# An Effective Model of the Retinoic Acid Induced HL-60 Differentiation Program

Ryan Tasseff, Holly A. Jensen, Wei Dai, Katherine Rogers, Adithya Sagar, Rodica P. Bunaciu[†], Johanna Congleton[†], Andrew Yen[†], and Jeffrey D. Varner[*]

Robert Frederick Smith School of Chemical and Biomolecular Engineering and [†]Department of Biomedical Sciences, Cornell University, Ithaca NY 14853

**Running Title:** Effective modeling of HL-60 differentiation

**To be submitted:** *Frontiers in Systems Biology*

[*]Corresponding author:

Jeffrey D. Varner,

Professor, Robert Frederick Smith School of Chemical and Biomolecular Engineering,

244 Olin Hall, Cornell University, Ithaca NY, 14853

Email: jdv27@cornell.edu

Phone: (607) 255 - 4258

Fax: (607) 255 - 9166

## Abstract

In this study, we present an effective model All-Trans Retinoic Acid (ATRA)-induced differentiation of HL-60 cells. The model describes a key architectural feature of ATRA-induced differentiation, positive feedback between an ATRA-inducible signalsome complex involving many proteins including Vav1, a guanine nucleotide exchange factor, and the activation of the mitogen activated protein kinase (MAPK) cascade. The model, which was developed by integrating logical rules with kinetic modeling, was significantly smaller than previous models. However, despite its simplicity, it captured key features of ATRA induced differentiation of HL-60 cells. We identified an ensemble of effective model parameters using measurements taken from ATRA-induced HL-60 cells. Using these parameters, model analysis predicted that MAPK activation was bistable as a function of ATRA exposure. Conformational experiments supported ATRA-induced bistability. These findings, combined with other literature evidence, suggest that positive feedback is central to a diversity of cell fate programs.

# Introduction

Understanding the architecture of differentiation programs is an important therapeutic challenge. Differentiation induction chemotherapy (DIC), using agents such as the vitamin A derivative all-trans retinoic acid (ATRA), is a promising approach for the treatment of many cancers (1–3). For example, ATRA treatment induces remission in 80–90% of promyelocytic leukemia (APL) PML-RAR$\alpha$-positive patients (4), thereby transforming a fatal diagnosis into a manageable disease. However, remission is sometimes not durable and relapsed cases exhibit emergent ATRA resistance (5, 6). To understand the basis of this resistance, we must first understand the ATRA-induced differentiation program. Toward this challenge, lessons learned in model systems, such as the lineage-uncommitted human myeloblastic cell line HL-60, could inform our analysis of the more complex differentiation programs occurring in patients. Patient derived HL-60 leukemia cells have been a durable experimental model since the 1970's to study differentiation (7). HL-60 undergoes cell cycle arrest and either myeloid or monocytic differentiation following stimulation; ATRA induces G1/G0-arrest and myeloid differentiation in HL-60 cells, while 1,25-dihydroxy vitamin D3 (D3) induces arrest and monocytic differentiation. Commitment to cell cycle arrest and differentiation requires approximately 48 hr of treatment, during which HL-60 cells undergo two division cycles.

Sustained mitogen-activated protein kinase (MAPK) activation is a defining feature of ATRA-induced HL-60 differentiation. ATRA drives sustained MEK-dependent activation of the Raf/MEK/ERK pathway, leading to arrest and differentiation (8). MEK inhibition results in the loss of ERK and Raf phosphorylation, and the failure to arrest and differentiate (9). ATRA (and its metabolites) are ligands for the hormone activated nuclear transcription factors retinoic acid receptor (RAR) and retinoid X receptor (RXR) (10). RAR/RXR activation is necessary for ATRA-induced Raf phosphorylation (9), and the formation of an ATRA-inducible signalsome complex at the membrane which drives MAPK activation

through a yet to be identified kinase activity. While the makeup of the signalsome complex is not yet known, we do know that it is composed of Src family kinases Fgr and Lyn, PI3K, c-Cbl, Slp76, and KSR, as well as IRF-1 transcription factors (11–15). Signalsome formation and activity is driven by ATRA-induced expression of CD38 and the putative heterotrimeric Gq protein-coupled receptor BLR1 (16, 17). BLR1, identified as an early ATRA (or D3)-inducible gene using differential display (18), is necessary for MAPK activation and differentiation (17), and is also involved with signalsome activity. Studies of the BLR1 promoter identified a 5' 17bp GT box approximately 1 kb upstream of the transcriptional start that conferred ATRA responsiveness (17). Members of the BLR1 transcriptional activator complex, e.g. NFATc3 and CREB, are phosphorylated by ERK, JNK or p38 MAPK family members suggesting positive feedback between the signalsome and MAPK activation (19). BLR1 overexpression enhanced Raf phosphorylation and accelerated terminal differentiation, while Raf inhibition reduced BLR1 expression and differentiation (20). BLR1 knock-out cells failed to activate Raf or differentiate in the presence of ATRA (20). Interestingly, both the knockdown or inhibition of Raf, also reduced BLR1 expression and functional differentiation (20). Thus, the expression of signalsome components e.g., BLR1 was Raf dependent, while Raf activation depended upon the signalsome. A recent computational study of ATRA-induced differentiation in HL-60 cells suggested that the BLR1-MAPK positive feedback circuit was sufficient to explain ATRA-induced sustained MAPK activation, and the expression of a limited number of functional differentiation markers (21). Model analysis also suggested that Raf was the most distinct of the MAPK proteins. However, this previous study developed and analyzed a complex model, thus leaving open the critical question of what is the minimal positive feedback circuit required to drive ATRA-induced differentiation.

In this study, we explored this question using a minimal mathematical model of the key architectural feature of ATRA induced differentiation of HL-60 cells, namely positive

feedback between an ATRA-inducible signalsome complex and MAPK activation. The ATRA responsive signalsome-MAPK circuit was then used to drive a downstream gene expression program which encoded for the expression of functional differentiation markers. The effective model used a novel framework which integrated logical rules with kinetic modeling to describe gene expression and protein regulation, while largely relying upon biophysical parameters from the literature. This formulation significantly reduced the size and complexity of the model compared to the previous study of Tasseff et al., while increasing the breadth of the biology described (21). The effective model, despite its simplicity, captured key features of ATRA induced differentiation of HL-60 cells. Model analysis predicted the bistability of MAPK activation as a function of ATRA exposure; conformational experiments supported ATRA-induced bistability. Model simulations were also consistent with measurements of the influence of MAPK inhibitors, and the failure of BLR1 knockout cells to differentiate when exposed to ATRA. Lastly, we showed by through immunoprecipitation studies, that the guanine nucleotide exchange factor Vav1 is potentially a new ATRA-inducible member of the signalsome complex. Taken together, these findings when combined with other literature evidence, suggested that positive feedback architectures are central to differentiation programs generally, and necessary for ATRA-induced differentiation.

## Results

We constructed an effective model of ATRA-induced HL-60 differentiation which described signaling and gene expression events following the addition of ATRA (Fig. 1). The model connectivity was developed from literature and the studies presented here (Table 1). We decomposed the ATRA program into three modules; a signal initiation module that sensed and transformed the ATRA signal into activated cRaf-pS621 and the ATRA-RXR/RAR (Trigger) signals (Fig. 1A); a signal integration module that controlled the expression of upstream transcription factors given cRaf-pS621 and activated Trigger signals (Fig. 1B); and a phenotype module which encoded the expression of functional differentiation markers from the ATRA-inducible transcription factors (Fig. 1C). Each component of these modules was described by a mRNA and protein balance equation. Additionally, the signal initiation module also described the abundance of activated species e.g., Trigger and cRaf-pS621 whose values were derived from unactivated Trigger and cRaf protein levels. Lastly, because the population of HL-60 cells was dividing (at least before ATRA-induced cell cycle arrest), we also considered a dilution term in all balance equations. The signal initiation module contained nine differential equations, while the signal integration and phenotype modules were collectively encoded by 54 differential equations. Model parameters were taken literature (Table 2), or estimated from experimental data using heuristic optimization (see materials and methods).

