A dynamical model of genetic networks describes cell differentiation

Marco Villani*°, Alessia Barbieri* & Roberto Serra*°

*Modelling and Simulation Laboratory, Economics Department, University of Modena and Reggio Emilia, via Allegri 9, 41121 Reggio Emilia (Italy)

°European Centre for Living Technology, San Marco 2940, 30124 Venice (Italy)

Abstract. Cell differentiation is a complex phenomenon whereby a stem cell becomes progressively more specialized and eventually gives rise to a specific cell type. Differentiation can be either stochastic or, when appropriate signals are present, it can be driven to take a specific route. Induced pluripotency has also been recently obtained by overexpressing some genes in a differentiated cell. Here we show that a stochastic dynamical model of genetic networks can satisfactorily describe all these important features of differentiation, and others. The model is based on the emergent properties of generic genetic networks, it does not refer to specific control circuits and it can therefore hold for a wide class of lineages. The model points to a peculiar role of cellular noise in differentiation, which has never been hypothesized so far, and leads to non trivial predictions which could be subject to experimental testing.

Introduction

Differentiation is a really complex phenomenon, or rather a set of interrelated phenomena: in organisms it most often displays a deterministic, signal-driven character[1], but stochastic differentiation has also been described [2-4]. In the following we will use the term "deterministic differentiation" to refer to the former case, when necesssary, to distinguish the two alternatives. Differentiation is usually irreversible,

proceeding from a less to a more differentiated state, but the reverse has also been observed in experiments where pluripotency has been induced by overexpressing some genes in differentiated cells[5,6].

Therefore it is not obvious that a single model can describe all these phenomena. Some previous models of differentiation are able to describe some of them[4,7,8]; they make use of a continuum description and, in part because of computational limitations, are bound to take into account the contribution of only few genes. Here we hypothesize that the robust properties of differentiation are rather the outcome of the interaction of very many genes, so our model is based on a simplified dynamical model of genetic regulatory networks, namely noisy random Boolean networks (NRBNs for short), which actually allow simulations of large networks[9]. NRBNs represent an extension of the well-known model of random Boolean networks[10-13] (RBNs) that, in spite of their approximations, have been able to describe important experimental facts concerning gene expression[14-16].

A classical RBN (see Supplementary Fig.1) is a directed graph with N nodes (genes), which can assume binary values 0 or 1 (inactive/active); time is discrete, with synchronous updating of all the node values. Each node has exactly k input connections chosen randomly with uniform probability among the remaining N-1 nodes (prohibiting multiple connections). To each node a (randomly chosen) Boolean function is associated, which determines its value at time t from the values of its inputs at the previous time step. Both the topology and the Boolean function associated to each node do not change in time. For reasons described in the legend to Supplementary Fig.1 we concentrate our study on so-called critical networks with k=2 and p=1/2[13, 17-19].

The network dynamics is discrete and synchronous, so fixed points and cycles are the only possible asymptotic states in finite networks. It would be natural to identify the attractors of RBNs with cell types, since they correspond to different coherent

dynamical states of activation, with the same genome (i.e. topology and Boolean functions)[11-13]. However attractors of RBNs are unstable with respect to noise even at low levels. Consider for example a transient flip of a randomly chosen node when the system is in a state of one of its attractors: even if the flip lasts for a single time step one sometimes observes transitions from that attractor to another one (see Supplementary Fig. 2)

We will therefore investigate the asymptotic dynamics of the network subject to random noise, modelled as above, i.e. by the transient flip of a randomly chosen node which lasts for a single time step; after that the node follows the rules of the network deterministic dynamics. This is indeed the smallest possible random fluctuation affecting a Boolean system. It will also be assumed that the noise level is small enough to allow the system to relax to an attractor before a new flip occurs[1]. This hypothesis allows us to make use of the knowledge of the attractors of the deterministic system to analyze the behaviour of its noisy version, thereby strongly simplifying the description of the asymptotic dynamics of the stochastic system.

