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Data-driven inference of Boolean networks from transcriptomes to predict cellular differentiation and reprogramming

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1 ABSTRACT

2 **Boolean networks provide robust explainable and predictive models of cellular dynamics, especially for cellular differenti-**
3 **ation and fate decision processes. Yet, the construction of such models is extremely challenging, as it requires integrating**
4 **prior knowledge with experimental observation of transcriptome, potentially relating thousands of genes. We present a**
5 **general methodology, implemented in the software tool BoNesis, for the qualitative modeling of gene regulation behind**
6 **the observed state changes from transcriptome data and prior knowledge of the gene regulatory network. BoNesis allows**
7 **computing ensembles of Boolean networks, where each of them is able to reproduce the modeled differentiation process.**
8 **We illustrate the scalability and versatility of BoNesis with two applications: the modeling of hematopoiesis from single-cell**
9 **RNA-Seq data, and modeling the differentiation of bone marrow stromal cells into adipocytes and osteoblasts from bulk**
10 **RNA-seq time series data. For this later case, we took advantage of ensemble modeling to predict combinations of repro-**
11 **gramming factors for trans-differentiation that are robust to model uncertainties due to variations in experimental repli-**
12 **cates and choice of binarization method. Moreover, we performed an in silico assessment of the fidelity and efficiency of**
13 **the reprogramming, and conducted preliminary experimental validation.**

14 INTRODUCTION

15 Mathematical models have demonstrated their utility in elucidating experimental findings that might challenge intuitive compre-
16 hension. Through meticulous depiction of interactions within intricate signaling pathways and by contextualizing the dynamics of
17 gene expression, these models provide a systematic approach to unveil the regulatory mechanisms that control cellular processes
18 and their dysregulation in diseases. Among the various mathematical formalisms, the Boolean network (Boolean network) is a sim-
19 ple but expressive formalism that relies on pragmatic rules to qualitatively simulate essential systems' features. It is notably valuable
20 in poorly understood large-scale systems as it can be employed for systems with hundreds of components and as the inference of
21 Boolean network models, contrary to quantitative models (typically ordinary differential equation (ODE)-based models), does not
22 require kinetic parameters derived from in-depth and often unavailable knowledge.

23 Consequently, Boolean networks are increasingly used to capture the interaction dynamics within complex signaling pathways and
24 regulatory mechanisms governing cellular behaviors. They have been inferred from high-throughput data for modeling a range
25 of biologically meaningful phenomena such as the mammalian cell cycle (19), cell differentiation and specifications (53, 57, 16,
26 30, 36), stress/aging-related cell behaviors (55, 75), cell apoptosis (50) and cancer cell functions (91, 15, 87, 58). Recent endeav-
27 ors have focused on enhancing the quantitative interpretation of the resulting models, incorporating probabilistic approaches to
28 effectively simulate heterogeneous cell populations and dynamical interacting populations (74, 76), and introducing a semantics
29 that offers the formal guarantee of completely capturing any behavior achievable by any quantitative model (multilevel or ODE) fol-
30 lowing the same logic (69).

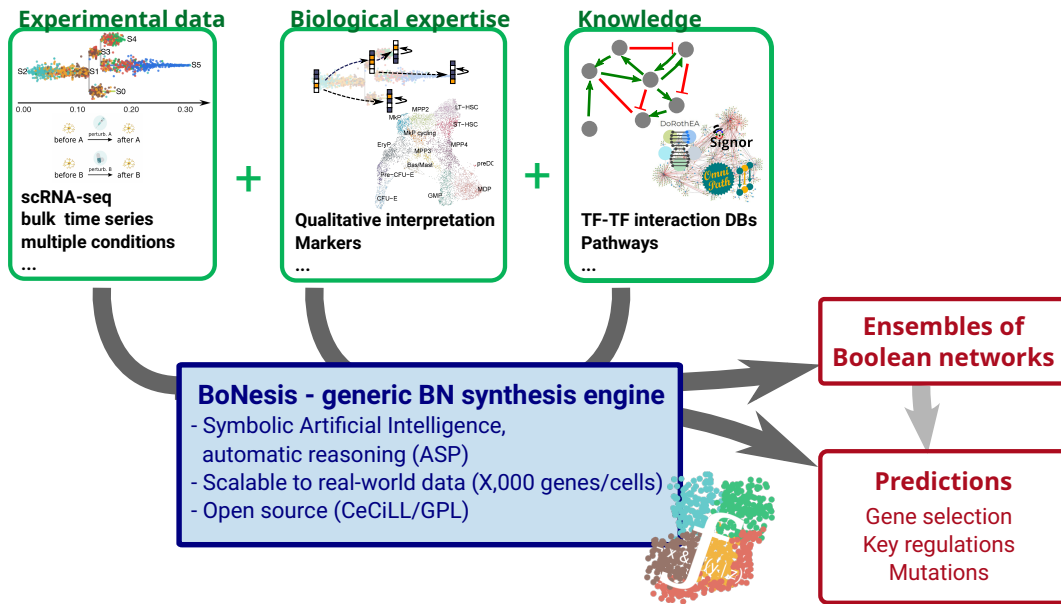


Figure 1: General principle of the conducted inference of ensembles of Boolean networks. By offering a generic modeling language, BoNesis enables integrating prior knowledge on regulation mechanisms with different type of experimental data, after qualitative interpretation, which may depend on biological hypothesis and experimental system. These inputs specify what an admissible model is. Then, employing logic programming, BoNesis can generate ensembles of Boolean networks that fulfill the structural and dynamical properties and, by combining with combinatorial optimization technologies, enables to predict important genes and derive predictions for attractor reprogramming.

31 A Boolean network consists of logical rules that are associated to each variable and that predict how their state evolves with time.
 32 The regulators of a variable are combined with logical connectors *or*, *and*, and *not* and define conditions for a variable to be at 0
 33 (false/inactive/absent) or (1/true/active/present). Through structural and dynamic analyses along with simulations under perturba-
 34 tions, Boolean networks provide versatile opportunities for exploring mechanisms underlying biological phenomena. This versatility
 35 allows them to serve as standalone informative tools but also, for specific modeling contexts and needs such as in systems phar-
 36 macology, to lay the groundwork for more detailed pharmacokinetic/pharmacodynamic and quantitative systems pharmacology
 37 (QSP) models using ODEs (38, 13, 6, 70).

38 One the prominent challenge for applications of Boolean networks in biology is the design of their logical rules. Indeed, besides the
 39 inference of the underlying gene regulatory network, the search for logical rules faces a double combinatorial explosion: the num-
 40 ber of possible logical rules for a single variable is exponential with the number of its regulators, and checking whether a candidate
 41 Boolean network possesses the desired dynamical features (steady states, trajectories, ...) can involve analysis that take time and
 42 memory exponential with the number of variables. Thus, in most applications of the literature, the Boolean networks have been
 43 manually designed from expert knowledge and by re-utilizing previously published models. Nevertheless, there has been recent
 44 progress on the inference of Boolean models from data (81, 67, 60, 25, 68, 24, 85, 1). These methods address the inference prob-
 45 lem with different restrictions, either on the type of data and their interpretation in order to obtain simpler dynamical properties, or
 46 on restricting the set of logical rules to those having a particular structure, in order to reduce the combinatorics of candidate mod-
 47 els. However, these methods remain difficult to scale above hundreds of variables, and typically enforces rather specific way of inter-
 48 preting the experimental data in terms of Boolean properties.

49 In this paper, we present a general methodology for the inference of Boolean networks from knowledge, data, and expert interpre-
 50 tation of data, and the computation of prediction from the resulting models. The methodology, summarized in Figure 1, builds on
 51 the following steps:

- 52 1. The modeling of the knowledge, essentially in terms of admissible structure for the models.
- 53 2. The qualitative modeling of the data in terms of expected dynamical properties of the model. This steps depends on the bio-
 54 logical expertise of the system, and relies on data analysis, notably to classify gene expression into binary values.
- 55 3. The tool BoNesis (12), which integrates (1) and (2) and, using logic programming and combinatorial optimization algorithms,

56 infers ensembles of Boolean networks that are compatible with modeled static and dynamical properties.

57 4. The analysis of sampled ensembles of models to perform predictions, including key genes and reprogramming mutations.

58 The modeling steps, that define the inference problem, allow a versatile pipeline, that is not tied to specific type of data, or a specific
59 interpretation of them. In some sense, our approach aims at moving the modeling effort from the design of Boolean rules to the
60 specification of the expected features of the model. Then, we employ symbolic artificial intelligence technologies to automatically
61 construct models that satisfy the desired properties.

62 We illustrate the applicability of the pipeline on two extensive case studies: the inference of Boolean networks from scRNA-seq data
63 of hematopoiesis, with the identification of key genes and the analysis of families of candidate models; and the prediction of repro-
64 gramming targets for adipocyte to osteoblast conversion from bulk RNA-seq time series data.

65 Importantly, these case studies demonstrate that our approach is scalable to TF-scale networks, by starting from complete TF-TF
66 regulatory networks and, through the inference of Boolean networks, are able to automatically identify sub-networks that can ex-
67 plain the observed dynamics.

68 RESULTS

69 Case study 1: Ensemble modeling of hematopoiesis from scRNA-seq data

70 We applied our inference pipeline (Figure 2A) to the identification of key genes and Boolean rules that can explain the hematopoiesis
71 observed in a mouse sample using scRNA-seq data from Nestorowa et al. (62). Employing trajectory reconstruction and binariza-
72 tion methods, we derived a logical specification of the differentiation dynamics. Then, using BoNesis, we considered any Boolean
73 network employing TF regulations referenced in the DoRothEA database, and automatically identified the sparsest among them
74 that are able to reproduce the differentiation dynamics. We compared the selected genes of importance with an export model of
75 the literature, showing a substantial overlap. Finally, we highlighted the advantage of the ensemble modeling by analyzing the vari-
76 ability of Boolean models compatible with the input data. We notably performed clustering of sampled models, resulting in clear 3
77 sub-families of models that can be distinguished on specific features of Boolean rules.

78 Biological context and experimental data

79 Hematopoiesis is a crucial differentiation process of blood cells for immune system regeneration. It has been extensively studied, in-
80 cluding with mathematical and logic dynamical models (30, 16, 57, 32).

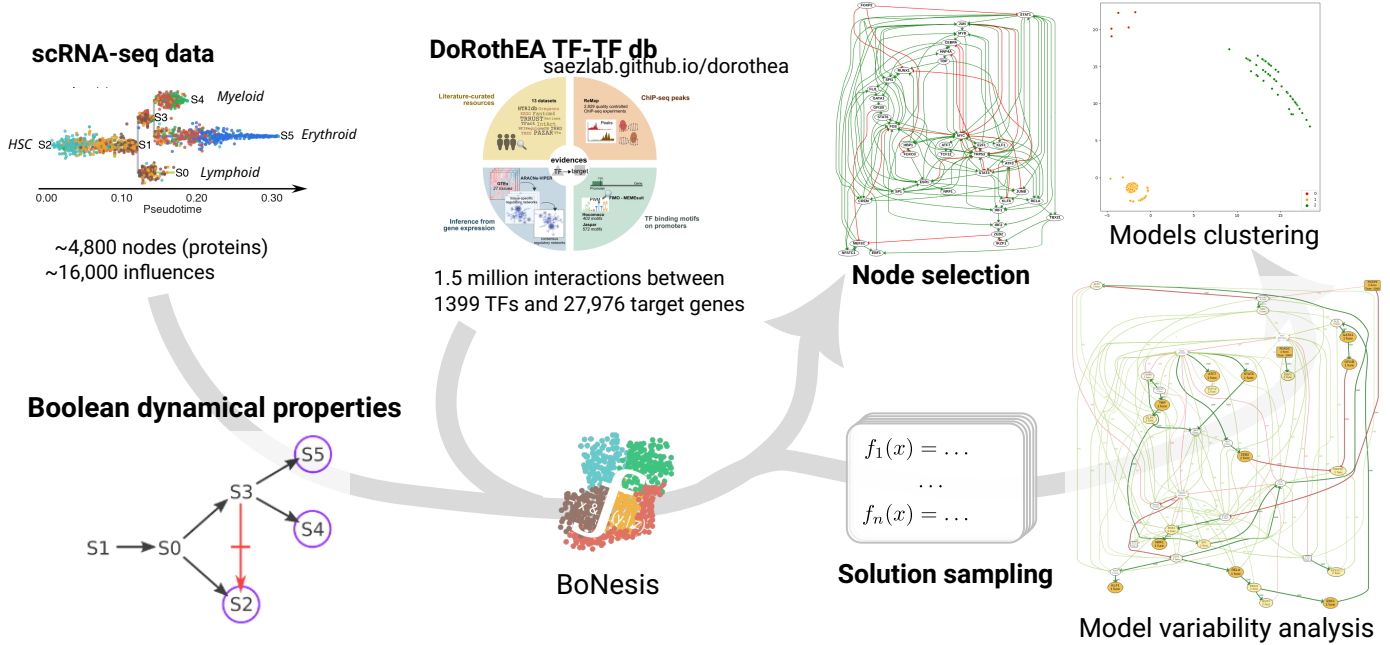
81 We focused on data-driven modeling of the early differentiation of mouse hematopoietic stem cells (HSCs) from scRNA-seq data of
82 Nestorowa et al. (62). The data shows heterogeneity of cells during HSCs differentiation, including lympho-myeloid primed progen-
83 itors (LMPPs) and common myeloid progenitors (CMPs), further differentiated into granulocyte-monocyte progenitors (GMPs) and
84 megakaryocyte-erythrocyte progenitors (MEPs). We performed hyper-variable gene selection and trajectory reconstruction using
85 STREAM (8). The resulting trajectory has the shape of a tree with two bifurcations visible in Figure 2B, having as root the endpoint
86 that concentrates the hematopoietic stem cells.

