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Cell dynamics and gene expression control in tissue homeostasis and development

Pau Rué & Alfonso Martinez Arias*

Abstract

During tissue and organ development and maintenance, the dynamic regulation of cellular proliferation and differentiation allows cells to build highly elaborate structures. The development of the vertebrate retina or the maintenance of adult intestinal crypts, for instance, involves the arrangement of newly created cells with different phenotypes, the proportions of which need to be tightly controlled. While some of the basic principles underlying these processes developing and maintaining these organs are known, much remains to be learnt from how cells encode the necessary information and use it to attain those complex but reproducible arrangements. Here, we review the current knowledge on the principles underlying cell population dynamics during tissue development and homeostasis. In particular, we discuss how stochastic fate assignment, cell division, feedback control and cellular transition states interact during organ and tissue development and maintenance in multicellular organisms. We propose a framework, involving the existence of a transition state in which cells are more susceptible to signals that can affect their gene expression state and influence their cell fate decisions. This framework, which also applies to systems much more amenable to quantitative analysis like differentiating embryonic stem cells, links gene expression programmes with cell population dynamics.

Keywords development; differentiation; homeostasis; stochastic cell fate; transition state

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Introduction

The total number of cells of an organism is a tightly regulated variable. For instance, a human being contains more than $10^{13}$ cells (Bianconi et al., 2013), while a male Caenorhabditis elegans nematode worm is composed of exactly 1,031 cells (Sulston, 1976; Sulston & Horvitz, 1977). These cells are organized in tissues and organs with two important properties. First, their final size is well defined, emerges during development and is species specific denoting the existence of an ‘internally driven final state’ (García-Bellido & de Celis, 1992; García-Bellido, 2009), which represents a growth target during development and therefore requires precisely organized proliferation of cells. Second, although most tissues are subject to a continuous loss of cellular mass due to wear and tear (Spalding et al., 2005), their size is maintained approximately constant for the lifetime of the organism and, in many cases, can be restored in response to severe injury. This second property suggests the existence of a self-regulated steady state or ‘homeostasis’ that keeps the number of cells constant through a balance of cell loss and proliferation. In both cases, it is not only the total number of cells that is accounted for but also their identities which, in many instances, need to be balanced for the correct function of tissues or organs. While knowing the genes associated with these events is important, it is the link between the genes and the population dynamics that will allow elucidating the molecular mechanisms that underlie these systems.

Here, we review the current knowledge on the dynamics of cell populations during homeostasis, highlight recent findings on universal patterns associated with this process and explore whether and how can these be extrapolated to developing tissues. We conclude that a key element of cell populations in homeostasis and development is the regulation of the dynamics of gene expression during the transition between different fates. This regulation takes place at the level of single cells and acts on what we call the ‘transition state’. This state provides the substrate to link population cell dynamics and gene expression.

Cell division and differentiation: basic mechanisms and principles

There are three ways to coordinate cell division and cell identity/fate. The first one is exemplified by eutelic organisms like C. elegans, in which every division is associated with the generation of two different cells and this process is iterated over time (Sulston, 1976; Sulston & Horvitz, 1977). The number of divisions is exquisitely regulated, such that each tissue is the result of a defined lineage built from a sequence of asymmetric cell divisions, that is, each gives rise to two different cells, and underpinned by a hardwired genetic programme (Gönczy, 2008; Knoblich, 2008). This strategy can also be found in other systems like the embryonic central nervous system (CNS) of insects (Kohwi & Doe, 2013) where each neuroblast...
sequentially divides asymmetrically to self-renew and generates a differentiating ganglion mother cell, which can further divide and generate two differentiated neural cells (Fig 1A). The whole process is associated with a gene expression programme running on each neuroblast, which involves the sequential expression of Hunchback, Kruppel, Pdm and Cas. These highly deterministic systems are
usually associated with small and fast-developing embryos and have little regulative capacity: when a cell is lost, it is not replenished. At the other extreme, there are situations in which a group of cells make copies of themselves over a period of time and are given specific identities as the tissue grows by virtue of global cues, for example the imaginal discs of *Drosophila* (Wartlick et al., 2011). In these cases, there is no recognizable pattern relating cellular proliferation and fate assignment. In between these two extremes, there is a collection of dynamic behaviours exemplified by systems of growth driven by stem/progenitor cells, which divide asymmetrically to generate a cell that remains undifferentiated and thus sustains a naive state, and another cell that differentiates (Fig 1B). In some instances, in tissues with wear and tear, these same cell populations maintain tissue homeostasis and have received increased attention over the last few years (Pellettiieri & Sánchez Alvarado, 2007; Simons & Clevers, 2011).

The possible interactions between the dynamics of cell proliferation and differentiation allow for a rich collection of behaviours that are exploited in the biology of organs and tissues and that can be modelled using simple mathematical models (see Box 1). These models indicate that, to fulfil the requirements of developing and homeostatic systems, the rates of proliferation and differentiation need to be extremely well balanced and coupled to the events that determine cell fates.

**From single cells to populations: the structure and dynamics of adult stem cell populations**

**Stochastic structure of clonal growth in homeostatic systems**

Strictly deterministic lineages cannot easily explain homeostasis in the case of tissues consisting of large and indeterminate numbers of cells that, nevertheless, maintain a defined size, such as the haematopoietic system. Every day an average human being replenishes 1% of the red blood cells in the bloodstream. This represents $>10^9$ cells, each of which is the result of a carefully controlled lineage tree that contributes to maintaining homeostasis (Bryder et al., 2006). Part of the answer to how this is achieved lies in the work of J. Till and E. McCulloch in the 60s (Till & McCulloch, 1961), who restored the ability of irradiated mice to make blood, by injecting bone marrow cells. In these experiments, Till and McCulloch observed in the spleen of the injected mice the appearance of colonies resulting from a founding effect of haematopoietic stem cells (SCs). The colonies exhibited a large disparity in the number of cells, and this led them to propose a stochastic birth and death model for SC activity (Till et al., 1963, see Box 1), namely each dividing SC would either give rise to two SCs (birth event) or differentiate and exit self-renewal (death event, see Box 1). This simple model, based on the assumption that there might be loose control of cell fate assignment during division, was able to explain the observed variability in the number of stem cells per colony. However, the model also illustrated the need for a control mechanism that regulates the probabilities of division versus differentiation at the population level: any slight divergence between proliferation and loss would lead in the short term to an exponentially growing imbalance.

Rapidly cycling ‘solid tissues’, such as skin, the intestine or the epithelium covering mucous membranes, also require a constant supply of differentiated cells (DD) in defined proportions. Analysis of these tissues (Leblond, 1981; Potten & Loeffler, 1990) led to a general model, the SC/TA model, in which a population of slow-cycling SCs maintains a population of rapidly dividing progenitor cells, the transit-amplifying (TA) compartment (Fig 1C), that protects the SCs from being used up and serves as a substrate for differentiation (Potten, 1974, 1981). Further considerations in the context of the population size that these cells maintain suggested the existence of *population asymmetry*, with some cells differentiating and others dividing symmetrically to generate two proliferative cells that could complement strictly asymmetric cell divisions at the level of single cells (Watt & Hogan, 2000).

A long-term study of the time evolution of clones in the skin of the mouse tail failed to support the well-established SC/TA compartment model and established a paradigm which is a universal feature of homeostatic systems (Clayton et al., 2007). Instead of confined finite-sized growth compartments, as predicted by the SC/TA model (Potten, 1974), the study revealed large variation in the number of clones and their individual size. Furthermore, the experiment revealed a continuous loss of clones that was counterbalanced by the expansion of the surviving clones, which, on average, expanded at a constant rate. A crucial observation was that the shape of the long-term distribution of clone sizes is time invariant, even though it becomes stretched according to the average size of persisting clones, that is, the clone distribution scales. Such scaling behaviour in the distribution of clone sizes is a signature of phenomena undergoing neutral drift dynamics, whereby random loss of clones due to depletion of stem cells is balanced with expanding clones (Box 1, Klein & Simons, 2011). This led to the suggestion that rather than being maintained by a population of slow-cycling stem cells, the skin of the mouse tail is maintained by a population of progenitors which divide stochastically to generate one of the three outcomes: two progenitors (PP), two DD or one of each (PD) with fixed probabilities. The stochastic structure of clonal growth is reminiscent of the notions introduced by Till and McCulloch, but the realization of the clone scaling and the calculation of the ratios of the different division types indicate the existence of a simple and reproducible process underlying the renewal of this tissue.

Similar observations have since been made in other homeostatic systems in vertebrates (Klein et al., 2010; Lopez-Garcia et al., 2010; Snipperl et al., 2010; Kent et al., 2013; Kozar et al., 2013) as well as in *Drosophila* (De Navascués et al., 2012) suggesting the existence of universal patterns, that is, robust signatures shared between systems, of stem cell self-renewal (see Box 1 for details). This means that the shape of the size distribution for each of these classes is fixed by the particular mode of cellular balance and the geometry of the tissue (see Klein & Simons, 2011 for a detailed review).

**Models of stem and progenitor cell dynamics**

These observations suggest the existence of competition between equipotent progenitors, leading to a neutral drift of the clones similarly to allele drift in a population (Kimura, 1984). The models indicate that analysing the long-term behaviour of the distribution of clone numbers and sizes reveals the neutral drift (Box 1). Random drift of equipotent clones can also lead to clone fixation if the number of cells in a population is small. Monoclonality has been observed in the mammalian intestine, a tissue that has become a benchmark to analyse the dynamics of stem cell populations in homeostasis (Lopez-Garcia et al., 2010; Snipperl et al., 2010). The continuous loss of cells from the villi on the intestine membrane is compensated by constant...
Box 1: Symmetric/asymmetric cell divisions and stochastic differentiation of homeostasis

Stochastic models of stem/progenitor cell dynamics

**A Population asymmetry model**

Division mode | Probability
--- | ---
| p | q | r

\[ p + q + r = 1 \]

**B Distribution of clone sizes**

Population asymmetry

<table>
<thead>
<tr>
<th>Time</th>
<th># clones</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clone size</td>
<td></td>
</tr>
</tbody>
</table>

Invariant asymmetry

<table>
<thead>
<tr>
<th>Time</th>
<th># clones</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clone size</td>
<td></td>
</tr>
</tbody>
</table>

**C**

<table>
<thead>
<tr>
<th>Time</th>
<th>Average clone size</th>
</tr>
</thead>
<tbody>
<tr>
<td>Scaled clone size</td>
<td></td>
</tr>
</tbody>
</table>

**D**

<table>
<thead>
<tr>
<th>Time</th>
<th>Scaled # clones</th>
</tr>
</thead>
<tbody>
<tr>
<td>Scaled clone size</td>
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</table>

**E Neutral drift**

Epithelium

Intestinal crypts

Homeostatic stem and progenitor cell populations are examples of indeterminate systems in which cells can divide and differentiate continuously. According to the fate adopted by the daughter cells, their divisions can be classified as symmetric proliferating (PP), symmetric differentiating (DD) or asymmetric (PD) (Potten & Loeffler, 1990) with a distribution of frequencies \( p, q \) and \( r \), respectively (\( p + q + r = 1 \), see panel A), that can be estimated experimentally. The outcome of each individual division is a priori unpredictable and thus can be deemed to be stochastic. The model that results from these considerations is known as the population asymmetry model (panel A). The dynamics of these simple rules of division and differentiation can be accounted statistically by means of branching processes as first introduced by Till, McCulloch and Siminovich (Till et al., 1963), whom only considered...
replenishment from a stem cell population located in crypts at the base of each villus. This population has a very well defined structure, with only a few active SCs giving rise to TA cells, which further differentiate into, among others, enterocytes and secretory cells (Lopez-Garcia et al., 2010; Snippert et al., 2010). Clone labelling of mice intestinal stem cells confirmed prior evidence that crypt SC clonality is attained in just a few weeks (Griffiths et al., 1988; Winton et al., 1988) and provided quantitative insights into the dynamics of the process. Interestingly, a simple model with a finite number of stem cells undergoing stochastic division can accurately account for the fraction of crypts that become monoclonal over time (Snippert et al., 2010). Continuous clonal labelling also indicates that at any given time, only a subset of stem cells in each crypt contributes to tissue homeostasis. The actively dividing stem cells per crypt turn out to be as few as five to seven, which roughly corresponds to 30–50% of the lower crypt (Kozar et al., 2013). Moreover, their replacement rate is kept approximately constant for at least 2 years of age, indicating that such cells do not suffer exhaustion (Kozar et al., 2013).

In these models, the probabilities of each class of division are either deemed to be intrinsic to the cells or locally balanced by means of cell-extrinsic mechanisms (Klein & Simons, 2011). In either case, the models indicate that the fates of the two daughter cells in each division are somehow linked: the frequencies of PP, PD and DD divisions predicted by the models are far from what would be expected if the fates of the two cells were independently assigned. These models are stochastic and, in homeostatic conditions, require an exquisite balance of the parameters controlling the cellular dynamics (i.e. are poised at criticality). Furthermore, they assume that the drivers of the system are cell intrinsic and do not address the associated biochemical mechanisms. However, the observation that, when perturbed, these experimental systems respond by changing parameters such as the fraction of PP divisions suggests that there exist control mechanisms that might go beyond those considered implicitly in these models. As discussed in the context of the olfactory epithelium in Box 2, these control systems might be central to the dynamics of the population and provide a link with the molecular and signalling mechanisms that underlie the process (Lander et al., 2009).

### Molecular mechanisms underlying homeostatic systems

**The existence of a ‘transition state’**

Accounting for the dynamics of homeostatic populations does not explain how different fates arise, or how they are maintained and propagated. Understanding this requires a description of the molecular events associated with fate assignment in those lineages and, more specifically, with the maintenance and differentiation of stem cell populations. A first approximation to this problem is currently being pursued by isolating stem and progenitor cells from different systems and performing single-cell gene expression studies. To date, these studies have failed to identify stereotypic profiles specific to stem cells and instead have revealed broad heterogeneous distributions of gene expression often associated with the tissue to which the given stem cell contributes (e.g. Tang et al., 2010; Izkovitz et al., 2012b; Muñoz et al., 2012; Pina et al., 2012; Guo et al., 2013; Kent et al., 2013; Tan et al., 2013; Yan et al., 2013; Turner et al., 2014a). In some instances, such as the mammalian intestine and the follicular and interfollicular epidermis, there seem to be different populations of stem cells with different profiles which effectively support the same function (Tan et al., 2013; Schepelet et al., 2014). The main conclusion so far is that rather than being a well-defined homogeneous profile of gene expression, the signature of a stem cell is a heterogeneous ensemble of gene expression patterns specific to the particular associated cell type. In these ensembles, cells express many differentiation genes at low and variable levels.

One interpretation of this observation is provided by the notion of multilineage priming (Hu et al., 1997). According to this notion, a characteristic of a stem cell population is the expression of markers of multiple lineages at low levels, which creates a landscape of differentiation potential (Moignard & Göttgens, 2014). A
Box 2: Proliferation/differentiation control in homeostasis and development

A General diagram of a feedback control system

Input → Process → Output

Controller

\[
\begin{align*}
\text{State} & \quad \text{Setpoint} \\
\text{Deviation} & \quad \text{Time}
\end{align*}
\]

B Feedback control in the mammalian olfactory epithelium

\[ p_1 = f(D) \]

SC (TA) → INP (TA) → ORN (D)

C Control on proliferation/differentiation ratio of INPs

\[ p_1 = f(D) \]

Cell number vs. Time

\[ p_0, \lambda_0, \lambda_1 \]

D Optimal growth and mouse intestinal crypts

\[ p(t), 1-p(t) - r(t), r(t) \]

SC, NSC, ORN

Biological systems share some features with engineered ones, in particular their tendency to aim towards and then operate within a set point, which, often, is optimal for a specific function. Homeostasis and development are examples of this behaviour and exhibit analogies with systems built to accomplish defined tasks under strict rules of robustness and optimality in performance. For instance, systems whose operation requires maintaining a particular magnitude to a fixed set point are engineered through feedback regulation whereby information on the state of the process (output) is used by a controller that feeds back to the system (input) in order to correct any deviations of the state from the set point level (panel A). If the output is off, the input will be modified to attain this set value; this guarantees the steadiness of the fixed parameter that regulates its behaviour (right plot in panel A, see Doyle et al, 1990). Similar mechanisms have been postulated to operate in biological systems, and it is possible to draw a useful analogy between set point and both developmental and homeostatic systems, where the principal ‘performance objectives’ are to rapidly achieve and robustly maintain a specified size or number of different cell types (Reeves & Fraser, 2009). This analogy has been used to investigate organ size control in homeostasis disruption and during development within the mammalian olfactory epithelium (OE) (Lander et al, 2009). This tissue undergoes constant and rapid neurogenesis by means of a well-defined linear multistage lineage in which terminally differentiated olfactory receptor neurons (ORNs) are sustained by a transit-amplifying compartment of immediate neuronal precursor (INP) cells which in turn are sustained by a stem cell (SC) compartment (panel B). The dynamics of this system can be characterized by the probabilities of self-renewal of SCs and INPs, \( p_0 \) and \( p_1 \), respectively, as well as their cell division rates \( \lambda_0 \) and \( \lambda_1 \). Analysis of quantitative data suggests that if the differentiated neurons negatively fed back onto the probabilities of proliferation/differentiation of the INPs (parameter \( p_1 \) in schema B and right panel C) the steady state of the system becomes robust and it could react orders of magnitude faster to perturbations such as tissue damage [left panel C, cf. the recovery of damaged DD (solid yellow line) to the case with no feedback (pale.
Box 2 (continued)

teal line]. Production of CDF11, a ligand of the TGFβ superfamily produced by neuronal cells and known to inhibit the production of ORNs, is capable of such feedback control (Lander et al., 2009). Thus, the dynamic response to punctual perturbations of an otherwise balanced system can uncover some aspects about the regulation of the homeostasis.

Optimal control theory (Donald, 1970) provides strategies towards efficiency in the achievement of specific aims and can be used to understand certain developmental systems, for example how to achieve a certain size within the shortest period of time. This question has been addressed in the context of the developing intestinal crypts of infant mice (Itzkovitz et al., 2012a) looking for the optimal temporal progression of the probabilities of each type of division, p(t), q(t) and r(t) (panel D, adapted from Itzkovitz et al., 2012a). When the SC population is not allowed to overshoot, its solution consists of two differentiated phases: one of exponential proliferation of SCs, with all divisions being symmetric, and a second one in which divisions become purely asymmetric and non-stem cells (NCs) are generated and multiply (panel D). Any strategy other than this performs suboptimally. Remarkably, the strategy fits the data to a great extent (right bottom panel D; Itzkovitz et al., 2012a). This type of analyses provides deeper understanding of the cell dynamics during development and opens new question such as how to reconcile this progression towards purely asymmetric divisions during crypt maturation with the population asymmetry behaviour observed in adult mice. Further investigation with control engineering tools will surely cast some light to this problem.

related view is contained in the ‘transition state’ (Fig 2A), a concept derived from the observation that when cells change state during development, the decision is taken by individual cells from a ‘transition state’ (TS), in which a cell transiently exhibits a mixed identity between the states we could call origin (o) and destination (d) (Fig 2A; Martinez Arias & Hayward, 2006; Muñoz-Descalzo & Martínez Arias, 2012). At the TS, a cell has a probability of returning to o or moving to d, and its mixed identity is reflected in simultaneous, though variable, expression of genes from both states in the same cell. Once a cell moves from the TS towards the d state, the progression becomes irreversible. In a population undergoing a state transition between two states (o and d), this results in a mixture of cells in one of three states: o, TS and d. Such heterogeneous patterns of gene expression are often observed in developmental systems. If one associates a self-renewal rate to cells in o and TS and balances the ratios of transition of this self-renewal with differentiation, the result is something that formally resembles a stem cell population, which thus could be construed as a self-replicating transition state (Muñoz-Descalzo et al., 2012). The TS is a crucial step during the cell fate decision process, and this contrasts with the notion of lineage priming that merely describes a population in a steady state.

