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Phenotypic stability and plasticity in GMP-derived cells as determined by their underlying regulatory network

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ABSTRACT

Motivation: Blood cell formation has been recognized as a suitable system to study cellular differentiation mainly because of its experimental accessibility, and because it shows characteristics such as hierarchical and gradual bifurcated patterns of commitment, which are present in several developmental processes. While hematopoiesis has been extensively studied and there is a wealth of molecular and cellular data about it, it is not clear how the underlying molecular regulatory networks define or restrict cellular differentiation processes. Here, we infer the molecular regulatory network that controls the differentiation of a blood cell subpopulation derived from the Granulocyte-Monocyte Precursor (GMP), comprising monocytes, neutrophils, eosinophils, basophils, and mast cells.

Results: We integrate published qualitative experimental data into a model to describe temporal expression patterns observed in GMP-derived cells. The model is implemented as a Boolean Regulatory Network (BRN), and its dynamical behavior is studied. Steady states of the network can be clearly identified with the expression profiles of monocytes, mast cells, neutrophils, basophils, and eosinophils, under wild-type and mutant backgrounds.

Supplementary Information Supplementary data is available at *Bioinformatics* online.

1 INTRODUCTION

A major issue in biology is to explain how specific temporal expression patterns observed in cells arise during differentiation. Blood cell formation, or hematopoiesis, is an excellent experimental model system to study differentiation processes (Doulatov *et al.*, 2012). There is a wealth of molecular and cellular data regarding hematopoiesis, and thus we face the task of integrating such information into coherent and predictive models. In particular, network modeling has been successfully used to integrate qualitative data to explain the appearance, stability, heterogeneity, and plasticity of specific expression profiles in blood cell subpopulations (Mendoza, 2006; Naldi *et al.*, 2010; Bonzanni *et al.*, 2013; Martinez-Sanchez *et al.*, 2015; Méndez and Mendoza, 2016; Collombet *et al.*, 2017).

In mice, the subpopulation known as the Granulocyte-Monocyte Precursor (GMP) is comprised by cells with the profile $Lin^{-}Sca^{-}CD34^{+}c\text{-}KIT^{+}Fc\gamma R^{+}$, having the potential to give raise to neutrophils ($MPO^{+}NE^{+}LF^{+}$), eosinophils ($Fc\epsilon RI\alpha^{+}CCR3^{+}c\text{-}KIT^{-}$), basophils ($Fc\epsilon RI\alpha^{+}CD11b^{+}c\text{-}KIT^{-}$), mast cells ($Fc\epsilon RI\alpha^{+}MMCP6^{+}c\text{-}KIT^{+}$), and monocytes ($M\text{-}CSFR^{+}$) (Akashi *et al.*, 2000). These cells play important roles in inflammation processes, allergic reactions, and immune responses against a wide range of pathogens (Galli *et al.*, 2011). Neutrophils and monocytes are important innate immune cell effectors because they can ingest and kill possible dangerous microorganisms, processing antigens to help mounting memory immune responses (Dale *et al.*, 2008). Eosinophils, basophils, and mast cells are necessary for the clearance of multicellular parasites (Stone *et al.*, 2010). Finally, mast cells and basophils have clinical importance due to their predominance in the regulation of allergic reactions (Sawaguchi *et al.*, 2012).

The differentiation of GMP-derived cells is regulated by key regulatory molecules. Specifically, the transcription factors (TFs) C/EBPα and PU.1 are necessary for proper maturation of GMP-derived lineages (McKercher *et al.*, 1996; Heath *et al.*, 2004), while GATA-1/2 and MITF-1 are crucial for the formation of eosinophils, basophils, and mast cells (Migliaccio *et al.*, 2003; Iwasaki *et al.*, 2006; Nei *et al.*, 2013). Although it is known that these transcription factors, as well as other molecules, are necessary for the correct differentiation of all GMP-derived cells, there is no consensus on how these molecules determine the developmental programs for each lineage.

Previous works have implemented regulatory network models to describe the general expression patterns of monocytes and granulocytes (Krumsiek *et al.*, 2011; Laslo *et al.*, 2006). However, such models are not able to describe the expression profiles observed in granulocyte subpopulations such as eosinophils, basophils, and mast cells. In this work we present a regulatory network model inferred from experimental murine systems that is able to determine the basic qualitative molecular patterns of expression observed in GMP-derived cells, as well as showing plasticity.