87 Qualitative interpretation of the data

88 To transform obtained trajectories into properties over Boolean states, we considered *six states* that must correspond to the start
89 and end of branches (named S0 to S5 in Figure 2B). Moreover, in order to reduce the sensitivity bias of single-cell observations, we
90 choose to consider observations formed by the union of several cells. This resulted in six clusters of a few tens to hundred of cells,
91 corresponding to initiation (root), two bifurcations points, and three leaves, we considered to be steady states of the Boolean model.
92 We classified the activity of each gene of each cluster using PROFILE (7) on individual cells and an aggregation by majority of value
93 among 0, 1, and ND (not determined).

94 Then, we specified the expected dynamical properties of a Boolean network corresponding to the data as follows. There must ex-
95 ist trajectories linking with states following the STREAM trajectories: e.g., there must exist a trajectory from a Boolean state cor-
96 responding to the root S1 to the Boolean state matching with S0, then from that later Boolean state to a Boolean state matching

A Illustration of the pipeline employed for the scRNA-seq hematopoiesis case study



B Gathering cells at key stages of the blood cell differentiation trajectory for building observations.



C Excerpt of synthesis specification using BoNesis

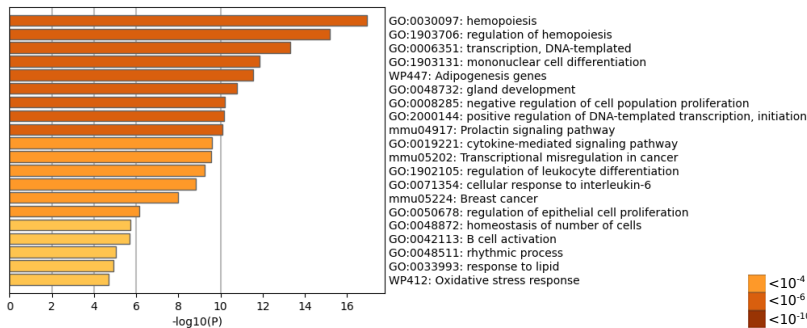
```
# steady states
s2 = fixed(~obs("S2"))
s4 = fixed(~obs("S4"))
s5 = fixed(~obs("S5"))

# trajectories
~obs('S1') >= ~obs('S0') >= s2
~obs('S0') >= ~obs('S3') >= s4
~obs('S3') >= s5

# bifurcation
~obs("S3") / s2

# universal constraints
~obs('S1') >> "fixpoints" ^ \
{obs(s) for s in ['S2', 'S4', 'S5']}
~obs('S3') >> "fixpoints" ^ \
{obs(s) for s in ['S4', 'S5']}
```

D Histogram of the 20 most enriched similar terms sets from the list of the 39 genes automatically selected by BoNesis, colored according to 3 p-value thresholds and with, per set, the representative term of the set of terms.



E Venn diagram showing the number of genes in common between our automatic selection with BoNesis and the state-of-the-art models (30), (16) and (57).

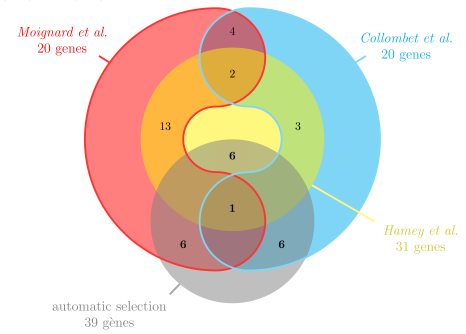


Figure 2: Overview of the case study on scRNA-seq data driven modeling of hematopoiesis.

97 with S2, and so forth. Moreover, we requested that the Boolean states corresponding to the leaves (S2, S4, S6) must be steady state
98 of the Boolean model, and that any steady state reachable from S3 must match with S5 and S4, and any steady state reachable
99 from the root state must match with S2, S4, and S5. [Figure 2C](#) summarizes the declared properties.

100 **Gene selection with BoNesis and DoRoTHEA**

101 Besides the expected dynamical properties of the model, BoNesis requires an *a priori* GRN from which it will reconstruct Boolean
102 rules that (1) employ only genes and regulations referenced in the input GRN, and (2) form a Boolean network that possess the ex-
103 pressed dynamical properties. Moreover, BoNesis is able to identify the genes that can have a constant binary expression towards
104 the whole dynamics, and can thus be ignored in the final model. In the scope of this case study, we took as a priori GRN the full
105 DoRoTHEA TF regulation database (34) with regulation p to confidence level C, comprising 2,777 regulations among 1,001 genes,
106 849 of which have an expression measurement in our dataset. We performed a multi-stage combinatorial optimization proce-
107 dure to identify the largest number of TF genes that cannot be considered as constant, and for each there exists a Boolean network
108 having the desired dynamical properties. It resulted in the selection of 39 TF genes and 137 TF-TF interactions shown in Supple-
109 mentary [Figure 1](#). A gene set enrichment analysis is performed with METASCAPE (92) shows a clear enrichment of terms related to
110 hematopoiesis ([Figure 2D](#)), with the top term being hematopoiesis, followed by others representing more specific biological pro-
111 cesses included in hematopoiesis.

112 Moreover, we compared the data-driven selected genes with three expert art logical models of hematopoiesis by Hamey et al. (30),
113 Collombet et al. (16) and Moignard et al. (57). These three models, composed of 20 to 31 genes, include a total of 53 genes and
114 it is worth noticing that there is no consensus on the interactions implied in the regulation of this process since only 2 genes are
115 common to these three models. The Venn diagram of [Figure 2E](#) shows the intersection between the components we have auto-
116 matically selected thanks to BoNesis and the three state-of-the-art hematopoiesis models. Each of state-of-the-art model shares 6
117 genes with our selection (for a total of 10 distinct genes in common):

- 118 · With Hamey et al. (30): FLI1, GATA1, GFI1B, IKZF1, MYB, RUNX1;
- 119 · with Moignard et al. (57): FLI1, GATA1, GFI1B, IKZF1, MYB, SPI1;
- 120 · with Collombet et al. (16): CEBPA, EFB1, IKZF1, MEF2C, RUNX1, SPI1.

121 **Ensemble analysis of compatible models**

122 From the TF-TF subnetwork extracted in the previous step (Supplementary [Figure 1](#)), we employed BoNesis to sample 1,000 dis-
123 tinct Boolean networks that all respect the qualitative dynamical properties described previously. The sampling has been performed
124 using heuristics to range over models with diverse logical rules. Each of the sampled Boolean network is able to reproduce the qual-
125 itative differentiation dynamics. Moreover, the universal constraints on the reachable steady states, ensure by design, that all trajec-
126 tories from the root state end in one of the three observed differentiated type. Furthermore, we verified a posteriori that none of the
127 sampled network possess cyclic attractors.

128 **Variability analysis shows highly preserved logic rules for two thirds of selected genes** For each selected gene, we analyzed
129 how many Boolean functions has been assigned to it in the sampled ensemble of 1,000 Boolean networks (Supplementary [Fig-](#)
130 [ure 2](#)). It resulted that 12 genes always received the same Boolean function, 12 other genes received only 2 to 3 distinct Boolean
131 functions. This suggest that a large part of the logic rules are highly preserved in all compatible models. Most of the diversity in the
132 sampled ensembles is essentially focused on gene FOS (682 different Boolean functions) and TRP53 (44 Boolean functions).

133 **Clustering identifies 3 sub-families of compatible Boolean models** Clustering the models according to the similarity of their
134 functions can highlight different possible pathways for a process regulation and also point some biologically irrelevant groups. We
135 performed a multidimensional scaling clustering (MDS) of sampled Boolean networks, using distance based on the inequality of
136 Boolean functions: given two Boolean networks f and g of size n , $d(f, g) = \sum_{i=1}^n \mathbb{1}_{f_i \neq g_i}$. MDS results highlight 3 groups of mod-
137 els (Supplementary [Figure 3](#)). It should be kept in mind that the number of models in each group does not reflect their biological

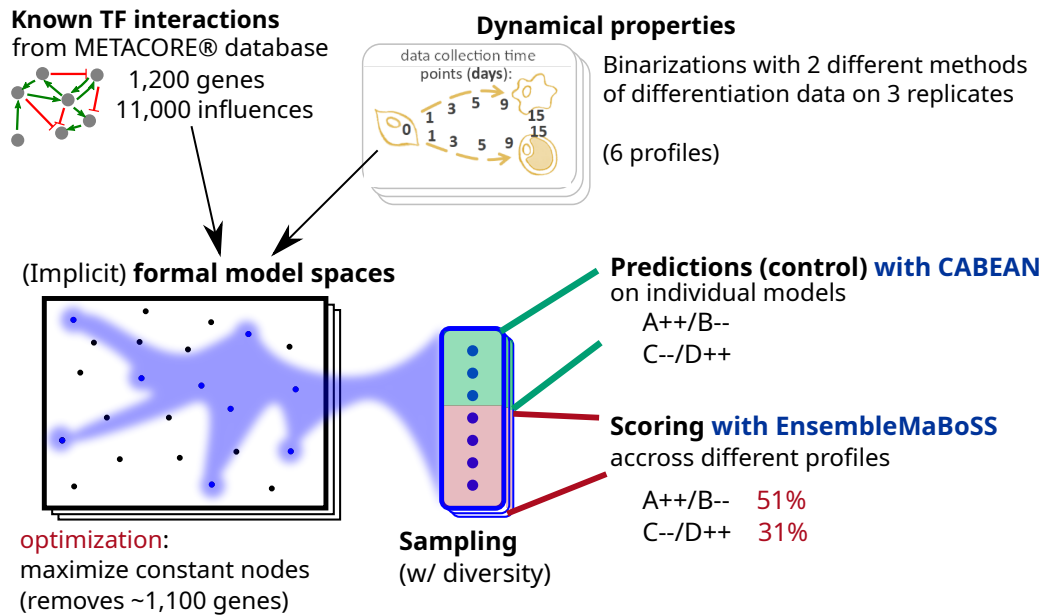


Figure 3: Illustration of the pipeline employed for case study 2. We combined prior knowledge TF-TF interactions extracted from METACORE(r) database with variants of Boolean dynamical properties extracted from time series bulk RNA-seq data. We employed BoNesis to sample Boolean networks fulfilling these properties. Then, we performed prediction of reprogramming determinants using CABEAN on a subset of these sampled models. The identified reprogramming determinants are combinations of gene knock-outs and constitute activations. The predictions are then assessed on the other sampled Boolean models using EnsembleMaBoSS simulation, leading to a scoring in terms of fidelity and efficiency, and thus a ranking of most robust predictions.

138 relevance, as the group size may result from underlying combinatorial aspects of compatible models, and may also relate to which
 139 some close solutions may be easier to find. By comparing the complexity of logical functions across the different cluster, we identi-
 140 fied that one of the cluster (the one in red in Supplementary Figure 3C) encompasses Boolean networks with much simpler rules
 141 (single activation condition), compared to the two others (Supplementary Figure 4-6).
 142 Thus, the ability to cluster the models and analyze their variability enable to pinpoint model features that can be challenged with
 143 expert knowledge are further experimental studies in order to discriminate among sub-families of models.

144 Case study 2: Prediction of reprogramming targets for adipocyte to osteoblast conversion from bulk 145 RNA-seq time series data

146 This case study demonstrates a full pipeline going from experimental bulk RNA-seq time series data and background knowledge
 147 on TF-TF networks to prediction of genetic mutations for trans-differentiation and preliminary experimental validation. The predic-
 148 tions have been obtained by combining inference of Boolean networks ensembles with formal methods for control and ensemble
 149 simulations for scoring (Figure 3).

150 Biological context and experimental data

151 Bone marrow stromal progenitor cells (MSCs) are multipotent cells capable of differentiating either into osteoblasts to form bone
 152 tissue or into bone marrow adipocytes, that play an important role in the hormonal homeostasis of the bone marrow (2). These
 153 cells are reciprocal in their differentiation and correct balance is important for bone health, with increased adipogenesis observed
 154 in obesity and during aging. Previous studies have sought to understand the gene regulatory networks underlying MSC differenti-
 155 ation and improved understanding of these networks could allow for identification of efficient reprogramming targets and better
 156 control of bone marrow cell composition (54). We have previously performed transcriptomic profiling of ST2 cells, a mouse MSC
 157 cell line, across multiple time points of parallel differentiations into adipocytes and osteoblasts using RNA-seq (23). Both differen-
 158 tiations were performed for 15 days with RNA samples collected from undifferentiated cells (ST2D0) and at 5 different time points
 159 (D1, D3, D5, D9, and D15) of each differentiation. Osteoblastogenesis (OD1-OD15) was performed with one composition of differ-
 160 entiation medium for the entire duration of 15 days while for adipogenesis (AD1-AD15) two different media were used, with media
 161 composition changed on the third day of differentiation (AD3). *In vitro* differentiations are known to vary between experiments (26)

162 and therefore three independent differentiation experiments were performed to capture the robustly reproducible gene expres-
163 sion changes. Please see Materials and Methods for further details. The obtained dataset formed a rich resource of dynamic gene
164 expression profiles towards two different trajectories from the same starting point.

165 **Qualitative modeling of bulk RNA-seq time courses triplicates**

166 **Bulk RNA-seq binarization** We employed two different binarization methods by classifying gene activity with respect to back-
167 ground RNA-seq data on a range of tissues, either by bootstrapping parametric distributions (RefBool), or by a simpler statistical
168 procedure applying gene-specific cutoffs, referred to as MUQ in the following. Each gene of each time point is thus assigned to a
169 Boolean or undefined value for each binarization method and each replicate. In total, 1560 genes received a binary value in at least
170 one binarization method and one replicate. For a fixed binarization method, we observed opposite classifications between repli-
171 cates for up to 29 genes, while up to only 2 genes when comparing across binarization methods. This can be explained by the fact
172 that, in general, RefBool classifies less genes than MUQ, and genes classified by RefBool are classified by MUQ similarly. In order to
173 account for this variance in binarizations, we considered each of the 6 profiles (2 binarization methods times 3 replicates) as alter-
174 nate model specifications. Our rationale was to constitute ensembles of models for each of these profiles, and study the robustness
175 of reprogramming prediction across them.

