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Venus-trap in the mouse embryo reveals distinct molecular dynamics underlying specification of first embryonic lineages

Jens-Erik Dietrich, Laura Panavaite, Stefan Gunther, Sebastian Wennekamp, Anna C Groner, Anton Pigge, Stefanie Salvenmoser, Didier Trono, Lars Hufnagel and Takashi Hiiragi

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Please note that this manuscript was originally submitted to the EMBO Journal where it was peer-reviewed. It was then transferred to EMBO reports with the original referees' comments attached. (Please see below)

Original referees' comments – EMBO Journal

Referee #1:

In their manuscript, "Venus-trap in the mouse embryo reveals distinct molecular dynamics underlying specification of its first lineages," Dietrich and colleagues present a lentivirus-based gene trapping approach to create reporters that could be used to live-image mouse early development. The approach is highly novel, and promises to yield very interesting new tools and insights, and the effort is truly admirable. Unfortunately, however, neither the rigour of the current study nor the novelty of the findings presented constitute "a significant and novel contribution to our understanding of a developmental mechanism," which would be a crucial requirement for publication in The EMBO Journal.

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The rigour of the study is compromised for two reasons. First, there is no evidence presented that the reporter lines faithfully recapitulate the expression patterns of the genes to which they map. Knowing that the new gene trap lines faithfully report gene expression is essential to knowing the biological significance of anything observed with these lines. Second, there is no molecular analysis to confirm that each line is associated with specific cell types of the blastocyst. Thus, the map of expression patterns presented in Figure 2A, that relies on morphological criteria alone, seems a bit preliminary. Finally, the figures include a lot of measurements, and the authors highlight the quantitative aspects of their study as a novelty of their approach. However, the analysis does not seem more computational than other publications.

For several reasons, the study does not contribute new insight into a developmental mechanism. First, there is no information about the functional role of the new genes identified in the gene trap screen. The authors acknowledge this shortcoming, stating that the purpose of the screen was to identify reporters, not to study loss of function phenotypes. However, not knowing the function of the trapped genes limits what we can learn from the trapped lines. For example, the authors place some of the new genes within the current hierarchy of trophectoderm development, showing that the two genes on which they focus for the remainder of the paper (GM13152 and Tmem50b) are downstream of Cdx2. However, without knowing the GM13152 or Tmem50b loss of function phenotypes, it is unclear whether descriptions of these genes' expression patterns tells us something new and different about what we already know of Cdx2.

Second, the main observations of the paper, regarding the dynamics of gene expression, do not seem mechanistic or novel, but descriptive and reminiscent of existing knowledge. The authors show that the trophectoderm-expressed Tmem50b follows the dynamics of Cdx2 expression, as previously published by the Rossant, Zheng, and Sasaki labs, while the inner cell mass-expressed GM13152 line behaves like a gene that is negatively regulated by CDX2, such as Oct4 or Nanog. Therefore, the study appears to focus on two reporters of CDX2 activity, but this has not revealed any new biology of the blastocyst.

Detailed remarks:

"to quantitatively charachterise the expression dynamics during blastocyst development..." (p. 7) - please clarify - the expression dynamics of what?

Figure 3: the expression patterns of the reporters in Tead4 -/- embryos should be compared to wild type embryos with the equivalent cell number, not at the equivalent temporal stage.

Figure 4: colors need to be defined in the merged panels. Also, it is not entirely clear what this figure is meant to show - examples of live imaging stills? Perhaps it would be helpful to show cell pairs that had undergone symmetric or asymmetric division.

Figure 5: "The lineage map of mouse pre-implantation development" appears to be based on 2 embryos. In addition, this title reveals that the study is descriptive because it does not describe what was discovered. Some arrow pairs are missing. Finally, the color coding makes it seem as though some 8-cell embryos have inside and outside cells - is this what the authors mean?

Throughout the figures, colour scheme could be confusing since blue and red are used to demarcate lineages and gene traps. Another color pair could be introduced.