Gene expression patterns in dynamic stem cell populations

These concepts have been illustrated and studied in mouse embryonic stem (ES) cells, clonal populations derived from mouse preimplantation blastocysts that are pluripotent and can be differentiated in vitro into all cell types (Smith, 2001; Nichols & Smith, 2011). ES cells can be stably propagated in culture and are characterized by heterogeneous gene expression with individual cells expressing a spectrum of genes from pluripotent to differentiation (see Fig 2B and Chambers et al., 2007; Hayashi et al., 2008). It has been suggested that this heterogeneity is driven by the noisy expression of a small network of transcription factors centred on Nanog, Oct4, Sox2 and Esrrb that are central to the maintenance of pluripotency (Chambers et al., 2007; Singh et al., 2007; Chambers & Tomlinson, 2009; Kalmar et al., 2009; Young, 2011; Abranches et al., 2014; Singer et al., 2014). The activity of this network can be read out in the distribution of Nanog expression (Kalmar et al., 2009; Abranches et al., 2014), which is characterized by three populations in dynamic equilibrium (Fig 2B) with a dominance of a high Nanog pluripotent self-renewing population and a low Nanog population.

While both populations contain cells that are lineage primed, only the low Nanog population includes cells that are committed to differentiation (Chambers et al., 2007; Kalmar et al., 2009; Luo et al., 2013; Munoz Dascalzo et al., 2013). Remarkably, several experiments have shown that this distribution is robustly maintained and that its profile can be reconstituted even from small populations of ES cells (Chambers et al., 2007; Singh et al., 2007; Kalmar et al., 2009; Canham et al., 2010; Abranches et al., 2013). These states are thus dynamic and interconvertible (though the transition rates may vary with the culturing conditions), enabling cells to sample different molecular states (Kalmar et al., 2009; Abranches et al., 2013, 2014). It has been suggested that, at the molecular level, this dynamic state is fuelled by the time average of a loose connectivity of the elements of the network which creates a number of microstates [i.e. one of the many permitted states of gene expression (Garcia-Ojalvo & Martinez Arias, 2012; MacArthur & Lemischka, 2013)], some of which are compatible with self-renewal (high Nanog expression) and others with differentiation [low Nanog expression (Kalmar et al., 2009; Trott et al., 2012; Munoz-Dascalzo et al., 2013)]. The fraction of different populations is approximately constant for a given condition but changes with signalling, suggesting that it is regulated (Luo et al., 2013; Munoz Dascalzo et al., 2013).

At the phenotypic level, this situation is analogous to what is observed in homeostasis, where cell subpopulations are maintained in a dynamic equilibrium and therefore can be described as a system of homeostatic heterogeneities. The connection between the ES system and adult stem cells is emphasized by the observation that the signals that regulate the dynamics and structure of ES cell populations are FGF, Wnt and BMP which often appear as regulators of adult stem cell populations (Turner et al., 2014b).

A similar organization into dynamically balanced populations has been described in a compartment of the haematopoietic system, the erythroid/myeloid progenitor, characterized by the expression of the stem cell antigen 1, Sc1 (Pina et al., 2012). Although initially it was thought that this was a heterogeneous population in dynamic equilibrium (Huang et al., 2007; Chang et al., 2008), subdivision with additional markers, in particular the haematopoietic progenitor cell antigen CD34, reveals the existence of disparate subpopulations (Fig 2D) (Pina et al., 2012). Cells with high CD34 are capable of self-renewal regardless of their levels of Sc1. A different subpopulation, with low Sc1 and CD34 levels, is analogous to the low Nanog one in ES cells, cannot reconstitute the culture and is committed to
Dynamics and control during cell differentiation

(A) The concept of the transition state (TS). During a fate change, a cell goes through a TS for details see text), which implies the existence of kinetic constants governing the transitions between different states. (B) The TS can be observed in mouse ESCs. In this case, this is shown within the framework of Nanog expression, which is heterogeneously expressed with three distinguishable populations. a, representing ground state pluripotency; d, where it is possible to find cells committed to differentiation and TS where cells make a choice. (C) The coexistence of committed and uncommitted cells in the Nanog:GFP d population can be revealed by looking at a second pluripotency marker, Pecam or SSEA1 in this case (Canham et al. 2010; Lim, 2011). (D) A similar scenario has been recently observed in blood stem cells: cells with high levels of Sca1 can self-renew and are in a state analogous to the ‘o’ state. Sca1 low cells further subdivide into two populations, which can be identified by CD34. Sca1+/CD34+ have repopulation capacity and can revert to Sca1+ while the Sca1−/CD34− population consists of erythroid committed cells with no self-renewal capacity.

Similarities and differences between homeostatic and developmental systems: the example of the vertebrate retina

Homeostatic systems can be considered to be in a state of dynamic equilibrium, and the models that explain their behaviour account for this. However, the construction of a tissue or an organ is different from its maintenance. During organ development, fates are allocated to specific cell populations, sometimes in reproducible proportions and under specific genetic programmes that yield functional tissues. In some cases, the development of a tissue is associated with the activity of progenitors and stem cells that, in this case, display transient dynamics. A good example is the emergence of the vertebrate central nervous system from a population of progenitor cells that over time produces an array of neurons that create the sensory and motor systems (Wolpert et al., 2015). The number of final neurons is much bigger than the number of initial progenitors, and therefore, the progenitor population needs to be amplified. This has been studied in detail in the cortex (Qian et al., 2000; Shen et al., 2006) and, recently, the spinal cord (Kicheva et al., 2014). As the cell subpopulations play (progenitors and DD) are similar to those operating in homeostatic systems, the question arises of how many of the principles applying to the dynamics of these populations also apply to developmental systems (see Box 2) and, specifically, whether stochastic processes apply. If the latter were the case, a reasonable question is how can the large variability introduced by such processes, which one might expect to be exponentially amplified by the net growth of the system, achieve a very stereotyped and
reproducible final target. This issue has been explored in the vertebrate retina (He et al., 2012), and the results highlight some similarities but also some important differences between homeostatic and developmental systems.

**Cell growth and differentiation dynamics during retina development**

The retina is a structure with millions of cells structurally diversified into seven main functionally distinct cell types that are allocated in specific proportions and positions to generate a functioning organ (Masland & Raviola, 2000; Masland, 2001). The structure emerges over time during embryogenesis and continues growth after birth from a collection of retinal progenitor cells (RPC, P) which display two essential behaviours (Fig 3A, Livesey & Cepko, 2001; Centanin et al., 2011; Centanin & Wittbrodt, 2014). The first one relates to the patterns of divisions which early on amplify the P compartment (P→PP, Fig 3A and B) while towards the end of development lean towards terminally differentiating symmetric divisions (P→DD) (Livesey & Cepko, 2001; Rapaport et al., 2004) and in between exhibit a mixture of symmetric and asymmetric (P→PD) divisions, with the corresponding proportions varying over time. The second behaviour is a reproducible sequence of fates adopted by differentiating cells; although to date there is no way to reliably predict the fate of a specific cell when it divides, it is known that, at a given time, a cell always chooses among a restricted number of fates and that the repertoire of available fates changes with time (Cepko et al., 1996; Livesey & Cepko, 2001, Fig 3B and C). These observations led to a model suggesting that as development proceeds, an intrinsically defined competence to obtain particular fates changes and this is what determines the fate of a differentiating cell. According to this model, a pattern of differentiating (DD) divisions is superimposed upon this shifting window of competence resulting in a loose but reproducible sequence of fates (Livesey & Cepko, 2001).

This classical model is similar to those suggested for the development of the cortex (Qian et al., 2000; Shen et al., 2006) and has been given quantitative substance through a detailed analysis of several lineages in the frog and rat retina in culture and the zebrafish retina.

![Diagram of vertebrate retinogenesis](image)
in vivo (Wong & Rapaport, 2009; Gomes et al., 2011; He et al., 2012). These studies have provided support for an intrinsic mechanism of the fate assignment, the difficulty to assign specific fates to specific lineages and the need to balance over time the PP, PD and DD divisions in order to get the organ within a defined size. However, the studies in rat and fish (Gomes et al., 2011; He et al., 2012) have suggested that stochasticity, rather than a regulated programme, is the main driver in the fate assignment and tissue growth (Boije et al., 2014). While it is clear that it is not possible to predict the fate of a specific cell at a given time and that there is no simple pattern in the reported lineages, there is ample evidence for a reproducible sequence of fate assignment and for a restriction of the fates available to a cell at any given time: mixing or transplanting progenitors from different ages highlights the time restricted fate choice of the cells (Cepko et al., 1996; Belliveau & Cepko, 1999; Belliveau et al., 2000; Rapaport et al., 2001; Wong & Rapaport, 2009). Further support for a temporal programme of fate assignment is provided by the association of the expression of Ikaros with early and not late fates and by the complex, but numerous, sequence of expression of transcription factors (Mu et al., 2005; Wang & Harris, 2005; Ohsawa et al., 2001; Andreazzoli, 2009). Regarding stochasticity, there is evidence that fate decisions are associated with heterogeneous gene expression (Trimarchi et al., 2008), but these might reflect priming for specific fates or transition states that cells pass through when they make decisions, rather than an open and unrestricted fate choice at any given time. In fact, in the context of a developing tissue, the TS could be crucial in determining how a population is subdivided, since the cells that return to ‘o’ would have the opportunity to adopt a new fate (Fig 4D). Thus, in the retina, if the TS is short, a cell might not have time to make a decision, would remain in ‘o’ and would have to wait to another entry in the TS for a new fate to be desired. Depending on the programme of gene expression that is running, the choice the next time will be the same or different. This possibility provides an explanation for the perceived stochasticity of the system: it is during this transition state of finite time that the appropriate genes must be expressed in order to make the choice. The entries and exits of the TS would happen independently of the state of origin, this might have changed in nature; this might account for many of the observations during retina development.

**Figure 4. Molecular mechanisms of mediating fate decisions in development at the level of single cells.**

(A) The transition state (TS, see Fig 3) represents the basic unit for fate decisions. (B) During a fate transition, each cell executes a change of connectivity of their gene regulatory network from A to B. In this process, the cell will sample over time different configurations (microstates) of the available gene regulatory networks (GRN); many of these networks will resemble A, and therefore, the cell might have a chance to revert to the state of origin. When the network associated with fate B is connected, the cell moves to fate B. Within the TS, we suggest that cells are more susceptible to respond to signals that can bias their transcriptional state by affecting the connectivity (see text for details). (C) The TS state is an inherently noisy state, dominated by stochastic gene expression and affected by complex combinations of signals. As a result, the commitment or reversion event can be deemed as unpredictable at the level of individual cells. (D) The paradigm of the TS can be applied to pluripotent embryonic stem cells as well as to each differentiation step within the development of a tissue or organ. In the latter case, the TS is also controlled by a cell-autonomous genetic programme that establishes the order of appearance of the cellular fates, and thus, if a cell reverts to the state of origin, this might have changed in nature; this might account for many of the observations during retina development.
and simultaneously in many cells, and therefore, given the dimensionality of the system (with multiple genes and programmes of gene expression involved), a low sampling could give an impression of stochasticity.

In a recent review, Boije et al (2014) emphasize stochasticity, in the sense that all fates are available to all cells at any one time. We stress, however, that even though each individual cell might stochastically differentiate into one of the limited fates available, there are strong signs of tight regulation of the differentiation process, with most cell sub-types involved in the control of their temporal restriction. Thus, the complexity of this differentiation process might largely account for the reported lack of predictability of the fates adopted by individual cells, which the authors ascribe to stochasticity (see Fig 3C).

A molecular framework underlying fate decisions in developmental systems

The vertebrate retina shares a number of features with other developmental systems that need to be integrated and explained at the molecular level: (1) an asymptotic tendency towards a defined and reproducible size; (2) a temporal sequence of fate assignments; and (3) a certain degree of linkage between fate decision-making and the cell cycle. While stochastic models of fate assignment with no explicit regulation might account for the dynamic equilibrium of some homeostatic populations (Klein & Simons, 2011), it is not clear that they can be applied without modification to developmental systems (He et al, 2012). An important reason for this, which could also apply to homeostasis, is the need for coordinated control of the mode of cell division and fate adoption (Lander et al, 2009 and Box 2).

So far, there are a few lessons that can be learned from the analysis of the favoured competence model for the retina (Livesey & Cepko, 2001) and others that have been suggested for haematopoeisis (Pina et al, 2012) and ES cells (Trott et al, 2012). Here, we summarize these in the form of a set of premises that should be considered in any model for developmental systems:

a. There exist ‘programmes’ of gene expression within single cells. Such ‘programmes’ are not a linear sequence of expression patterns but, rather, a network of gene interactions encoding combinatorial regulation through the differential activity and interactions of the network components over time.

b. At each transition point of the programme, there are reversible intermediate transition states (TSs), where cells explore the space of available transcriptional networks (Fig 4A and B).

c. In the TS, individual cells make a choice for irreversible fate commitment at defined points of the programme (Fig 4C).

d. Signal-mediated cell interactions affect the progression to and from the TS and the choices made by individual cells (Fig 4C).

Conclusions: the transition state as the central element for fate decisions at the single cell level

With these premises in mind, we envision the TS as the central element of cell fate decisions at the single cell level (Martinez Arias & Hayward, 2006; Muñoz-Descalzo et al, 2012). This situation is similar to that described above for populations of stem cells, but in this case, the ‘0’ state is a particular intermediate in a developmental programme, say A, and the ‘d’ state is a new state, B, that is, the process is of the type A → B (notice that if A → A or B, we have a stem/progenitor cell population Fig 4D). From this, it follows that underlying each cell fate decision, there exists a TS promoted by the transcriptional programme that leads from A to B and thus enables the fate choice. In the TS, a cell needs to dismantle the network associated with the A state and connect the corresponding B network. This process will require the connectivity of several nodes only a few of which will lead to a stable state B, and therefore, a cell will exit the TS when the B network is in place (Fig 4A and B). The nodes of the networks that define these states are transcription factors and signalling effectors. While the transcription factors that define A initiate the transition by activating the B network, signalling might act by affecting the connectivity of the system in individual cells which will have an effect on the rates of state conversion (o/A → TS, TS → o/A, TS → d/B). As most signals are secreted, this will allow for a coordination of gene regulation at the population determining the number of cells that undergo a fate transition. In certain cases, some nodes might also include cell cycle-related proteins that, as recently shown in human ES cells (Paulkin & Vallier, 2013), can participate in cell fate choice, thus creating an opportunity to link the fate decision to the cell cycle (Rue et al, 2014).

The model that we propose to explain fate assignment from progenitor populations during development can be extrapolated to homeostatic systems on the premise that these are trapped TSs (Muñoz-Descalzo et al, 2012 and Fig 4). The model also provides a framework to link transcriptional events with lineages by suggesting that the parameters of the TS, the rates of the gene regulatory networks at the transition state, determine the behaviour of a cell population. This notion suggests that the transition state could be an effective target for the balance of cell populations in development, homeostasis and, more important, pathological situations in which imbalances emerge between proliferation and differentiation.

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Author contributions

PR and AMA conceived and wrote the article using information from their own research and the literature.

Conflict of interest

The authors declare that they have no conflict of interest.

References


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Pau Rued & Alfonso Martinez Arias
Dynamics and control during cell differentiation


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Cell shape and the microenvironment regulate nuclear translocation of NF-κB in breast epithelial and tumor cells

Julia E Sero¹*, Heba Zuhair Sailem¹, Rico Chandra Ardy¹, Hannah Almuttaqi¹, Tongli Zhang² & Chris Bakal¹**

Abstract

Although a great deal is known about the signaling events that promote nuclear translocation of NF-κB, how cellular biophysics and the microenvironment might regulate the dynamics of this pathway is poorly understood. In this study, we used high-content image analysis and Bayesian network modeling to ask whether cell shape and context features influence NF-κB activation using the inherent variability present in unperturbed populations of breast tumor and non-tumor cell lines. Cell–cell contact, cell and nuclear area, and protrusiveness all contributed to variability in NF-κB localization in the absence and presence of TNFα. Higher levels of nuclear NF-κB were associated with mesenchymal-like versus epithelial-like morphologies, and RhoA–ROCK-myosin II signaling was critical for mediating shape-based differences in NF-κB localization and oscillations. Thus, mechanical factors such as cell shape and the microenvironment can influence NF-κB signaling and may in part explain how different phenotypic outcomes can arise from the same chemical cues.

Keywords  Bayesian, breast cancer; morphology, NF-κB; RhoA
Subject Categories  Quantitative Biology & Dynamical Systems; Signal Transduction
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Introduction

Cell morphology reflects the balance of internal and external chemical and physical cues, which in turn provide the context for signaling events. The shape of a cell can regulate differentiation, proliferation, survival, and motility (Chen et al., 1997; McBeath et al., 2004; Discher et al., 2009; Sero et al., 2011), and the role of morphology in tumor progression has become apparent in recent years. For example, cell shape is greatly influenced by the stiffness of the extracellular matrix (ECM), which correlates with metastasis and prognosis in breast cancer (Kass et al., 2007; Mousse et al., 2014). Indeed, the morphology of cells has long been the primary method of diagnosing and staging tumors in the clinic. While mechanosensitive transcriptional regulators, such as YAP, have been shown to link shape information with gene expression (Cordenonsi et al., 2011; Dupont et al., 2011; Aragona et al., 2013), the mechanisms by which cellular fates are determined by cell shape are still poorly understood.

Nuclear factor kappa B (NF-κB) is a key transcription factor involved in mediating inflammation, cellular stress responses, and tumor progression. NF-κB also controls cell and tissue morphogenesis, including proliferation and branching of the mammary gland (Brantley et al., 2001). Chronic expression of the inflammatory cytokine tumor necrosis factor-alpha (TNFα), a potent NF-κB activator (Van Antwerp et al., 1996), can drive cancer metastasis by inducing epithelial-to-mesenchymal transition (EMT) and tumor cell migration (Wu & Zhou, 2010). NF-κB dimers are sequestered in the cytoplasm by inhibitory IκB until activated by cellular stress or cytokines (Gilmore, 2006). Binding of TNFα to cell surface receptors leads to recruitment of adaptors (TRAFs and RIP) and activation of IKK kinases, which phosphorylate IκB and target it for proteolytic degradation. Once released from inhibition, NF-κB can translocate to the nucleus, bind DNA, and induce the expression of target genes, including IκB.

Following TNFα stimulation, the nuclear to cytoplasmic ratio of NF-κB has been shown to exhibit damped oscillations (Ashall et al., 2009; Kalita et al., 2011; Zambrano et al., 2014). A number of studies have demonstrated that the dynamics of these oscillations determine its physiological effects. For example, target gene expression depends on the persistence of NF-κB oscillations (Sung et al., 2009; Tay et al., 2010), and NF-κB dynamics have been reported to encode gene-specific transcription patterns (Lee et al., 2014). Notably, however, recent studies appear to suggest these oscillations may be cell type or context specific, suggesting that biophysical factors can...
impact NF-κB dynamics (Kearns et al, 2006). Moreover, NF-κB activation and dynamics are heterogeneous on the single cell level, even in isogenic populations (Nelson et al, 2004). This heterogeneity may be due to stochastic noise and/or may be regulated deterministically. Mechanical forces can activate NF-κB in many cell types, including muscle, lung, and vascularizing (Chen et al, 2003; Copland & Post, 2007), and this signaling pathway is sensitive to perturbation of F-actin and microtubules (MT), the main components of the cytoskeleton (Rosette & Karin, 1995; Nemeth et al, 2004). While these findings indicate that NF-κB activity is affected by cellular architecture, the exact mechanisms that underpin its mechanosensitivity are unclear. In this study, we asked whether cell shape and context can influence NF-κB and whether differences in shape can explain any of the cell-to-cell variability observed in its activation. We used high-content analysis (HCA) to quantitatively measure cell morphology and transcription factor (TF) localization in hundreds of thousands of single cells per experiment in cell lines derived from human breast tumor and non-tumor tissues. Using this compendium of data, we built statistical Bayesian network models that exploit intrinsic heterogeneity present in cellular populations to uncover dependencies between cell shape, context, and TF intensity features. We then tested predictions from network models using chemical, physical, and genetic methods. This study reveals that cell shape and microenvironmental factors are determinants of NF-κB translocation dynamics that contribute to cell-to-cell heterogeneity. We propose that cell shape-mediated regulation of NF-κB plays a key role in breast epithelial development and tumor progression.