176 **Dynamical properties** Our main modeling hypothesis was that the Boolean model must be able to reproduce the observed mat-
177 uration trajectories from fixed cellular environments. For instance, due to the change of treatment between days 1 and 3 of adipocyte
178 culture, we did not require the existence of a trajectory from **AD1** to **AD3**. From the experimental protocol, this resulted in the speci-
179 fication of two trajectories: the maturation of adipocytes, modeled as the existence of a Boolean trajectory going through **AD3** → **AD5**
180 → **AD9** → **AD15**; and the maturation of osteoblasts, modeled as the existence of a Boolean trajectory going through **OD1** → **OD3** → **OD5** →
181 **OD9** → **OD15**.

182 Moreover, we assumed that **ST2D0**, **AD15**, and **OD15** are observations of cells in steady states. At last, we modeled the observed cellular
183 differentiation process by denying the existence of trajectories across the two branches, nor reverting to the precursor state: there
184 must not exist a trajectory from **AD3** to **OD15**, from **AD3** to **ST2D0**, from **OD1** to **AD15**, nor from **OD1** to **ST2D0**.

185 **Gene selection and sampling of ensemble of diverse Boolean models**

186 We performed selection of genes and sampling of diverse ensembles of Boolean networks from the qualitative modeling of RNA-
187 seq data. We followed a workflow similar to use case 1, that we repeated for each replicate and for each binarization method. Then,
188 as the gene selection resulted in several optimal solutions, the sampling of Boolean networks has been repeated on each of them.
189 The prior GRN was consisting of TF-TF interactions extracted from METACORE database from all the TFs of the RNA-seq dataset. It
190 resulted in a signed digraph comprising 1,027 genes with 11,159 regulations, 169 of which were with an undetermined sign. This
191 prior GRN served to define the set of candidate Boolean networks. Because several genes have more than one hundred referenced
192 regulators, we restricted to the Boolean networks whose activation functions can be expressed with at most 32 disjunctive clauses,
193 without any limit on the size of the clauses. Thus, while we permit a gene to depend on all of its potential regulators, we limited the
194 number of activation contexts.

195 On the 1,027 genes of the prior GRN, one can expect that only a fraction of them are involved in the observed differentiation pro-
196 cess. As in case study 1, we employed BoNESIS to perform gene selection by identifying Boolean networks reproducing the dynam-
197 ics of as much as varying gene as possible, while assigning as much as activation function as possible to a constant value. Depend-
198 ing on the replicate and binarization method, the optimization results in different optimal sets of genes to preserve, ranging from
199 49 to 79 genes. Finally, we performed the diverse sampling of 264 distinct Boolean networks for each of the six profiles, resulting in
200 1,584 Boolean networks verifying the qualitative dynamical properties corresponding to at least one replicate and one binarization
201 method, and using one of the optimal set of genes.

202 Prediction of reprogramming targets with high fidelity and efficiency

203 Our objective was to predict combinations of perturbations of gene expression to trigger a transdifferentiation of adipocytes into
204 osteoblasts. In terms of Boolean network, this corresponded to identifying control strategies to enforce the reachability of the **OD15**
205 state from the **AD15** state. In order to account for candidate model heterogeneity, our approach was to compute reprogramming tar-
206 gets on individual Boolean networks from a subset of the sampled ensemble, and evaluate them on the full ensemble. In the end,
207 we aimed at selecting the perturbations predicted to be most effective on a range of models reconstructed from different binariza-
208 tion and replicates.

209 **Reprogramming computation from subsampled individual models with CABEAN** We selected the CABEAN tool (77) to com-
210 pute combinations of temporary gene knock-out and constitutive activations enforcing the convergence to **OD15** state from **AD15**
211 state for a given individual Boolean network. For each of the 6 qualitative profiles, we applied CABEAN on 24 of the 264 Boolean
212 networks sampled in the inference part. CABEAN failed on 15 of these 144 Boolean networks due to memory issue. On the re-
213 maining 129 models, CABEAN identified combinations of up to 5 simultaneous perturbations leading to a reprogramming from
214 **AD15** to **OD15**. Because we are interested in perturbations that can be effective on as many models as possible, we kept combinations
215 of perturbations that have been identified in at least 10% of the individual models given to CABEAN. This short list of candidates
216 contained 34 different combinations of 2 to 4 simultaneous perturbations.

217 **Simulation of reprogramming with ENSEMBLEMABOSS** The reprogramming perturbations computed by CABEAN are guaran-
218 teed to be effective on their input individual Boolean network. Our objective was to assess the robustness of these (combination of)
219 perturbations the Boolean network ensembles inferred from different qualitative interpretation of the data. To do so, we extended
220 the MABOSS stochastic Boolean network simulator to sample trajectories from the Boolean network ensembles: each Boolean net-
221 work of the ensemble is simulated k times from the corresponding the state corresponding to **AD15** while enforcing the given re-
222 programming perturbation. For each candidate combination of perturbations, we obtain an estimation of the distribution of the
223 steady states of Boolean networks of the ensembles after enforcing the perturbation from the **AD15** state.

224 **Evaluation of reprogramming efficiency and fidelity** We defined scores to evaluate reprogramming candidates from their sim-
225 ulation on Boolean network ensembles for their ability to reprogram to the osteoblast phenotype, as observed at **OD15**. Inspired by
226 usual cellular reprogramming assessments, we considered two measures: the *efficiency* relates to the proportion of cells (models)
227 that show all the prior knowledge adipocyte gene markers (ADIPOQ, FABP4, CEBPA, LPL) and none of the osteoblast prior knowl-
228 edge marker gene (ALPL, HEY1, SP7). Then, the *fidelity* relates to the similarity of those cells to the full **OD15** state.

229 For a given reprogramming perturbation, we write S the set of states resulting from the ENSEMBLEMABOSS simulation, and for each
230 state $s \in S$, we denote by p_s its estimated steady state probability. Moreover, we define $\mathbf{1}_{\text{Ost}}(s)$ as being equal to 1 whenever each
231 osteoblast marker gene is active in s and each adipocyte marker gene is inactive in s , otherwise, $\mathbf{1}_{\text{Ost}}(s)$ is equal to 0.

232 The reprogramming efficiency is computed from the estimated steady state distribution after perturbation of Boolean network en-
233 sembles as the fraction of simulations ending in a state having all the osteoblast marker genes active and all the adipocyte marker
234 genes inactive:

$$\text{efficiency}(S) = \sum_{s \in S} p_s \cdot \mathbf{1}_{\text{Ost}}(s) \quad (1)$$

235 The reprogramming fidelity employs a similarity measure between a full Boolean state $s \in S$ and the binarized state β_{OD15} of the
236 corresponding qualitative interpretation. Because the binary state of some genes of β_{OD15} can be non-determined, the similarity is
237 defined as the proportion of binarized genes in β_{OD15} that have the same value in s . In the end, the fidelity is the expected similarity
238 of the Osteoblast steady states of Boolean networks in the ensemble after reprogramming from **AD15**:

$$\text{fidelity}(S) = \left(\sum_{s \in S} p_s \cdot \mathbf{1}_{\text{Ost}}(s) \cdot \text{similarity}(s, \beta_{\text{OD15}}) \right) / \text{efficiency}(S) \quad (2)$$

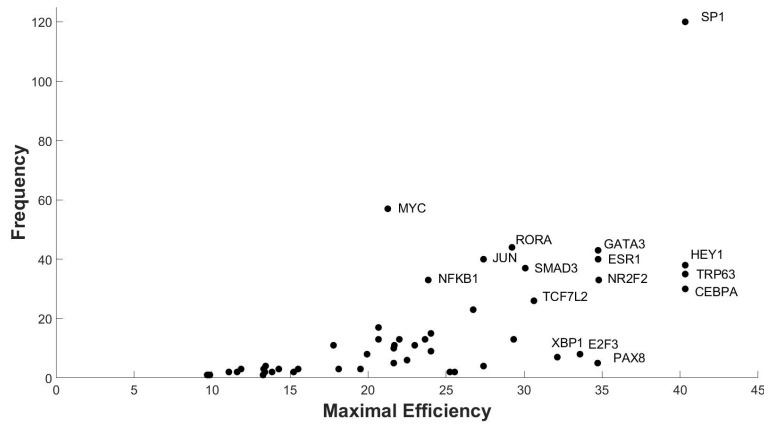


Figure 4: Specific TFs occur more often and with higher efficiency in the predicted reprogramming determinants. Frequency (y-axis) and maximal efficiency (x-axis) of the TFs included in the predicted reprogramming determinants applying permanent stimulation are reported. Names of most promising TFs are given.

239 Validation of node selection and reprogramming targets with literature

240 The TFs included in the predicted reprogramming determinants were ranked by frequency and maximal predicted efficiency (Fig-
 241 ure 4). Analysis of the literature related to the predicted top TFs revealed their existing association with regulation of osteoblasto-
 242 genesis, thereby providing indirect validation for our approach. For example, HEY1, a well-known target of Notch signaling(83) and
 243 a regulator of osteogenesis(72) was among the factors with highest predicted efficiency score. Similarly, CEBPA, SP1, and TRP63
 244 have been associated with adipogenesis, osteogenesis, and bone formation (45, 63, 27), respectively. With repression of CEBPA
 245 likely to act to reverse adipogenic gene expression program. Likewise, the repression of NR2F2 or GATA3 were predicted to lead to
 246 high efficiency of conversion, something that is also supported by literature (88, 40, 42, 86). One of the top TFs included TCF7L2, a
 247 mediator of WNT signaling (Figure 4). Consistently, WNT signaling is known to positively regulate osteoblastogenesis (17, 3), with
 248 the effect mediated by TCF7L2 (90). Finally, also MYC genes have been previously shown to contribute to reprogramming of fibrob-
 249 lasts towards osteoblasts (89, 56). However, some of the well established regulators of osteoblastogenesis, such as SP7 (also known
 250 as Osterix) or RUNX2 (89, 56), were not included among the predicted reprogramming determinants. This could be related to, at
 251 least in case of RUNX2, its already abundant expression in undifferentiated ST2 cells (23).
 252 Based on the predicted combinatorial efficiencies, and the existing literature evidence, three combinations of three determinants
 253 each were selected for experimental validation. Reprogramming experiment 1 (*RE1*) involved up-regulation of TRP63 and down-
 254 regulation of CEBPA and SP1. *RE2* involved up-regulation of TCF7L2 and TRP63 and down-regulation of SP1. And *RE3* involved
 255 up-regulation of TCF7L2 and down-regulation of CEBPA and SP1.

256 Preliminary experimental validation

257 In order to experimentally test the different combinations of reprogramming determinants selected for *RE1*, *RE2* and *RE3*, we took
 258 advantage of lentiviral overexpression in combination with siRNA-mediated gene repression. Overexpression constructs were syn-
 259 thesized at Sirion Biotech and were designed to coexpress either green fluorescent protein (GFP) and red fluorescent protein (RFP)
 260 to confirm a successful lentiviral transduction and to allow to separate between different constructs (specifically co-transduction
 261 of TCF7L2 and TRP63). Day 9 adipocytes were transduced with lentivirus of interest and 24 h later transfected with the relevant
 262 siRNAs to achieve the respective combinations for each RE. In parallel control cells were transduced with a negative control virus
 263 overexpressing only GFP and subsequently transfected with a scrambled control siRNA (please see Materials and Methods for ad-
 264 ditional details). Using reverse transcription real-time quantitative PCR (RT-qPCR) TCF7L2 and TRP63 were confirmed to be 8- and
 265 15-fold overexpressed in undifferentiated cells, respectively, while siRNAs against SP1 and CEBPA led to 35-68% decrease in their
 266 mRNA expression (Supplementary Figure 7). Following transfections, the adipocytes were cultured in osteoblast medium for 9 days
 267 before collection of the cells for single cell RNA-sequencing (scRNA-seq). After the sequencing and data processing, transduced
 268 cells were identified based on the presence of the mRNA sequence encoding for GFP and/or RFP and the relative changes in the

269 transcriptomes were analysed. Differentially expressed genes (DEGs) were obtained in the single-cell expression data based on the
270 Wilcoxon rank-sum test with $FDR < 0.05$. Directionality of the observed change was determined with the common language effect
271 size, with a > 0.5 indicating up-regulation (see Supplementary Table 1). The precision for the upregulated single-cell DEGs com-
272 pared against the bulk DEGs (Day 15: Adipocyte vs Osteoblast, adjusted p-value < 0.05) is **0.57**, **0.51**, and **0.33** for the three different
273 reprogramming experiments *RE1-RE3*. This means that for *RE1* 57% of the genes which were called up-regulated in the single-cell
274 experiment were also up-regulated in osteoblasts compared to adipocytes in the bulk data (23), indicating an initial consistent ef-
275 fect of the applied reprogramming determinant. The respective hypergeometric p-values for enrichment of the true positives in the
276 single-cell DEGs are $4.4e-14$, $1.1e-12$, and **0.057**, respectively. This promising tendency is also confirmed by visual inspection of the
277 bulk RNA-seq data of DEGs obtained in the single-cell experiment (for *RE1* see Supplementary Figures 8 and 9). The very low re-
278 call (< 0.02) in all experiments however indicates that only few of the relevant genes responded to this perturbation in the relatively
279 short time frame of the experiment.

280 **DISCUSSION**

281 **Overview on the methodology**

282 We demonstrated our methodology on two complementary case studies for a combination of data-driven and expert-driven in-
283 ference of Boolean models from scRNA-seq and bulk RNA-seq data. The essence of our approach is the explicit modeling of the
284 inference problem, with the specification of the prior knowledge, typically extracted from databases of TF-TF interactions, and the
285 specification of the expected properties of the model. These latter must reflect the expert and qualitative interpretation of the ex-
286 perimental data. We relied on the BoNESIS engine which enables to specify and combine a broad range of dynamical properties,
287 notably related to trajectories and steady states. Then, ensembles of compatible models can be sampled and further analyzed. We
288 took advantage of ensemble modeling to analyze candidate model variability with model clustering (case study 1), as well as to pro-
289 vide robust reprogramming predictions (case study 2).