Referee #2:

In this manuscript, the authors reported new reporter mouse lines for lineage-tracing in mouse pre-implantation embryos and the results of their analyses. They applied a gene-trap strategy to generate Tg mouse lines in which the Venus reporter was introduced by a lentivirus vector system expressed in a lineage-specific manner by the endogenous promoter upstream of the insertion site. They succeeded to establish several reporter mouse lines in which the Venus reporter is expressed in either the inner cell mass (ICM) or the trophectoderm (TE) cell lineage respectively and specifically.

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Using these lines, they analyzed lineage specification pattern by time-lapse imaging and found that the trophectoderm cell lineage is specified at a relatively early stage whereas the inner cell mass fate appears to fluctuate.
Lineage specification is a process in which the specific cell fate is determined in an irreversible manner. To follow this event, proper selection of lineage-specific marker(s) is absolutely required. Here the authors identified Gm13152 and Tmem50b as ICM and TE-specific lineage marker.

However, there is no confirmation whether their expression are actually linked to the lineage specification event. This would have to be studied in much more detail, as for instance analysing the expression of known lineage marker such as Oct4, Nanog, Cdx2 and nuclear localization of Yap in relation to the trapped-genes, preferably also under conditions of their genetic perturbation. This brings me to the second problem of the analysis: it lacks functional significance of these new marker genes and thus remains mostly descriptive. Since the reporter lines were generated by gene-trap, the authors should comment/report on functional impairment of the trapped alleles. If it is the case for these marker genes, the authors should establish the phenotypes of homozygous embryos for these gene-trap insertions. Does the lack of the description in Table S1 mean there are no phenotypes observed?
Additional points are listed in below.

1. The authors used Venus as a reporter. It was known that the wild-type GFP protein is quite stable and the fluorescent signal can remain stable after transcriptional repression. Has this been studied/considered in case of Gm13152 and Tmem50b? Do the trapped lines reflect transcriptional changes correctly?
2. In the case of Tmem50b, the expression looks not too specific for TE because some ICM cells seem to have Venus signal as shown in Figure 2b and Figure 4a. Has this been studied in time-lapse imaging?
3. The maps of the insertion sites of the gene-trap vectors for Gm13152 and Tmem50b would add to the understanding/potential functional perturbations of gene function and should thus be presented.
4. Because of the reasons described above, the results obtained by the analyses of these reporter mouse lines should be carefully interpreted in relation to the lineage specification. The statement by the authors summarized in Figure 7 appears preliminary/too strong based on the presented data.

1st Editorial Decision 03 February 2015

Thank you for transferring your manuscript to EMBO reports. Based on the existing referee comments, we can offer to publish it as methods/resource paper if you can address the more technical concerns. Further functional insight into the role of the trapped genes will not be required.

Basically, co-stainings with known ICM and TE markers, the maps of the insertion sites, and evidence that the reporter lines faithfully represent the expression of the trapped genes should be provided.

We would thus like to invite you to revise your manuscript with the understanding that the referee concerns must be fully addressed and their suggestions taken on board, except for the request for further functional insight. However, please include all information that you have on the trapped genes and whether they are disrupted by the Venus integration. Acceptance of the manuscript will depend on a positive outcome of a second round of review. It is EMBO reports policy to allow a single round of revision only and acceptance or rejection of the manuscript will therefore depend on the completeness of your responses included in the next, final version of the manuscript.

Revised manuscripts should be submitted within three months of a request for revision; they will otherwise be treated as new submissions. Please contact us if a 3-months time frame is not sufficient for the revisions so that we can discuss the revisions further. Given that this study will be published as a methods paper, there is no need to bring it into our short format. However, please keep the text and number of figures at a minimum. Please also change the reference style to the numbered EMBO reports style, which will further help to reduce the overall character count. Commonly used materials and methods can be moved to the supplementary information, however, please note that materials and methods essential for the understanding of the experiments described in the main text must remain in the main manuscript file.

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Regarding data quantification, please specify the number "n" for how many independent experiments were performed (and please also indicate how many embryos or cells were used), the bars and error bars (e.g. SEM, SD) and the test used to calculate p-values in the respective figure legends. This information is currently incomplete and must be provided in the figure legends.

