# Signalling-modulated gene regulatory networks in early mammalian development ${ }^{\text {d }}$ 

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#### Abstract

Early mammalian embryo is a paradigm of dynamic, self-organised process. It involves gene expression, cell division and intercellular signalling. How these processes interact to ensure reproducible development is being often investigated by modelling, which allows to dissect the mechanisms controlling cell fate decisions. In this work, we present two models based on ordinary differential equations describing the first and second specification processes in the mouse embryo. Together, they describe the cell fate decisions leading to the first three cell lineages which form the blastocyst 4.5 days after fertilisation: the trophectoderm, the epiblast and the primitive endoderm. Both specifications rely on multistability, and signalling allows the selection of the appropriate steady-state. In addition to the gene regulatory network, the first specification process is indeed controlled by the Hippo pathway, which is itself controlled by cell polarity and cell-to-cell contacts. This leads to a spatially organised arrangement of cells. The second specification process is controlled by Fgf signalling and leads to a salt and pepper distribution of the two cell types. We discuss the respective mechanisms and their physiological implications.


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## 1. Introduction: cell fate specification as a choice between multiple coexisting stable steady states

Embryogenesis is a dynamic process that involves gene expression, cell division, signalling and mechanical forces. Before implantation in the uterus at embryonic day E4.5, the early mouse embryo undergoes a series of divisions and differentiation processes leading to a blastocyst composed of $\sim 128$ cells of three different types. These three cell types are the trophectoderm (TE), the epiblast (Epi) and the primitive endoderm ( PrE ). Epi cells are pluripotent and will give rise to the embryo itself, while $\operatorname{PrE}$ and TE cells form extra-embryonic structures such as the placenta (Lanner et al., 2014; Rivera-Pérez et al., 2014; Frum et al., 2015; Chazaud et al., 2016).

In mammals, blastocyst formation is a self-organized process, which takes place independently of the maternal environment (Deglincerti et al., 2016). Theoretical approaches have early emphasized that cell specification is best described as the evolution towards one of the multiple stable steady states of the gene regulatory network (GRN) that describes the interactions between specific transcription factors (Huang et al., 2005, 2007; Moris et al., 2016). Thus, theoretical studies of regulatory circuits of the GRN

[^0]are frequently used to back up experimental investigations in the field of embryonic development.

GRN are modulated by signalling and the formation of tissues and organs during the embryonic period is governed by the orchestrated actions of multiple signalling pathways. In the mouse, the first cell fate decision is sensitive to Hippo signalling, which allows the transduction of the positional cues into the nucleus (Bedzhov et al., 2014; Sasaki, 2015, 2017). In response to signalling, polarized outer cells will become TE while apolar, inner cells will adopt the ICM (inner cell mass) fate. Importantly, both the polarity and the level of compaction -which is higher for inner than for outer cells - can be perceived by the cell and can synergistically modulate Hippo signalling. In the second differentiation step, the ICM cells specify into Epi or PrE cells depending on their level of Fgf/Erk signalling. The specification of PrE requires activation of Fgf/Erk signalling between E2.5 and E4.5. In contrast, a low level of Fgf/Erk signalling largely favours the appearance of Epi cells (Artus and Chazaud, 2014; Chazaud et al., 2016). While positional cues regulate Hippo signalling, and thus lead to a spatially organized repartition of the TE and ICM cells in the embryo, Fgf secretion is modulated by the GRN itself. As a result, Epi and PrE cells are arranged in a typical salt and pepper pattern after specification. Later, they are sorted into their correct positions by active cell migration and selective apoptosis.

The core GRN driving the first two specification steps involves both mutual inhibition and auto-activation (Niwa et al., 2005). For
the TE/ICM fate choice, the Cdx2 transcription factor that promotes the TE cell fate inhibits the expression of Oct3/4 (simply referred to as Oct4 here below), which regulates the ICM cell fate. Cdx2 and Oct4 amplify their own expression. By a combination of modelling and experimental approaches, Holmes et al. (2017) have shown that this system can exhibit bistability, with high Cdx2 and low Oct4 expression corresponding to the TE state, and high Oct4 and low Cdx2 expression corresponding to the ICM state. The authors, who did not consider Hippo signalling in the model, predict that noise plays an important role in the maintenance of cellular plasticity. Other models for the TE/ICM specification similarly considered the Cdx2/Oct4 GRN and spatial interactions with a static representation of signalling (Chickarmane and Peterson, 2008; Krupinski et al., 2011). In this work (Section 2), we extended the core model proposed by Holmes et al. (2017) to take Hippo signalling into account. We investigated how polarity and inter-cellular adhesion dynamically regulate cell fate choice. We formalized the hypothesis that both cues converge at the level of Hippo signalling and investigated their respective impact on the existence of bistability and on the dynamics of specification into the TE or ICM states. On the other hand, as the two positional cues converge at the level of Hippo signalling, TE/ICM specification could in principle be the result of a switch occurring at a given value of Hippo signalling. We took advantage of the model to investigate the possible physiological advantage of bistability -whose existence in the embryo has been highlighted by Holmes et al. (2017) - with respect to a simple switch-like mechanism.

