OPINION

# Transition states and cell fate decisions in epigenetic landscapes

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Abstract | Waddington's epigenetic landscape is an abstract metaphor frequently used to represent the relationship between gene activity and cell fates during development. Over the past few years, it has become a useful framework for interpreting results from single-cell transcriptomics experiments. It has led to the proposal that, during fate transitions, cells experience smooth, continuous progressions of global transcriptional activity, which can be captured by (pseudo) temporal dynamics. Here, focusing strictly on the fate decision events, we suggest an alternative view: that fate transitions occur in a discontinuous, stochastic manner whereby signals modulate the probability of the transition events.

The development of a multicellular organism with its array of organs and tissues is a reproducible event that, upon detailed observation, shows coordination between two processes: an increase in cellular mass and phenotypic diversification of the expanding cell populations. Classical studies of organisms with fixed lineages and numbers of cells established that there is an order to these processes in which cells adopt different identities with exquisite spatiotemporal precision in parallel to the increase in numbers[1,2]. In the case of *Caenorhabditis elegans*, the degree of this association is extreme, as the outcome of every cell division is largely predictable in terms of the identity, fate and relative position of the emerging cells[1]. This reproducibility suggests the existence of an underlying programme: a sequence of instructions towards an end point, which in this case is a specific cell type or cell fate. These programmes are implemented by gene regulatory networks (GRNs), which are fundamental units of molecular activity that build and maintain functioning tissues by promoting sequential, and largely irreversible, patterns of gene expression that link genes to cellular lineages[3,4]. The universality of this notion is supported by the observation that tissues and organs of insects and vertebrates, which do not have fixed numbers of cells, also emerge through lineages — in these cases, polyclonal lineages[5] — associated with ordered sequences of gene expression[3,6,7].

Programmes of gene expression are cell autonomous in that they operate independently and reproducibly in individual cells. Examples of this phenomenon are the lineages of stem cells in several tissues[8,9] and, most clearly, the differentiation processes of embryonic stem (ES) cells, which closely resemble the events in embryogenesis[10,11]. A formal consideration of the relationship between cell lineages and genetic programmes during development highlights some essential elements of any process of cell fate specification: a sequence of instructions associated with a given fate, specific decision events, directionality and a means of apportioning defined numbers of cells to particular fates to generate proportionate tissues. The most crucial feature of any cell fate specification process is the mechanism by which cells make a choice, the so-called cell fate decision. For the most part, this process is binary[12] and steers cells to adopt one of two cell states, thus enabling an isogenic population to assume divergent states over time, each with a specific profile of gene expression.

Many attempts have been made to understand the connection between genetic programmes and cell fate decisions in developing organisms and to identify the underlying molecular mechanisms. One of the most enduring is Conrad Waddington's notion of the 'epigenetic landscape' (REFS 13,14), which focused on the activity of single cells and tried to conceptualize the emergence of developmental choices as the result of intrinsic constraints (regulatory interactions) shaped during evolution. With the recent rise in single-cell transcriptional methods and the accompanying statistical and computational analytical tools, we have a greater ability than ever before to begin to unravel some of the complexity of cell fate decisions and to challenge long-standing paradigms.

In this Opinion article, we discuss the relationship between Waddington's epigenetic landscape and genetic programmes in the light of recent advances in genomics and, specifically, single-cell technologies. We focus particularly on the decision events within cell lineages; using examples and illustrations from recent literature, we review the relationship between the data and the abstract representations that exist for their interpretation. We suggest that there might be a fundamental flaw in the reinterpretation of Waddington's landscape in terms of an assumption of continuous trajectories that branch smoothly at cell fate decisions, and instead propose the notion of a transition state as an alternative that accounts for the gene expression heterogeneity in terms of discontinuities in the mechanisms of fate choice.

## Landscapes and dynamical systems

Waddington drew several representations of his famed landscape[15], but two (FIG. 1a,b) have come to be identified with his views on development and evolution. In the first and most popular one, a cell, reimagined as a pebble, begins at the top of a hill and follows existing paths in the landscape driven by a gravitational force that leads it into one of several possible fates represented as valleys. In principle, the decisions about fate are stochastic, so if there are many cells rolling down the landscape they will go one way or another independently. Once a cell makes a decision, it is restricted in its subsequent decisions by the route it has taken, representing decreased cellular potential