290 The flexibility of the workflow facilitates the comparison of different modeling choices and hypotheses. For instance, in case study 2,
291 we considered different binarization methods and different replicates. We took advantage of the variability to generate ensemble
292 of models spanning the different hypotheses and identify consensus predictions. The choice of the input GRN can also impact the
293 results, and it could be assessed in a similar fashion. Such an analysis is out of the scope of this paper, as our objective was not in the
294 benchmark of prior knowledge data nor binarization methods.

295 Remarkably, the inference pipeline was tractable on TF-scale networks. By employing logic programming and relying on recent
296 complexity breakthrough in Boolean network analysis (69), we have been able to fully account for thousands of transcription fac-
297 tors. Then, we took advantage of combinatorial optimization technologies to automatically prune non-necessary variables and iden-
298 tify sub-networks that drive the observed dynamics.

299 **Case study 1**

300 With this case study, we leveraged ensemble modeling to analyze diversity of models that can explain the observed differentiation
301 process. The experimental scRNA-seq data has been processed with trajectory inference methods from which we extracted both
302 clusters of cells corresponding to initiation, bifurcation, and differentiated states, and dynamical properties related to existence of
303 trajectories linking those states.

304 From a full TF-TF interaction database, we have been able to identify a subset of core TFs from which can be drawn Boolean net-
305 works reproducing the qualitative differentiation, that present some intersection with expert models from the literature. By analyz-
306 ing the ensemble of sampled models, we identified three families of models that diverge by the complexity of their logical rules. The
307 variability analysis also emphasized model patterns that are preserved across the ensemble.

308 As we performed a partial enumeration, we are not assured not to miss models with characteristics different from those of the three
309 highlighted groups. However, partial enumerations of 250 and 500 models already highlight the three groups and suggest that in-
310 creasing the number of models only leads to an increase of the size of the 3 groups, without highlighting any new type of models.

311 This motivates the chosen number of 1000 models. MDS done with 250 and 500 models are presented in Supplementary [Fig-](#)
312 [ure 3A](#) and [Figure 3B](#).

313 This modeling of hematopoiesis from a causal network automatically inferred from the dynamics of the data already provides av-
314 enues for further exploration of the mechanisms of its regulation, with the possibility of ensemble simulation of models with Ma-
315 BoSS ([76](#)).

316 **Case study 2**

317 The applied pipeline yielded promising predictions for the reprogramming of adipocytes to osteoblasts, as confirmed by our litera-
318 ture validation, highlighting its utility in identifying novel targets from time-series RNA-seq data. Notably, transcription factors such
319 as SP1, HEY1, and TRP63 frequently appeared in both temporary and permanent perturbation lists, suggesting central roles for
320 them in adipocyte-to-osteoblast conversion. The successful prediction included factors like CEBPA and SP1, known to be crucial
321 for adipocyte and osteoblast functions as modulators of the activity of the respective master regulators of these lineages, PPARC
322 and SP7 (also known as Osterix) ([49](#), [64](#)). These TFs' selection as reprogramming targets is consistent with their involvement in
323 these regulatory networks, supported by their high occurrence in prediction lists and established biological roles in these processes
324 ([46](#), [5](#)).

325 The reliability of the predictive model is evidenced by its consistency with existing literature on differentiation of mesenchymal cells.
326 Factors such as ATF4 and NR2F2, previously implicated in positive and negative control of osteoblast differentiation, respectively,
327 are found among our predictions ([51](#), [41](#)). Similarly, the positive effect of HEY1 and TRP63 on bone formation in the literature ([73](#),
328 [28](#)) highlights the utility of our approach. The predictions also drew attention to the role of less commonly studied factors like AHR
329 and RARA, which are implicated in sensing the cellular microenvironment crucial for differentiation. AHR, predicted for upregula-
330 tion in two combinations, is known to shift the gene regulatory network towards a less specialized cell state, potentially facilitating
331 the reprogramming process ([29](#)). Similarly, RARA, noted for its negative regulatory role in adipogenesis, is part of the reprogram-
332 ming predictions ([59](#)), suggesting that its modulation could inhibit adipocyte traits while promoting osteoblast characteristics.
333 Follow-up studies of our findings could benefit the understanding of bone-related diseases like osteoporosis. By utilizing knowl-
334 edge of specific gene targets that promote osteoblast differentiation, new therapeutic avenues may be explored that enhance bone
335 regeneration and repair ([66](#), [14](#)). Additionally, manipulating adipocyte-to-osteoblast conversion could benefit obesity-related dis-
336 eases where abnormal adipogenesis is prevalent ([9](#)).

337 Nevertheless, for now our validation experiments do face limitations. While gene expression changes upon reprogramming per-
338 turbations were enriched for genes involved in osteoblastogenesis, as observed by scRNA-seq, the overall efficiency of gene deliv-
339 ery and knockdown would need to be improved to be able to better address the quality of our predictions. Usage of stable and in-
340 ducible systems that would be independent of chemicals such as doxycycline, that might perturb cellular systems beyond intended
341 effects, should optimally be used. Additionally, the complexity of gene and protein interactions, including the necessity of consid-
342 ering interaction partners like beta-catenin with TCF7L2 or post-translational modifications like phosphorylation of factors like JUN
343 and FOS, suggests a greater number of (also non-genetic) perturbations may be required for effective reprogramming than initially
344 predicted. Integrating multi-omics data with transcriptomic profiles could provide a more comprehensive understanding of the re-
345 programming processes, allowing for the examination of post-translational modifications and protein activity states crucial in the
346 transcriptional state changes.

347 **MATERIALS AND METHODS**

348 **Boolean network inference methods**

349 **Knowledge-based (bottom-up), data-based (top-down), and combinations** The manual approach is a bottom-up modeling
350 (also called forward modeling) that designs a model through expert and literature-based knowledge to determine the Boolean
351 functions from known and suspected interactions ([19](#), [82](#), [91](#), [50](#), [53](#), [15](#), [16](#), [55](#), [75](#), [36](#), [87](#)). The resulting model is validated or re-
352 fined in an iterative process according to the fitting between its dynamics features and observations of the biological phenomenon

353 (for example its attractors correspond to known phenotypes). This time-consuming approach requires a deep understanding of
354 the biological system and does not ensure that all possible regulations leading to the observed behavior are explored. In contrast,
355 methods have been developed to propose a top-down (reverse) modeling approach that derives from experimental data both the
356 topology of the network, namely the causalities between the biological components, and the logical rules of nodes activation, con-
357 stituting the Boolean network (18, 57, 84, 30, 47, 35, 71, 20, 33). This purely data-based inference approach can suffer from over-
358 fitting to a dataset and circular reasoning: a dataset generates a network, which then predicts the dataset. It also confronts the vast-
359 ness of the solution space without considering prior knowledge about the phenomena. Consequently, other methods have been
360 developed midway between these previously mentioned top-down and bottom-up modeling approaches (81, 67, 60, 25, 68, 10,
361 24, 85, 1), which encompasses the method presented here. The purpose is to leverage both experimental observations and prior
362 knowledge on interactions related to the biological phenomenon to model. Each tool combining data- and knowledge-based mod-
363 eling considers as input both experimental data, typically expression profiles, and a gene regulatory network (GRN) potentially ex-
364 tended with some features. The GRN, also named influence graph or regulation graph, structures the prior knowledge about inter-
365 acting components according to the following definition: a directed graph that represents regulatory interactions between biologi-
366 cal components, interactions categorized in the simplest case as activations and inhibitions (52). A high number of tools have been
367 developed to address these issues because they perform the Boolean network inference through different algorithms and are de-
368 signed for different types of experimental data and different biological interpretations of it.

369 **Partial vs exhaustive exploration of the space of solutions** Exploring the space of possible Boolean networks and their dynam-
370 ics is a challenge because there can be millions of combinations of logical rules that can be formulated. This is why, depending on
371 the algorithm implemented by the tool, the output models can be either Boolean networks that exactly reproduce the desired
372 properties, or Boolean networks optimized to best match these properties with no guarantee of optimality. In the first case, the
373 exploration of solutions can be exhaustive. It should also be taken into account that this search for models (exact or by optimiza-
374 tion) can be limited to a subspace of solutions if Boolean function patterns are imposed. **Optimization problems vs satisfiabil-
375 ity problems.** If we focus on the way solutions are explored, we can distinguish between optimization-based methods (44, 68, 47,
376 81, 1, 35, 20) (heuristic, identify a Boolean network that best aligns with the observations and prior knowledge, based on a defined
377 set of criteria or objective functions) and decision-based methods on satisfiability problems (67, 30, 10, 1, 85) (exact, determine
378 whether there exists a Boolean network consistent with the observations and prior knowledge – based on constraints on its topol-
379 ogy and dynamics – and find such network if it exists). The methodology we present in this paper, based on previously published
380 works (10, 11), belongs to this latter category. According to the chosen approach, some methods output (near-)optimal solutions
381 with no guarantee of reaching a global optimum, while others can theoretically explore the solution space exhaustively to output
382 the whole set of Boolean networks that comply exactly with the biological constraints (in practice often limited to a subset of these
383 Boolean networks as the network size increases and the number of solutions becomes tremendous). This latter ability of inferring
384 an ensemble of models is highly informative. It enables variability analysis across the models to identify common patterns of in-
385 teractions, supporting the formulation of hypotheses that benefit both the modeling process and the biological exploration of key
386 components. **Patterns of Boolean functions.** Beyond this distinction between categories of algorithms, limitations on the formu-
387 lation of the Boolean rules that are considered by the methods also impact which solutions are explored. It can be a fixed limit on
388 the number of regulators (e.g., a maximum of 6 regulators including 4 activators and 2 inhibitors (30, 33)) or a certain pattern of
389 rules (e.g., a component is expressed if all its activators are expressed and none of its inhibitors, i.e., *and* logic gate between activa-
390 tors and *or* between inhibitors (18, 33)). Because of the combinatorial explosion, methods designed to consider all possible Boolean
391 functions can also offer a limit on the size of the Boolean function to enable dealing with dozens of components and complex reg-
392 ulations. This limit can be on the number of regulators or, like the choice we made, on the number of different combinations of reg-
393 ulators (i.e. a limit on the number of clauses that can compose the Boolean functions). This latter choice seems to be biologically
394 consistent: the number of direct regulators for a gene or protein can vary significantly, often being numerous. Whereas the number
395 of functional states, reflected by specific combinations of these regulators, is typically more constrained, ensuring system resilience

396 and controlled responses to a manageable range of conditions.

397 **Different qualitative interpretation of experimental data** Regardless the implemented algorithm, the Boolean network in-
398 ference tools can also be judiciously compared by focusing on the types of data that they can consider for the modeling: bulk or
399 single-cell expression profiles, at cellular steady states or covering cellular evolutions, linear evolutions, or with bifurcations (e.g., lin-
400 eage differentiation), observed with time series limited to two time points or longer, with/out perturbations on components, etc.
401 This is directly linked to the dynamical features they are able to model. **Steady-state data.** Measurements acquired at assumed
402 stable cellular states are commonly interpreted as attractors in the Boolean network dynamics. On this basis, some inference tools
403 such as CellNOptR (81), Griffin (60) and BONITA-RD (68) are specifically designed for inferring Boolean networks that respect con-
404 straints on their attractors (focusing mostly on fixed points), constraints that are derived from a set of steady-states data. **Time se-**
405 **ries data.** A range of tools extends the modeling scope by addressing time series of expression measurements, representing a
406 succession of cellular states ensuing one after the other. Among them is CellNOptR-dt (81) which can fit time course data using
407 a synchronous updating scheme. The modeling of this data is widened to asynchronous semantics with ASKEed (85) and IQCELL
408 (33) that model time series as trajectories in which the succession of measurements constitutes a succession of transitions (reacha-
409 bility in a single dynamic step). Caspo-TS (67), also asynchronous, relaxes the constraint of observing each transition as it considers
410 the reachability property between the observed cellular states. It is worth noting that none of these methods takes into account
411 the modeling of bifurcations/branching in the trajectories. **Single-cell data.** Among the methods specially designed for inferring
412 Boolean networks from single-cell expression data, we can distinguish different interpretations of their dynamical features. SCNS
413 (57), BTR (44) and SgpNet (20) interpret the set of single-cell measurements as a state space reached from an initial state in an
414 asynchronous Boolean network. These tools seek, through optimization, a Boolean network for which the set of states contained
415 in its transition graph (the "model state space") is close to the set of binarized expression data (the "data state space"). However, the
416 strategy presented in (71) is based on another interpretation: the state of each single-cell measurement is assumed to be a po-
417 tential predecessor or successor of the state of any other single-cell measurement coming from the same sample. Consequently,
418 a solution is here a synchronous Boolean network whose dynamics matches a random set of two-time-points time series, namely
419 couples of single-cell measurements. The previously mentioned IQCELL (33) proposes a third interpretation of this data, consider-
420 ing a pseudo-time ordering of the cells seen as a time series and searching for an asynchronous Boolean network whose dynamics
421 includes a sequence of states compatible with the cell ordering. **Complex dynamics.** A tool that can consider a diversity of dynam-
422 ical features can exploit different types of data and address the modeling of varied and complex cellular behaviors. Three methods
423 stand out for the complexity of the behaviors that can be modeled. The tool BRE:IN (25) allows for more nuanced and complex dy-
424 namic behaviors than the previously mentioned tools as it supports complex temporal logic specifications (CTL and LTL), both in
425 synchronous and asynchronous semantics. However, the high computational cost of checking these traces prevents scaling to net-
426 works of biological interactions of dozens of components: the larger networks for benchmarks (25) had 16 nodes with synchronous
427 semantics and 11 with asynchronous. Similarly, Boolean network sketches (4) also performs the inference with the help of model-
428 checking methods, in asynchronous semantics, but with the richer logic HCTL (hybrid extension of the branching-time temporal
429 logic CTL) allowing more expressive specifications. Unlike BRE:IN, the exploration of the Boolean functions is not limited by patterns,
430 and this method was tested in a network with more than 321 nodes.