2 METHODS

2.1 Molecular basis of the regulatory network

For the reconstruction of the regulatory network we used a bottom-up approach, identifying regulatory interactions with an extensive literature search, as well as following annotations found in STRING, NetPath, and DAVID (Huang *et al.*, 2009; Kandasamy *et al.*,

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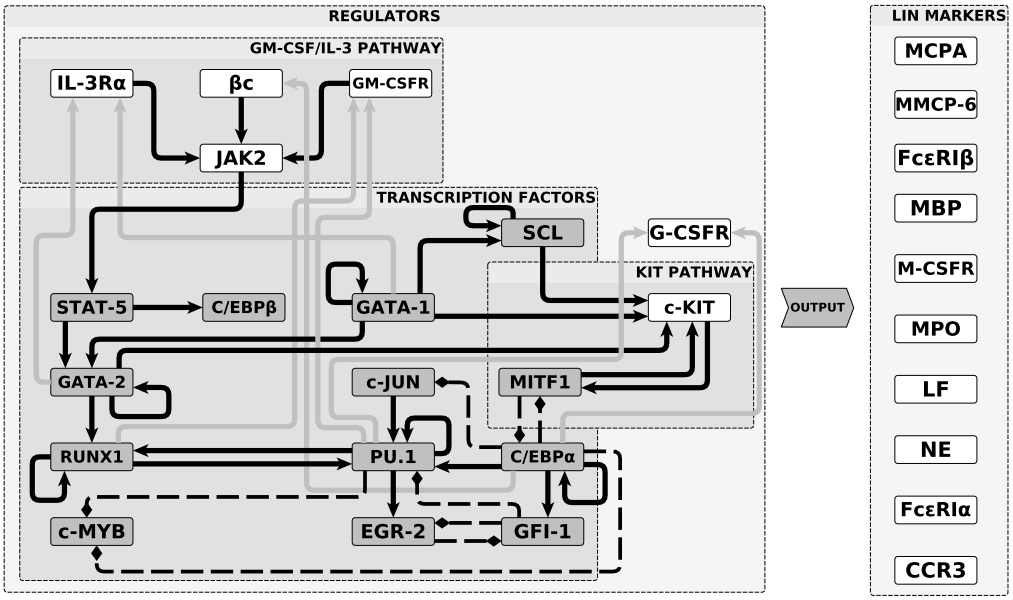

Fig. 1: **The Regulatory Network that controls the differentiation of GMP-derived cells**. Continuous and discontinuous blunt arrows represent positive and negative regulatory interactions, respectively. Transcription factors are shown as gray nodes. Some interactions are shown as thick black arrows to highlight key regulatory circuits. For simplicity, the regulation of lineage (LIN) markers are collectively represented as the output obtained from the dynamic of the network of regulators (see Section 3.1 for details, and Supplementary Figure 5 for the full network).

2010; Szklarczyk *et al.*, 2011). The molecular information was mostly obtained from mice, except in cases where it is explicitly stated otherwise. We manually curated all interactions to avoid the introduction of weakly supported regulatory interactions.

We wanted to asses if a regulatory network module comprised of key elements was enough to describe the temporal patterns of activation observed during GMP differentiation. Hence, we constructed the regulatory network in two phases. In the first phase we only take into account direct, well supported interactions. Namely, we considered regulatory interactions satisfying at least one of the following criteria: *i*) corroborated by different molecular biology techniques; *ii*) tested in different cell systems; or *iii*) reproduced by different groups. These regulatory interactions are compiled in Supplementary Table 1, defining the network shown as Supplementary Figure 1. Furthermore, the inferred regulatory rules are shown in Supplementary Table 2. In the second phase, we considered interactions for which there is indirect evidence, and we also added some proposed interactions. The resulting network is shown in Figure 1, based upon the interactions presented in Supplementary Table 3. The logical rules for this second version model are given in Supplementary Table 4. The information used to reconstruct the regulatory network is briefly summarized in the following paragraphs. Furthermore, the reader may find a table containing more detailed information regarding each regulatory interaction in the Supplementary File *modelInteractions.csv*.

C/EBPα positively regulates PU.1, GFI-1, and itself (Laslo *et al.*, 2006; Lidonnici *et al.*, 2010; Timchenko *et al.*, 1995). In combination, these TFs are able to activate neutrophil markers such as lactoferrin (LF), myeloperoxidase (MPO), and neutrophil elastase (NE) (Oelgeschläger *et al.*, 1996; Ford *et al.*, 1996; Khanna-gupta *et al.*, 2000). At the other hand, simultaneous expression of C/EBPα, PU.1, RUNX-1, and EGR-2 induce monocyte lineage determination by targeting monocyte markers (Laslo *et al.*, 2006; Hu *et al.*, 2011; Behre *et al.*, 1999). GFI-1 and EGR-2 regulate the reinforcement of commitment in the monocyte *versus* granulocyte decision, respectively (Laslo *et al.*, 2006); this effect is carried out by direct mutual inhibition (Laslo *et al.*, 2006). Additionally, GFI-1 has been shown to downregulate PU.1 during G-CSFR treatment of multipotential hematopoietic cells to skew neutrophil formation (Dahl *et al.*, 2007). The cell surface marker GM-CSFRα is upregulated by PU.1 and RUNX-1 in both monocyte and granulocytes (Hu *et al.*, 2011; Pahl *et al.*, 1993).

