Chapter 12

Discrete Logic Modeling of Cell Signaling Pathways

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Abstract

Cell signaling pathways often crosstalk generating complex biological behaviors observed in different cellular contexts. Frequently, laboratory experiments focus on a few putative regulators, alone unable to predict the molecular mechanisms behind the observed phenotypes. Here, systems biology complements these approaches by giving a holistic picture to complex signaling crosstalk. In particular, Boolean network models are a meaningful tool to study large network behaviors and can cope with incomplete kinetic information. By introducing a model describing pathways involved in hematopoietic stem cell maintenance, we present a general approach on how to model cell signaling pathways with Boolean network models.

Key words Boolean networks, Dynamic modeling, Discrete logics, Long-term behavior, Niche induced homeostasis, Signaling cascades, Pathway prediction, Hematopoietic stem cell, Qualitative model, External stimulation

1 Introduction

Signaling pathways involved in cell maintenance form complex networks with multiple regulators [1, 2]. The latter cannot be completely understood only by molecular studies based on single interactions. Hence, a holistic system approach is demanded to unravel the dynamic behavior of complex cell signaling pathways [1, 2]. Boolean networks are among the most straightforward approaches to investigate complex biological behaviors [3, 4]. By implementing the bimodal activation (ON/OFF switching behavior) from Boolean logics, these models can be set up also if only qualitative knowledge about the system of interest is available. In particular, these models can cope with the absence of precise kinetic parameters. In Boolean networks, each node is regulated by a Boolean function summarizing the effect of incoming regulators. The dynamic behavior of the model is studied by updating the Boolean functions in discrete time steps [3, 4]. Based on the time update scheme and bimodal activation of components, Boolean networks are considered discrete models (Fig. 1). In fact,

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continuous variables as time and concentration are simplified in stepwise updates with limited activation patterns [5]. This simplification allows the scalability of these models towards a high number of components. However, relaxations of this discrete assumption are possible in order to consider levels of activation. Fuzzy logic can be applied to relax the "ON/OFF" activation assumption by considering gene activation propensities [6–8]. Therefore, instead of "ON/OFF" there will be a plethora of discrete activation states obtained by a generalization of Boolean operators with either min-max logic or product-sum logic [6–8] (Fig. 1). Again, to generate these multiple activation states, there is no need for further biological information and still, the network size scales up well. As a drawback, this modeling approach results in functions that are generally not differentiable and difficult to be translated with ordinary differential equations (ODE) methods [8]. Hence, translating Fuzzy logic models to ODE might be more difficult than for Boolean models. Finally, for small systems, it is possible to apply ODE modeling if enough kinetic parameters are available [5, 6, 8]. Here, both concentration and time are considered as continuous variables, and regulatory functions are summarized by differential equations [9–11]. As also shown by Cantone and colleagues [6], discrete modeling approaches are particularly applicable when modeling large interaction networks (Fig. 1). For this reason, discrete modeling and its relaxations are particularly interesting when studying regulatory cascades, normally involved in multiple crosstalks [5].

Boolean networks were applied to study various processes, from homeostasis, to development, to disease and aging [12–17]. In these studies, Boolean networks were simulated not only to recapitulate phenotypes but also to suggest molecular interventions and pathway regulatory mechanisms. A striking example of these complex and nuanced regulations is depicted in hematopoietic stem cells (HSCs). HSCs are a restricted blood cell population able to both self-renew and give rise to all types of differentiated blood cells of the body [18–21]. To preserve the integrity of HSCs, they are mainly maintained in a quiescent state and only activated under certain stimuli [22–25]. Here, the specialized environment called niche where the stems are found to reside plays a significant role. Hence, a complex mechanism is involved in the homeostatic regulation of HSCs involving crosstalk among signaling pathways and the niche environment [26–32]. In particular, the stem cell niche instructs the maintenance of HSCs by a combination of quiescence stimuli (such as transforming factor beta (TGF-β) and hypoxia) and cycling stimuli (such as thrombopoietin (TPO) and stem cell factor (SCF)) [26–32]. Different models of HSCs differentiation fate have been developed [33–36]. However, HSCs function does not rely only on their ability to differentiate. In fact, no

