

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

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Abstract

Mammalian development begins with the segregation of embryonic and extra-embryonic lineages in the blastocyst. Recent studies revealed cell-to-cell gene expression heterogeneity and dynamic cell rearrangements during mouse blastocyst formation. Thus, mechanistic understanding of lineage specification requires quantitative description of gene expression dynamics at a single-cell resolution in living embryos. However, only a few fluorescent gene-expression reporter mice are available and quantitative live-image analysis is limited so far. Here, we carried out a fluorescence gene-trap screen and established reporter mice expressing Venus specifically in the first lineages. Lineage tracking, quantitative gene-expression and cell-position analyses allowed us to build a comprehensive lineage map of mouse pre-implantation development. Our systematic analysis revealed that, contrary to the available models, the timing and mechanism of lineage specification may be distinct between the trophectoderm and the inner cell mass. While expression of our trophectoderm-specific lineage marker is upregulated in outside cells upon asymmetric divisions at 8- and 16-cell stages, the inside-specific upregulation of the inner-cell-mass marker only becomes evident at the 64-cell stage. This study thus provides a framework toward systems-level understanding of embryogenesis marked by high dynamicity and stochastic variability.

Keywords:

gene-trap/lineage map/live-imaging/mouse/pre-implantation development

Introduction

Mammalian development is characterised by the pre-implantation stage during which the blastocyst forms with a single-layer epithelium, the trophectoderm (TE), surrounding a fluid-filled cavity and an eccentric inner cell mass (ICM). Upon implantation TE will give rise to extra-embryonic tissues, whereas the epiblast cells within ICM will form the embryo proper. Two models have been proposed for the mechanism underlying the first lineage segregation between TE and ICM in the mouse embryo. The inside-outside model suggests that cell position in the morula regulates cell fate [1]; the inside cells with circumferential cell contacts contribute to the ICM, whereas cells facing the outside surface of the embryo form TE. The cell polarity model proposes that the apico-basal polarity of the blastomeres underlies cell fate differentiation [2]. Depending on the cleavage plane orientation relative to the apico-basal polarity, the 8- to 16- and 16- to 32-cell divisions generate either symmetric (two TE) or asymmetric (ICM and TE) descendants. Notably, in both models, the ICM vs TE cell fate decision is binary and acquisition of molecular lineage-identity was considered to be regulated in a reciprocal manner [3]. Recent studies, however, revealed overlapping mosaic expression of the transcription factors that confer molecular identity to the first lineages [4-6]. In particular the inside cells of the early blastocyst exhibit stochastic cell-to-cell variability in gene expression [7,8]. These data are incompatible with the above-mentioned models and thus the mechanism underlying the first lineage specification remains to be further investigated.

Recent developments in live imaging of the early mouse development revealed unprecedented dynamics during early mouse embryogenesis [9-13]. Live imaging analysis was enhanced by imaging transgenic embryos expressing fluorescence-labeled nuclei, that allowed tracking of all cells during pre-implantation development and generating lineage-trees [14,15]. Given the dynamicity and stochastic molecular heterogeneity, mechanistic understanding of the first lineage specification requires quantitative description of the gene expression dynamics in living embryos during morphogenesis at single-cell resolution [16]. However, to date, only a limited number of fluorescence gene-expression reporter mice suitable for live-imaging are available [5,17-19], quantitative analysis of gene expression dynamics has begun only recently [19], and it has not been systematically integrated with cell lineage segregation pattern.

To analyse endogenous gene and protein expression dynamics, targeted knock-in of fluorescence-fusion reporters are often ideal [20]. However, this can result in transgenic embryos expressing low-level fluorescence signal, which is unsuitable for live imaging and subsequent image analysis. On the other hand, gene-trap introduces random integration of a fluorescence reporter into the genome and the transgenic embryos suitable for live imaging can be selected by microscopic screenings. Gene-trap screenings have been carried out in various model organisms [21-23] and have generated valuable resources for live imaging embryonic development. However, mouse gene-traps have thus far been primarily designed for genetic loss-of-function studies and expression analysis in fixed (for example by LacZ reporter), but not in live, tissues [24,25]. This may partly be because in mouse, embryonic development can be observed through live imaging only for a limited period of time. However, recent development of live imaging systems for various stages of mouse development and for different tissues [26-32] raised an interest in

fluorescent reporters that quantitatively reflect the endogenous expression of lineage specific genes.

In this study, we have carried out an *in vivo* fluorescence gene-trap screen, to our knowledge for the first time in the mouse embryo, using lentiviral transgenesis and generated reporter mice that express Venus specifically in one of the first lineages to be established during mammalian development. These newly established resources, when combined with live imaging microscopy, allowed us to directly connect gene expression dynamics with morphogenetic cellular processes. Taking advantage of this, we present a pipeline to integrate quantitative 4D image analysis into an enhanced lineage map, which allowed us to identify lineage-specific gene regulation. This will lay the foundation for an integrative analysis of mouse embryonic development.

Results

Gene-trap screen generated fluorescence reporter mice for lineage-specific gene expression

We wished to establish an experimental system for monitoring expression dynamics of lineage-specific genes during early mouse embryogenesis. In order to acquire transgenic mice expressing a fluorescent gene-expression reporter that allows quantitative expression analysis integrating morphogenetic information, we carried out a fluorescence gene-trap screen in the early mouse embryo using lentiviral transgenesis. One of the goals of this pilot study was to generate fluorescence reporter mice for each of the cell lineages in the blastocyst, specifically TE and ICM that includes epiblast and primitive endoderm.

A lentiviral gene-trap vector was designed with a promoter-less Venus reporter (thus the screen was named as Venus-trap) so that upon integration into the transcribed region of an active gene, Venus would be expressed under the control of the promoter and enhancers of this gene (Fig 1A). Furthermore, Venus is tagged with a nuclear localisation signal (NLS) to concentrate the fluorescent signal. To achieve efficient viral transduction while minimizing the degree of mosaicism, we transduced 2-cell stage embryos with the resulting vector (see Materials and Methods for details).

The screen was performed in two steps (Fig 1B). First, expression of the Venus fluorescent reporter was examined in the blastocyst at embryonic day 4.5 (E4.5), that is, three days after lentiviral transduction. Blastocysts yielding a positive signal upon brief microscopic inspection (21.8%; n = 462 blastocysts of n = 2121 transduced 2-cell embryos; Fig 1C) were then transferred to the uterus of the foster mothers. This resulted in the generation of a total of n = 107 founder mice (F0) named VET or fVET after Venus-trap for male or female founders respectively. In a second screen, blastocysts of F1 generation for VET, or of F2 generation for fVET, were screened for fluorescence patterns. This screen identified 16 founder mice carrying at least one gene-trap integrant that, after germ-line transmission, express detectable levels of Venus in the blastocyst.

Southern-blot analyses revealed that the 16 founder mice carry a total of 65 integrations with an average of 4.1 integrations per founder (Fig 1C and D). Integrations derived from one founder were labelled alphabetically in ascending order with "A" ascribed to the largest fragment. Integration sites, distributed over the entire chromosomes (Fig 1E), were cloned using ligation-mediated (LM)-PCR or Tail-PCR, and trapped transcripts were cloned by 5'RACE (Appendix Table S1). Cloned sequences were annotated using UCSC BLAT and Ensembl [33,34]. When single-integrant VET lines were derived by breedings with wild-type (wt) mice over generations, 23 of 65 integrations (35.4%) turned out to express the Venus reporter at a detectable level in the blastocyst (Fig 1C, Appendix Table S1).

To characterise the overall change in expression during pre-implantation development, we imaged live embryos of each of the 23 transgene-expressing single-integrant lines at E1.5, 2.5, 3.5 and 4.5 (Fig 2A). The images revealed distinct patterns of Venus expression among the active traps, indicating that the trapped genes are under specific spatio-temporal control during embryonic development. Importantly, specific expression patterns were maintained over more than 10 generations. They can be classified as: (1) ubiquitous, with similar fluorescence-levels in all lineages (n = 15), (2) specific to TE (n = 3), (3) specific to ICM (n = 2), (4) restricted to TE and

primitive endoderm (PrE; $n = 2$), or (5) overall heterogeneous, with reporter expression only in a subset of blastomeres but without specificity for any particular lineage ($n = 1$). Altogether we were able to establish 9 of 23 gene-trap mouse lines (39.1%) in which the Venus reporter is expressed in a lineage-specific manner at the blastocyst stage, for example $\text{Tmem50b}^{\text{Gt(Venus)fVET17B}}$ (hereafter written as $\text{Tmem50b}^{\text{Gt}}$) specific to TE (Fig 2B, Appendix Figure S1A and Appendix Movie S1) and $2610305\text{D13Rik}^{\text{Gt(Venus)VET53A}}$ (hereafter written as $2610305\text{D13Rik}^{\text{Gt}}$) specific to ICM (Fig 2C, Appendix Fig S1B and Appendix Movie S2; see Fig EV1 and Materials and Methods for more information on the identity of the gene trapped in VET53A, and Fig EV2 for additional lines with non-ubiquitous expression patterns). Lineage specific expression of two of the trapped genes ($\text{Tmem50b}^{\text{Gt}}$ and $2610305\text{D13Rik}^{\text{Gt}}$) was also confirmed by qRT-PCR (Appendix Fig S2). In four of those lines, reporter expression transiently exhibited a heterogeneous pattern before being confined to specific lineages (see the image of the E3.5 blastocyst for $2610305\text{D13Rik}^{\text{Gt}}$ in Fig 2C, for Cd2ap^{Gt} in Fig EV2B, for $\text{Rbfox2}^{\text{Gt}}$ in Fig EV2F and for Cers6^{Gt} in Fig EV2G), with one exception (VET53C) having heterogeneous expression throughout the pre-implantation phase (see Fig EV2D).

Depending on the locus of genomic integration, a homozygous gene-trap may result in a loss-of-function mutation [35,36]. This was observed with $\text{Ctnna1}^{\text{Gt/Gt}}$, for which the blastocyst cavity failed to expand ($n = 15$ embryos, $n = 6$ experiments; Fig EV3A1 and B1), a phenotype observed in an earlier gene-trap study [37]. We noted that fluorescent intensities are correlated with, and could be used to determine the genotype of live VET embryos (Fig EV3A1 and A2, Kruskal-Wallis H test $p = 3 \times 10^{-5}$; Fig EV3A3 and A4, Kruskal-Wallis H test $p = 2 \times 10^{-6}$). Interestingly, $\text{Spt6}^{\text{Gt/Gt}}$ homozygotes died during pre-implantation development ($n = 15$ embryos, $n = 4$ independent experiments; Fig EV3A3 and B3). Loss of protein expression was confirmed by immunostaining (Fig EV3B1 and B2, Kruskal-Wallis H test $p = 10^{-5}$; Fig EV3B3 and B4, Kruskal-Wallis H test $p = 0.003$) and genotyping PCR of single embryos. These data demonstrate that VET insertions can produce functional KO by disrupting protein formation of the trapped gene. Furthermore, the Venus-trap screen allowed us to identify a new gene, *Spt6*, required for mouse pre-implantation development.

ICM and TE reporter expression is controlled under the lineage-specific genes

We examined the potential regulation of the newly identified lineage reporters through key factors of the emerging regulatory networks [38] by analysing Venus expression of $\text{Tmem50b}^{\text{Gt}}$ or $2610305\text{D13Rik}^{\text{Gt}}$ embryos in $\text{Cdx2}^{-/-}$ or $\text{Tead4}^{-/-}$ backgrounds. *Tead4* and *Cdx2* are required to maintain the TE lineage and its gene regulatory network [39-41]. Expression of the TE reporter $\text{Tmem50b}^{\text{Gt}}$ was reduced in a $\text{Tead4}^{-/-}$ background (Fig 3A). Quantification of the mean fluorescence intensity of the $\text{Tmem50b}^{\text{Gt}}$ reporter in individual nuclei of outer cells of $\text{Tead4}^{+/+}$ ($n = 4$ embryos, $n = 16$ nuclei), $\text{Tead4}^{+/-}$ ($n = 4$ embryos, $n = 16$ nuclei) and $\text{Tead4}^{-/-}$ embryos ($n = 5$ embryos, $n = 19$ nuclei) revealed that the TE reporter $\text{Tmem50b}^{\text{Gt}}$ is positively regulated by *Tead4* (Fig 3B; Kruskal-Wallis H test, $p = 10^{-8}$). Similarly, quantification of the $\text{Tmem50b}^{\text{Gt}}$ mean fluorescence intensity in individual nuclei of outer cells of $\text{Cdx2}^{+/+}$ ($n = 2$ embryos, $n = 9$ nuclei), $\text{Cdx2}^{+/-}$ ($n = 2$ embryos, $n = 10$ nuclei) and $\text{Cdx2}^{-/-}$ embryos ($n = 4$ embryos, $n = 22$ nuclei) revealed a positive regulation of $\text{Tmem50b}^{\text{Gt}}$ by *Cdx2* (Fig 3C and D; Kruskal-Wallis H test, $p = 2 \times 10^{-6}$). In contrast, the ICM marker $2610305\text{D13Rik}^{\text{Gt}}$ was negatively regulated by *Tead4* and *Cdx2* as

measured in individual nuclei of outer cells in *Tead4*^{+/+} (n = 3 embryos, n = 17 nuclei), *Tead4*^{+/-} (n = 4 embryos, n = 15 nuclei) and *Tead4*^{-/-} embryos (n = 5 embryos, n = 16 nuclei) (Fig 3E and F; Kruskal-Wallis H test, $p = 5 \times 10^{-5}$); and in *Cdx2*^{+/+} (n = 4 embryos, n = 15 nuclei), *Cdx2*^{+/-} (n = 4 embryos, n = 16 nuclei) and *Cdx2*^{-/-} embryos (n = 4 embryos, n = 14 nuclei, Fig 3G and H; Kruskal-Wallis H test, $p = 0.01$).