GATA-1/2, PU.1 along with C/EBPα expression are related to eosinophil and basophil formation (Iwasaki *et al.*, 2006). Eosinophil markers are positively regulated by C/EBPα/ϵ, PU.1, GATA-1/2, and c-JUN (Nishiyama *et al.*, 2002; Du *et al.*, 2002). The regulation of the eosinophil marker MBP by C/EBPα/β, PU.1, and GATA-1, have been consistently observed in humans (Du *et al.*, 2002; Yamaguchi *et al.*, 1998, 1999). Furthermore, a regulatory sequence of the MBP gene has conserved binding sites for these three factors (Gombart *et al.*, 2003). Therefore, it seems very likely that these interactions are also present in mice. Additionally, forced expression of C/EBPα and GATA-2 in GMP mice cells increases MBP mRNA production (Iwasaki *et al.*, 2006).

TF autoregulation is a frequent feature in this regulatory network. In particular, C/EBPα, GATA-1, GATA-2, PU.1, RUNX-1, and SCL present self-activation (Christy *et al.*, 1991; Tsai and Orkin, 1997; Grass *et al.*, 2003; Martowicz *et al.*, 2005; Okuno *et al.*, 2005; Leddin *et al.*, 2011; Nottingham *et al.*, 2007).

There is contradictory evidence regarding the regulation of GATA-2 by GATA-1. Specifically, GATA-1 and GATA-2 are coexpressed in GMP cells in mice and humans (Hirasawa *et al.*, 2002; Moignard *et al.*, 2013; Qiu *et al.*, 2009), suggesting a positive regulatory interaction. However, it has been shown in an erythroid context that GATA-2 is inhibited by GATA-1 (Grass *et al.*, 2003; Martowicz *et al.*, 2005). This could be explained by the fact that the regulation of GATA factors is different in granulocyte and erythroid cells (Ohmori *et al.*, 2012). Given that our model tries to reflect the molecular context of GMP cells, we incorporated to the model a positive regulatory interaction from GATA-1 to GATA-2.

Basophil specification is poorly characterized (Dahlin and Hallgren, 2014). It is known that C/EBPα activation in a bipotential progenitor (which gives raise to mast cells and basophils) favors basophil formation (Arinobu *et al.*, 2005; Qi *et al.*, 2013). GATA-2 has been proposed as a critical marker of basophil formation in humans and mice (Baba *et al.*, 2012; Ohmori *et al.*, 2015; Iwasaki *et al.*, 2006). In fact, some basophil subpopulations express GATA-2 and low levels of GATA-1 as observed by northern blot assays (Zon *et al.*, 1993), and recently in single cell transcriptomic analysis (Paul *et al.*, 2015). The temporal order of expression of C/EBPα and GATA-2 factors has also been proposed to determine basophil formation, but the molecular mechanism underlying this phenomenon is not known (Iwasaki *et al.*, 2006). RUNX-1 is recognized as an important factor for basophil development, because RUNX-1 null mutants have reduced numbers of these cells (Mukai *et al.*, 2012). In the case of mast cells, the activation of cell markers such as c-KIT, MMCP6, and MMCPA, requires MITF-1 expression (Phung *et al.*, 2011; Morii *et al.*, 1996). Additionally, GATA-1 is able to promote c-KIT, but only in combination with SCL (Tripic *et al.*, 2009; Munugalavadla *et al.*, 2005). MITF-1 and C/EBPα directly inhibit the expression of each other, and their expression favor mast cell or basophil formation, respectively (Qi *et al.*, 2013).

2.2 The network as a discrete dynamical system

We transformed the regulatory network into a dynamical system in the form of a Boolean Network following standard procedures (Abou-Jaoudé *et al.*, 2016). Briefly, each node in a BN is in one of two possible values: 0/OFF or 1/ON. The value of a node x_i is determined by a Boolean function f_i of the nodes regulating x_i: $x_{i(t+1)} = f_i(x_1(t), ..., x_k(t))$; where $x_1(t), ..., x_k(t)$ is the set of values of the k regulators of x_i at time t, . The set of all f_is of the BN model consisting of only direct interactions and the full BN model are given in Supplementary Table 2 and 4, respectively. A detailed table with the description of the experimental findings underlying the Boolean functions of the models is in Supplementary File *modelFunctions.csv*. Additionally, the full set of equations is available as Supplementary File *GMPModel.sbml*, in SBML qual format. The model is publicly available at https://thecellcollective.org#5705 (Helikar *et al.*, 2012).

