{-/-} mice developed to term and appeared grossly normal at birth. In contrast, the Dnmt3b-null allele resulted in embryonic lethality, indicating a more prominent role for Dnmt3b during embryogenesis (Okano et al., *Cell* 1999). Third, there is only one known promoter for the Dnmt3b gene, but there are two separate promoters for the Dnmt3a gene (Chen et al., *J. Biol. Chem.* 2002), which can complicate analysis. For example, the level of the full-length Dnmt3a protein is similar between Dicer^{-/-} embryonic stem cells (ESCs) and wildtype ESCs, while the level of the smaller Dnmt3a protein, which originates from a downstream intronic promoter, is appreciably lower in Dicer^{-/-} ESCs (Sinkkonen et al., *Nat. Struct. Mol. Biol.* 2008). Clearly, the regulatory activities at the two promoters can be different. Further work is needed to tease apart the regulation occurring at the two distinct Dnmt3a promoters, which is beyond the scope of the present study. We have also described the reasons why Dnmt3a was left out of the analysis in the discussion section of the main text (Page 20).

3. The wide variation in gene expression is worrisome and points to stochastic variation in gene expression or the measurement. One would expect that genes repressed by oct4 and Nanog to go up upon KD and genes that are positively regulated by Oct4 etc to go down.

Transcription factor-target gene relationships are typically studied using experiments that are averaged over cell populations. For example, one may knockdown Oct4 in a plate of ESCs, collect all the cells, and then perform experiments on them to determine the transcript levels of some known target genes. Such measurements represent mean values over a few million cells. Hence, on average, it is true that genes repressed by a transcription factor go up upon knockdown of the regulator and vice versa.

However, gene regulation is often probabilistic in nature, especially since transcription factors may not be present in high copy numbers. In some cells, the transcription factor may be present but by chance, it does not find the correct promoter. Alternatively, the transcription factor may bind the correct promoter, but other essential proteins may not have bound at the same time. Hence, when the regulator is knocked down in these cells, there will be no change in the expression of the target genes and this can only be detected in single cell gene expression profiling experiments.

We are indeed interested in stochastic gene expression. The actual concentration of a transcription factor can vary significantly from cell-to-cell or from embryo-to-embryo. Yet, there are some biological processes that have to be precisely controlled. How does a cell or an embryo take a variable regulatory input and convert it into a precise or reproducible output? There have been multiple studies in the past that investigated this important question (for example, see He et al., *Dev. Cell* 2008). In our study, we showed that a feed-forward network motif helps to buffer the expression of Dnmt3b (output) against fluctuations in the level of the pluripotency factors (input) in wildtype preimplantation embryos in order to achieve a reliable outcome (developmental progression). Furthermore, the differences in gene expression variability between control and knockdown cells is unlikely to be a result of measurement errors because the measurement error profile should be similar across different cell populations or different experimental conditions.

4. The equations defining the feed forward regulatory mechanisms are hard to validate and as

a whole are unconvincing in establishing the cause of the noisy gene expression observed in the experiments.

We disagree with the reviewer on this point. Every mathematical model is an abstraction of some complex underlying biological process and has to be experimentally validated. Our model is no exception and we have validated it in two different ways. First, we broke the feed-forward loop and demonstrated that the expression of Dnmt3b did become noisier, as predicted by the model (Pages 13-14, Figure 6E, and Supplementary Figure S17). Second, we knocked down Oct4 or Sall4 and showed that the noise buffering mechanism was lost, which was also predicted by the model (Pages 14-16 and Figure 7).

5. An important control the affectiveness of KD of target proteins by the various MOs should not be relegated to the supplemental data. They should be a crucial first figure.

We thank the reviewer for the suggestion. As requested, we have moved our immunostaining images to the main manuscript (Figure 1A).

6. The authors do not discuss their results in relation to other published data in the area.

We apologize for missing out some related publications. In our revised manuscript, we have now cited previous works on gene expression profiling in mouse preimplantation embryos, including the first genome-wide transcriptome profiling efforts by Wang et al. (2004) and Hamatani et al. (2004) as well as the first comprehensive transcriptome study at the single-cell resolution by Tang et al. (2011). We also compared our results with some of these previously published datasets. First, we found that 376 genes that were up-regulated during the maternal-embryonic transition in our microarray data for uninjected embryos had previously been shown to be activated between the 2-cell stage and the 8-cell stage in wildtype mouse embryos (see Supplementary Information Page 3 and Figure S5A). Second, we found that the gene expression variability of our uninjected cells is comparable to the variability observed in the real-time PCR dataset from Tang et al. (2011) (the standard deviations of Ct values are 1.00 and 1.19 respectively). Interestingly, out of all the genes that are common between the two datasets, the gene that showed the largest variability in its expression level is the same in both cases. We speculate that this gene, *Fgf4*, may play a role in biasing blastomeres from the same embryo towards different cell fates (see Supplementary Information Page 5).