Altogether, these data place the newly identified genes, *Tmem50b* and *2610305D13Rik*, under the control of the gene regulatory network establishing the TE and ICM cell fates in the pre-implantation mouse embryo.

Establishment of the lineage segregation map of mouse pre-implantation development

To establish a map of lineage segregation between ICM and TE in association with gene expression dynamics, we time-lapse imaged one representative VET line for each lineage, *Tmem50b*^{Gt} for TE and *2610305D13Rik*^{Gt} for ICM, and followed gene expression dynamics in embryos developing from the 4-cell to expanding blastocyst stage (64-cell). Under the established imaging condition, 98.1% of the live-imaged embryos (n = 53/54 embryos, n = 6 independent experiments) successfully developed to the expanded blastocyst stage, suggesting that the established lineage map would reflect the physiological situation *in vivo*.

Combining our gene-trap lines with a transgenic mouse line ubiquitously expressing histone H2B tagged with mCherry (R26-H2B-mCherry [42]), we normalised the Venus signal to the mCherry signal thereby correcting for systematic error arising from nuclear position within the embryo. Furthermore, to rightly assign a given cell position and fate [32], we used mTmG to obtain membrane signal (hereafter written as mT in this study; [43]). A cell is defined as outside or TE if some part of the cell membrane is exposed to the outside, whereas inside or ICM cells are entirely surrounded by cell-cell contacts [4]. Cell membrane signal also helped identifying individual nuclei when the distance between them becomes small. Altogether we prepared a triple transgenic embryo for quantitative gene-expression analysis: a VET line combined with R26-H2B-mCherry and mT.

A representative image of the *Tmem50b*^{Gt}-Venus x R26-H2B-mCherry x mT embryo developing from the 8- to 64-cell stage is shown in Fig 4A. The *Tmem50b*^{Gt} reporter expression became detectable at 8- to 16-cell stage in all blastomeres. Subsequently, expression was specifically upregulated in the outside cells at around the E3.5 (>32-cell) stage, and, ultimately in the E4.5 blastocyst (≥ 64 -cell), was restricted to the outside TE cells (Fig 4A, Appendix Movie S3). On the other hand, *2610305D13Rik*^{Gt}-Venus x R26-H2B-mCherry x mT embryos exhibited heterogeneous Venus expression pattern from 8- to 16-cell until E3.5 blastocyst, irrespective of any specific position of the cell within the embryo (Fig 4B, Appendix Movie S4). The ICM-specific expression became restricted only at late E4.5 (≥ 128 -cell) blastocyst.

To quantitatively characterise the expression dynamics of the VET reporters for *Tmem50b* and *2610305D13Rik* genes during blastocyst development, we segmented the nuclei, tracked their trajectories through divisions, measured the signal intensity for Venus and normalised it against that of H2B-mCherry. This allowed us to fully track and quantify the dynamic change in expression of *Tmem50b*^{Gt} (n = 3 embryos) and *2610305D13Rik*^{Gt} (n = 3 embryos) throughout 4 cell cycles over a 3

days period. A representative set of Venus expression data after tracking of all 8 blastomeres and its descendants in a single embryo is shown for Tmem50b^{Gt} (Fig EV4A) and 2610305D13Rik^{Gt} (Fig EV4B). Using the mT membrane signal, a binary information on cell position was assigned at each time point as either “inside” (red line), when entirely surrounded by other cells, or “outside” (blue line), when part of the cell surface is exposed to the outside. In many cases blastomeres did not stop in their position immediately after mitosis but progressively moved towards their final position during interphase (see tracks changing from “blue” to “red” during interphase in Fig 5, EV4, Appendix Fig S3, S4), in agreement with recent live-imaging studies [32,44-46].

Finally, all the extracted lineage relationships, gene expression dynamics and binary (inside or outside) cell-positional information were integrated into a comprehensive lineage map starting from the beginning of 8-cell until the late 32- or late 64-cell stage (Fig EV5). To supplement the binary cell-positional information, a relative position of nuclei along the embryonic radial axis was included in the map. The extracted nuclear position is effectively in agreement with the manually annotated binary cellular position. Altogether we have established a complete lineage map of early mouse development in which lineage tracks are integrated with gene expression dynamics, absolute cell- and relative nuclei-position (Fig 5, one representative map is shown for each lineage reporter, for which the expression dynamics are presented in Fig EV4; two more maps for each lineage are presented in Appendix Fig S3 for Tmem50b^{Gt} and in Appendix Fig S4 for 2610305D13Rik^{Gt}).

Quantitative expression analysis reveals distinct mechanisms underlying ICM and TE lineage specification

Using the established lineage maps, we wished to better understand the potential mechanism underlying ICM and TE lineage specification. For each lineage marker, we sought for the cue in the map that would allow us to predict the lineage, ICM or TE, at the earliest possible time in embryonic development.

First, we investigated the potential contribution of the division pattern to the regulation of lineage-specific gene expression. The orientation of the 8- to 16-cell division in relation to the cellular apico-basal axis was proposed to play a key role in the ICM vs. TE lineage segregation [2]. Indeed we noted that many asymmetric divisions, defined in this study as a division generating one inside- and one outside-positioned descendant, are followed by an upregulation of Tmem50b^{Gt} in the outer descendant. Out of 48 divisions from 8- to 16-cell stage for all 6 embryos analysed (3 each for Tmem50b^{Gt} and 2610305D13Rik^{Gt}), we identified 31 asymmetric divisions (64.6%) and out of 96 divisions from 16- to 32-cell stage, we identified 18 asymmetric divisions (18.8%). To examine the relationship between division pattern and gene expression, we have assembled the trajectories for all asymmetric divisions ($n = 16$ for 8- to 16-cell stage, and $n = 7$ for 16- to 32-cell stage for Tmem50b^{Gt}, and $n = 15$ for 8- to 16-cell stage and $n = 11$ for 16- to 32-cell stage for 2610305D13Rik^{Gt}) and collectively compared the change in gene expression upon 8- to 16-cell and 16- to 32-cell divisions between the outer and inner descendants (Fig 6A, B and C). This pairwise analysis indicated that the Tmem50b^{Gt} expression is upregulated upon asymmetric division, and becomes significantly higher in the outer cell as compared to the inner sister for both - 8- to 16-cell and 16- to 32-cell - divisions (Fig 6A and C; $p = 0.0011$ and $p = 0.0019$ for 8- to 16- and 16- to 32-cell divisions, respectively, by

two-sided Wilcoxon rank sum test). In contrast, no such temporal change could be observed for 2610305D13Rik^{Gt} expression in the inside cells upon asymmetric division (Fig 6B and C; $p = 0.0158$ and $p = 0.0943$ for 8- to 16- and 16- to 32-cell divisions, respectively). The expression of 2610305D13Rik^{Gt} in outside cells becomes higher upon asymmetric 8- to 16-cell divisions, however the overall level of expression remains low during 8- to 32-cell stages (see Fig EV4B) and the biological significance of this temporal change remains to be investigated. Taken together, these data suggest that asymmetric division drives the upregulation of a TE lineage marker expression specifically in the outer descendants, but it does not drive a reciprocal upregulation of an ICM lineage marker in the inner descendant.

Since the asymmetric 8- to 16-cell or 16- to 32-cell divisions did not upregulate the ICM reporter expression in the inside cells, we sought for other possible cues that would correlate with differential expression of the ICM reporter. From our VET reporter, the dynamics of gene expression could be more faithfully reflected in their increase than in their decrease, because dynamics of degradation of Venus-NLS protein may be different than those of the endogenous protein. As anticipated, live imaging of maternal Venus protein decay in wt embryos derived from a gene-trap line (Ctnna1^{Gt/+}) gave an estimate for the relevant half-life of 17 ± 3 h ($n = 16$, Appendix Fig S5). Thus, instead of the total level, we analysed the average rate of the expression change for the Tmem50b^{Gt} reporter in outside and inside cells during 16- ($n = 32$ and $n = 16$) and 32-cell stages ($n = 56$ and $n = 40$), and for the 2610305D13Rik^{Gt} reporter in outside and inside cells during 16- ($n = 33$ and $n = 15$), 32- ($n = 53$ and $n = 41$) and 64-cell stages ($n = 106$ and $n = 84$; Fig 6D). As an alternative to the orientation of cell division, we investigated the possible role of cell position within the embryo (inside or outside) [1], in the regulation of lineage reporter expression. The data indicate that while the TE-reporter Tmem50b^{Gt} is upregulated more strongly in outside cells than inside cells as early as at the 32-cell stage ($p = 2 \times 10^{-5}$), higher expression of the ICM reporter 2610305D13Rik^{Gt} in inside cells as compared to outside cells becomes evident only at the 64-cell stage ($p = 0.025$). Furthermore in many cells that change position within the embryo from outside to inside the expression of TE-reporter remains low, whereas their sibling cells that remain outside upregulate the expression (Fig 5A, marked with yellow arrows). Therefore, cell position within the embryo indeed correlates with differential expression of our lineage reporters. Together, our lineage map allows us to faithfully allocate outer cell fate earlier than inner cell fate, in agreement with a recent study[32].

Discussion

In this study we successfully generated novel lineage-specific fluorescent reporters by a fluorescence gene-trap screen in the mouse pre-implantation embryo. The screen was primarily designed to identify fluorescent reporter lines suitable for live imaging of pre-implantation development, while the possibility of examining the functional requirement of the trapped genes was subsidiary to the main goal. Generally, it would be ideal to generate a knock-in fluorescence-fusion reporter that would reflect the endogenous dynamics and function [18]. However, the successful use of such lines will crucially depend on the endogenous expression level of the gene and on the sensitivity of live imaging microscopy, precisely the signal detection efficiency relative to the laser illumination, since the mammalian embryo is particularly sensitive to light exposure [47]. In the present study we adjusted the spatio-temporal resolution to enable manual tracking, positional annotation and segmentation of nuclei for signal quantitation while ensuring efficient development of the embryo. More advanced microscopy, for example selective-plane illumination microscope, SPIM [48,49], will allow live imaging of knock-in fluorescence-fusion reporters and with higher resolution for automated tracking and segmentation in the future. Using an unbiased approach, we aimed at discovering new markers for the first lineages in the early mouse embryo and fully characterised the expression dynamics of two genes with previously unknown specificity to ICM or TE lineage. This will be helpful to further study the gene network regulating lineage specification in the blastocyst.

We also present here advanced image analysis tools and strategies to include the increasing amount of information into lineage tracks to facilitate an integrative understanding of embryonic development and lineage segregation [16]. We developed a pipeline to establish a lineage map of embryonic development by live imaging the newly established VET lines and integrating gene expression dynamics and cell-positional information into lineage trees. These maps allowed us to analyse comprehensively and quantitatively gene expression dynamics as the first lineages are progressively segregated during mouse blastocyst development. Thus our image analysis revealed gene expression dynamics in relation to particular morphogenetic processes. In light of the available models [1,2], we focused our analysis to the possible link between the cell division pattern or cell position, and upregulation of lineage-specific gene expression. The systematic analyses presented here suggest that the expression of TE and ICM lineage markers is not initiated in a reciprocal and antagonistic manner as suggested earlier [2,3] but rather with different timings and through distinct mechanisms (Fig. 7). Namely, the up-regulation of a TE lineage marker begins earlier and may be largely driven by asymmetric division, while that for the ICM marker may not be following the same mechanism. The mechanism regulating the ICM marker expression remains elusive and the initiation of expression appears stochastic in the morula and early blastocyst stages without obvious correlation to position and the future lineage [4,8]. This finding is in line with our recent single-cell gene expression profiling study demonstrating genome-wide stochastic gene expression in the inner cells of the 32-cell stage blastocyst [7].