In the second specification step during which ICM cells differentiate, Nanog and Gata6 are the characteristic transcription factors of the Epi and the PrE state, respectively. As in the previous step, the two factors cross-inhibit and self-amplify. Moreover, Fgf secretion is under the control of Nanog and Gata6, and Fgf stimulates the expression of Gata6. In previous studies (Bessonnard et al., 2014; De Mot et al., 2016; Tosenberger et al., 2017), we have shown that this Fgf-modulated GRN can display tristability and that specification of ICM cells into Epi and PrE cells arranged in the salt and pepper pattern is best described by the self-modulated evolution of the cell population towards one of the three stable steady states. In Section 3, we review the main features of these computational approaches and compare this specification mechanism to the regulatory processes related to the TE/ICM cell fate choice.

## 2. Hippo signalling controls the bistable Cdx2/Oct4 gene regulatory network driving the $\mathrm{TE} / \mathrm{ICM}$ cell fate specification

### 2.1. Model

ICM and TE cells are characterized by the expression of the Oct4 and Cdx 2 transcription factors, respectively. Based on the observation that both transcription factors amplify their own expression but inhibit each other, Holmes et al. (2017) have proposed the following equations to describe the Oct4/Cdx2 transcriptional system and have shown its good agreement with experimental observations:
$\frac{1}{\mu} \frac{d C d x 2}{d t}=k\left(S^{\prime}+\frac{C d x 2^{n}}{C d x 2^{n}+K^{n}}\right)\left[(1-I)+I \cdot \frac{K^{n}}{O c t 4^{n}+K^{n}}\right]-C d x 2$
$\frac{1}{\mu} \frac{d O c t 4}{d t}=k\left(b+\frac{O c t 4^{n}}{O c t 4^{n}+K^{n}}\right)\left[(1-I)+I \cdot \frac{K^{n}}{C d x 2^{n}+K^{n}}\right]-O c t 4$
Parameter I allows the tuning of the extent of cross-inhibition, a parameter that was investigated in the original study. Here, its value is fixed at 0.6 as it was found by Holmes et al. (2017) to give
best agreement with experimental data. Parameters S' and b are basal rates of expression of Cdx2 and Oct4, respectively. Parameter $\mu$, which is added in the present study, allows the modulation of the respective time scales of the GRN and of signalling (see below).

In the early embryo, Hippo signalling controls the level of Cdx2 expression (see Fig. 1B and Biggins et al., 2015; Sasaki, 2015, 2017 for reviews). More specifically, in the absence of signalling, Yap and its homolog Taz (shortened as just Yap from here on) can enter the nucleus and activate, together with TEAD4, the transcription of Cdx2 (Nishioka et al., 2009; Home et al., 2012). When Hippo signalling is active, the Lats $1 / 2$ kinases get phosphorylated. In this active state, Lats1/2 phosphorylate Yap. Phosphorylated Yap cannot translocate into the nucleus and is degraded in the cytosol. Thus, Cdx2 expression is reduced, which in turn allows the expression of Oct4 (Hirate et al., 2012). To take Yap into account in the model, we write S' appearing in Eq. (1) as
$S^{\prime}=S+\frac{Y a p}{Y a p+K_{a}}$
For simplicity, to describe the evolution of Yap, we consider that phosphorylated Lats activates Yap degradation. Thus:
$\frac{d Y a p}{d t}=v_{3}-k_{4} \cdot Y a p-v_{4} \cdot L a t s P \cdot \frac{Y a p}{K_{4}+Y a p}$
LatsP stands for the fraction of Lats1/2 that is phosphorylated. To further simplify the above equations, we neglect the compartmentalisation: the translocation of Yap into the nucleus is not explicitly modelled.

The Amot protein makes the link between Lats1/2 activity on one hand and positional cues on the other hand (Hirate et al., 2013). In newly derived inner cells, Amot is present throughout the entire plasma membrane, while in outer cells it remains mainly restricted to the apical domain. More precisely, when bound to the cell adhesion molecules E-cadherins, Amot becomes active. In polar cells, Amot is trapped by the Par-aPKC complex in the apical membrane and thus cannot be activated (Fig. 1A). In the following equations, Ecadh and aPKC are two parameters representing the amount of E-cadherins and Par-aPKC in a blastomere, respectively. An inner cell corresponds to a large value of Ecadh and ParaPKC $=0$. An outer cell is characterised by a low value of Ecadh and a high value of Par-aPKC. In the equations, we assume that once bound to Ecadh, Amot is immediately phosphorylated and thus write:
$\frac{d A m o t P}{d t}=k_{a 1} \cdot$ Amot $\cdot$ Ecadh $-k_{d 1} \cdot$ AmotP
Phosphorylated Amot phosphorylates Lats $1 / 2$, yielding the following equation for the fraction of phosphorylated Lats (LatsP):
$\frac{d \text { Lats } P}{d t}=v_{1} \cdot \operatorname{Amot} P \cdot \frac{\text { Lats }}{K_{1}+\text { Lats }}-v_{2} \cdot \frac{\text { Lats } P}{K_{2}+\text { Lats } P}$
with Lats $=1-$ LatsP.
The fraction of Amot trapped at the apical membranes, written AmotM, evolves following the equation:
$\frac{d A m o t M}{d t}=k_{a 2} \cdot$ Amot $\cdot a P K C-k_{d 2} \cdot A m o t M$
with the fraction of free Amot: $A m o t=1-\operatorname{Amot} P-A m o t M$.
Eqs. (1)-(7), with the parameter values given in Table 1, formalize our model describing ICM/TE specification.