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

REFEREE REPORTS:

Referee #1

In their manuscript, "Venus-trap in the mouse embryo reveals distinct molecular dynamics underlying specification of its first lineages," Dietrich and colleagues present a lentivirus-based gene trapping approach to create reporters that could be used to live-image mouse early development. The approach is highly novel, and promises to yield very interesting new tools and insights, and the effort is truly admirable. Unfortunately, however, neither the rigour of the current study nor the novelty of the findings presented constitute "a significant and novel contribution to our understanding of a developmental mechanism," which is the requirement for publication in The EMBO Journal.

The rigour of the study is compromised for two reasons. First, there is no evidence presented that the reporter lines faithfully recapitulate the expression patterns of the genes to which they map. Knowing that the new gene trap lines faithfully report gene expression is essential to knowing the biological significance of anything observed with these lines. Second, there is no molecular analysis to confirm that each line is associated with specific cell types of the blastocyst. Thus, the map of expression patterns presented in Figure 2A, that relies on morphological criteria alone, seems a bit preliminary. Finally, the figures include a lot of measurements, and the authors highlight the quantitative aspects of their study as a novelty of their approach. However, the analysis does not seem more computational than other publications.

For several reasons, the study does not contribute new insight into a developmental mechanism. First, there is no information about the functional role of the new genes identified in the gene trap screen. The authors acknowledge this shortcoming, stating that the purpose of the screen was to identify reporters, not to study loss of function phenotypes. However, not knowing the function of the trapped genes limits what we can learn from the trapped lines. For example, the authors place some of the new genes within the current hierarchy of trophectoderm development, showing that the two genes on which they focus for the remainder of the paper (GM13152 and Tmem50b) are downstream of Cdx2. However, without knowing the GM13152 or Tmem50b loss of function phenotypes, it is unclear whether descriptions of these genes' expression patterns tells us something new and different about what we already know of Cdx2.

Second, the main observations of the paper, regarding the dynamics of gene expression, do not seem mechanistic or novel, but descriptive and reminiscent of existing knowledge. The authors show that the trophectoderm-expressed Tmem50b follows the dynamics of Cdx2 expression, as previously published by the Rossant, Zheng, and Sasaki labs, while the inner cell mass-expressed GM13152 line behaves like a gene that is negatively regulated by CDX2, such as Oct4 or Nanog. Therefore, the study appears to focus on two reporters of CDX2 activity, but this has not revealed any new biology of the blastocyst.

Detailed remarks:

"to quantitatively charachterise the expression dynamics during blastocyst development..." (p. 7) - please clarify - the expression dynamics of what?

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Figure 4: colors need to be defined in the merged panels. Also, it is not entirely clear what this figure is meant to show - examples of live imaging stills? Perhaps it would be helpful to show cell pairs that had undergone symmetric or asymmetric division.

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Throughout the figures, colour scheme could be confusing since blue and red are used to demarcate lineages and gene traps. Another color pair could be introduced.

Referee #2

In this manuscript, the authors reported new reporter mouse line for lineage-tracing in mouse pre-implantation embryos and the results of their analyses. They applied gene-trap strategy to generate Tg mouse lines in which the Venus reporter introduced by lentivirus vector system expresses in lineage-specific manner by the endogenous promoter upstream of the insertion site. They succeeded to establish several reporter mouse lines in which the Venus reporter expresses in either inner cell mass (ICM) or trophectoderm (TE) cell lineages specifically. Using these lines, they analyzed lineage specification pattern by time-lapse imaging and found that trophectoderm cell lineage is specified at early stage whereas the inner cell mass fate is fluctuated.
Lineage specification is a process that the specific cell fate is determined in irreversible manner. To follow this event, proper selection of the lineage-specific marker is absolutely required. Here the authors identified Gm13152 and Tmem50b as ICM and TE lineage markers. However, there is no confirmation whether their expressions are actually linked to the lineage specification. This is due to the lack of the data for the expression of the known lineage markers such as Oct4, Nanog, Cdx2 and nuclear localization of Yap in the reporter mouse embryos. Another problem is the lack of the functional significance of the expressions of these new marker genes. Since the reporter lines were generated by gene-trap, it could cause functional abolishment of the trapped allele. If it is the case for these marker genes, the authors should report the phenotypes of homozygous embryos for these gene-trap insertions. Is the lack of the description in Table S1 meant no phenotype?
Additional points for revision are listed in below.