Results

Experimental settings and image analysis

We used HCA to quantitatively profile the morphology and NF-κB nuclear localization (which serves as a proxy for its activation), in 17 breast cancer and two non-tumor cell lines (Table 1). Nuclei and cell bodies were stained with fluorescent labels and imaged by confocal microscopy (see Materials and Methods). Cellular regions (nucleus, cytoplasm, membrane, perinuclear ring) were automatically segmented and measured (Fig 1A, top). All cells were grown in ‘base’ medium, and the non-tumor lines MCF10A and MCF12A were also cultured ‘complete’ medium, as MCF10A cells required EGF to proliferate (see Materials and Methods). This dataset contained 307,643 cells, and a total of 77 shape and context features were measured for each cell (Fig 1A). These include geometric features such as area, roundness, length/width; measurement of protrusions and ‘ruffliness’, which detects variations in membrane intensity; measures of cell polarity, such as the distance between the centroids of the nucleus and the cell body (centers distance); measures of context including colony size and neighbor fraction (NF), the proportion of a cell’s boundary that is in contact with other cells; and fluorescence intensity in each subcellular region. On the third day in culture, cells were treated with or without TNFα for 1 h or 5 h to capture the first peak and later steady state of NF-κB activation (see Fig 4E). NF-κB activation state can be inferred by the ratio of nuclear to cytoplasmic (perinuclear, i.e., ring region) p65/RelA fluorescence intensity.

Table 1. Breast tumor and non-tumor cell lines.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Genetic subtype</th>
<th>ER</th>
<th>PR</th>
<th>Her2</th>
<th>TP53</th>
<th>N-cadherin</th>
<th>Shape cluster</th>
</tr>
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<tbody>
<tr>
<td>hs5787T</td>
<td>BaB</td>
<td>−</td>
<td>−</td>
<td>+M</td>
<td>++</td>
<td>B</td>
<td></td>
</tr>
<tr>
<td>MCF10A (complete medium)</td>
<td>BaB</td>
<td>−</td>
<td>−</td>
<td>+/−WT</td>
<td>++</td>
<td>L/B</td>
<td></td>
</tr>
<tr>
<td>MCF10A (base medium)</td>
<td>BaB</td>
<td>−</td>
<td>−</td>
<td>+/−WT</td>
<td>++</td>
<td>L2</td>
<td></td>
</tr>
<tr>
<td>MDAMB157</td>
<td>BaB</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>++</td>
<td>B</td>
<td></td>
</tr>
<tr>
<td>MDAMB231</td>
<td>BaB</td>
<td>−</td>
<td>−</td>
<td>++M</td>
<td>−</td>
<td>B</td>
<td></td>
</tr>
<tr>
<td>SUM149</td>
<td>BaB</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>+</td>
<td>B</td>
<td></td>
</tr>
<tr>
<td>SUM159</td>
<td>BaB</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>++</td>
<td>L/B</td>
<td></td>
</tr>
<tr>
<td>HCC1143</td>
<td>BaA</td>
<td>−</td>
<td>−</td>
<td>++M</td>
<td>−</td>
<td>B</td>
<td></td>
</tr>
<tr>
<td>HCC1964</td>
<td>BaA</td>
<td>−</td>
<td>−</td>
<td>+/−</td>
<td>−</td>
<td>L1</td>
<td></td>
</tr>
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<td>++M</td>
<td>−</td>
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<td>+/WT</td>
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<td></td>
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<tr>
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<td>Lu</td>
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<td>−</td>
<td>+</td>
<td>−</td>
<td>L2</td>
<td></td>
</tr>
<tr>
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<td>+</td>
<td>−</td>
<td>+/−</td>
<td>−</td>
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<td></td>
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<tr>
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<td>[+]</td>
<td>+/−WT</td>
<td>−</td>
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<td></td>
</tr>
<tr>
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<td>−</td>
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<td>−/WT</td>
<td>−</td>
<td>L2</td>
<td></td>
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<tr>
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<td>Lu</td>
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<td>−</td>
<td>[+</td>
<td>+</td>
<td>B</td>
<td></td>
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<tr>
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<td>Lu</td>
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<td>[+]</td>
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<td>−</td>
<td>−</td>
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<td>JIMT1</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>++</td>
<td>−</td>
<td>L1</td>
<td></td>
</tr>
</tbody>
</table>

Gene cluster: Lu = luminal, BaA = Basal A, BaB = Basal B, ER/PR/HER2/TP53 status: +/− from protein and mRNA expression; [−] inferred from mRNA expression; M = mutant, WT = wild-type (Neve et al, 2006). N-cadherin status: +/− from mRNA expression and immunofluorescence (Supplementary Fig S1B). Shape clusters assigned from hierarchical clustering shown in Fig 1D.
Morphology regulates NF-κB in breast cells

A. Features extracted from image analysis

B. NF-κB label

C. Cell lines clustered by morphology

D. NF-κB activation across genetic subtypes

E. Cell lines clustered by NF-κB ratios

Figure 1. Characterization of cell morphology and NF-κB activation in breast cell lines.

A. Cell segmentation and shape features.
B. NF-κB staining in MCF10A cells (left) and nuclear/cytoplasmic ratios mapped as grayscale pixel values to segmented cells (right). Scale bar = 20 μm.
C. Hierarchical clustering of the first 8 principal component scores for each cell line based on geometric and context features. Genetic subtype is indicated by color. Representative images (20×) of cells from each cluster are shown at the right (red: DAPI, green: NF-κB, blue: DHE). Scale bar = 30 μm.
D. NF-κB ratios (TNFα 1 h) in cell lines of different genetic subtypes (box and whisker plot). 14 replicates wells per cell line. Basal A: 3 lines, Basal B: 7 lines, Luminal: 8 lines.
E. Cell lines clustered by nuclear/cytoplasmic NF-κB ratio in the presence or absence of TNFα.

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which we hereafter refer to as the ‘NF-κB ratio’ (Nelson et al., 2004) (Fig 1B).

**Characterization of cell lines by morphology**

The cell lines profiled represent a variety of phenotypes. While some can be easily designated epithelial-like or mesenchymal-like, others have intermediate phenotypes or consist of a mixture of shapes. Principal component analysis (PCA) was performed on well averages of geometric, protrusion, polarity, and context features to reduce the dimensionality of the data. The average scores of the first eight PCs for each cell line, including MCF10A and MCF12A cells in different media, were grouped by hierarchical clustering (centered correlation, centroid linkage) (Fig 1C). Replicate wells also clustered together, showing good reproducibility of feature measurements (Supplementary Fig S1C).

Two major groups emerged from this clustering. The first major cluster (B) contained mostly myoepithelial Basal B cell lines, and the second (L) contained mostly luminal lines (Neve et al., 2006) (Fig 1C). The Basal B group was characterized by significantly larger cell area (2,269 ± 672 μm² [mean ± SD]), low nuclear/cytoplasmic area (Ånucl/Åcyto: 0.15 ± 0.029), low NF (0.36 ± 0.1), and high ruffliness (0.215 ± 0.023) compared with the luminal lines (P < 0.01) (Supplementary Fig S1). The luminal cluster contained three sub-groups with epithelial-like morphologies. The first subgroup (L1) contained lines with classic ‘cobblestone’ epithelial morphology, such as MCF7, that grew in colonies with extensive cell–cell contacts (NF = 0.56 ± 0.038) and were significantly larger in area (1,788 ± 308 μm²) than the L2 group (Fig 1D and Supplementary Fig S1G). The L2 sub-group contained smaller cells (835 ± 173 μm²) that grew in colonies (e.g. HCC70) or clusters (e.g. CAMA1) (NF = 0.46 ± 0.087). Non-proliferative MCF10A cells (base medium) were also in this group. The final sub-group (L/B) contained two Basal B lines with luminal morphologies, SUM159, and proliferative MCF10A cells. This group may represent an intermediate or progenitor phenotype, as these lines are derived from the myoepithelial layer and contain stem cell-like subpopulations (Fillmore & Kuperwasser, 2008).

Membership in a morphological cluster was not correlated with ER, PR, HER2, or TP53 status. However, clusters did correspond to expression of N-cadherin, a marker of EMT and poor prognosis in breast cancer (Andrews et al., 2012) (Table 1 and Supplementary Fig S1B). None of the luminal cell lines were positive for N-cadherin, whereas both cell lines of the L/B group and most of the lines in the B group were. Furthermore, the only Basal A line in the B morphological cluster, HCC1143, was N-cadherin positive. Morphological profiling was therefore not only able to recapitulate molecular subtypes determined by gene expression profiles (basal/luminal), but also discriminated between myoepithelial-derived basal cell lines that were positive or negative for an important EMT marker and pointed to a further distinction between Luminal cell lines.

**NF-κB activation in response to TNFα scales with subtype and cell shape**

Ranking cell lines by NF-κB ratio shows that Basal B lines stimulated with TNFα for 1 h (roughly the first peak of NF-κB activation) had predominantly nuclear staining (nuclear/perinuclear ratio > 0.8), compared with luminal (0.2–0.8) and Basal A lines (0.1–0.6). One-way ANOVA with a multi-comparison procedure confirmed significant differences in NF-κB localization between genetic subtypes (P < 0.01) (Fig 1D). Importantly, we also observed a relationship between cell morphology and NF-κB response within genetic subtypes. Luminal cell lines with cobblestone morphology (L1) had significantly lower unstimulated NF-κB ratios and greater fold change after TNFα stimulation than cell lines in the L2 sub-group (P < 0.01) (Fig 1E). Clustering cell lines by stimulated and unstimulated NF-κB ratios shows the distinction between L1 and L2 sub-groups, as these lines did not cluster together (Fig 1E). Furthermore, NF-κB activation in the Basal A cell lines followed the morphological trend: the B group HCC1143 cells had the highest NF-κB ratio after TNFα, followed by the L1 line HCC1954, and finally the L2 line HCC70 (Fig 1E and Supplementary Fig S2A).

**Relationship between NF-κB and morphology in single cells**

Given the differences in NF-κB activation between differently shaped breast cell lines, we next set out to determine whether NF-κB activation was related to morphology on the level of single cells. The distribution of NF-κB ratios varied between cell lines and conditions (Fig 2A (left) and Supplementary Fig S2A), and in some cases, the cell-to-cell differences ranged over orders of magnitude (log ratio –1 to > 1). Moreover, all cell lines were morphologically heterogeneous. Figure 2A (right) and Supplementary Fig S2B show the distribution of first principal component (PC1) scores of single cells based on 77 geometric shape and context features. Cell shape distributions were multi-modal, suggesting the existence of a finite number of morphological states (Yin et al., 2013; Sailer et al., 2014), and each cell line showed a different degree of heterogeneity.

We first asked whether any shape and context features differed significantly between cells with high and low NF-κB ratios in each cell line. Interestingly, many features varied consistently in cell lines (Supplementary Fig S2C and Supplementary Materials and Methods). Unstimulated cells with nuclear NF-κB (high ratio) tended to have significantly higher ruffliness, lower nuclear roundness, smaller nuclear and cell areas, and fewer neighboring cells than those with low NF-κB ratios in many cell lines (Supplementary Fig S2C). However, linear correlations between NF-κB ratios and morphological features were poor (Supplementary Fig S2D), which suggests that the relationship between NF-κB and cell morphology is complex and non-linear.

**Bayesian network models**

To investigate the relationship between cell shape, microenvironmental factors, and NF-κB, we used Bayesian network modeling to identify dependencies between variables. Bayesian networks are a class of graphical models that can capture non-linear relationships between interacting components using conditional probabilities (Friedman et al., 2000; Sachs et al., 2005). Network models were built for each cell line ± TNFα using the 17 significant features that were most frequently significantly different between cells with high and low NF-κB ratios across cell lines, resulting in 60 network models (Supplementary Fig S2C). An
A  

**Nuc/Cyto NF-κB (log)**

Cell shape (PCA)

<table>
<thead>
<tr>
<th></th>
<th>HCC70</th>
<th>MCF7</th>
<th>SUM159</th>
</tr>
</thead>
<tbody>
<tr>
<td>unstimulated</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TNFα 1h</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>TNFα 5h</td>
<td></td>
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**B  **

**NF-κB network Model: MCF7**

**C  **

**Frequency of NF-κB dependency on morphology features in breast cell lines**

**D  **

**NF-κB dependencies**

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**Figure 2. Bayesian network dependency models infer connections between shape and transcription factor localization using intrinsic heterogeneity of single cells.**

A. Left: Distribution of nuclear/cyttoplasmic NF-κB ratios in single cells. Blue = unstimulated, red = TNFα 3 h, green = TNFα 5 h. Right: Distribution of first principal component (PC) of morphology features showing multi-modal distribution of cell shapes.

B. Example Bayesian network (MCF7). Edge (line/arrow) color denotes treatment conditions. Arrows indicate direction of dependency, and lines indicate interactions where direction cannot be determined. Numbers denote confidence (see Materials and Methods).

C. Frequency of NF-κB ratio dependencies by cell line for the most commonly connected features = TNFα (1 h).

D. NF-κB dependencies on morphological factors detected in each condition. Red = dependency detected in unstimulated condition. Asterisk = dependency detected in TNFα-stimulated cell (1 h).

The statistical dependency of NF-κB localization on shape features varied between cell lines and conditions (Fig 2C and D), but some dependencies were more frequent than others. The most commonly connected features in unstimulated cell lines were cell area (18 cell lines), ruffliness (15), nuclear area (11), \( \frac{A_{\text{nuc}}}{A_{\text{cyt}}} \) (11), and nuclear roundness (8 lines) (Fig 2C). In TNFα-stimulated cells, the most common dependencies were nuclear area (15), \( \frac{A_{\text{nuc}}}{A_{\text{cyt}}} \) (10), ruffliness (10), NF (9), and nuclear roundness (9). In some cell lines, NF-κB ratio was more dependent on morphological factors before stimulation (Basal A lines HCC70 and HCC1954), while in other lines it was more dependent on shape after addition of TNFα (Basal B lines MDA-MB-157 and hS578T).

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**Example network model (MCF7) is shown in Fig 2B, where each feature is represented as a node and interactions by lines or arrows. An arrow pointing from A to B indicates that B is dependent upon A; that is, changes in A affect the value of B. Numbers denote the confidence of the interaction (see Supplementary Materials and Methods). Undirected arrows indicate an interaction where the dependency cannot be determined. In MCF7 cells (Luminal genetic subtype, L1 morphological group), NF-κB ratio was dependent on cell area, nucleus area, ruffliness, \( \frac{A_{\text{nuc}}}{A_{\text{cyt}}} \), and NF in unstimulated cells, as well as on centers distance (distance between cell and nuclear centroids) after TNFα stimulation (confidence > 0.6).**
TNFα stimulation reduced the frequency of dependency on cell area and ruffliness, but increased dependency on nuclear morphology and NF. These findings indicate that the effects of cell morphology on NF-κB localization are largely conserved across cell lines but can vary depending on whether cells are responding to cytokine stimulation (± TNFα).

YAP, but not jun, activation is dependent on cell morphology

To test the validity of this approach, we asked whether Bayesian network models could detect connections between cell shape features and localization of YAP, another transcription factor that is activated by nuclear translocation and is known to be regulated by cell density, spreading, and mechanical force (Fig 3A, left) (Halder et al., 2012). Nuclear/cytoplasmic YAP ratios varied widely across ten cell lines (Fig 3B) and were not significantly affected by 1-h TNFα treatment (Supplementary Fig S3A). YAP ratio was dependent on many of the same shape and context features as NF-κB ratio, specifically NF, ruffliness, and nuclear morphology features (7/10 lines each), but the networks were not identical. In MDA-MB-231 cells (genetic subtype Basal B, morphological group B), for example, YAP ratio was dependent on cell roundness, whereas NF-κB ratio was not (Figs 2D and 3C). In MCF7 cells (Luminal, L1), NF-κB ratio was also highly connected to shape, but YAP ratio was only dependent on nuclear area (Figs 2 and 3D). YAP ratio was most often dependent on NF and ruffliness. These data demonstrate that our experimental and analytical approach captures the known relationships between YAP nuclear localization and cell shape. Moreover, we find that the amount of nuclear YAP and NF-κB is dependent on overlapping, but not identical, aspects of cell morphology.

In order to determine whether the activity of other branches of the TNFα signaling pathway are dependent on cell shape, we measured levels of phosphorylated Jun, a transcription factor that is activated downstream of TNFR ligand binding by phosphorylation of Jun N-terminal kinase JNK (Supplementary Fig S3B). While phosphorylated Jun accumulated in cell nuclei following TNFα stimulation, Bayesian models did not infer dependency between nuclear pJun intensity and any morphological features in any of the ten cell lines tested (Supplementary Fig S3C). Furthermore, nuclear/cytoplasmic pJun and NF-κB ratios were not correlated in

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**Figure 3. The statistical dependency of YAP on cell shape.**

A. MCF10A cells stimulated with TNFα labeled for YAP and phospho-Jun. Scale bar = 20 μm.

B. Frequency of YAP ratio dependencies by cell line for the most commonly connected features.

C. Example Bayesian network model for YAP ratio (MDA-MB-231 cells).

D. Dependencies of YAP ratio on the features that are most frequently connected to YAP ratio in unstimulated conditions. Red = dependency detected.
TNFα-stimulated MCF10A cells expressing GFP-p65/RelA ($R^2 < 0.05$, $n = 594$ GFP-positive cells) (Supplementary Fig S3D). (In comparison, the $R^2$ for NF-kB and YAP ratios was typically 0.3 in unstimulated cells and 0.5 in TNFα-stimulated MCF10A cells.) These data indicate that although NF-kB-xenotransplant downstream of TNFα is shape sensitive, Jun phosphorylation downstream of TNFR is not.

Importantly, the finding that Jun activation is not dependent on cell shape or microenvironment also suggests that the statistical correlations between shape and NF-kB activation are not simply due to the fact that some cells express more TNFα than others. This notion is supported by the fact that we found no strong correlations ($R^2 > 0.5$) between NF-kB ratios and mRNA levels of TNFR, or other members of the NF-kB signaling pathway (i.e., RelA, RelB, RelB, NFKB1, NFKB2, TRAF2, TRAF5, TNIK, or four IKK genes ($\alpha$, $\beta$, $\gamma$, $\epsilon$) in any treatment condition (unstimulated, 1 h, or 5 h TNFα) using microarray data generated from 18 of the 19 cell lines used in this study (Supplementary Table S1) (Grigoriadis et al., 2012) (no data were available for AU565 cells, which were obtained from ATCC). These results suggest that the differences in NF-kB localization between and within cell lines are not simply due to cells’ ability to detect TNFα.