### 2.2. Results: TE/ICM specification upon developmental changes in cellular adhesion and polarity

We first studied the steady-state behaviour of the model describing ICM/TE specification defined by Eqs. (1)-(7). Bifurcation diagrams are shown in Fig. 2. Fig. 2A shows the levels of expression


Fig. 1. Angiomotin (Amot) positioning regulates Hippo signalling.
(A) Schematic view of Amot position in inner and outer cells. Amot (green) is sequestered at the apical domain (blue) in polar outer cells. In apolar inner cells, Amot is found all over the cell membrane, where it interacts with E-Cadherin (red) through a multi-protein complex including Nf2 and catenins
(B) The Hippo signalling pathway and its interaction with the Cdx2/Oct4 switch. E-Cadherin and Par-aPKC compete for Amot. The binding of Amot to E-Cadherin triggers the successive phosphorylation of Amot, Lats1/2, and YAP. Non-phosphorylated YAP (together with TEAD4 that is not represented for clarity) activates the transcription of Cdx2, while phosphorylated YAP is excluded from the nucleus and degraded. In the text and in the equations, AmotP refers to the concentration of the complex between E-cadherin and phosphorylated Amot, while AmotM stands for the concentration of the complex between Amot and Par-aPKC.

Table 1.
List of parameter values of the TE/ICM specification model defined by Eqs. (1)-(7). Units are arbitrary.

| Core |  |  |  | Cdx2/Oct4 | model (Holmes et al., 2017) |
| :--- | :--- | :--- | :---: | :---: | :---: |
| $S$ | 0.15 | In original model: "transcription bias" on Cdx2, representing position/polarity. Here, basal transcription rate of Cdx2 |  |  |  |
| $B$ | 0.7 | Basal transcription rate of Oct4 |  |  |  |
| $I$ | 0.6 | Cdx2/Oct4 reciprocal inhibition strength |  |  |  |
| $k$ | 0.7 | Transcription rate |  |  |  |
| $K$ | 0.5 | Threshold constant of auto-activation and cross-inhibition |  |  |  |
| $n$ | 4 | Hill coefficient of auto-activation and cross-inhibition |  |  |  |
| Hippo pathway model |  |  |  |  |  |
| $k_{a 1}$ | 1 | Amot/E-Cadherin association kinetic constant |  |  |  |
| $k_{d 1}$ | 0.05 | Amot/E-Cadherin dissociation kinetic constant |  |  |  |
| $k_{a 2}$ | 1 | Amot/Par-APKC association kinetic constant |  |  |  |
| $k_{d 2}$ | 0.05 | Amot/Par-APKC dissociation kinetic constant |  |  |  |
| $v_{1}$ | 1 | Lats phosphorylation rate |  |  |  |
| $v_{2}$ | 1 | Lats-P dephosphorylation rate |  |  |  |
| $K_{1}$ | 0.05 | Michaelis-Menten constant for Lats phosphorylation |  |  |  |
| $K_{2}$ | 0.5 | Michaelis-Menten constant for Lats-P dephosphorylation |  |  |  |
| $v_{3}$ | 0.4 | Yap production rate |  |  |  |
| $k_{4}$ | 0.1 | Kinetic constant of basal degradation of Yap |  |  |  |
| $v_{4}$ | 2 | Yap phosphorylation rate |  |  |  |
| $K_{4}$ | 0.1 | Michaelis-Menten constant for YAP phosphorylation/degradation |  |  |  |
| $K_{a}$ | 0.3 | Half-maximal concentration of Yap for Cdx2 induction |  |  |  |
| $\mu$ | 0.05 | Scaling factor (applied to the Holmes model) |  |  |  |