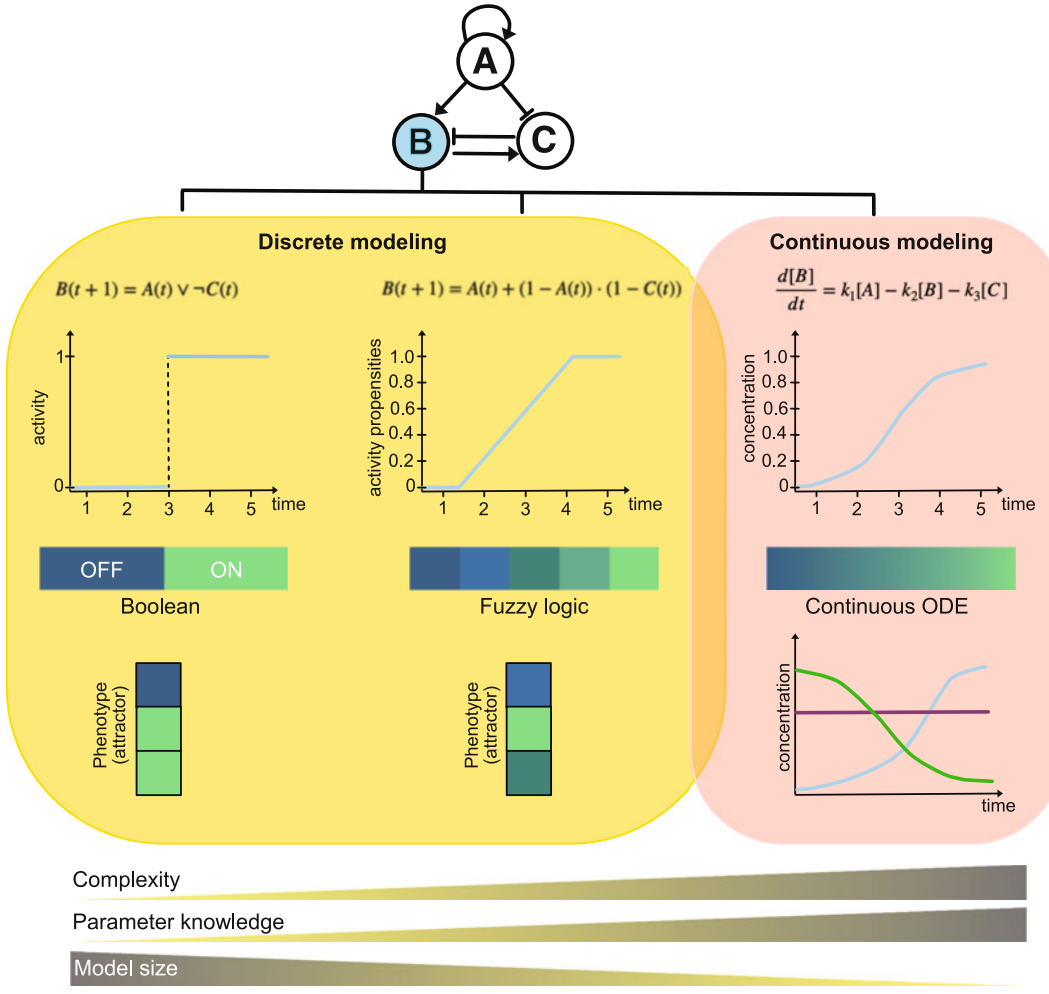

Fig. 1 Discrete and continuous modeling approaches. Depending on the biological question, network size, and kinetic details available, different modeling approaches are possible. Discrete models as Boolean networks can either describe activation by "ON/OFF" switch or by applying fuzzy logic. Both rely on attractors for phenotype description. Continuous modeling is applicable for small networks, where enough parameter knowledge is known. Here, interactions are described as differential equations, depending on concentrations and time as continuous variables. Phenotype description is depicted by concentration of components through time

previous model has described HSCs maintenance in virtue of niche stimulation.

In the following chapter, we will introduce strategies on Boolean modeling for cell signaling pathways by presenting our model on HSC maintenance regulation.

2 Materials

The presented procedure can be reproduced by using the freely available software R [37] and the R-package BoolNet [38]. Besides specific commands for simulation, BoolNet will also need the Boolean function describing the regulatory interaction parsed as a .txt file or SBML file (*see* **Note 1**). Furthermore, comprehensive guidelines are available describing the use of BoolNet with small examples (guidelines available at https://CRAN.R-project.org/package=BoolNet). Also, different alternative tools offer more user-friendly interfaces [3, 39]. Exemplarily, the open-source tool ViSiBooL and its extension are available [40, 41].

3 Methods

In the following, we will introduce the reader to Boolean network modeling strategies by presenting a recently published model describing HSC homeostatic maintenance in virtue of niche stimulation [15]. In particular, we will present our work by mimicking the steps followed in a real case scenario modeling approach. Therefore, we will first present mathematical insights on Boolean modeling together with strategies to collect, organize, and summarize data in Boolean functions. We will move to model simulations and interpretations of resulting long-term behaviors and cascades studies. Further, we will show how an established model can be applied to investigate perturbations. Finally, we will present how to assess biological meaningfulness through network robustness evaluation.

3.1 Boolean Network Models: A Brief Mathematical Description

For Boolean network models, a set of n genes, proteins, or compounds are represented by a set of variables $x_i \in \mathbb{B}$ that can exist in a binary state (active or inactive)—one variable x_i for each compound [42]. Regulatory interactions between compounds in the network are described as a set of Boolean functions $F = \{f_1, \ldots, f_n\}, f_i : \mathbb{B}^n \to \mathbb{B}$ [39]. The assignment of each variable at a specific point in time t is determined by applying its Boolean function f_i [43]. Depending on the value of its regulatory compounds, the assignment of each variable might change over time. The state of the complete network model at one particular point in time t is defined by a binary vector $\vec{x}(t) \in \mathbb{B}^n$ which comprises the actual assignment of each compound at this point in time. Consecutively, dynamic simulations consist of updating the state of the Boolean network in discrete time steps (*see* **Note 2**). In synchronous updating schemes, all Boolean functions are updated at each step-in time [44] (for further information, *see* **Note 3** on updating schemes). Due to the deterministic behavior of the synchronous

update strategy and the finite state space (2^n), at a certain point in time, the network simulation will enter a previously encountered state [44]. Once reached, the recurring sequence of one or multiple states cannot be exit without applying external perturbations [3]. We call this sequence attractor. Attractors depict the long-term behavior of a network and have been connected to biological phenotypes [44].

3.2 Constructing a Boolean Network Model of Cell Signaling Pathways Based on Literature

In order to give an introduction on how to construct a Boolean network model based on literature, we will depict a step-by-step procedure by applying it to the HSC case study [15]. For alternative strategies to set up a Boolean network model, *see* **Note 4**.

3.2.1 Literature Collection and Evaluation

The first step in the model setup is getting a rudimentary understanding of the involved important signaling pathways and their components. Therefore, we suggest starting with reading review papers (*see* **Note 5**). Already for this step, it is important to stick to the particular cellular context (*see* **Note 6**). In the case of the HSC model [15], we collected general reviews by querying in PubMed and Google Scholar entries such as "HSC quiescence," "HSC maintenance," "HSC regulation," or "HSC stem cell niche". From this research, we inferred the main players in stem cell maintenance. In particular, we considered cell cycle regulation, cellular metabolism regulation, and survival regulation as pillars of HSC maintenance. For what concerns the niche, we divided regulatory inputs into two main subgroups: quiescence inducing stimuli (TGF-β, hypoxia) and cycling inducing stimuli (TPO and SCF) [26–32] (*see* also **Note 7**).

3.2.2 From Literature to Boolean Functions

After collecting an initial set of information, we started to combine this information into Boolean functions. To describe biological interactions, logical connectives such as AND, OR, and NOT are used. These are often abbreviated by "∧" or " & " for AND, "∨" or "|" for OR, and "¬" or "!" for NOT. In the following, we will give small examples of how to translate biological information into Boolean functions taken from the rules of the HSC model [15].