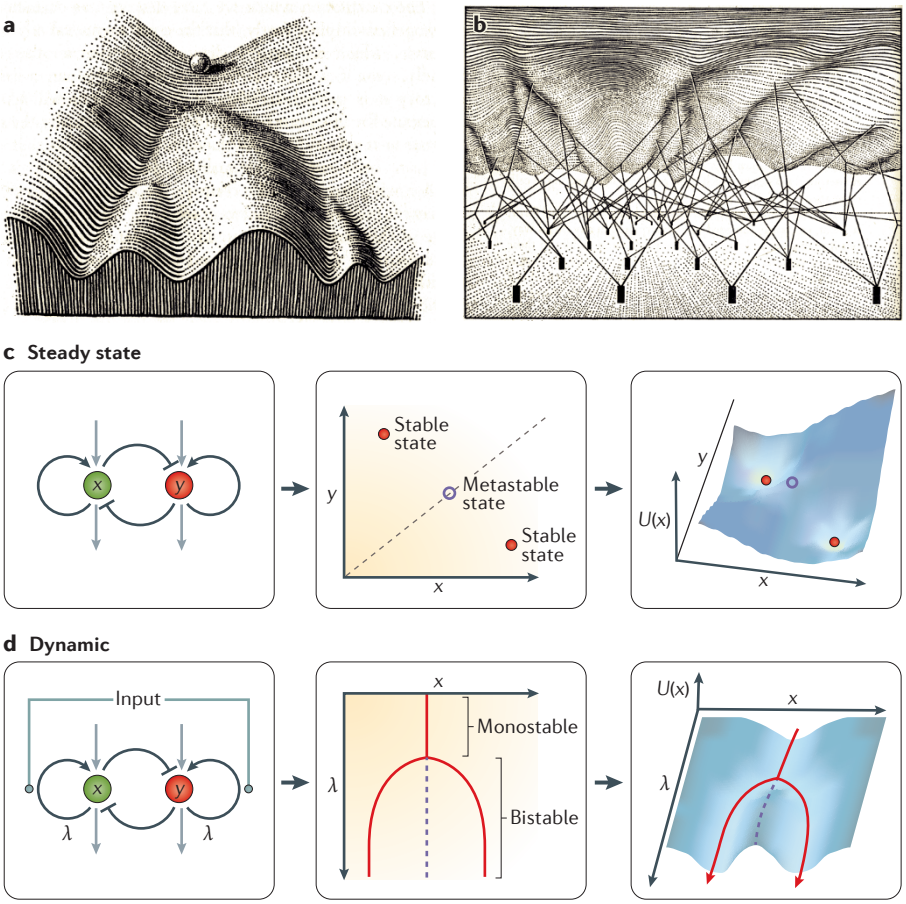


Figure 1 | **Waddington's epigenetic landscape and modern representations. a** | The classical view in which a cell, represented as a pebble, starts at the top of a hill and rolls down the landscape through a series of branching points that represent decision events. **b** | Waddington conceived that the landscape was underpinned by the activity of genes, represented as pegs underneath the hills and valleys. **c** | Cell fate decisions are coordinated through the regulatory interactions between genes (*x* and *y*). Simple network motifs such as the bistable switch (left panel) can be modelled, and phase planes can be drawn, which identify the stable (filled red circles) and metastable (empty purple circle) points of the system (middle panel). With this framework, quasipotential values for each potential state of the system can be calculated and plotted as a third dimension (right panel). **d** | By invoking a time-dependent parameter of the network (λ), the dynamic response of the system can be examined. Such parameter changes represent biological situations in which cellular signalling inputs alter the network and affect fate decisions (left panel). At critical parameter values, the topology of the system can change, for instance by converting a monostable system with one stable point (red line) into a bistable one with two stable (red lines) and one metastable points (purple dashed line; middle panel). Calculating the quasi-potential values in this system can give a dynamic landscape (right panel), which is highly reminiscent of Waddington's original epigenetic landscape. Parts **a** and **b** are from *The Strategy of the Genes*, C. H. Waddington © Allen & Unwin 1957. Reproduced by permission of Taylor & Francis books UK.

and fate restriction. In a second image, Waddington presciently implies that there are genetic regulatory mechanisms responsible for the shape of the landscape. Any modern interpretation of Waddington's landscape needs to consider three elements that are built into his vision: the discreteness of the final states or fates, the smooth paths down which cells travel as they move towards these states and the decision events that occur at binary branches in the landscape.

Although Waddington was aware of some dynamical systems theory, he used the epigenetic landscape more as a metaphor for development, without being explicit about its details. A formal underpinning of the landscape was first suggested by Kaufmann[16,17] in 1969: he formalized the landscape by identifying the valleys with attractors in Boolean networks. Later, Huang and colleagues[18,19] developed this notion further into a multidimensional dynamical systems framework. In this formalism, Waddington's intuition of genetic control of the landscape is brought to light using gene expression profiles projected onto an *n*-dimensional phase space, with vector fields where stable states act as attractors[16,18]. It is these frameworks that have perhaps become most popular when invoking the Waddington landscape as a reference to interpret gene expression data in development and disease.

The epigenetic landscape is without a doubt a compelling and prescient analogy for how the static information encoded in the genome is translated dynamically into tissues and organs and how it is used to drive cell fate decisions. However, despite its popularity, when examined in closer detail, it harbours problems of formalism and interpretation. Although a good metaphor, it is difficult to implement formally; potentials cannot realistically be calculated analytically for more than one or two dimensions[20], so interpretations of the *z* coordinate in graphical outputs need to be treated with care, especially as cellular states are often characterized by differences in many thousands of genes. More specifically, as a system far from chemical equilibrium, with no conservation of energy and in which the energy effort to move from A to B depends on the exact path, rather than the difference in elevation (non-gradient), the true potential landscape is unobtainable and alternatives (discussed later) require some assumptions. Furthermore, an essential feature of developmental processes is their dynamic behaviour, with properties that can make their accurate representation difficult. For instance, developmental systems often show overall directionality with some local reversibility, are associated with high levels of control such that cells can make decisions that are seemingly deterministic rather than stochastic, or display specific temporal dynamics.

The most critical challenge to Waddington's vision arises specifically at the branching points in the landscape, where cell fate decisions take place. In Waddington's representation, although the final states are clearly discrete, at decision points smooth channels simply branch off as states diverge, implying that cells passively 'slide' into the decision event (FIG. 1a,b). Alternative theories posit that the initial and final states do not converge and that the decision is discontinuous[21] or that cells must actively 'jump' to overcome barriers between discrete fates[22]. This distinction has broad implications for the interpretation of experimental data sets at cell fate decisions.

## Gene regulatory networks and cell state

The genetic analysis of development in model organisms led to the identification of genes associated with particular cell fates and, through epistasis analysis, to the arrangement of those genes into 'developmental pathways', such as the segmentation cascade of *Drosophila melanogaster*[23,24] or the vulva pathway in *C. elegans*[25]. The interpretation of these pathways rests on two important assumptions that permeate gene expression analyses of similar processes: first, given that development proceeds over time, the chains of gene activity must reflect continuous temporal sequences of events; and, second, the process of diversification proceeds through binary choices. As different studies uncovered an increasing number of genes involved in particular processes and identified many of these as transcription factors, pathways became linked to the activity of GRNs[3]. In its basic structure, a GRN captures a collection of interactions between transcription factors and their targets, and can be represented in network diagrams with genes or proteins as nodes and their input–output relationships as edges that, when possible, have a directional component[26]. The signs (+ or –) and strengths of the edges are identified either genetically or biochemically and provide a rational description of the regulatory relationships of a system. In its ideal form, a GRN should contain a complete molecular description of a specific process and provide insights into the manner in which multiple transcription factors create a unique 'code' for a particular cell fate and, if possible, for cell fate decisions. However a complete GRN — as the sum total of transcription factors associated with a fate — is impractical to model; just deriving all of the parameters of each interaction across a transcriptome is an insurmountable challenge in most biological systems. Fortunately, there are ways to reduce this complexity and, in the process, potentially identify the elements that drive biological events. The ability to do so lies in the observation that GRNs are organized in hierarchical and highly modular structures[27–29].

Typically, high-throughput transcriptional studies generate large correlational structures, which, although useful for identifying markers or signatures of various cell types, are difficult to link to dynamic processes. Alternatively, such data can be used together with perturbation experiments to identify small, regulatory circuits of the system, often arranged in common structural patterns known as network motifs[26,30], which are far more amenable to modelling. The simplest GRN structure that might give rise to a directional state transition event is linear, whereby a signalling input increases the expression of gene A, which in turn regulates the expression of gene B, and so on, meaning that an input regulates a cascade of downstream genes that results in the equivalent of a 'gene avalanche' (REF. 20). Although this model is sufficient to cause systemic directionality, it proves lacking for other contexts in which signalling, rather than having a singular defined cell-type output, works pleiotropically to regulate diverse fate decisions at varying times during development. Instead of simplistic linear structures, there is evidence for core feedback loops in regulatory networks that control development, effectively creating circular structures that are adapted to produce stable, discrete cellular states[29,31,32]. Thus, GRNs can be constructed as interactomes of network motifs, which provide a more insightful and functional representation of the processes under consideration[30].

## Waddington's potential landscape

An advantage of a network motif formalism of GRNs is that landscapes similar to Waddington's epigenetic landscape can be reconstructed largely using dynamical systems representations (FIG. 1). For example, bistable switches have many of the properties required for stable fate decisions and have been implicated in various systems in which a decision results in two discrete states[33–35]. Such network motifs can be

### Glossary

**Bifurcation theory**
A branch of mathematics associated with dynamical systems that accounts for the evolution of a physical or biological system according to a control parameter.

**Cell fate**
The developmental destination of a cell if left undisturbed in its environment. The fate of a cell is more restricted than its potential.

**Cell states**
The transcriptional output of a gene regulatory network, with a variable degree of stability; development is characterized by sequences of cell states that culminate in specific fates.