Since attractors (this term will always be used here for those of the deterministic system) are unstable with respect to noise (which is known to play a role in key cell processes[20-23]), they can no longer be associated to cell types. A possible way out was proposed by Ribeiro and Kauffman[24] who observed that there exist sets of attractors, which they called ergodic sets, which entrap the system in the long time limit, so the system continues to jump between attractors which belong to the set. It would then be

---

[1] Several simulations have indeed shown that, while the transient from a random initial state to an attractor may be long, the transitions between two different attractors almost always require a small number of steps.

natural to associate cell types to such ergodic sets, but unfortunately it turns out that most NRBNs have just one such set (at most 2 of them have been observed in very many simulations). This strong limitation on the number of ergodic sets rules out the possibility to associate them to cell types.

A possible solution to this problem was proposed in [ref. 9] and is briefly summarized in the next section, where it is also shown that by a proper interpretation it can describe in an elegant way the fact that there exist different degrees of differentiation, and that it provides a natural way to simulate stochastic differentiation. In the following section we show that the same model describes also deterministic differentiation, when appropriate signals are provided. In a further section we show that it also accounts for induced pluripotency and other related phenomena. Finally, in the last section we discuss the biological meaning of a key variable, the implications of the model and possible experimental tests.

Threshold ergodic sets and stochastic differentiation

Observe that the kind of noise which is taken here into account is fairly intense, as it amounts to silencing an expressed gene or to express a gene which would otherwise be inactive; therefore it is an event which is much less frequent than, say, typical molecular scale fluctuations. Consider now the case where the transition between two attractors occurs only when a single specific node is flipped. This may well be an event too rare to happen with significant probability in the cell lifetime. Therefore we will introduce a threshold, and will consider as acceptable only those transitions that may happen by a number of flips above the threshold. Note that here we are not considering multiple flips (these would be even rarer) but independent paths that lead from one attractor to another. A formal definition of the threshold has been given elsewhere[9] and is summarized in Supplementary Fig. 3.

It is intuitively clear that the threshold could be related to the level of noise in the cell. It was indeed shown[9] that the threshold scales with the reciprocal of the frequency of flips, i.e. the noise level. A more thorough discussion of the biological significance of the threshold will be deferred to the final section.

The important notion is that of the Threshold Ergodic Set (briefly TES) which is also formalized in Supplementary Fig. 3. Informally, a TES is the analogous of an Ergodic Set when one takes into account only the transitions that have a chance to happen larger than the threshold. In this case, a TES is a set of attractors that entrap the system in the long time limit, so the system continues to jump between attractors belonging to the set.

Let us now consider what happens by gradually increasing the threshold. At $\theta=0$ one typically has a unique TES but, by increasing the threshold, it breaks into some disjoint TESs. By further increasing the threshold these TESs in turn break into smaller ones until, at high enough levels of the threshold, all attractors are also TESs (i.e. they cannot be abandoned). The process is shown in Fig. 1 (see also Supplementary Fig. 3). It was shown elsewhere[9] that the ratio between the total number of TESs and the total number of attractors increases as the threshold is increased, and that for each network there is a value such that, when θ exceeds that value, all the attractors are TESs.

We propose to associate cell types to TESs. They represent indeed coherent stable ways of functioning of the same genome (i.e. connections and Boolean functions) even in the presence of noise. The problem that hampered the straightforward association of cell types to ergodic sets is no longer present in this case, since there may be several TESs in the same network.

The degree of differentiation is supposed to be related to the possibility for the cell, in its asymptotic state, to wander in a portion of phase space, which should be smaller for a more differentiated cell. In the present framework, a convenient proxy for the available portion of phase space is the number of attractors belonging to the TES. Therefore, a totipotent cell should be associated to the TES_0 (i.e. the one found when $\theta=0$), while as the threshold is increased more differentiated forms appear (pluripotent or multipotent cells), corresponding to smaller TESs like those shown in Fig. 1. At high enough threshold values all the attractors are TESs, and these should describe the fully differentiated cells[2].

In order to describe differentiation, in the present framework it is assumed that it implies a change in the threshold, which in turn implies a change in the noise level. Differentiation increases if the threshold increases, i.e. the noise level decreases, and this latter effect could be related to an improvement in the mechanisms whereby fluctuations are kept under control. The association of differentiation to changes in the threshold level represents the most striking outcome of this model, and is in principle amenable to experimental test, as it will be discussed in the final section. For the time being let it suffice to remark that a higher noise level in undifferentiated cells, with respect to more differentiated forms, has been actually reported[25-27].