7. The paper can be significantly shortened in every section.

We thank the reviewer for the comment. We have made every effort to write the manuscript as concisely as possible without sacrificing clarity. The character count of the manuscript (including spaces) is now ~60,000, which meets the requirement of the journal.

Reviewer #6

In the revised paper by Tan et al., the authors have included additional experiments to thoroughly show the specificity of the morpholino targeting to TFs and have addressed many other issues raised by previous reviewers. The work is substantial and provides an excellent resource for future study.

We thank the reviewer for highlighting the interest in this work.

However, the revised work is repetitive in many places and needs to be extensively revised to be more concise. The authors need to rigorously evaluate how to re-structure their paper in order to emphasize and highlight the appropriate points. Otherwise, the excessive verbiage distracts readers from the "bottom-line message" and dilutes away main points.

We thank the reviewer for the comment. We have tried our best to re-structure the paper, so as to bring out the key biological results. We have also moved all supporting material to the Supplementary Information.

Specific Comments:

1. The authors shall consider to remove the Dnmt3b result/discussion, which was a bit disjointed compared to other data surrounding TFs Oct4, Sall4, and Nanog. Although interesting, the study on Dnmt3b expands to the epigenetic mechanism underlying preimplantation development. One obvious question concerns on how the effect of Dnmt3b is compared to the role of Dnmt3a (another de novo DNA methylase with known overlapping function to Dnmt3b), its functional relevance to shaping the preimplantation methylome (see Smith et al., 2012. Nature PMID:22456710). Thus, revising this paper as a TF-centric study

will help shorten the paper, re-focus main points, and afford the authors more space for in-depth commentary.

We thank the reviewer for the suggestion. However, we feel that the Dnmt3b results form an important component of the manuscript. Hence, we have tried to present these results more concisely and clearly instead.

Dnmt3b appears to perform a more important role than Dnmt3a during embryogenesis. In our BioMark experiments on single embryos, we analyzed the expression of both Dnmt3a and Dnmt3b. However, only Dnmt3b allowed us to distinguish early-arrested embryos from late-arrested embryos. The expression of Dnmt3b was 2.1-fold higher in embryos that progressed further to the 4-cell and multicell stages than in embryos that arrested at the 2-cell and 3-cell stages ($p=1.29 \times 10^{-20}$), while the expression of Dnmt3a was only 1.1-fold higher ($p=0.00296$). This result is in agreement with existing knockout mouse models. Dnmt3a^{-/-} mice develops to term and appears grossly normal at birth. In contrast, the Dnmt3b-null allele results in embryonic lethality, which supports a more prominent role for Dnmt3b during development (Okano et al., *Cell* 1999).

Understanding how Dnmt3a and Dnmt3b contribute to shaping the preimplantation methylome is both an interesting and also an important question. Two sets of experiments are necessary to address the question. First, chromatin immunoprecipitation coupled with high throughput DNA sequencing (chIP-seq) using anti-Dnmt3a and anti-Dnmt3b antibodies will allow us to map the genome-wide locations of the enzymes. Second, depletion or knockdown of Dnmt3a/Dnmt3b followed by bisulfite sequencing will allow us to determine which DNA methylation marks are dependent on Dnmt3a and which marks are dependent on Dnmt3b. However, these experiments are quite involved and such a systematic study is beyond the scope of the current project. Nevertheless, we have included a discussion of Dnmt3a vs. Dnmt3b in the main text (Page 20).