**Cellular potential**
Biologically, potentials represent the range of fates into which a cell can develop. It is reduced during development and is obscured in, for example, lineage-tracing experiments, which only reveal fates. In physics, potential can be described as the ability to do work and represents an amount of energy stored for that purpose. In both biology and physics, it represents an ability to do something.

**Dynamical systems**
Systems defined by a number of related variables that evolve in time according to certain rules. A gene regulatory network is an example of a dynamical system in which the variables are the transcription factors that represent the nodes.

**Epistasis analysis**
A genetic technique in which analysis of the phenotype of double mutants allows an ordering of the temporal activity of the wild-type products of the mutated genes. This works best, and often only, in linear processes.

**Gene expression heterogeneity**
Variability in the expression of a gene or a group of genes across a population at single-cell resolution.

**Gene regulatory networks**
(GRNs). GRNs represent units of interacting proteins that are functionally constrained by defined regulatory relationships. These interactions provide a structure and determine an output in the form of a pattern of gene expression. GRNs are usually represented by nodes (proteins) and edges (their interactions).

**Genetic programmes**
Temporally ordered interactions between proteins, usually transcription factors, associated with the emergence of cell types.

**Macrostate**
A notion derived from statistical mechanics that defines the macroscopic state of a system (for example, a particular volume or temperature) and, in the case of a biological system, a functional state. Importantly, a macrostate can be observed and measured.

**Microstate**
A notion derived from statistical mechanics that defines a configuration of the elements that are associated with a particular macrostate of the system: for example, a molecular configuration associated with a particular volume or temperature. Any given macrostate may be associated with many different microstates. We surmise that gene expression profiles can be related to microstates in a biological context. These are often inferred.

**Phase space**
A geometrical representation of the possible states of a dynamical system as a function of the value of its variables. A simple example is the states of water in terms of pressure, temperature and volume. In a cell state, the 'phenotype' is represented by the levels of expression of the genes that are active in that state.

**Pseudotime**
A notion derived from the analysis of single-cell transcriptomes in a cell population that allows the ordering of individual cells based on minimal differences of their transcriptomes. It has an implicit assumption that the resulting order reflects a smooth and continuous change in the state of the cell and aims to relate this change to changes in gene expression.

**Transition state**
An intermediate state during cell fate decisions in which a cell exhibits a mixed identity between two or more states, which often represents the state of origin (that is, the initial state the cell is in) and that of destination (that is, the identity that the cell is adopting). It is highly unstable and reversible.

modelled and projected onto a phase plane that identifies the stable points of the system and the vectors that govern the motion of any state. If one could calculate the potential of every position on the phase plane, an extra dimension could be added that would generate a landscape. However, it is important to remember that for non-equilibrium, non-gradient systems such as genetic networks, it is not possible to obtain a strict potential function[20]. The alternative is to evaluate the steady-state probabilities of each state or to decompose the vector field and approximate the quasipotential landscape[19,20]. Using these methods, a value of quasipotential can be calculated for every state that is inversely correlated to the probability that a cell will assume that state, meaning that local 'low-energy states' are more densely populated[36]. Changes in the expression profile of any cell are then associated with altered coordinate positions, meaning that dynamic state transitions can be directly mapped onto the landscape.

In the extreme, there are two means by which cells can change their state: by stochastic fluctuations that cause cells to jump around the landscape without requiring any parameter changes, or by extrinsic inputs to the system that alter the parameters of the network and change the landscape geometry (the position, shape or size of landscape elements) or topology (the number of landscape elements)[37,38] (FIG. 1d).

Stochastic fluctuations appear to be an intrinsic feature of some biological systems, especially with regard to transcription in which low numbers of transcription factors and DNA molecules can cause sporadic engagement of RNA polymerase and lead to discontinuous mRNA synthesis[39,40], resulting in deviations from the stable attractor state. Usually, these perturbations are small enough that the force of the attractor will draw the system back to its original stable state, but occasionally cells might cross a barrier in the landscape that divides attractor basins, causing a spontaneous state-switching event. Biologically, these events could represent stochastic fluctuations, perhaps of transcription factor levels, over a threshold that proves sufficient to promote cell identity changes[41]. Such events have been described in bacteria, in which they have been shown to have functional significance[42]. Stochastic fluctuations might also apply to stem cells in eukaryotes and, in particular, ES cell populations[43–45]. Such noise-induced transition events can be modulated by the landscape geometry such that deep attractor basins, or high barriers between basins, can reduce the probability of spontaneous transition events and even impose directionality on the transitions[20,46].

Alternatively, the change in state can be associated with a transformation of the landscape, associated with variations in some parameters of the system[38] and often mediated by input stimuli on the networks. In a biological context, these inputs could represent extracellular signalling associated with cell fate decisions, such as fibroblast growth factor (FGF), Nodal or bone morphogenetic protein (BMP) signalling. At critical parameter values, the landscape can change qualitatively: for example, converting a monostable system, with one stable state or attractor, to a bistable system, with two. These dynamical system changes can be related to bifurcations (BOX 1). This formalism is important as it can predict systemic behaviour from an underlying GRN, providing a testable assessment of various models. Identifying which model best approximates the biological behaviour of cell fate decisions requires experimental evidence of sufficient resolution as to be able to test predictions, ideally using dynamic data from single cells.

## Single-cell resolution of fate decisions

Over the past 10 years, there have been remarkable advances in our ability to monitor the transcriptional activity of single cells either for specific sets of genes by multiplexed quantitative reverse transcription PCR or for whole transcriptomes by RNA sequencing (RNA-seq)[47,48]. The output of these experiments is a gene expression matrix of *n* genes across *m* cells that reveals how the genome is expressed within individual cells, and from which the corresponding distribution pattern of expression across a population can be estimated. A complementary technique measures protein levels at single-cell resolution by labelling antibodies with heavy-metal tags (CyTOF)[49]. Applications of these technologies to an ever increasing number of case studies have revealed that although a cell type is indeed largely characterized by differential expression of particular genes, when observed at the level of individual cells, even phenotypically homogeneous cell types display a high degree of heterogeneity in the expression of individual genes. Indeed, the crucial difference between ensemble and single-cell-level transcriptional assays lies resolutely in the ability to observe heterogeneity; the challenge is to interpret this heterogeneity in a meaningful way (reviewed in REFS 48,50,51).

Sources of observed heterogeneity can be broadly divided into two groups: those associated with the experimental protocol or those that are an integral element of the process under study. Although technical noise is indeed a concern in the analysis of the data, accurately quantifying technical noise remains challenging. Nevertheless, there are experimental and analytical means of reducing the impact of such occurrences, including RNA spike-ins of known concentrations, or through application of compensatory models[52,53]. If noise is suitably accounted for, the remaining variability can be associated with biological processes that may or may not have functional relevance. Over the past few years, mouse ES cells have been well studied in this context, and there is now ample evidence that, in this system, broad distributions of many transcription factors have functional significance[44]. For example, in the case of Nanog, its expression distribution has been shown to be dynamic, with individual cells capable of re-forming the original distribution, suggesting some overall control of the heterogeneity[43,54,55]. Importantly, cells with different expression levels of Nanog have different functional capabilities for self-renewal and differentiation[43,56], implying that dynamic heterogeneity is functionally relevant to cellular decision making.