While in the current study image analysis was mostly performed manually, future development of automatic image processing will allow more comprehensive analysis with a large amount of image sets. This might help seeing through the apparently stochastic expression of the ICM markers. The fluorescent signals in a live embryo and its live imaging-analysis will also allow to isolate a cell specifically

expressing one, or eventually multiple, lineage marker(s) at a particular stage, and perform single-cell transcriptome analysis [7] to acquire comprehensive information of gene expression [16].

This study represents the first step in such an integrative approach, and its further development will enable us to understand the mechanisms underlying the early lineage segregation in the mammalian embryo.

Materials and Methods

Animal works, embryo recovery and culture

All animal works were performed in the animal facility at the Max Planck Institute for Molecular Biomedicine or at the European Molecular Biology Laboratory, according to the permission from the *Landesamt für Natur, Umwelt und Verbraucherschutz NRW*, Germany (projects #9.93.2.10.36.07.168 and #87-51.04.2010.A223) or to the permission by the institutional veterinarian overseeing the operation (ARC number TH11 00 11), respectively. The animal facilities are operating according to international animal welfare rules (Federation for Laboratory Animal Science Associations guidelines and recommendations). No statistical method was used to estimate the sample size for animal experiments.

Isolation and culturing of embryos was performed as described previously [4]. Embryos were isolated from superovulated (C57BL/6xC3H) F1 female mice mated with (C57BL/6xC3H) F1 male mice. Superovulation was induced by intraperitoneal (ip) injection of 5 international units (IU) pregnant mare's serum gonadotropin (PMSG, Intervet Intergonan), followed by ip injection of 5 IU human chorionic gonadotropin (hCG, Intervet Ovogest 1500) 44-48 hours later. Embryos were washed with FHM (Millipore, MR-024-D) several times and cultured in a 10 μ l microdrop of KSOM (Millipore, MR-121-D) covered with mineral oil (Sigma, M8410 or Acros Organics) in 5% CO₂ atmosphere at 37°C.

Zygotes were recovered from oviduct ampullae 18 to 22 hours after hCG injection. Cumulus cells were removed by briefly washing in 300 μ g/ml Hyaluronidase (Sigma, Typ IV-S, H4272) in FHM. Two-cell embryos to morulae were flushed from oviducts on day 1.5 to 2.5 after hCG injection with a fine canule (Acufirm, 1400 LL 23). Blastocysts were flushed from uteri on day 3.5 or 4.5 with a wide canule (Acufirm, 1400 LL 18). Embryos were handled using an aspirator tube (Sigma, A5177-5EA) or a hand pipette holder (Vitrolife, Sweden) equipped with a glass pipette pulled from Pasteur pipettes.

For the analysis shown in Fig 3 expression of Venus in *Cdx2* backgrounds was analysed at E4.5. At E4.5 embryos reached the transition from 7th to 8th cell-cycle (containing 65-to-128 cells to 129-to-256 cells). *Cdx2*^{+/+} embryos had 122.0 \pm 23.3 cells (n = 4), *Cdx2*^{+/-} embryos had 136.0 \pm 13.9 cells (n = 4) and *Cdx2*^{-/-} embryos had 118.5 \pm 21.8 cells (n = 4) (average \pm s.d.). Expression of Venus in *Tead4* embryos was analysed at E3.5, because many cells of *Tead4*^{-/-} embryos began to show signs of apoptosis at E3.5 with fragmented chromosomes and could not be analysed later. At E3.5 embryos reached the beginning of the 7th cell-cycle (65-to-128 cells). *Tead4*^{+/+} embryos had n = 72.2 \pm 7.8 cells (n = 5), *Tead4*^{+/-} embryos had n = 65.4 \pm 11.4 (n = 6) and *Tead4*^{-/-} embryos had 66.7 \pm 3.1 cells (n=6) (average \pm s.d.).

Lentiviral transgenesis

Delivery of the gene-trap cassette into the mouse genome was achieved using self-inactivating lentiviruses (sinLVs; [50]). The pRRL-based lentiviral vector carries a gene-trap cassette with Venus as a fluorescent reporter (sinLV-pRRL-K; Fig. 1A). To enhance the signal, Venus is coupled to the 39 nucleotides SV40-T-antigen nuclear localization signal (NLS) domain [51], followed by the Woodchuck hepatitis post-transcriptional regulatory element (WPRE; [52] and the bovine growth hormone gene polyadenylation signal (pA; [53]). The gene-trap cassette utilizes a 3' splice consensus

sequence from the type II adenovirus major late transcript intron 1/exon 2 (splice acceptor, SA;[54] to trap 5' splice consensus sequences (splice donors) from endogenous transcripts. Stop codons in all three reading frames prevent formation of a fusion protein and the encephalomyocarditis virus (EMCV) internal ribosomal entry site (IRES; [55] guides translation of Venus-NLS. Additional elements non-essential for the gene-trap cassette are FRT sites [56] flanking the gene-trap cassette, as well as a LOXP2272 [57] and an AttB element [58] 5' of the pA.

Lentiviruses were produced and harvested according to the published procedure [52], and were concentrated by ultracentrifugation to titers of 1 to 5.4×10^9 viral genomes per ml. Preliminary transduction of the mouse zygote using sinLV pRRL-K was inefficient in producing embryos with detectable signal at the late blastocyst stage ($n = 1 / 54$ in three independent trials), whereas efficient transduction was achieved with the embryos infected at the 2-cell stage.

Two-cell embryos were prepared for transgenesis by removal of the zona pellucida through incubation in pronase (0.5 % w/v Protease; Sigma, P8811) for 2-3 minutes. The denuded embryos were co-cultured with concentrated virus (2×10^8 to 1×10^9 HeLa-transducing units/ml) in FHM for 6-8 h. After 3 washes in FHM the embryos were transferred to KSOM and cultured for 3 days. To prevent aggregation of embryos, individual denuded embryos were placed in micro-wells prepared with an aggregation needle (BLS) directly inside the micro-drops of medium.

Identification of proviral integrations and the trapped genes

Genomic DNA was isolated from tail tissue, as described by The Jackson Laboratory (http://jaxmice.jax.org/support/genotyping/tail_phenol.html), by digestion with Proteinase K (Sigma, P2308, 0.5 mg/ml in 50 mM Tris-HCl (pH 8.0), 100 mM EDTA (pH 8.0), 100 mM NaCl, 1% SDS) at 55°C followed by purification with Phenol-Chloroform-Isoamylalcohol (25:24:1, Roth, A156.1). The aqueous phase was separated from the organic phase using phase lock gels (5 Prime, VWR International, 713-2534). The gDNA was then precipitated with 100% ethanol (Roth, 9065.3), washed in 70% ice-cold ethanol, and resuspended in 100 μ l buffer TE (Invitrogen, 12090-015).

Proviral integration sites were detected by Southern blot. Southern blots were performed with a α - 32 P-dCTP-labelled probe against Venus (711bp *NcoI*/*BsrGI* fragment). Ten microgram of gDNA were digested with 250 U *EcoRI* (NEB, R0101M) for 12 hours and subsequently loaded in 10x1 mm pockets on a 20x25 cm 0.8% Agarose gel (Lonza). After 6-7 hours at 120 V the DNA in the gel was depurinated in 0.25 N HCl for 20 min and denatured in 0.5 M NaOH, 1.5 M NaCl for 2 times 15 minutes, before blotting the DNA onto a Nylon membrane (GE Healthcare, Nylon Hybond N, RPN203N). The membrane was neutralized in 0.05 M $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$ at pH 6.5 for 30 minutes and then slightly dried between blotting papers (Schleicher & Schuell, GB005). The gDNA was then cross-linked to the membrane 2 times at 0.125 Joule. The cross-linked gDNA was pre-hybridized for 2 hours at 65°C in pre-hybe buffer (5x SSC, 5x Denhardt's, 0.025 M $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$ at pH 6.5, 0.1% SDS, 0.1 mg/ml fish ssDNA in ddH_2O). This solution was then replaced with hybe buffer (pre-hybe buffer supplemented with 10% Dextran sulfate Na-salt) and α - 32 P-dCTP-labelled Venus and incubated overnight. To radioactively label Venus, 75 ng DNA per 45 μ l TE were processed with 2.5 μ l α - 32 P-dCTP (10mCi/ml, Perkin-Elmer, Redivue) using the Rediprime II kit (GE Healthcare, RPN 1633) and

purified using the ProbeQuant G-50 microcolumn (GE Healthcare, 28-9034-08) according to the manufacturer's protocol. Membranes were then washed two times for 15 minutes at room temperature in 2x SSC with 0.1% SDS, and two times for 15 minutes at 65°C in 0.5x SSC with 0.1% SDS. Membranes were then exposed to Calcium Tungstate intensifying screens (Hyperscreen, GE Healthcare, RPN1663) at room temperature and analyzed on a phosphorimager (FLA-7000, Fujifilm). Additionally, membranes were exposed to films (Amersham Hyperfilm ECL, GE Healthcare, 28906837) at -80°C and developed (Agfa Curix 60).

Proviral integration sites were identified by Ligation-mediated (LM)-PCR (modified from [59]). One microgram genomic DNA was *Mse*I (60 U, NEB R0525L) digested and purified (Promega Wizard SV A 2360). *Mse*-linker oligos (*Mse*-linker+: 5'-GTAATACGACTCACTATAGGGCTCCGCTTAAGGGAC-3', *Mse*-linker-: 5'-PO₄-TAGTCCCTTAAGCGGAG-NH₄ (C7)-3', 20 μM each) were hybridized by heating to 90°C and then decreasing the temperature 1°C every 3 minutes to a final temperature of 20°C. In a total reaction volume of 20 μl, 3.5 μl of 20 μM *Mse*I-linker were ligated with 400U T4 ligase (NEB, M0202S) to 13.5 μl *Mse*I digested gDNA. To remove bacterial contamination and intra-proviral fragments the ligation was digested with 40 U *Dpn*I (NEB, R0176) and 40 U *Sac*I (R0156) in 50 μl total volume for 4 hours. A first PCR was performed in 25 μl total reaction volume using 5 μl processed gDNA, 0.3 μM of primers *Mse*L1 MKL-3 (5'-CTTAAGCCTCAATAAAGCTTGCCTTGAG-3') and *Mse*L1 MKL-3 (5'-GTAATACGACTCACTATAGGGC-3') each, 0.2 mM dNTP mix, 0.5 μl *Taq* Polymerase (Advantage 2, TaKaRa 639207) and 1x buffer. The thermal cycler settings were 1 minute at 94°C (hot start), 7 rounds of 2 seconds at 94°C and 1 minute at 72°C, followed by 37 rounds of 2 seconds at 94°C and 1 minute at 67°C and a final elongation step of 4 minutes at 72°C. Nested PCR was performed on 1 μl of a 1:200 dilution of the first PCR, 0.3 μM of primers *Mse*L2 (5'-AGGGCTCCGCTTAAGGGAC-3') and MKL-5 (5'-TGA CTCTGGTAACTAGAGATCCCTCAG-3') each, 0.2 mM dNTP mix, 0.5 μl *Taq* Polymerase (Advantage 2) and 1x buffer. The thermal cycler settings for the nested PCR were 1 minute at 94°C (hot start), 5 rounds of 2 seconds at 94°C and 1 minute at 75°C, followed by 20 rounds of 2 seconds at 94°C and 1 minute at 72°C and a final elongation step of 4 minutes at 72°C. The nested PCR product was run on a 0.8% agarose gel and purified (Qiagen, 28706). The isolated fragment was TOPO-TA cloned into pCR4, transformed into TOP-Ten or DH5α *E. coli* and the bacteria plated according to the manufacturer's instructions (Invitrogen, TOPO-TA, K4575J10). Colonies were picked the following day and grown in LB medium with 50 μg/ml ampicillin overnight at 37 °C. Plasmids were purified using the Qia-Prep Spin system (Qiagen, 27106) and subsequently sequenced using M13 reverse primer.

Alternatively, integration sites were identified using thermal asymmetric interlaced (TAIL)-PCR [60,61] performed as described by [62], using the arbitrary degenerate primers and cycling conditions proposed there. Gene specific primers were designed to identify either the region adjacent to the 3'-end of the integration (ISP-provirK-31 (5'-CTTTCCCCCTGGCCTTAACCGAATTT-3'), ISP-provirK-32 (5'-TTTTCCCATCGCGATCTAATTCTCCC-3'), ISP-provirK-33 (5'-GCTTAATACTGACGCTCTCGCACCCA-3')) or the region adjacent to the 5'-end of the integration (ISP-provirK-51 (5'-GGGGATCAATTCGAGCTCGGTACGA-

3'), ISP-provirK-52 (5'-GGAACCTTCACCGGTATTTGGGGGATC-3'), ISP-provirK-53 (5'-GGGATCAATTCGAGCTCGGTACCTTT-3').