The vector $(x_i, ..., x_n)$ containing the state of activation of all nodes at a given time t is the network state. For n nodes, the state space is formed by 2^n network states. We analyzed the behavior of the network by studying the dynamical behavior starting from all possible (*i.e.* $2^{29} = 536,870,912$) initial states using asynchronous updating.

In addition to the wild-type behavior of the network, we also analyzed mutants and perturbations. The simulation of loss- and gain-of-function mutants in the model was performed by fixing node values to 0 or 1 throughout the simulation, respectively. To simulate possible transitions between steady states driven by deterministic perturbations, we flipped the value of a node and let the system evolve until it converged to an attractor. This procedure was repeated for each attractor in every node. Given that using asynchronous updating trajectories vary among simulations, we repeated the perturbation analysis 1000 times to obtain the statistical behavior.

For the simulation of the effect of fixed environments, some node values were kept constant. Since we were interested in finding the wild type steady states that are preserved after the environment conditions are switched, we used the wild type attractors as initial states for the simulation of change in the environment.

Finally, we addressed whether each interaction in the network is necessary to recover the GMP patterns. We achieved this objective by systematically removing interactions (one-by-one) from the model, finding the resulting steady states, and comparing the results with the original model.

3 RESULTS AND DISCUSSION

3.1 A regulatory network of direct interactions is not sufficient for recovering the main GMP-derived patterns

We started with a version of the network containing only well-documented direct interactions (Supplementary Figure 1 and Supplementary Table 1). Our purpose was to asses whether the regulatory network of transcription factors was able to determine the main expression patterns of GMP-derived cells, following the analysis carried out by (Martinez-Sanchez *et al.*, 2015). We had to include, additionally to the TFs, markers of mature lineage patterns to give a biological interpretation of the steady states. Nonetheless, this small version containing only direct interactions was not able to recover all the patterns of GMP-derived cells. Specifically, basophil and monocyte patterns were not recovered (Supplementary Figure 2). Instead, mixed patterns were found. The reason for this behavior is that this version of the model does not include sufficient nodes to discriminate among all granulocytes. Specifically, the profile of basophils is FcϵRIα^+ CD11b$^+$c-KIT$^-$, and the absence of CD11b in the network would require to identify basophils by the FcϵRIα^+c-KIT$^-$ molecular signature. However, this is not sufficient because such patterns are also observed in eosinophils (Akashi *et al.*, 2000).

As a next step we added interactions which have been inferred from epistatic experiments, and thus might not be direct regulatory interactions. Specifically, C/EBPα suppresses the protein expression of c-MYB (Soliera *et al.*, 2008). EGR-2 downregulates LF, since the expression of a shRNA that targets EGR-2 causes LF induction as evaluated by RT-PCR (Laslo *et al.*, 2006).

The following interactions were reported in human cell models. C/EBPα and PU.1 activate the G-CSFR gene promoter (Radomska *et al.*, 1998; Smith *et al.*, 1996). GFI-1 inhibits MBP expression during G-CSFR stimulation of granulocytes (Liu and Dong, 2012). C/EBPα and PU.1 synergistically activate the βc gene promoter.

In the first version of the network we could not associate any steady state to basophil lineages, thus we needed to add more markers associated with this lineage. Thus, we added

CCR3, IL-3Rα, and used as basophil signature the experimentally observed profile expression IL-3Rα^+, CCR3$^-$ along with GATA-2$^+$, C/EBPα^+, and RUNX-1 transcriptions factors. CCR3 is an important eosinophil marker not expressed in basophils, which is activated by GATA-1, PU.1 and RUNX-1 (Kim *et al.*, 2010).

IL-3, IL-5, and GMCSFR are important cytokines for eosinophil, basophil, and mast cell formation. In mice, IL-5Rα skews eosinophil formation, while IL-3Rα promotes basophil and mast cell proliferation (Ohmori *et al.*, 2009; Roboz and Rafii, 1999). The receptors of these cytokines are related because they share a common β chain (βc) subunit that activates the JAK-STAT pathway (Hercus *et al.*, 2013). Of these, only IL-3Rα is known to signal the core of transcription factors of the network, activating GATA-2 or C/EBPβ gene via JAK2-STAT5 transducers (Xu *et al.*, 2003; Li *et al.*, 2015).

Finally, we propose some interactions. Specifically, we added a GATA-1/2 positive regulation to IL-3Rα based in the fact that the IL3-Rα gene promoter region has potential binding sites for GATA factors (Miyajima *et al.*, 1995). KIT signaling by MITF-1 and c-KIT could be important for MCCPA expression since c-KIT null mutation cause downregulation of this marker Ishijima *et al.* (2012). We also assumed a negative regulation of EGR-2 on MPO and NE neutrophil markers, since this regulator has been reported to globally shut off the granulocyte gene expression program (Laslo *et al.*, 2006). GATA factors favor eosinophil, basophil and mast cell formation when they are transduced into GMP cells, decreasing neutrophil and monocyte markers expression (Iwasaki *et al.*, 2006). Hence, we assumed that GATA-1 and -2 downregulate neutrophil markers such as MPO, NE and LF. Readers may find more details on the proposed functions in the Supplementary File *modelFunctions.csv*.