Attempts to ascribe meaning to observed heterogeneities in gene expression, alongside the sheer growth in popularity of single-cell methods, have necessitated the development of statistical and computational analyses that are capable of accessing the wealth of information harboured within the patterns of these heterogeneities. Many of these techniques were borrowed from other disciplines, whereas others have been devised specifically for the application to such data types (TABLE 1). The simplest methods seek to cluster cells on the basis of the similarities of their expression profiles and to distinguish them on the basis of differences, thereby revealing underlying structures within the population. Many of these methods execute weighted dimensionality reductions that turn the *n*-dimensional space into a number of components or dimensions that enable visualization and identification of the population structure. Classification of single cells into clusters by mapping them to gene expression patterns can identify the subpopulation structure within the data set. Clustering analysis of single-cell data is particularly valuable in identifying very rare or transient populations of cells that might not have been previously observable from low-dimensional or ensemble assays[57,58].

Box 1 | **Bifurcations and cell fate decisions**

A gene regulatory network (GRN) can be construed as a dynamical system in which the temporal evolution of the system is a function of the parameters of the network (for example, the rate constants of the gene interactions). An important consequence is the ability to apply 'bifurcation theory', a geometrical formalism that identifies critical parameter values at which qualitative changes in systemic behaviour occur. This is particularly important in the context of fate decisions, as such an analysis could identify the drivers of the system and dynamics of the decision-making process.

There are several types of bifurcation[108], each with specific features that produce different behaviours. Here, we focus on three that are pertinent to the study of cell fate decisions[37,38,94]. The simplest is the supercritical pitchfork bifurcation (see the figure, part **a**), which maps well to the classical Waddington landscape, as it allows for a temporal diversification of cell states by binary fate decisions. Cells in a locally monostable regime on a landscape undergoing a supercritical pitchfork bifurcation would gradually move into one of two new stable states after the bifurcation, and as a result fate decisions appear smooth and continuous (in the mathematical sense), as a cell remains in a stable basin throughout the decision event. Although this bifurcation type is favoured by many models of fate decision, it is crucially unable to account for a key feature of cell fate decisions: their irreversibility[94] (see the main text). In the supercritical pitchfork bifurcation, the temporal dynamics of the landscape depend entirely on the parameter change: if the parameter were reversed, cells from both of the two final states would simply slide back to the initial state. This reversibility is a challenge in the context of cell fate decisions given that state reversibility is not often observed, instead requiring experimental manipulation.

Alternatively, irreversibility can be achieved through a saddle-node bifurcation[94] (see the figure, part **b**). In this case, rather than the creation of new stable states, all stable states pre-exist in the system and a state is removed as the parameter value changes, when an unstable point (the 'saddle') and a stable state (the 'node') meet, effectively destabilizing the initial state. Similarly to the supercritical pitchfork bifurcation, the saddle-node bifurcation is capable of reproducing the temporal progression of cell states, but it also has the intrinsic properties of irreversibility and hysteresis because reversing the parameter levels in a deterministic system will retain cells in their final state, as a barrier between states exists. Importantly, the initial and final states (A and B or C) never converge, meaning that the temporal state change is itself discrete.

A third type of bifurcation, the subcritical pitchfork bifurcation[87,108] (see the figure, part **c**), contains elements of both the supercritical pitchfork bifurcation and the saddle-node bifurcation, and has clear parallels with the cell fate decision process. Like the supercritical pitchfork bifurcation, this bifurcation begins with a locally monostable regime (A) and resolves into a bistable regime (B and C), except that it does so through a transient period of tristability, which is initiated through two saddle-node bifurcations. For a range of parameter values, there is therefore an overlap between the initial and final states. This model has interesting features, including directionality and hysteresis (that is, a cell that transitions to a final state reverts back to the original state at a different parameter value to the one at which it transitioned). But the key feature of this bifurcation is that, in a stochastic system, there will be heterogeneity and local interchangeability between states within the tristable parameter range, as the force of each attractor is weak and corresponding barriers between the states are relatively low. Such transient tristability has previously been linked to cell fate decisions, and could represent a transition state-promoting regime within the system (see the main text). Importantly, the subcritical pitchfork bifurcation also maintains discrete states throughout the range of parameter values (A, B and C), reiterating that the transition is not a gradual or continuous state separation but comprises discrete state-switching events.