The primary PCR was conducted using 3 μ L gDNA in a total reaction volume of 20 μ L (1x buffer, 1.5 mM MgCl₂, 0.2 mM dNTP (each), 0.15 μ M ISP 1.3 μ M AD primer, 1 U Taq DNA polymerase). 2 μ L of a 1:20 dilution of this PCR were used as template in the secondary PCR with a total volume of 20 μ L (1x buffer, 1.5 mM MgCl₂, 0.2 mM dNTP (each), 0.2 μ M ISP 2, 2 μ M AD primer, 1 U Taq DNA polymerase). Of this secondary PCR, 10 μ L of a 1:10 dilution were used as template in the tertiary PCR with a total volume of 100 μ L (1x buffer, 1.5 mM MgCl₂, 0.2 mM dNTP (each), 0.2 μ M ISP 3.2 μ M AD primer, 1 U Taq DNA polymerase). DNA fragments from tertiary reactions were purified, subcloned and amplified like DNA fragments from LM-PCR, using the pGEM®-T Easy Vector System (Promega, Fitchburg, USA) instead of the TOPO-TA system.

The cloned sequences were cleaned from vector, primer and proviral sequences to perform a BLAT search against genomic sequences of the GRCm38/mm10 (December 2011) or GRCm38.p3 (release 78; December 2014) assembly of the mouse (*mus musculus*) genome (Fig EV1). In a few cases it was difficult to unequivocally identify the integration site: the integration site for VET53A includes highly repetitive sequences that have an equally high homology to 2610305D13Rik, Rex2, Zfp600, Gm13145, Gm13242 and Gm13152. RT-PCRs however 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, thus the trapped gene was tentatively assigned as 2610305D13Rik (marked by an asterisk in Fig 2A). The integration site for VET53C is assigned as "X" (hidden) and the Venus signal was always detected in males, suggesting that the integration site is on Y chromosome for which the available sequence information is limited. The integration sites were mapped to karyotype ideograms (Fig 1E) using the Ensembl Karyoview. Trapped transcripts were detected by 5'RACE as described below and searched against cDNAs (transcripts/splice variants; BLASTN) or genomic sequences (BLAT) of the GRCm38.p3 (release 78; December 2014) assembly of the mouse genome (Fig EV1).

For genotyping VET mice proviral integrants were detected by PCR on genomic DNA using a pair of primers specific for Venus (5'-TGAACCGCATCGAGCTGAAG-3') and WPRE (5'-GCAATGCCCCAACCAGT-3') yielding a 671bp fragment. Known integration sites were exponentially amplified by multiplex integration-site specific PCR using a set of three primers, one inside the provirus (5'-GGACTACTGCGCCCTACAGA-3') and two specific for genomic sequences flanking the provirus. Specific primers are listed in Appendix Table S1. Reactions were performed in a total volume of 20 μ l containing 50 to 100 ng gDNA, 0.7x to 1.0x PCR buffer (+KCl / -MgCl₂), 1.5 mM to 2.5 mM MgCl₂, 0.2 mM dNTP-mix (Fermentas, R0192), 0.4 μ M of each primer and 0.5 U Taq Polymerase (Fermentas, EP0402). The reaction was initiated at 94°C for 4 minutes, followed by 35 rounds of 1 minutes at 94°C, 1 minutes at 52°C to 62°C and 1 minutes at 72°C (see Appendix Table S1 for details). An additional final elongation was performed for 7 minutes at 72°C.

5' Rapid amplification of cDNA ends (RACE)

First-strand cDNA was synthesized from purified total RNA using 400 U Reverse Transcriptase (SuperScript III, Invitrogen, 18080), 5 mM DTT, 1 x buffer, 2 pmol specific primer IRES-2 (5'-AACTCACAACGTGGCACTGG-3'), 30 U RNase Inhibitor (Qiagen, 129918), 0.5 mM dNTPs (Fermentas, R0192) in a total Volume of 20 μ l. RNA was then degraded using Ribonuclease H (Fermentas, EN0201) and subsequently purified using the Qiaex II Gel Extraction Kit (Qiagen, 20021) following the manufacturers protocols. First-Strand cDNA was tailed using 30 U TdT (Invitrogen, 10533-065) in 1 x buffer and 0.2 mM dATP (Invitrogen, 10216-018) in a total Volume of 20 μ l. First PCR was performed on 2 μ l tailed cDNA using primers QA (5'-CCAGTGAGCAGAGTGACGAGGAC-3') and IRES-(-) (5'-CCTCACATTGCCAAAAGACGGC-3') at 0.4 μ M and QT (5'-CCAGTGAGCAGAGTGACGAGGACTCGAGCTCAAGCTTTTTTTTTTTTTTTTTTTT T-3') at 0.02 μ M in the PCR Mastermix (Genaxxon, M3014) in a total Volume of 50 μ l. Second PCR was performed on 1 μ l of a 1 : 50 dilution of the first PCR product using primers QB (5'-GACGAGGACTCGAGCTCAAGC-3') and O-IRES-1b-(-) (5'-CAAACGCACACCGGCCTTATTC-3') at 0.4 μ M and QT at 0.02 μ M in the PCR Mastermix (Genaxxon, M0314). The second PCR product was purified from 1% agarose gel and TOPO-TA cloned before sequencing as described above.

Genotyping of single embryos

After live-imaging Tmem50b^{Gt} or 2610305D13Rik^{Gt} embryos in *Tead4*^{-/-} or *Cdx2*^{-/-} backgrounds, embryo genotypes were assessed by genomic PCR of single embryos. Embryos were transferred to lysis buffer containing Proteinase K (0.2 μ g/ μ l) and 1x PCR buffer (with KCl, Fermentas, EP0402) in H₂O. Lysis was performed at 55°C for 1 hour and 96°C for 10 minutes. For PCR 2 to 5 μ l of the lysate were used. *Cdx2*<tm1Fbe> (MGI: 1857928) and *Tead4*<IRES-lacZ> (RIKEN accession number: CDB0409K) embryos were genotyped as described elsewhere [41,63], whereas 2610305D13Rik^{Gt}, Tmem50b^{Gt}, Ctnna1^{Gt} and Spt6^{Gt} were genotyped as described above.

Total RNA isolation from placenta

For homogenization placentas were washed in PBS, snap-frozen in the vapor phase of liquid nitrogen and after addition of 600 μ l lysis buffer RLT (Qiagen, RNeasy Mini Kit, 74104) treated with a Precellys 24 using 1.4 mm ceramic beads at 5000rpm for 15 seconds. Total RNA was isolated using the RNeasy Mini Kit (Qiagen, 74104) using the manufacturers protocol.

Isolation of trophectoderm cells by manual bisection of the blastocyst

TE cell samples were collected from E4.5 embryos after they hatched from the zona pellucida. E4.5 – E4.75 blastocysts were manually bisected at the ICM-blastocyst cavity boundary by cutting with a 30Gx1/2 needle (BD, 305771) and removing polar TE cells and the ICM cells. The remaining mural TE cells were immediately transferred to 100 μ l TRI reagent (Ambion, AM9738) at 4°C and stored at -20°C until RNA extraction.

RNA isolation from trophectoderm cells and reverse transcription

To extract RNA, 20% v/v of trichloromethane was added to TE samples in TRI reagent, followed by 15 minutes centrifugation at 4°C at 12000 rpm. The supernatant

was transferred into a fresh tube together with 50% v/v 2-propanol and 3 μ l Glycogen (Ambion, AM9510). Samples were incubated for 15 minutes on ice, centrifuged for 30 min at 4°C at 12000 rpm and the supernatant was discarded. The pellet was washed with 80% Ethanol and dried. Reverse transcription was performed using SuperScript® III First-Strand Synthesis System for RT-PCR (Life Technologies, 18080-051) according to the manufacturers' instructions, using Oligo(dT)₂₀ priming method. The cDNA synthesis was performed for 1 hour.

qRT-PCR

Single-cell cDNA samples for qRT-PCR were prepared by Y. Onishi and inside-outside position determined as described in [7]. qRT-PCR was performed using SYBR® Green PCR Master Mix (Life Technologies, 4309155) on ABI StepOne devices (Life Technologies, 4376357, Ser. No. 272006386) according to the manufacturers' instruction with 5 μ M primer concentration in 10 μ l final sample volume, run for 40 cycles. Each sample was run in duplicates with a negative control and Gapdh as a housekeeping gene control, all in the same experiment. ES cell cDNA samples were added to each experiment in two dilutions to ensure normalised amplification between experiments. The primers sequences were as follows: Cdx2 (5'-ATTGTTTGCTGCTGTTCGAGTC-3', 5'-CGACTTCCCTTCACCATACAAC-3'), Gapdh (5'-ATGAATACGGCTACAGCAACAGG-3', 5'-CTCTTGCTCAGTGTCCTTGCTG-3'), Gm13145 (5'-TGGACTTCTCATCGGAGGAA-3', 5'-TGTTCCACGACCTTCTCCTT-3'), Gm13152 (5'-TAATCCGGGCAACAAGAATG-3', 5'-GCACAATGACCTCTGAGCAA-3'), Gm13242 (5'-CTGCCAGATCAGAGGTCTCC-3', 5'-TGGTGAGGAGCAAACATTCA-3'), Oct4 (5'-GGCTCCTGATCAACAGCATCAC-3', 5'-GATGCTGTGAGCCAAGGCAAG-3'), Rex2 (5'-TGCTCAGTCAACTCTCCGTAA-3', 5'-TGGTGAGGAGCAAACATTCA-3'), Sox2 (5'-CATGAGAGCAAGTACTGGCAAG-3', 5'-CCAACGATATCAACCTGCATGG-3'), Tmem50b (5'-GCCGTGGCTTCTCTATTCAT-3', 5'-AAGAGAACCGTGTGCATTTT-3'), Zfp600 (5'-CATGGATGTGATGTTGGAGAA-3', 5'-TGTGCTGTGTTTCTTGTTCCA-3'), 2610305D13Rik (5'-TGAATGTTTGCTCCTCACCA-3', 5'-TGAGACATTCCCATTCTCTG-3'). Raw data was processed using StepOne Software (Life Technologies, version 2.3). A threshold for determining C_t values was set in the linear phase of amplification, at an equal value for the same gene in different experiments. The baseline was selected automatically and controlled manually for any software algorithm-induced mistakes.

After confirming sample and experiment quality by assessing negative control, ES cell cDNA amplification, and Melt Curves, average values of the technical replicates were calculated for each gene. No replicates had to be excluded from the calculation. The Δ C_t value was calculated by subtracting the average C_t value of Gapdh from the average C_t value of the gene of interest for the same sample within the same experiment. Within a category (EPI, PE or TE cells) Δ C_t values for a single gene were used to calculate the average and standard deviation, as described in Appendix Fig S2. In negative controls and samples where cDNA for the gene in question was not present, the C_t value of one or both duplicates could not be determined by the StepOne Software algorithm. The average C_t value in such samples was set to the maximum experiment cycle number and used to calculate average and

standard deviation for Appendix Fig S2A. Where Melt Curve analysis indicated a shift of the product melting temperature (T_m) to a different value (such as for 2610305D13Rik), the samples resulting from qRT-PCR reaction were additionally run on an agarose gel to identify the size of the product. The samples in which the product size did not correspond to that of control samples were marked by an asterisk in Fig S4 and the C_t values were not used to calculate average and standard deviation. For Appendix Fig S2B, ΔC_t value of samples marked with asterisk were set to maximum value on the plot for representation purposes and does not reflect an actual ΔC_t as explained above. No statistical analysis of data distribution could be performed for samples in Appendix Fig S2 as no numerical value is available for several cells.