3.2 The regulatory network

The network comprises 29 nodes and 83 regulatory interactions among them, as shown in Figure 1. Only 19 nodes are regulators (Figure 1, left) and the rest are lineage markers (Figure 1, right). While the markers are not important for the dynamic of the model, they are important to associate steady state patterns to GMP phenotypes. For clarity, the regulatory interactions on the lineage markers were omitted in Figure 1, but the full network can be seen as Supplementary Figure 5.

The network consist of a core of TFs made of RUNX1, MITF-1, c-JUN, c-MYB, GATA-1, GATA-2, C/EBPα, C/EBPβ, SCL, GFI-1, PU.1, STAT5, and EGR-2. Subsets of this module have been previously studied Krumsiek *et al.* (2011); Laslo *et al.* (2006). We included MITF-1, an important transducer of the c-KIT pathway. A negative regulatory feedback is formed between C/EBPα and MITF-1. This circuit is important to determine basophil *versus* mast cell commitment Qiu *et al.* (2009). We also added part of the GM-CSF and IL-3 signaling pathways. These two routes share the βc subunit, JAK2 and STAT-5 transducers. STAT-5 positively regulates GATA-2 and C/EBPα. These interactions are important since they link the core of TF with extracellular IL-3 and GM-CSFR pathways.

3.3 Boolean Network steady states match expression patterns found in GMP derived cells

We explored the state space of the regulatory network to obtain the steady states of the dynamical system, and found 22 fixed point steady states (Figure 2) using asynchronous updating. In addition, four cyclic attractors of length 2 were found when using synchronous updating simulation, characterized by alternative EGR-2 and GFI-1 expression (see the Supplementary File *attractors.txt*). These cycles can be considered an artifact of synchronous updating since they are not observed in asynchronous regimen. Only fixed points attractors were found in the asynchronous exhaustive search. Fixed point attractors are the same in both updating schemes and they coincide with asynchronous steady states shown in Figure 2 (compare with fixed points in *attractors.txt*). Therefore, we chose asynchronous updating for all simulations. Finally, since all steady states in this model are fixed points, we use the terms *attractor* and *steady state* interchangeably in the rest of this work (Boeing, 2016; Strogatz, 2014).

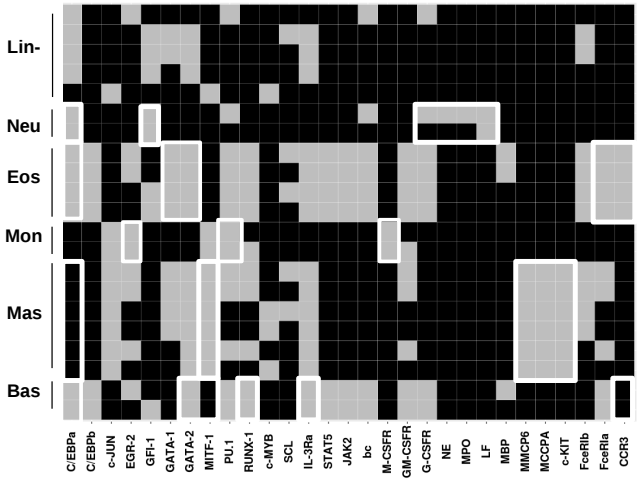

Fig. 2: **Steady states of the BN model**. Active and inactive nodes are depicted as gray and black boxes, respectively. Steady states, shown in rows, are grouped in classes according to the cellular phenotype they represent, as explained in Section 3.2. Labels at the left indicate: lineage negative (Lin-), neutrophils (Neu), eosinophils (Eos), monocytes (Mon), mast cells (Mas), and basophils (Bas). Lineage signatures as given in Supplementary Table 5 are highlighted using white borders.

These steady states can be grouped into classes, according to their associated cellular phenotype (see Supplementary Table 5 and Figure 2). The steady states that comprise the neutrophil class express C/EBPα, GFI-1, MPO, NE, and LF (Ma *et al.*, 2014; Egesten *et al.*, 1994; Laslo *et al.*, 2006). Monocyte steady states express PU.1, EGR-2, and M-CSFR (Laslo *et al.*, 2006; Ma *et al.*, 2014). The eosinophil class is characterized by MBP, FcϵRIα, CCR3, GATA-1/2, C/EBPα, and PU.1 (Iwasaki *et al.*, 2005). The class of basophils expresses C/EBPα, GATA-2, RUNX-1, IL-3Rα, and FcϵRIα (Arinobu *et al.*, 2005; Mukai *et al.*, 2012). Finally, the mast cells class expresses c-KIT, MITF-1, MMCPA, MMCP6, IL-3Rα, and does not express C/EBPα (Arinobu *et al.*, 2005; Qi *et al.*, 2013). The above mentioned molecular signatures are highlighted in Figure 2 for each class.