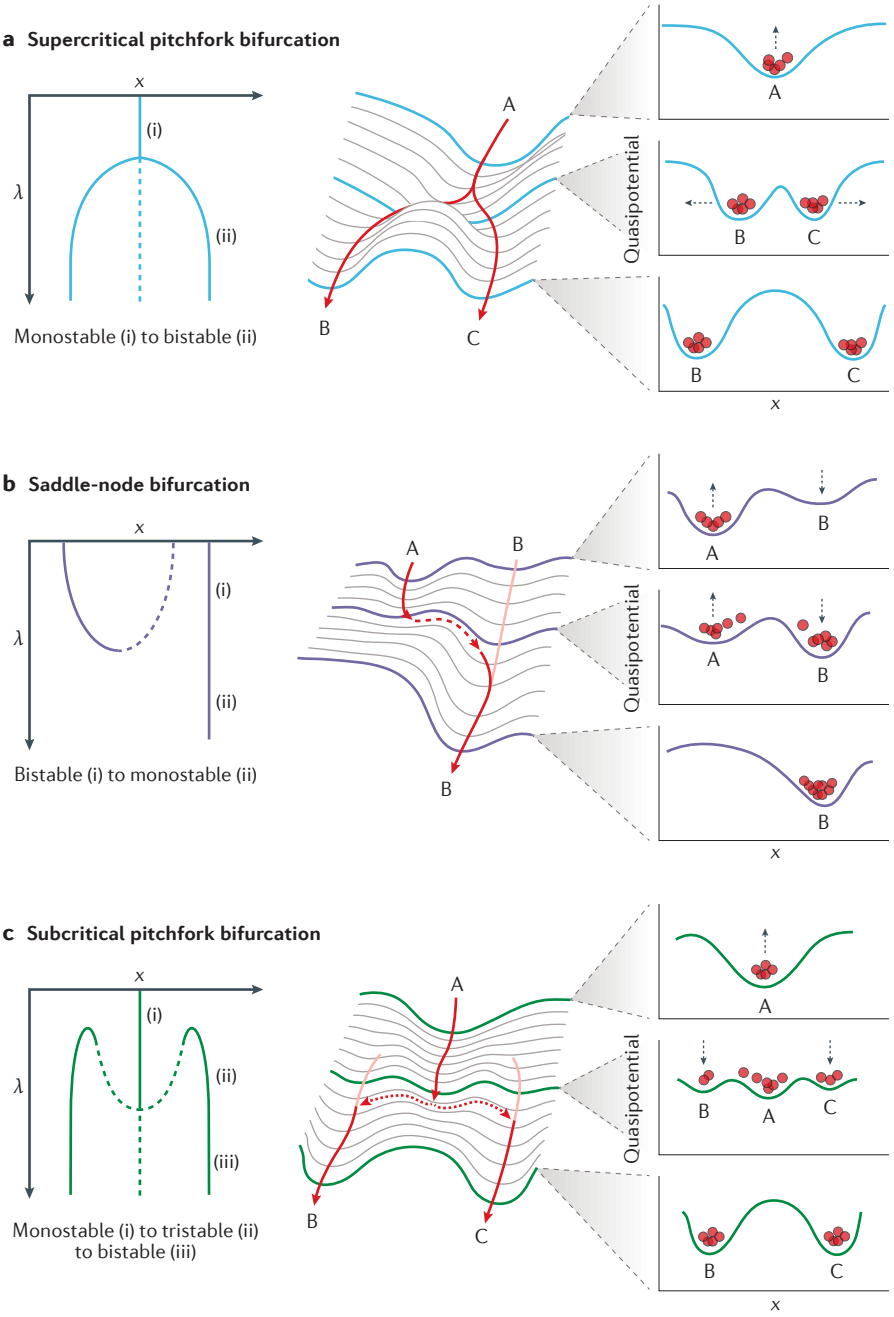

Table 1 | **Single-cell transcriptional analysis tools**

| Application | Tools | Description |
|---|---|---|
| Dimensionality reduction techniques | • Component analysis, including principle (PCA), independent (ICA) and multiresolution (MCA)<br>• Multidimensional scaling (MDS)[109]<br>• t-distributed stochastic neighbour embedding (t-SNE)[110]<br>• Diffusion maps[111] | Reduces multidimensional data to a minimal number of dimensions for visualization by identifying those dimensions that capture the important information in the data structure |
| Gene clustering | Self-organizing maps (SOM)[112,113] | SOMs are unsupervised neural network learning algorithms that organize genes into biologically relevant clusters that can then be compared between samples |
| Clustering methods | Various, including hierarchical, *k*-means, shared-nearest neighbour and many others | A collection of methods that attempt to group observations based on similarity. With single-cell transcriptional data, these can be used to find populations of cells or cohorts of genes |
| Dynamic clustering | Time-variant clustering[114] | Using statistical methods, cells can be spatially clustered at each time point and the relationship of clusters across time points described. Can be used to generate 'branching' patterns of cell clusters over time |
| Trajectory reconstruction | Pseudotime methods, with tools including Wanderlust[68] and Monocle[69] | Orders cells by progress through a dynamic process based on similarity and arranges them into a 'trajectory' |
| Network analysis | Gene regulatory network inference methods[89,115] | These methods seek to identify the underlying gene regulatory network that is responsible for the observed transcriptional patterns |

Subpopulation characterization based on multidimensional gene expression data can also relate back to original estimates of population structure based on alternative methods, to validate or challenge it. In haematopoietic progenitors, sorting strategies based on cell surface markers are often used to capture distinct functional cell types[59,60]. However, a recent study identified new myeloid progenitor subpopulations from single-cell gene expression clusters, suggesting that traditional sorting strategies might result in mixed populations and showing that the transcriptionally defined subpopulations better predicted the functional capacity of cells[61,62]. These findings provide a transcriptional regulatory framework in which to place observations made a decade ago that challenged the routes of lineage specification[63–65] suggested by the classical haematopoietic hierarchy[66].

The power of single-cell transcriptomic data does not only lie in their potential to reveal structures within populations, but in the possibility that these data contain information about the dynamics of the GRNs during fate decisions. Assuming that gene expression profiles provide an estimate of the coordinates for a phase space of the dynamical system that defines the state of a cell, heterogeneities can be used to infer the mechanisms of transitions between different states of a system. The potential of this approach has been shown using a stochastic model of lineage commitment in the haematopoietic system[67]. By using the combined transcriptional patterns of three transcriptional regulators in individual self-renewing and committed cells, the model calculates the probability of transition from self-renewal to the committed state associated with each individual transcriptional pattern, and successfully recapitulates *in silico* the dynamics of a differentiation culture system. In this example, cells assayed came from a source in constant asynchronous flux, such that cells were observable at all points of the dynamic process. This makes a key assumption of ergodicity: that a snapshot of cell identities at one time point is equivalent to a longitudinal observation of one cell over time. In other examples, cells originate from relatively synchronized populations, with observations recorded at different temporal intervals, such as different developmental stages or times after a differentiation cue.

When time-resolved data have been collected, the major challenge is to extract the salient features in a manner that reveals the underlying dynamic processes (FIG. 2). These challenges are ongoing, but progress has been made towards this end with recently devised algorithms derived from dimensionality reduction techniques that have been used to create a sequence of ordered events in a dimension referred to as pseudotime (two of the most common algorithms are Wanderlust[68] and Monocle[69]; see also REF. 70). These methods seek to minimize the variability conferred by cellular heterogeneity by ordering cells by similarity (FIG. 2b). Any heterogeneity of cells during a decision is minimized as average continuous trajectories are drawn. Any remaining observed heterogeneity between cells at a snapshot in time might then be reduced to asynchronous traversal of a fixed pathway or lack of synchrony in decisions. These methods have already been applied to systems including primary human neuroblasts[69], human B cell lymphopoiesis[68] and haematopoietic stem cells[71].

It is unclear how much the output of these methods tells us about the specific process of cell fate decisions, as there is a considerable assumption that influences the pseudotime interpretation — that the transition between states follows a continuous trajectory. This view has been compared to a temporal interpretation of Waddington's landscape, but it is not implicit in the original formulation of the landscape and need not reflect the actual course of events, particularly decision events in which changes of state might be mediated by discontinuous mechanisms. These mechanisms would be obscured by methods attempting to linearize heterogeneities into a continuous, convergent pathway.

## The transition state

Pioneering single-cell studies of fate decisions in the haematopoietic system revealed that cells with multilineage potential could co-express genes typically associated with each of their alternative lineage fates[72]. For the most part, expression was infrequent and at low levels that varied from cell to cell[72]. Similar observations have since been made in several different systems[73–76], specifically in single cells within populations undergoing fate changes[77,78] and, notably, in stem cell populations in which these genes are thought to have a role in the balance between differentiation and self-renewal[79–81]. These observations suggest that heterogeneities might represent a general feature of changes in state[44,82,83] and have led to the proposal that heterogeneous patterns of expression are associated with the cell fate decision event at the level of single cells[44,72,84–86]. The varied expression of genes associated with each alternative fate in single cells can act as a

substrate for selection by signals[87], or as an exploration of a phase space where changes in the levels of the regulators can lead to stochastic fate change[67,81]. In either case, when cells make a decision, they upregulate the expression levels of the gene cohort of their chosen fate and downregulate those of the alternative one, as has been seen in many systems[88,89].