1. The authors used Venus as a reporter. It was known that the wild-type GFP protein is quite stable and the fluorescent signal can retain after the transcriptional repression. How about in the case of Gm13152 and Tmem50b? Do they monitor the transcriptional levels correctly?
2. In the case of Tmem50b, its expression looks not so specific to TE because some ICM cells possesses Venus signal as found in Figure 2b and Figure 4a. Is it observed in time-lapse imaging?
3. The maps of the insertion sites of the gene-trap vectors for Gm13152 and Tmem50b will be required.
4. Because of the reasons described above, the results obtained by the analyses of these reporter mouse lines should be carefully interpreted in relation to the lineage specification. The statement by the authors summarized in Figure 7 sounds too strong.

1st Revision - authors' response 03 May 2015

Editorial comments:

… Based on the existing referee comments, we can offer to publish it as methods/resource paper if you can address the more technical concerns. **Further functional insight into the role of the trapped genes will not be required.**

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Basically, **co-stainings with known ICM and TE markers, the maps of the insertion sites, and evidence that the reporter lines faithfully represent the expression of the trapped genes should be provided**.

We would thus like to invite you to revise your manuscript with the understanding that the referee concerns must be fully addressed and their suggestions taken on board, except for the request for further functional insight. However, please **include all information that you have on the trapped genes and whether they are disrupted by the Venus integration.** …

Co-immunostaining of Venus with Cdx2 (for TE) or Sox2 (for ICM) was performed and included as a **new Supplementary Fig S2 and new Supplementary movies S1 and S2.**

The maps of the insertion sites for all traps are provided as a **new Supplementary Fig S1**. All information that we have on the trapped genes is provided in **Supplementary Table S1**, including, for those VET lines examined, whether the embryo with homozygous trap allele has a phenotype during pre-implantation development.

qRT-PCR of single cells or pooled cells of the blastocyst was performed and presented as a **new Supplementary Fig S4**. The results demonstrated that the reporter lines, Tmem50b^{Gt} for TE and 2610305D13Rik^{Gt} for ICM, faithfully represent the expression of the trapped genes.

… Regarding data quantification, please specify the number "n" for how many independent experiments were performed (and please also indicate how many embryos or cells were used), the bars and error bars (e.g. SEM, SD) and the test used to calculate p-values in the respective figure legends. This information is currently incomplete and must be provided in the figure legends. …

All the requested information is provided in the figure legends.

Referee #1:

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The rigour of the study is compromised for two reasons. First, **there is no evidence presented that the reporter lines faithfully recapitulate the expression patterns of the genes to which they map.** Knowing that the new gene trap lines faithfully report gene expression is essential to knowing the biological significance of anything observed with these lines.

To examine whether the two reporter lines mainly used in this study, Tmem50b^{Gt} for TE and 2610305D13Rik^{Gt} for ICM, faithfully represent the expression of the trapped genes, we performed qRT-PCR using single or pooled TE and ICM cells derived from the blastocyst. The results, presented as a **new Supplementary Fig S4**, show that these reporter lines faithfully represent the expression of the trapped genes.

We have extensively tried to generate specific mAb and pAb against Tmem50b and 2610305D13Rik over years without success, thus we were unable to examine the expression at the protein level.