GMP cells are Lin$^-$Sca$^-$CD34$^+$c-KIT$^+$FcγR$^+$ (Akashi *et al.*, 2000). Of these markers, only regulatory interactions of c-KIT are

known. But since this molecule is also expressed in mast cells (Arinobu *et al.*, 2005), we could not use it as a unique pattern signature to identify the GMP class. However, we noted that Lin^- steady states resemble immature subpopulations, as evidenced by their response to perturbations (see Section 3.5). Importantly, Lin^- steady states do not have an activated c-KIT although GMP subpopulations do. This difference could be attributed to the nature of the Boolean model. A node is in an "OFF" state to represent either total absence or low activity. Now, c-KIT is expressed at high levels only in mast cells (Arinobu *et al.*, 2005). Thus an inactive c-KIT node in Lin^- steady states means that the expression of c-KIT is low; namely, below a certain arbitrary threshold. Furthermore, C/EBPα is present in GMPs (Arinobu *et al.*, 2005; Iwasaki *et al.*, 2006), and is a negative regulator of c-KIT; thus keeping c-KIT at low levels. Low expression of c-KIT in GMP cells are also observed in single cell transcriptomic measures when compared to HSC and erythroid progenitors (Moignard *et al.*, 2013). Another interesting expression pattern is that of MBP node, which is traditionally though as a protein maker of mature eosinophil cells (Hirasawa *et al.*, 2002). However, MBP can be expressed in basophils progenitors in mice (Arinobu *et al.*, 2005). This is consistent with MBP expression in eosinophil and basophil steady states of the model.

As stated in Section 2.1, there is contradictory evidence regarding the sign of the interaction of GATA-1 over GATA-2 (Martowicz *et al.*, 2005; Grass *et al.*, 2003). While we decided to incorporate a positive interaction in our model, we explored the implications of using a negative interaction instead. We did this by using the following rule: GATA-2 = NOT GATA-1 AND (GATA-2 AND STAT-5). In this case, all GMP phenotypes are recovered (see the Supplementary File *attractors.csv*). However, in this variant of the model the expression of GATA-1 and GATA-2 is mutually exclusive, which is in direct contradiction with the experimental evidence (Moignard *et al.*, 2013; Hirasawa *et al.*, 2002; Qiu *et al.*, 2009). Therefore, our model suggests that GATA-1 is a positive regulator of GATA-2.

It is important to note that some nodes show variability in steady states comprising a single class. Namely, the SCL transcription factor has only been observed to be expressed in Granulocytes-Monocytes progenitors and mature mast cells (Dey *et al.*, 2010; Babina *et al.*, 2005). Given the absence of known regulators of SCL, other than itself, it is possible that the variation of the state of this node among stationary states is due to missing regulatory interactions.

The expression of PU.1 has been linked to neutrophil maturation (Anderson *et al.*, 1998, 1999). Hence, neutrophil steady states with an active PU.1 can be interpreted as mature neutrophil stages. This stage also present an active LF node, which is a known secondary granule protein marker of neutrophil maturation (Khanna-gupta *et al.*, 2000).

The four steady states associated to eosinophils vary in EGR-2, GFI-1, and MBP. In a single-cell transcriptomic study there was evidence of variability in GFI-1 levels (Paul *et al.*, 2015). At the other hand, interleukin-5 stimulation has been observed to induce EGR-2 and MBP (Byström *et al.*, 2004; Temple *et al.*, 2001; Byström *et al.*, 2004). Therefore, steady states which have active EGR-2 and MBP, but inactive GFI-1 can be associated to stimulated eosinophil stages.

Variation of GM-CSFR node values in the monocyte class agrees with the fact that this receptor can be modulated by a wide variety of stimuli (Cannistra *et al.*, 1990). Furthermore, our model describes variable levels of RUNX-1 in monocytes, which has been reported to be expressed in this lineage (Paul *et al.*, 2015).

In the case of the mast cell class, a subpopulation of these cells expressing EGR-2 was observed when stimulated with IL-33 or Ag-IgE cross-linking (Chhiba *et al.*, 2017). In a similar study, individual mast cells progenitors were found to have higher levels of MCCPA with respect to mature cells (Franco *et al.*, 2010). Interestingly, they found GFI-1 and GATA-2 invariantly downregulated and upregulated, respectively; in agreement with the steady states of our model. Variability of GATA-1, RUNX-1, PU.1, c-MYB, GM-CSFR, FcϵRIα, and FcϵRIβ in mast cells requires further assessment.