In general, when constructing a Boolean function, alternative activators of a node will be combined by an OR logical operator. All inhibitors instead are added with an AND NOT operator. This approach allows a first organization of the functions.

3.2.3 Practical Examples on How to Solve Modeling-Related Issues

In the following subsections, we will give operative examples on how to approach common modeling issues arising during network construction.

1. Combining activators and inhibitors: An example of this approach of construction is the rule of mouse double minute

2 MDM2 (Table 1). Here, Myeloid Elf-1-like Factor 4 (MEF) and tumor protein p53 (TP53) are alternative transcriptional activators of MDM2 [45–49]. Hence, we combined them with an OR function. Cyclic dependent kinase inhibitor 2D (CDKN2D) and ataxia telangiectasia mutated (ATM) are instead inhibitors of MDM2 activity and therefore were added by using AND NOT [45, 50–52]. Attention should be paid for the use of brackets. Following the rules of Boolean algebra, logical operators have a binding hierarchy: NOT > AND > OR. Therefore, without claps, our two inhibitors would only inhibit MEF and not TP53. To preserve the meaning of our function, we would write it as *MDM2(t+1) = (TP53 | MEF) & !CDKN2D &!ATM*.

2. Combining independent processes: The general construction assumption described above holds true until specific information about cooperation is available. An example is the Boolean function for senescence. Here, two main independent axes for the induction of senescence are considered and connected by an OR operator. In both cases stressors (reactive oxygen species (ROS)) are needed. On one side activation of cyclin dependent kinase inhibitor 2A (CDKN2A) together with ROS induces cell cycle arrest [53–55]. On the other side, TP53 expression concomitant with cyclin dependent kinase inhibitor 1A (CDKN1A) and ROS [53–55]. These independent axes would either inhibit Cyclin D (CCND1) or Cyclin E (CCNE1). In each axis, all regulators need to be present in order to induce senescence. This leads to the Boolean function of *Senescence(t+1) = (CDKN2A & ROS) | (TP53 & ROS & CDKN1A)*. In fact, the presence of ROS alone does not per se mean induction of senescence or apoptosis. As a further sustain to construction of Boolean function, we provide a complete set of mathematical laws to consider when modeling (Table 2).
3. Evaluating concentration-dependent activities: Another alternative construction strategy in the HSC model has been made for the regulation of ROS. Studies on HSCs connected high ROS levels to both cycling stimulation and adverse events such as senescence or apoptosis. Hence, a precise leveling of this stressor is not available. If this knowledge would be available, one could consider introducing helper nodes (*see* **Note 8**). Therefore, we considered an equal weighting of all factors reported to activate ROS [50, 56–60]. This results in the Boolean function of *ROS(t+1) = Mitochondria | !ATM | !FOXO3A | !BMI1 | !TP53*. Further regulators in the HSC model distinguish between the two scenarios in ROS activation, further indicating that the final outcome of their presence is context dependent. This underlines the relevance of a holistic approach. For further information about the general

description of our regulatory mechanism, please refer to Ikonomi et al. [15].

4. Combining linear connections: Finally, in order to keep the model as precise as possible, linear connections can be summarized in one regulation. A striking example is the activation of protein kinase B (AKT) by phosphoinositide 3-kinase (PI3K). Here, multiple phosphorylation steps from the PI3K complex are required to finally activate AKT [18, 54]. However, since this process takes place in one complex block of modifications, it is reasonable to summarize the interaction simply as $AKT(t+1) = PI3K$.
5. Dealing with uncertainty—the concept of iteration: In any case, if problems in the formulation of a Boolean function still arise, truth tables can be applied (*see* **Note 9**). As shown most of the time, translating collected knowledge into Boolean regulations is not a straightforward process [1, 2]. It rather ends up in an iterative process that requires different steps of simulations, refinements, and further literature research to match attractors with expected biological behaviors (*see* **Note 10**). In our case, further cross-regulations were inferred by looking for specific publications and querying databases such as BioGRID [61] or MetaCore (from *Clarivate Analytics*) in the HSC cellular context. Further strategies on how to optimize literature screening are provided in **Note 5**. The Boolean functions of the final model are depicted in Table 1.

3.3 Studying the Model Dynamics: Attractors and Biological Interpretation

Once established a model, the next step will be to analyze its dynamics. Here, it is important to consider and analyze the whole attractor landscape. Therefore, it is suggested to perform an exhaustive search through the whole state space. The aim is to correlate each attractor to a biological phenotype. It is possible to exclude attractors because of unrealistic biological conditions. However, this should be limited to a few attractors.

In the case of the HSC model, we performed an exhaustive search by applying the SAT-solver function [62] available in BoolNet [38]. From our analysis, we obtained four single-state attractors (Fig. 2). The resulting attractors were matched to three possible phenotypes of the HSC. Three attractors were matched to respectively: long term (LT-), short term (ST-), and cycling HSCs according to the activity of nodes stated in Table 3. Here, we could distinguish between dormant HSCs (LT-HSC) with any metabolic activation and activated HSCs (ST-HSC) with activation of metabolism and partial loss of cell cycle inhibitors. The latter is described as a more active HSC and results from the presence of cycling and quiescence stimuli. When only cycling stimuli are present, the resulting attractor matched the phenotype of a cycling HSC, with activation of synthesis phase (S-phase) node. The fourth

Table 1
Boolean functions for the HSC model taken from Ikonomi et al. [15] with permission