**Drug-induced cytoskeleton modification affects cell shape and NF-kB**

We next asked whether inducing cell shape changes by chemically altering the cytoskeleton would affect NF-kB. Non-tumor MCF10A cells were treated with Y-27632 (Y27), H1152, blebbistatin (Blebb), and/or low doses of nocodazole (Noc) prior to TNFα stimulation. Y27 and H1152 induce F-actin depolymerization by inhibiting the activity of ROCK, a RhoA effector, and Blebb disrupts F-actin contractility by blocking myosin II ATPase (Kovacs et al., 2004). Noc inhibits MT polymerization and can induce activation of RhoA via GEF-H1 (Chang et al., 2008). Y27, H1152, and Blebb treatment induced cell spreading and disassembly of cell-cell adhesions, whereas Noc induced rounding up and an increase in E-cadherin staining at cell-cell contacts (Fig 4A and Supplementary Fig S4D). The first PC scores of shape and context features (well averages) illustrate the opposite effects of Y27/H1152/Blebb and Noc on cell morphology (Fig 4B). These drugs also had contrasting effects on NF-kB. Y27, H1152, and Blebb increased, whereas Noc decreased, NF-kB nuclear localization after TNFα stimulation (Fig 4B and C, Supplementary Fig S4A and B). Interestingly, pre-treatment with Y27 and Noc together resulted in similar cell shape to controls (by PCI score) and ‘rescued’ NF-kB activation. Consistent with this model, stabilizing Mts with a high dose of taxol also induced cell rounding and reduced NF-kB activation (Supplementary Fig S4A–C). These data demonstrate that chemical modulation of cytoskeletal tension, and the concomitant effects on cell shape, can influence NF-kB localization in response to TNFα.

**Prediction of changes in NF-kB from changes in morphology**

To further validate that the effect of cytoskeleton-modifying drugs affected NF-kB activation through shape changes, we treated 10 cell lines (nine breast cell lines plus HeLa cells for comparison) with drugs that were expected to alter cell shape and cytoskeletal tension, including Y27, Noc, and Blebb (Supplementary Tables S2 and S3). In general, NF-kB ratios increased in cells treated with ROCK inhibitors and decreased in cells treated with nocodazole, but different lines showed different sensitivities to drugs (see Supplementary Dataset S3). We used multilinear regression with tenfold cross-validation to predict the fold change in NF-kB ratio from the fold change in morphological features (see Supplementary Materials and Methods), resulting in the following equation:

$$\text{ΔNF-kB ratio} = 0.055(\text{ΔRuffles}) - 0.72(\text{ΔNuc/Acyto}) - 0.3(\text{ΔNuc}) + 1.99$$

NF-kB ratio was negatively associated with $\text{ΔNuc/Acyto}$ and NF and positively associated with ruffles. This model was able to predict the fold difference in average NF-kB with $R^2 = 0.37$, low error variance (0.033), and a very significant $P$ value ($2.25 \times 10^{-17}$) (Fig 4D). The average error between cross-validation samples was 0.0172 (±0.0077), and residuals were normally distributed. Changes in NF-kB were explained by changes in shape in the majority of cases. The overall goodness of fit in this statistical model strongly suggests that cell area, protrusiveness, and cell–cell contact all impact NF-kB activation. Only seven cases were not within the 95% confidence interval of the predicted value (Fig 4D, circled). Three of these, in which NF-kB ratios were higher than expected based on changes in cell morphology, were Y27-treated HCC1954 cells (Basal A, L1) stimulated with TNFα. The cases with lower than predicted NF-kB ratios were HCC1954, JIMT1 (unclassified, L1), and T47D (Luminal, L1) cells treated with nocodazole. HCC1954 cells had very low NF-kB activation compared with other L1 morphology group lines in the absence of ROCK inhibitor, which may indicate an inhibitory effect of RhoA signaling on NF-kB in these cells.
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Molecular Systems Biology

Figure 4

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Figure 4

Figure 4.

Figure 4.
Cell shape and the microenvironment regulate NF-κB translocation dynamics

To investigate how changes in cell shape affect the dynamic behavior of NF-κB, MCF10A cells were transiently transfected with GFP-p65, selected by FACS, and imaged over 6 h at 5-min intervals after addition of TNFα (Fig 4E and Supplementary Movies). NF-κB ratios (nuclear/perinuclear GFP intensity) were measured for 40 cells in each condition. Y27 treatment caused an increase in nuclear NF-κB immediately after addition of TNFα, whereas Noc treatment significantly decreased the amplitude of the first peak (Fig 4F). Unexpectedly, the initial wave of nuclear localization was more rapid and less variable in Y27-treated cells (Fig 4G). Consistent with reports in other cell types, damped oscillations with a period of 110–120 min were observed in all conditions, with higher amplitudes in Y27-treated and lower amplitudes in Noc-treated cells (Fig 4H–J) (Ashall et al., 2009; Zambrano et al., 2014). The first peak, at around 30 min, was followed by a rapid (30–40 min) then steady (60–80 min) decrease in nuclear NF-κB.

Y27 treatment resulted not only in greater amplitude but also less variability in oscillations, whereas Noc had the opposite effect. To compare NF-κB translocation dynamics in different conditions, wavelet analysis was used to estimate the instantaneous periods of oscillation at each time point (t = 300 min shown in Fig 4K and L). Control cells showed a high frequency of 110–120 min oscillations, with smaller peaks at 60 and 90 min (Fig 4K and L, black bars). Y27 increased the frequency of 120-min oscillations and reduced the frequency of shorter periods (Fig 4K). In contrast, Noc treatment reduced the frequency of 110–120 min oscillations and increased the frequency of shorter periods (Fig 4L). These data suggest that Noc treatment could drive NF-κB toward a non-oscillating steady state, whereas Y27 treatment could enhance the cycle of nuclear import and export. Thus, NF-κB nuclear translocation dynamics are sensitive to cell shape and the actin cytoskeleton.

Network models predict NF-κB and YAP sensitivity to cell–cell contact

Although statistical modeling showed that differences in NF-κB nuclear localization could be explained by changes in shape, we cannot exclude the possibility that drugs had other effects on upstream signaling. We therefore used non-chemical methods to alter cell morphology. To test dependencies on cell–cell contact (neighbor fraction; NF), a subset of six cell lines were seeded at four different densities and stimulated with TNFα for 1 h and stained for both NF-κB and YAP (Fig 5A). This gave a wider distribution of NF values and enriched populations in phenotypes that were rare in the initial screen, such as MDA-MB-231 cells with high NF and MCF7 cells with low NF (Supplementary Fig S5A and B).

Binning all single cells by NF shows the relationship between the extent of cell–cell contact and nuclear TF localization (Fig 5B–E). In MCF10A cells, NF-κB and YAP ratios were negatively correlated with NF in both low-density and high-density wells (Fig 5B and C). Importantly, there was no significant difference in average NF-κB ratio for cells with comparable NF between low- and high-density wells (Fig 5B). Average YAP ratios, however, were significantly lower in high NF cells (> 0.9) in high-density wells (Fig 5C). MCF10A cells continue to divide upon reaching confluence, which results in reduced spreading and cell–ECM contact. Consistent with previous reports (Aragona et al., 2013), these data indicate that nuclear YAP continued to decrease after confluence as cells became more tightly packed (Supplementary Fig S4B and C). Cell–cell contact therefore suppressed nuclear localization of both NF-κB and YAP, but YAP was more sensitive to cell density.

Bayesian networks detected NF-κB dependency on NF in non-tumor MCF10A (genetic subtype Basal B, morphological group L/B), MCF7 (Luminal, L1), SUM159 (Basal B, L/B), and MDA-MB-231 (Basal B, B) cells, but not in HCC1954 (Basal A, L1) and AU565 (Luminal, L2) cells stimulated with TNFα (Fig 2D). Indeed, NF-κB ratios were inversely correlated with NF in the former but not the latter cell lines (Supplementary Fig S5D and F), and the strength of the relationship (the slope of the regression line between NF-κB ratio and NF) was lower in AU565 and HCC1954 cells (Fig 5D). YAP ratios, on the other hand, did decrease with NF in these cell lines (Fig 5E and Supplementary Fig S5E and F). These findings confirm that NF-κB regulation was uncoupled from cell–cell contact in AU565 and HCC1954 cells, as predicted by network models, whereas YAP localization remained sensitive to cell density in these lines.

MCF7 was the only one of these lines in which no YAP ratio dependency on NF was detected. Although a negative linear correlation was observed between YAP ratio and NF in single MCF7 cells (Supplementary Fig S5E), the strength of the relationship was low (Fig 5E). These data indicate that Bayesian network models identified only the most robust relationships between features and also suggest that YAP regulation by cell–cell contact may be abnormal in MCF7 cells.

NF-κB sensitivity to cell density requires Rhoa and ROCK

Because NF-κB nuclear localization was higher in MCF10A cells with few neighbors, we next asked whether inducing an ‘edge’ morphology in MCF10A cells affected NF-κB ratios and, if so, whether this effect was mediated through cytoskeletal tension. TNFα was added immediately after scrape wounding confluent
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**Figure 5.**

- **A** Representative images of TNFα-stimulated MCF10A (left) and MDA-MB-231 (right) cells under different conditions.
- **B** and **C** Slope of linear regression for NF-κB and YAP ratios by NF in MCF10A cells.
- **D** and **E** Strength of association between NF-κB and NF, and YAP and NF, respectively.
- **F** NF-κB activation in scrape wound (control).
- **G** NF-κB activation in scrape wound + Y27.
- **H** Actin staining in control and RhoA k/d conditions.
- **I** Nuclear/Cytoplasmic NF-κB (+TNFα) ratio.
monolayers of cells starting from 8 h before fixation. At all time points, edge cells showed higher NF-κB ratios than non-edge cells (Fig 5F). Pre-treatment with the ROCK inhibitor Y27, however, reduced the difference in NF-κB ratios between edge and non-edge cells, especially at 1 h (Fig 5G). As before, Y27-treated cells had a higher first peak of nuclear NF-κB ratios than controls (0.51 vs 0.36 for edge cells, 0.49 vs 0.3 for non-edge cells) and caused a steeper decrease in NF-κB ratio from 1 to 2 h in edge cells (24% vs 11%). Importantly, Y27 treatment eliminated the difference between edge and non-edge cells at 1 h.

To confirm that the effect of cell density on NF-κB required Rho-ROCK signaling, we knocked down RhoA in MCF10A cells and repeated the plating density experiment. RhoA knockdown (k/d) cells had an elongated morphology similar to Y27-treated cells (Fig 5H) and significantly higher NF-κB ratios than control cells at all NF values (P < 0.01) (Fig 5I) and NF-κB was less sensitive to NF in RhoA-depleted cells (slope of linear regression: control = 0.21, RhoA k/d = −0.05). RhoA knockdown also eliminated the NF dependency observed in wild-type MDA-MB-231 (Basal B, B) and SUM159 (Basal B, L/B) cells stimulated with TNFα (1 h) (Supplementary Fig S5B). However, NF-κB ratio dependency on ruffling was observed in these lines (confidence > 0.9), as well as in RhoA-depleted AU565 (Luminal, L2) and T47D (Luminal, L1) cells, in which NF-κB was not connected to NF. Taken together, these data suggest that the RhoA-ROCK pathway is involved in mediating the negative regulation of NF-κB by cell–cell contact and that this link may be broken in some cancer cells.

**NF-κB is sensitive to substrate stiffness**

Based on these findings, we hypothesized that cortical F-actin could couple NF-κB activation to protrusion and spreading. Specifically, nuclear translocation could be enhanced in cells that are under low cortical tension, such as protrusive cells or cells at the edge of a colony, and suppressed in round or non-protrusive cells with high cortical tension (Thoumine et al, 1999; Maddox & Burridge, 2003). To test this hypothesis, we cultured MCF10A and MDA-MB-231 cells on fibronectin-coated glass or flexible polyacrylamide (PA) gels with Young’s moduli (a measure of stiffness) of 35 and 16 kPa (Tse & Engler, 2010) and measured cell shape and NF-κB localization in cells stimulated with TNFα. PA gels have long been used to fabricate substrates with more physiologically relevant elasticity than glass (Pelham & Wang, 1997). Substrate stiffness can control differentiation, motility, and cytoskeleton organization as cells sense and respond to matrix compliance (reviewed by Discher et al, 2005). Moreover, cells can tune their internal stiffness to match that of the extracellular matrix by controlling F-actin cross-linking and contraction (Solon et al, 2007).

Substrate flexibility altered morphology and NF-κB localization in both cell lines. MCF10A cells adhered and spread on PA gels but were significantly smaller in area and in tighter colonies than cells on glass (P < 0.01) (Supplementary Fig S6A). Nuclear area decreased in MCF10A cells only on the most flexible (16 kPa) gels (P < 0.01) (Supplementary Fig S6B). MDA-MB-231 morphology was extremely sensitive to substrate flexibility. Cells on glass ranged from large and flat to small and round, whereas cells spread poorly on 35 kPa gels and hardly at all on 16 kPa gels, indicating high levels of actin contractility and/or poor ECM adhesion. Cell and nucleus area decreased with stiffness (Supplementary Fig S6C and D), and nuclei in round, poorly spread cells were often crescent or cup shaped (Fig 6A, inset). NF-κB ratios decreased dramatically
with substrate stiffness in both cell lines (Fig 6B). In fact, about 5% of MDA-MB-231 cells on 16 kPa gels had negative log ratios, indicating little or no NF-κB activation. NF-κB ratios were also strongly associated with $\Delta_{\text{nuc}}/\Delta_{\text{cyto}}$ ($R^2 = 0.98$) (Fig 6C). Because cell spreading represents the balance between internal contractile forces and external resistance, these data indicate that NF-κB regulation is sensitive to cytoskeletal tension.

**N-cadherin depletion induces changes in cell shape and NF-κB localization**

Finally, we asked whether N-cadherin expression, which was associated with mesenchymal-like morphology and high NF-κB activation, affected NF-κB localization. MCF10A cells were transfected with siRNA against N-cadherin or mock-transfected (control) and seeded at varying densities. N-cadherin, but not E-cadherin, levels were significantly reduced after 3 days ($P < 0.01$) (Supplementary Fig S7A and B), and N-cadherin depletion resulted in a dramatic change in cell morphology (Fig 7A). Cells lacking N-cadherin grew exclusively in colonies, so NF was significantly higher in N-cadherin k/d wells than in control wells of comparable density (0.88 ± 0.05 vs 0.58 ± 0.04 for medium-density wells; $P < 0.01$) (Supplementary Fig S7C). Nuclear area and $\Delta_{\text{nuc}}/\Delta_{\text{cyto}}$ were also significantly higher in N-cadherin knockdowns ($P < 0.01$) (Supplementary Fig S7D and E). N-cadherin depleted cells had strikingly thick F-actin fibers, many of which terminated at cell membranes and appeared to be contiguous with fibers in adjacent cells (Fig 7B, arrow). NF-κB ratios were significantly lower in TNFα-stimulated N-cadherin k/d cells with NF ≥ 0.5, i.e. non-edge cells (Fig 7C). These data suggest that N-cadherin may modulate NF-κB activation in response to cytokine stimulation via effects on cell shape and cytoskeletal organization.

**Discussion**

Cell shape, in particular the cortical cytoskeleton and curvature of the plasma membrane, has been described as a ‘repository of information’ that can modulate signal transduction (Rangamani et al., 2013). We speculated that shape variation could account for some of the heterogeneity observed in NF-κB localization in breast cancer cells. We exploited the naturally occurring variation in cellular populations to uncover relationships between morphology and transcription factor activation in breast tumor and non-tumor cell lines, and tested model predictions using different techniques. To our knowledge, this is the first time that cell shape and context have been extensively quantified and linked to NF-κB regulation in breast cancer cells.

The effects of cell shape on NF-κB signaling were revealed through Bayesian analysis of single-cell datasets. In addition to NF-κB, Bayesian network models identified dependencies between morphological features and nuclear localization of YAP, a well-known shape-regulated TF (Dupont et al., 2011; Aragona et al., 2013). In contrast, Jun activation downstream of TNFα was not dependent on cell shape, which suggests that the dependencies we identified were specific to the NF-κB pathway and did not simply scale with differences in TNFR ligation. Whether shape has a role to play in regulating the activity of other TFs remains unclear. We speculate that TFs whose function is regulated largely through subcellular localization (i.e., are inhibited through cytoplasmic or membrane sequestration) are likely to be influenced by cell shape, which would couple transcriptional outputs to morphological inputs. In contrast, TFs that are constitutively localized to the nucleus and are regulated transcriptionally or via phosphorylation may be less dependent on morphological cues.

Well-spread, protrusive cells with few cell-cell contacts (mesenchymal-type morphologies) tended to be more responsive to TNFα than cells that were poorly spread and/or had many neighbors (epithelial-type morphologies). Because NF-κB drives expression of EMT genes, we propose that this difference in responsiveness could have the effect of reinforcing mesenchymal morphology in cancer cells, thus generating a positive feedback loop. In support of this model, chemically, physically, or genetically inducing cells to adopt mesenchymal-like shapes resulted in greater NF-κB activation.
Conversely, inducing cell rounding and/or strengthening cell-cell adhesions reduced NF-kB nuclear localization. Furthermore, expression of N-cadherin, an EMT marker (Andrews et al., 2012), was associated with mesenchymal-like morphology and high NF-kB activation in cancer lines, and its depletion induced an epithelial-like phenotype and suppressed NF-kB activation in MCF10A cells.

Bayesian networks predicted that NF-kB was decoupled from cell-cell contact after stimulation with TNFα in the tumor breast cell lines HCC1954 and AU565, and these results were confirmed by forcing cells into high and low NF by altering plating densities. This suggests that TF regulation in some cancer cells might become insensitive to cell morphology and context, which would result in inappropriate proliferation, survival, or migration. More work is needed to investigate how such decoupling might occur and what its consequences might be in the context of cancer.

Our data point to a critical role for cortical actomyosin tension in NF-kB regulation. Spreading cells and protrusive cells at the edge of a scrape wound experience a decrease in cortical tension, whereas cells rounding up due to loss of cell-matrix adhesion, entry into mitosis, or MT depolymerization have stiff actin cortices (Thoumine et al., 1999; Maddox & Burridge, 2003). We observed that cells with few neighbors, cells at wound edges, and cells in which RhoA-ROCK-myxosin II signaling was blocked had increased levels of nuclear NF-kB in response to TNFα. On the other hand, cells with rounded morphologies induced by nocodazole or taxol treatment, flexible substrates, or high plating density had less nuclear NF-kB. In addition, the difference in NF-kB activation between wound edge and non-edge cells was lost when actomyosin-mediated contractility was suppressed by ROCK inhibition, and the suppressive effect of high neighbor fraction was lost in the absence of RhoA.

Although I-kB is reported to bind to dynein (Crepeaux et al., 1997) and MTs (Chi et al., 2012), our observations do not suggest that NF-kB translocation depends on MT-mediated transport. Treating cells with noc and Y27 together shifted both cell shape and NF-kB ratios toward control values, and stabilizing MTs with taxol induced both rounded morphology and low NF-kB activation. Instead, we propose that MT depolymerization suppresses NF-kB by increasing cortical tension. Dynamic MTs provide mechanical resistance to actomyosin tension at the membrane (Wang et al., 2001), and Noc treatment can induce RhoA activation by releasing its activator GEF-H1 (Chang et al., 2008). Thus, the effects of ROCK inhibitors and Noc could cancel each other out. These results may help explain conflicting reports regarding the role of the cytoskeleton in NF-kB regulation, as they imply that drug effects depend on cell tension and shape (Rosette & Karin, 1995; Nemeth et al., 2004; Mackenzie & Oteiza, 2006; Ishitahara et al., 2013). Drug effects may also depend on which NF-kB pathways are activated. We found that low doses of nocodazole increased NF-kB nuclear localization in unstimulated cells (Supplementary Fig S4A), similar to Rosette and Karin (1995). This effect could be due to the induction of cellular stress responses to drug treatment. Cytoskeleton-modifying drugs affected cancer cell lines to different degrees, but the changes in cell shape they caused were consistently predictive of changes in NF-kB ratio (Fig 4D).