Immunohistochemistry

Embryos were fixed in 2% (for Spt6 and Ctnna1) or 4% (for other stainings) paraformaldehyde (Sigma, P6148) in PBS for 10 to 15 minutes. For stainings with anti-GFP antibody, the embryos were sorted into Venus positive and negative groups prior to fixation and remained separated throughout the procedure. After fixation, the embryos were washed with 0.1% Tween 20 (Sigma, P7949) in PBS (PBS-T) and permeabilized in 0.25% (for Spt6 and Ctnna1) or 0.5% (for other stainings) Triton X-100 (Sigma, T8787) in PBS for 30 minutes. Subsequently, the embryos were washed in PBS-T, blocked with 3% BSA in PBS-T (blocking solution), and incubated with the following primary antibodies in blocking solution at 4°C overnight: rabbit polyclonal anti-Spt6 (Abcam, ab32820, 1:2000 dilution), rabbit monoclonal anti-Ctnna1 (Abcam, ab51032, 1:100 dilution), polyclonal rabbit anti-GFP (MBL, Code No. 598, 1:500 dilution) and polyclonal goat anti-Sox2 (Y-17, sc-17320, Santa Cruz Biotechnology, 1:100 dilution) or monoclonal mouse anti-Cdx2 (Biogenex, MU392-UC, 1:200 dilution). After washing in blocking solution, embryos were incubated with secondary antibodies as follows: for Spt6 and Ctnna1, 1 hour with Alexa Fluor 546 donkey anti-rabbit (Life Technologies, A10040, 1:100 dilution) and Alexa Fluor 647 donkey anti-rabbit (Life Technologies, A31573, 1:100 dilution), respectively; for others, 2 hours at room temperature with Alexa Fluor 488 donkey anti-goat (Life Technologies, A11055, 1:200 dilution) or Alexa Fluor 488 donkey anti-mouse (Life Technologies, A21202, 1:200 dilution) in addition to Alexa Fluor 546 donkey anti-rabbit (Life Technologies, A10040, 1:200 dilution) and Phalloidin 633 (Life Technologies, A22284, 1:50 dilution), all in blocking solution. When nuclear staining was required, the embryos were washed with PBS-T containing DAPI (Life Technologies, D3571, 1:2000 dilution), and imaged immediately in the same solution. All incubation, washing and imaging steps were performed at room temperature unless stated otherwise. The stained embryos were imaged with LSM780 (Zeiss) equipped with a 40x water-immersion C-Apochromat 1.2 NA objective lens (Zeiss), using Zen 2010 software (Zeiss). The images were processed using Fiji running ImageJ (NIH, USA)

Live imaging microscopy

For microscopic analysis and live-imaging, embryos were cultured in 5 μ l drops prepared on a 0.17 mm glass-bottom dish (ZOG3, Elekon Science or MatTek, P35G-1.5-20C) in a 5% CO₂ chamber (PeCon). Temperature was maintained by a Tempcontrol 37-2 digital (Zeiss) at 37.5°C in a plastic chamber incubator XL (Zeiss) and a heatable mounting frame M-H (Zeiss), attached to a Zeiss Axiovert 200M with Narishige manipulators. Fluorescence was detected with a Zeiss AxioObserver

equipped with a 25x/0.8 LCI Plan Neofluar water immersion objective lens (Zeiss). The light source was either a Colibri LED at 505 nm combined with a 60 HE filter set or a HXP lamp with a 46 HE filter set (Zeiss). For 4D imaging, LSM780 (Zeiss) was used with a 40x water-immersion C-Apochromat 1.2 NA objective lens (Zeiss). Triple transgenic embryos carrying H2B-mCherry and membrane-tdTomato in addition to a Venus-trap reporter were prepared and imaged from 2- to 4-cell up to 128-cell stage (about 72 hours). Venus was excited using a 514 nm Argon laser, while mCherry and tdTomato a 561 nm DPSS laser. Z-stacks were recorded every 20 minutes and reconstructed from up to 55 images at 2 μ m distance at 8-bit and 512 x 512 pixel resolution. Zen 2010 software (Zeiss) was used for the acquisition of images.

Half-life of the Venus-NLS protein was determined as decay of maternal Venus-NLS in wt embryos derived from mating heterozygous *Ctnna1*^{Gt} mice (see Fig EV4). Four-cell stage blastomeres were dissociated in calcium- and magnesium-free KSOM medium (1/4-cell), as described previously [4] and imaged until 4/16-cell stage.

Image analysis

Image analysis was performed using AxioVision (Zeiss), Imaris (Bitplane), Fiji running ImageJ (NIH, USA) or Matlab (The MathWorks).

For lineage mapping (Fig 5, EV4, and Appendix Fig S3, S4) a total of 364 cells were tracked until early 64-cell ($n = 3$ embryos for *Tmem50b*^{Gt}) or late 64-cell ($n = 3$ embryos for *2610305D13Rik*^{Gt}; 94.8% of $n = 384$ cells). Due to blastocyst expansion 14 cells were lost during tracking. A total of 54 cells (14.8%), in particular 47 of 83 ICM cells (56.6%) during the 64-cell stage, underwent nuclear DNA fragmentation, indicative of apoptosis [5].

Fluorescence signal in Fig 3 and EV3 was quantified using Fiji by manually selecting the area to be analyzed and extracting sum intensities from single confocal sections. Fluorescence intensities were normalized to the highest level of expression. Quantitation in Fig 5, 6, EV4 and Appendix Fig S3, S4, S5 was done after 4D lineage tracking. Segmentation was done manually or, if applicable, automatically using Imaris (Bitplane). Spots of 6 to 10 μ m diameter were assigned to all nuclei based on Venus and/or H2B-mCherry signal. For Appendix Fig S4 Venus mean fluorescence intensities were normalized with the highest level of expression. For Fig 5, 6, EV4, and Appendix Fig S3, S4 Venus mean fluorescence intensities were normalized using nuclear mCherry mean fluorescence intensities. In the case of lineage maps shown in Fig 5, Appendix Fig S3, S4 fluorescence intensity was further normalized to the maximum expression intensity of all cells in the same cell-cycle stage of an embryo. In Fig 6, EV4 the normalized Venus intensity was additionally normalized to the highest level of expression within the displayed set of data.

Binary information of cell position (inside or outside) was determined based on mT signal. Blastomeres with circumferential contacts to cells were defined to be inside, whereas blastomeres lacking circumferential contacts to cells to be outside (see Fig EV5). Furthermore, relative position of nuclei was determined for each time point. For that the approximate surface of the entire embryo was determined by finding the center of mass and the radius of a fitted hollow sphere, whose surface was closest to the convex hull of all nuclear positions. Nuclear positions were manually annotated using Imaris as described above. For each individual nucleus the distance to the surface was calculated. Distant scales are defined in units of 1.2 times the radius

of the calculated convex hull. Blue corresponds to nuclei close to the surface, red to nuclei deep inside the embryo. Nuclei outside the calculated convex hull with surface distances larger than 0.2 times radius were assigned the distance 0 (dark blue) as well. To generate lineage trees spot-segmented nuclei were manually or, if applicable, automatically connected to tracks.

For the analysis shown in Fig 6A and B the individual cell divisions of the same cell-cycle stage were identified separately for all embryos carrying TE and ICM lineage markers. For each stage the expression of the parent and the two descendant cells was plotted only if parent cells were found to be outside (blue) and one of the descendant cells was found to switch inside (red) within the following cell-cycle stage. Time zero indicates the time point of division. The Venus expression intensity was normalized to the maximum intensity of all lineages within an embryo. In Fig 6C, for each pair of descendant blastomeres in Fig 6A and B the difference in cumulative expression from 0 to 7 hours post division was measured for those tracks for which the expression data are available for the first 7 hours (see inset in Fig 6C). For Fig 6D, Venus expression intensity was extracted as described for Fig 6A, except that expression curves of all descendant cells were included in the analysis. In this analysis the curves rate of change was measured and averaged between 0 to 6 hours for those tracks for which the expression data are available for the first 6 hours.

Statistical analysis

Boxplot graphs were generated and statistical analyses were performed using IBM SPSS Statistics (Version 22) and Matlab. No statistical methods were used to ensure adequate power to detect a pre-specified effect size. Samples were either chosen randomly for analysis without pre-established criteria or all available data were used. No randomization was applied and outcome assessment was not blinded.

For statistical analysis data were first analysed for normality using Shapiro-Wilks test. Variance was either assessed using Levene's test or judged based on box-plots. Non-parametric significance tests for independent samples were performed for the analysis in Fig 3B, D, F, H and Fig 6C, D and Fig EV3A2, A4, B2, B4, since many data sets were not normally distributed and variance was not always similar. For the analysis presented in Fig 3B, D, F, H and Fig EV3A2, A4, B2, B4 significance tests were performed pairwise using Kruskal-Wallis H tests, because more than two groups were compared. For the analysis presented in Fig 6C and D the two-sided Wilcoxon rank sum test was performed. In Fig 6C the sample distribution tested against was derived by calculating a set of pairwise differences in cumulative Venus expression intensity of all descendant cells with all other descendant cells, effectively ignoring family relationships and inside-outside associations. The same test was performed pairwise in Fig 6D comparing inside and outside blastomeres.

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All mouse lines were deposited to and are available at the RIKEN BioResource Center (Tsukuba, Japan).

Author contributions

J.-E.D. and T.H. conceived and designed the study, and J.-E.D. performed the experiments presented in Fig 1-6, EV1-5 and Appendix Fig 8-10 with help of S.W., A.P. and S.S, and L.P. performed the experiments presented in Appendix Fig S1 and 2 with help of S.S. S.G., J.-E. D. and L.H. performed image analyses presented in Fig 5, 6, EV4, 5 and Appendix Fig S3, S4. A.G. and D.T. helped J.-E.D. to generate lentiviral vectors and to analyse the integration sites. T.H. wrote the paper, except for Materials and Methods and Figure legends written by J.-E.D. and L.P., with inputs from all authors.

Conflict of interest

The authors declare that they have no conflict of interest.

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Figure Legends

Figure 1

Design and outcome of the fluorescence gene-trap screen

(A) Design of the fluorescence gene-trap vector. The gene-trap cassette consists of a splice acceptor (SA) followed by stop codons in all three reading frames and an internal ribosomal entry site (IRES). Venus is tagged with a nuclear localisation signal (NLS). The essential virus-specific elements include: self-inactivating long terminal repeat (LTR) consisting of U5, R and truncated U3 element (Δ U3); Woodchuck hepatitis post-transcriptional regulatory element (WPRE); central poly-purine tract (cPPT); Rev-responsive element (RRE); and Rous-Sarcoma Virus promoter (RSV). (B) Design of the screenings. Denuded 2-cell stage embryos were transduced with the self-inactivating virus for 6-8 hours. Blastocysts were first screened for fluorescence, indicating successful trapping of a gene after 3 days of *in vitro* culture. Positive embryos were then transferred to foster mothers to give rise to adult founders. Germline transmission of fluorescent signal-positive proviral integrants was tested in a fluorescence-based 2nd screen of expanded blastocysts of subsequent generations. (C) Efficiency of the Venus-trap screen. (D) Number of integrations identified in the founders positive in the 2nd screen. (E) Distribution of integration sites among mouse chromosomes. Yellow triangles mark identified integration sites positive for Venus expression at the blastocyst stage (n = 22 of 23).

Figure 2

Spatio-temporal characterisation of gene-trap mouse embryos

(A) The trapped gene and the Venus expression pattern of the established VET mouse lines, summarised as a result of $n \geq 3$ independent experiments for each line. *The trapped gene for VET53A is tentatively assigned as 2610305D13Rik (see Materials and Methods for details). (B,C) Brightfield and confocal live-images of the developing embryos at E2.5 (8- to 16-cell stage morula), E3.5 and blastocyst cultured beyond E4.5 derived from the (B) TE-specific line *Tmem50b^{Gt}* and (C) ICM-specific line *2610305D13Rik^{Gt}*. Scale bars are 50 μ m. All fluorescent images are single confocal sections, representative for $n \geq 3$ independent experiments.

Figure 3

ICM and TE reporter expressions are controlled under the emerging lineage-specific genes

(A) Brightfield and confocal live-images of Venus expression in *Tmem50b^{Gt}* in *Tead4^{+/+}* or *Tead4^{-/-}* background at E3.5 (n = 109 embryos in n = 3 experiments). (B) Box-plots depicting *Tmem50b^{Gt}* Venus fluorescence intensities in individual nuclei of *Tead4^{+/+}* (n = 16 nuclei from n = 4 embryos), *Tead4^{+/-}* (n = 16 nuclei from n = 4 embryos) or *Tead4^{-/-}* embryos (n = 19 nuclei from n = 5 embryos; Kruskal-Wallis H test, $p = 10^{-8}$). (C) Brightfield and confocal live-images of Venus expression in individual nuclei of *Tmem50b^{Gt}* in a *Cdx2^{+/+}* or *Cdx2^{-/-}* background at E3.5 (n = 57 embryos in n = 5 experiments). (D) Box-plots depicting *Tmem50b^{Gt}* Venus intensities in individual nuclei of *Cdx2^{+/+}* (n = 9 nuclei from n = 2 embryos), *Cdx2^{+/-}* (n = 10 nuclei from n = 2 embryos) or *Cdx2^{-/-}* embryos (n = 22 nuclei from n = 4 embryos; Kruskal-Wallis H test, $p = 2 \times 10^{-6}$). (E) Brightfield and confocal live-images of

Venus expression in 2610305D13Rik^{Gt} in a *Tead4*^{+/+} or *Tead4*^{-/-} background at E3.5 (n = 68 embryos in n = 3 experiments). (F) Box-plots depicting 2610305D13Rik^{Gt} Venus intensities in individual nuclei of TE cells in *Tead4*^{+/+} (n = 17 nuclei from n = 3 embryos), *Tead4*^{+/-} (n = 15 nuclei from n = 4 embryos) or *Tead4*^{-/-} embryos (n = 16 nuclei from n = 5 embryos; Kruskal-Wallis H test, $p = 5 \times 10^{-5}$). (G) Brightfield and confocal live-images of Venus expression in 2610305D13Rik^{Gt} in a *Cdx2*^{+/+} or *Cdx2*^{-/-} background at E3.5 (n = 95 embryos in n = 4 experiments). (H) Box-plots depicting 2610305D13Rik^{Gt} Venus intensities in individual nuclei of TE cells in *Cdx2*^{+/+} (n = 15 nuclei from n = 4 embryos), *Cdx2*^{+/-} (n = 16 nuclei from n = 4 embryos) or *Cdx2*^{-/-} embryos (n = 14 nuclei from n = 4 embryos; Kruskal-Wallis H test, $p = 0.01$). Venus intensities in B, D, F and H are normalized to the highest level of expression. Statistical significance is indicated as * ($p < 0.05$) or ** ($p < 0.001$). Scale bars are 50 μ m. Venus expression images are sum intensity projections with a fire LUT. A fire LUT from 0 to 255 grey scales is shown in Fig. 3A.