Pathway association	Node	Boolean function
Input	External quiescence	External quiescence
	External cycling	External cycling
Metabolism	PI3K	RAS
	TSC1/2	¬AKT
	mTORC1	¬TSC1/2
	FOXO3A	External quiescence ∧ ¬AKT
	ATM	FOXO3A
	ROS	Mitochondria ∨ ¬ATM ∨ ¬FOXO3A ∨ ¬BMI1 ∨ ¬TP53
	Mitochondria	mTORC1
	Autophagy	FOXO3A ∧ ROS ∧ ¬mTORC1
Cell Cycle	RAS	External cycling
	ETS	RAS ∧ ¬MEF
	MEF	RAS
	GSK3B	¬AKT
	CTNNB1	¬GSK3B
	cMYC	CTNNB1 ∧ ¬GSK3B
	BMI1	cMYC ∨ (FOXO3A ∧ ATM)
	MDM2	(TP53 ∨ MEF) ∧ ¬CDKN2D ∧ ¬ATM
	TP53	¬MDM2
	CDKN1C	External quiescence ∨ FOXO3A
	CDKN1A	(TP53 ∨ FOXO3A ∨ External quiescence ∨ GFI1) ∧ ¬cMYC
	CDKN1B	FOXO3A
	GFI1	TP53
	RB	¬CCND1 ∧ ¬CCNE1
	E2F	¬RB ∧ ¬GFI1
	CCND1	¬CDKN2A ∧ ¬CDKN1C ∧ cMYC
	CCNE1	¬CDKN1C ∧ ((¬CDKN1A ∧ ¬CDKN1B) ∨ CCND1) ∧ E2F
	S-phase	E2F ∧ CCNE1
Survival	AKT	PI3K
	CDKN2A	(ETS ∨ ROS) ∧ ¬BMI1
	CDKN2D	(E2F ∨ ROS) ∧ ¬BMI1
	Pro-apoptotic proteins	ROS ∧ TP53 ∧ ¬AKT
	Anti-apoptotic proteins	(RAS ∨ External quiescence) ∧ ¬ GSK3B
	CYCS	Pro-apoptotic proteins ∧ ¬ Anti-apoptotic proteins
	Apoptosis	CYCS ∧ ¬AKT
	Senescence	(CDKN2A ∧ ROS) ∨ (TP53 ∧ ROS ∧ CDKN1A)

Nodes are abbreviated according to accepted nomenclature. Boolean functions represent regulatory interactions and are summarized by logical connectives AND (∧), OR (∨), and NOT (¬)

Table 2
Mathematical rules that have to be considered for construction of Boolean functions

Mathematical rule	Example 1	Example 2
Absorption law	$A \wedge (A \vee B) = A$	$A \vee (A \wedge B) = A$
Associative law	$(A \wedge B) \wedge C = A \wedge (B \wedge C)$	$(A \vee B) \vee C = A \vee (B \vee C)$
Commutative law	$A \wedge B = B \wedge A$	$A \vee B = B \vee A$
Distribution law	$(A \vee B) \wedge C = (A \wedge C) \vee (B \wedge C)$	$(A \wedge B) \vee C = (A \vee C) \wedge (B \vee C)$
De Morgan rule	$\neg(A \wedge B) = (\neg A \vee \neg B)$	$\neg(A \vee B) = (\neg A \wedge \neg B)$
Double negation	$\neg(\neg(A)) = A$	
Exclusion law	$A \wedge \neg A = 0$	$A \vee \neg A = 1$
Idempotence law	$A \wedge A = A$	$A \vee A = A$

attractor, instead, was excluded from further investigation since it represents the absence of external stimulation. This combination of inputs is not plausible in a niche condition, that is constantly affected by balances of cycling and quiescence inducing stimuli.

When possible or necessary, another strategy to match attractors to biological phenotypes is the use of expression data (*see* **Note 11**).

3.4 From Matching Phenotypes to Molecular Mechanisms: How to Approach Study of Cascades

After assessing the biological meaningfulness of obtained attractors, it is possible to study cascades leading to the switching of attractors. This type of simulation allows us to investigate the molecular mechanisms behind complex molecular behaviors. Technically, this approach starts from the assumption that one phenotype can turn into another depending on switching conditions. In our case, we considered that a dormant HSC (LT-HSC) can get activated into a ST-HSC and finally enter the cell cycle in dependence of niche stimulations. To do so, the attractor pattern is used as the starting state of the simulation, and cascades are triggered by activating or inactivating input nodes. In the HSC model, we started from the LT-HSC attractor and switched on the external cycling node. This leads, as expected, to the ST-HSC attractor. Finally, from this start state, we inactivated external quiescence and ended up in the cycling HSC attractor (Fig. 3).

The advantage of working with cascades is that patterns of activation and inhibitions can be investigated. In our case, we could show that the switch from an LT- and ST-HSCs is triggered by activation of mTOR complex 1 (mTORC1) that finally leads to ROS activation and activation of rat sarcoma (RAS) that inhibits TP53 and different cell cycle inhibitors. However, ST-HSC are still quiescent due to the activation of CDKN1C directly maintained from the niche (*see* Fig. 3 and Table 3). When only cycling