Note that highly repetitive sequences surrounding the integration site of VET53A made it very difficult to unequivocally identify the trapped gene. The trapped sequence has an equally high homology to 2610305D13Rik, Rex2, Zfp600, Gm13145, Gm13242 and Gm13152. Now, however, qRT-PCR using primers designed to distinguish these transcripts, showed that only 2610305D13Rik exhibits the expression specific to ICM cells in E4.5 blastocysts but not in E3.5 blastocysts, in a manner consistent with the Venus expression pattern. This led us to tentatively assign the gene trapped in VET53A as 2610305D13Rik in this study. All these data are presented in a **new Supplementary Fig S4**, the full description is in Materials and Methods, and the tentative annotation in Fig 2A is marked with *.

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Second, there is **no molecular analysis to confirm that each line is associated with specific cell types of the blastocyst**. Thus, the map of expression patterns presented in Figure 2A, that relies on morphological criteria alone, seems a bit preliminary.

Co-immunostaining of Venus/GFP with Cdx2 (for TE) or Sox2 (for ICM) was performed for Tmem50b^{Gt} and 2610305D13Rik^{Gt} embryos. The results confirm that each line is associated with specific cell types of the blastocyst, and are now added as a **new Supplementary Fig S2 and new Supplementary movies S1 and S2.**

Finally, the figures include a lot of measurements, and the authors highlight the quantitative aspects of their study as a novelty of their approach. However, the analysis does not seem more computational than other publications.

Addressed below discussing specific publications.

For several reasons, the study does not contribute new insight into a developmental mechanism. First, there is no information about the functional role of the new genes identified in the gene trap screen. The authors acknowledge this shortcoming, stating that the purpose of the screen was to identify reporters, not to study loss of function phenotypes. However, not knowing the function of the trapped genes limits what we can learn from the trapped lines. For example, the authors place some of the new genes within the current hierarchy of trophectoderm development, showing that the two genes on which they focus for the remainder of the paper (GM13152 and Tmem50b) are downstream of Cdx2. However, without knowing the GM13152 or Tmem50b loss of function phenotypes, it is unclear whether descriptions of these genes' expression patterns tells us something new and different about what we already know of Cdx2.

The aim of this study is to identify and generate lineage reporters useful for live-imaging. We show that Tmem50b^{Gt} and 2610305D13Rik^{Gt} are also downstream targets of known GRN. However, functional characterisation of these newly identified lineage-markers is not the primary aim and is out of the scope of this study.

Indeed, depending on the locus of genomic integration, a gene-trap may result in a loss-of-function mutation, and some of our VET insertions, i.e., fVET3A and fVET5A for Ctnna1 and Supt6, produced functional KO by disrupting protein formation of the trapped gene. This indicates that the Venus-trap screen allowed us to identify a new gene required for mouse pre-implantation development, and the results are now presented as a **new Supplementary Fig S5**.

Unfortunately for those two genes Tmem50b and 2610305D13Rik that we focused in this study, VET insertion did not allow us to characterize its loss-of-function phenotype. For Tmem50b the insertion is located at 3' of the coding sequence, thus Tmem50b$^{Gt/Gt}$ embryos did not show any phenotype during the pre-implantation stage. On the other hand, highly repetitive sequences surrounding 2610305D13Rik prevented us from designing PCR primers that allow distinguishing 2610305D13Rik$^{Gt/Gt}$ from 2610305D13Rik$^{Gt/+}$.

Second, the main observations of the paper, regarding the dynamics of gene expression, do not seem mechanistic or novel, but descriptive and reminiscent of existing knowledge. The authors show that the trophectoderm-expressed Tmem50b follows the dynamics of Cdx2 expression, as previously published by the Rossant, Zheng, and Sasaki labs, while the inner cell mass-expressed GM13152 line behaves like a gene that is negatively regulated by CDX2, such as Oct4 or Nanog. Therefore, the study appears to focus on two reporters of CDX2 activity, but this has not revealed any new biology of the blastocyst.

In fact none of the studies published by the Rossant, Zheng or Sasaki lab quantitatively measured the dynamics of gene expression by live-imaging mouse embryos. This was the challenge in the field – to characterise gene expression dynamics directly linked with morphogenesis, such as cell position and division pattern. Thus the unique and novel aspect of this study is that we have carried out quantitative gene expression analysis during morphogenesis and lineage segregation, and that we built a comprehensive lineage map of mouse pre-implantation development. This systematic analysis led us to identify the correlation between asymmetric division and up-regulation of a TE reporter expression in outside cells. This study will also serve as a framework for future studies aiming for systems-level analysis of embryogenesis.