The signal initiation module recapitulated sustained signalsome and MAPK activation following exposure to 1$\mu$M ATRA (Fig. 2A-B). An ensemble of effective model parameters was estimated by minimizing the difference between simulations and time-series measurements of BLR1 mRNA and cRaf-pS621 following the addition of 1$\mu$M ATRA. We focused on the S621 phosphorylation site of cRaf since enhanced phosphorylation at this site is a defining characteristic of sustained MAPK activation in HL-60. The effective model captured both ATRA-induced BLR1 expression (Fig. 2A) and sustained phospho-

rylation of cRaf-pS621 (Fig. 2B) in a growing population of HL-60 cells. Together, the reinforcing positive feedback between the signalsome and MAPK led to sustained activation over multiple cellular generations. However, the effective model failed to capture the decline of BLR1 message after 48 hr of ATRA exposure. This suggested that we captured the logic leading to the onset of differentiation, but failed to describe program shutdown. Next, we tested the response of the signal initiation module to different ATRA dosages.

The signal initiation model was bistable with respect to ATRA induction (Fig. 2C-D). Phaseplane analysis predicted two stable steady-states when ATRA was present below a critical threshold, and only a single steady-state above the threshold (Fig. 2C). In the lower stable state, neither the signalsome nor cRaf-pS621 were present (thus, the differentiation program was deactivated). However, at the high stable state, both the signalsome and cRaf-pS621 were present, allowing for sustained activation and differentiation. Interestingly, when ATRA was above a critical threshold, only the activated state was accessible (Fig. 2D). To test these findings, we first identified the ATRA threshold. We exposed HL-60 cells to different ATRA concentrations for 72 hr (Fig. 2E). Morphological changes associated with differentiation were visible for ATRA $\geq$ 0.25 $\mu$M, suggesting the critical ATRA threshold was near this concentration. Next, we conducted ATRA washout experiments to determine if activated cells remained activated in the absence of ATRA. HL-60 cells locked into an activated state remained activated following ATRA withdraw (Fig. 3). This sustained activation resulted from reinforcing feedback between the signalsome and the MAPK pathway. Thus, following activation, if we inhibited or removed elements from the signal initiation module we expected the signalsome and MAPK signals to decay. We simulated ATRA induced activation in the presence of kinase inhibitors, and without key circuit elements. Consistent with experimental results using multiple MAPK inhibitors, ATRA activation in the presence of MAPK inhibitors lowered the steady-state value of signalsome (Fig. 3A). In the presence of BLR1, the signalsome and cRaf-pS621

signals were maintained following ATRA withdraw (Fig. 3B, gray). On the other hand, BLR1 deletion removed the ability of the circuit to maintain a sustained MAPK response following the withdraw of ATRA (Fig. 3B, blue). Lastly, washout experiments in which cells were exposed to 1$\mu$M ATRA for 24 hr, and then transferred to fresh media without ATRA, confirmed the persistence of the self sustaining activated state for up to 144 hr (Fig. 3C). Thus, these experiments confirmed that reinforcing positive feedback likely drives the ATRA-induced differentiation program. Next, we analyzed the ATRA-induced downstream gene expression program following signalsome and cRaf activation.

The signal integration and phenotype modules described ATRA-induced gene expression in wild-type HL-60 cells (Fig. 4). The signal initiation module produced two outputs, activated Trigger and cRaf-pS621 which drove the expression of ATRA-induced transcription factors, which then in turn activated the phenotypic program. In particular, Trigger, which is a surrogate for the RAR$\alpha$/RXR transcriptional complex, regulated the expression of the transcription factors CCATT/enhancer binding protein $\alpha$ (C/EBP$\alpha$), PU.1, and EGR1. In turn, these transcription factors, in combination with cRaf-pS621, regulated the expression of downstream phenotypic markers such as CD38, CD11b or P47Phox. We assembled the connectivity of the signal integration and phenotypic programs driven by Trigger and cRaf-pS621 from literature (Table 1). We estimated the parameters which appeared in the control laws regulating these programs from steady-state and dynamic measurements of transcription factor and phenotypic marker expression following the addition of ATRA (22–25). However, the bulk of the remaining model parameters were taken from directly from literature (26) and were not estimated in this study (see materials and methods). The model simulations captured the time dependent expression of CD38 and CD11b following the addition ATRA (Fig. 4A), and the steady-state for signal integration and phenotypic markers (Fig. 4B). Lastly, we used the *predicted* values of the p21 and E2F protein abundance to estimate a blackbox model of ATRA-induced G0 arrest (Fig.

5). The phenotype module predicted p21 expression significantly increased and E2F expression decreased, in response to ATRA exposure (Fig. 5A). We then used the ratio of these values in a polynomial model to calculate the fraction of HL-60 cells in G0 arrest following the addition of ATRA (Fig. 5B). The third-order polynomial model captured the trend in measured G0-arrest values as a function of time, and wash robust to uncertainty in the measured data (Fig. 5B, gray). Taken together, the output of the signal integration and phenotypic modules was consistent with time-series and steady-state measurements, thereby validating the assumed molecular connectivity. Moreover, outputs from the phenotype module described the trend in ATRA-induced G0 cell cycle arrest. Next, we explored which nodes and interactions between nodes in the signal integration module most influenced the system response.

The PU.1 and AP1 proteins were important regulators of ATRA-induced signal integration and phenotypic change (Fig. 6). We conducted pairwise knock simulations of genes in the signal integration and phenotype modules to estimate which nodes controlled the processing of the Trigger and cRaF-S621 signals. The difference between the system state with and without the gene knockouts (encoded as a state displacement matrix) was decomposed using Singular Value Decomposition (SVD). All simulations were conducted using the best fit parameter set. The first four modes described $>$99% of the gene knockout variance, with the most important components of these modes being the PU.1 and AP1 proteins, and to a much lesser extent Gfi1 and C/EBPa (Fig. 6A). To better understand which connections involving the PU.1 and AP1 proteins were important, we simulated the pairwise deletion of interactions between these proteins and their respective regulatory targets. SVD decomposition of the state displacement matrix assembled from the pairwise deletion of interactions, suggested the first five modes accounted for $>$99% of the variance resulting from deletion of the interactions. The most sensitive interactions for the PU.1 protein involved the C/EBPa-dependent regulation of P47Phox expression,

and to a lesser extent AP1 and EGR1 expression (Fig. 6B). On the other other, the most sensitive connections for AP1 involved the C/EBPa-dependent regulation of p21 expression, and the mutual activation of PU.1 and AP1 expression. Taken together, these results suggested that the PU.1 and AP1 proteins acted as important self-reinforcing regulators for both the signal integration and phenotype modules. The analysis suggested that the PU.1 signaling axis promoted the formation of the neutrophil NADPH oxidase (through p47Phox), while AP1 was responsible for cell cycle arrest (through p21). However, this analysis did not give insight into which upstream components of the signal initiation module were important. Toward this question, we explored the composition and regulation of the signalsome complex by experimentally interrogating a panel of possible Raf interaction partners.

The composition of the signalsome, and the kinase ultimately responsible for mediating ATRA-induced Raf activation is currently unknown. To explore this question, we conducted immunoprecipitation and subsequent Western blotting to identify physical interactions between Raf and 19 putative interaction partners. A panel of 19 possible Raf interaction partners (kinases, GTPases, scaffolding proteins etc) was constructed based upon known signaling pathways. We did not consider the most likely binding partner, the small GTPase RAS, as previous studies have ruled it out in MAPK activation in HL-60 cells (20, 27). Total Raf was used as a bait protein for the immunoprecipitation studies. Interrogation of the Raf interactome suggested Vav1 was involved with ATRA-induced initiation of MAPK activity (Fig. 7). Western blot analysis using total Raf and pS621 Raf specific antibodies confirmed the presence of the bait protein, total and phosphorylated forms, in the immunoprecipitate (Fig. 7A). Of the 19 proteins sampled, Vav1, Src, CK2, Akt, and 14-3-3 precipitated with Raf, suggesting a direct physical interaction was possible. However, only the associations between Raf and Vav1 and Raf and Src were ATRA-inducible (Fig. 7). Furthermore, the Vav1 and Src associations were correlated with pS621 Raf