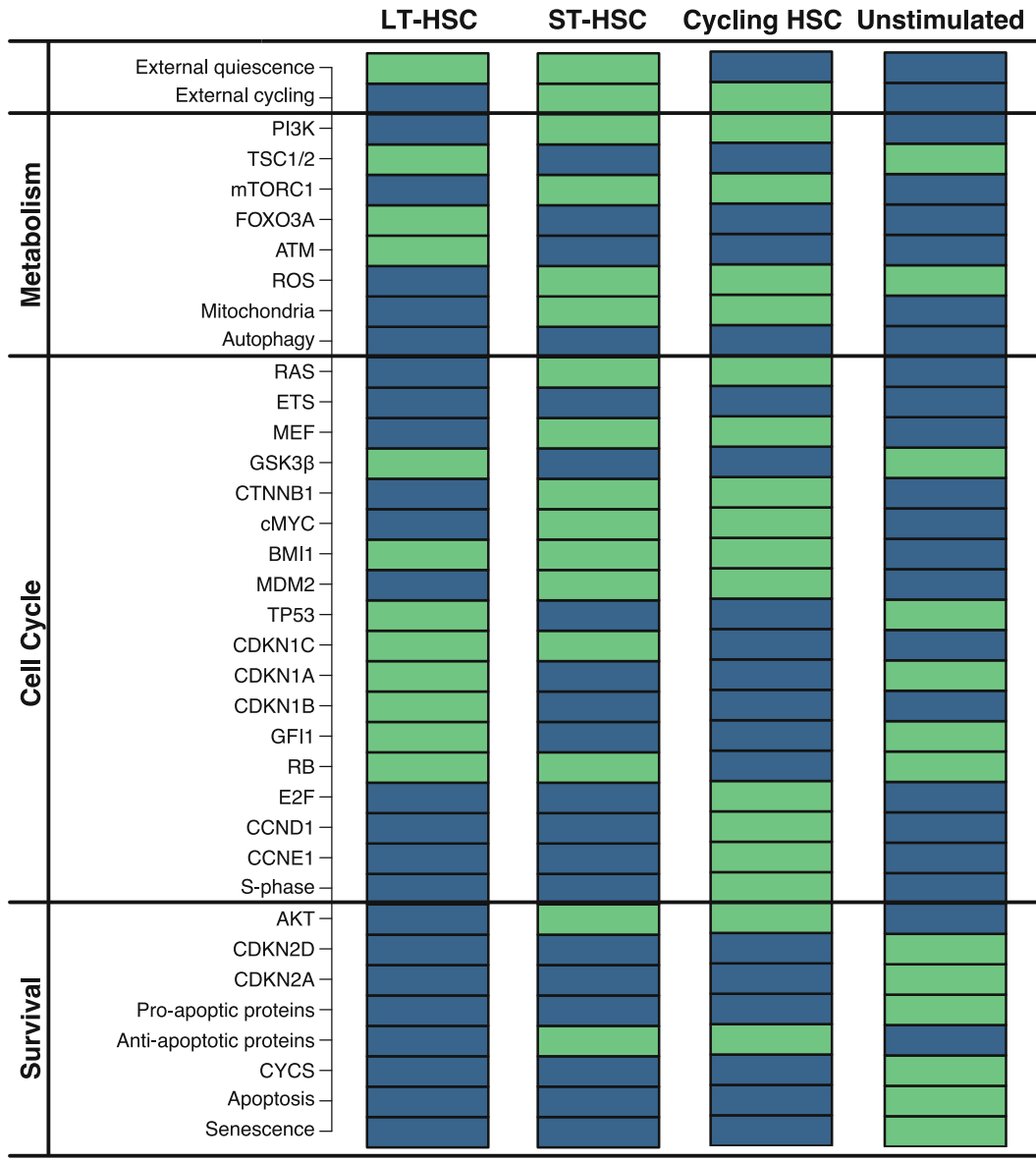

Fig. 2 Network attractors. Dynamic analyses of the HSC model revealed four attractors representing the long-term behavior of the model. According to external stimulations, four different activation patterns have been identified and matched to a biological phenotype. (Figure has been taken from Ikonomi et al. [15] with permission)

stimulations are present, all cell cycle inhibitors are lost and the stem cell enters in a few time steps the cell cycle (activation of S-phase).

Finally, approaching modeling with the study of cascades is still poorly applied by modelers. However, we believe that this type of simulation truly empowers the understanding of complex

Table 3
Attractor summary

Attractor	Process	Phenotypical description	Associated HSC phenotype
LT-HSC	Metabolism	Inactive ROS	Quiescent HSC
	Metabolism	Inactive mTORC1	
	Metabolism	Active FOXO3A	
	Cell cycle	Inactive MYC	
	Cell cycle	Active TP53	
	Cell cycle	Active CDKN1C	
	Cell cycle	Active CDKN1A	
	Cell cycle	Active CDKN1B	
	Cell cycle	Active GFI1	
	Cell cycle	Inactive S-phase	
ST-HSC	Metabolism	Active ROS	Activated HSC
	Metabolism	Active mTORC1	
	Metabolism	Inactive FOXO3A	
	Cell cycle	Active MYC	
	Cell cycle	Inactive TP53	
	Cell cycle	Active CDKN1C	
	Cell cycle	Inactive S-phase	
Cycling HSC	Cell cycle	Active CCND1	Proliferating HSC
	Cell cycle	Active CCNE1	
	Cell cycle	Active S-phase	
Unstimulated	External Stimuli	–	–

Depicted are the activity of nodes that are responsible for different HSC phenotypes. Table has been taken from Ikonomi et al. [15] with permission

regulations. In fact, with wet laboratory experiments, it would not be possible to have such a systems perspective of the network behavior after stimulation, underlining the power of a systems holistic approach in studying signaling pathways and their crosstalk.

3.5 Applying the Established Model to Predict Behaviors Under Perturbations

Besides assessing unknown molecular mechanisms of complex signaling pathways, models can be applied to predict interventions' outcome. These predictions allow us to guide laboratory experiments and may serve the costs of unnecessary experiments that do not reveal the desired outcome since complex mechanisms have not been considered. This type of approach applies particularly in models describing disease or aging processes (e.g., tumor development). Here, intervention screening can be applied to revert or improve attractors connected to disease-related phenotypes. Exemplarily, this strategy was applied in Meyer et al. [13] where knockout interventions were aimed to rescue senescent behavior. Here, a new target, NF-kappa-B essential modulator (NEMO), able to prevent senescence secretory phenotype after DNA damage could be identified. Laboratory experiments also confirmed this new target. Nevertheless, perturbation screening can also be applied to