431 **Binarization of transcriptome data**

432 **Case study 1**

433 From the full scRNA-seq dataset, we employed PROFILE (7) to classify in each cell each gene to either 0, 1, or undetermined, by
434 comparing its expression to the distribution in all cells. Then, each cluster identified using STREAM (see main text) has been bina-
435 rized using a majority rule: a gene is classified following the majority of values the gene has been classified to in the individual cells
436 of the cluster. Among the 4 768 hyper-variable genes selected by STREAM, 1 519 have been classified with different binary values
437 in at least two different clusters, and 1 369 are classified as binary value in all clusters.

438 Case study 2

439 We previously performed experiments for acquiring RNA sequencing data in populations of ST2 cells at different stages of differ-
440 entiation towards adipocytes and osteoblasts (23). Adipocyte differentiation was induced using a medium of isobutylmethylxan-
441 thine, dexamethasone, and insulin for 2 days, followed by a change of medium with rosiglitazone insulin (Sigma-Aldrich, I9278) un-
442 til 9 days. Differentiation towards osteoblasts was induced using bone morphogenetic protein-4 until 15 days. Each experiment
443 has been replicated 3 times. At days 0, 1, 3, 5, 9, 15, a subpopulation of the cells has been sequenced genome-wide. In the scope
444 of this project, we focused on the activity of transcription factors (TFs) in the different stages of the ST2 differentiation. To enable
445 building of qualitative models for the differentiation process, an automated method was applied for transforming the quantitative
446 RNA-seq measurements of TF activities into qualitative assessment of their activity: active (1), inactive (0), or undetermined (inter-
447 mediate). Two different methods of binarization were employed and compared: RefBool (37) for binarization with respect to back-
448 ground RNA-seq data collected in a range of different cell types; and a statistical analysis developed in the scope of the project, for
449 binarization with respect to the collected RNA-seq data. For this statistical analysis, representative background distributions of gene
450 expressions in 63 mouse tissues were generated from ArchS4 (39) data [<https://maayanlab.cloud/archs4/>, Kallisto raw read counts,
451 retrieved 2019]. Independent vertex sampling was performed per tissue to remove correlated samples. Samples were further fil-
452 tered for overall read counts between 10 and 100 Mio and a median ≥ 1 to remove unusual distributions and outliers. This back-
453 ground data was merged with our own data (after Kallisto alignment) and then quantile normalized and converted to TPM (gene
454 length normalization and TPM scaling). The gene-specific background distributions were then applied for discretization of the own
455 data as follows: Gene expressed in own data below median of background was discretized to 0 and above upper quartile to 1. In
456 addition, genes with TPM < 1 are discretized to 0. Furthermore, genes with large expression differences over all samples and time-
457 points were identified via k-means-clustering (2 clusters), with a minimum three-fold-change of centroid locations, at least three
458 data points per cluster, and full-filling a ttest2 (MATLAB©) between the two clusters. 36 genes were found accordingly. The upper
459 cluster was discretized to 1 and the lower cluster to 0.

460 Prior knowledge on gene regulation

461 Case study 1

462 We extracted TF-TF and TF-gene interactions referenced in the DoRothEA database (21) with confidence levels A to C. It resulted
463 in a signed directed graph with 5 186 components and 12 895 regulations. We filtered the components to keep only genes with
464 classified binary values, reducing to 1 001 components and 2 777 regulations.

465 Case study 2

466 We extracted from the METACORE© database (in 2019) all known interactions (Transcription regulation, Influence on expression,
467 and Binding) between TFs (31) and seven known marker genes which show clear expression changes from adipocytes to osteoblasts
468 in our own expression data: Four high in adipocytes (ADIPOQ, FABP4, CEBPA, LPL) and three high in osteoblasts (ALPL, HEY1, SP7).
469 Only measured nodes and their interactions were kept. This resulted in a network of 1,027 nodes (almost only TFs) with 11,000
470 pairwise interactions.

471 Boolean networks

472 Inference of Boolean networks from structural and dynamical properties

473 The strategy behind BoNesis is to describe, in the form of a logical problem to be solved, the search for a Boolean network compat-
474 ible with a network architecture and with given dynamic properties. To do so, BoNesis integrates prior knowledge influence graph
475 and observations within the same logic program, so that any solution of this program is a Boolean network made up of possible in-
476 teractions given the PKN whose dynamic properties are compatible with the behavior of the observations.

477 The logic program is written in Answer-Set Programming. It describes the biological data (the prior influence graph as well as the
478 observations and the dynamical properties linking them) and the modeling formalism (the Boolean network and the computation
479 of its dynamics in Most Permissive Semantics) via predicates and constraints. The constraints are necessary and sufficient condi-

480 tions that guarantee that any solution of the problem is a Boolean network compatible with the biological data, i.e., a Boolean net-
481 work included in the architecture of the prior influence graph and whose dynamic properties are compatible with the behavior of
482 the observations. Solutions are obtained using an answer-set solver, *clasp* (22): the models are the answer-sets satisfying the logic
483 program.

484 **Component selection**

485 Building a prior knowledge influence graph specific to a biological process is a complex and delicate expert task. Yet, determining
486 which interactions are to be considered when building a model is an essential step in the preparation of data before synthesizing
487 models. It is important not to miss components that are essential to the regulation mechanism in order to be able to explain the
488 observations, but also not to consider components that do not play any role and hence penalize the construction and understand-
489 ing of the system. If the interaction graph contains components that are not involved in the regulation of the observed biological
490 behavior, many different functions can be attributed to these components without impacting the compatibility of the Boolean net-
491 work with the observations. These components without importance for the behavior then strongly increase the number of solu-
492 tions without bringing any information. Conversely, if no combination of functions can reproduce the data because key compo-
493 nents and interactions of the process are missing, no model can be found. The complexity of this task currently limits the use of
494 modeling.

495 With BoNesis, we propose a way to assist in the design of a relevant interaction graph with regard to observations. It offers to select,
496 among a large interaction graph (as it can be extracted from a public interaction database such as DoRothEA (34) or Signor (43)),
497 the components that can be included in a model to explain the observations.

498 To this end, we set two optimization criteria. Firstly, we want the solver to search for a compatible boolean network that maximizes
499 the number of components in the models. An optimal solution is then a biggest boolean network, composed of components com-
500 ing from the large original interaction graph, that can reproduce the observations. Yet, we also want the solution to maximize the
501 number of components of a particular type, called *strong constants*. A strong constant is a component to which a constant func-
502 tion is assigned, and whose value remains constant to reproduce the observations within the dynamics of the Boolean network.
503 Thus, within a Boolean network compatible with biological data, a node A is a strong constant if and only if $f(A) = v$ with $v \in \{-1, 1\}$
504 and that within the dynamics of the Boolean network it is possible to reproduce the data with the node A always equal to v (all the
505 configurations x associated with the observations have $x_A = v$). In other words, it is a component having neither activator nor in-
506 hibitor and whose value can remain unchanged without preventing the Boolean network from being compatible with the obser-
507 vations. The particularity of these strong constants is that they can be removed from the domain without impacting compatibility
508 with data behavior. Once the domain of interactions has been reduced to components that can explain the dynamics of the ob-
509 servations and that are not strong constants, we can limit ourselves to the maximum strongly connected component of this graph,
510 which is particularly interesting to focus on the interactions that regulate the observed process.

511 **Model synthesis**

512 **Exhaustive enumeration** BoNesis searches for all the Boolean networks of the same size than the input interaction network and
513 whose dynamics exactly reproduce those of the data (via the defined dynamical constraints). Hence, when BoNesis is used for model
514 synthesis without optimisation criteria, all the Boolean networks output from BoNesis are models of the same relevance with re-
515 spect to the dynamics to be modeled.

516 **Partial enumeration with diversity** For biological applications, it is frequent that an exhaustive enumeration of models is not ju-
517 dicious given that biological observations are rarely constraining enough considering a large size interaction network. Indeed, it is
518 sufficient that a few components have few dynamic information for having an explosion of the number of models.

519 **Specifics for case study 1**

520 We first performed component selection (see previous section) from the 1 001 genes prior knowledge influence graph and the
521 dynamical constraints of Figure 2C. We extracted the largest strongly connected component of the resulting graph, leading to se-

522 lecting 39 genes and 137 regulations. Then, from this 39 genes influence graph, we performed diverse sampling of 1 000 Boolean
523 networks fulfilling the dynamical properties of [Figure 2C](#).

524 **Specifics for case study 2**

525 As described in the main text, the dynamical properties consisted of (1) existence of trajectories, (2) stability properties, and (3) ab-
526 sence of trajectories. Moreover, we add prior knowledge markers of the two differentiated cellular types: **ADIPOQ**, **FABP4**, **CEBPA**, **LPL** for
527 adipocytes, and **ALPL**, **HEY1**, **SP7** for osteoblasts.

528 In a first stage, we performed gene selection on the METACORE(r) prior knowledge influence graph by identifying Boolean net-
529 works that maximize, by decreasing priority, the inclusion of a prior knowledge markers, the number of genes whose dynamics can
530 be explained, and the number of strong constants, that will be removed (see previous section). For this first staged, we ignored con-
531 straints on the absence of trajectory for complexity reasons. We performed this gene selection for each replicate and each binariza-
532 tion method.

533 We then analyzed the binary valued inferred by BoNesis for the selected genes that have not been classified by binarization meth-
534 ods in the stable phenotypes. We identified 19 genes (**ATF2**, **CLOCK**, **CTCF**, **CUX1**, **GATA6**, **NFYB**, **REL**, **RELA**, **SMAD1**, **SMAD2**, **SMAD4**, **STAT3**, **TGIF1**,
535 **TRP53**, **TTF1**, **USF1**, **USF2**, **YY1**, **ZFP148**) that have been inferred to have different binary states, while the data show no clear statistical
536 ground for supporting these different qualitative states. We performed again the gene selection stage with the additional con-
537 straint that the binary value of these 19 genes must be equal in all phenotypes. Depending on the binarization method and repli-
538 cates, different sets of genes have been selected, with, in some cases, multiple optimal solutions. Then, for each optimal solution,
539 we extracted the sub-GRN consisting of the identified non-constant genes, and performed diverse sampling of Boolean networks
540 taking into account the whole dynamical constraints, including on the absence of trajectories.

541 **Boolean network clustering and complexity analysis (Case study 1)**

542 **Complexity of the models per clusters** To explore the three highlighted groups, we study if the complexity of the functions of
543 a model is characteristic of the group to which the model belongs (Supplementary [Figures 4](#) and [5](#)). Specifically, Supplementary
544 [Figure 4](#) shows, for each group, the percentage of the functions of its models according to the number of clauses constituting the
545 functions. Supplementary [Figure 5](#) shows, for each group, the percentage of clauses constituting its model functions according to
546 the number of components in the clauses. We observe that red models are significantly less complex than those of the other two
547 groups, with functions composed of a smaller number of clauses, themselves composed of a smaller number of components com-
548 pared to the orange and green group models.

549 **Complexity of the invariable functions of the models per clusters** Among the functions constituting the models, some are
550 invariable between models of the same group beyond the 12 invariable functions which are common to all groups. We therefore
551 have three sets of invariable functions that seem to indicate three key patterns of interactions to reproduce the data dynamics. Sup-
552 plementary [Figure 6](#) shows, for each group, the percentage of its invariable functions according to their number of clauses. Again, a
553 clear difference in complexity between the red group and the others is highlighted. All the functions shared by the red models have
554 a single clause, whereas it represents for green and orange models respectively less than half and one third of the functions (some
555 of their invariable functions having even respectively up to 7 and 8 clauses). Hence, the red group models appear to be more parsimo-
556 nious explanations of the regulation mechanism of hematopoiesis than the two other groups.

557 **Computational prediction of reprogramming determinants (case study 2)**

558 The prediction of the combinations of perturbations for the case study 2 has been performed via the software CABEAN ([77](#)). CABEAN
559 implements several methods for the source-target control of asynchronous Boolean networks, and these methods can be used
560 to identify the minimal and exact control sets of perturbations that ensure the inevitable reachability of the target attractor from
561 a given source attractor. Based on the application time of the perturbations, CABEAN supports several types of controls: instantane-
562 ous control applies perturbations instantaneously; temporary control applies perturbations for sufficient time and then releases
563 them to retrieve the original dynamics; and permanent control applies the control for all the following time steps. All these control

564 methods (80) are based on the computation of strong and weak basins of attractors, which explores both the structure and dynam-
565 ical properties of asynchronous Boolean networks. More specifically, an instantaneous control drives the network dynamics from
566 the source attractor to a state in the strong basin of the target attractor, from which there only exist paths to the target attractor.
567 On the other hand, temporary control and permanent control can make use of the spontaneous evolutions of the network dynam-
568 ics by moving into first the weak basin of the target attractor, from which there exist paths to the target attractor. To ensure the in-
569 evitable reachability of the target attractor, a temporary control should drive the network dynamics to a state in the strong basin of
570 the target attractor at the end of control, while a permanent control stirs the network from the source state to a state in the strong
571 basin of the target attractor in the resulting transition system under control. More recently, CABEAN (78) has been extended with
572 target control methods for asynchronous Boolean networks (79), which can be used to identify perturbations that can drive the dy-
573 namics of a Boolean network from any initial state to the desired target attractor.

574 In this way, CABEAN fits well with the objective for case study 2, i.e., enforcing the convergence to **OD15** state from **AD15** state. CABEAN
575 was applied to each inferred Boolean networks from BoNesis to compute combinations of temporary perturbations (gene knock-
576 out), and it identified combinations of up to 5 simultaneous perturbations leading to a reprogramming from **AD15** to **OD15**. We kept
577 combinations of perturbations that have been identified in at least 10% of the individual models given to CABEAN. In the end, we
578 have obtained 34 different combinations of 2 to 4 simultaneous perturbations, which are given as input for the next analysis steps.