abundance in the precipitate. Others proteins e.g., CK2, Akt and 14-3-3, generally bound Raf regardless of phosphorylation status or ATRA treatment. The remaining 14 proteins were expressed in whole cell lysate (Fig. 7B), but were not detectable in the precipitate of Raf IP. Treatment with the Raf kinase inhibitor GW5074 following ATRA exposure reduced the association of both Vav1 with Raf and Src with Raf (Fig. 7), although the signal intensity for Src was notably weak. However, GW5074 did not influence the association of CK2 or 14-3-3 with Raf, further demonstrating their independence from Raf phosphorylation. Interestingly, the Raf-Akt interaction qualitatively increased following treatment with GW5074; however, it remained unaffected by treatment with ATRA. Src family kinases are known to be important in myeloid differentiation (28) and their role in HL-60 differentiation has been investigated elsewhere (11). Given the existing work and variable reproducibility in the context of the Raf immunoprecipitate, we did not investigate the role of Src further in this study. Taken together, the immunoprecipitation and GW5074 results implicated Vav1 association to be correlated with Raf activation following ATRA-treatment. Previous studies demonstrated that a Vav1-Slp76-Cbl-CD38 complex plays an important role in ATRA-induced MAPK activation and differentiation of HL-60 cells (13). Here we did not observe direct interaction of Raf with Cbl or Slp76; however, this complex could be involved upstream. Next, we considered the effect of the Raf kinase inhibitor GW5074 on functional markers of ATRA-induced growth arrest and differentiation.

Inhibition of Raf kinase activity modulated MAPK activation and differentiation markers following ATRA exposure (Fig. 7D-F). ATRA treatment alone statistically significantly increased the G1/G0 percentage over the untreated control, while GW5074 alone had a negligible effect on the cell cycle distribution (Fig. 7D). Surprisingly, the combination of GW5074 and ATRA statistically significantly increased the G1/G0 population (82 $\pm$ 1%) compared with ATRA alone (61 $\pm$ 0.5%). Increased G1/G0 arrest following the combined treatment with GW5074 and ATRA was unexpected, as the combination of ATRA and the

MEK inhibitor (PD98059) has been shown previously to decrease ATRA-induced growth arrest (8). However, growth arrest is not the sole indication of functional differentiation. Expression of the cell surface marker CD11b has also been shown to coincide with HL-60 cells myeloid differentiation (29). We measured CD11b expression, for the various treatment groups, using immuno-fluorescence flow cytometry 48 hr post-treatment. As with G1/G0 arrest, ATRA alone increased CD11b expression over the untreated control, while GW5074 further enhanced ATRA-induced CD11b expression (Fig. 7E). GW5074 alone had no statistically significant effect on CD11b expression, compared with the untreated control. Lastly, the inducible reactive oxygen species (ROS) response was used as a functional marker of differentiated neutrophils (16). We measured the ROS response induced by the phorbol ester 12-O-tetradecanoylphorbol-13-acetate (TPA) using flow cytometry. Untreated cells showed no discernible TPA response, with only $7.0 \pm 3.0\%$ ROS induction (Fig. 7F). Cells treated with ATRA had a significantly increased TPA response, $53 \pm 7\%$ ROS induction 48 hr post-treatment. Treatment with both ATRA and GW5074 statistically significantly reduced ROS induction ($22 \pm 0.6\%$) compared to ATRA alone. Interestingly, Western blot analysis did not detect a GW5074 effect on ATRA-induced expression of p47phox, a required upstream component of the ROS response (Fig. 7F, bottom). Thus, the inhibitory effect of GW5074 on inducible ROS might occur downstream of p47phox expression. However, the ROS producing complex is MAPK dependent, therefore it is also possible that GW5074 inhibited ROS production by interfering with MAPK activation (in which case the p47Phox marker might not accurately reflect phenotypic conversion and differentiation).

## Discussion

In this study, we presented an effective model of ATRA-inducible differentiation of HL-60 cells which encoded positive feedback between the ATRA-inducible signalsome complex and the MAPK pathway. Despite its simplicity, the model captured key features of the ATRA induced differentiation such as sustained MAPK activation, and bistability with respect to ATRA exposure. We also reported a new ATRA-inducible component of the signalsome, Vav1. Vav1 is a guanine nucleotide exchange factor for Rho family GTPases that activate pathways leading to actin cytoskeletal rearrangements and transcriptional alterations (30). The Vav1/Raf association correlated with Raf activity, was ATRA-inducible and decreased after treatment with GW5074. The presence of Vav1 in Raf/Grb2 complexes has been shown to correlate with increased Raf activity in mast cells (31). Furthermore, studies on Vav1 knockout mice demonstrated that the loss of Vav1 resulted in deficiencies of ERK signaling for both T-cells as well as neutrophils (32, 33). While its function in the signalsome is unclear, Vav1 has been shown to associate with a Cbl-Slp76-CD38 complex in an ATRA-dependent manner; furthermore, transfection of HL-60 cells with Cbl mutants that fail to bind CD38, yet still bind Slp76 and Vav1, prevented ATRA-induced MAPK activation (13). Thus, interaction of Cbl-Slp76-Vav1 and CD38 appears to be required for transmission of the ATRA signal by the signalsome.

Analysis of the signal integration and phenotype modules suggested that PU.1 and AP1 regulated distinct phenotypic axes following ATRA exposure. In particular, deletion of PU.1 and AP1 was predicted to lead to largest shift in the system state.

Immunoprecipitation studies identified a limited number of ATRA-dependent and -independent Raf interaction partners. While we were unable to detect the association of Raf with common kinases and GTPases such as PKC, PKA, p38, Rac and Rho, we did establish potential interactions between Raf and key partners such as Vav1, Src, Akt, CK2 and 14-3-3. All of these partners are known to be associated with Raf activation

or function. Src is known to bind Raf through an SH2 domain, and this association has been shown to be dependent of the serine phosphorylation of Raf (34). Thus, an ATRA inducible Src/Raf association may be a result of ATRA-induced Raf phosphorylation at S259 or S621. We also identified an interaction between Raf and the Ser/Thr kinases Akt and CK2. Akt can phosphorylate Raf at S259, as demonstrated by studies in a human breast cancer line (35). CK2 can also phosphorylate Raf, although the literature has traditionally focused on S338 and not S621 or S259(36). However, neither of these kinase interactions were ATRA-inducible, suggesting their association with Raf alone was not associated with ATRA-induced Raf phosphorylation. The adapter protein 14-3-3 was also constitutively associated with Raf. The interaction between Raf and 14-3-3 has been associated with both S621 and S259 phosphorylation and activity (37). Additionally, the association of Raf with 14-3-3 not only stabilized S621 phosphorylation, but also reversed the S621 phosphorylation from inhibitory to activating (38). Finally, we found that Vav1/Raf association correlated with Raf activity, was ATRA-inducible and decreased after treatment with GW5074. The presence of Vav1 in Raf/Grb2 complexes has been shown to correlate with increased Raf activity in mast cells (31). Furthermore, studies on Vav1 knockout mice demonstrated that the loss of Vav1 resulted in deficiencies of ERK signaling for both T-cells as well as neutrophils (32, 33). Interestingly, while an integrin ligand-induced ROS response was blocked in Vav1 knockout neutrophils, TPA was able to bypass the Vav1 requirement and stimulate both ERK phosphorylation and ROS induction (33). In this study, the TPA-induced ROS response was dependent upon Raf kinase activity, and was mitigated by the addition of GW5074. It is possible that Vav1 is downstream of various integrin receptors but upstream of Raf in terms of inducible ROS responses. Vav1 has also been shown to associate with a Cbl-Slp76-CD38 complex in an ATRA-dependent manner; furthermore, transfection of HL-60 cells with Cbl mutants that fail to bind CD38, yet still bind Slp76 and Vav1, prevents ATRA-induced MAPK activation (13). The literature

suggest a variety of possible receptor-signaling pathways, which involve Vav1, for MAPK activation; moreover, given the ATRA-inducible association Vav1 may play a direct role in Raf activation.