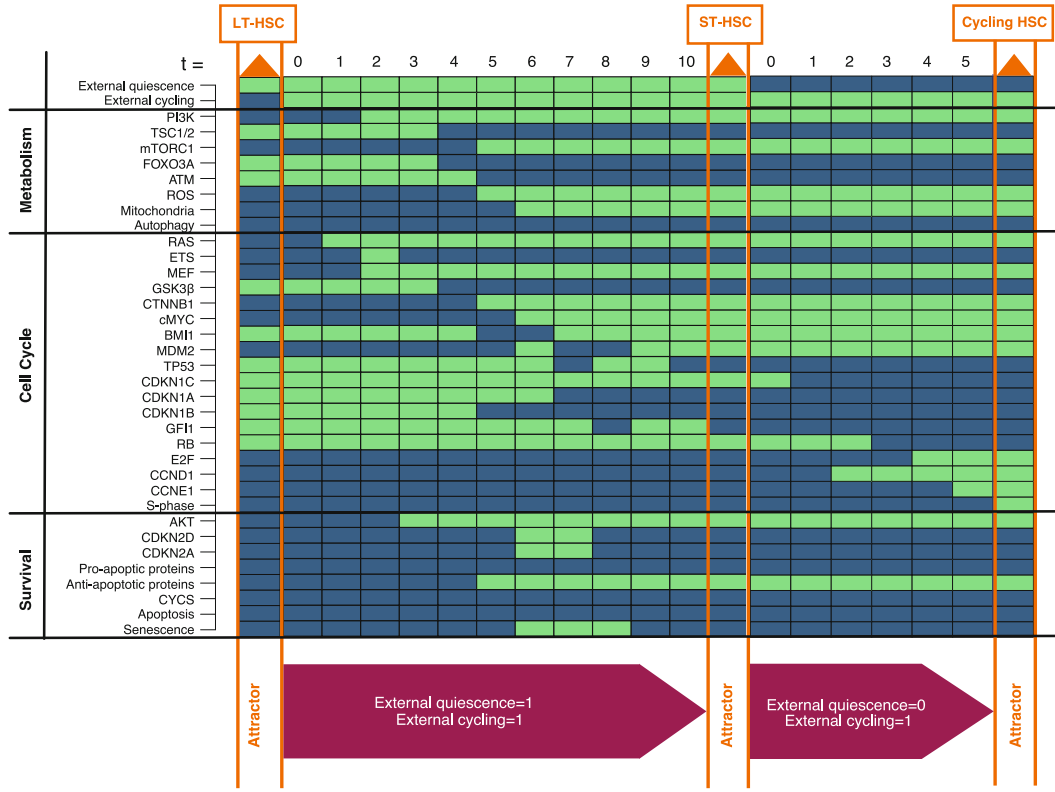

Fig. 3 Simulations of pathway cascades. The figure shows the progression from LT-HSC to ST-HSC to cycling HSC triggered by the switch of external stimulation inputs. (Figure has been taken from Ikonomi et al. [15] with permission)

show the effects of the loss of relevant regulators and confirm predicted mechanisms of regulation hypothesized by studying cascades. For example, if a regulator shows to be relevant for quiescence maintenance, then its loss should lead to a perturbed quiescent state.

The latter strategy was put in place in the HSC model [15], where perturbations have been applied to further investigate alterations of the HSC homeostatic maintenance. In fact, based on the perturbation experiments, we could not only match results from knockout mouse models but also formalize a general hypothesis of the molecular regulation of the HSC maintenance (*see* **Note 11**). For the perturbation experiments, we constitutively knocked out a node of interest in the network and performed attractor search afterward. We considered both alterations that cause perturbation of quiescence by loss of stems (like the polycomb ring finger protein (BMI-1) knockout) or impaired activation and cell cycle entry (like MEF knockout). By studying the effects of these knockouts together with mechanistic insights from signaling cascades, we could propose a mechanism of regulation of HSC maintenance [15].

3.6 Stability Assessment

Biological systems are assumed to be relatively insensitive to both environmental and internal changes [1, 2]. Hence, robustness analyses can be applied to assess the biological meaningful networks. For this purpose, the standard approach is to apply noise to the network dynamics and see if this can be overcome by the system. In all our experiments noise is considered by applying random bit flips [63, 64], which are alterations of the state of a node for one point in time.

In the HSC model, we assessed robustness by two different strategies.

3.6.1 Stability of Phenotypes: Can Noise Induce Shift of Attractors?

First, we tested if a certain starting state can reach the same attractor even under perturbed noisy conditions. Therefore, we created a set of one million randomly generated starting states and perturbed in independent experimental sets one, two, or three assignments of randomly selected nodes for one point in time. Each experimental setup was repeated three times. A robust network dynamic is expected to still be able to reach the same attractor even if noise is applied. Simulating the HSC model, we obtained that each perturbed state could reach the expected attractors in respectively 95%, 89%, and 85% of cases depending on having one, two, or three perturbations.

3.6.2 Comparing the Network Stability to Randomly Generated Networks

Another approach to assess stability is to compare the resistance of the Boolean networks to noise. To do so, the dynamic perturbation of the established network was compared to a set of randomly generated networks of the same size [65]. To measure the difference between two states in a Boolean network, the normalized Hamming distance is computed [65]. The Hamming distance counts the number of differing bits in two vectors of equal length (here, states of the network). For normalization, the difference is divided by the length of the vectors. To measure the robustness of trajectories in the state graph, one can perturb a state of the network $\vec{x}$ by flipping (inverting) a randomly selected set of assignments. Next, a specific number of state transitions is applied for the original state $\vec{x}$ and its perturbed copy $\vec{x}'$. Finally, the computed successors of original and perturbed states are compared using Hamming distances. The smaller the Hamming distances, the more similar these two states are. For instance, a Hamming distance of *zero* indicates the two states are identical. Consequently, the applied amount of noise was absorbed by the network, leading back to identical states after a certain amount of time steps.

For the HSC model one thousand randomly generated states were mutated by applying one flip. Their successor state is then computed and compared to the successor state of the unperturbed progression. The same strategy was conducted with the states of one thousand randomly generated networks. The performance in