It is clear that the above hypothesis explains in a straightforward way the fact that there are different degrees of differentiation, related to different threshold values. But note that also stochastic differentiation[25,28] can be described by the model. Indeed, the fate of a given cell depends on the particular attractor where it is found at the moment when the threshold is increased: the new type will be the one described by the TES to which that attractor belongs, at the higher threshold value (see Fig. 1).

---

[2] A TES with a single attractor will be called a single-TES, while a TES with two or more attractors is a multi-TES

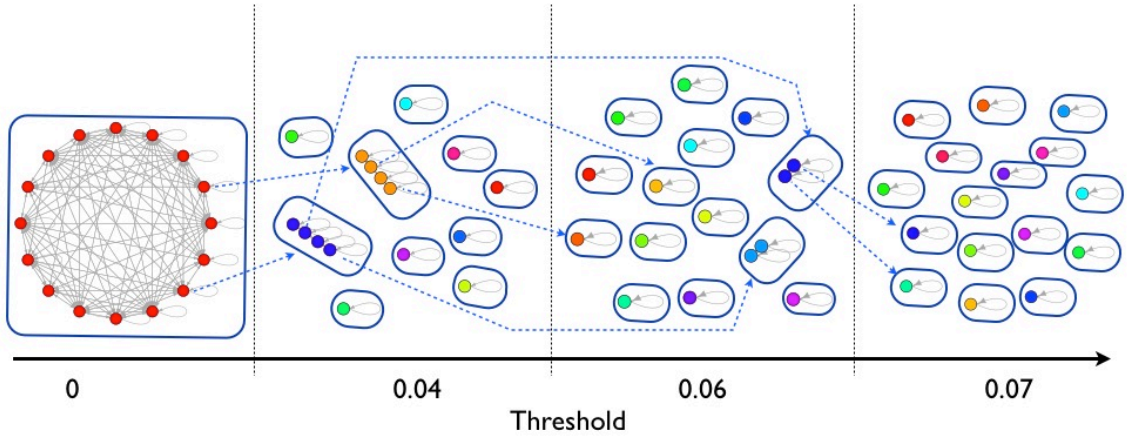

Figure 1 | TESs and stochastic differentiation

Switch nodes determine the cell fate

There exist processes, e.g. during the embryogenesis, in which cell differentiation is not stochastic but it is driven by specific chemical signals, which activate or silence some genes. These signals are thus represented in the model by permanent perturbations of a node[3], which fix its state to 1 or 0. In order to describe these deterministic differentiation processes in our model we couple these permanent perturbations with an increase of the threshold (which by itself would lead to the stochastic differentiation shown in Fig.1).

The model will be considered able to describe deterministic (signal-driven) differentiation if one can demonstrate the existence of *switch* genes, whose permanent activation or inhibition always leads the system through the same differentiation pathway, i.e. nodes that uniquely determine to which TES the system will evolve. *Switches* are precisely defined as follows: starting from a certain TES, if perturbing a node from all phases of each attractors of that TES the system goes always in the same attractor, then the perturbed node is a switch (in that TES). The existence of switch

[3] For reasons of simplicity we will consider the fixing of the value of a single node at a time

nodes has actually been verified to be a widespread property (found in about 1/3 of the nets), thereby proving the effectiveness of the model[4].

In Fig. 2 one can see an example of differentiation, from a multi-TES_0 to a set of single-TESs, which shows a remarkable qualitative similarity with differentiation diagrams of real cell lineages, like e.g. hemopoietic cells.