We hypothesized that Vav1 is a member of an ATRA-inducible complex which propels sustained MAPK activation, arrest and differentiation. Initially, ATRA-induced Vav1 expression drives increased association between Vav1 and Raf. This increased interaction facilitates phosphorylation and activation of Raf by pre-bound Akt and/or CK2 at S621 or perhaps S259. Constitutively bound 14-3-3 may also stabilize the S621 phosphorylation, modulate the activity and/or up-regulate autophosphorylation. Activated Raf can then drive ERK activation, which in turn closes the positive feedback loop by activating Raf transcription factors e.g., Sp1 and/or STAT1 (39–42). We tested this working hypothesis using mathematical modeling. The model recapitulated both ATRA time-course data as well as the GW5074 inhibitor effects. This suggested the proposed Raf-Vav1 architecture was at least consistent with the experimental studies. Further, analysis of the Raf-Vav1 model identified bistability in ppERK levels. Thus, two possible MAPK activation branches were possible for experimentally testable ATRA values. The analysis also suggested the ATRA-induced Raf-Vav1 architecture could be locked into a sustained signaling mode (high ppERK) even in the absence of a ATRA signal. This locked-in property could give rise to an ATRA-induction memory. We validated the treatment memory property predicted by the Raf-Vav1 circuit experimentally using ATRA-washout experiments. ERK phosphorylation levels remained high for more then 96 hr after ATRA was removed. Previous studies demonstrated that HL-60 cells possessed an inheritable memory of ATRA stimulus (43). Although the active state was self-sustaining, the inactive state demonstrated considerable robustness to perturbation. For example, we found that 50x overexpression of Raf was required to reliably lock MAPK into the activated state, while small perturbations had almost no effect on ppERK levels over the entire ensemble. CD38

expression correlated with the ppERK, suggesting its involvement in the signaling complex. Our computational and experimental results showed that positive feedback, through ERK-dependent Raf expression, could sustain MAPK signaling through many division cycles. Such molecular mechanisms could underly aspects of cellular memory associated to consecutive ATRA treatments.

Several engineered, or naturally occurring systems involved in cell fate decisions incorporate reinforcing feedback and bistability (44, 45). One of the most well studied cell fate circuits is the Mos mitogen-activated protein kinase cascade in *Xenopus* oocytes. This cascade is activated when oocytes are induced by the steroid hormone progesterone (46). The MEK-dependent activation of p42 MAPK stimulates the accumulation of the Mos oncoprotein, which in turn activates MEK, thereby closing the feedback loop. This is similar to the signal initiation module presented here; ATRA drives signalsome formation, which activates MAPK, which in turn leads to more signalsome activation. Thus, while HL-60 and *Xenopus* oocytes are vastly different biological models, their cell fate programs share a similar architectural feature. Reinforcing feedback and bistability has also been implicated in hematopoietic stem cell fate determination. Laslo et al showed that the counter antagonistic repressors, Gfi-1 and Egr-1/2 (whose expression is tuned by PU.1 and C/EBPa), encodes a bistable switch that results in a macrophage, neutrophil or a mixed lineage population depending upon PU.1 and C/EBPa expression (45). Other unrelated cell fate decisions such as programmed cell death have also been suggested to be bistable (47). Still more biochemical networks important to human health, for example the human coagulation or complement cascades, also feature strong positive feedback elements (48). Thus, while positive feedback is often undesirable in human engineered systems, it may be at the core of a diverse variety of cell fate programs and other networks important to human health.

There were several issues that can be explored further with the effective ATRA differ-

entiation model. First, there was likely missing connectivity in the effective differentiation circuit. Decreasing BLR1 expression with simultaneously sustained cRaf-pS261 activation was not captured by the current network architecture. This suggested that signalsome, once activated, had a long lifetime as decreased BLR1 expression did not impact cRaf-pS261 abundance. We could model this by separating signalsome formation into an inactive precursor pool that is transformed to a long-lived activated signalsome by MAPK activation. We should also explore adding additional downstream biological modules to this skeleton model, for example the upregulation of reactive oxygen markers such as p47Phox or cell cycle arrest components to capture the switch from an actively proliferating population to a population in G0-arrest. Next, the choice of max/min integration rules or the particular form of the transfer functions could also be explored. Integration rules other than max/min could be used, such as the mean or the product, assuming the range of the transfer functions is always $f \in [0, 1]$. Alternative integration rules might have different properties which could influence model identification or performance. For example, a mean integration rule would be differentiable, allowing derivative-based optimization approaches to be used. The form of the transfer function could also be explored. We choose hill-like functions because of their prominence in the systems and synthetic biology community. However, many other transfer functions are possible.

## Materials and Methods

*Effective gene expression model equations.* We decomposed the ATRA-induced differentiation program into three modules; a signal initiation module that sensed and transformed the ATRA signal into activated cRaf-pS621 and the ATRA-RXR/RAR (activated Trigger) signals; a signal integration module that controlled the expression of upstream transcription factors given cRaf-pS621 and activated Trigger signals; and a phenotype module which encoded the expression of functional differentiation markers from the ATRA-inducible transcription factors. The output of the signal initiation module was the input to the gene expression model. For each gene $j = 1, 2, \ldots, \mathcal{G}$, we modeled both the mRNA ($m_j$), protein ($p_j$) and signaling species abundance:

$$\frac{dm_j}{dt} = r_{T,j} - (\mu + \theta_{m,j})\, m_j + \lambda_j \tag{1}$$

$$\frac{dp_j}{dt} = r_{X,j} - (\mu + \theta_{p,j})\, p_j \tag{2}$$

$$\mathbf{g}\,(p_1, \ldots, p_{\mathcal{G}}, \kappa) = \mathbf{0} \tag{3}$$

The terms $r_{T,j}$ and $r_{X,j}$ denote the specific rates of transcription, and translation while the terms $\theta_{m,j}$ and $\theta_{p,j}$ denote first-order degradation constants for mRNA and protein, respectively. The specific transcription rate $r_{T,j}$ was modeled as the product of a kinetic term $\bar{r}_{T,j}$ and a control term $u_j$ which described how the abundance of transcription factors, or other regulators influenced the expression of gene $j$. The kinetic transcription term $\bar{r}_{T,j}$ was modeled as:

$$\bar{r}_{T,j} = V_T^{max} \left(\frac{L_{T,o}}{L_{T,j}}\right) \left(\frac{G_j}{K_T + G_j}\right) \tag{4}$$

where the maximum gene expression rate $V_T^{max}$ was defined as the product of a characteristic transcription rate constant ($k_T$) and the abundance of RNA polymerase ($R_1$),

$V_T^{max} = k_T(R_1)$. The $(L_{T,o}/L_{T,j})$ term denotes the ratio of transcription read lengths; $L_{T,o}$ represents a characteristic gene length, while $L_{T,j}$ denotes the length of gene $j$. Thus, the ratio $(L_{T,o}/L_{T,j})$ is a gene specific correction to the characteristic transcription rate $V_T^{max}$. The degradation rate constants were defined as $\theta_{m,j}$ and $\theta_{p,j}$ denote characteristic degradation constants for mRNA and protein, respectively. Lastly, the $\lambda_j$ term denotes the constitutive rate of expression of gene $j$.

The gene expression control term $0 \leq u_j \leq 1$ depended upon the combination of factors which influenced the expression of gene $j$. If the expression of gene $j$ was influenced by $1, \ldots, m$ factors, we modeled this relationship as $u_j = \mathcal{I}_j(f_{1j}(\cdot), \ldots, f_{mj}(\cdot))$ where $0 \leq f_{ij}(\cdot) \leq 1$ denotes a regulatory transfer function quantifying the influence of factor $i$ on the expression of gene $j$, and $\mathcal{I}_j(\cdot)$ denotes an integration rule which combines the individual regulatory inputs for gene $j$ into a single control term. In this study, the integration rule governing gene expression was the weighted fraction of promoter configurations that resulted in gene expression (49):

$$u_j = \frac{W_{R_1,j} + \sum_n W_{nj} f_{nj}}{1 + W_{R_1,j} + \sum_d W_{dj} f_{dj}} \tag{5}$$

The numerator, the weighted sum (with weights $W_{nj}$) of promoter configurations leading to gene expression, was normalized by all possible promoter configurations. The likelihood of each configuration was quantified by the transfer function $f_{nj}$ (which we modeled using hill like functions), while the lead term in the numerator $W_{R_1,j}$ denotes the weight of constitutive expression for gene $j$. Given this formulation, the rate of constitutive expression was then given by:

$$\lambda_j = \bar{r}_{T,j} \left( \frac{W_{R_1,j}}{1 + W_{R_1,j}} \right) \tag{6}$$

If a gene expression process had no modifying factors, $u_j = 1$. Lastly, the specific trans-

lation rate was modeled as:

$$r_{X,j} = V_X^{max} \left( \frac{L_{X,o}}{L_{X,j}} \right) \left( \frac{m_j}{K_X + m_j} \right) \tag{7}$$

where $V_X^{max}$ denotes a characteristic maximum translation rate estimated from literature, and $K_X$ denotes a translation saturation constant. The characteristic maximum translation rate was defined as the product of a characteristic translation rate constant ($k_X$) and the Ribosome abundance ($R_2$), $V_X^{max} = k_X (R_2)$. As was the case for transcription, we corrected the characteristic translation rate by the ratio of the length of a characteristic transcription normalized by the length of transcript $j$.