579 **Stochastic simulations of ensembles of Boolean networks (case study 2)**

580 To perform simulations on our ensembles of models, we developed a new version of MaBoSS, the stochastic Boolean simulator(76),
581 called Ensemble-MaBoSS(11). MaBoSS usually takes a single Boolean model and performs a large number of simulations to obtain
582 many stochastic trajectories. It later uses this set of trajectories to compute time-dependent probabilities for every visited Boolean
583 state. During these simulations, it also stores every fixed point observed and produces the set of fixed points observed in all these
584 simulations. To perform the simulation of an ensemble of models, we need to compute an equal set of trajectories for every model
585 of the ensemble. We can then use the consolidated set of trajectories from all the models, and compute the time-dependent prob-
586 abilities specific to the whole ensemble of models. We can also produce the same list To analyze the composition of the ensemble,
587 we also compute the time-dependent probabilities for each model. This way, we get individual results, that can then be used for en-
588 semble analysis such as model clustering.

589 **Wetlab experiments (case study 2)**

590 **Cell culture**

591 The mouse bone marrow-derived stroma cell line ST2 was established from Whitlock-Witte type long-term bone marrow culture
592 of BC8 mice (65). The ST2 cells were cultured in growth medium: Roswell Park Memorial Institute (RPMI) 1640 medium (Gibco,
593 32404-014) supplemented with 10% fetal bovine serum (FBS) (Gibco, 10270-106) and 1% L-Glutamine (Lonza, BE17-605E) at
594 37°C, 5% CO₂. All experiments were carried out with cells less than 10 passages. For differentiation of adipocytes, ST2 cells were
595 seeded 2 days before differentiation. The cells reached 100% confluency after 24 hours of culture, and were further maintained
596 in growth medium for 24 hours (Day 0). Adipogenic differentiation was initiated on Day 0 by culturing in adipogenic differentia-
597 tion medium I consisting of growth medium, 0.5 mM isobutylmethylxanthine (IBMX) (Sigma-Aldrich, I5879), 0.25 μ M dexametha-
598 sone (DEXA) (Sigma-Aldrich, D4902) and 5 μ g/mL insulin (Sigma-Aldrich, I9278). From Day 2, the cells were cultured in adipogenic
599 differentiation medium II consisting of growth medium, 500 nM rosiglitazone (RGZ) (Sigma-Aldrich, R2408) and 5 μ g/mL insulin
600 (Sigma-Aldrich, I9278). The adipogenic differentiation medium II was replaced every second day.
601 For differentiation of osteoblasts, ST2 cells were seeded 2 days before differentiation, and reached 100% confluency after 24 hours
602 of culture, and were further maintained in growth medium for 24 hours (Day 0). Osteogenic differentiation was initiated on Day 0
603 by culturing in osteogenic differentiation medium consisting of growth medium and 100 ng/mL bone morphogenetic protein-4
604 (BMP4) (PeproTech, 315-27). The osteogenic differentiation medium was replaced every second day.

605 RNA extraction and cDNA synthesis

606 Total RNA was extracted from cells using Quick-RNATM MiniPrep (Zymo Research, R1055). RNA concentration was measured by
607 Nanodrop 2000c (Thermo Fisher Scientific, E597). cDNA was synthesized with the following cocktail: 1 μg total RNA, 0.5 mM dNTPs
608 (Thermo Fisher Scientific, R0181), 2.5 μM oligo dT-primer (Eurofins GmbH), 1 U/ μL Ribolock RNase inhibitor (Thermo Fisher Scien-
609 tific, E00381), and 1 U/ μL RevertAid Reverse transcriptase (Thermo Fisher Scientific, EP0352). The cocktail was maintained at 42°C
610 for 1 hour, following 70°C for 10 min to stop the reaction.

611 Reverse transcription real-time quantitative PCR (RT-qPCR)

612 RT-qPCR was performed to measure the RNA expression using the Applied Biosystems 7500 Fast Real-Time PCR System. Each re-
613 action contained the following cocktail: 5 μL of cDNA, 5 μL of primer mix (forward and reverse primers, both in 2 μM concentration),
614 and 10 μL of Absolute Blue qPCR SYBR Green Low ROX Mix (Thermo Fisher Scientific, AB4322B). The PCR reaction were the fol-
615 lowing: 95°C for 15 min and repeating 40 cycles of 95°C for 15 s, 55°C for 15 s, following 72°C for 30 s. The gene expression level
616 was calculated using the 2 $^{-\Delta\Delta\text{Ct}}$ method (48). The $\Delta\Delta\text{Ct}$ refers to ($\Delta\text{Ct}(\text{target gene}) - \Delta\text{Ct}(\text{housekeeping gene})$) from the treat-
617 ment - ($\Delta\text{Ct}(\text{target gene}) - \Delta\text{Ct}(\text{housekeeping gene})$) from the control. Rpl13a was used as the housekeeping gene and the primer
618 sequences can be found in the Supplementary Table 2.

619 Viral transduction

620 Day 9 adipocytes were transduced with lentivirus (Sirion Biotech GmbH; details can be found in the Supplementary Table 2). For a
621 well of 48-well plate, the estimated cell number was 200 000. The amount of lentivirus was calculated to achieve expected mul-
622 tiplicity of infection (MOI). The transduction cocktail was as followed: lentivirus stock diluted with RPKM and 8 $\mu\text{g}/\text{mL}$ polybrene
623 transfection reagent (EMD Millipore, TR-1003-G) to achieve a final volume of 100 μL . The culture medium was removed, and the
624 cells were washed once with 1xDPBS (Gibco, 14190-094), then supplemented with the transduction cocktail. The cells were kept
625 in the transduction cocktail for 6 hours. After the 6-hour transduction, the transduction cocktail was removed, and the cells were
626 supplemented with the growth medium. The transduction efficiency was controlled by observed GFP and RFP levels by microscopy.

627 RNA interference

628 On Day 1 post-transduction (Day 1 PT), the cells were transfected with siRNA according to manufacturer's recommendation (Hori-
629 zon Discovery; details can be found in the Supplementary Table 2). In brief, siRNA was diluted to 5 μM solution in DNase/RNase free
630 water (Invitrogen, 10977-035). In separate tubes, siRNA and DharmaFECT 1 transfection reagent (Horizon Discovery, T-2001-03)
631 were diluted with RPMI. To prepare the transfection cocktail for 1 well of 48-well plate, in Tube 1, 25 μL of the siRNA in serum-free
632 medium was prepared by adding 1.25 μL of 5 μM siRNA to 23.75 μL of RPMI. In Tube 2, 1 μL of the DharmaFECT 1 in serum-free
633 medium was prepared by adding 1 μL of DharmaFECT 1 to 24 μL of RPMI. The mixture was incubated for 5 min, then two tubes
634 were mixed following incubation of 20 min. After incubation, 200 μL of growth medium was added to achieve a final volume of
635 250 μL . The culture medium was removed, and the cells were supplemented with the transfection medium for 24 hours. For sin-
636 gle and double siRNA transfection, the final concentration was maintained at 100 nM.

637 Single cell RNA-seq

638 The single cell RNA-seq (scRNA-seq) was performed according to Chromium Next GEM Single Cell 3' Reagent Kits v3.1 Review D
639 (CG000204). The ChromiumTM Next GEM Single Cell 3' GEM, Library Gel Bead Kit 3.1 (1000128) consisted the following: Chromium
640 Next GEM Single Cell 3' Gel Bead Kit v3.1, 4 (1000129), Chromium Next GEM Single Cell 3' GEM Kit v3.1 (1000130), Chromium
641 Next GEM Single Cell 3' Library Kit v3.1 (1000158), Single Index Kit T SetA (1000213), DynabeadsTM MyOneTM SILANE (2000048).
642 To achieve single cell suspension, the cells were first treated with 1 mg/mL Collagenase A (Roche, 10103586001) for 15 min. After
643 Collagenase A treatment, the cells were trypsinised with 150 μL Trypsin (Lonza, BE17-161E) for 5 min. The trypsin was quenched
644 with 500 μL growth medium. Wide-bore tips (Thermo Scientific ART 1000G Self-Sealing Barrier Pipet Tips, 2079G) were used to
645 pipet up and down. The suspension was centrifuged for 1000 rpm for 5 min, and the supernatant was removed. Single cell suspen-
646 sion was achieved by adding 400 μL growth medium and pipetted up and down with wide-bore tips. The cell number was deter-
647 mined with C-CHIP (NanoEntek, DHC-N01). The suspension was centrifuged again for 1000 rpm for 5 min, and the supernatant

648 was removed and replaced with 1xDPBS + 0.04% BSA to achieve the target cell number. The suspension was filtered through 40
649 μ M Flowmi Cell strainer (Merck, BAH136800040-50EA). The cell number was determined again with C-CHIP, which would be used
650 to determine the input for scRNA-seq. In brief, cells was loaded with a targeted recovery rate of 10 000 cells per sample. scRNA-seq
651 library quality were assessed by Agilent DNA High sensitivity Bioanalyzer chip (Agilent, 5067-4626) and further sequenced on a
652 150 cycles High Output Kit using Illumina NextSeqTM 500 with targeted sequencing depth of 20 000 read pairs per cell.

653 Data and code availability

654 The software BoNESIS, employed for Boolean network inference, is available at <https://bnediction.github.io/bonesis> under the
655 GPLv3-compatible free software license CeCILL. The software CABEAN, employed for reprogramming prediction, is available at <https://satoss.uni.lu/software/CABEAN>. Both tool are available through the CoLoMoTo Docker distribution (61) at <https://colomoto.github.io/colomoto-docker/> with persistently archived Docker images. *Case study 1*: data and scripts are available at https://github.com/StephanieChevalier/notebooks_for_bonesis/tree/main/applications/hematopoiesis. *Case study 2*: Matlab code for statistical analy-
657 sis and binarization of bulk RNA-seq data is available at <https://github.com/sysbiolux/algorecell>; data and scripts for Boolean net-
658 work inference and control predictions are available at <https://sdrive.cnrs.fr/s/nrB72LpBspeKHEp>.

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AUTHOR COMPETING INTERESTS

The authors declare no competing interests.