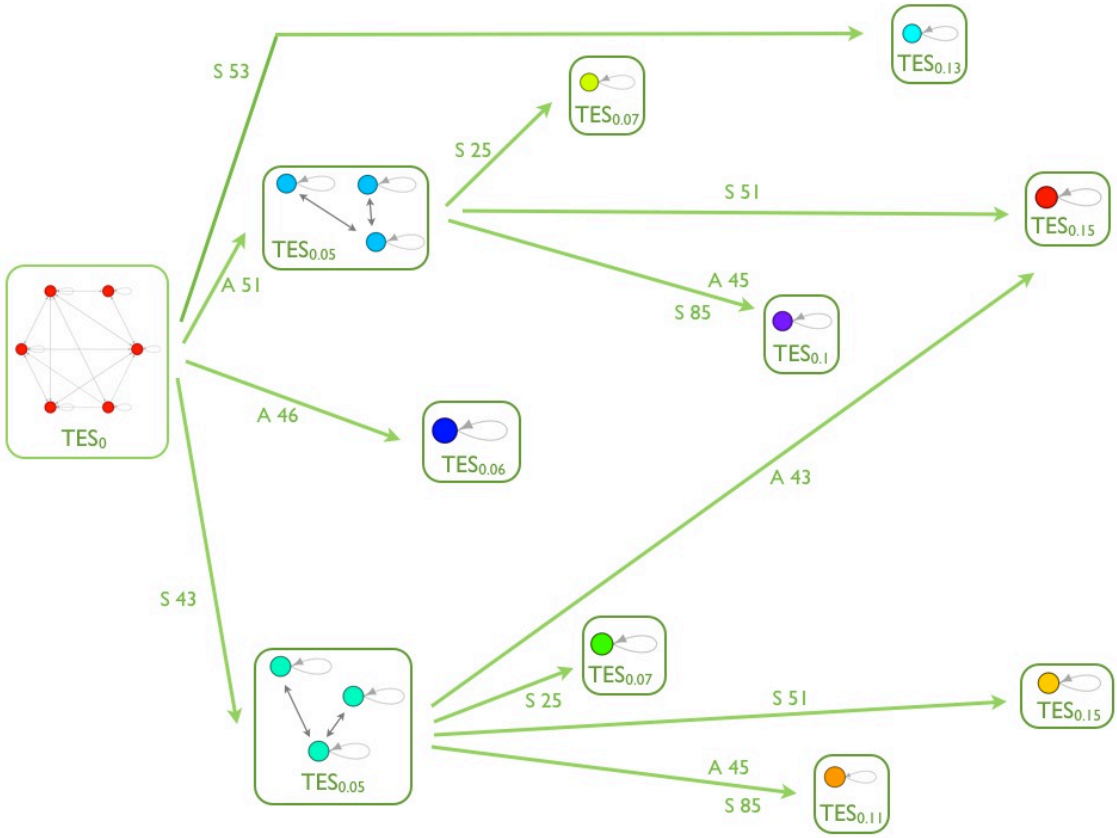

Figure 2 | a case of deterministic differentiation

Some considerations arise from the experiments we performed: first of all, this case represents just one possible diagram obtained from simulations; the system shows a very rich and complex landscape of possible behaviours, as in biological

[4] Note that it is not necessary to prove that switches exist for all the NRBNs, it is indeed sufficient to show that they are present in a significant fraction if them, so that natural selection can pick up the "good" ones.

differentiation. Second, the activation order of the switches matters: acting first on switch *a* and then on switch *b* leads to different fates than acting first on *b* and then on *a* (not shown here). This is coherent with the fact that in real systems different consequences may arise from the action on a gene in different physiological moments. Moreover, there exist cases in which a final single-TES can be reached from the same multi-TES acting on different switches (as shown by double labels associated to the same arrow). Finally there are cases in which a single final type can be reached from different pathways (as in the case of the red single-TES, which can be reached either from the azure or from the turquoise multi-TES).

Simulating induced pluripotency

In recent years considerable attention has been raised by the discovery of induced pluripotency, where overexpression of a few transcription factors (from 1 to 4) in differentiated cells can make them "come back" to a less differentiated state[5,6,29]. Simulating such a process of dedifferentiation by a decrease of the threshold would be straightforward but, since there is no evidence that such a process actually takes place in experiments, we checked whether dedifferentiation can be achieved without modifying the threshold, by simply fixing the value of a gene to 1 permanently so to simulate its overexpression (of course this makes sense on those genes which are not always active).