*Signaling model equations.* The signal initiation, and integration modules required the abundance of cRaf-pS621 and ATRA-RXR/RAR (activated Trigger) as inputs. However, our base model described only the abundance of inactive proteins e.g., cRaf or RXR/RAR but not the activated forms. To address this issue, we estimated pseudo steady state approximations for the abundance of cRaf-pS621 and activated Trigger (shown generally as Eq (3)). The abundance of activated trigger ($x_{a,1}$) was estimated directly from the RXR/RAR abundance ($x_{u,1}$):

$$x_{a,1} \sim x_{u,1} \left( \frac{\alpha \cdot \mathrm{ATRA}}{1 + \alpha \cdot \mathrm{ATRA}} \right) \tag{8}$$

where $\alpha$ denotes a gain parameter; $\alpha = 0.0$ if ATRA is less than a threshold, and $\alpha = 0.1$ if ATRA is greater than the differentiation threshold. The abundance of cRaf-pS621 was estimated by making the pseudo steady state approximation on the cRaf-pS621 balance. The abundance of an activated signaling species $i$ was given by:

$$\frac{dx_i}{dt} = r_{+,i} (\mathbf{x}, \mathbf{k}) - (\mu + k_{d,i}) x_i \qquad i = 1, \ldots, \mathcal{M} \tag{9}$$

The quantity $x_i$ denotes concentration of signaling species $i$, while $\mathcal{R}$ and $\mathcal{M}$ denote the number of signaling reactions and signaling species in the model, respectively. The term $r_{+,i}(\mathbf{x}, \mathbf{k})$ denotes the rate of generation of activated species $i$, while $\mu$ denotes the specific growth rate, and $k_{d,i}$ denotes the rate constant controlling the non-specific degradation of $x_i$. We neglected deactivation reactions e.g., phosphatase activities. We assumed that signaling processes were fast compared to gene expression; this allowed us to approximate the signaling balance as:

$$x_i^* \simeq \frac{r_{+,i}(\mathbf{x}, \mathbf{k})}{(\mu + k_{d,i})} \qquad i = 1, \ldots, \mathcal{M} \tag{10}$$

The generation rate was written as the product of a kinetic term ($\bar{r}_{+,i}$) and a control term ($v_i$). The control terms $0 \leq v_j \leq 1$ depended upon the combination of factors which influenced rate process $j$. If rate $j$ was influenced by $1, \ldots, m$ factors, we modeled this relationship as $v_j = \mathcal{I}_j(f_{1j}(\cdot), \ldots, f_{mj}(\cdot))$ where $0 \leq f_{ij}(\cdot) \leq 1$ denotes a regulatory transfer function quantifying the influence of factor $i$ on rate $j$. The function $\mathcal{I}_j(\cdot)$ is an integration rule which maps the output of regulatory transfer functions into a control variable. In this study, we used $\mathcal{I}_j \in \{min, max\}$ and hill transfer functions (50). If a process had no modifying factors, $v_j = 1$. The kinetic rate of cRaf-pS621 generation $\bar{r}_{+,cRaf}$ was modeled as:

$$\bar{r}_{+,cRaf} = k_{+,cRaf} x_s \left( \frac{x_{cRaf}}{K_{+,cRaf} + x_{cRaf}} \right) \tag{11}$$

where $x_s$ denotes the signalsome abundance, and $K_{+,cRaf}$ denotes a saturation constant governing cRaf-pS621 formation. The formation of cRaf-pS621 was regulated by only a single factor, the abundance of MAPK inhibitor, thus $v_{+,cRaf}$ took the form:

$$v_{+,cRaf} = \left( 1 - \frac{\mathrm{I}}{K_D + \mathrm{I}} \right) \tag{12}$$

where $\mathrm{I}$ denotes the abundance of the MAPK inhibitor, and $K_D$ denotes the inhibitor affinity.

*Estimation of gene expression model parameters.* We estimated parameters appearing in the mRNA and protein balances, the abundance of polymerases and ribosomes, transcription and translation rates, the half-life of a typical mRNA and protein, and typical values for the copies per cell of RNA polymerase and ribosomes from literature (Table 2). The saturation constants $K_X$ and $K_T$ were adjusted so that gene expression and translation resulted in gene products on a biologically realistic concentration scale. Lastly, we calculated the concentration for gene $G_j$ by assuming, on average, that a cell had two copies of each gene at any given time. Thus, the bulk of our gene expression model parameters were based directly upon literature values, and were not adjusted during model identification. However, the remaining parameters, e.g., the $W_{ij}$ appearing in the gene expression control laws, or parameters appearing in the transfer functions $f_{dj}$, were estimated from the protein expression and signaling data sets discussed here.

Signaling and gene expression model parameters were estimated by minimizing the squared difference between simulations and experimental protein data set $j$. We measured the squared difference in the scale, fold change and shape for protein $j$:

$$E_j(\mathbf{k}) = \Big(\mathcal{M}_j\left(t_-\right) - \hat{y}_j\left(t_-, \mathbf{k}\right)\Big)^2 + \sum_{i=1}^{\mathcal{T}_j}\Big(\hat{\mathcal{M}}_{ij} - \hat{y}_{ij}(\mathbf{k})\Big)^2 + \sum_{i=1}^{\mathcal{T}_j}\Big(\mathcal{M}'_{ij} - y'_{ij}(\mathbf{k})\Big)^2 \qquad (13)$$

The first term in Eqn. (13) quantified the initial *scale* error, directly before the addition of ATRA. In this case, $\mathcal{M}_j\left(t_-\right)$ (the approximate concentration of protein $j$ before the addition of ATRA) was estimated from literature. This term was required because the protein measurements were reported as the fold-change; thus, the data was normalized by a control value measured before the addition of ATRA. However, the model operated on a physical scale. The first term allowed the model to capture physically realistic changes

following ATRA addition. The second term quantified the difference in the *fold-change* of protein $j$ as a function of time. The terms $\hat{\mathcal{M}}_{ij}$ and $\hat{y}_{ij}$ denote the scaled experimental observations and simulation outputs (fold-change; protein normalized by control value directly before ATRA addition) at time $i$ from protein $j$, where $\mathcal{T}_j$ denoted the number of time points for data set $j$. Lastly, the third term of the objective function measured the difference in the *shape* of the measured and simulated protein levels. The scaled value $0 \leq \mathcal{M}'_{ij} \leq 1$ was given by:

$$\hat{\mathcal{M}}_{ij} = \left(\mathcal{M}_{ij} - \min_i \mathcal{M}_{ij}\right) / \left(\max_i \mathcal{M}_{ij} - \min_i \mathcal{M}_{ij}\right) \tag{14}$$

where $\mathcal{M}'_{ij} = 0$ and $\mathcal{M}'_{ij} = 1$ describe the lowest (highest) intensity bands. A similar scaling was used for the simulation output. We minimized the total model residual $\sum_j E_j$ using a heuristic direct-search optimization procedure, subject to box constraints on the parameter values, starting from a random initial parameter guess. Each downhill step was archived and used for ensemble calculations. The optimization procedure (a covariance matrix adaptation evolution strategy) has been reported previously (51).