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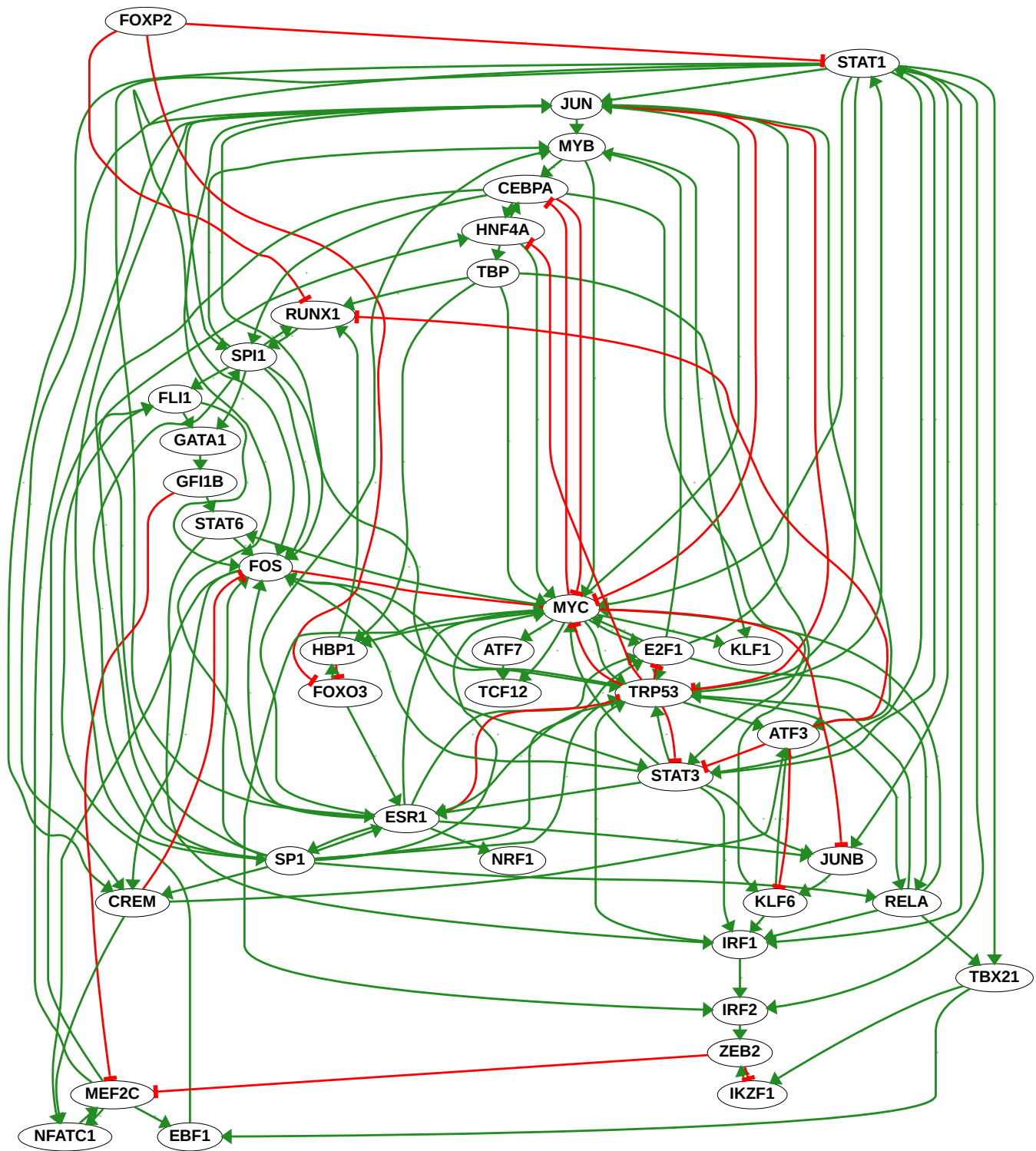
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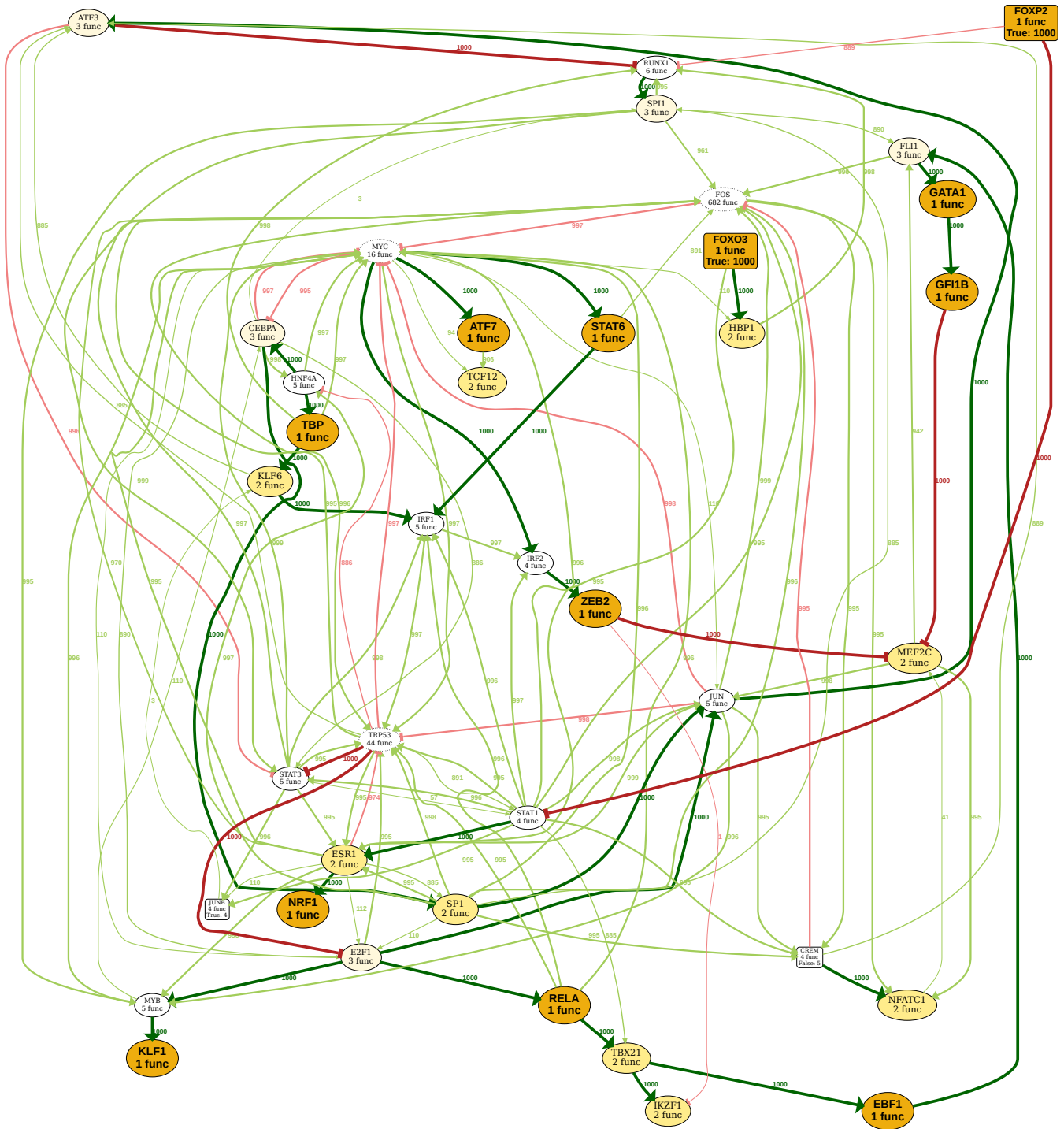
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SUPPLEMENTARY FILES

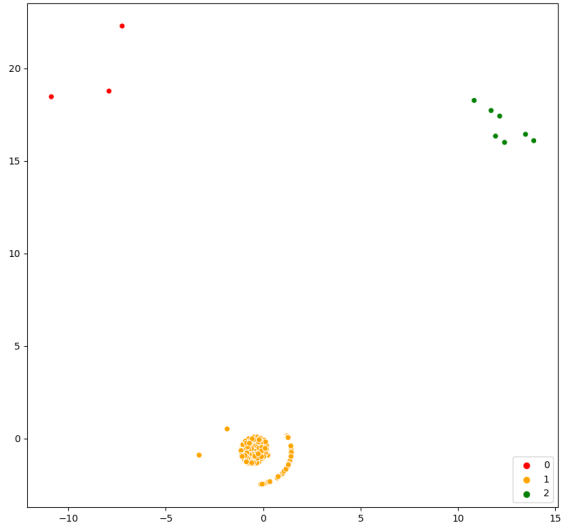
- Supplementary Table 1 (**SupplTable1.xlsx**). Differential gene expression analysis from scRNA-seq RE1 experiment.
- Supplementary Table 2 (**SupplTable2.xlsx**). Primer sequences, siRNAs, and viral constructs used in the study. The primers, siRNAs, and lentiviral constructs are detailed in the indicated work sheets, respectively.



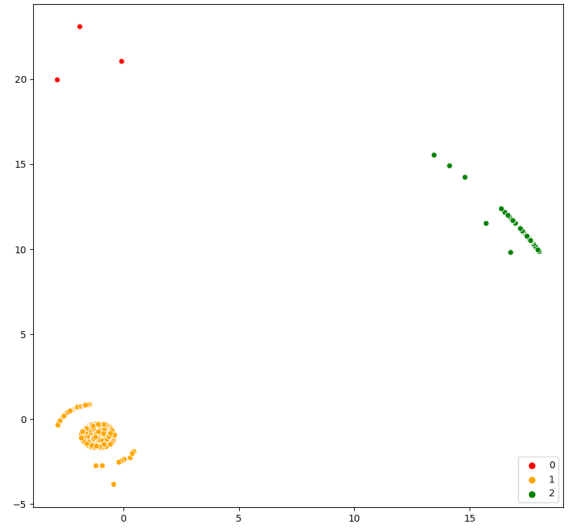
Supplementary Figure 1: Network of 39 components and 137 arcs obtained by component selection using BoNesis.



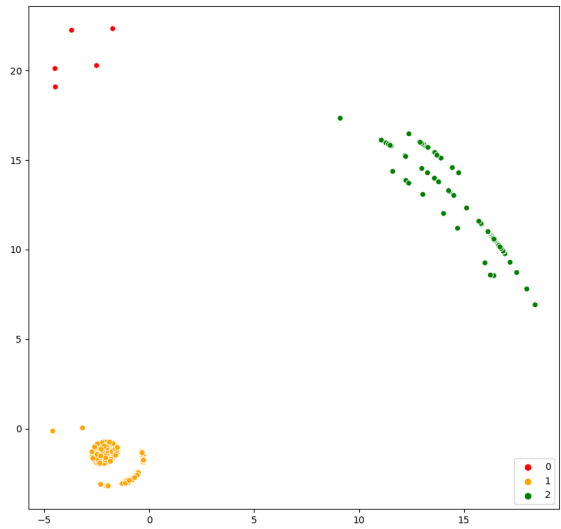
Supplementary Figure 2: Variability analysis Boolean functions in sampled ensemble of models. The label of each node includes the number of different local Boolean functions in the sampled Boolean network set. Orange indicates a unique shared function, yellow only 2 different functions. Edge edge is labeled with the number of Boolean networks that utilizes the influence (over the 1000 sampled), with its thickness scaled accordingly.



A MDS with 250 models.

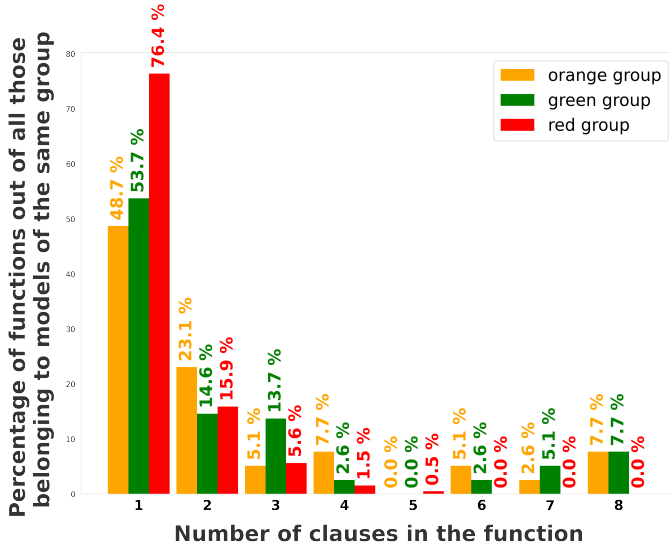


B MDS with 500 models.

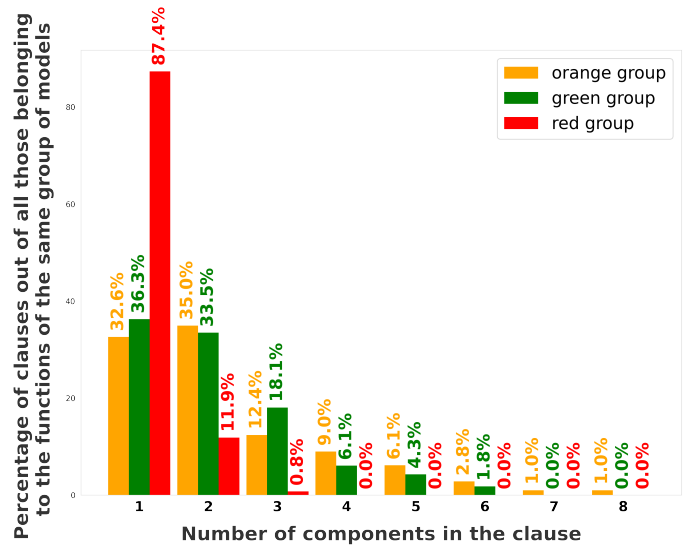


C MDS with 1000 models.

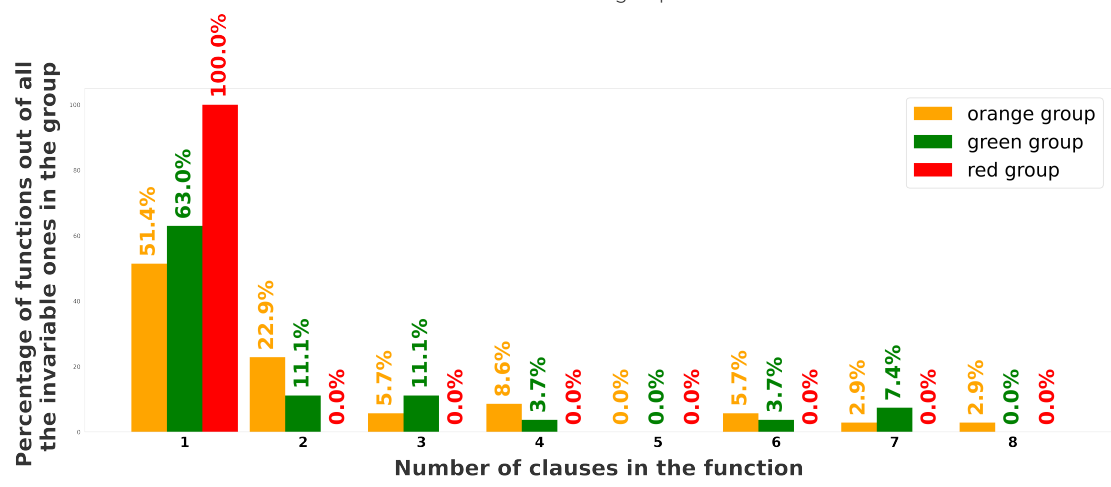
Supplementary Figure 3: MDS highlights 3 groups of models.



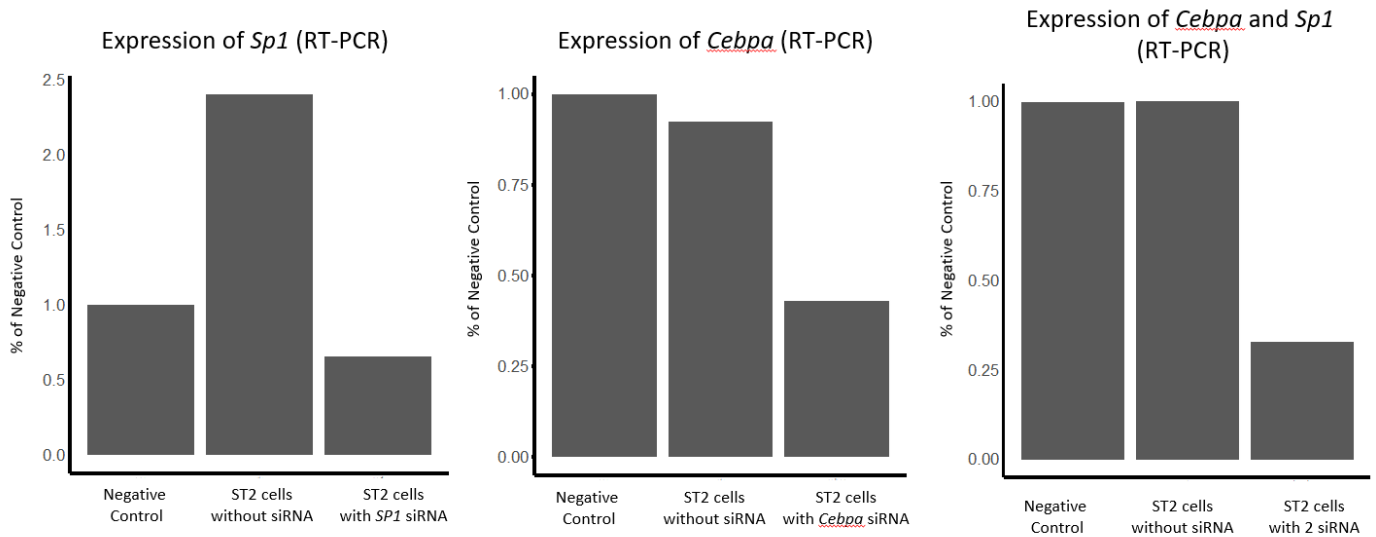
Supplementary Figure 4: Distribution of the functions of the models according to the number of clauses they are made up, by group.