2. There is a glaring omission of proper references that make the paper appear more novel than it actually is. Morpholino expression has been performed by others (also in mouse preimplantation embryos), yet there is no mention of prior works, nor details on how they carried out morpholino injection. Secondly, some seminal works on gene expression profiling in mouse preimplantation embryos (Wang et al., 2004 and Hamatani et al., 2004 both published in *Dev. Cell*) as well transcription network analyses (Xie et al., 2010, *Genome Res*) need to be addressed. Moreover, gene profiling in mouse preimplantation embryos at the single-cell resolution has been performed by Tang et al., 2011, *PLoS One*. The authors should make conclusion in the context of these works. It would be very helpful to cross-reference their current datasets to these previous studies as a verification of the authors' dataset. For example, the authors argue that the discrepancy between their results and genetic knockout studies is that maternal RNAs, but not the zygotic genome, are capable of driving preimplantation development. As morpholinos targeting splicing event can knock-down newly synthesized RNAs from ZGA, the authors should be able to use this approach to assess the requirement of ZGA for the gene regulatory network of Oct4/Nanog/Sall4 in driving preimplantation development.

We apologize for missing out some relevant publications and have included them in the revised manuscript.

(i) Morpholinos. In our paper, we did not claim any novelty on the usage of morpholinos in mouse preimplantation embryos. Nevertheless, we have now mentioned some prior works that reported the application of morpholinos to mouse embryos (see Supplementary Information Page 13). Details on how we carried out the microinjections are present in the Materials and Methods section (Page 22).

(ii) Expression profiling in mouse preimplantation embryos. We have highlighted the major publications that profiled the transcriptome during preimplantation developmental stages in the Introduction section of our manuscript (Page 4). In addition, we compared our microarray data for uninjected embryos with one of the previously published datasets. Specifically, we found that 376 genes that were up-regulated during the maternal-embryonic transition in our data for uninjected embryos had previously been shown to be activated between the 2-cell stage and the 8-cell stage in wildtype mouse embryos (see Supplementary Information Page 3).

(iii) Requirement of Oct4/Sall4/Nanog in zygotic genome activation or ZGA. We analyzed how the transcriptome changes in the embryos between our 20 hour timepoint (before ZGA) and our 44 hour timepoint (after ZGA). Interestingly, we found that depletion of Oct4, Sall4, or Nanog reduced the extent to which the embryonic genome was activated. The number of up-regulated genes in knockdown embryos was 26.7-37.4% less than that in uninjected embryos (Supplementary Figure S5B). In contrast, the number of activated genes in control-injected embryos was nearly identical to

that in uninjected embryos. Hence, our results suggest that the maternally-deposited pluripotency factors may play an important role in ensuring the full activation of the zygotic genome. We have included this analysis in the Supplementary Information (Page 3).

(iv) Transcriptional network analysis. In the paper pointed out by the reviewer (Xie et al., *Genome Res.* 2010), the authors first performed microarray analysis of the transcriptome in wildtype embryos of three different species and then intersected the zygotic activated genes with the genes whose promoters were bound by Oct4 and Nanog in ESCs. In contrast, we intersected genes that were differentially expressed upon knockdown of Oct4/Nanog with genes whose promoters were bound by Oct4/Nanog. Conceptually, the two approaches are different and it would be informative to compare them. Unfortunately, the only relevant data that are publicly available for the Xie et al. (2010) paper are the microarray datasets. We further requested for the analysis files from the authors but were informed that they no longer have those files. Hence, we were unable to cross-reference our work with their network analysis. In any case, the network deconstructed by Xie et al. (2010) was based purely on association with no evidence of causality. Furthermore, the authors only identified the activated genes but not the repressed genes. Hence, there are likely to be some differences between our network and the network presented by Xie et al. (2010).

(v) Single-cell profiling. We have highlighted the paper that reported gene expression profiling at single-cell resolution (Tang et al., *PLoS One* 2011) in the Introduction section of our manuscript (Page 4). In addition, we have compared our single-cell data with the authors' dataset and reported the results in the Supplementary Information (Page 5). Briefly, the gene expression variability of our uninjected cells is comparable to the variability observed in the authors' real-time PCR dataset (the standard deviations of Ct values are 1.00 and 1.19 respectively). Interestingly, out of all the genes that are common between the two datasets, the gene that showed the largest variability in its expression level is the same in both cases. We speculate that this gene, *Fgf4*, may play a role in biasing blastomeres from the same embryo towards different cell fates.