Figure 4

4D live-imaging of the pre-implantation development of ICM and TE lineage reporter mice

(A) Representative images of a *Tmem50b*^{Gt}-Venus x R26-H2B-mCherry x mT embryo developing from the 8- to 64-cell stage (n = 27 embryos in n = 3 experiments). Upper row panels show Venus expression, middle panels mT and H2B-mCherry expression, and lower panels all fluorescent channels merged. (B) Representative images of a 2610305D13Rik^{Gt}-Venus x R26-H2B-mCherry x mT embryo developing from the 16- to 128-cell stage (n = 27 embryos in n = 3 experiments). Upper panels show Venus expression, middle panels mT and H2B-mCherry expression, and lower panels all fluorescent channels merged. Scale bars are 50 μ m. All images are single confocal sections and representative for n \geq 3 independent experiments. Venus expression images are shown using a fire LUT, mT and mCherry are shown using a grey scale LUT.

Figure 5

A lineage map of mouse pre-implantation development

(A, B) Lineage segregation maps of all cells of two embryos indicating cell position and a lineage reporter expression. (A) Lineages from the 8- to 32-cell stage based on live-imaging a *Tmem50b*^{Gt}-Venus x R26-H2B-mCherry x mT embryo. Venus expression intensity is normalized first to mCherry, and subsequently to the maximum expression intensity of all cells in the same cell-cycle stage of an embryo. Each box in the lineage tree ranges from 0 to 1 in normalized expression. Blue and red lines indicate outside and inside cells, respectively. Blue-white-red scale indicates relative distance of nuclei to the surface of the embryo. Dagger marks cells undergoing apoptosis and cell lineages lost during imaging are labelled with an "L". (B) Lineages from the 8- to 64-cell stage based on live-imaging of a 2610305D13Rik^{Gt}-Venus x R26-H2B-mCherry x mT embryo. Arrows highlight representative descendant pairs as a result of asymmetric divisions (white: essentially no difference in the expression level; yellow: higher expression in the outside cell than in the inside cell).

Figure 6

Quantitative analyses of the expression dynamics of TE and ICM lineage markers

(A) Venus expression dynamics, normalized to mCherry signal, in cells of Tmem50b^{Gt}-Venus x R26-H2B-mCherry x mT embryos dividing asymmetrically from 8- to 16-cell (n = 16 divisions in n = 3 embryos) and 16- to 32-cell stage (n = 7 divisions in n = 3 embryos; see Text and Materials and Methods for details). Blue and red lines indicate outside and inside cells, respectively. Time-point of division is set at t = 0 hour. (B) Venus expression, normalized to mCherry signal, in cells of 2610305D13Rik^{Gt}-Venus x R26-H2B-mCherry x mT embryos dividing asymmetrically from 8- to 16-cell (n = 15 divisions in n = 3 embryos) and 16- to 32-cell stage (n = 11 divisions in n = 3 embryos). (C) A pairwise difference of cumulative Venus expression between the descendants of asymmetric divisions (outside cell minus inside cell) at the 16- and 32-cell stages in Tmem50b^{Gt} (blue box plots; expression dynamics depicted in A; two-sided Wilcoxon rank sum test; p (16-cell) = 0.0011; p (32-cell) = 0.0019) and 2610305D13Rik^{Gt} embryos (red box plots; depicted in B). Inset depicts an example taken from Fig 6A in which the measured value is highlighted in grey. (D) Mean rate of change of the normalized Venus intensity from t = 0 to 6 hours after division in outside and inside cells for 16- (n = 32 and n = 16) and 32- (n = 56 and n = 40) cell stage in Tmem50b^{Gt} (left; Wilcoxon rank sum test, p (32-cell) = 2×10^{-5}) and for 16- (n = 33 and n = 15), 32- (n = 53 and n = 41) and 64- (n = 106 and n = 84) cell stages in 2610305D13Rik^{Gt} (right; Wilcoxon rank sum test, p (64-cell) = 0.025) embryos. Blue and red median bars mark outside and inside cells respectively. Statistical significance is indicated as * ($p < 0.05$), ** ($p < 0.001$).

Figure 7

A new model for TE and ICM lineage segregation

Distinct mechanisms for the acquisition of the TE or ICM molecular identity. TE or ICM molecular identity is acquired in a non-binary fashion and at different developmental times. The specific expression of a TE fate marker (blue) is established through asymmetric divisions from 8- to 16-cell and 16- to 32-cell stage, as quantified by our reporter mice. The ICM-specific gene (red) expression is non-reciprocal to the TE marker and only becomes ICM-specific at the late 64-cell stage.

Expanded View Figure Legends

Figure EV1

The maps of the proviral integration sites of the VET mouse lines

Maps showing the genomic structures of the trapped genes (black), the sequence identified by LM- or TAIL-PCR representing the proviral integration site (red) and the sequence identified by 5'-RACE representing the trapped exons (green). The maps were established using Ensembl BLAT/BLASTN search against the mouse (*mus musculus*) DNA databases of the GRCm38.p3 assembly (release 78) with standard settings. LM- and TAIL-PCR sequences were BLAT searched against genomic sequences, 5'RACE sequences were BLASTN and BLAT searched against cDNAs (transcripts/splice variants) or genomic sequences respectively. Gene structures

represent a representative splice variant of the Ensembl/Havana merge transcripts or Havana transcripts.

Figure EV2

Venus expression pattern of the established VET mouse lines

VET lines with non-ubiquitous Venus expression during the pre-implantation stage. Brightfield and confocal live-images of the developing embryos at E1.5 (2- to 4-cell stage), E2.5 (8- to 16-cell stage morula), E3.5 and E4.5 blastocyst derived from (A) $\text{Polg}^{\text{Gt(Venus)VET25A}}$, (B) $\text{Cd2ap}^{\text{Gt(Venus)VET36C}}$, (C) $\text{Itpkc}^{\text{Gt(Venus)VET50A}}$, (D) VET53C line, (E) $\text{Ctnna1}^{\text{Gt(Venus)fVET3A}}$, (F) $\text{Rbfox2}^{\text{Gt(Venus)fVET6C}}$ and (G) $\text{Cers6}^{\text{Gt(Venus)fVET12A}}$. Arrowheads mark the expression positive cells of the presumptive PrE. Scale bars are 50 μm . All fluorescent images are representative single confocal sections from $n \geq 3$ embryos ($n \geq 2$ experiments).

Figure EV3

Venus-trap screen identified a novel gene essential for the pre-implantation development

(A) Fluorescence intensity is correlated with the genotype of VET embryos. (1) $\text{Ctnna1}^{+/+}$ ($n = 6$ embryos), $\text{Ctnna1}^{\text{Gt/+}}$ ($n = 10$ embryos) and $\text{Ctnna1}^{\text{Gt/Gt}}$ embryos ($n = 7$) at E3.5 shown as brightfield images (upper row panels) and for Venus expression (lower panels) ($n = 2$ experiments). (2) Box-plots depicting Venus fluorescence intensity in individual nuclei of $\text{Ctnna1}^{+/+}$ ($n = 5$ nuclei from $n = 1$ embryo), $\text{Ctnna1}^{\text{Gt/+}}$ ($n = 10$ nuclei from $n = 3$ embryos) and $\text{Ctnna1}^{\text{Gt/Gt}}$ ($n = 10$ nuclei from $n = 2$ embryos) embryos ($n = 2$ experiments). Kruskal-Wallis H test $p = 3 \times 10^{-5}$. (3) $\text{Spt6}^{+/+}$ ($n = 11$ embryos), $\text{Spt6}^{\text{Gt/+}}$ ($n = 29$ embryos) and $\text{Spt6}^{\text{Gt/Gt}}$ embryos ($n = 15$) at E3.5 shown as brightfield images (upper panels) and Venus expression (lower panels) ($n = 4$ experiments). (4) Box-plots depicting Venus fluorescence intensity in individual nuclei of $\text{Spt6}^{+/+}$ ($n = 10$ nuclei from $n = 3$ embryos), $\text{Spt6}^{\text{Gt/+}}$ ($n = 10$ nuclei from $n = 2$ embryos) and $\text{Spt6}^{\text{Gt/Gt}}$ ($n = 10$ nuclei from $n = 2$ embryos) embryos. Kruskal-Wallis H test $p = 2 \times 10^{-6}$. (B) Venus-trap insertions disrupt the formation and function of the protein of the trapped gene. (1) Immunostaining of CTNNA1 protein in $\text{Ctnna1}^{+/+}$ ($n = 20$ embryos), $\text{Ctnna1}^{\text{Gt/+}}$ ($n = 17$ embryos) and $\text{Ctnna1}^{\text{Gt/Gt}}$ ($n = 8$ embryos) embryos at the E4.5 blastocyst stage ($n = 4$ experiments). White arrowheads indicate accumulation of the CTNNA1 signal on cell-cell adhesions that was lost in $\text{Ctnna1}^{\text{Gt/Gt}}$ embryos. (2) Box-plots indicating the fluorescence intensity at cell-cell contacts of $\text{Ctnna1}^{+/+}$ ($n = 10$ cell-cell contacts from $n = 2$ embryos), $\text{Ctnna1}^{\text{Gt/+}}$ ($n = 15$ contacts from $n = 3$ embryos) and $\text{Ctnna1}^{\text{Gt/Gt}}$ embryos ($n = 11$ contacts from $n = 3$ embryos). Kruskal-Wallis H test $p = 10^{-5}$. (3) SPT6 distribution in $\text{Spt6}^{+/+}$ ($n = 4$ embryos), $\text{Spt6}^{\text{Gt/+}}$ ($n = 10$ embryos) and $\text{Spt6}^{\text{Gt/Gt}}$ ($n = 2$ embryos) embryos at E3.5 ($n = 1$ experiment). (4) Box-plots indicating the fluorescence intensity in individual nuclei of $\text{Spt6}^{+/+}$ ($n = 4$ nuclei from $n = 1$ embryo), $\text{Spt6}^{\text{Gt/+}}$ ($n = 8$ nuclei from $n = 1$ embryo) and $\text{Spt6}^{\text{Gt/Gt}}$ ($n = 6$ nuclei from $n = 1$ embryo) embryos at E3.5. Kruskal-Wallis H test $p = 0.003$. Venus expression images in Figure EV3A are sum intensity projections and in Figure EV3B are single confocal sections. Venus expression level is shown using a fire look-up table (LUT). Scale bars are 50 μm . Fluorescence intensities in EV3A2, A4, B2 and B4 are normalized to the highest level of fluorescence. Statistical significance is indicated as * ($p < 0.05$), ** ($p < 0.001$).

Figure EV4

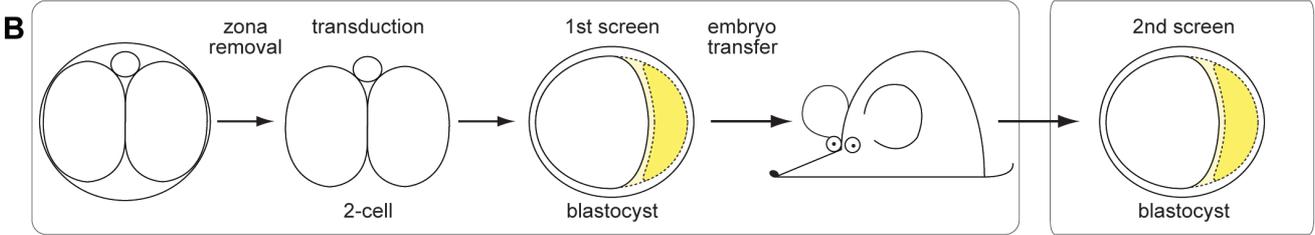
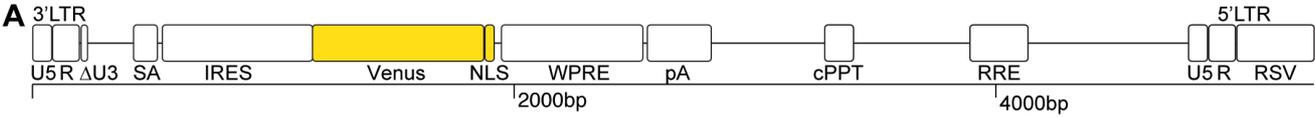
The expression dynamics of lineage markers

(A) A representative track for the expression dynamics of Tmem50b^{Gt}-Venus expression based on live imaging of a Tmem50b^{Gt}-Venus x R26-H2B-mCherry x mT embryo. Note that these data are integrated in the lineage map shown in Fig 5A. (B) A representative track for the expression dynamics of 2610305D13Rik^{Gt} Venus expression based on live imaging of a 2610305D13Rik^{Gt}-Venus x R26-H2B-mCherry x mT embryo. Note that these data are integrated in the lineage map shown in Fig 5B. Blue and red lines indicate outside and inside cells, respectively. Venus expression is normalized to mCherry signal.