Further support for this model comes from the effects of N-cadherin depletion on cell shape and NF-kB in MCF10A cells. N-cadherin adhesions are less resistant to pulling forces than E-cadherin adhesions (Chu et al., 2006), so cells expressing only E-cadherin would exert more tensile force on one another. N-cadherin k/d cells are therefore predicted to be in a state of extremely high tension, and this is evident from the presence of thick F-actin fibers terminating at cell–cell contact points (Fig 7B, arrow). If actomyosin tension suppresses NF-kB activation, we would predict that N-cadherin k/d cells in the middle of colonies, with extensive cell–cell contacts (high NF), would have even lower NF-kB ratios than control cells, and in fact, this is what we observed. N-cadherin depleted cells also had significantly larger nuclei than controls, which could also be due to actin-mediated connections between the nuclei and cell–cell/matrix adhesions (Khatau et al., 2009). Moreover, NF-kB ratio in TNFα-stimulated cells was dependent on nuclear area in 15/19 cell lines, including MCF10A (Fig 2). Thus, N-cadherin expression could enhance NF-kB activation in cancer cells through its effects on cell morphology.

Altering cell shape with cytoskeleton-modifying drugs also affected the synchronicity and duration of NF-kB cycling in and out of the nucleus. Heterogeneity in nuclear-cytoplasmic shuttling may arise from differences in negative feedback caused by I-kB transcription, IKK activation, or the availability of NF-kB (Kearns et al., 2006; Ashall et al., 2009; Paszek et al., 2010; Kalita et al., 2011), and our data suggest that cell shape is also an important source of cell-to-cell variation. Heterogeneity may have evolved to ensure population robustness and reduce tissue sensitivity to inflammatory signals (Paszek et al., 2010). We speculate that shape-mediated differences in NF-kB shuttling could therefore have profound effects on how healthy, wounded, and pathological tissues respond to cytokines. While some models of oscillation have been proposed which take morphology into account (Terry & Chaplain, 2011; Sturrock et al., 2012), more work is needed to determine how cell shape impacts NF-kB cycling. Further high-content studies that incorporate live cell GFP-p65 and shape measurements will overcome the acyclic nature of Bayesian networks and elucidate whether a feedback exists from NF-kB to cell shape and provide insight into these mechanisms.

This work demonstrates the key role of shape and the microenvironment in regulating signal transduction and gene expression. Shape-dependent regulation of NF-kB may have evolved by facilitating the ability of isogenic cell types to achieve different responses to a uniform signal. For example, this mechanism could enable an inflammatory response in cells at a wound site while limiting the response in cells distal to the wound. The effects of cell shape on NF-kB signaling could also drive metastatic processes if cells at the invasive edge of tumors are more likely to activate NF-kB in response to inflammatory cytokines such as TNFα. Decoupling of NF-kB from the cell context and morphology could also drive tumorigenesis through increasing survival and proliferation.

Finally, these studies illustrate the utility of Bayesian network modeling for uncovering complex relationships between cell form and function, and they highlight the importance of context in determining cell behavior. Cellular context was shown to be an important source of variation in virus infectivity and endocytosis, and accounting for such differences in cellular states could explain much of the heterogeneity observed within cellular populations (Snijder et al., 2009). These findings indicate that cell shape and context are also important determinants of transcription factor regulation and cells’ response to chemical signals. Thus, care should be taken to consider factors such as density when interpreting data from experiments where culture conditions may vary, including RNAi screens, drug screens, and comparisons between cell lines.
Materials and Methods

Cell culture and staining

All cancer cell lines were grown in DMEM:F12 (Gibco) plus 5% heat-inactivated FBS (Gibco). MCF10A cells were maintained in DMEM:F12 medium (Gibco) supplemented with 100 ng/ml EGF, insulin, 0.5 mg/ml hydrocortisone, and cholera toxin (Sigma). Imaging experiments were performed in 384-well Cell Carrier plates (Greiner) on cells cultured for 3 days unless otherwise specified. For the initial screen, 1,000 cells/well were seeded in 14 replicate wells per cell line per plate. Live cell imaging was performed using a climate control chamber (37°C, 5% CO₂, 70% humidity).

Cells were labeled with 10 μM DHE (Invitrogen) 30 min prior to fixation in 4% PFA. Cells were permeabilized in 0.1% Triton X-100 and stained with anti-p65 antibody (Abcam), secondary antibody (Alexa®488 or Alexa®647 anti-rabbit, Invitrogen) and DAPI or Hoechst. In later experiments, segmentation of cell bodies was performed using anti-p65 or mouse anti-YAP (Santa Cruz) labels. Rabbit phospho-c-Jun (Ser63) antibody was from New England Biolabs. Mouse anti-N-cadherin was from Abcam.

Cytokine, drug treatments, and transfection

Human recombinant TNFα (Life Technologies) was added to a final concentration of 10 ng/mL. Y-27632 (Sigma) was used at 10 μM unless otherwise specified, and nocardazole was used at 0.1 μg/mL unless otherwise specified. Blebbistatin, taxol, and DMSO were obtained from Sigma. H1152 was from Tocris Bioscience. Plasmid transfection with GFP-p65/RelA (Addgene; plasmid ID 23255; unless otherwise specified). Blebbistatin, taxol, and DMSO were used at the indicated times before fixation. Cells were transfected with GFP-p65/RelA (Addgene; plasmid ID 23255; unless otherwise specified).

Plating density and scrape wound assays

Cells were seeded at 500, 1,000, 2,000, and 4,000 cells/well for TNFα/YAP density experiments and 500–8,000 cells/well for cadherin and RhoA knockdown experiments, and all cells were cultured for 3 days before fixing and staining. Scrape wounds were made using a pipet tip in confluent monolayers of cells cultured for 3 days; then, medium was removed and replaced with fresh medium containing TNFα at the indicated times before fixation.

Polyacrylamide gel fabrication and imaging

Flexible substrates were made by casting solutions containing different concentrations of acrylamide and bis-acrylamide (Sigma) as described by Tse and Engler (2010) onto glass coverslips functionalized with NaOH followed by APTMS and glutaraldehyde (Sigma). Cured gels were incubated with 25 μM sulfo-SANPAH (Thermo-Fisher) in 50 mM HEPES (pH 8.5) under UV light for 10 min, washed, and incubated with 50 μg/ml fibronectin (Sigma) in PBS overnight at 4°C. Glass coverslips were also coated with 50 μg/ml fibronectin. Cells were plated on PA gels for 3 days, fixed and stained, mounted using Fluoromount G (Southern Biotech), and imaged using a Nikon Eclipse epifluorescence microscope (20x). Analysis was performed using Columbus software (PerkinElmer).

Wavelet analysis

We extracted the characteristic periods and amplitudes from the oscillation data at 5-min intervals using the WAVOS Matlab toolbox (Harang et al., 2012).

PCA, clustering, and statistical tests

Principal component analysis was carried out using Cluster 3.0 and MATLAB software based on Z-scores [(value-mean)/SD]. Hierarchical clustering was performed with Cluster 3.0 and visualized using Java Tree View. P values were determined using Student’s t-test and ANOVA (Excel and MATLAB). R and R² values were determined using Excel or MATLAB (Pearson correlation unless otherwise specified).

Bayesian network and multivariate linear regression modeling

See Supplementary Materials and Methods for details and methods.

Data availability

Single cell data used to generate Bayesian network models for 19 cell lines ± TNFα (Supplementary Dataset S1), explanation of morphological features (Supplementary Dataset S2), and data used

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for multivariate linear regression (fold change compared to control for each cell line) (Supplementary Dataset S3) are provided as Supplementary Datasets S1, S2, and S3.

Image datasets for the cell lines used for morphological profiling are available from DRYAD: http://dx.doi.org/10.5061/dryad.tc5g4.

Supplementary information for this article is available online: http://msb.embopress.org

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Author contributions

JES and HZS designed experiments, developed image analysis scripts, and analyzed the data. JES, HZS, and CB prepared the manuscript. JES performed cell culture, immunostaining, PA gel fabrication, and microscopy. HS performed Bayesian network modeling and multivariate regression analysis. RCA performed qPCR and N-cadherin RNAi experiments. HM performed scrape wound experiments. TZ performed wavelet analysis.

Conflict of interest

The authors declare that they have no conflict of interest.

References


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Julia E Sero et al Morphology regulates NF-κB in breast cells


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A multi-scale approach reveals that NF-κB cRel enforces a B-cell decision to divide

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Abstract

Understanding the functions of multi-cellular organs in terms of the molecular networks within each cell is an important step in the quest to predict phenotype from genotype. B-lymphocyte population dynamics, which are predictive of immune response and vaccine effectiveness, are determined by individual cells undergoing division or death seemingly stochastically. Based on tracking single-cell time-lapse trajectories of hundreds of B cells, single-cell transcriptome, and immunofluorescence analyses, we constructed an agent-based multi-modular computational model to simulate lymphocyte population dynamics in terms of the molecular networks that control NF-κB signaling, the cell cycle, and apoptosis. Combining modeling and experimentation, we found that NF-κB cRel enforces the execution of a cellular decision between mutually exclusive fates by promoting survival in growing cells. But as cRel deficiency causes growing B cells to die at similar rates to non-growing cells, our analysis reveals that the phenomenological decision model of wild-type cells is rooted in a biased race of cell fates. We show that a multi-scale modeling approach allows for the prediction of dynamic organ-level physiology in terms of intra-cellular molecular networks.

Keywords apoptosis; B-lymphocyte; cell cycle; cell fate decision; NF-κB cRel
Subject Categories Quantitative Biology & Dynamical Systems; Signal Transduction; Autophagy & Cell Death

Introduction

B-lymphocytes are central to immune responses as producers of antibodies and mediators of immunological memory. Upon recognition of specific antigens or pathogen-derived ‘danger’ signals, B cells may enter a proliferative program (Murphy et al., 2007). This physiological process can be recapitulated ex vivo using agonists of the B-cell receptor or Toll-like receptors (TLRs), which recognize specific pathogen-derived substances. Such agonists elicit a dynamic population response in which individual cells may undergo several rounds of cell division, exit the cell cycle and/or die by programmed cell death (Rawlings et al., 2012). Indeed, while the population response is generally robustly reproducible and predictable, the behavior of individual cells is seemingly stochastic. In each generation, only a fraction of cells divide, while others die, and the timing of division and death is highly variable, typically well-modeled by long-tailed log-normal distributions and resulting in a spectrum of many generations at any given time point after stimulation (Hawkins et al., 2007b, 2009).

Given the physiological and pathological importance of the B-cell response, the underlying biochemical processes involved in transducing receptor signals, cell growth, cell cycling, and programmed cell death by apoptosis have been well studied (recently reviewed in Browne, 2012; Gerondakis & Siebenlist, 2010; Link & Hurlin, 2014; Renault & Chipuk, 2013). Their involvement in B-cell expansion has been characterized by measuring population cell numbers or apoptotic cells, bulk replicative activity (by measuring DNA synthesis), or distributions of generational cell counts at given time points (by dye dilution studies coupled to FACS). For example, deficiency in the NF-κB transcription factor cRel was reported to result in a substantially reduced B-population response, due to deficiencies in cell-cycle entry and cell survival (Gerondakis et al., 1998; Grumont et al., 1998). Further, potential cRel-dependent mediators of these processes have been identified, such as the genes coding for CyclinD (Wang et al., 1996; Guttridge et al., 1999; Huang et al., 2001), Myc (Duyao et al., 1990), and BclXL (Chen et al., 1999). Yet, how these
functions coordinately produce the dynamics of the population response, the generation-specific distributions, or fate control at the individual cell level remains poorly understood.

Previous studies have shown that the time to the first division is substantially longer than that of subsequent divisions, and the timing of cell death is also generation dependent (Hawkins et al., 2009). Yet there are competing theories for how fate (i.e., whether the cell divides or dies) is determined. Some studies invoke a molecular race hypothesis, which posits that processes leading to cell division and apoptosis are proceeding concurrently within cells, with the faster of the two determining the outcomes (Hawkins et al., 2007b; Duffy et al., 2012); however, other observations support the notion that cells decide their fate prior to it being manifest (Hawkins et al., 2009; Shokhirev & Hoffmann, 2013). In particular, the cyton (Hawkins et al., 2007b) and cyton (Shokhirev & Hoffmann, 2013) age- and generation-structured models describe lymphocyte population dynamics assuming a molecular race or decision between division and death, respectively. Further, it is unclear what the determinants are for the variability in timing which in the former but not the latter model underlies the variability of cell fate determination. Previous studies offer evidence that the inherent variability in timing of receptor-induced apoptosis of transformed liver cells is caused primarily by cell-to-cell variability in the steady state (Gaudet et al., 2012).

Recent advances in single-cell analysis and modeling render answers to these questions within reach. Flow cytometry and immunofluorescence microscopy, which provide snapshots of a few attributes of the cells within a population, may be complemented with single-cell mRNA sequencing, which provides transcriptome-wide measurements, and live cell microscopy, which provides longitudinal information at single-cell resolution; however, challenges in data analysis and integration persist. Interestingly, kinetic models that capture the dynamic control of molecular networks can function as platforms for data integration and provide a predictive understanding; for example, iterative experimental and modeling studies have delineated numerous negative and positive feedback loops that control the dynamics of NF-κB (Basak et al., 2012), or identified determinants of cell-cycle progression (Conradie et al., 2010) and cell death/survival fate decisions (Loriaux et al., 2013).

**Results**

Here, we aimed to construct a multi-modular mathematical model that accounts for B-cell population dynamics in terms of intra-cellular molecular network dynamics. Starting at the B-cell population scale, we employed carboxyfluorescein succinimidyl ester (CFSE) flow cytometry and live time-lapse microscopy tracking of cell lineages to characterize the model topology and parameters at the cell biological scale. Starting at the molecular network scale, we used single-cell RNAseq and quantitative immunofluorescence to characterize the connections between several regulatory molecular network modules (Fig 1).

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**Figure 1.** Developing a multi-scale understanding of the B-cell immune response.

We employed a multi-scale approach to studying the B-cell response. Time-lapse microscopy observations of B-cell populations revealed cellular growth trajectories, distribution of division and death time, as well as the fraction of cells responding in each generation. Single-cell molecular assays provided insights into the upregulation of key molecular players upon activation within individual cells. The number of cells in each generation was measured by the division tracking dye CFSE and deconvoluted into maximum-likelihood cellular parameters using the FlowMax computational tool. We used our observations to parameterize a multi-scale agent-based mathematical model consisting of established modules for signaling, apoptosis, and the cell cycle which allowed us to mechanistically study molecular perturbations on population dynamics.
Multi-scale modeling of B-lymphocyte dynamics

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Time-lapse microscopy reveals generation-specific single-cell behavior

In order to obtain cell lineage information that accounts for the population response, we tracked 1,295 live primary B cells using a time-lapse microscopy pipeline (Fig 2A). We developed a semi-automated image analysis method, combining the advantages of computational automation and human input to minimize errors (see Materials and Methods). Analysis of wild-type B cells responding to high Cpg stimulation confirmed the expected population expansion followed by a contraction period (Fig 2B). After cells that died from mechanical death in the initial phase (Hawkins et al, 2007a) were filtered out (Supplementary Fig S1A and B), we found that approximately 38% of the starting, ‘generation 0’, cells divided; then, 85% of generation 1 cells divided with subsequent generations, showing a steady decrease in this fraction such that only 9% of cells divided in generation 6 (Fig 2C). To quantify the cellular response, we classified cell size trajectories into two categories: (i) cells that grew by at least 350 μm² (representing at least two standard deviations on average, Supplementary Fig S1C) or reached a final size of at least 800 μm² (based on the bimodal size distribution (Supplementary Fig S1D) and to ensure that large generation 1+ cells are included), dubbed ‘growers’ and (ii) cells that did not meet these criteria, dubbed ‘non-growers’ (Fig 2D). To test the sensitivity of the growth threshold, we repeated the quantification with a 25% lower and higher growth threshold, revealing that few cells exhibited ambiguous growth (Supplementary Fig S1E). Averaging the growth trajectories of ‘growers’ (Fig 2E) and ‘non-growers’ (Fig 2F) in each generation normalized by percent cell lifespan revealed that progenitors (generation 0) that grew exhibited a growth delay followed by rapid growth to approximately fivefold their starting size, while generation 1+ growers did not exhibit the delay phase, and started growing immediately after mitosis. Furthermore, ‘grower’ cells generally grew to the same size on average in all generations except prior to their final division. While ‘non-growers’ by definition did not exhibit significant growth (as defined above), they nevertheless typically exhibited some growth.

Distinguishing between race and decision models in cell fate determination

To further characterize the underlying cellular mechanisms, we next tested whether cell cycle and apoptosis were parallel racing processes (Fig 3A), as previously suggested (Duffy et al, 2012), or
whether the growth phase was indicative of a prior decision to assume the division fate instead of the death fate (Fig 3B). For each generation, we counted the fraction of ‘growers’ that divided and died within a 24-h period: 12–36 h in generation 0 or 0–24 h in generations 1+ (Fig 3C), as well as the fraction of ‘non-growers’ that divided and died within the same periods for each generation (Fig 3D). Our results indicate that virtually all ‘growers’ in the first four generations divided, supporting the notion of an early decision that predisposes B cells to a particular fate (Fig 3B). Interestingly, following the first generation, there was a significant fraction of cells that divided that had been classified as ‘non-growers’; however, such poor growth almost always occurred in the last division (Supplementary Fig S2). To further test this important distinction, we noted the time point at which growth starts (Tgro), the time to division (Tdiv), and time to death (Tdie) of progenitor cells and calculated the expected lower-bound probability that a dying cell would have grown, provided a ‘molecular race’ or ‘decision’ model (Fig 3E and F). Our analysis revealed that even under relatively relaxed assumptions, the data are inconsistent with both processes occurring simultaneously in cells (i.e., race). A decision model, which commits cells to either fate, is more consistent with the observed behavior. In other words, because time to death is typically earlier than time to division (Fig 3E), and because time to start growing, Tgro, is typically much earlier than Tdiv or Tdie, our analysis predicts most cells would grow prior to death if the two processes were indeed running in parallel as implied by the ‘race’ model.

Next, we tested this hypothesis with an alternate method, using computational deconvolution of flow cytometric measurements of the generational populations at specific time points (Supplementary Fig S3A). CFSE-stained B cells were stimulated with CpG, and fluorescence histograms indicative of each generation were analyzed by the software tool FlowMax (Shokhirev & Hoffmann, 2013) to identify maximum-likelihood cellular parameters. Employing either the cyton model (which assumes that responding cells may die, Supplementary Fig S3B) or the fcyton model (which assumes that they do not, Supplementary Fig S3C), we asked which derived cellular parameters best agreed with those observed by time-lapse microscopy (Supplementary Fig S3D). While both models accurately fit the CFSE time course (Supplementary Fig S3E), the race (cyton) model requires a much longer distribution for Tdie0 than the decision (fcyton) model (Supplementary Fig S3F); shorter Tdie0 parameters are more consistent with the experimental microscopy dataset.
While our results suggest that cell fate is determined early, it is unclear what contributes to the cell-to-cell variability. Recent studies have shown that extrinsic variability in cell states rather than intrinsic signaling noise can account for variability in cell fate decision in mammalian cells (Spencer et al., 2009, 2013; Lee et al., 2010). As recently divided sibling cells have more similar cell states, we determined whether fate and timing are more correlated between related cells (Supplementary Fig S4). Indeed, sister cells were observed to undergo the same fate approximately 90% of the time, while cousin cells were more likely to experience different fates in all generations (Supplementary Fig S4A). Further, the timing of the decision process, interdivision time, and to a smaller extent lifespan were significantly correlated between sister cells: Pr (ΔTgro ≤ 4 h) = 0.90, R^2 = 0.74, and R^2 = 0.39, respectively (Supplementary Fig S4B–D). Furthermore, the correlations decreased with a subsequent division (i.e., between cousins): Pr (ΔTgro ≤ 4 h) = 0.77, R^2 = 0.44, and R^2 = 0.38, respectively, consistent with mixing times on the order of hours to days, more consistent with variability in molecular network states rather than genetic or epigenetic sources of cell-to-cell variability (Spencer et al., 2009).