*Estimation of an effective cell cycle arrest model.* We formulated an effective N-order polynomial model of the fraction of cells undergoing ATRA-induced cell cycle arrest at time t, $\hat{\mathcal{A}}(t)$, as:

$$\hat{\mathcal{A}}\left(t\right) \simeq a_0 + \sum_{i=1}^{N-1} a_i \phi_i\left(\mathbf{p}(t), t\right) \tag{15}$$

where $a_i$ were unknown parameters, and $\phi_i\left(\mathbf{p}(t), t\right)$ denotes a basis function. The basis functions were dependent upon the system state; in this study, we assumed $N = 4$ and basis functions of the form:

$$\phi_i\left(\mathbf{p}(t), t\right) = \left(\frac{t}{T} + \frac{p21}{E2F}\bigg|_t\right)^{(i-1)} \tag{16}$$

The parameters $a_0, \ldots, a_3$ were estimated directly from cell-cycle measurements (biological replicates) using least-squares.

*Availability of model code.* The signaling and gene expression model equations, and the parameter estimation procedure, were implemented in the Julia programming language. The model equations were solved using the `ODE23s` routine of the ODE package (52). The model code and parameter ensemble is freely available under an MIT software license and can be downloaded from http://www.varnerlab.org.

*Cell culture and treatment* Human myeloblastic leukemia cells (HL-60 cells) were grown in a humidified atmosphere of 5% $CO_2$ at 37$^o$C and maintained in RPMI 1640 from Gibco (Carlsbad, CA) supplemented with 5% heat inactivated fetal bovine serum from Hyclone (Logan, UT) and 1$\times$ antibiotic/antimicotic (Gibco, Carlsbad, CA). Cells were cultured in constant exponential growth (53). Experimental cultures were initiated at $0.1 \times 10^6$ cells/mL 24 hr prior to ATRA treatment; if indicated, cells were also treated with GW5074 (2$\mu$M) 18 hr before ATRA treatment. For the cell culture washout experiments, cells were treated with ATRA for 24 hr, washed 3x with prewarmed serum supplemented culture medium to remove ATRA, and reseeded in ATRA-free media as described. Western blot analysis was performed at incremental time points after removal of ATRA.

*Chemicals* All-Trans Retinoic Acid (ATRA) from Sigma-Aldrich (St. Louis, MO) was dissolved in 100% ethanol with a stock concentration of 5mM, and used at a final concentration of 1$\mu$M (unless otherwise noted). The cRaf inhibitor GW5074 from Sigma-Aldrich (St. Louis, MO) was dissolved in DMSO with a stock concentration of 10mM, and used at a final concentration of 2$\mu$M. HL-60 cells were treated with 2$\mu$M GW5074 with or without ATRA (1$\mu$M) at 0 hr. This GW5074 dosage had a negligible effect on the cell cycle distribution, compared to ATRA treatment alone.

*Immunoprecipitation and western blotting* Approximately $1.2 \times 10^7$ cells were lysed using 400$\mu$L of M-Per lysis buffer from Thermo Scientific (Waltham, MA). Lysates were cleared by centrifugation at 16,950 $\times$ g in a micro-centrifuge for 20 min at 4$^o$C. Lysates were pre-cleared using 100$\mu$L protein A/G Plus agarose beads from Santa Cruz Biotechnology (Santa Cruz, CA) by inverting overnight at 4$^o$C. Beads were cleared by centrifugation and total protein concentration was determined by a BCA assay (Thermo Scientific, Waltham, MA). Immunoprecipitations were setup by bringing lysate to a concentration of 1g/L in a total volume of 300$\mu$L (M-Per buffer was used for dilution). The anti-Raf antibody was added at 3$\mu$L. A negative control with no bait protein was also used to exclude the direct interaction of proteins with the A/G beads. After 1 hr of inversion at 4$^o$C, 20$\mu$L of agarose beads was added and samples were left to invert overnight at 4$^o$C. Samples were then washed three times with M-Per buffer by centrifugation. Finally proteins were eluted from agarose beads using a laemmli loading buffer. Eluted proteins were resolved by SDS-PAGE and Western blotting. Total lysate samples were normalized by total protein concentration (20$\mu$g per sample) and resolved by SDS-PAGE and Western blotting. Secondary HRP bound antibody was used for visualization. All antibodies were purchased from Cell Signaling (Boston, MA) with the exception of $\alpha$-p621 Raf which was purchased from Biosource/Invitrogen (Carlsbad, CA), and $\alpha$-CK2 from BD Biosciences (San Jose, CA).

*Morphology assessment* Untreated and ATRA-treated HL-60 cells were collected after 72 hr and cytocentrifuged for 3 min at 700 rpm onto glass slides. Slides were air-dried and stained with Wright's stain. Slide images were captured at 40X (Leica DM LB 100T microscope, Leica Microsystems).

## Competing interests

The authors declare that they have no competing interests.

## Author's contributions

J.V and A.Y directed the study. R.T, H.J, R.B and J.C conducted the cell culture measurements. J.V, R.B, W.D, K.R and A.S developed the reduced order HL-60 models and the parameter ensemble. W.D and J.V analyzed the model ensemble, and generated figures for the manuscript. The manuscript was prepared and edited for publication by W.D, A.Y and J.V.

## Acknowledgements

We gratefully acknowledge the suggestions from the anonymous reviewers to improve this manuscript.

## Funding

We acknowledge the financial support to J.V. by the National Science Foundation CAREER (CBET-0846876) for the support of R.T. and H.J. In addition, we acknowledge support to A.Y. from the National Institutes of Health (CA 30555, CA152870) and a grant from New York State Stem Cell Science. Lastly, we acknowledge the financial support to J.V. and A.Y. from the National Cancer Institute (#U54 CA143876). The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Cancer Institute or the National Institutes of Health.

## References


1. Bushue N, Wan YJY (2010) Retinoid pathway and cancer therapeutics. Adv Drug Deliv Rev 62: 1285-98.
2. Tang XH, Gudas LJ (2011) Retinoids, retinoic acid receptors, and cancer. Annu Rev Pathol 6: 345-64.
3. Cheung FSG, Lovicu FJ, Reichardt JKV (2012) Current progress in using vitamin d and its analogs for cancer prevention and treatment. Expert Rev Anticancer Ther 12: 811-37.
4. Nilsson B (1984) Probable in vivo induction of differentiation by retinoic acid of promyelocytes in acute promyelocytic leukaemia. Br J Haematol 57: 365-71.
5. Warrell RP Jr (1993) Retinoid resistance in acute promyelocytic leukemia: new mechanisms, strategies, and implications. Blood 82: 1949-53.
6. Freemantle SJ, Spinella MJ, Dmitrovsky E (2003) Retinoids in cancer therapy and chemoprevention: promise meets resistance. Oncogene 22: 7305-15.
7. Breitman TR, Selonick SE, Collins SJ (1980) Induction of differentiation of the human promyelocytic leukemia cell line (HL-60) by retinoic acid. Proc Natl Acad Sci U S A 77: 2936–2940.
8. Yen A, Roberson MS, Varvayanis S, Lee AT (1998) Retinoic acid induced mitogen-activated protein (MAP)/extracellular signal-regulated kinase (ERK) kinase-dependent MAP kinase activation needed to elicit HL-60 cell differentiation and growth arrest. Cancer Res 58: 3163–3172.
9. Hong HY, Varvayanis S, Yen A (2001) Retinoic acid causes MEK-dependent RAF phosphorylation through RARalpha plus RXR activation in HL-60 cells. Differentiation 68: 55–66.
10. Mangelsdorf DJ, Ong ES, Dyck JA, Evans RM (1990) Nuclear receptor that identifies a novel retinoic acid response pathway. Nature 345: 224–229.