of Cdx2 and Oct4 as a function of the value of Par-aPKC, which reflects the polarity of the cell (Plusa et al., 2005). The value of Ecadh is set to 0.5 , which corresponds to an intermediate level of cellular adhesion. For low values of Par-aPKC, ECadh > Par-aPKC and the stationary state corresponds to a state where Hippo signalling is active, because of the high level of phosphorylated Amot that in turn activates Yap phosphorylation by Lats. In these conditions, Cdx2 expression is not stimulated, which allows the expression of Oct4 and the establishment of the ICM state. In contrast, when ParaPKC \gg ECadh (i.e., for a polar cell), Amot remains bound to the Par-aPKC system, Hippo is not activated and a high Cdx2 TE fate is established. These situations are observed for extreme values of Par-aPKC and ECadh. For intermediate values of these parameters, two stable steady states characterized by a high level of Cdx2 and a low level of Oct4 (TE) or the opposite (ICM) can coexist. In
this domain, the steady-state that is reached depends on the initial conditions and on the path followed by the cell in the (Ecadh, Par-aPKC) plane. As seen in the 2-parameter bifurcation diagram shown in Fig. 2B, the domain of bistability is delimited by straight lines as there is a linear relation between the values of Par-aPKC and ECadh at the saddle node bifurcation points (see Appendix). The two lines intersect on the $Y$ axis at $-k_{d 2} / k_{a 2}$. Thus, for very low values of Ecadh and Par-aPKC, i.e., if there is no compaction, cells will always evolve towards a TE-like state.

We next studied the dynamics of cell fate specification (Fig. 3). During early development, the compaction occurring at the 8-cell stage corresponds to an increase in intercellular adhesion, which is modelled as an increase in the value of ECadh. Upon compaction, each blastomere becomes polarized along the apicobasal axis. In the model, this corresponds to an increase in Par-aPKC. After di-


Fig. 2. Bifurcation analysis of the model of TE/ICM specification.
(A) Bifurcation diagram of Oct4 and Cdx2 as a function of Par-aPKC activity. Results have been obtained with Eqs. (1)-(7) and the values of parameters listed in Table 1 (and Ecadh $=0.5$ ), using XPPAUT (Ermentrout, 2002). Dashed lines represent instability while solid lines represent stability.
(B) Two-parameter bifurcation diagram showing the bistable zone as a function of Par-aPKC and E-Cadherin activities. Results have been obtained as in (A).


Fig. 3. Simulations of the specification of cells towards the ICM or the TE fate during compaction and division.
(A, D) Temporal evolution of Par-aPKC and E-Cadherin. Both initially rise during compaction. (A) After division, Par-aPKC falls and E-Cadherin rises in the apolar daughter cell. (D) Par-aPKC rises and E-Cadherin falls slightly in the polar daughter cell.
(B, E) Trajectory of the system in parameter space (purple), with the boundaries of the bistable zone shown in black.
(C, F) Temporal evolution of Cdx2 and Oct4. During compaction, the system initially goes towards a high Cdx2, moderate Oct4 state. (C) The apolar daughter cell switches to ICM fate (low Cdx2). (F) The polar daughter cell further develops towards TE state (high Cdx2).
vision towards the 16 -cell stage, some blastomeres occupy inner positions and thus lose polarity (decrease in Par-aPKC), while the second wave of compaction further increases the adhesion of inner cells. The time evolution of ECadh and Par-aPKC are shown in Fig. 3A and D. Fig. 3A corresponds to a cell that will move to and eventually stay at an inner position. Fig. 3D corresponds to a cell that will move to and eventually stay at an outer position. Time units are arbitrary, but we considered that transitions (corresponding to cell divisions) only last for a small fraction of the time
interval between the $8-16$ and the $16-32$ cell divisions. The corresponding trajectories in the (Ecadh, Par-aPKC) plane are shown in Fig. 3B and E. Initially, due to the increase in E-Cadh and ParaPKC, both Cdx2 and Oct4 increase, although the increase of Cdx2 is larger than that of Oct4. Hippo signalling is indeed only slightly activated as a significant fraction of Amot (AmotM $\approx 0.45$ ) remains bound to Par-aPKC. From this state on, in the case of an asymmetric division (at $t=1000$ ), one daughter cell will lose its polarity and increase its level of adhesion (inner cell, upper row of Fig. 3), while


Fig. 4. Behaviour of a modified model with a very small bistable region.
(A) Parameter space trajectory (purple) with the boundaries of the bistable domain shown in plain black for the original version, dashed black for the modified version. (B) Temporal evolution of Cdx2 (blue) and Oct4 (red) levels, shown in plain lines for the original version and dashed lines for the modified version. An unrealistic cell fate switch is observed in the case of the modified version that has a very small region of bistability.
the other one will become more polarized and decrease its level of adhesion (outer cell, lower row of Fig. 3). In the former case, the cell ends up in the region where only the ICM state is stable, while in the latter case, only the TE state is available.