Regarding the set of steady states associated to basophils, there is a variation in the states of EGR-2 and MBP. Of these, only EGR-2 has been found to be modulated in these cells by Ag-IgE stimulation (Chhiba *et al.*, 2017).

Single-cell gene expression experiments have found variability in GATA-1, GATA-2, GFI-1, MITF-1, and SCL in GMPs (Moignard *et al.*, 2013). This is in agreement with the variability in the steady states of our model. However, in the same study RUNX-1 and PU.1 were uniformly expressed in GMPs, which do not agree with our model, suggesting the possibility of missing interactions.

3.4 Analysis of mutants and perturbations

We simulated all the loss- and gain-of-function single mutants in the network (see Supplementary File *attractors.txt*), and compared the obtained steady states with reported experimental results in the literature (Supplementary Table 6). The model qualitatively agrees with a series of experimentally described mutants. Specifically, there is no formation of granulocytes, but monocytes can be found in C/EBPα null mutant mice (Zhang *et al.*, 1997). Donor liver progenitor cells from c-JUN mutant mice can reconstitute granulocytes of irradiated recipients (Eferl *et al.*, 1999). GFI-1 mice mutants lack normal neutrophils (Hock *et al.*, 2003). Monocytes can be derived from liver cells from GATA-$2^{-/-}$ mice (Tsai and Orkin, 1997). EGR-2 has been reported to be part of an important regulatory circuit that determines monocyte *versus* neutrophil commitment experimentally. So, it is interesting that in our mutant simulations monocytes pattern are still found in EGR-2 null mutants. This is in accordance with the observation that EGR-$2^{+/-}$ heterozygous mice have a skew to neutrophil differentiation, although they still have monocytes at lower levels (Laslo *et al.*, 2006).

There is a multiple deficiency in GMP-derived lineages in PU.$1^{-/-}$ mice (Scott *et al.*, 1994; Olson *et al.*, 1995). Mice heterozygous for the GATA-2 allele still produces monocytes (Tsai and Orkin, 1997). MITF and c-KIT null mutants have a deficiency of mast cell production (Kim *et al.*, 1999; Grimbaldeston, 2005). Finally, RUNX-1 mice mutants have no basophil development (Mukai *et al.*, 2012). IL-3 Receptor α null mutants have normal hematopoiesis (Hara *et al.*, 1995). The same result is observed in βc mutants (Nishinakamura *et al.*, 1995). Contradictory results have been obtained while evaluating GATA-$1^{-/-}$ mutants (Hirasawa *et al.*, 2002; Dyer *et al.*, 2007). The model supports results from (Hirasawa *et al.*, 2002) who observed an eosinophil lineage specific development deficiency.

There are also some mutant simulations that do not quite agree with experimental results, pointing to aspects of the network model to be improved. Specifically, SCL and c-MYB null mutants in the model recover all lineage steady states, but experimentally these null mutants are reported to have deficiencies in the production of some lineages (Lieu and Reddy, 2009; Robb *et al.*, 1995). c-MYB and SCL null mutants seem to cause deficiencies during early hematopoiesis stages, which is beyond the scope of this model bounded to the GMP differentiation process. Therefore, more complete models of hematopoiesis are necessary to recover these mutants.

c-JUN model mutant does not reach the monocyte attractor, but the experimental mutants do (Eferl *et al.*, 1999). This can be explained by the fact that other TFs —like JUNB— not taken into account in this network are redundant to c-JUN in some contexts and can substitute its function *in vivo* (Passegué *et al.*, 2002). Thus, the JUN family of transcription factors and their regulation most be added in future model versions.

Finally, experiments show that GATA-2 null mutants do not have mast cells (Tsai and Orkin, 1997), but the simulation of this mutant does (Supplementary File *attractors.csv*, and Supplementary Table 6). This discrepancy might suggest a stronger positive dependency of GATA-2 on mast cells markers such as MMCPA or c-KIT, as has been suggested elsewhere (Zon *et al.*, 1991; Maeda *et al.*, 2010)

A systematic deletion of network interactions showed that 46 interactions in the network are necessary to maintain wild type steady states (see the Supplementary File *removedInteractions.csv*). We analyzed for each removed interaction the number of missing wild type stationary states, and the total of missing GMP patterns, or classes. For example, deletion of the positive regulation of GATA-2 over M-CSFR causes the disappearance of three wild type steady states, all belonging to only one GMP class. Remotion of any of the other 37 interactions did not cause disappearance of any steady state with respect to the wild type model. Therefore, we conclude that the model is relatively robust to the deletion of a single regulatory interaction.