11. Congleton J, MacDonald R, Yen A (2012) Src inhibitors, PP2 and dasatinib, increase retinoic acid-induced association of Lyn and c-Raf (S259) and enhance MAPK-dependent differentiation of myeloid leukemia cells. Leukemia 26: 1180-8.
12. Shen M, Bunaciu R, Congleton J, Jensen H, Sayam L, et al. (2011) Interferon regulatory factor-1 binds c-Cbl, enhances mitogen activated protein kinase signaling and promotes retinoic acid-induced differentiation of HL-60 human myelo-monoblastic leukemia cells. Leuk Lymphoma 52: 2372-9.
13. Shen M, Yen A (2009) c-Cbl tyrosine kinase-binding domain mutant G306E abolishes the interaction of c-Cbl with CD38 and fails to promote retinoic acid-induced cell differentiation and G0 arrest. J Biol Chem 284: 25664–25677.
14. Yen A, Varvayanis S, Smith J, Lamkin T (2006) Retinoic acid induces expression of SLP-76: expression with c-FMS enhances ERK activation and retinoic acid-induced differentiation/G0 arrest of HL-60 cells. Eur J Cell Biol 85: 117–132.
15. Marchisio M, Bertagnolo V, Colamussi ML, Capitani S, Neri LM (1998) Phosphatidylinositol 3-kinase in HL-60 nuclei is bound to the nuclear matrix and increases during granulocytic differentiation. Biochem Biophys Res Commun 253: 346-51.
16. Congleton J, Jiang H, Malavasi F, Lin H, Yen A (2011) ATRA-induced HL-60 myeloid leukemia cell differentiation depends on the CD38 cytosolic tail needed for membrane localization, but CD38 enzymatic activity is unnecessary. Exp Cell Res 317: 910–919.
17. Wang J, Yen A (2004) A novel retinoic acid-responsive element regulates retinoic acid induced BLR1 expression. Mol Cell Biol 24: 2423 - 2443.
18. Yen A (1990) HL-60 cells as a model of growth and differentiation: the significance of variant cells. Hematology Review 4: 5-46.
19. Yang T, Xiong Q, Enslen H, Davis R, Chow CW (2002) Phosphorylation of NFATc4 by p38 mitogen-activated protein kinases. Mol Cell Biol 22: 3892–3904.

20. Wang J, Yen A (2008) A MAPK-positive Feedback Mechanism for BLR1 Signaling Propels Retinoic Acid-triggered Differentiation and Cell Cycle Arrest. J Biol Chem 283: 4375–4386.
21. Tasseff R, Nayak S, Song S, Yen A, Varner J (2011) Modeling and analysis of retinoic acid induced differentiation of uncommitted precursor cells. Integr Biol 3: 578 - 591.
22. Jensen HA, Styskal LE, Tasseff R, Bunaciu RP, Congleton J, et al. (2013) The src-family kinase inhibitor pp2 rescues inducible differentiation events in emergent retinoic acid-resistant myeloblastic leukemia cells. PLoS One 8: e58621.
23. Jensen HA, Bunaciu RP, Ibabao CN, Myers R, Varner JD, et al. (2014) Retinoic acid therapy resistance progresses from unilineage to bilineage in hl-60 leukemic blasts. PLoS One 9: e98929.
24. Jensen HA, Bunaciu RP, Varner JD, Yen A (2015) Gw5074 and pp2 kinase inhibitors implicate nontraditional c-raf and lyn function as drivers of retinoic acid-induced maturation. Cell Signal 27: 1666-75.
25. Jensen HA, Yourish HB, Bunaciu RP, Varner JD, Yen A (2015) Induced myelomonocytic differentiation in leukemia cells is accompanied by noncanonical transcription factor expression. FEBS Open Bio 5: 789-800.
26. Milo R, Jorgensen P, Moran U, Weber G, Springer M (2010) Bionumbers–the database of key numbers in molecular and cell biology. Nucleic Acids Res 38: D750-3.
27. Katagiri K, Hattori S, Nakamura S, Yamamoto T, Yoshida T, et al. (1994) Activation of ras and formation of gap complex during tpa-induced monocytic differentiation of hl-60 cells. Blood 84: 1780–1789.
28. Miranda MB, Johnson DE (2007) Signal transduction pathways that contribute to myeloid differentiation. Leukemia 21: 1363–1377.
29. Hickstein DD, Back AL, Collins SJ (1989) Regulation of expression of the cd11b and

633 cd18 subunits of the neutrophil adherence receptor during human myeloid differen-
634 tiation. J Biol Chem 264: 21812–21817.
635 30. Hornstein I, Alcover A, Katzav S (2004) Vav proteins, masters of the world of cy-
636 toskeleton organization. Cell Signal 16: 1-11.
637 31. Song JS, Gomez J, Stancato LF, Rivera J (1996) Association of a p95 vav-containing
638 signaling complex with the fcepsilonri gamma chain in the rbl-2h3 mast cell line.
639 evidence for a constitutive in vivo association of vav with grb2, raf-1, and erk2 in an
640 active complex. J Biol Chem 271: 26962–26970.
641 32. Costello PS, Walters AE, Mee PJ, Turner M, Reynolds LF, et al. (1999) The rho-
642 family gtp exchange factor vav is a critical transducer of t cell receptor signals to the
643 calcium, erk, and nf-kappab pathways. Proc Natl Acad Sci U S A 96: 3035–3040.
644 33. Graham D, Robertson C, Bautista J, Mascarenhas F, Diacovo M, et al. (2007)
645 Neutrophil-mediated oxidative burst and host defense are controlled by a Vav-
646 PLCgamma2 signaling axis in mice. J Clin Invest 117: 3445–3452.
647 34. Cleghon V, Morrison DK (1994) Raf-1 interacts with fyn and src in a non-
648 phosphotyrosine-dependent manner. J Biol Chem 269: 17749–17755.
649 35. Zimmermann S, Moelling K (1999) Phosphorylation and regulation of raf by akt (pro-
650 tein kinase b). Science 286: 1741–1744.
651 36. Ritt DA, Zhou M, Conrads TP, Veenstra TD, Copeland TD, et al. (2007) Ck2 is a
652 component of the ksr1 scaffold complex that contributes to raf kinase activation.
653 Curr Biol 17: 179–184.
654 37. Hekman M, Wiese S, Metz R, Albert S, Troppmair J, et al. (2004) Dynamic changes
655 in c-raf phosphorylation and 14-3-3 protein binding in response to growth factor stim-
656 ulation: differential roles of 14-3-3 protein binding sites. J Biol Chem 279: 14074–
657 14086.
658 38. Dhillon AS, Yip YY, Grindlay GJ, Pakay JL, Dangers M, et al. (2009) The c-terminus

of raf-1 acts as a 14-3-3-dependent activation switch. Cell Signal 21: 1645–1651.

39. Kim HS, Lim IK (2009) Phosphorylated extracellular signal-regulated protein kinases 1 and 2 phosphorylate sp1 on serine 59 and regulate cellular senescence via transcription of p21sdi1/cip1/waf1. J Biol Chem 284: 15475–15486.
40. Milanini-Mongiat J, Pouyss?gur J, Pag?s G (2002) Identification of two sp1 phosphorylation sites for p42/p44 mitogen-activated protein kinases: their implication in vascular endothelial growth factor gene transcription. J Biol Chem 277: 20631–20639.
41. Zhang Y, Cho YY, Petersen BL, Zhu F, Dong Z (2004) Evidence of stat1 phosphorylation modulated by mapks, mek1 and msk1. Carcinogenesis 25: 1165–1175.
42. Li Z, Theus MH, Wei L (2006) Role of erk 1/2 signaling in neuronal differentiation of cultured embryonic stem cells. Dev Growth Differ 48: 513–523.
43. Yen A, Reece SL, Albright KL (1984) Dependence of hl-60 myeloid cell differentiation on continuous and split retinoic acid exposures: precommitment memory associated with altered nuclear structure. J Cell Physiol 118: 277–286.
44. Ferrell J (2002) Self-perpetuating states in signal transduction: positive feedback, double-negative feedback and bistability. Curr Opin Cell Biol 14: 140-8.
45. Laslo P, Spooner CJ, Warmflash A, Lancki DW, Lee HJ, et al. (2006) Multilineage transcriptional priming and determination of alternate hematopoietic cell fates. Cell 126: 755-66.
46. Xiong W, Ferrell J (2003) A positive-feedback-based bistable 'memory module' that governs a cell fate decision. Nature 426: 460-5.
47. Bagci EZ, Vodovotz Y, Billiar TR, Ermentrout GB, Bahar I (2006) Bistability in apoptosis: roles of bax, bcl-2, and mitochondrial permeability transition pores. Biophys J 90: 1546-59.
48. Luan D, Zai M, Varner JD (2007) Computationally derived points of fragility of a

human cascade are consistent with current therapeutic strategies. PLoS Comput Biol 3: e142.