This phenomenon can actually be observed in some networks, as shown in Fig. 3. This behaviour is not generic, and it is found rarely, but also in biological systems there are just a few genes that can give rise to induced pluripotency. Note also from Fig. 3 that most of the attractors of the TES_θ reached in this way are identical (apart from the perturbed node) to the original TES_0, a situation which can be summarized by saying that the two TESs are similar to each other - and this closely parallels what has been experimentally observed. Note also that the above description belongs to the set of so-

called stochastic models of iPSC that seem in accordance with known experimental facts[30].

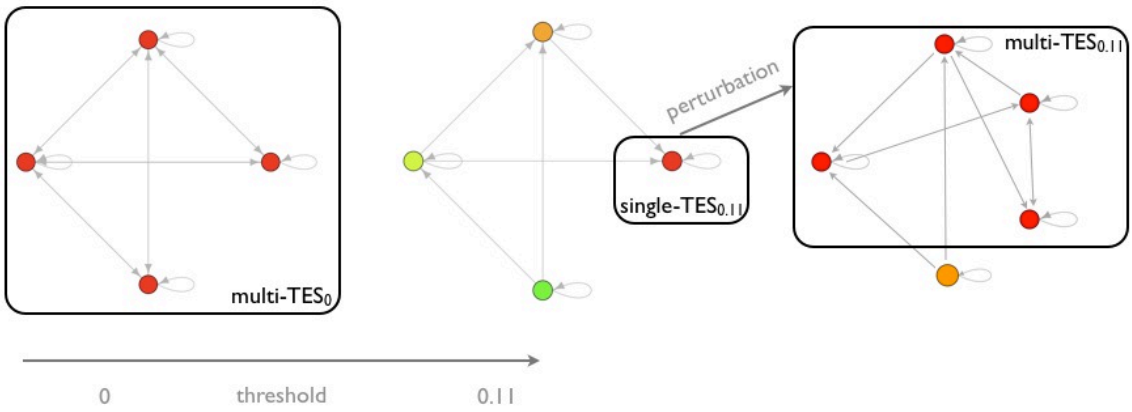

Figure 3 | Yamanaka-like in silico experiment

Discussion

The mathematical model describes the main features of differentiation, namely the existence of different degrees of differentiation, of stochastic and deterministic differentiation, and of induced pluripotency. Indeed, it is actually able to describe an even wider set of phenomena, including the well-known existence of both committed and determined cells (Supplementary Fig. 4) and possible transitions between two differentiated cell types (Supplementary Fig. 5) as the one that has been recently reported between fibroblasts and neurons[31].

Another interesting feature of the model is that the explanation of differentiation makes use of the global properties of a generic dynamical system, without resorting to detailed hypotheses concerning very specific control circuits. The fact that differentiation is linked to sets of attractors of a large network, rather than to a specific kind of interaction between few genes, is also worth noticing.

The most striking result obtained here concerns the importance of the threshold: if we permanently modify the expression of one or a few genes without acting on the

threshold, the breakup of a TES into smaller disjoint ones is not observed. This statement is in principle subject to experimental testing, provided that we define the biological meaning of the threshold. As it has been repeatedly stressed, this could be related to the level of noise in the cell, as a back-of-the-envelope calculation shows[9].

If the threshold is related to the noise level, and if differentiation requires a change of the threshold, then differentiation should be accompanied by a change in the noise level. It is important to remark that flips (active/inactive) similar to those adopted here have actually been observed[32] as well as to make reference to some works which show that in stem cells more genes are usually active than in differentiated ones, albeit at a lower level[25,26]. Since this entails a smaller number of copies of the key molecules (say, m-RNA) per cell, and since the relative role of fluctuations is higher when the number of exemplars is lower, this indicates that noise can indeed be higher in stem cells than in differentiated one. It is also particularly interesting to observe that it has recently been reported[3,26,27] that the state of gene expression levels of (at least some) stem cells can be described as slowly itinerating among several quasi-stable states, a description which fits that of a TES.

The deterministic differentiation processes which are observed e.g. in embryo growth require that the threshold of a cell can change when needed. It is natural to suppose that the threshold itself is under genetic control, so that it can be modified when appropriate. Among the various mechanisms, which may be involved in such control, let us mention that *i)* the folding/unfolding of chromatin can modify the level of noise of many genes[33] and *ii)* the production of miRNA can silence genes which are expressed at low levels, thereby making expression noise vanish[34]. These two mechanisms can suppress noise around the inactive state of the genes. Other mechanisms can be at work to stabilize the active state, for example by producing more copies of m-RNA per unit

time[35], by reducing the degradation rate of the proteins or by using buffer circuits to keep constant nonzero activation values[34].