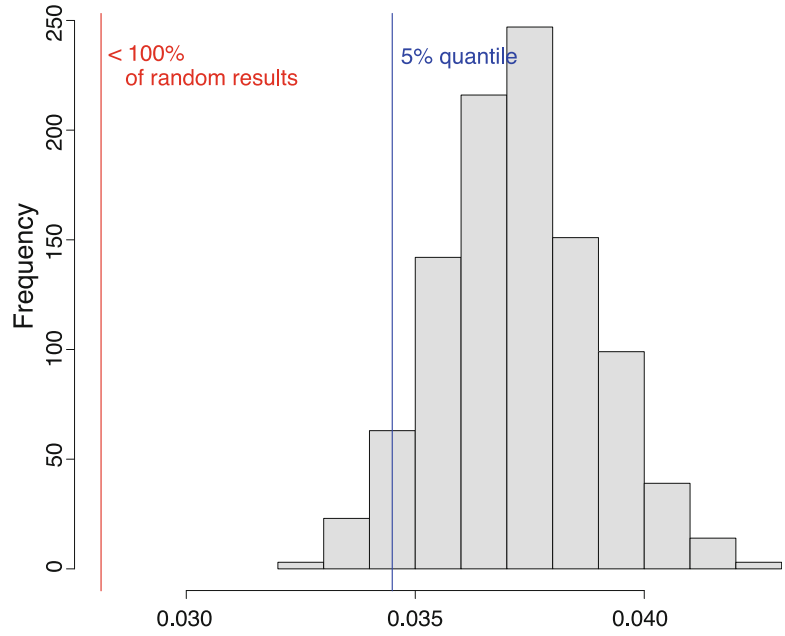

Fig. 4 Robustness analysis. The histogram shows the distribution of Hamming distances obtained from one thousand randomly generated networks. The blue line indicates the 5% quantile of the distribution. The red line instead depicts the hamming distance of HSC model. (Figure has been taken from Ikonomi et al. [15] with permission)

terms of Hamming distance was compared between the HSC and the randomly generated networks. The results of this computer intensive tests show that the Hamming distance of the HSC model is significantly smaller than 95% of the ones computed from the randomly generated networks. A strategy to perform this analysis is included in BoolNet [38], which also returns a graphical representation of the test (Fig. 4).

4 Notes

1. *Format of the model:* A variety of different formats to represent Boolean network models exist. Among these formats, SBML qual is an extension of the System Biology Markup Language (SBML) [66], which was set up to provide a standardized format to describe qualitative models such as Boolean networks. SBML qual is a machine-readable, XML-based format which is supported by a variety of different tools. However, for the modeling process itself, other formats such as the text-based BoolNet-format [38] might be preferable as they are easier to read. In this text format, the first line of a Boolean network model contains the header which has to be "targets,

factors." It is followed by the Boolean functions for each node in a separate line. Each target node is only allowed to be specified once and is always denoted first in a new line. The target node is then followed by its regulatory Boolean function after a comma. Boolean functions can be expressed using the logical operators " & " (AND), "|" (OR), and "!" (NOT). Avoid using Greek symbols, backslashes, spaces, or minus signs for the naming of nodes. BoolNet [38] or ViSiBooL [40] can also be used to translate this text-based representation to a network in SBML-representation after finishing to construct the model.

2. *Discrete time steps:* Note that time is considered as a discrete variable [4]. This also means in a broader interpretation that time steps can have different length. For example, if one update describes gene expression and another phosphorylation, these two processes will have significantly different timings in real scenarios [67]. However, since attractors are considered to represent long-term behavior of biological processes, timing of single functions has a less severe impact in the final behavior of the system.
3. *Updating schemes:* Similar assumptions about time can be adapted to updating schemes. For example, the asynchronous update is thought to resemble more closely biological timings and is based on random update of nodes at each step-in time [68]. However, choosing between synchronous and asynchronous updates depends on the final task. In the context of modeling HSCs behavior, both modeling approaches have been applied. In fact, Krumsiek et al. [34] applied asynchronous updating to model differentiation decisions on HSCs based only on transcription factors cross-regulations. The authors motivate this choice since differentiation includes "randomness" in the decision of which path to undergo [34]. In our context instead, we considered different levels of regulations for each interaction (transcriptional and post-translational regulations). Here, synchronous update allows to consider these different levels of dependencies in an ordered fashion. Exemplarily, updating first a node involved in phosphorylation than transcription regulation would not be realistic. As a second point to consider, asynchronous updating has the effect of getting rid of cyclic attractor, whereas single state attractors are normally preserved between the two updating schemes. Moreover, if cyclic attractors are preserved, asynchronous update can exacerbate oscillatory behaviors, leading to very long multiple state attractors. This points in two different directions: first we suggest evaluating the regulation landscape considered during modeling. Second, asynchronous update can anyways be used as a stability assessment for cycling attractors. It is of relevance,

in fact, to take these artifacts attractors into account when modeling. For example, if your simulation results in a burst of cyclic and single state attractors this might be a sign of instability of the final model.

4. *Data-driven reconstruction:* The automatic reconstruction of Boolean networks from data is an alternative approach to model Boolean networks. Time series of data can be used to reconstruct Boolean regulatory functions for compounds in a system of interest. There exists a variety of different approaches that infer regulatory dependencies [69, 70], Boolean networks from scratch [71–73, 84], or adapt prior networks [74]. Besides the reduced modeling effort, one benefit of this data-driven approach is that it is not biased by a modelers knowledge but only the information derived from the data. On the other hand, time series are usually sparse and noisy, which may lead to false-positive interactions. Additionally, numerous Boolean functions might recapitulate a time series equally well [16], and consequently, the reconstruction results may be a set of potential Boolean networks instead of one ground-truth network. Depending on the data's time-resolution, interactions in reconstructed Boolean networks might not reflect true interactions between certain compounds. However, the networks recapitulate the measured time series and can, thus, be used for more general, systemic analyses.
5. *Selecting papers:* Reading review papers will not only help to understand the topic, but also will give an idea of the gold standard papers relevant for the field. This will help to get a core set of highly cited papers that can then be enlarged with more recent or minorly cited publications. According to our experience, this approach allows to keep a rigorous method in selecting both relevant and updated literature. In this context, it is further suggested to pay particular attention on the raw results before diving into the written paper. This avoids being biased by the interpretation of the authors and helps especially when the number of considered papers rises and a more systemic view gets into place. For example, authors might highlight more some results than others or give interpretation that are biased by their experimental set. Moreover, while selecting literature contradicting information might appear. This should not be considered as a limitation. In fact, modeling can help overcoming contrasting results. Alternative regulations can be independently integrated in the model and attractors can be evaluated to choose the best fitting one to expected behaviors. When two alternatives are equally plausible, the model can be applied to focus wet laboratory validation. The model can already point out which targets might be differently regulated, reducing experimental effort in testing.