3. How does miRNA and pluripotency network identified by Rick Young's group compare with the current findings?

We have compared our findings with publicly available chIP datasets in ESCs, which included results from Richard Young's laboratory (see Pages 7-9, Figure 3C, Supplementary Figures S6 and S7, as well as Supplementary Files S6, S13, and S15). Briefly, the overlaps between our microarray results and the chIP results were 20.4% for down-regulated genes and 18.8% for up-regulated genes. In addition, 33.3% of the miRNAs that were differentially expressed during knockdown of a pluripotency factor in embryos also had regulatory regions that were bound by the corresponding factor in ESCs.

4. The forward-feed loop is not well explained and suffers from lack of citations of previous works that may be similar or have employed this method. The authors might discuss whether the model will perform in the case of cellular reprogramming, where expressions of Oct4, Sall4, Nanog, Dnmt3b are staggered between partially and fully reprogrammed cells.

We apologize for the lack of clarity. We have included a short description in the figure legends and cited three publications from Uri Alon's laboratory that studied network motifs and the feed-forward loop in great detail as well as other relevant publications that may enhance the reader's understanding of transcriptional networks (Page 35).

We interpret "cellular reprogramming" as nuclear reprogramming of somatic cells to pluripotent stem cells. As the reviewer pointed out, distinct markers are activated or repressed sequentially over the course of reprogramming. After a series of intermediate stages that are still not clearly understood, endogenous pluripotency factors such as Oct4 and Nanog are activated with the concomitant formation of ESC-like colonies. Even though the expression of *Dnmt3b* rises slightly during the intermediate stages, it increases by more than four-fold after activation of the pluripotency genes (Stadtfeld et al., *Cell Stem Cell* 2008), suggesting that *Dnmt3b* expression relies predominantly on the endogenous pluripotency factors. Furthermore, introduction of the miR-290 cluster of miRNAs significantly improved reprogramming efficiency (Judson et al., *Nat. Biotechnol.* 2009), while knockdown of the *Rbl2* transcription factor also enhanced reprogramming, albeit more modestly (Subramanyam et al., *Nat. Biotechnol.* 2011). Hence, the feed-forward loop may represent a gene module that regulates the transcription of *Dnmt3b* during the formation of induced pluripotent stem cells. We have included this discussion in the Supplementary Information (Pages 10-11).

5. Statistical significance are still lacking in both texts and in figures, including figures 1b, 6C-D, 7A.

We apologize for the oversight. As suggested, we have included the statistical significance for Figures 1C (previously Figure 1B), 5A, 5C, 5D, 6E, and 7A as well as for Supplementary Figure S3.

As another reviewer mentioned, motif discovery and miRNA target prediction are both algorithms that give more results than are actually true. The authors may perform statistics on the overlap between two algorithms and determine whether the list of intersect is more significant than background. This is needed for data on pages 8, 10, etc.

The Oct4 motif that we presented is almost identical to the well established Oct4-Sox2 binding site, which has already been experimentally validated (Loh et al., *Nat. Genet.* 2006). For Nanog, we asked if we could recover the same (or at least comparable) motifs that were presented in Figure 2E using a different program. To this end, we performed a *de novo* search for *cis*-regulatory elements using SCOPE (Chakravarty et al., *BMC Bioinformatics* 2007; Carlson et al., *Nucleic Acids Res.* 2007) and found that the top motifs resembled the TGACCTT core sequence that was predicted by CisGenome (Ji et al., *Nat. Biotechnol.* 2008) (Supplementary Figure S8).

To increase confidence in the miRNA-target predictions, we asked which of the miRNAs outputted by miRanda (Betel et al., *Nucleic Acids Res.* 2008) were also predicted by at least one other program, including DIANA-microT (Maragkakis et al., *BMC Bioinformatics* 2009; Maragkakis et al., *Nucleic Acids Res.* 2009), miRDB (Wang et al., *Bioinformatics* 2008; Wang, *RNA* 2008), miRWalk (Dweep et al., *J. Biomed. Inform.* 2011), PITA (Kertesz et al., *Nat. Genet.* 2007), RNA22 (Miranda et al., *Cell* 2006), and TargetScan (Lewis et al., *Cell* 2005; Friedman et al., *Genome Res.* 2009) (Page 9). Besides the original files containing all the miRNAs predicted by miRanda only (Supplementary Files S12-S13), we have now provided additional files containing the miRNAs that are predicted by at least two different algorithms (Supplementary Files S14-15). In these additional files, each listed miRNA is followed by [x], where x is the number of programs that predicted a particular miRNA-target pair and ranges from 2 to 7.