3.5 Transitions between steady states resemble GMP derived cells plasticity

In the Supplementary Figure 3, transitions between steady states derived by single transient perturbations observed in the simulations are given. They are tagged with some of the node perturbations that cause the transition. A full list of perturbations is given in the Supplementary File *steadyStateTransitions.csv*.

The following transitions observed in the model agree with experiments. As mentioned above, the Lin^- steady states class has a pattern of transitions similar to GMP cells since it can give rise to monocytes by PU.1 upregulation (Laslo *et al.*, 2006), neutrophils by increasing C/EBPα levels (Dahl *et al.*, 2007), basophil and eosinophils by IL-3 stimulation (Ohmori *et al.*, 2009; Takamoto and Sugane, 1995). Lastly, c-KIT or MITF-1 induction causes Lin^- differentiation to mast cells (Tsai *et al.*, 1991). Interestingly, the transcriptional factor signature C/EBPα^+PU.1$^+$GATA-1/2$^-$ is observed in GMP cells by western blot bulk assays, and also using single cell transcriptomics (Arinobu *et al.*, 2005; Iwasaki *et al.*, 2006; Moignard *et al.*, 2013). This molecular signature was found in one Lin^- stationary state, which is shown as the first attractor in Figure 2, and is tagged as Lne (Lineage negative) in the Supplementary File *steadyStatesTransitions.csv*. This attractor when perturbed can give rise to monocytes and granulocyte patterns but it can not transit to mast cells steady states (see transitions from the Lne steady state and compare it with those of Lne 1-4 steady states. This transition pattern is consistent with experimental evidence showing that mast cells are originated by non-GMP Lin^- subpopulations (Chen *et al.*, 2005).

Traditionally, transitions between subpopulations were thought to be directional, from progenitors to more committed cells. However, in the last years there have been reports documenting transitions from mature lineages to less committed progenitors, or even between different lineages (Graf, 2002; DuPage and Bluestone, 2016). These transitions have been collectively called *plasticity* events. However, a precise definition of the term is still lacking (Lakshmipathy and Verfaillie, 2005). In murine models, plasticity in GMP-derived cells has been observed. For example, monocyte committed leukemia cell lines can be forced to express erythroid markers (Yamaguchi *et al.*, 1998). In the context of regulatory network models, plasticity may be rigorously defined as a transition from one basin of attraction to another due to the effect of a perturbation in the system. Transitions between steady states from mature lineages to Lin^- classes or between mature lineages patterns observed in the simulations (Supplementary Figure 3) could correspond to potential plasticity predicted by the model as observed in other BRN models (Naldi *et al.*, 2010; Martinez-Sanchez *et al.*, 2015; Bonzanni *et al.*, 2013).

Extra- and intra-cellular environmental clues (such as cytokines and transcription factors) are important for guiding the type of cellular response (Doulatov *et al.*, 2012). Hence, we analyzed the change in steady state patterns in response to different fixed extracellular environments defined as follows: pro neutrophil (C/EBPα^+ PU.1$^+$ G-CSFR$^+$)(Laslo *et al.*, 2006), pro monocyte (C/EBPα^+ PU.1$^+$ M-CSFR$^+$)(Laslo *et al.*, 2006), pro mast cell (IL-3Rα^-c-KIT$^+$)(Dvorak *et al.*, 1994; Qi *et al.*, 2013), pro eosinophil (C/EBPα^+GATA-1$^+$FcϵRIα^+)(Iwasaki *et al.*, 2006), and pro basophil (C/EBPα^+GATA-2$^+$RUNX-1$^+$IL3Rα^+)(Qi *et al.*, 2013), see methods. We found that a fixed environment skews the appearance of molecular patterns to its expected phenotype (Supplementary Figure 4). Additionally, we simulated the effect of certain intracellular states. In the absence of the main TFs expression only Lin^- are found, but neutrophils and basophils are still found in the absence of cytokine receptors expression. This is in accordance with a permissive function for cytokines *versus* an instructive role for TFs as some reports have pointed out (Robb, 2007).

4 CONCLUSIONS

We used a Boolean regulatory network model to test whether the available information regarding interactions of key elements of the network involved in GMP differentiation were sufficient to determine the expression patterns observed in cells derived from the Granulocyte-Monocyte Progenitors. We found that a model containing only direct, well-recognized regulatory interactions was insufficient to recover the observed expression patterns. Nonetheless, we were able to infer a regulatory network that includes indirect experimental evidence that *does* recover the observed patterns in wild type and mutant cells. Furthermore, by

systematically perturbing the system we found complex patterns of transitions between steady states classes that can be associated to commitment transitions, as well as plasticity events observed in GMP derived cells. Therefore, our model is a valuable tool for the elaboration of hypothesis regarding the existence, or not, of certain regulatory interactions. Indeed, our model provides experimentalist with a set of regulatory interactions that need to be further studied in the process of GMP differentiation.

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