On the theoretical side, there are several aspects that are worth exploring, including those concerning the generality of our results. The general picture of the cell as a dynamical system, and the idea that differentiated cells are more constrained in their wandering in phase space can be applied also to other models of gene and cell dynamics, and the question can be raised concerning the possibility of obtaining similar results also with these other gene network models.

We have modelled here only a single cell, lumping the effect of the other neighbouring cells in a "signal" which sets the value of a particular gene; it would be interesting to explore along these lines also the role of the interactions among communicating cells in differentiation.

Other research directions include the use of variations of the classical RBN model, motivated by increasing knowledge of the actual properties of biological systems (like e.g. scale-free networks, modular networks, different updating schemes, three-valued models, etc.)

Let us finally remark that the availability of sophisticated system-level models like this can lead to a deeper understanding of the process and can provide impulse to the experiments by suggesting testable hypotheses, in particular those concerning the importance of controlling the noise level in differentiation.

Methods Summary

The simulations concerning RBNs were made using a software developed in house, written in C++. Different network sizes were tested, and most of the results refer to

nodes with 100 networks (a few smaller networks with 10 and 20 nodes were also simulated, as well as some larger ones with 200 nodes).

Except for the 10-node and 20-node networks, exhaustive testing of the possible initial conditions is impossible, so in networks of 100 or more nodes attractors were found starting by 10.000 randomly chosen initial conditions. The search was performed with an algorithm able to find attractors with periods not larger than 500 time steps (and a maximum transient of 1000 steps). It turns out that these search parameters allow one to find an attractor for all the initial conditions in about 99% of the random networks.

The transition graph between different attractors was obtained by perturbing (independently) each node of each state of each attractor. For each perturbation the new attractor was found, thereby determining the weights of the links of the attractor transition graph (see Supplementary Fig. 2)

The search for TESs was made using a software developed in house, written in C++. The algorithm was based on the search for the strongly connected components of the attractor transition graph (taking into account the level of the threshold). For each strongly connected component it was then checked whether it actually entrapped the system, a necessary condition for it to be a TES.

The results concerning the switches have been obtained as follows, starting from critical RBNs with 100 nodes. In order to describe cells with the same genome, i. e. the same structure of the RBN, which can evolve to different fates we limited our analysis to networks with more than one switch and where there are at least two switches leading to different asymptotic states. Starting from TES_0 we searched for a switch and, when we found one, we fixed its value and grew up the threshold to obtain a $TES_{x>0}$ composed by a smaller number of attractors. Then we repeated the procedure starting from the newly found $TES_{x>0}$ to find a $TES_{x>y}$ with an even smaller number of

attractors, until we found a single-TES (i.e. a fully differentiated cell). In this way we explored just one of the possible paths, only a tree branch, so in order to obtain a complete picture of the possible fates we iterated the procedure for all the branches of the root (the initial multi-TES_0) and all possible sub-branches. Eventually we found all the possible system fates, which can be represented e.g. as in Fig. 2.

References

1. Wolpert, L. Do we understand development? *Science* **266**, 571–572 (1994).
2. Hume, D. A. Probability in transcriptional regulation and its implications for leukocyte differentiation and inducible gene expression. *Blood* **96**, 2323–2328 (2000).
3. Chang, H. H., Hemberg, M., Barahona, M., Ingber, D. E., Huang, S. Transcriptome-wide noise controls lineage choice in mammalian protenitor cells. *Nature* **453**, 544–548 (2008).
4. Huang, S. Reprogramming cell fates: reconciling rarity with robustness. *Bioessays* **31 (5)**, 546–560 (2009).
5. Takahashi, K., Yamanaka, S. Induction of pluripotent stem cells from mouse embryonic and adult fibroblasts cultures by dfined factors. *Cell* **126 (4)**, 663–676 (2006).
6. Takahashi, K. *et al*. Induction of pluripotent stem cells from adult human fibroblasts by defined factors. *Cell* **131 (5)**, 861–872 (2007).
7. Kaneko, K. LIfe: *An Introduction to Complex System Biology*. Springer-Verlag, Berlin, New York (2006).
8. Huang, S., Guo, Y., May, G., Enver, T. Bifurcation dynamics in lineage-commitment in bipotent progenitor cells. *Dev. Biol.* **305 (2)**, 695–713 (2007).