In the context of the premise that fate decisions typically occur between discrete states (that is, attractor states), the heterogeneities in gene expression as cells change state suggest a general principle: cells within a discrete attractor state may experience a degree of transcriptional stochasticity that can, with different probabilities, result in various transcriptional profiles characterized by the expression of genes associated with one or more independent cell identities. Such expression profiles, which are transient, distinct from cell to cell and manifest as heterogeneities at the population level, endow cells with varied probabilities of effecting a cell fate transition. We have called this collection of transcriptional profiles a 'transition state', and suggest that it represents a substrate for cell fate decisions by facilitating state switching while retaining a reverse transition probability (FIG. 3). An important element in the notion of the transition state is that the passage from one state to another need not be smooth and continuous as portrayed in representations of pseudotime. Furthermore, a transition state can be characterized by bistability or, in certain instances, tristability, because a cell can have access to either two or, if they still express some genes of the initial state, three states.

The term 'transition state' refers to an analogy with the well-known transition state in chemistry, which was introduced to provide a mechanistic underpinning for the progress of a chemical reaction[90]. The induction of a reaction between two substances, by a new reactant or a catalyst, triggers a short-lived intermediate in which the molecules involved engage into a number of configurations exhibiting intermediate characteristics between reactants and products. It represents a state of maximal potential energy as a chemical potential barrier is traversed, often using catalytic factors to reduce the intervening barrier height (FIG. 3a). In an analogous manner, we surmise that during a fate transition, a GRN can be seen as a reactant that receives a new input, from a signal or from crossing a threshold of its own activity, that leads to either a new network or to a new pattern

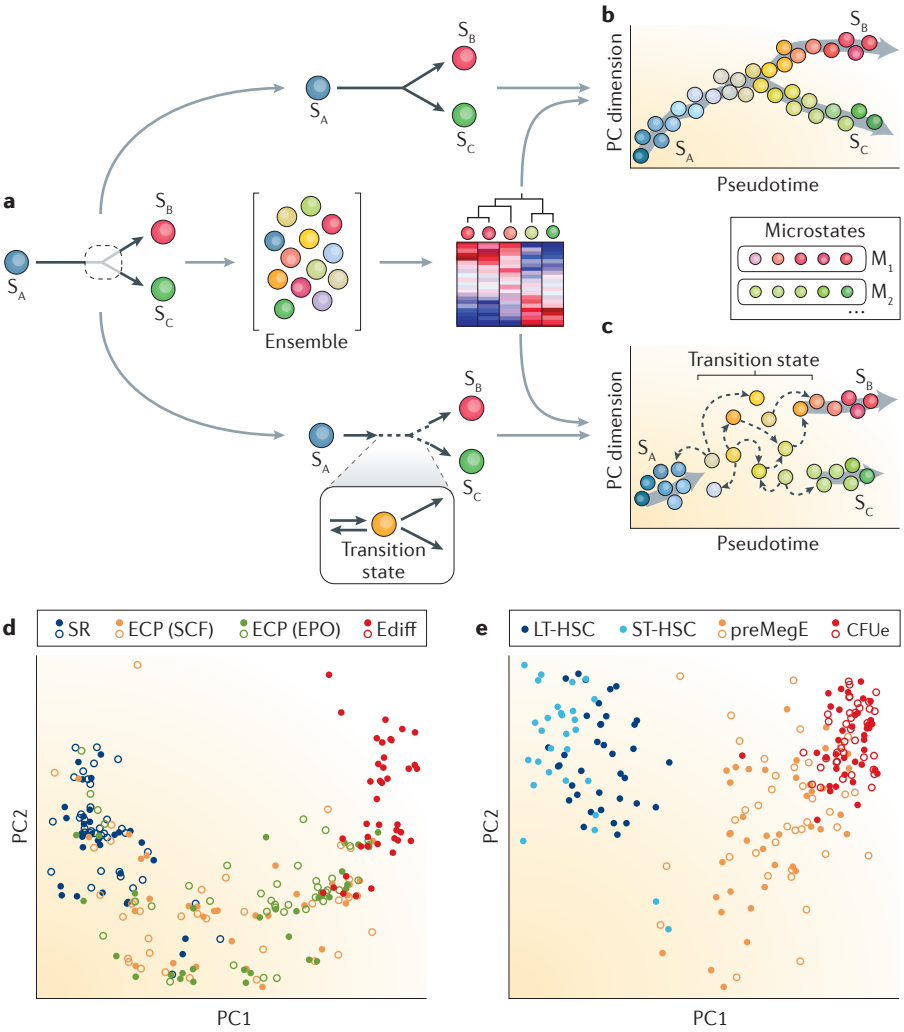


Figure 2 | **Continuous and discrete analysis of cell fate decisions from single-cell gene expression data. a** | In a fate transition, a cell may traverse the transcriptional phase space in a smooth, continuous manner before reaching a decision point. Single-cell transcriptional data from cell ensembles can provide a snapshot of the population structure, and transcriptional profiles of each cell can be arranged into hierarchical clusters of varying similarity, but the data might also contain information about the dynamics and mechanisms of the decision event. There are two alternative interpretations of this information. **b** | If the transition between states is assumed to be continuous, cells are ordered along a sequence that assumes that more similar cells should be closer together on the trajectory of differentiation; a sequence often referred to as 'pseudotime'. **c** | Instead, if the transition between states is deemed to be discontinuous, as in the case of the transition state, cells might cluster into a number of functional 'macrostates' (shown as $M_1$ and $M_2$), each with a number of corresponding 'microstates' (see the main text for details). Each of these microstates has a different probability of transitioning to another state, but this is not necessarily directly correlated to similarities between a given microstate and the final state. Although there may be continuous processes leading up to and after decision events, the decision itself is discontinuous and stochastic, in which each state has an associated probability of transitioning to any other state. **d**,**e** | Experimental evidence of a broad transcriptional space in haematopoietic cells undergoing commitment decisions. Principal component (PC) analysis plots of single-cell quantitative reverse transcription PCR data for cultured, erythroid myeloid lymphoid (EML; part **d**) and primary mouse bone marrow (BM; part **e**) cells undergoing erythroid commitment and differentiation decisions. Plots are a comprehensive representation of the data in Pina *et al.*[81], and highlight the point that early committed cells (erythroid-committed population (ECP) obtained in two distinct cytokine conditions, in the case of EML and pre-megakaryocytic/erythroid progenitors (preMegE), in the case of primary BM) are more heterogeneous in their transcriptional programmes than the multipotent self-renewing cells (SR; such as EML, BM, long-term reconstituting haematopoietic stem cells (LT-HSCs) and short-term HSCs) they originate from, or the differentiated progeny (EML, differentiated erythroid cells (Ediff); BM and colony-forming unit erythroid (CFUe)) they give rise to. A similar observation was made by Jaenisch and collaborators[77] when analysing transcriptional programmes of individual cells undergoing early fate transitions in induced pluripotent stem cell reprogramming (see REF. 81 for details). EPO, erythropoietin; SCF, stem cell factor.

of connectivity of the existing network. By analogy with a chemical reaction, the cell fate decision process has an intermediate during which the networks explore the state space (FIG. 3b), and this manifests as heterogeneities in gene expression at the level of single cells. Although the analogy between the chemical and biological transition states holds value in its description of an intermediate state, care should be taken not to extend the analogy too far, especially as the biological transition state is limited by its thermodynamic properties that prevent a formal calculation of potential.