Reviewer #7

In this version, the authors have spent considerable effort to address the potential problem of MOs having off target effects through an approach of co-injection of the MO with the corresponding mRNA for rescue. One of the most interesting results in the manuscript is the demonstration of existence of a feed-forward loop and its importance in controlling variability of the gene expressions. Here are some minor comments.

We thank the reviewer for highlighting the interest in this work.

1) Bottom of page 15 - "the same Hill coefficient and Michaelis constant.". What exactly did the authors mean by this? It is important to list H1, H2, ..., H6 used for the simulation in the supplement because those numbers dictate the switch-like behavior.

We apologize for the confusion. We meant that we used the same numbers for all the common parameters between the feed-forward loop and the simple two-component model (pluripotency factors and Dnmt3b only). We have adjusted the text (Page 12 and Supplementary Information Page 36) and provided all parameters used for the simulations in Supplementary Files S17-S18.

2) Page 18 - "In this case, robustness is lost .. appreciably". The statement is way too strong: The system only becomes a bit more sensitive with minor change in the slope of its response curves with or without the feed-forward loop.

The reviewer has misunderstood the statement. In the paragraph that contains the statement, we were not comparing the response curve for the feed-forward loop with the response curve for the simple two-component system without the feed-forward loop. Instead, we were describing how the slope of the same response curve (blue graph in Figure 6C only) changes at different levels of the pluripotency factors. At high levels of the pluripotency factors (i.e. right side of the response curve), the slope is shallow and Dnmt3b expression is robust with respect to intrinsic fluctuations in the levels of the factors. However, at low levels of the factors (i.e. left side of the same response curve), the gradient increases dramatically and Dnmt3b expression becomes much more sensitive to changes in the levels of the factors. We have rewritten the paragraph in order to improve its clarity (Page 14).

3) From the biological point of view, is there an intuitive way to argue that feed-forward loop can usually make an output response less switch-like? From the modeling point of view, as long as the feed-forward term (the 2nd term of the equation 3 in Fig. 6A) dominates, the overall slope of the response curve will be dictated by H3 and the strength of this term relative to the others in the equation.

An intuitive argument from the biological point of view is as follows. The ultrasensitivity in the system originates from the Oct4-miRNA-Rbl2-Dnmt3b pathway. The Rbl2 3'UTR contains multiple binding sites for the miR-290-295 cluster of miRNAs (Sinkkonen et al., *Nat. Struct. Mol. Biol.* 2008). It is possible that the binding of the miRNAs is cooperative. For example, the 3'UTR may be highly structured, such that the miRNAs have limited access to their binding sites. Nevertheless, when the first miRNA manages to bind, it opens up the double-stranded structure to allow other miRNAs to bind more easily. Hence, the concentration of the miRNAs may have to exceed a certain threshold to bring about rapid degradation of the Rbl2 mRNA or efficient translation blockage of the Rbl2 transcripts.

However, when the direct Oct4-Dnmt3b pathway is added on to create the feed-forward loop, it provides a means to bypass the indirect regulatory pathway, from which the ultrasensitivity originates. Thus, even when the miRNAs have not accumulated beyond the requisite threshold to relieve the Dnmt3b promoter from repression by Rbl2, Oct4 itself is already impacting on the transcription of Dnmt3b, thereby short-circuiting the process. As a result, the output response becomes less switch-like. We have included this argument in the Supplementary Information (Pages 9-10).

4) What is the importance of the cooperative term (4th term of the equation 3)? How did you choose H5 and H6?

We investigated the importance of the cooperative term (fourth term of the third equation) by varying the parameter, β_c . We observed that the precise value of β_c does not affect the steepest gradient of the input-output curve appreciably, i.e. the sensitivity of the dynamical system seems to be unaffected. Instead, the curve appears to move to the left with smaller values of β_c or move to the right with larger values of β_c (Supplementary Figure S15). Hence the cooperative term determines where the switch in the response curve occurs.

We chose H5 and H6 to be equal to H3 and H4 respectively. These Hill coefficients were set to either 1 or 2, which are commonly used values (Supplemental File S17).

5) All legends of the figures can be enlarged to be more readable.

We apologize for the small font and have tried to increase the font size in the figures wherever possible to improve readability.

6) Bottom of page 4: "unprecedented insight"?

We apologize for the lack of clarity. We have changed the phrase to "novel and unexpected insights" (Page 4).