9. Serra, R., Villani, M., Barbieri, A., Kauffman S. A., Colacci, A. On the dynamics of random Boolean networks subject to noise: attractors, ergodic sets and cell types. *J. Theor. Biol.* **265**, 185–193 (2010).

10. Kauffman, S. A. Metabolic stability and epigenesis in randomly constructed genetic nets. J. Theor. Biol. 22 (3), 437–467 (1969).

11. Kauffman, S. A. *The Origins of Order*. New York, Oxford University Press (1993).

12. Kauffman, S. A. *At Home in the Universe*. New York, Oxford University Press (1995).

13. Aldana, M., Coppersmith, S., Kadanoff, L. P. Boolean dynamics with random couplings. In: Kaplan, E., Marsden, J.E., Sreenivasan, K. R. (Eds), *Perspectives and Problems in Nonlinear Science*. 23–89. A Celebratory Volume in Honor of Lawrence Sirovich. Springer Applied Mathematical Sciences Series (2003).

14. Serra, R., Villani, M., Semeria, A. Genetic network models and statistical properties of gene expression data in knock-out experiments. *J. Theor. Biol.* **227 (1)**, 149–157 (2004).

15. Serra, R., Villani, M., Graudenzi, A., Kauffman, S. A. Why a simple model of genetic regulatory networks describes the distribution of avalanches in gene expression data. *J. Theor. Biol.* **249 (3)**, 449–460 (2007).

16. Shmulevich, I., Kauffman, S. A., Aldana, M. Eukaryotic cells are dynamically ordered or critical but not chaotic. *PNAS* **102 (38)**, 13439–13444 (2005).

17. Aldana, M., Balleza, E., Kauffman, S.A., Resendiz, O. Robustness and evolvability in genetic regulatory networks. J. Theor. Biol. 245, 433–448 (2007).

18. Langton, C. G. Life at the edge of chaos. In: Langton, C.G., Taylor, C., Farmer, J.D., Rasmussen, S. (Eds.), Artificial Life II. Addison-Wesley, Reading MA, pp. 41–91 (1992).

19. Ramo, P., Kesseli, J., Yli-Harja, O. Perturbation avalanches and criticality in gene regulatory networks. J. Theor. Biol. 242 (1), 160–164 (2006).

20. McAdams, H. H., Arkin, A. Stochastic mechanisms in gene expression. *PNAS* **94**, 814–819 (1997).

21. Swain, P. S., Elowitz, M. B., Siggia, E. D. Intrinsic and extrinsic contributions to stochasticity in gene expression. *PNAS* **99**, 12795–12800 (2002).

22. Blake, W. J., Kærn, M., Cantor, C. R., Collins, J. J. Noise in eukaryotic gene expression. *Nature* **422**, 633–637 (2003).

23. Raj, A., van Oudenaarden, A. Nature, Nurture, or Chance: Stochastic Gene Expression and Its Consequences. *Cell* **135 (2)**, 216–226 (2008).

24. Ribeiro, A. S., Kauffman, S. A. Noisy attractors and ergodic sets in models of gene regulatory networks. *J. Theor. Biol.* **247 (4)**, 743–755(2007).

25. Hu, M.*et al*. Multilineage gene expression precedes commitment in the hemopoietic system. *Genes Dev.* **11 (6)**, 774–785 (1997).

26. Furusawa, C., Kaneko, K. Chaotic expression dynamics implies pluripotency: when theory and experiment meet. *Biol. Direct* **4 (1)**, 17 (2009).

27. Hayashi, K., de Sousa Lopes, S. M. C., Tang, F., Surani, M. A. Dynamic equilibrium and heterogeneity of mouse pluripotent stem cells with Distinct Functional and Epigenetic States. *Cell Stem Cell* **3 (4)**, 391–440 (2008).