In the simulations shown in Fig. 3, we assumed that the final, specified states of the cells belong to a region of monostability. We indeed reasoned that the cell should not be able to change fate in response to internal fluctuations that could be rather large in the developing embryo. The question thus arises as to the possible physiological advantage for the cell to transit through a domain of bistability before fixing its fate. To answer this question, we took advantage of the fact that by changing parameter $I$ appearing in Eqs. (1) and (2), one can modulate the size of the domain of bistability. This parameter measures the extent of cross-inhibition between Cdx2 and Oct4. If $I=0.45$ instead of 0.6 as used before, the domain of bistability is much reduced (Fig. 4A). Then we compared the evolution of the expression levels of Cdx 2 and Oct4 for the two values of $I$. As shown in Fig. 4B, when the region of bistability is small, Cdx2 is transiently repressed and Oct4 transiently expressed in a cell headed towards the TE state. This situation, which would correspond to a fate switch, does not comply with experimental observations. Moreover, if determination processes were initiated during this transient period, they could lead to errors in fate determination. Thus, by allowing the two cell fates to be stable for overlapping domains of values of parameters, bistability provides robustness in the dynamics of cell fate specification.

The results shown in Fig. 4 show that bistability provides robustness with respect to the external noise due to the random variations in the positions of the cells in the embryo and in the levels of compactness. One might wonder if bistability also enhances the robustness of cell fate specification in presence of internal noise, i.e., molecular fluctuations. To investigate this question, a stochastic version of the model was developed using the Gillespie's algorithm Gillespie, 1977). The stochastic version of the model is given in Table S1. Simulations were performed in the same conditions as in Fig. 3. Time series are shown in Fig. 5A-D. We observe a higher variability in the levels of Cdx 2 and Oct4 when the domain of bistability is much reduced ( $I=0.45$ in Eqs. (1) and (2), see Fig. 4B). Fig. 5E shows the (Cdx2,Oct4) values every time unit for 18,000 time units once specification has occurred. While the sets of points corresponding to the two fates are clearly separated in
the bistable case, they nearly overlap when the region of bistability is very small ( $I=0.45$ in Eqs. (1) and (2), see Fig. 4B). Altogether, results shown in Figs. 4 and 5 suggest that bistability in ICM/TE specification provides robustness with respect to noise, originating from internal or external sources.

## 3. Fgf-Erk signalling controls cell fate specification in the tristable Nanog-Gata6 gene regulatory network

Following specification of TE, the ICM must differentiate into Epi and PrE. From the early blastocyst stage ( $\sim$ E3.0), Nanog and Gata6 are increasingly co-expressed in pluripotent Oct4-expressing ICM cells (Guo et al., 2010; Frankenberg et al., 2011). However, Nanog and Gata6 expression progressively become mutually exclusive, leading to the salt and pepper pattern of expression in the inner part of the embryo. Signalling also plays a crucial role here, as Fgf/Erk activity can balance cell identity toward the PrE (high Erk) or the Epi (low Erk) fate. This process is self-regulated, as Fgf4 secretion itself is under the regulation of Nanog and Gata6.

In this section, we review our previously proposed model of Epi/PrE specification, emphasizing the similarities and dissimilarities with the model of TE/ICM specification proposed here above.

### 3.1. Model

The model of the GRN driving Epi/PrE specification is schematized in Fig. 6A and has been described in details in previous publications (Bessonnard et al., 2014; De Mot et al., 2016; Tosenberger et al., 2017). The Nanog (N) and Gata6 (G) cross-inhibition and selfactivation lie at the core of this network. The evolution of their concentrations is described by the following equations:

$$
\begin{align*}
\frac{1}{f(t)} \frac{d G}{d t}= & {\left[v s g 1 \cdot \frac{E r k^{r}}{K a g^{r}+E r k^{r}}+v s g 2 \cdot \frac{G^{s}}{K a g 2^{s}+G^{s}}\right] } \\
& \times \frac{K i g^{q}}{\operatorname{Kig}^{q}+N^{q}}-k d g \cdot G \tag{8}
\end{align*}
$$

$$
\begin{align*}
\frac{1}{f(t)} \frac{d N}{d t}= & {\left[v \operatorname{sn} 1 \cdot \frac{\operatorname{Kin} 1^{u}}{\operatorname{Kin}^{u}+E r k^{u}}+v \operatorname{sn} 2 \cdot \frac{N^{v}}{\operatorname{Kan}^{v}+N^{v}}\right] } \\
& \times \frac{\operatorname{Kin2}^{w}}{\operatorname{Kin}^{w}+G^{w}}-k d n \cdot N \tag{9}
\end{align*}
$$