Molecular determinants of cell fate decision processes

To characterize the molecular connections that underlie fate decision processes, we turned to single-cell molecular assays (Fig 4A). Following CpG stimulation of B cells for 24 h, we sequenced the transcriptomes of five large and five small cells using a single-cell autopen system, which allowed us to image and measure the size of individual B cells trapped in a microfluidic chip (Fig 4B). After normalizing transcript counts to RNA spike-in controls, we identified 369 upregulated and 121 downregulated genes in large versus small cells (Fig 4C). Using pathway enrichment tools, we identified pathways that were significantly upregulated in large cells, including metabolism, the control of apoptosis and the G1-to-S transition, and NF-κB, a known key regulator of B-cell expansion (Fig 4D). Further, we performed a transcription factor enrichment analysis on the upregulated and downregulated gene sets and found that binding motifs of nine transcription factors that are known NF-κB target genes, as well as NF-κB itself, were enriched among the genes upregulated in big cells (Fig 4E), whereas p53 was the only known NF-κB target gene transcription factor enriched in the set of genes downregulated in big cells.

Next, for immunofluorescence, we stained stimulated B cells for cRel, and measured average fluorescence as a function of cell area (Fig 4F). We found that compared to a 0 h control, B cells were larger (63% of cells) and had higher cRel abundance (63% of cells) after 24 h of stimulation. Furthermore, 68% of large cells had upregulated cRel at 24 h. To confirm the specificity of our analysis, we showed that cRel-deficient B cells had no detectable cRel fluorescence at 24 h (Fig 4G). Similarly to NF-κB cRel, approximately 50% of cells showed significantly increased levels of NF-κB RelA after 24 h, with approximately 56% of large cells showing increased NF-κB RelA abundance after 24 h of stimulation (Supplementary Fig S5A). Staining for Myc, a master transcriptional regulator of cell growth and known NF-κB target gene, we revealed that 57% of cells had upregulated Myc levels at 24 h compared to 0 h, and 62% of large cells had elevated Myc levels (Fig 4H). Upon NF-κB cRel deletion, only 35% of cells had elevated Myc levels (Fig 4I). Similarly, BcLXL, a known NF-κB cRel target gene and anti-apoptotic regulator, was found to be elevated primarily in large cells (Fig 4J). Whereas BcLXL was upregulated in 85% of large cells, only 8% of all large cells upregulated BcLXL in the absence of cRel (Fig 4K). Quantitative RT–PCR confirmed that BcLXL was upregulated at the mRNA level and that NF-κB cRel contributes about two-thirds of the BcLXL expression at 20 h (Supplementary Fig S5B).

Repeating the immunofluorescence analysis in the presence of 1 ng/ml rapamycin, a known mTORC1 inhibitor (Supplementary Fig S5C), we found the same fraction of cells had upregulated cRel abundance after 24 h of stimulation, though the fraction of large cells was reduced, suggesting that cRel upregulation is independent of mTORC1. Conversely, we tested the role of NF-κB cRel in regulating mTORC1 and found that the abundance of p-S6, an indicator of mTORC1 activity, by immunoblot was reduced by approximately a factor of 2 in NF-κB cRel-deficient B cells (Fig 4K), though cell growth itself was not substantially diminished presumably due to compensatory mechanisms including NF-κB family members RelA and RelB. Our analysis supports a model in which NF-κB is a regulator of both cell survival and cell growth.

Constructing a multi-scale model to predict B-cell population dynamics

The described analyses of CFSE time courses, time-lapse microscopy, and molecular studies led us to test whether B-cell population dynamics may be accounted for with a mathematical model of intracellular molecular networks that exist in cell-specific steady states due to biochemical variability. We implemented established ordinary differential equation (ODE) kinetic models of the NF-κB signaling system (Alves et al., 2014), apoptotic control network (Loriaux et al., 2013), and the cell cycle (Conradie et al., 2010) (Supplementary Fig S6A). Introducing sources of extrinsic variability, we found that variability in protein levels alone was sufficient to produce cell-to-cell variability in nuclear NF-κB concentration, cell-cycle duration, and lifetime typically observed (Supplementary Fig S6B). Importantly, the cell-cycle model with added sources of extrinsic noise produced relatively short cell-cycle durations of ~10–20 h, similar to generation 1+ cells, but did not readily account for the generation 0 delay (Fig 2). Further, we found that introducing extrinsic protein variability resulted in substantial cell growth variability.

Based on our molecular analysis, we constructed an integrated ODE model (Fig 5A and Supplementary Methods) with NF-κB-controlled synthesis of BcLXL, a key regulator in the apoptosis module, as well as NF-κB-controlled synthesis of CycD, a key regulator in the cell-cycle module. Furthermore, in the cell-cycle module, growth is controlled by general machinery (GM), which represents the ribosomes and other cellular components that promote the accumulation of cell mass. Mass in turn promotes the growth of general machinery, creating a positive feedback loop that results in exponential growth and cellular progression through the cell cycle. However, since we observed B cells to delay growth prior to the first division (Fig 2), we needed to model the control of general machinery (GM) in more detail. Hence, we incorporated NF-κB-controlled synthesis of Myc, a transcription factor that promotes cell growth,
which is typically low in quiescent cells but a known NF-κB target gene. To obtain population dynamics, the integrated ODE model was incorporated into cellular agents (Fig 5B), which kept track of their generation, age, and independent set of starting synthesis/ degradation or total protein concentrations, which were drawn from normal or log-normal distributions, respectively. The models were solved until the agent died [defined as (cPARP) 25,000 mole-] or completed mitosis [(cdh1) > 25,000 mole-]. Pathways in Small Cells

<table>
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Pathways in Big Cells

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Figure 5. Multi-scale agent-based modeling of B-lymphocyte dynamics. A–L Naïve purified B cells were stimulated with 250 nM CpG for 24 h and analyzed using single-cell RNA sequencing (A top). Five small and five large B cells were captured in a microfluidics chip (B), and their transcriptomes were sequenced to reveal sets of genes typically upregulated in large cells (C, red) or small cells (C, green). Pathway analysis on genes upregulated in large B cells (D, top) and small cells (D, bottom) was performed. (E) Transcription factor motif enrichment analysis on the genes upregulated in large B cells (E, top) and small cells (E, bottom) was performed and filtered to show only significantly upregulated (P < 0.05) and known NF-κB target genes or NF-κB itself. NF-κB cRel abundances of purified naïve B cells stimulated with 250 nM CpG for 24 h were obtained by quantifying average fluorescence in fixed B cells stained with anti-cRel antibody conjugated to fluorophore, or anti-BclXL antibody bound to a fluorescent secondary antibody (A). Quadrants in (F–H). Immunoblot for p-S783: BclXL of purified naïve B cells stimulated with 250 nM CpG for 24 h (P outcomes). NF-κB cRel-rich (F), growth regulator Myc (H), and anti-apoptotic regulator BclXL (I) were obtained by quantifying average fluorescence in fixed B cells stained with anti-cRel antibody conjugated to fluorophore, or anti-BclXL antibody bound to a fluorescent secondary antibody (A). Quadrants in (F–K) indicate the proportion of cells at 24 h compared to 0 h. Growth was manually defined as a cell area > 100 pixels to avoid cell selection bias in images.

Figure 4. Molecular assays suggest that NF-κB enforces an upstream fate decision.

A–L Naïve purified B cells were stimulated with 250 nM CpG for 24 h and analyzed using single-cell RNA sequencing (A top). Five small and five large B cells were captured in a microfluidics chip (B), and their transcriptomes were sequenced to reveal sets of genes typically upregulated in large cells (C, red) or small cells (C, green). Pathway analysis on genes upregulated in large B cells (D, top) and small cells (D, bottom) was performed. (E) Transcription factor motif enrichment analysis on the genes upregulated in large B cells (E, top) and small cells (E, bottom) was performed and filtered to show only significantly upregulated (P < 0.05) and known NF-κB target genes or NF-κB itself. NF-κB cRel abundances of purified naïve B cells stimulated with 250 nM CpG for 24 h were obtained by quantifying average fluorescence in fixed B cells stained with anti-cRel antibody conjugated to fluorophore, or anti-BclXL antibody bound to a fluorescent secondary antibody (A). Quadrants in (F–H). Immunoblot for p-S783: BclXL of purified naïve B cells stimulated with 250 nM CpG for 24 h (P outcomes). NF-κB cRel-rich (F), growth regulator Myc (H), and anti-apoptotic regulator BclXL (I) were obtained by quantifying average fluorescence in fixed B cells stained with anti-cRel antibody conjugated to fluorophore, or anti-BclXL antibody bound to a fluorescent secondary antibody (A). Quadrants in (F–K) indicate the proportion of cells at 24 h compared to 0 h. Growth was manually defined as a cell area > 100 pixels to avoid cell selection bias in images.
was incorporated into cellular agents (Fig 5B), which kept track of the gene. To obtain population dynamics, the integrated ODE model, which is typically low in quiescent cells but a known NF-κB target gene, was implemented and combined into one integrated model. Blue, green, and red colors represent NF-κB, apoptosis, and cell-cycle modules, respectively.

Figure 5. Multi-scale agent-based modeling of the B-cell response.

A Established ordinary differential equation models for NF-κB signaling (Alves et al, 2014), apoptosis (Loriaux & Hoffmann, 2012), and the cell cycle (Conradie et al, 2010) were implemented and combined into one integrated model. Blue, green, and red colors represent NF-κB, apoptosis, and cell-cycle modules, respectively, while bolded species represent active IKK (input), cleaved PARP (death readout), and cdh1 abundance (mitosis readout).

B Instances of the integrated model were incorporated into cellular agents, extrinsic noise was introduced to mimic cell-to-cell variability, and the agent-based model was solved one generation at a time, with division resulting in the creation of two new agents, and death resulting in the removal of the agent from the population.

C–F A comparison of agent-based modeling solutions to the time-lapse microscopy dataset is shown. Cell counts normalized to start count (C), fraction of cells dividing or dying in each generation (D), average size of growers in each generation as a function of % lifetime (E), and average size of non-growers in each generation as a function of % lifetime (F) are compared. Error bars represent SEM or 1 SD.

was removed or replaced by two new daughter agents, respectively. We subjected daughter agents to extrinsic re-mixing noise to account for loss of correlation with successive generations. When training the model on our results from the wild-type condition, we retained the value of all published NF-κB, cell-cycle, and apoptosis parameters, leaving a set of free parameters specifying BclXL, CycD, and Myc transcript synthesis and degradation, as well as parameters controlling the growth and survival of cells (Supplementary Methods and Supplementary Table S9). Remarkably, we were able to recapitulate the observed population dynamics (Fig SC), the fraction of cells dividing or dying in each generation (Fig SD), as well as the growth trajectories of growing and non-growing cells in each generation (Fig SE and F) by fitting just these free parameters from within biologically plausible ranges to a set of features (Supplementary Tables S9 and S10, and Supplementary Methods).

Model-enabled perturbation studies: NF-κB cRel enforces the execution of the cell fate decision by biasing a fate race of growing cells against death

We next asked whether the model could be used for studies of genetic or pharmacological perturbations. In particular, we examined the population behavior in B cells exposed to reduced stimulus concentrations in the absence of NF-κB cRel, or when treated with the cell growth inhibitor rapamycin (Fig 6A). Model predictions were compared to time-lapse microscopy experiments in which the
same conditions were applied. First, we simulated the low dose stimulation condition by allowing for a faster decay of the active IKK species (see Supplementary Table S9 and Supplementary Methods). The model predicted a dramatic decrease in the total B-cell population (Fig 6B), resulting from a decrease in the fraction of cells that divide in generations 3+ (Fig 6C); however, cell size trajectories (Supplementary Fig S7B and C) and fate timing (Supplementary Fig S7D–F) were unaffected. An equivalent analysis of subsequent time-lapse experiments confirmed these predictions (Fig 6B–D, Supplementary Fig S7), although the model predicted a later peak in total B-cells (Supplementary Fig S7B and C) and fate timing (Supplementary Fig S7D), although the model predicted a later peak in total B-cell abundance resulted in dramatic changes to the population dynamics (Fig 6I), was recapitulated well by simply decreasing the global protein translation rate by 30% (Fig 6A, H–J). Importantly, this also resulted in longer delays prior to division (Fig 8D), as long as the positive regulators were among those lacking cRel (Fig 8D compare to B), suggesting that growth and division, or death processes (Supplementary Fig S7D of cRel-deficient cells confirmed these predictions (Supplementary Table S11). Finally, treatment with rapamycin, the inhibitor of mTOR, which results in defective cell growth and ribosome biosynthesis, as well as a decrease in cells that divide more than once (Fig 6I), was recapitulated well by simply decreasing the global protein translation rate by 30% (Fig 6A, H–J). Importantly, this also resulted in longer delays prior to division (Fig 8D), as long as the positive regulators were among those lacking cRel (Fig 8D compare to B), suggesting that growth and division, or death processes (Supplementary Fig S7D–F), but that a higher percentage of growing cells would die (Fig 6G). A side-by-side comparison with the results from experimental cell tracking of cRel-deficient cells confirmed these predictions (Supplementary Table S11). Finally, treatment with rapamycin, the inhibitor of mTOR, which results in defective cell growth and ribosome biosynthesis, as well as a decrease in cells that divide more than once (Fig 6I), was recapitulated well by simply decreasing the global protein translation rate by 30% (Fig 6A, H–J). Importantly, this also resulted in longer delays prior to division (Fig 8D), as long as the positive regulators were among those lacking cRel (Fig 8D compare to B), suggesting that growth and division, or death processes (Supplementary Fig S7D–F), but that a higher percentage of growing cells would die (Fig 6G). A side-by-side comparison with the results from experimental cell tracking of cRel-deficient cells confirmed these predictions (Supplementary Table S11). Finally, treatment with rapamycin, the inhibitor of mTOR, which results in defective cell growth and ribosome biosynthesis, as well as a decrease in cells that divide more than once (Fig 6I), was recapitulated well by simply decreasing the global protein translation rate by 30% (Fig 6A, H–J). Importantly, this also resulted in longer delays prior to division (Fig 8D), as long as the positive regulators were among those lacking cRel (Fig 8D compare to B), suggesting that growth and division, or death processes (Supplementary Fig S7D–F), but that a higher percentage of growing cells would die (Fig 6G). A side-by-side comparison with the results from experimental cell tracking of cRel-deficient cells confirmed these predictions (Supplementary Table S11). Finally, treatment with rapamycin, the inhibitor of mTOR, which results in defective cell growth and ribosome biosynthesis, as well as a decrease in cells that divide more than once (Fig 6I), was recapitulated well by simply decreasing the global protein translation rate by 30% (Fig 6A, H–J). Importantly, this also resulted in longer delays prior to division (Fig 8D), as long as the positive regulators were among those lacking cRel (Fig 8D compare to B), suggesting that growth and division, or death processes (Supplementary Fig S7D–F), but that a higher percentage of growing cells would die (Fig 6G). A side-by-side comparison with the results from experimental cell tracking of cRel-deficient cells confirmed these predictions (Supplementary Table S11). Finally, treatment with rapamycin, the inhibitor of mTOR, which results in defective cell growth and ribosome biosynthesis, as well as a decrease in cells that divide more than once (Fig 6I), was recapitulated well by simply decreasing the global protein translation rate by 30% (Fig 6A, H–J). Importantly, this also resulted in longer delays prior to division (Fig 8D), as long as the positive regulators were among those lacking cRel (Fig 8D compare to B), suggesting that growth and
death were no longer mutually exclusive. The increased probability was still lower than the minimum probability expected for a complete loss of decision enforcement, calculated using observed distributions for the time to start growing, divide, and die (Fig 7C). A lack of decision enforcement was not seen when a lower dose of the stimulus (Fig 7E and F) or rapamycin drug treatment (Fig 7G and H) was used, confirming NF-κB cRel’s specific role. These studies suggest that the phenomological cell fate decision is mediated at the molecular level by cRel, which biases a cell fate race in growing cells against cell death, rendering them pre-determined for division.

Extrinsic molecular network noise determines the magnitude of the population response

Utilizing the multi-scale model, we explored how the average and the variability of protein abundances within the molecular network may affect the population response. In this analysis, we distinguished between negative regulators of NF-κB signaling (the IκBs), the positive regulators (IKK and the NF-κB monomers RelA, p50, and cRel), or both, as well as apoptosis and cell-cycle regulators, or all proteins (Fig 8A). Increased average abundance (Fig 8B) was achieved by increasing the translation rate or the total protein abundance (if constant) by 10 or 50%, respectively, while increased protein variability (Fig 8C) was achieved by doubling the coefficient of variation (CV) of the translation rate or total protein abundance (if constant). As expected, moderately increasing the average protein abundance resulted in dramatic changes to the population dynamics (Fig 8D), as long as the positive regulators were among those affected (blue, purple, and gray conditions). Our analysis indicates that this is primarily caused by an increase in the number of division rounds that progenitors underwent (Fig 8E), as well as due to typically shorter interdivision times (Fig 8F). Meanwhile, increasing the expression of negative regulators of NF-κB (IκBs) decreased the population response (Fig 8D), decreased propensity to divide (Fig 8E), and resulted in typically longer cell-cycle duration (Fig 8F). Furthermore, increasing the positive regulators alone and to a lesser extent the cell-cycle/apoptosis proteins resulted in an accumulation of non-dividing and surviving cells (Fig 8G; blue, orange), while increasing negative regulators (IκBs) tended to decrease survival (Fig 8G; red versus green, purple versus blue, gray versus orange).

However, when manipulating the variability of expression only, we found that increased variability in negative regulators of NF-κB and non-NF-κB proteins resulted in increased cell counts over time, due to accumulation of non-dividing surviving cells (Fig 8K; red, orange, gray). Increasing the CV of both the positive and negative regulators resulted in modest increases in the number of times a progenitor divided (Fig 8I); however, doubling the CV of negative regulators also resulted in increased survival (Fig 8K). Increased variability for apoptosis and cell-cycle proteins also resulted in higher survival (Fig 8K; orange, gray); however, on average cells experienced fewer division rounds (Fig 8I), resulting in broader population dynamics, indicating that cell-cycle regulation is sensitive to relatively large increases in protein variability (Fig 8H). Thus, the multi-scale model enabled us to test the role that extrinsic variability plays in a module-specific manner, revealing that extrinsic noise in the expression of negative regulators of NF-κB can lead to hyper-proliferative phenotypes due in part to long-term cell survival, while positive regulators of NF-κB determine the number of divisions.

Discussion

The complexity and inherent heterogeneity of the B-cell population response poses serious challenges to predicting modes of disease action and the potential efficacy of drugs. In this study, a
combination of single-cell molecular assays, single-cell time-lapse microscopy, and population flow cytometry allowed us to construct a multi-scale model, in which the intra-cellular network of NF-κB signaling, cell-cycle, and apoptosis control accounts for the cell population dynamics in response to mitogen, which provides a framework for genetic and pharmacological perturbation studies that begin to link molecular scale perturbations to organ-level phenotypes and function.

Agent-based multi-scale modeling of the B-cell immune response

Agent-based models (ABMs) explicitly describe autonomous entities within a system and provide a natural computational framework for modeling immune processes (recently reviewed in An et al., 2009; Narang et al., 2012). As such, ABMs have been successfully utilized to provide insights into the dynamics of the NF-κB signaling system (Pogson et al., 2006), wound healing (Walker et al., 2004), the multi-scale effects of acute inflammation (An, 2008), the implications of transgenerational epigenetic inheritance (Jiao et al., 2012), and the evolution of aging (Shokhirev & Johnson, 2014).