Figure EV5

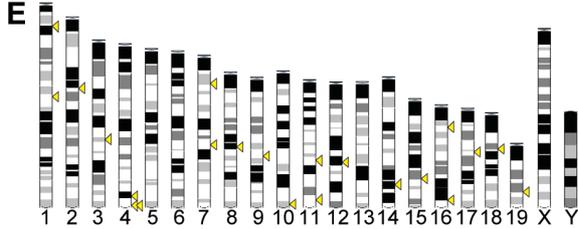
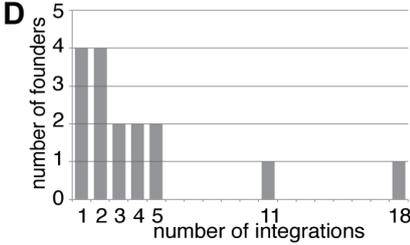
Establishment of an enhanced lineage map of mouse pre-implantation development

Lineage maps are generated by tracking all nuclei of an embryo over several divisions. Binary cellular position, that is inside (red) or outside (blue) position, as judged by the membrane contacts, and relative nuclear position, scaled by blue-white-red-gradient, were combined with fluorescence intensity of the VET-reporter measured in nuclei and incorporated into the lineage map.

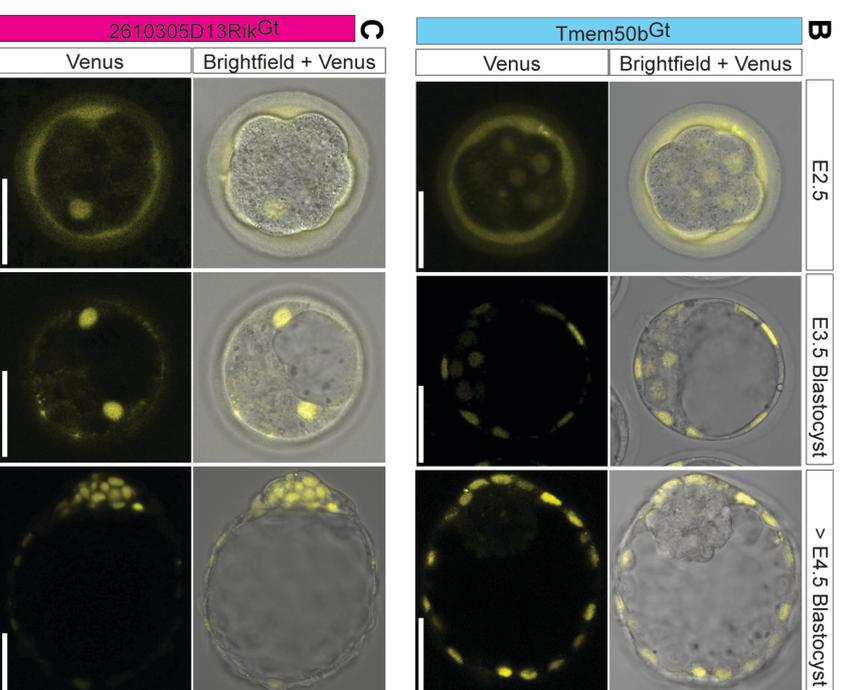
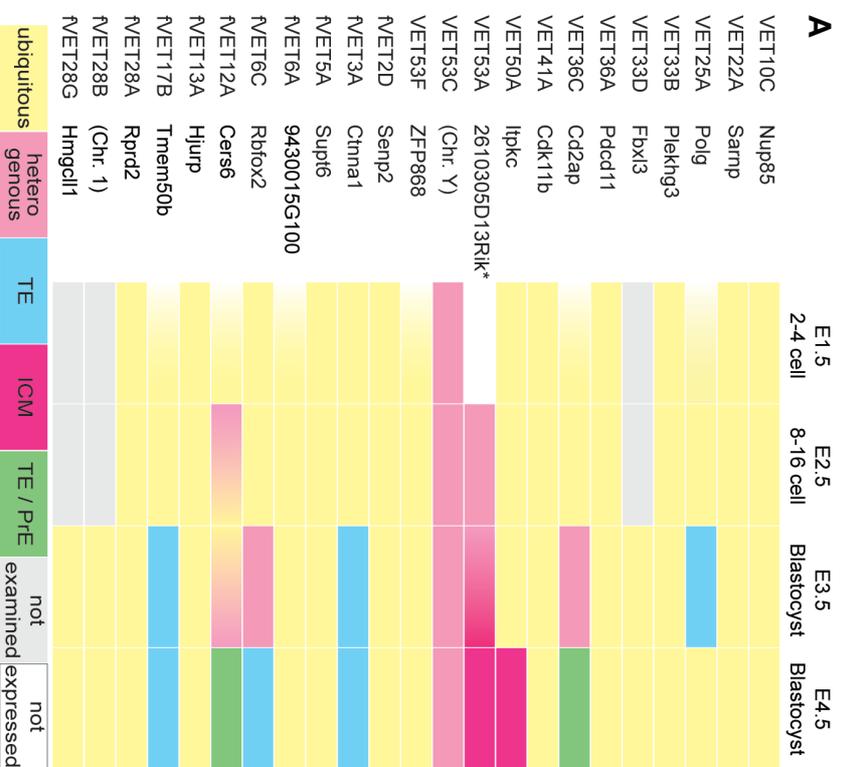


C

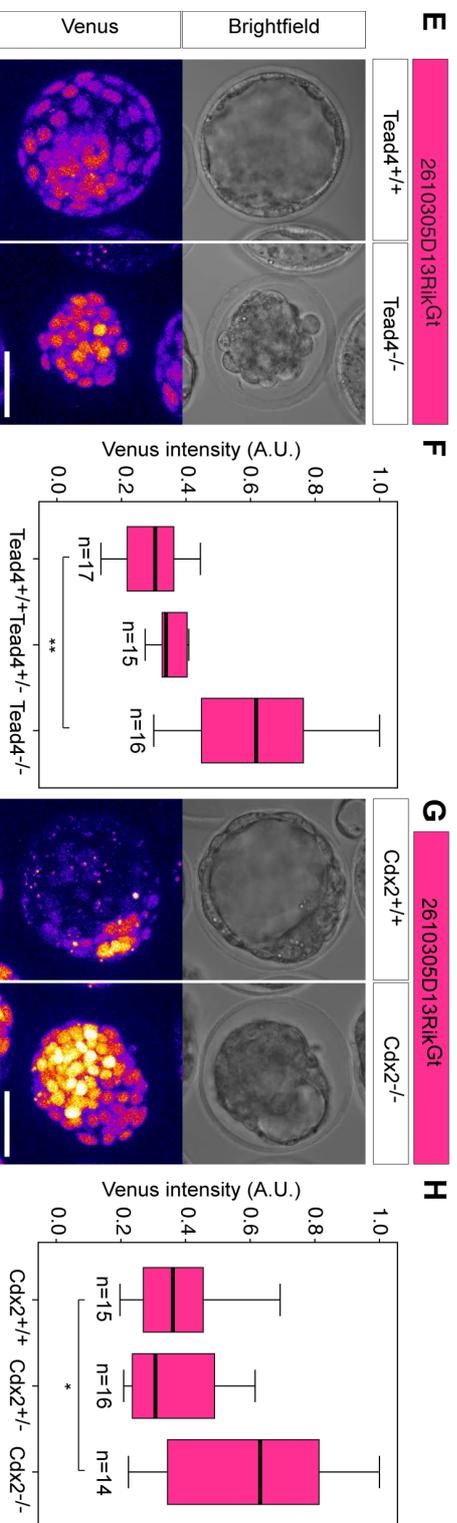
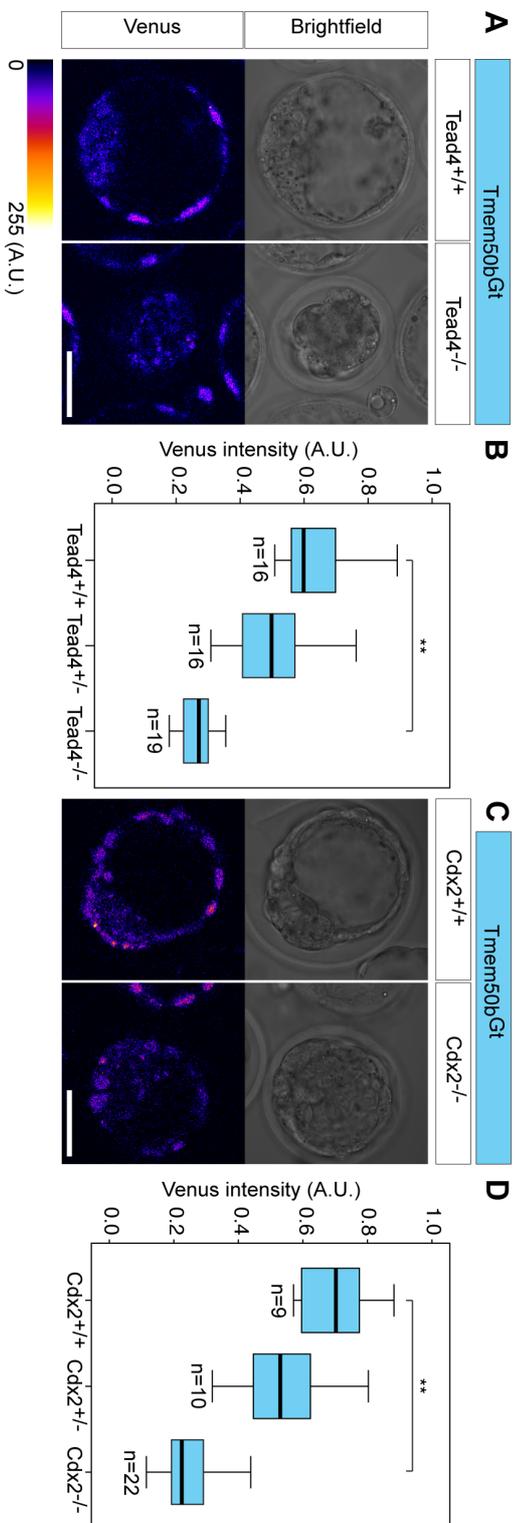
number of embryos	transduced	2121
	1st screen positive	462
number of animals	born	107
	2nd screen positive	16
number of integrations	in positive founders	65
	expressed in pre-implantation stage	23



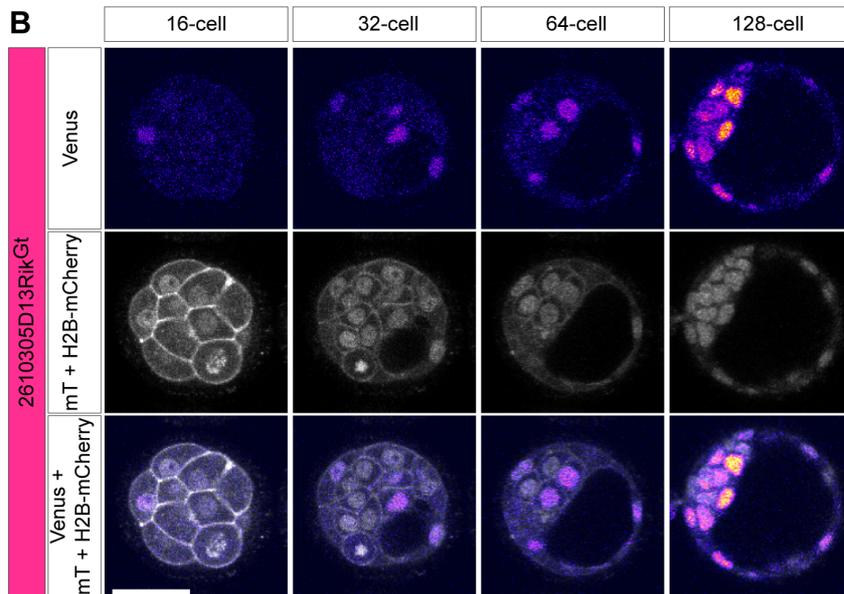
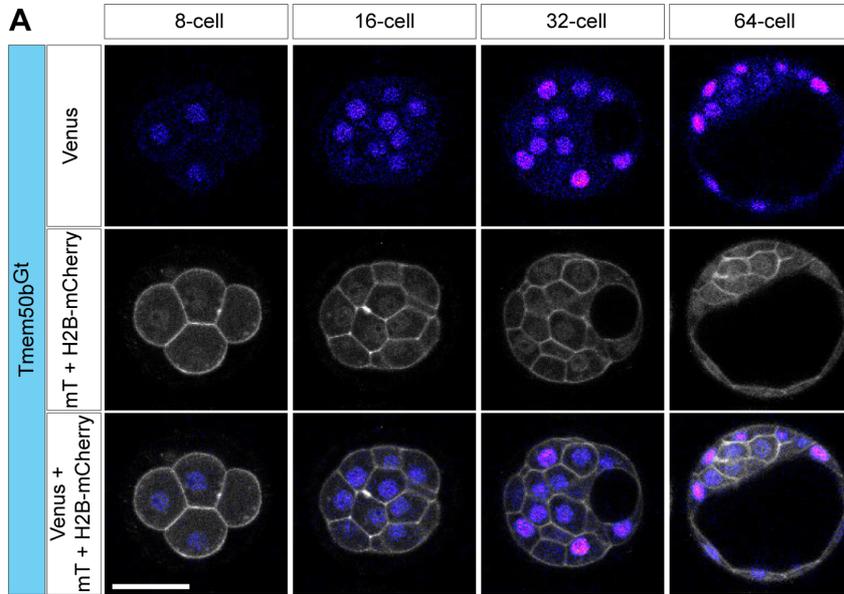
Dietrich_Fig2