Fig. 5. Impact of bistability on the robustness of the ICM and TE cell fates in presence of molecular fluctuations.
(A)-(D) Time series of the stochastic simulations corresponding to the panels shown in Fig. 3A and D when the domain of bistability is large ( $I=0.6$ ) or very limited ( $I=0.45$ ). (E) Phase space view of the Cdx2/Oct4 pair values obtained in the 4 Gillespie simulations shown in panels (A)-(D). For each simulation, one point every time unit between 2000 and 20,000 time units is plotted. The deterministic attractors are shown in white, with plus signs for $I=0.6$ and crosses for $I=0.45$. In the bistable case, the two attractors are clearly separated while they almost overlap when the domain of bistability is very small. Details of the Gillespie's simulations are given in Table S1. $\Omega=50$.
in which Erk represents the level of active Erk signalling. In contrast to what happens for the Cdx2/Oct4 network described by Eqs. (1) and (2), the regulation of this network by signalling is symmetrical: Fgf/Erk indeed activates Gata6 expression and inhibits that of Nanog. As another difference, cross-inhibition is here supposed to be a stronger regulation as it counteracts the two terms representing gene expression. As for Eqs. (1) and (2), the timing of the GRN is scaled. Here, this scaling factor $f(t)$ is a function of time to account for a progressive expression of the transcription factors, corresponding to their increasing rates of expression from the 8 -cell stage (Schrode et al., 2014). As expected, this network can also dis-
play bistability. However, the GRN contains more regulations. As Gata6 activates the expression of Fgf receptors and as the resulting increase in Erk signalling stimulates the expression of Gata6, it provides an additional positive circuit. In a symmetric manner, Nanog inhibits Erk signalling, which itself inhibits Nanog expression, also providing a positive circuit. The full model encompassing these additional regulations contains three additional evolution equations, for the concentration of Fgf receptors, for the level of Fgf/Erk signalling (Erk) and for the amount of secreted Fgf4 (see Fig. 1 of Tosenberger et al., 2017 for the equations). This model can display tristability for a range of intermediate concentrations of ex-


Fig. 6. Model for the specification of cells of the inner cell mass into cells of the epiblast or the primitive endoderm.
(A) Schematic representation of the GRN model of Epi/PrE specification (Tosenberger et al., 2017).
(B) Bifurcation diagram of the GRN model (Bessonnard et al., 2014; De Mot et al., 2016; Tosenberger et al., 2017) for Fgf4/Nanog and Fgf4/Gata6, showing the tristability of the system for intermediate concentrations of extracellular Fgf4. Dashed lines represent instability while solid lines represent stability.
(C), (D) Cell specification towards the Epi or PrE state in the simplified 2 cell model. Cells 1 and 2 direct themselves towards the ICM-like state, before cell 1 is finally attracted by the Epi-like state, and thus increases its rate of Fgf4 secretion. In consequence, cell 2 goes to the PrE-like state and stops producing Fgf4. Results have been obtained by numerical integration of the Eqs. (1)-(5) given in Fig. 1 of Tosenberger et al. (2017) with the same values of parameters, except for $f(t)=1$.
ternal Fgf4 (Fig. 6B). The three steady states correspond to the Epi (high Nanog, low PrE), PrE (high Gata6, low Nanog) and ICM (intermediate levels of Nanog and Gata6) states, which are all three observed in the developing embryo.

### 3.2. Results: Epi/PrE specification due to self-modulated variations in the local Fgf4 environment of the ICM cells

Most of the Fgf4 present in the extracellular medium is secreted by the blastocyst itself. Secretion of this growth factor is indeed under the dual control of Nanog and Gata6. We have shown previously that the main contribution to this regulation is most probably the inhibition of Fgf4 secretion by Gata6 (Tosenberger et al., 2017). The contribution of Fgf4 secretion to the dynamics of cell specification is best apprehended by the study of a 2-cell system. In this case, the two cells are described by the same GRN and secrete Fgf4 at a rate that depends on their levels of Gata6. The concentration of Fgf4 in the extracellular medium ([Fgf4] $]_{\text {extra }}$ ) is defined as the average between the amounts of Fgf4 secreted by the 2 cells. To introduce some heterogeneity between the 2 cells, it is assumed that one cell perceives [Fgf4] extra $(1+\gamma)$ and the other
[Fgf4] $]_{\text {extra }}(1-\gamma)$, where $\gamma$ represents a small, random perturbation (3\%). Fig. 6 (C and D) illustrates the results of such a simulation: from very low values of Nanog and Gata6, both cells will first evolve towards the ICM state, which is stable because $[\mathrm{Fgf4} 4]_{\text {extra }}$ is relatively high as none of the cells express Gata6. Then, as a result of the Gata6 increase, [Fgf4] extra starts decreasing. When reaching the left limit point of the branch of the ICM state (see Fig. 6B), the cell that perceives a bit less external Fgf4 will jump to the Epi state. As in this state, it does not express Gata6 anymore, it will secrete more Fgf4. In consequence, [Fgf4] extra will increase again, which will finally induce the other cell to pass the right limit point of the branch of the ICM state and jump to the branch of the PrE state. As both the Epi and PrE branches are stable on extended ranges of $[\mathrm{Fgf4}]_{\text {extra }}$, further variations of external Fgf4 will not induce cell fate changes.