6. *Cellular context:* Note that context matters when withdrawing information. Especially in heterogeneous populations, attention should be paid to the cellular context. Exemplarily, it is not said that regulations found in lung tumors could also fit for stem cells. It is possible to consider regulations out of the cellular context; however, in this case we suggest using well-established homeostatic regulations that are not dependent on heterogeneous populations such as tumors are. Moreover, we still recommend sticking to the given context for first phases of modeling, and eventually integrate information in case of missing interactions or not expected behaviors.
7. *Inputs:* It is relevant when modeling to distinguish between different stimulations. In this regard, it has to be noted that increasing dramatically the number of input nodes in the network will lead first to an increase of the state space, and second to a plethora of different attractors representing combination of these inputs. The latter might lead to identical attractors differing only in the different combination of input nodes. In these cases, it is desirable to try to collapse redundant stimulations into one input node as shown in the HSC model. In the specific case, stimulation on the HSC can either promote its cycling or maintenance of quiescence. As an example, activation of CDKN1C is sustained by redundant stimulations from TGF-β signaling and hypoxia [75–78]. On the other hand, cytokines such as TPO, SCF, and Fibroblast Growth Factor (FGF) all stimulate cycling by the RAS/PI3K axis [26–32]. This approach is particularly effective especially in cases where signals are compartmentalized (like in the niche) or when multiple ligands are known to activate the same receptors/pathways (e.g., wingless (Wnt) ligands family [79]). In the latter, it would still be possible to distinguish ligands into canonical and non-canonical Wnt ligands and corresponding receptors in case of proof evidence of different regulatory impact.
8. *Helper nodes:* Boolean modeling gives various advantages when it comes to compounds representation. When for example a protein has a different behavior depending on environmental conditions, this particular condition can be set in the Boolean function. This was the approach for example for TP53 modeling, where this protein is known to have different functions in stress and homeostatic conditions [80]. However, an alternative approach is to divide the protein of interest in subnodes. For example, if the compound of interest has a certain activity on the cytoplasm, one could divide it in two nodes "X_cytosolic" and "X_nuclear." Similarly, if only high concentrations of the compound are known to trigger a certain activation or inhibition one could think of having nodes such as "X_high"

and "X_low." In any case, we suggest approaching this type of modeling only when precise information is known about the regulation. Unfortunately, this is often not the case. Examples given exact concentration ranges are almost never available in functional studies. In the same direction, various modifications like multiple phosphorylation sides are frequently not deeply investigated. On the other hand, information as if X is present, Y activates Z is way more common and helps modeling in the context-dependent direction. Thus, the risk is to dissect too much the information inside of the model and force too many sub-behaviors which may lead to an overestimation of the model. Note also that the model can always be refined if new information is available.

9. *Truth table:* It can happen that a Boolean function is not immediately intuitive to define. In this case using truth tables might come in support to the modeler. With truth tables it is possible to formalize all combination of activation and inhibitions on a single node and their final outcome. This will help in order to better handle the balance of logic operator. Moreover, a strategy to infer unknown Boolean functions from truth tables is the application of the disjunctive normal form (DNF) and the conjunctive normal form (CNF). The unknown function is therefore describing either as the disjunction (OR) of all combinations that yield a final activation of the target node or as the conjunction (AND) of all negated regulations that yield an inhibition of the target node.
10. *Problem oriented modeling:* Constructing a Boolean model is not a one-shot resolvable problem. It goes, instead, more in the direction of an iterative process. Once a first draft is available, simulations should be compared to available data to help refine the model and check for its prediction power. Moreover, we suggest approaching modeling by functional blocks. In the HSC model, different regulations on cellular behavior are described. It is preferable, in order to not get lost in interactions and literature research, to investigate first a part of the process and start modeling it. For example, one could start by describing cell cycle regulation. Once we have a good sub-model describing cell cycle with meaningful attractors, we can move to expanding it to survival and metabolism. This approach helps to refine the model in an ordered and rigorous fashion and to not get overwhelmed during the modeling process.
11. *Matching attractors to biological phenotypes:* Interpreting and matching resulting attractors to biological behaviors is probably the crucial point to evaluate the established model's goodness. However, this task is not always straightforward. First, we recommend if possible, comparing the simulated results to

independent publications from the ones that were used for constructing the model. Thus, an independent learning and training set of observations may prevent overfitting to certain behaviors. Comparison of binary attractors to biological behaviors or measurements can be difficult. Here, some common reasons coming from our experience might be helpful to be considered. First, consider if you are looking at the same level of regulation. For example, it might be that a gene is expressed but inactivated at the protein level. The other way around, it is not said that a decrease in mRNA levels will obviously lead to a completely inactive protein. The comparison can become even more tricky when it gets to in vivo models. For example, a certain knockout can have a different effect on young and old mice. In this case it might be that the attractor might match a long-term behavior that is depicted in later stages of life. This is the example of the forkhead box protein 3A (FOXO3A) knockout that shows more active stems in young mice with still activation of CDKN2A, CDKN2D, cyclin-dependent kinase inhibitor 1B (CDKN1B), TP53, and other cycle arrest markers [58]. The further decrease of stems in older mice leads to the hypothesis of a cumulative effect that finally will lead to senescence, that would match also the behavior with the similar knockout of ATM in older mice [58]. Hence, there is no "automized" way to interpret attractors without an expert look at the biological processes. Moreover, one has to be aware of what the Boolean function is actually describing and at which level of regulation. In general, for researches approaching to the field, we suggest getting into a holistic perspective: authors of single research papers may not have noticed or underlined parts of their results that a modeler can glimpse due to its traversal knowledge. Contradictions are part of process; however, with experience they can be interpreted and tackled. Besides, if available literature is not enough, one can validate the presented model by designing own experiments to proof suggested interventions or regulatory data. Another approach we suggest is to compare attractor patterns to binarized expression data. Expression values, e.g., for time series can be binarized, for instance, based on clustering approaches [81]. Other approaches such as BASC-A/BASC-B aim at fitting step-functions to find an optimal binarization threshold [82]. The R-package called BiTrinA allows for binarization with these approaches [83].

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