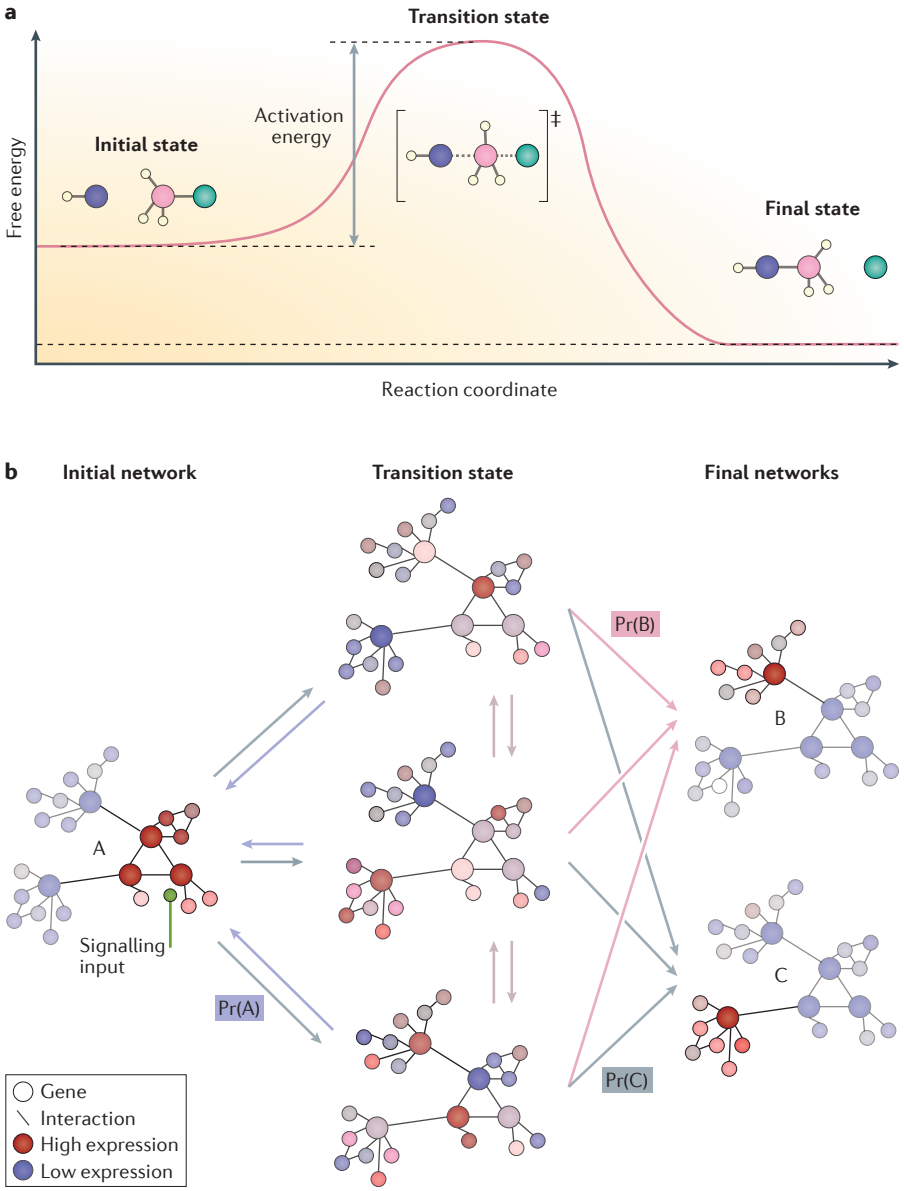


Figure 3 | **The transition state. a** | In chemistry, a transition state is a short-lived intermediate that arises in a chemical reaction, in which reactant atoms assume a configuration that is intermediate between the initial and final products and which explores an available space of potential energy. In endergonic reactions, the free energy of the initial state must overcome an activation energy before assuming the lower-energy final state with the products of the reaction. Additional components such as catalysts can alter the height of the activation energy, changing the likelihood of transition. **b** | By analogy, biological gene regulatory networks could be deemed to undergo similar transition dynamics as they make cell fate decisions. The activation of a specific node within a gene regulatory network leads the system to assume a transition state, in which a number of network states are represented that are intermediate between the initial and final states of the network. Each has identical network structure, but with different gene expression levels (represented by node colour) and with an associated probability (Pr) of transitioning to any other state, including a transition back to the original state. We surmise that the transition state represents a heterogeneous substrate for network selection and fate decisions by factors such as signalling pathways.

A corollary of the notion of the transition state is that any given phenotypic state might not be associated with just one network state. If GRNs involved in cell fate decisions are hierarchical, it might be that there are key configurations of specific network motifs or small networks that are sufficient to trigger a particular state, independently of additional or downstream gene activity. Pursuing a chemical analogy further, a cell state or fate can be described by its macrostate (the core GRN connectivity that gives rise to a specific phenotypic state), which can be represented by more than one microstate (one of many connectivities of a network compatible with a particular macrostate)[21,91] (FIG. 2c). The number of potential microstates depends on the number of nodes of the network, but whether a cell will assume any given microstate is dependent on the network configuration and its biological relevance (for instance, a cell can only assume a positive value of a transcription factor level). In a transition state, cells may explore a higher number of microstates. This notion has been formally discussed in the context of mouse ES cells[21] but is, in principle, applicable to any cell fate transition.

***Landscapes and transition states.*** The notion of the transition state leads to the corollary that heterogeneities in gene expression, which are observed with single-cell transcriptomics in systems undergoing fate decisions, need not align through time along an ordered continuum, and could instead be interpreted as a reflection of systemic features that allow a cell to stochastically rearrange its networks by exploring the local phase space. We do not venture to suggest that cellular trajectories do not exist at all; merely that at the decision event, the actual transitions between states might be discontinuous, and that the observed transcriptional heterogeneity reflects the existence of a dynamic array of transcriptional states, with varied probability of transitions. It is highly likely that downstream of the decision, external signalling or intrinsic programmes of the GRN result in continuous trajectories through geometric landscape changes that lead to the next decision point in a manner that is consistent with the temporal continuity implicit in the notion of pseudotime. The distinction between the two notions arises at the point at which cellular decisions are made (FIG. 2). There are hints that discontinuous fate decisions do

occur in some contexts: for instance, in the haematopoietic system, particularly in the case of early myelo-erythroid choices in mouse haematopoiesis[81] and in human haematopoietic stem cells that can give rise to differentiated cells directly[92] without going through multilineage intermediates. But the framework could be applied to many binary decisions, such as the neuromesodermal progenitor in the mammalian embryo, which generates neural and mesodermal progenitors from a common precursor that co-expresses neural and mesodermal genes[93].