28. Miyamoto, T. *et al*. Myeloid or Lymphoid Promiscuity as a Critical Step in Hematopoietic Lineage Commitment Dev. *Cell* **3**, 137–147 (2002).

29. Giorgetti, A. *et al*. Generation of Induced Pluripotent Stem Cells from Human Cord Blood Using OCT4 and SOX2 *Cell Stem Cell*. **5 (4)**, 353–7 (2009).

30. Yamanaka, S., Elite and stochastic models for induced pluripotent stem cell generation. *Nature* **460**, 49–52(2009).

31. Vierbuchen, T. *et al*. Direct conversion of fibroblasts to functional neurons by defined factors. *Nature* **463**, 1035– 1041 (2010).

32. Kuznetsov, V. A., Knott, G. D., Bonner, R. F. General Statistics of Stochastic Process of Gene Expression in Eukaryotic Cells. *Genetics* **161**, 1321–1332 (2002).

33. Raser, J. M., O'Shea, E. K. Control of Stochasticity in Eukaryotic Gene Expression. *Science* **304**, 1811–1814 (2004).

34. Hornstein, E., Shomron, N. Canalization of development by microRNAs. *Nature Genetics* **38** (Supplementary), S20–S24 (2006).

35. Thattai, M., van Oudenaarden, A. Intrinsic noise in gene regulatory networks. *PNAS* **98**, 8614–8619 (2001).

Supplementary Information accompanies the paper on **www.nature.com/nature**.

Correspondence and requests for materials should be addressed to R.S. (e-mail: roberto.serra@unimore.it).

Acknowledgements We are deeply indebted to Stuart Kauffman for his inspiring ideas and for several conversations on various aspects of random Boolean networks. We also gratefully acknowledge useful discussions on the model and its biological significance with Annamaria Colacci, Chiara Damiani, Alex Graudenzi and Alessandro Filisetti. We also benefited from discussions on various topics related to this work with Sui Huang, Kunihiko Kaneko and Andre Ribeiro. This work has been supported in part by the Italian Miur Project MITICA (FISR nr. 2982/Ric) and by the DICE project of the Fondazione di Venezia.

Author contributions This work grew out of a tight collaboration among the three authors, so it is difficult to precisely identify their roles. Keeping this caveat in mind, it is worth mentioning that M.V. first proposed to study the influence of random noise, contributed to the development and testing of the software, deepened the analysis of the biological meaning of the threshold, that A.B., besides developing all the software tools necessary for the simulations and the analyses, performed the actual *in-silico* experiments, explored the study of the model behaviours and found networks endowed with the properties

described in the text, that R.S. introduced the concept of threshold, formalized the notion of TES, proposed the association of the number of attractors of a TES with the degree of cell differentiation and the notion of switch. The three authors equally contributed to the analysis of the results of the simulations.

Legends to Figures

Figure 1 | **TESs and stochastic differentiation**. As the threshold θ is increased (from left to right) one sees that the initial TES_0, which comprises in this case all the attractors, breaks into a set of smaller disjoint TESs, which describe more differentiated cells. As θ is further increased the remaining multi-TESs break into single-TESs, which describe fully differentiated cells. The arrows show some transitions: the fate of a cell described by a multi-TES (e.g. the initial one) depends upon the particular attractor where it is found when the threshold is increased, and this happens at random. Only some transitions are shown.

Figure 2 | **a case of deterministic differentiation**: each box represents a TES_θ while circles represent attractors. Arrows indicate possible paths of differentiation; labels on arrows denote both the number of the node that acts as a switch and whether it is effective when set to 1 (A) or to 0 (S). The details are described in the Methods summary section.

Figure 3 | Yamanaka-like *in silico* experiment: we take an initial network whose TES_0 (left most graph) represents the totipotent stem cell. By increasing the threshold value we reach a situation where there is a single-TES, which represents a fully differentiated state (centre). In this state, we permanently

modify the value of a gene fixing it to 1. We have found some cases where this permanent perturbation leads the system, without changing the threshold, to a multi-TES, as shown above (rightmost graph). This is not in contradiction with what has been observed elsewhere, since by fixing the value of a gene we have actually modified the network itself.