In the absence of an established framework for multi-scale B-cell modeling, we took a parsimonious approach toward model construction. Since the number of parameters typically scales nonlinearly with the size of the model, our strategy was to use previously established models and manually parameterize the connections between them based on experimental studies following genetic or pharmacological perturbations. While numerous regulators of B-cell signaling and proliferation have been identified, we
focused here (for the purposes of this first version of a B-cell ABM) on the stimulus-responsive NF-κB signaling system as a key determinant of B-cell population dynamics (Pohl et al., 2002). Several important regulators are known NF-κB target genes (Duyao et al., 1990; Wang et al., 1996; Chen et al., 1999; Gattridge et al., 1999; Huang et al., 2001); however, how they function together to produce the observed population dynamics remained poorly understood. We took both an unbiased approach by sequencing the transcriptomes of small cells and growing cells, and a targeted approach via single-cell measurements of key proteins by immunofluorescence. While there was significant cell-to-cell transcriptome variability (Supplementary Table S2), there was a clear NF-κB signaling signature in large but not small cells (Fig 4), indicating that the fate variability may originate upstream of the NF-κB system. Indeed, NF-κB cRel and RelA are upregulated after 24 h of stimulation in large but not small cells (Fig 4F and Supplementary Fig S5A), causing the upregulation of the growth regulator Myc (Fig 4H and I) and the anti-apoptotic regulator BclXL (Fig 4J and K) and the activity of the metabolic regulator mTORc1 (Fig 4L). These studies quantified the connectivity of NF-κB with downstream effector functions, enabling us to parameterize the relative contributions of NF-κB cRel, RelA, and non-NF-κB transcriptional regulators toward the activation of these downstream effectors. This placed NF-κB in a position of biasing the fate of growing cells toward division over death.

In addition, we were also able to confirm that fate decisions and division and death times are correlated between sibling cells (Supplementary Fig S4), which is consistent with apparent cell-to-cell variability being due to differences in protein turnover processes (Gaudet et al., 2012; Flusberg et al., 2013) resulting in distributions of single-cell proteomes within a population. Such variability constitutes noise that is extrinsic to the molecular processes explicitly represented in the model, thus justifying an ordinary differential equation formulation with distributed initial states to model a population of B cells, akin to previous studies (Spencer & Sorger, 2011; Loriaux & Hoffmann, 2012).

The resulting simulations recapitulated major features of the cellular and population responses with an accuracy that was surprising given that the three models were connected with a minimum number of reactions (Supplementary Methods, Supplementary Table S9). In particular, the maximum relative cell count (Fig 5C), the characteristic total population expansion and contraction curve (Fig 5C), the number of divisions observed (Fig 5C and D), the fraction of cells responding in each generation (Fig 5D), and the average growth trajectories of growing (Fig 5E) and non-growing (Fig 5F) cells were captured by the model, among others (see Supplementary Tables S9 and S10). The model was then used to predict population dynamics following a number genetic and pharmacological perturbations that yielded a first set of biological insights.

**NF-κB cRel enforces a cell fate decision by protecting growing cells against death**

Previous studies have described the B-cell response as a molecular race between division and death processes (Hawkins et al., 2007b; Duffy et al., 2012), while others have argued for an early decision process (Hyrien et al., 2010; Shokhirev & Hoffmann, 2013; Chakravorty et al., 2014). A decision model is consistent with the observation that a subset of generation 0 cells prepare for several rounds of rapid divisions by simultaneously deactivating quiescence (Yusuf & Fruman, 2003; Hawkins et al., 2009) and activating growth pathways such as Myc and mTOR (Grumont et al., 2002; Wang et al., 2011). In contrast, cell death is a default pathway as unstimulated B cells will undergo apoptosis in vitro (Supplementary Fig S8), though cell lifetime may be extended by expressing anti-apoptotic regulators as a consequence of signaling (recently reviewed in Renault & Chipuk, 2013).

To probe whether the division or death fate was a result of a fate race or a decision, we tracked B cells in long time course microscopy studies to characterize several key properties of the response. There is a pronounced but variable delay in growth initiation prior to the first division, while generation 1+ cells start growing immediately (Fig 2D). Tracking cell size trajectories and their eventual fate allowed us to show that B cells that had entered the growth phase were protected from death (Fig 3). Further, a mathematical model which assumed a race between division and death (Hawkins et al., 2007b), applied to flow cytometry data, could not account for the death time distribution observed in microscopy experiments (Supplementary Fig S3), even when early death (within the first 12 h), potentially caused by mechanical manipulation of cells, is filtered out (Supplementary Fig S1).

Using the multi-scale model, we explored NF-κB’s role in determining B-cell population dynamics. As expected, in silico knockout of NF-κB cRel substantially reduced the population response (Fig 6E), allowing for fewer divisions (Fig 6F). This was due to a greater fraction of growing cells dying (Fig 6G), but fate timing and growth trajectories were predicted to and remained largely unchanged (Supplementary Fig S7). Importantly, time-lapse microscopy experiments confirmed these model predictions (Fig 6E-G, Supplementary Fig S7). Further, model simulations predicted and experimental studies confirmed that in the absence of cRel, cells that have entered the growth phase may not be committed to divide, but instead are prone to death (Fig 7D). Thus, cRel’s function may be described as enforcing a decision to divide, with the population response of cRel-deficient cells resembling that of a molecular race more closely than that of wild-type cells. Indeed, our work with cRel-deficient models and cells suggests that the fate decision at the cell biological scale may be described as a fate race that is highly biased against death by NF-κB cRel. Other NF-κB members such as RelA and RelB may contribute as well, and their combined function is likely critical for promoting entry into the growth phase also.

**The population response is sensitive to extrinsic noise in the signaling module**

The present model version could be used to explore how molecular-level perturbations affect cell population dynamics (Fig 8). It may not be surprising that increasing the abundance of negative regulators of NF-κB diminished the population response; however, the sensitivity to small increases in the positive regulators was striking (Fig 8D), affirming the strategy for searching for cancer-causing mutations that alter NF-κB control (Staudt, 2010). We also found an increased population response (Fig 8H) due to enhanced survival (Fig 8K) if instead the variability but not the average of protein abundances was increased in the model. Our data suggest that deregulation (i.e., increased variability) of negative regulators was particularly important (Fig 8H-K). Increased
cell-to-cell variability of cell-cycle and apoptosis proteins resulted in accumulation of long-lived cells, decreasing the fraction of dividing cells in each generation (Fig 8I) and increasing the length of the cell cycle (Fig 8J). Our analysis points to the importance of quantitative data at the single-cell level (e.g., the distributions of protein abundances, even when average measurements remain unaltered) in the diagnosis and prognosis of disease using single-cell technologies (Chattopadhyay et al., 2014; Macaulay & Voet, 2014).

In sum, the multi-scale model we present here is a first attempt at connecting molecular networks to B-cell population dynamics and demonstrates that much of the population behavior, including the observed biasing of cell fate, emerges when NF-kB is allowed to affect mammalian models for cell cycling and apoptosis. This model enables in silico molecular perturbation studies, allows the testing of many molecular factors and mechanisms simultaneously, and can serve as a framework for refinement within the iterative Systems Biology approach.

Materials and Methods

B-cell purification and incubation

Primary splenocytes were isolated from 6- to 8-week-old mice, and naïve B cells purified using magnetic bead separation (Miltenyi Biotech) and stimulated with 250 nM, or 10 nM CpG ODN 1668 (Invivogen). mTORC1 inhibition was achieved by 1 h pretreatment Biotec) and stimulated with 250 nM, or 10 nM CpG ODN 1668 systems biology approach.

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Time-lapse microscopy

Purified naïve B cells were grown in 1,536 flat-bottom tissue-culture-treated microwells (Greiner Bio-One). Images were acquired on an Axio Observer Z1 inverted microscope (Carl Zeiss Microscopy GmbH, Germany) with a 10×, 0.3 NA air immersion objective to a CoolSnap HQ2 CCD camera (Photometrics, Canada) using ZEN imaging software (Carl Zeiss Microscopy GmbH). Environmental conditions were maintained at 37°C, 10% CO2 with a heated enclosure, and CO2 controller (Pecon, Germany). Phase-contrast images were taken every minute for 6 days.

Cell tracking

A semi-automated computational approach was used to track B cells in phase-contrast images. First, image intensities were normalized to maximize contrast. Next, edges were identified using a Sobel transformation and global thresholding. Cells were identified using a customized Hough transformation assuming cells were approximately circular. Next, approximate linear paths were manually drawn for each cell until the cell was observed to divide, die, or leave the field of view (also manually annotated to ensure accuracy). Cells entering the field of view after 24 h of stimulation (i.e., potentially after the first division) and debris were tracked but removed from the subsequent analysis. After all paths were drawn, all cell boundaries were optimized simultaneously from frame to frame. During automatic optimization, cells were modeled as deformable two-dimensional polygons with forces acting upon each vertex that ensured the polygons did not grow/shrink too quickly, did not overlap other polygons, were attracted to edges in the image, and were attracted to their respective manually curated path. The relative magnitudes of the forces were manually calibrated to ensure appropriate behavior. Cell size trajectories were fitted using a piecewise function consisting of a linear no-growth period, followed by exponential growth:

\[ V(t) = \begin{cases} V_o & t \leq t_{gro} \\ V_o e^{kt} & t > t_{gro} \end{cases} \]

The quality of individual cell tracks were assessed by calculating RMSE from \( V(t) \), and the \( t_{gro} \) value was assumed to be the fitted inflection point in this function (i.e., when cells were predicted to start exponential growth). Growing cells were defined as having an average ending volume at least 350 µm^3 above the average starting volume, or if the final volume was at least 800 µm^3. Cells that grew but then decreased in size or that did not meet any of these conditions were labeled as non-growing. The Java platform-independent executable for tracking cells is included as Supplementary File S1. Tracked videos of WT 250 nM CpG, WT 10 nM CpG, NF-kB cRel deficient 250 nM CpG, and WT 250 nM CpG + Rapamycin treatment B cells are provided as Supplementary Files S2, S3, S4, and S5, respectively.

Calculating the expected probability that a dying cell would have started growing

For a detailed methodology and notes, please see Supplementary Methods. In short, if division and death are parallel biological processes running within a cell, it is possible to calculate the lower bound on the expected fraction of progenitor cells that would start growing and then die if the time to die is typically earlier than the time to division. We predict this lower bound from the observed distributions for the time to decide to grow (Tgro), time to die (Tdieu), time to divide (Tdive), and the observed fraction of dividing cells in generation 0, and then compare the predicted lower bound to the actual observed fraction of growing cells that die.

Single-cell RNAseq

Stimulated wild-type B cells were collected at 24 h post-stimulation and concentrated to 5 × 10^6 cells per ml. Cells were loaded onto a 10–17 µM primed C5 single-cell auto prep array IFC (Fluidigm), and phase-contrast images were taken of all viable cells as determined by the Live/Dead Viability/Cytotoxicity Kit (Invitrogen). ERCC RNA spikein controls (Life Technologies) were added to the lysis mix at a 1:200 dilution. Tube controls (bulk cell positive control and no cell negative control) were also prepared according to the Fluidigm protocol. Lysis, reverse transcription, and PCR were performed using the SMARTer Ultra Low RNA Kit (CloneTech) and Advantage 2 PCR Kit (CloneTech) on individual cells using the C5 Single-Cell Auto Prep System (Fluidigm). Cell size was manually determined.
from images using ImageJ software. Sample libraries for the five smallest and five largest cells along with the controls were prepared using the Nextera XT DNA Sample Preparation (Illumina), and library quality was assayed using the Quant-iT PicoGreen dye (Life Technologies) quantification on a Qubit 2.0 Fluorometer (Life Technologies) and by gel electrophoresis. Libraries were sequenced by the UCLA Broad Stem Cell Research Center High Throughput Sequencing Core on Illumina HiSeq 2000 sequencers according to manufacturer recommendations. Reads were aligned to the ENSEMBL NCBI m37 genome (Church et al., 2009) using rna-STAR (Dobin et al., 2013). To compute spike-in concentrations for normalization purposes, the 23 most abundant RNA spike-in concentrations (at least one read in all samples) were compared to the expected concentrations in log-log space, and the y-intercept in log-space was used to compute normalized spike-in concentrations for each sample, [Spikein]. The normalized expression of gene $i$, in sample $j$, was then computed as:

$$\text{Gene}_{ij} = \frac{\text{Gene}_{i}}{\text{Spikein}} + 100.$$  

A constant count of 100 was added because spike-ins with counts $<100$ were variable across samples. To assess the overall quality of each cell, we correlated their wide transcript rpmk values to the average across all cells as well as to the positive tube control. We found that one large cell had significantly lower correlation, so we omitted it from further analysis. To determine whether a particular gene was upregulated in big cells, we computed an expression score:

$$\text{Escore}_{i} = \frac{\sum_{\text{big}} f(\text{gene}_{i} > 300)}{\#\text{cells}_{\text{big}}} - \frac{\sum_{\text{small}} f(\text{gene}_{i} > 300)}{\#\text{cells}_{\text{small}}},$$  

where $f(\text{gene}_{i} > 300)$ is 1 if gene $i$ has above 300 read count in a particular sample. A gene with an expression score $\geq 0.5$ was considered upregulated in big cells, while a gene with an expression score $\leq -0.5$ was considered downregulated in big cells. These sets of upregulated and downregulated genes were analyzed for pathway enrichment and transcription factor motif enrichment using WebGestalt (Wang et al., 2013). Significant transcription factors were further filtered to remove non-NF-kB downstream targets as defined in Supplementary Table S1. The single-cell RNA seq feature counts and analysis are included as a Microsoft Excel file (Supplementary File S6).

**Immunofluorescence**

$\times 10^6$ cells were collected after 24 h incubation at 37°C and 5% CO$_2$, washed with cold 1 × PBS, resuspended in Annexin binding buffer containing 10 μl Annexin-V conjugated to AlexaFluor350 (Life Technologies), stained for 1 h at 25°C, fixed with 4% paraformaldehyde (Electron Microscopy Sciences) for 15 min, washed with 1 × PBS, and incubated in cold blocking buffer (1 × PBS, 5% normal goat serum, 0.4% Triton X-100, 0.02% SDS), washed with IF buffer, counted, and then incubated at 4°C overnight in IF buffer containing 1:100 primary antibody such that cell densities and antibody concentrations were normalized across all conditions. After incubation with primary antibody, cells were washed 3 × 5 min with IF buffer and incubated in IF buffer containing 1:1,000 secondary antibody for 1 h, washed with IF buffer (3 × 5 min), plated into μ-slide 8-well plates (ibidi), and visualized using an Axio Observer Z1 inverted microscope (Carl Zeiss Microscopy GmbH) with a 20×, 0.3 NA air immersion objective, acquired to a Coolsnap HQ2 CCD camera (Photometrics) using ZEN imaging software (Carl Zeiss Microscopy GmbH). Cells in images were manually identified in phase contrast (circular shape), and average fluorescence values were recorded after local background subtraction. Fluorescence from debris was manually excluded from calculations. Cell viability prior to fixation was confirmed with Annexin-V staining in the blue channel. Antibodies used for NF-kB RelA (Rabbit anti-p65, sc-372), cRel (anti-mouse cRel conjugated to PE, #12-6111-80), Myc (Rabbit anti-c-Myc, ab32072), and BclXL (Rabbit anti-BclXL, ab2568) were obtained from SantaCruz Biotechnology, eBioScience, AbCam, and AbCam, respectively. Goat anti-rabbit-conjugated secondary antibodies were obtained from Life Technologies (A-11001). To quantify changes in expression, we found significance thresholds for cell size and average cell fluorescence for the indicated protein after 24 h as compared to the 0 h control. Percentages (Fig 4H–K, and Supplementary Fig S5A and C) show the fraction of cells from the 24 h time point occupying each quadrant. Significant cell size was defined to be $>100$ pixels manually, while significant abundance was defined as a value that is greater than or equal to the 95th percentile abundance from the 0 h datasets. All immunofluorescence images and the custom Java software used to analyze the images are provided as a zipped file (Supplementary File S7).

**CFSE flow cytometry and FlowMax analysis**

Cells were removed from media, stained with 10 ng/ml propidium iodide, and measured using an Accuri C6 Flow Cytometer (Accuri Inc.) over a 6-day time course. CFSE histograms were constructed after software compensation for fluorescence spillover and manual gating on viable (PI-negative) B cells using FlowMax software. All measurements were performed in duplicate (B cells from the same spleen were cultured in separate wells, two wells per time point to ensure that each time course represented a single population of cells subject to only experimental variability). The FlowMax computational tool (Shokhirev & Hoffmann, 2013) was used to construct 1D log-transformed CFSE histograms of viable cells. After specifying the fluorescence of the undivided peak manually for each time point, maximum-likelihood cyton model parameter ranges were determined by filtering, and clustering 1,000 best-fit solutions and their corresponding sensitivity ranges. The top solution cluster was plotted by randomly sampling parameters from within the maximum-likelihood parameter ranges. To account for potential censorship of the fraction of dividing cells or division and death time distributions when both division and death processes were active simultaneously (i.e., cyton model), Monte Carlo sampling of cell populations was used to approximate population model parameters directly.

**Western blot analysis**

Whole-cell lysates were prepared using RIPA buffer lysis of B cells. The resulting lysates were resolved on a 10% SDS–PAGE and proteins detected using the Bio-Rad ChemiDoc XRS System and SuperSignal west femto substrate (Thermo Scientific). Antibodies...
used to identify the protein of interest as follows: S6 Ribosomal Protein (Cell signaling #2217) and α-tubulin (Santa Cruz sc-5286). Quantification was performed using ImageJ software using the 0 h protein levels for normalization.

**RT–PCR**

RNA extraction was performed using RNeasy Mini Kit (Qiagen). cDNA synthesis of purified RNA was done with iScript cDNA Synthesis kit (Bio-Rad). Quantitative RT–PCR was performed with SYBR Green PCR Master Mix reagent (Stratagene) and Eppendorf Mastercycler replexus system using the Δ(ΔCt) method with β-actin as normalization control.

**Multi-scale agent-based modeling**

Ordinary differential equation models of the cell-cycle (Conradie et al., 2010), apoptosis (Loriaux & Hoffmann, 2012), and NF-κB signaling (Alves et al., 2014) were implemented in Matlab (Mathworks), using the ode15s solver for stiff problems. Please refer to the Supplementary Methods for the list of model reactants, reactions, constants, and free parameters, as well as the fitting methodology and parameter sensitivities. The modules were connected by imposing cooperative Hill activation of the CyclinD, reactions, constants, and free parameters, as well as the fitting works), using the ode15s solver for stiff problems. Please refer to the Supplementary Methods for the list of model reactants, reactions, constants, and free parameters, as well as the fitting methodology and parameter sensitivities. The full set of model constants, reactions, fluxes, species, parameterizations, parameter sensitivity, fitting procedure, and model construction methods are described in the supplementary tables, and the model construction details and fitting routines are described in Supplementary Methods. The full model code is provided as a collection of zipped MATLAB files (Supplementary File S8).

**Predicting the role of extrinsic abundance noise for specific sets of proteins**

We used the multi-scale model to predict the effect of increasing the variability or mean protein levels in specific modules. To do this, we grouped proteins in the model into functionally distinct sets: the negative regulators of activation (IkBs), the positive regulators of activation (IKK and the NF-κB monomers), and all other proteins (cell-cycle and apoptosis). Then, we tested the effect of increasing the average abundance of the proteins in these functional sets by increasing the translation rate of the proteins (IkBs, NF-κBs, cell-cycle/apoptosis proteins) by 10%, or increasing the total concentration by 50% (for proteins which are assumed to be constant in the model such as IKK and some cell-cycle proteins). We also tested the effect of increasing the extrinsic noise of these protein sets by increasing the CV associated with the cell-to-cell protein variability in the synthesis/degradation rates (assumed to be drawn from a normal distribution with mean centered on the initial parameter value) or the total protein abundance (assumed to be log-normally distributed with mean equal to the initial starting concentration). We then quantified the mean relative population count throughout the simulation, the expected number of divisions that progenitors underwent, a summary statistic for the fractions of cells dividing in each generation (fs), the average interdivision time for generation 1+ cells (a measure of cell-cycle speed), and the relative number of cells remaining at the end of the simulation (accumulation of surviving non-dividing cells).

**Data availability**

Supplementary files, parameter tables, movies of tracked cells, code, raw images from time-lapse microscopy, and CFSE flow cytometry datasets (FC53.0 files) are available for download at http://www.signalingsystems.ucla.edu/max/. Model parameters are also available as Supplementary Dataset S1. Single-cell RNA sequencing datasets are available from GEO: GSE64156 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE64156).