To gain insight into the mechanism of emergence of the salt and pepper pattern of Epi and PrE cells, it is necessary to simulate a population of cells. Moreover, cell divisions and cell movements, which can affect the local environment of the cells, also need to be taken into account. Fig. 7 represents the results of such a


Fig. 7. Cell specification towards the Epi or PrE state in the model including cell division and movement.
(A) A 3D simulation of Epi/PrE specification from precocious ICM cells (Tosenberger et al., 2017), showing the formation of the salt and pepper pattern. The colour scheme indicates the corresponding levels of expression of Nanog and Gata6, i.e., the current state of the cell. The corresponding age of the embryo is expressed in embryonic dates and indicated at the left-hand side.
(B) Evolution of cells in (A) in the Fgf4/Nanog plane (dots) and the associated bifurcation diagram. In the bifurcation diagrams, red bold and black thin lines refer to stable and unstable steady states, respectively.


Fig. 8. Evolution of Fgf4 (left hand side), Nanog and Gata6 expressions (right hand side) in three cells with the final Epi (A), ICM (B) and PrE (C) fate. The three cells were taken from the simulation presented in Fig. 7.
simulation. Starting from 3-5 (3 in Fig. 7) precocious ICM cells, Nanog and Gata6 start increasing together in all cells. At $\sim$ E3.0, the second round of asymmetric divisions of outer cells generates additional precocious ICM cells. As external Fgf4 concentration follows a complex spatio-temporal evolution in the blastocyst, each cell perceives a slightly different concentration of Fgf4. In the simulation framework, this concentration is computed as the average between the Fgf4 secreted by the cell itself and all its nearest neighbours. As visible in Fig. 7A, this eventually leads to a mixture of Epi and PrE cells arranged in the salt and pepper pattern.

The dynamics of specification is best apprehended when superimposing the evolution of the GRN of the individual cells on the bifurcation diagram of Nanog as a function of Fgf4. In Fig. 7B, each dot represents a cell that is coloured according to the status of its GRN. Cells tend to acquire the level of Nanog given by the bifurcation curve, which represents the steady-state situation. It is visible that the specification scenario proposed in the 2 -cell model described above remains robust for a more realistic situation taking into account an appropriate number of cells, cell division and cell movement.

This scenario agrees with the in vivo dynamics of cell specification and is supported by a variety of observations (Bessonnard et al., 2014; De Mot et al., 2016; Tosenberger et al., 2017). For example, the model predicts that, in average, the Epi cells are specified earlier than the PrE progenitors. This prediction is not related to a specific choice of parameter values, but is inherent to the specification mechanism highlighted by the model. Indeed, it is the increased secretion of Fgf4 by the cells that initially become Epi that leads to a subsequent activation of Erk signalling in a subset of non-yet specified cells and triggers the PrE cell fate. In agreement with this prediction, the downregulation of Gata6 in the future Epi cells indeed occurs sooner than the downregulation of Nanog in the PrE precursors (Fig. 7). This is also verified in vivo as at E3.25 a bias can already be observed for a few cells that express Nanog at higher levels, indicating the beginning of differentiation. Moreover, at E3.5 many Epi cells are already specified, compared to future PrE cells (Bessonnard et al., 2014). Simulations of the model are indeed in agreement with experimental findings under various treatments where the levels of Erk signalling were manipulated either by the addition of exogenous Fgf4 or by the application of Erk inhibitors, both on wild-type (WT) mice and on different mutants. A detailed comparison of the effects of interfering with Erk signalling in simulations of the model and in embryos can be found in (De Mot et al., 2016) and in (Tosenberger et al., 2017).

In the model, the salt and pepper pattern is a consequence of the GRN as signalling by extracellular Fgf4 is modulated by the latter. In agreement with this mechanism of patterning, sustained exogenous Fgf4 fails to rescue the salt and pepper pattern observed in the Fgf4-l- mutant. Instead, embryos cultured in such conditions exhibit a unique cell type, the nature of which depends on the Fgf4 concentration (Kang et al., 2013). Clustering analyses also showed that the bimodal expression or Fgf4 is required for ICM lineage segregation (Ohnishi et al., 2014).

The model predicts that the dynamics of Fgf4 is different around each cell of the embryo. It is also rather noisy, given cell divisions and cell movements. Typical evolutions of local Fgf4 concentrations are shown in Fig. 8 (left column). Each row represents the evolution towards a different fate (A: Epi, B: a cell that remains on the ICM state, a situation that corresponds to a cell that will be eliminated by apoptosis, C: PrE). In all cases, [Fgf4]extra first increases because of the low level of Gata6. It then decreases because it is degraded in the extracellular medium and gradually less secreted as the overall level of Gata6 expression (right column, blue lines) increases. As it was also the case in the 2-cell model, an increase in the surrounding concentration of Fgf4 favours the occurrence of an Epi cell. When cells start to specify, the levels of [Fgf4] $]_{\text {extra }}$ become rather similar and their evolutions rather noisy. In particular, when cells divide (at E3.0 and at E3.5), this significantly affects the composition of their environment. It is thus necessary for the GRN to be robust with respect to fluctuations in Fgf4 concentration, to avoid cell fate switching. Thus, as in the case of the TE/ICM specification, multistability in the GRN allows for robustness with respect to inherent noise in signalling. However, in contrast to the TE/ICM specification for which positional cues are responsible for distinct signalling, noise in signalling is necessary to initiate Epi/PrE specification. As a matter of fact, alternative sources of noise may also contribute to trigger cell fate specification: simulations indeed indicate that uneven repartitions of molecules at division are also able to break the symmetry and to initiate specification (Tosenberger et al., 2017). The existence of the third stable steady state could reconcile the need for robustness with respect to noise during the developmental path (by providing an intermediate state with a large enough basin of attraction) with the possibility to escape from this state by perturbations that exceed a given threshold.