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During the revision of this study, one paper is published in which quantitative analysis of Nanog gene expression and dynamic cell behavior was performed using live-imaging (Xenopoulous et al. 2015 *Cell Reports* 10, 1508-1520). While this newly published study investigated EPI vs. PrE lineage segregation in the blastocyst, a topic distinct from our study, and did not generate a comprehensive linage map or perform the systematic image analysis as presented in our study, we cited this study in our revised manuscript and softened our claim on the novelty of our study.

Detailed remarks:

"to quantitatively charachterise the expression dynamics during blastocyst development..." (p. 7) - please clarify - the expression dynamics of what?

of the VET reporters for Tmem50b and 2610305D13Rik genes – the text is corrected accordingly.

Figure 3: the expression patterns of the reporters in Tead4 -/- embryos should be compared to wild type embryos with the equivalent cell number, not at the equivalent temporal stage.

We counted the cell numbers in Tead4$^{+/+}$, $^{+/-}$, $^{-/-}$ and Cdx2$^{+/+}$, $^{+/-}$, $^{-/-}$ embryos and they are comparable as presented in Materials and Methods.

Figure 4: colors need to be defined in the merged panels. Also, it is not entirely clear what this figure is meant to show - examples of live imaging stills? Perhaps it would be helpful to show cell pairs that had undergone symmetric or asymmetric division.

Yes, they are examples of live imaging stills for off-line readers. Color definition is given in figure legends.

Figure 5: "The lineage map of mouse pre-implantation development" appears to be based on 2 embryos. In addition, this title reveals that the study is descriptive because it does not describe what was discovered. Some arrow pairs are missing. Finally, the color coding makes it seem as though some 8-cell embryos have inside and outside cells - is this what the authors mean?

Throughout the figures, colour scheme could be confusing since blue and red are used to demarcate lineages and gene traps. Another color pair could be introduced.

The title of Figure 5 is corrected accordingly. Arrows in Figure 5 highlight “representative” descendant pairs as a result of asymmetric divisions, whereas the quantitative analysis shown in Figure 6 was performed for all asymmetric divisions as described in Materials and Methods in detail. Blue and red lines indicate outside and inside cells, respectively, as defined in figure legend, and all cells in the 8-cell stage embryos have only blue lines, indicating that all cells are located outside.

The colours demarcate lineages, red for ICM and blue for TE, and as a consequence the two VET lines. Red and blue are used for 2610305D13Rik (ICM reporter) and Tmem50b (TE reporter), respectively to make it easier for readers to remember for which lineage these lines serve as a reporter. We consider that it would be helpful rather than confusing; however if all reviewers unanimously suggest, we can remove the colour code for VET lines instead of adding another.

Referee #2:

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However, there is no confirmation whether their expressions are actually linked to the lineage specification. This is due to the lack of the data for the expression of the known lineage markers such as Oct4, Nanog, Cdx2 and nuclear localization of Yap in the reporter mouse embryos.

Co-immunostaining of Venus/GFP with Cdx2 (for TE) or Sox2 (for ICM) was performed for Tmem50b^{Gt} and 2610305D13Rik^{Gt} embryos. The results confirm that each line is associated with specific cell types of the blastocyst, and are now added as a **new Supplementary Fig S2** and **new Supplementary movies S1 and S2.**

Another problem is the lack of the functional significance of the expressions of these new marker genes. Since the reporter lines were generated by gene-trap, it could cause functional abolishment of the trapped allele. If it is the case for these marker genes, the authors should report the phenotypes of homozygous embryos for these gene-trap insertions. Is the lack of the description in Table S1 meant no phenotype?