**Supplementary information** for this article is available online: http://mmb.embopress.org
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Author contributions
MNS designed and performed experimental, bioinformatics, and computational modeling work. JA assisted in B-cell preparation and biochemical analysis. JD-T performed bioinformatics and modeling work. HB provided bioinformatics work. TM helped with single-cell RNAseq. JAV performed microscopy studies. AH designed and supervised the work. MNS and AH wrote the manuscript. MNS, AH, JA, HB, JD-T, and JAV edited the manuscript.

Conflict of interest
The authors declare that they have no conflict of interest.

References


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Single stem cell gene therapy for genetic skin disease

Jean-Christophe Larsimont¹ & Cédric Blanpain¹,²

Stem cell gene therapy followed by transplantation into damaged regions of the skin has been successfully used to treat genetic skin blistering disorder. Usually, many stem cells are virally transduced to obtain a sufficient number of genetically corrected cells required for successful transplantation, as genetic insertion in every stem cell cannot be precisely defined. In this issue of *EMBO Molecular Medicine*, Droz-Georget Lathion et al developed a new strategy for *ex vivo* single cell gene therapy that allows extensive genomic and functional characterization of the genetically repaired individual cells before they can be used in clinical settings.

See also: S Droz-Georget Lathion et al

Epidermolysis bullosa (EB) is a family of inherited genetic diseases that are characterized by an extreme skin fragility that causes skin blistering disorder (Carulli et al., 2013). This family further divides into four subgroups that arise from different mutations: epidermolysis bullosa simplex, junctional epidermolysis bullosa, dystrophic epidermolysis bullosa (DEB), and Kindler syndrome (Carulli et al., 2013). These different genetic disorders result in skin blistering due to the detachment of the epidermis from the dermis or a split within the epidermis (Coulombe et al., 2009). DEB can be either dominant or recessive (then called recessive dystrophic epidermolysis bullosa or RDEB), with mutations in gene coding for collagen type VII (*COL7A1*) that localizes to the anchoring fibrils, structures that ensure the adhesion of the basal lamina with the extracellular matrix (Bruckner-Tuderman et al., 1999). The disease severely impacts the patient’s quality of life and can even cause early lethality in the most severe forms due to defects in skin barrier function leading to infections and development of aggressive skin squamous cell carcinoma (Carulli et al., 2013).

Until recently, the only treatment available for EB was restricted to supportive care including pain management, treatment of wound infection and chronic wound, and prevention of mechanical stresses to avoid formation of new blisters.

The important morbidity and mortality associated with EBs stimulated different groups to try unconventional therapies such as type VII collagen administration or grafting of allogeneic fibroblasts with potentially encouraging but not long-lasting results (Carulli et al., 2013). In a seminal study, De Luca and colleagues modified the well-established protocol of skin reconstruction used to treat patients with severe burns, developed in the laboratory of Howard Green. In this method, keratinocytes are isolated from skin biopsies and expanded on irradiated fibroblasts, stimulated to differentiate into a functional epidermis before being grafted back to the burnt regions (Gallico et al., 1984). Mavillo et al cultured the epidermal stem cells of a patient presenting loss-of-function mutation in one of the isoforms of laminin 5, transduced these cells with a retrovirus expressing normal laminin 5, made skin equivalents with the *in vitro* expanded genetically corrected epidermal stem cells, and transplanted sheath of *in vitro* reconstituted skin to the patient. The results were spectacular! The genetically corrected skin presented a long-term functional engraftment with no particular side effects such as skin atrophy or skin cancers after 6.5 years of follow-up (Mavillo et al., 2006; Carulli et al., 2013). While the authors have analyzed the integration sites of transgene, because the cell population was not clonal, it was not possible to rule out that a minor clonal population of cells within the bulk of the cultured stem cells presented potentially harmful retroviral insertion site, other mutations or chromosome aberrations that were already present in the skin of the patients or induced by the culture conditions.

In this issue of *EMBO Molecular Medicine*, Droz-Georget Lathion et al developed a strategy that embraces international standards of good laboratory practices (GLP) and good manufacturing practices (GMP) for medical uses of stem cells and demonstrate the feasibility of single stem cell gene therapy to reconstituted epidermis (Droz-Georget Lathion et al., 2014). Barrandon and colleagues used similar approach to the one previously described, the main difference being that individual colonies arising after gene correction of single stem cells were cloned and expanded *in vitro* first.

This approach permitted the study the genomic integrity of the gene-corrected clones of stem cells in great details. They could precisely determine the exact number of copies and integration sites of transgene using LM-PCR and confirmed these observations by fluorescence *in situ* hybridization and next-generation sequencing. Second, this protocol allowed for monitoring the tumorigenic potential of the corrected clones. The authors made the observation that the corrected epidermal stem cells do not form tumor upon grafting into immuno-compromised mice, display normal karyotype and normal levels of cell cycle regulators. Finally, the presence of the transgene was assessed by PCR in different organs, which
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**EMBO Molecular Medicine**

Single-stem-cell gene therapy for genetic skin disease

Jean-Christophe Larsimont & Cédric Blanpain

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**Figure 1.** Strategy developed for safe ex vivo gene therapy.

Keratinocyte stem cells from RDEB patient are isolated and infected ex vivo with viruses carrying COL7A1 CDNA to correct their function, and colonies arising from a single transduced cell are cloned, selected for their ability to produce collagen 7, and expanded in vitro. Their safety is then evaluated by assessing their genomic integrity and oncogenic potential. The clones that expressed Col VII and did not present safety issues are stimulated to reconstruct the epidermis that will be grafted onto the recipient.

excluded the possibility that genetically engineered epidermal stem cells had disseminated in distant organs.

In addition, this approach also allowed for a full characterization of the functionalities of the corrected clones. They showed that reconstructed epidermis from keratinocyte stem cells transduced with the normal form of the defective gene could form an epidermis that engrafted onto immunocompromised mice and was able to produce functional collagen VII that localized to the basal lamina and corrected the blistering phenotype of RDEB.

Overall, the study of Droz-Georget Lathion demonstrates the feasibility of ex vivo single cell gene therapy that allows a careful characterization of the recombined cells prior to medical use. However promising, this study was performed on epidermal stem cells from a single patient and it will be necessary to reproduce this finding in other patients, and independently of the severity of the disease since this aspect might represent a limiting factor for obtaining a sufficient amount of epidermal stem cells that can grow and expand in vitro (Mavilio et al., 2006). Furthermore, it would be of great interest to evaluate the scalability of this single stem cell gene therapy for more applications to the treatment of other genetic diseases affecting other tissues. Novel strategy using patient-derived iPSCs in which Col7 has been genetically corrected, which are then differentiated into stratified epidermis and transplanted into the damaged site, appears as an interesting alternative for the treatment of genetic skin disorders (Sebastiano et al., 2014; Umegaki-Arao et al., 2014; Wenzel et al., 2014).

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Constant rate of p53 tetramerization in response to DNA damage controls the p53 response

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Quantification of the dynamics of p53 tetramers in single cells using a fluorescent protein-fragment complementation assay reveals that, while total p53 increases proportionally to the DNA damage strength, p53 tetramers are formed at a constant rate. This breaks the linear input–output relation and dampens the p53 response. Disruption of the p53-binding protein ARC led to a dose-dependent rate of tetramers formation, resulting in enhanced tetramerization and induction of p53 target genes. Our work suggests that constraining the p53 response in face of variable inputs may protect cells from committing to terminal outcomes and highlights the importance of quantifying the active form of signaling molecules in single cells.

Synopsis

Quantification of the dynamics of p53 tetramers in single cells using a fluorescent protein-fragment complementation assay reveals that, while total p53 increases proportionally to the DNA damage strength, p53 tetramers are formed at a constant rate.

- A fluorescent protein-fragment complementation assay is developed and used to quantify p53 tetramers in single living cells.
- In response to DNA damage, p53 total levels increase proportionally to the strength of the damage; however, p53 tetramers are formed at a constant rate across damage doses.
- The protein ARC is a key component of the “molecular throttle” that controls the rate of p53 tetramer formation and breaks the linear relationship between the input strength (DNA damage) and cellular output (active p53).
- In the absence of ARC, the rate of p53 tetramerization becomes dose dependent and the expression of p53 target genes is enhanced.
Loss of growth homeostasis by genetic decoupling of cell division from biomass growth: implication for size control mechanisms

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Growing cells adjust their division time with biomass accumulation to maintain growth homeostasis. Size control mechanisms, such as the size checkpoint, provide an inherent coupling of growth and division by gating certain cell cycle transitions based on cell size. We describe genetic manipulations that decouple cell division from cell size, leading to the loss of growth homeostasis, with cells becoming progressively smaller or progressively larger until arresting. This was achieved by modulating glucose influx independently of external glucose. Division rate followed glucose influx, while volume growth was largely defined by external glucose. Therefore, the coordination of size and division observed in wild-type cells reflects tuning of two parallel processes, which is only refined by an inherent feedback-dependent coupling. We present a class of size control models explaining the observed breakdowns of growth homeostasis.

Synopsis

Live microscopy of individual cells growing in conditions that decouple nutrient sensing from nutrient influx reveals independent regulation of biomass accumulation and cell division. Two distinct types of arrest are described with implications for models of cell size control.

- We use genetic manipulations that enable modulating glucose influx independently of external glucose to interrogate the coordination of biomass accumulation and cell division cycle observed under normal conditions.
- Cell size follows external glucose, whereas division rate is controlled by glucose influx, leading to the loss of growth homeostasis.
- Cells continuously increase or decrease their size until arresting, challenging existing models of cell size control.
From intracellular signaling to population oscillations: bridging size- and time-scales in collective behavior

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Collective behavior in cellular populations is coordinated by biochemical signaling networks within individual cells. Connecting the dynamics of these intracellular networks to the population phenomena they control poses a considerable challenge because of network complexity and our limited knowledge of kinetic parameters. However, from physical systems, we know that behavioral changes in the individual constituents of a collectively behaving system occur in a limited number of well-defined classes, and these can be described using simple models. Here, we apply such an approach to the emergence of collective oscillations in cellular populations of the social amoeba Dictyostelium discoideum. Through direct tests of our model with quantitative in vivo measurements of single-cell and population signaling dynamics, we show how a simple model can effectively describe a complex molecular signaling network at multiple size and temporal scales. The model predicts novel noise-driven single-cell and population-level signaling phenomena that we then experimentally observe. Our results suggest that like physical systems, collective behavior in biology may be universal and described using simple mathematical models.

Synopsis

A simple two-variable model in combination with quantitative in vivo measurements of single-cell and population signaling dynamics is used to analyze the emergence of collective cAMP oscillations in Dictyostelium discoideum.

- Single Dictyostelium cells are well described as excitable, oscillatory systems.
- A universal, top-down model reproduces single-cell and population-level behaviors.
- Model-based predictions are validated in individual cells and in cellular populations.
- Stochasticity drives the emergence and continued coordination of collective behavior.
Cell cycle population effects in perturbation studies

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Growth condition perturbation or gene function disruption are commonly used strategies to study cellular systems. Although it is widely appreciated that such experiments may involve indirect effects, these frequently remain uncharacterized. Here, analysis of functionally unrelated Saccharomyces cerevisiae deletion strains reveals a common gene expression signature. One property shared by these strains is slower growth, with increased presence of the signature in more slowly growing strains. The slow growth signature is highly similar to the environmental stress response (ESR), an expression response common to diverse environmental perturbations. Both environmental and genetic perturbations result in growth rate changes. These are accompanied by a change in the distribution of cells over different cell cycle phases. Rather than representing a direct expression response in single cells, both the slow growth signature and ESR mainly reflect a redistribution of cells over different cell cycle phases, primarily characterized by an increase in the G1 population. The findings have implications for any study of perturbation that is accompanied by growth rate changes. Strategies to counter these effects are presented and discussed.

Synopsis

Genetic, stress, or nutrient perturbation of yeast resulting in slower growth yields a common expression signature, previously known as the environmental stress response. This is largely due to a cell cycle population shift and is relevant to many perturbation-based studies.

- Yeast deletion strains with slower growth exhibit a common gene expression signature proportional to their degree of slow growth.
- The slow growth signature is also found in wild-type cells subjected to various environmental perturbations that result in slow growth including the environmental stress response (ESR).
- The ESR and the genetic perturbation slow growth signature can largely be explained by a redistribution of cells over cell cycle phases.
- Transformation of slow growth-affected data enriches for finding direct targets of the original perturbation.
Systematic identification of cell size regulators in budding yeast

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Cell size is determined by a complex interplay between growth and division, involving multiple cellular pathways. To identify systematically processes affecting size control in G1 in budding yeast, we imaged and analyzed the cell cycle of millions of individual cells representing 591 mutants implicated in size control. Quantitative metric distinguished mutants affecting the mechanism of size control from the majority of mutants that have a perturbed size due to indirect effects modulating cell growth. Overall, we identified 17 negative and dozens positive size control regulators, with the negative regulators forming a small network centered on elements of mitotic exit network. Some elements of the translation machinery affected size control with a notable distinction between the deletions of parts of small and large ribosomal subunit: parts of small ribosomal subunit tended to regulate size control, while parts of the large subunit affected cell growth. Analysis of small cells revealed additional size control mechanism that functions in G2/M, complementing the primary size control in G1. Our study provides new insights about size control mechanisms in budding yeast.

Synopsis

New regulators of cell size are identified using a mutant screen based on high-throughput time-lapse microscopy. A quantitative framework distinguishes direct regulators of size control from mutants whose size is altered due to reduced growth rate.

- High-throughput time-lapse microscopy identified 17 negative and dozens positive regulators of cell size at START.
- Negative regulators form a small genetic network centered around the mitotic exit network, suggesting that G1 length depends on processes occurring prior to cell separation.
- The small ribosomal subunit affects size control, while the large subunit influences cell growth only, suggesting a role for translation initiation in size control.
- A backup mode of size control that functions in the budded phase is suggested.
Robust synchronization of coupled circadian and cell cycle oscillators in single mammalian cells

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Circadian cycles and cell cycles are two fundamental periodic processes with a period in the range of 1 day. Consequently, coupling between such cycles can lead to synchronization. Here, we estimated the mutual interactions between the two oscillators by time-lapse imaging of single mammalian NIH3T3 fibroblasts during several days. The analysis of thousands of circadian cycles in dividing cells clearly indicated that both oscillators tick in a 1:1 mode-locked state, with cell divisions occurring tightly 5 h before the peak in circadian Rev-Erbα-YFP reporter expression. In principle, such synchrony may be caused by either unidirectional or bidirectional coupling. While gating of cell division by the circadian cycle has been most studied, our data combined with stochastic modeling unambiguously show that the reverse coupling is predominant in NIH3T3 cells. Moreover, temperature, genetic, and pharmacological perturbations showed that the two interacting cellular oscillators adopt a synchronized state that is highly robust over a wide range of parameters. These findings have implications for circadian function in proliferative tissues, including epidermis, immune cells, and cancer.

Synopsis

Single-cell time-lapse analyses in mouse cells show that circadian and cell cycles are robustly synchronized. This state reflects a predominant unilateral influence of the cell cycle on the circadian oscillator.

- Circadian and cell cycles in mouse NIH3T3 cells proceed in tight synchrony that is highly robust over a wide range of conditions.
- The synchronized state reflects predominant influence of the cell cycle on the circadian cycle.
- Timing of divisions relative to the circadian cycle is predicted by the period mismatch of the two cycles.
- Stochastic modeling of two interacting phase oscillators identifies the parameters of the coupling functions.
Fluctuations in intracellular molecule abundance can lead to distinct, coexisting phenotypes in isogenic populations. Although metabolism continuously adapts to unpredictable environmental changes, and although bistability was found in certain substrate-uptake pathways, central carbon metabolism is thought to operate deterministically. Here, we combine experiment and theory to demonstrate that a clonal *Escherichia coli* population splits into two stochastically generated phenotypic subpopulations after glucose-gluconeogenic substrate shifts. Most cells refrain from growth, entering a dormant persister state that manifests as a lag phase in the population growth curve. The subpopulation-generating mechanism resides at the metabolic core, overarches the metabolic and transcriptional networks, and only allows the growth of cells initially achieving sufficiently high gluconeogenic flux. Thus, central metabolism does not ensure the gluconeogenic growth of individual cells, but uses a population-level adaptation resulting in responsive diversification upon nutrient changes.
A single epidermal stem cell strategy for safe ex vivo gene therapy

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There is a widespread agreement from patient and professional organisations alike that the safety of stem cell therapeutics is of paramount importance, particularly for ex vivo autologous gene therapy. Yet current technology makes it difficult to thoroughly evaluate the behaviour of genetically corrected stem cells before they are transplanted. To address this, we have developed a strategy that permits transplantation of a clonal population of genetically corrected autologous stem cells that meet stringent selection criteria and the principle of precaution. As a proof of concept, we have stably transduced epidermal stem cells (holoclones) obtained from a patient suffering from recessive dystrophic epidermolysis bullosa. Holoclones were infected with self-inactivating retroviruses bearing a COL7A1 cDNA and cloned before the progeny of individual stem cells were characterised using a number of criteria. Clonal analysis revealed a great deal of heterogeneity among transduced stem cells in their capacity to produce functional type VII collagen (COLVII). Selected transduced stem cells transplanted onto immunodeficient mice regenerated a non-blistering epidermis for months and produced a functional COLVII. Safety was assessed by determining the sites of proviral integration, rearrangements and hit genes and by whole-genome sequencing. The progeny of the selected stem cells also had a diploid karyotype, was not tumorigenic and did not disseminate after long-term transplantation onto immunodeficient mice. In conclusion, a clonal strategy is a powerful and efficient means of by-passing the heterogeneity of a transduced stem cell population. It guarantees a safe and homogenous medicinal product, fulfilling the principle of precaution and the requirements of regulatory affairs. Furthermore, a clonal strategy makes it possible to envision exciting gene-editing technologies like zinc finger nucleases, TALENs and homologous recombination for next-generation gene therapy.
Scl binds to primed enhancers in mesoderm to regulate hematopoietic and cardiac fate divergence

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Scl/Tal1 confers hemogenic competence and prevents ectopic cardiomyogenesis in embryonic endothelium by unknown mechanisms. We discovered that Scl binds to hematopoietic and cardiac enhancers that become epigenetically primed in multipotent cardiovascular mesoderm, to regulate the divergence of hematopoietic and cardiac lineages. Scl does not act as a pioneer factor but rather exploits a pre-established epigenetic landscape. As the blood lineage emerges, Scl binding and active epigenetic modifications are sustained in hematopoietic enhancers, whereas cardiac enhancers are decommissioned by removal of active epigenetic marks. Our data suggest that, rather than recruiting corepressors to enhancers, Scl prevents ectopic cardiogenesis by occupying enhancers that cardiac factors, such as Gata4 and Hand1, use for gene activation. Although hematopoietic Gata factors bind with Scl to both activated and repressed genes, they are dispensable for cardiac repression, but necessary for activating genes that enable hematopoietic stem/progenitor cell development. These results suggest that a unique subset of enhancers in lineage-specific genes that are accessible for regulators of opposing fates during the time of the fate decision provide a platform where the divergence of mutually exclusive fates is orchestrated.

Synopsis

Previous work had revealed that Scl promotes hemogenic competence while repressing cardiac fate. Comprehensive genome-scale data presented here establish Scl-mediated ‘enhancer decommissioning’ as underlying molecular mechanism.

- Scl binds to hematopoietic and cardiac enhancers that have been epigenetically primed in multipotent cardiovascular mesoderm.
- Scl-regulated cardiac enhancers become decommissioned in blood cells by loss of active histone marks.
- A subset of Scl-bound enhancers can also be regulated by cardiac Gata and bHLH factors.
- Hematopoietic Gata factors are required for Scl-dependent gene activation but not repression.