## 4. Conclusions

The emergence of a blastocyst composed of three different cell types from a single population of nearly identical cells can be described as to successive specification steps controlled by two simple networks displaying multistability. The choice of the steady state reached by the system is not only imposed by the initial conditions, but also by signalling. While for the $\mathrm{TE} / \mathrm{ICM}$ specification Hippo signalling transduces positional cues, for the Epi/PrE specification Erk signalling is self-modulated in such a manner that the cross-signalling between the cells is required for the emergence of the two cell types. This mechanism generates a combinaison of Epi and PrE cells arranged in the salt and pepper pattern. An additional step of cell sorting is necessary to separate the epithelium of PrE cells from the blastocoel (Artus and Chazaud, 2014).

Another important point pertains to the existence of a zone of tristability in the model of Epi/PrE specification. The state with intermediate levels of Gata6 and Nanog indeed corresponds to the state that is observed for extended periods of time in the embryo and must thus correspond to a stable state. Interestingly, the unstable state characterized by intermediate levels of Cdx2 and Oct4 in the $\mathrm{TE} / \mathrm{ICM}$ specification step (Fig. 2) is relatively attractive and is easily observable in the numerical simulations for intermediate levels of Ecadh and Par-aPKC (not shown). However, the transient evolution towards this unstable steady state is not expected to occur when stochastic effects are taken into account, and thus also not in vivo. For both specification processes studied here, multistability allows the specification mechanism to be modulated by signalling in a highly flexible way, while avoiding the occurrence of cell fate switches in a fluctuating environment such as the developing embryo. Modelling however suggests that noise has an additional constructive role in initiating Epi/PrE specification where the intermediate stable steady state must be conserved, which is not necessary for the TE/ICM decision in which no such intermediate steady state is stable.

The two first specification processes studied here have also been modelled by an agent-based model of physically interacting cells (Nissen et al., 2017). In this study, rules governing the Epi/PrE specification step are exclusively based on Fgf4 signalling in a manner qualitatively equivalent to that described here above. Concerning the TE/ICM specification, the authors did not consider the effect of polarity and of cellular adhesion on Hippo signalling. Instead, cells with fewer than 5 nearest neighbours were assigned the TE fate and in turn acquired polarity. As physical interactions between polar cells are different, this allowed the formation of a single layer sheet of TE cells in the simulations. In the first approach considered in the present study, we have focussed on the GRN and on signalling aspects. It is however also possible that during asymmetric division, the most external daughter cell has received more Cdx2 mRNA (Jedrusik et al., 2008); this unequal repartition of transcription factors would be reinforced by the GRN and lead to the specification of the cell in the TE fate. This assumption is known as the polarity-based hypothesis. We also did not consider the important role played by surface contractility (Maître et al., 2016), nor recent results showing the key role played by the cell three-dimensional neighbourhood (Fischer et al., 2018). In the future, physical interactions could be considered in a computational framework similar to that developed for the Epi/PrE specification (Fig. 7 in Tosenberger et al., 2017). This would allow a realistic description of the temporal evolutions of our parameter Par-aPKC and Ecadh instead of the arbitrary functions chosen in the present study. Alternatively, the GRN modelled in this study could be included in highly realistic descriptions of 3D multi-cellular systems that consider cells as elastic and adhesive objects (Hoehme and Drasdo, 2010).

From a methodological point of view, our approach is based on the description of the dynamics of transcription factors and signalling by ordinary differential equations, as in generic models of multistability (Gardner et al., 2000). In very different contexts, similar approaches revealed a role for tristability in the control of the choice between erythroid and myelomonocytic fates (Huang et al., 2007) and in the epithelial-hybrid-mesenchymal fate determination that is regulated by micro-RNAs (Lu et al., 2013). The logical approach developed by René Thomas and his co-workers is also very useful to study cell differentiation from the modelling point of view (Thomas and Kaufman, 2001a, b). This method is indeed particularly well-adapted to the description of complex regulatory networks implying a large number of pathways and variables, as shown recently in the case of the differentiation of hematopoietic cells (Collombet et al., 2017) and pancreas cell differentiation (Zhou et al., 2016). Altogether, these studies clearly demonstrate the importance of looking at differentiation processes, not so much in terms of molecules, but rather from the point of view of regulatory circuits as emphasized in the pioneering researches of René Thomas.

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