The notion of a 'state' (the transition state), made up of a collection of cells, each with a different transcriptional profile, that are disconnected temporally is, at first sight, at odds with the intuitive, smooth and continuous interpretation derived from a cursory examination of Waddington's landscapes. However, by representing the process in terms of a computable dynamical system with parameter changes, we can apply bifurcation theory to examine systemic behaviours[37,38,94], particularly focusing on bifurcation events (BOX 1). In this framework, a subcritical pitchfork bifurcation[38] (BOX 1) captures many of the features of cell fate decisions that we have described as a transition state. Namely, the specific range of parameter values conferring tristability could correspond to a transient 'window' during which transitions are promoted; the system assumes a permissive topology in which both the initial and final states stably coexist and transcriptional exploration is favoured as the landscape assumes relatively high-energy (quasipotential) stable states with low intervening barriers. This bears a clear resemblance to our proposed transition state, as the landscape promotes increased heterogeneity across the population owing to the relatively low quasipotential force of each attractor. Like the supercritical pitchfork bifurcation (BOX 1), the initial and final states are discrete, although the subcritical pitchfork bifurcation suggests that transitions might occur through state-switching events rather than continuous divergence of states as implicated in Waddington's epigenetic landscape.

Importantly, the bifurcation diagram as drawn in FIG. 1d does not represent the evolution of the system as a function of time but as a function of a parameter or parameters that could themselves also vary with time, not necessarily in a deterministic or linear manner[37]. An example of these parameters could be extracellular signals. This means that the same system could easily be tuned to different circumstances — for instance, by applying high cooperativity to the input that alters the system's parameter — which would effectively reduce the time the system spends in the tristable regime. The inverse is also possible, with certain systems potentially tuned to elongate this transition-promoting phase, a possible example of which is ES cells. These cells exist in a pluripotent state that, in the developing embryo, represents the transition to differentiation, which is very short lived and not renewed, but which is stabilized and maintained indefinitely in certain culture conditions[95,96]. These cells might then be described essentially as a 'trapped' transition state[12,85,97].

***A molecular interpretation of the transition state.*** At the molecular level, a cell fate decision necessarily involves a change in the transcriptional state of a cell. Gene transcription requires a close interaction between three sets of components (each of which involves large multiprotein complexes): transcription factors that define a state by promoting spatiotemporal control of gene expression; histone modifiers and chromatin remodellers, which determine the accessibility of the transcription factors to the DNA and their binding stability; and the basal transcriptional machinery, which executes the transcriptional process and, to a first approximation, is unlikely to vary throughout the process. Proteins that interact with each and any of these machines have the potential to change the coordinates of the transcriptional state of a cell, but the effects, and the type of input that an individual protein will have on the process, are different in each case. For example, transcription factors can define the cellular state by activating specific genes and thus define which networks will be active; this could be a noisy process if the numbers of molecules of the participant proteins are limiting and vary from cell to cell[39,40,98–100], thus creating a substrate for regulation. The effectors of BMP–transforming growth factor-β (TGFβ) signalling, the SMAD proteins, would fall into this class, and their activity may result in a change in the parameters of the GRN as on their own they have weak outputs that are stabilized by association with other transcription factors[101,102] that change their binding kinetics and, by extension, the associated landscape. This is an example of how an interaction between the intrinsic cellular state (which is defined by extant transcription factors) and extrinsic signals (BMPs and TGFβ in this case) can sculpt the outline of the landscape in a synergistic manner: the interaction between the two results in the expression of a set of genes that neither of them alone can activate, and thus changes the landscape.

The role of the other components in fate decisions is less specific. They do not determine a cell state per se, but rather govern the robustness of the transcriptional process by modulating the binding kinetics of particular transcription factors (chromatin modifiers) or altering the frequency and amplitude of the transcriptional process (basal transcriptional machinery). Thus, any event that targets any of these will affect the efficiency of transition between states in individual cells[45]. In landscape terms, such interactions might change the potential barrier (that is, act as the catalysts in a chemical reaction) and, on the basis of their strength, determine the number of cells that would change state over time. We have suggested that WNT–β-catenin and FGF–extracellular signalling-regulated kinase (ERK) signalling pathways act in this manner[12,41,45]. This level of regulation is particularly important at the decision events and would allow tuning of the number of cells making fate decisions.

***The transition state in developmental populations.*** A valuable feature of the proposed framework is that it provides a mechanism for the control over the size of any given population, because some of the variables can act to bias the landscape towards certain fate decisions. This is because although decisions are taken at the level of single cells, at the population level, biases in the bifurcation landscape will result in differences in the number of cells apportioned to each state. Extrinsic factors (for example, signalling) or intrinsic factors (for example, basal transcriptional machinery and chromatin modifiers) that bias state-space exploration towards specific transcriptional states, or that change the probability of transition associated with each individual state, can ultimately alter the final number of differentiated cells and their relative proportions. This poses a potentially useful framework for the homeostatic regulation of the size of a population[86].

An example of this situation can be found in the partitioning of the inner cell mass of the pre-implantation mouse embryo into the embryonic epiblast and the extra-embryonic primitive endoderm. This binary decision occurs in a population of cells deemed to be in a transition state, as cells express variable levels of genes associated with both fates[103–105]. The heterogeneities are resolved through the activity of a small transcriptional network

involving GATA factors and Nanog, which is modulated by FGF signalling. The decision can be recapitulated in ES cells, in which the mechanisms involved in the decision can be explored in detail[41]. Modelling of this decision has suggested that it is driven by a tristable network[106,107] that can be reduced to a bistable one[41], which accounts for most of the observations. The outcome is two populations with balanced cell numbers that will interact later in development to steer the patterning of the embryo. How the precise and reproducible partitioning is coordinated is not fully understood, but there is evidence that it is achieved through time integration of FGF signalling by the transcriptional network. A population in a transition state is an ideal substrate for this integration, as it has an a priori equal probability of each cell adopting one of the two alternative fates. This system acts as a good model for similar decisions that occur during development and which result in precise and robust partitioning of populations.

## Perspectives

It will be interesting to see how general and useful the notion of the transition state is, not only in terms of its ability to represent data but, importantly, in its ability to frame the role and activity of signals at the decision events. In most representations of developmental decisions, signals simply contribute another node to the network, but in our view they represent parameters that can change the structure and the dynamics of the landscape that cells explore. To test these ideas, we shall have to go beyond the analysis of snapshot gene expression data and explore the dynamics of individual genes within individual cells. A validation of the hypothesis of the transition state will probably require a combination of quantitative live imaging of cellular dynamics through a decision event, with concurrent measurement of multiple genes predicted to be indicative of transition events. In the case of a continuous transition, as portrayed by pseudotime, cells at the same point of the fate transition will be transcriptionally very similar to each other, whereas in the case of a discontinuous one, they should be very dissimilar. By observing cells at different stages, according to the continuous pseudotime representation, all sampled intermediates between functional states will follow an identical sequence of cellular states. By contrast, the transition state theory predicts that cells will transition between states through a diverse array of dynamic mechanisms (FIG. 2).

We believe that the discussion of the contrasting frameworks that we have raised is important, as what is at stake is not simply a representation of the data derived from single-cell analysis, but the mechanisms underlying fate decisions and their impact on assigning different fates to cell populations, which create the building blocks for tissues and organs. In addition, we hope that this discussion will lead to reconsideration of the role of signals in fate decisions and how they impinge on the dynamics of cell populations rather than just on the fates during development. Importantly, we want to encourage an open-minded view of Waddington's epigenetic landscape, which, over the past few years, has become an icon for cell fate decisions in homeostasis and development.

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**Competing interests statement**
The authors declare no competing interests.