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Indeed, depending on the locus of genomic integration, a gene-trap may result in a loss-of-function mutation, and some of our VET insertions, i.e., fVET3A and fVET5A for Ctnna1 and Supt6, produced functional KO by disrupting protein formation of the trapped gene. This indicates that the Venus-trap screen allowed us to identify a new gene required for mouse pre-implantation development, and the results are now presented as a **new Supplementary Fig S5**.

Unfortunately for those two genes Tmem50b and 2610305D13Rik that we focused in this study, VET insertion did not allow us to characterize its loss-of-function phenotype. For Tmem50b the insertion is located at 3' of the coding sequence, thus Tmem50b$^{Gt/Gt}$ embryos did not show any phenotype during the pre-implantation stage. On the other hand, highly repetitive sequences surrounding 2610305D13Rik prevented us from designing PCR primers that allow distinguishing 2610305D13Rik$^{Gt/Gt}$ from 2610305D13Rik$^{Gt/+}$.

All information that we have on the trapped genes is provided in **Supplementary Table S1**, including, for those VET lines examined, whether the embryo with homozygous trap allele has a phenotype during pre-implantation development. Those VET lines not examined/determined for the null phenotype are marked as "n.d.".

Additional points are listed in below.

1. The authors used Venus as a reporter. It was known that the wild-type GFP protein is quite stable and the fluorescent signal can retain after the transcriptional repression. How about in the case of Gm13152 and Tmem50b? Do they monitor the transcriptional levels correctly?

We examined the stability of our Venus reporter, as described in page 9:

"From our VET reporter, the dynamics of gene expression could be more faithfully reflected in their increase than in their decrease, because degradation of Venus-NLS protein may be of different dynamics than the endogenous protein. As anticipated, live-imaging of the decay of maternal Venus protein in wt embryos derived from a gene-trap line (Ctnna1$^{Gt/+}$) gave an estimate for the relevant half-life of 17 ± 3 h (n = 16, Supplementary Fig S10). Thus, instead of the total level, we analysed the average rate of the expression change…"

2. In the case of Tmem50b, its expression looks not so specific to TE because some ICM cells possesses Venus signal as found in Figure 2b and Figure 4a. Is it observed in time-lapse imaging?

The Venus expression in Tmem50b^{Gt} becomes exclusive to TE later in the blastocyst stage. As a few ICM cells transiently express the reporter in earlier blastocysts, it would be fair to show such still images, given the aim of this study being quantification of gene expression dynamics, rather than binary classification of "positive" or "negative" expression.

3. The maps of the insertion sites of the gene-trap vectors for Gm13152 and Tmem50b will be required.

The maps of the insertion sites for all traps are provided as a **new Supplementary Fig S1**.

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4. Because of the reasons described above, the results obtained by the analyses of these reporter mouse lines should be carefully interpreted in relation to the lineage specification. The statement by the authors summarized in Figure 7 sounds too strong.

We toned down the conclusion accordingly.

2nd Editorial Decision 20 May 2015

Thank you for the submission of your revised manuscript to our journal. We have now received the enclosed reports from the referees that were asked to assess it. As you will see, both referees support publication of the revised manuscript in our journal and only have a few more minor suggestions that I would like you to incorporate before we can proceed with the official acceptance of your manuscript.

We will publish your paper as a full length article, and all materials and methods can and should therefore be moved to the main manuscript file. You can send us only the modified files, and we will upload everything for you, or you upload only the modified files as a new version of your manuscript.

I am looking forward to receiving the final version of your manuscript and to seeing it published. Your cover image suggestion looks great!