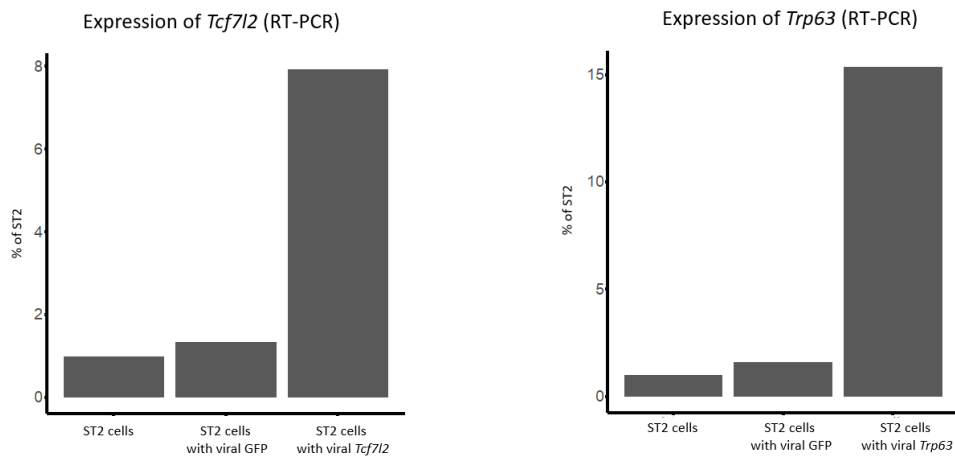
Supplementary Figure 5: Distribution of the clauses of the models according to the number of components they are made up, by group.



Supplementary Figure 6: Distribution of the invariable functions of the models according to the number of clauses they are made up, by group.



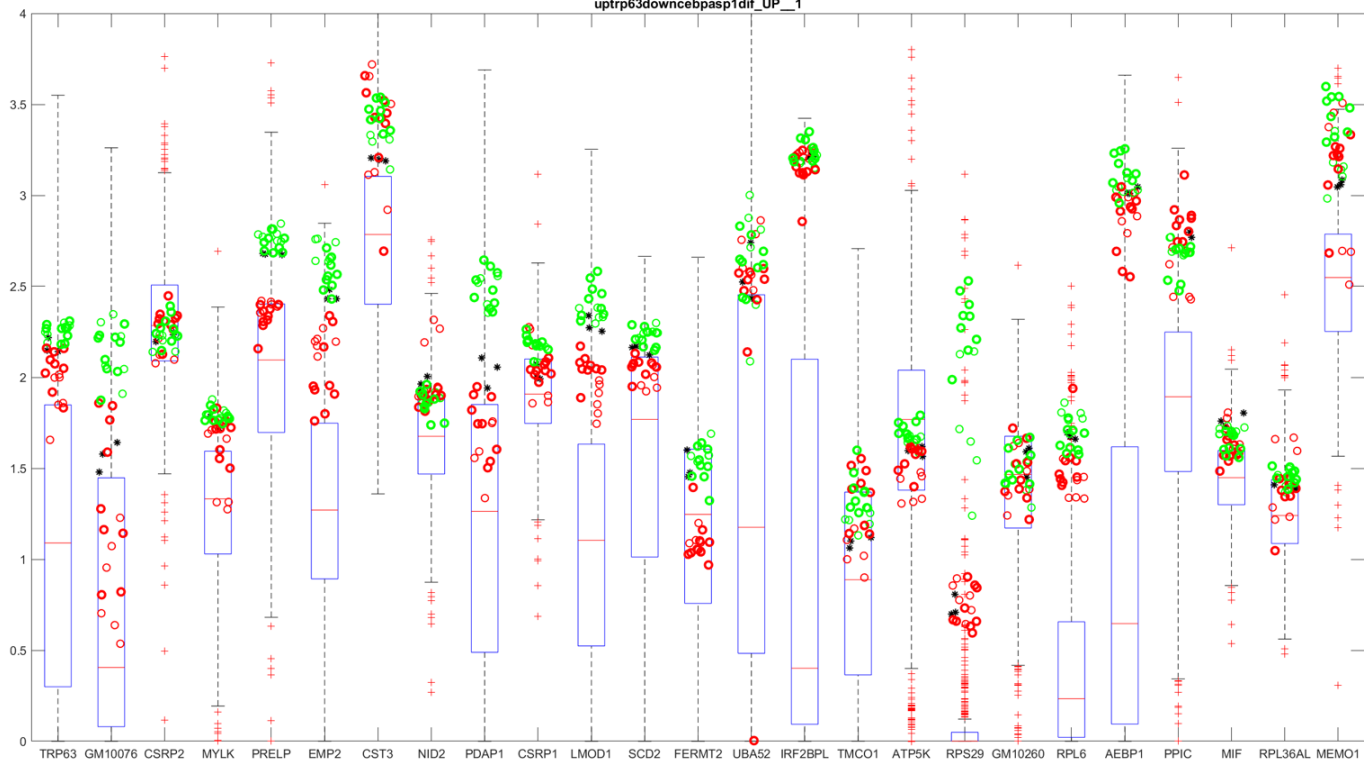
siRNA knockdown efficiency in ST2 (n=1): *Sp1*: 35%; *Cebpa*: 67%, *Cebpa* + *Sp1*: 68%



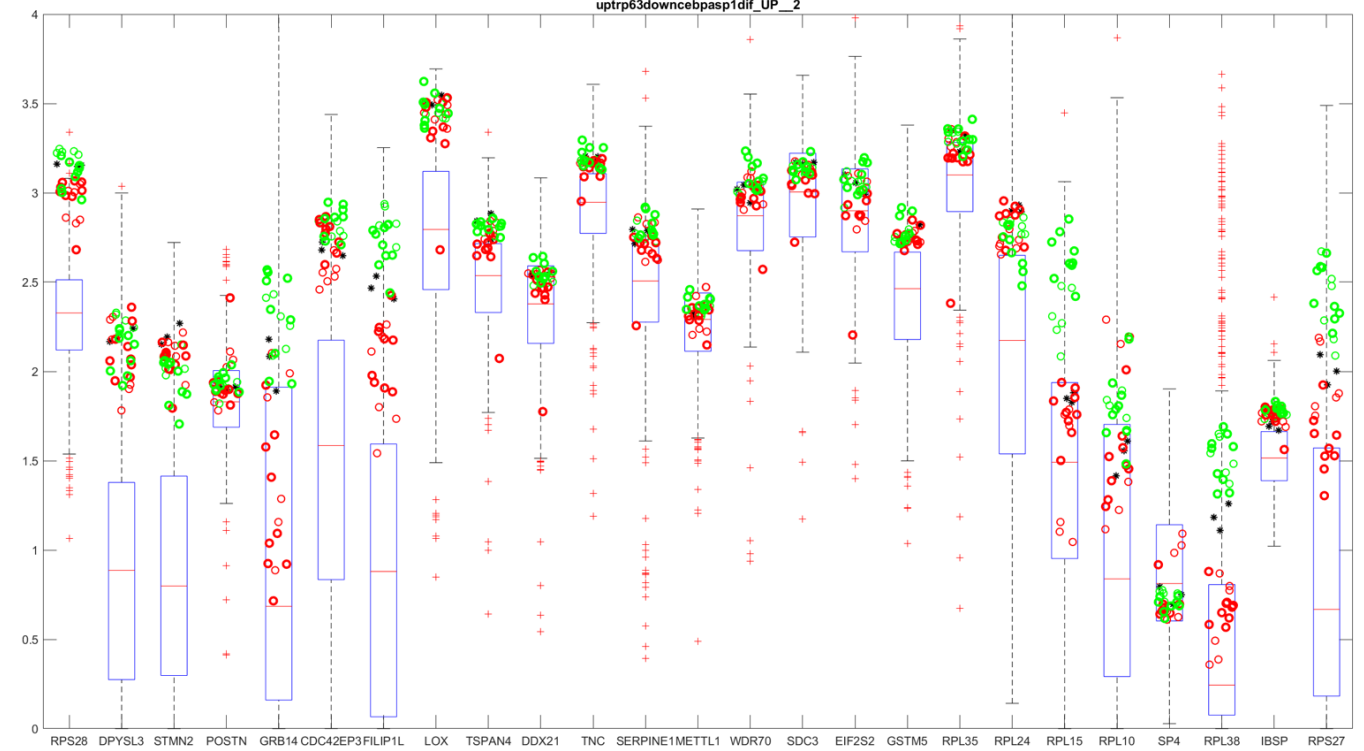
viral knock-in increase expression in ST2 (n=1): *Tcf7l2*: 8 fold; *Trp63*: 15 fold comparing to ST2

Supplementary Figure 7: Experimental validation of case study 2: siRNA and viral transfection test

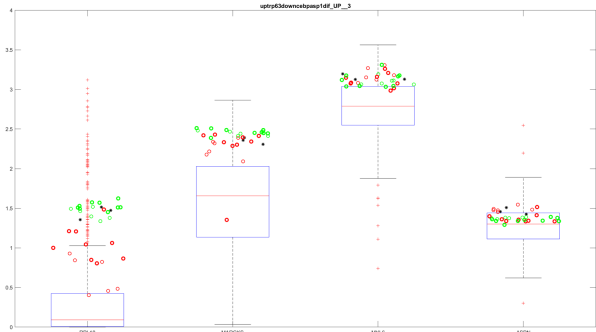
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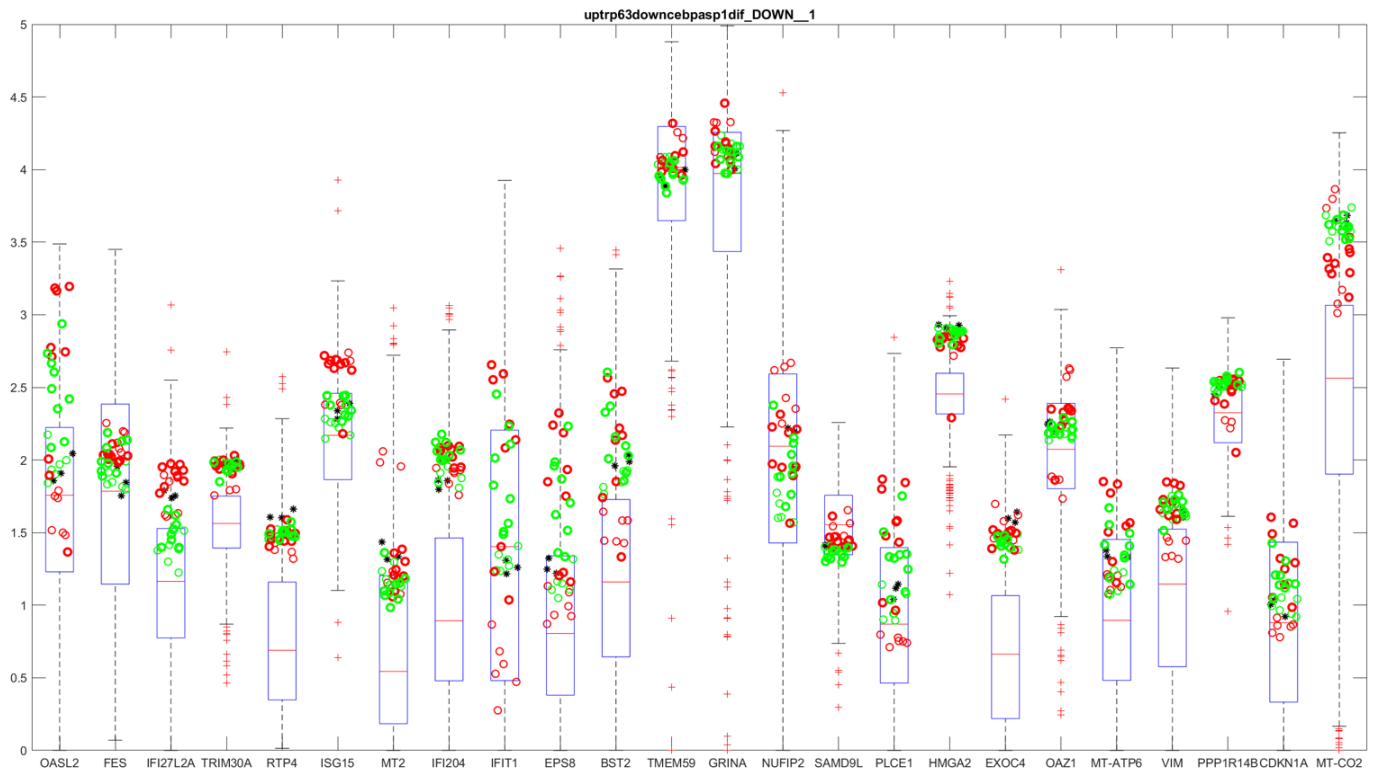


uptrp63downcebpasp1dif_UP_2



uptrp63downcebpasp1dif_UP_3





Supplementary Figure 9: Differentially expressed downregulated genes in the RE1 single cell experiment are partially also downregulated in the bulk RNA-seq data of osteoblasts vs adipocytes. Bulk RNA-seq data (TPM) of osteoblasts (green) and adipocytes (red) are plotted for downregulated sc DEGs (x-axis). Boxplots indicate the gene specific backgrounds from 63 mouse tissues (see methods).