Dietrich_Fig3



Dietrich_Fig4



Dietrich_Figs

A Trem50bGt

B 2610305D13RikGt

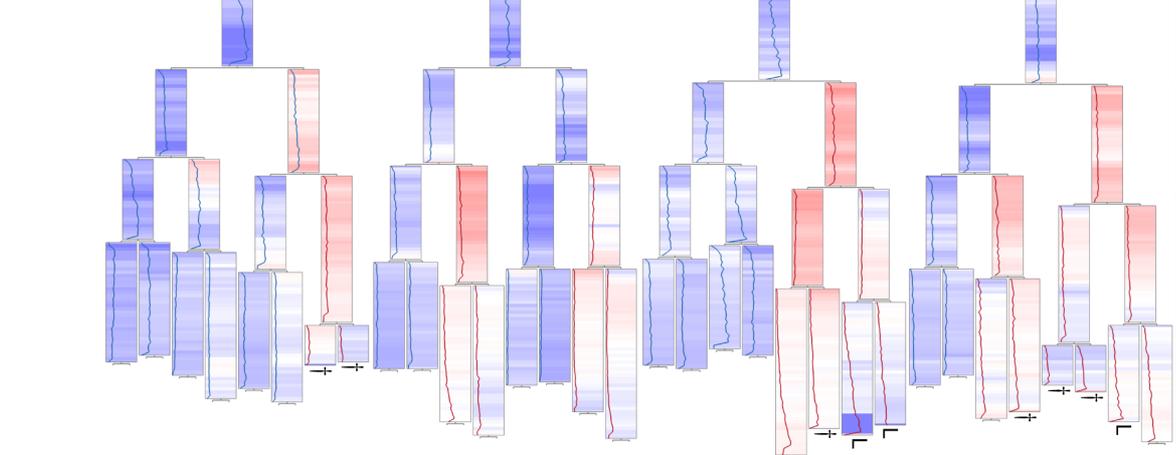
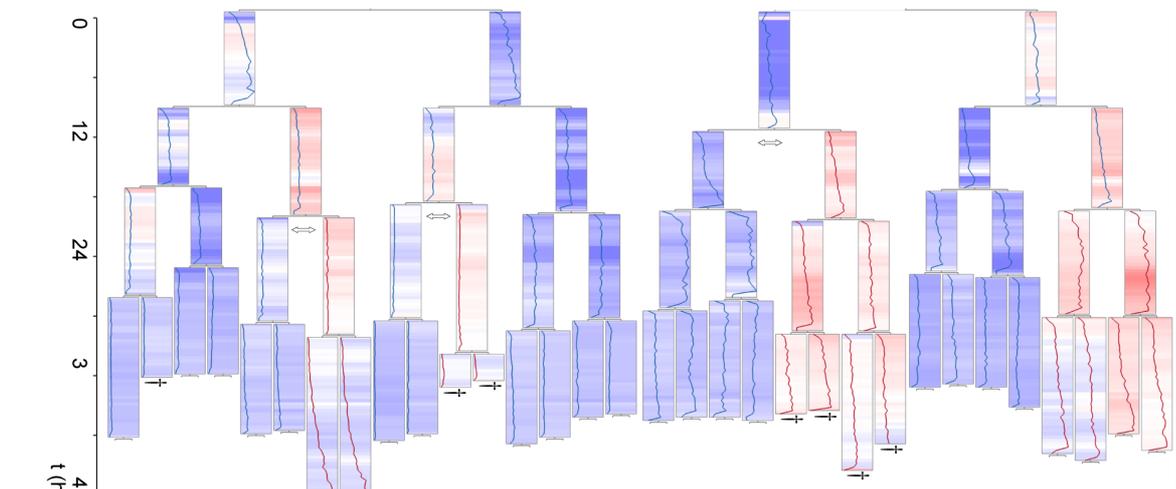
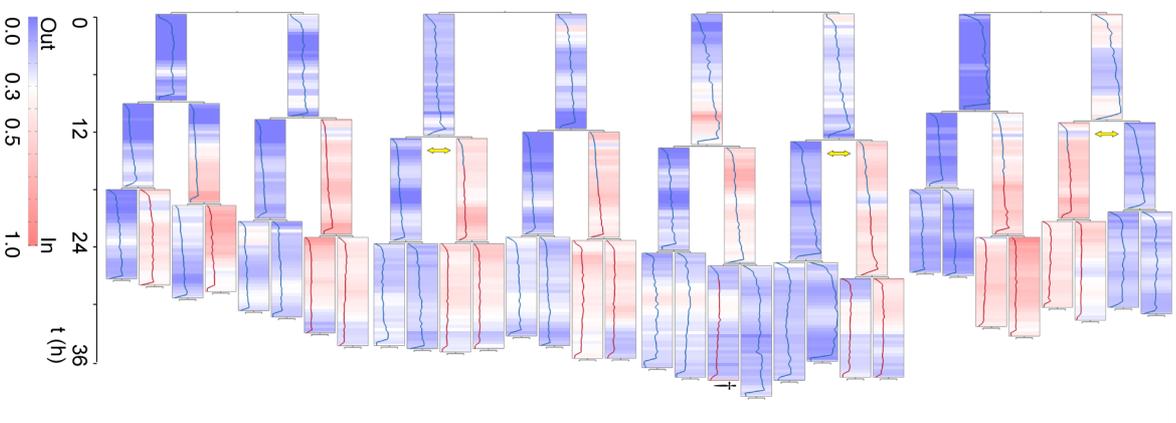
8- 16- 32- 64-cell

8- 16- 32-

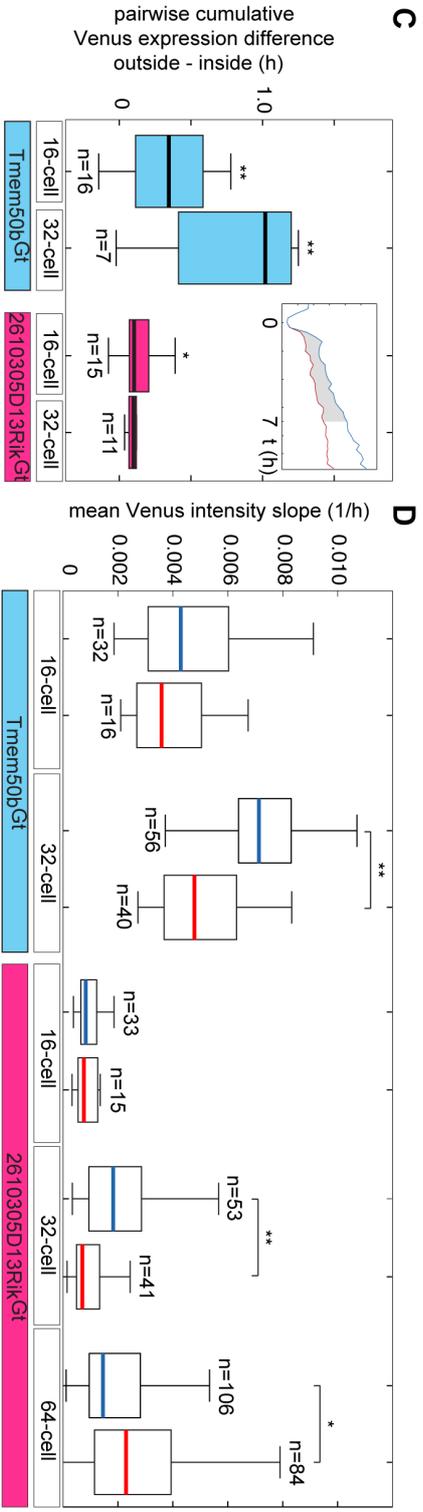
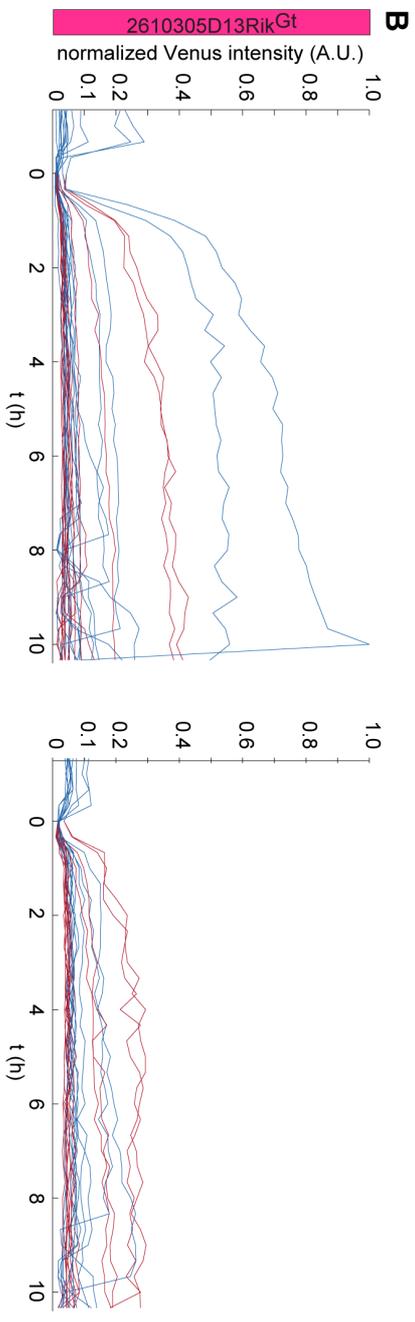
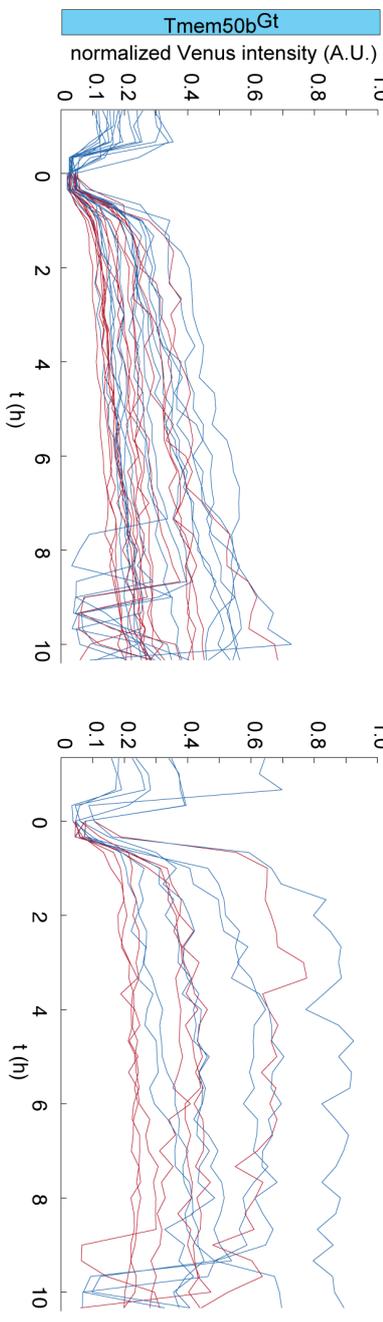
8- 16- 32-

8- 16- 32-

64-cell



A 8 - 16-cell 16 - 32-cell **Dietrich_Fig6**



Dietrich_Fig7