REFEREE REPORTS:

Referee #1:

The manuscript by Dietrich and colleagues has been greatly improved by their revision. The authors now include 1) the requested evidence that the gene mouse lines faithfully report expression of the trapped genes, 2) they show evidence that the reporters colocalize with cell fate markers in the blastocyst and 3) provide improved information regarding the genomic position of the viral insertions. The manuscript is now acceptable for publication, following attention to the following minor issues:

1. The great strength of the manuscript is in the novelty of the gene trap screen. Therefore, the authors should focus on this rather than other aspects, which are not as highly novel. The authors have revised their manuscript to lessen claims of novelty with regard to preimplantation live imaging. However, the abstract still contains a statement, the kind of which the authors, in their rebuttal had agreed, is overstated: "no quantitative live-image analysis has been carried out systematically." This claim is not consistent with a paper from the Hadjantonakis lab (Xenopoulos et al., 2015 Cell Reports). Similary, on p. 4 "which was never before possible..." The use of 'never' does not seem justified, especially considering the wealth of insight that has been contributed by static and live imaging approaches alike.
2. There are a buzzwords used throughout the manuscript that rather detract from the clarity of the narrative. For example, "advanced" live imaging (p. 4), "systems-level understanding" (abstract, p. 4, p. 10), "gene regulatory networks" (p. 6 and accompanying figure legend). The flow of the paper would be improved by use of simpler, more accurate terms: change "advanced live imaging" to "live imaging, change "systems-level understanding" to "dynamic understanding" or "time-resolved understanding", change "gene regulatory networks" to "genes."
3. On p. 10 the assertion that there is no marker that is ICM-specific prior to the 64 cell stage is not consistent with report from the Ralston Lab that Sox2 protein is detected specifically in the ICM starting the 16-cell stage, and the report from Robson lab that Sox2 mRNA is detected in the ICM at this stage as well. These two papers should be cited in this discussion (Wicklow et al., 2014 PLoS Genetics and Guo et al., 2010 Developmental Cell).
4. Fig. S4B - please define how cell position is known (based on expression level of Cdx2, Sox2, Oct4, etc.?)

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Referee #2:

Here the authors revised the manuscript as a resource paper with incorporating the answers to the criticisms made by the reviewers. Now they showed quite comprehensive data set, which looks sufficient as a resource paper, and gave prompt answers to my concern. Therefore, I agree with the publication of the present version of the manuscript in EMBO Reports.

However, I have one suggestion to the authors. As the authors mentioned, there are several reports for establishment of transgenic mice lines that allow monitoring lineage segregation such as Cdx2-Tg and Nanog reporter line. How about the similarity/difference between these lines and the lines established by Venus trap? It will be worth to discuss to emphasize the significance of this work.

2nd Revision - authors' response 02 June 2015

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Text is corrected accordingly.

2. There are a buzzwords used throughout the manuscript that rather detract from the clarity of the narrative. For example, "advanced" live imaging (p. 4), "systems-level understanding" (abstract, p. 4, p. 10), "gene regulatory networks" (p. 6 and accompanying figure legend). The flow of the paper would be improved by use of simpler, more accurate terms: change "advanced live imaging" to "live imaging, change "systems-level understanding" to "dynamic understanding" or "time-resolved understanding", change "gene regulatory networks" to "genes."

Text is corrected accordingly.

3. On p. 10 the assertion that there is no marker that is ICM-specific prior to the 64 cell stage is not consistent with report from the Ralston Lab that Sox2 protein is detected specifically in the ICM starting the 16-cell stage, and the report from Robson lab that Sox2 mRNA is detected in the ICM at this stage as well. These two papers should be cited in this discussion (Wicklow et al., 2014 PLoS Genetics and Guo et al., 2010 Developmental Cell).

Indeed given the study Wicklow et al. (2014), this discussion is no longer applicable and we removed it.

4. Fig. S4B - please define how cell position is known (based on expression level of Cdx2, Sox2, Oct4, etc.?)

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We used the same method as Ohnishi et al. (2014) and now added this explanation in Materials and Methods.

Referee #2:

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We discussed these reporters (Ref. 18 and 19) in Introduction and Discussion. For instance, in Discussion (page 10) “it would be ideal to generate a knock-in fluorescence-fusion reporter that would reflect the endogenous dynamics and function [18]”.

3rd Editorial Decision 02 June 2015

I am very pleased to accept your manuscript for publication in the next available issue of EMBO reports.

Thank you for your contribution to EMBO reports and congratulations on a successful publication. Please consider us again in the future for your most exciting work.

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