49. Moon TS, Lou C, Tamsir A, Stanton BC, Voigt CA (2012) Genetic programs constructed from layered logic gates in single cells. Nature 491: 249-53.
50. Wayman JA, Sagar A, Varner JD (2015) Dynamic modeling of cell-free biochemical networks using effective kinetic models. Processes 3: 138.
51. Igel C, Hansen N, Roth S (2007) Covariance matrix adaptation for multi-objective optimization. Evol Comput 15: 1-28.
52. Bezanson J, Edelman A, Karpinski S, Shah VB (2014) Julia: A fresh approach to numerical computing. CoRR abs/1411.1607.
53. Brooks SC, Kazmer S, Levin AA, Yen A (1996) Myeloid differentiation and retinoblastoma phosphorylation changes in HL-60 cells induced by retinoic acid receptor- and retinoid X receptor-selective retinoic acid analogs. Blood 87: 227–237.
54. Rishi AK, Gerald TM, Shao ZM, Li XS, Baumann RG, et al. (1996) Regulation of the human retinoic acid receptor alpha gene in the estrogen receptor negative human breast carcinoma cell lines skbr-3 and mda-mb-435. Cancer Res 56: 5246-52.
55. Mueller BU, Pabst T, Fos J, Petkovic V, Fey MF, et al. (2006) Atra resolves the differentiation block in t(15;17) acute myeloid leukemia by restoring pu.1 expression. Blood 107: 3330-8.
56. Friedman AD (2007) Transcriptional control of granulocyte and monocyte development. Oncogene 26: 6816-28.
57. Luo XM, Ross AC (2006) Retinoic acid exerts dual regulatory actions on the expression and nuclear localization of interferon regulatory factor-1. Exp Biol Med (Maywood) 231: 619-31.
58. Sylvester I, Schöler HR (1994) Regulation of the oct-4 gene by nuclear receptors. Nucleic Acids Res 22: 901-11.

711 59. Drach J, McQueen T, Engel H, Andreeff M, Robertson KA, et al. (1994) Retinoic
712 acid-induced expression of cd38 antigen in myeloid cells is mediated through
713 retinoic acid receptor-alpha. Cancer Res 54: 1746-52.
714 60. Liu M, Iavarone A, Freedman LP (1996) Transcriptional activation of the human
715 p21(waf1/cip1) gene by retinoic acid receptor. correlation with retinoid induction of
716 u937 cell differentiation. J Biol Chem 271: 31723-8.
717 61. Bunaciu RP, Yen A (2013) 6-formylindolo (3,2-b)carbazole (ficz) enhances retinoic
718 acid (ra)-induced differentiation of hl-60 myeloblastic leukemia cells. Mol Cancer 12:
719 39.
720 62. Balmer JE, Blomhoff R (2002) Gene expression regulation by retinoic acid. J Lipid
721 Res 43: 1773-808.
722 63. Rosen ED, Hsu CH, Wang X, Sakai S, Freeman MW, et al. (2002) C/ebpalpha in-
723 duces adipogenesis through ppargamma: a unified pathway. Genes Dev 16: 22-6.
724 64. Varley CL, Bacon EJ, Holder JC, Southgate J (2009) Foxa1 and irf-1 intermediary
725 transcriptional regulators of ppargamma-induced urothelial cytodifferentiation. Cell
726 Death Differ 16: 103-14.
727 65. Bruemmer D, Yin F, Liu J, Berger JP, Sakai T, et al. (2003) Regulation of the growth
728 arrest and dna damage-inducible gene 45 (gadd45) by peroxisome proliferator-
729 activated receptor gamma in vascular smooth muscle cells. Circ Res 93: e38-47.
730 66. Delerive P, De Bosscher K, Besnard S, Vanden Berghe W, Peters JM, et al. (1999)
731 Peroxisome proliferator-activated receptor alpha negatively regulates the vascular
732 inflammatory gene response by negative cross-talk with transcription factors nf-
733 kappab and ap-1. J Biol Chem 274: 32048-54.
734 67. Altiok S, Xu M, Spiegelman BM (1997) Ppargamma induces cell cycle withdrawal:
735 inhibition of e2f/dp dna-binding activity via down-regulation of pp2a. Genes Dev 11:
736 1987-98.

68. Fei J, Cook C, Gillespie M, Yu B, Fullen K, et al. (2011) Atherogenic $\omega$-6 lipids modulate ppar- egr-1 crosstalk in vascular cells. PPAR Res 2011: 753917.
69. Song EK, Lee YR, Kim YR, Yeom JH, Yoo CH, et al. (2012) Naadp mediates insulin-stimulated glucose uptake and insulin sensitization by ppar$\gamma$ in adipocytes. Cell Rep 2: 1607-19.
70. Szanto A, Nagy L (2005) Retinoids potentiate peroxisome proliferator-activated receptor gamma action in differentiation, gene expression, and lipid metabolic processes in developing myeloid cells. Mol Pharmacol 67: 1935-43.
71. Han S, Sidell N, Fisher PB, Roman J (2004) Up-regulation of p21 gene expression by peroxisome proliferator-activated receptor gamma in human lung carcinoma cells. Clin Cancer Res 10: 1911-9.
72. Von Knethen A, Brüne B (2002) Activation of peroxisome proliferator-activated receptor gamma by nitric oxide in monocytes/macrophages down-regulates p47phox and attenuates the respiratory burst. J Immunol 169: 2619-26.
73. Dispirito JR, Fang B, Wang F, Lazar MA (2013) Pruning of the adipocyte peroxisome proliferator-activated receptor $\gamma$ cistrome by hematopoietic master regulator pu.1. Mol Cell Biol 33: 3354-64.
74. Chen H, Ray-Gallet D, Zhang P, Hetherington CJ, Gonzalez DA, et al. (1995) Pu.1 (spi-1) autoregulates its expression in myeloid cells. Oncogene 11: 1549-60.
75. Steidl U, Rosenbauer F, Verhaak RGW, Gu X, Ebralidze A, et al. (2006) Essential role of jun family transcription factors in pu.1 knockdown-induced leukemic stem cells. Nat Genet 38: 1269-77.
76. Pahl HL, Scheibe RJ, Zhang DE, Chen HM, Galson DL, et al. (1993) The proto-oncogene pu.1 regulates expression of the myeloid-specific cd11b promoter. J Biol Chem 268: 5014-20.
77. Yuki H, Ueno S, Tatetsu H, Niiro H, Iino T, et al. (2013) Pu.1 is a potent tumor

763 suppressor in classical hodgkin lymphoma cells. Blood 121: 962-70.
764 78. Li SL, Schlegel W, Valente AJ, Clark RA (1999) Critical flanking sequences of pu.1
765 binding sites in myeloid-specific promoters. J Biol Chem 274: 32453-60.
766 79. Dahl R, Walsh JC, Lancki D, Laslo P, Iyer SR, et al. (2003) Regulation of macrophage
767 and neutrophil cell fates by the pu.1:c/ebpalpha ratio and granulocyte colony-
768 stimulating factor. Nat Immunol 4: 1029-36.
769 80. Timchenko N, Wilson DR, Taylor LR, Abdelsayed S, Wilde M, et al. (1995) Autoreg-
770 ulation of the human c/ebp alpha gene by stimulation of upstream stimulatory factor
771 binding. Mol Cell Biol 15: 1192-202.
772 81. Lidonnici MR, Audia A, Soliera AR, Prisco M, Ferrari-Amorotti G, et al. (2010) Ex-
773 pression of the transcriptional repressor gfi-1 is regulated by c/ebpalpha and is
774 involved in its proliferation and colony formation-inhibitory effects in p210bcr/abl-
775 expressing cells. Cancer Res 70: 7949-59.
776 82. D'Alo' F, Johansen LM, Nelson EA, Radomska HS, Evans EK, et al. (2003) The
777 amino terminal and e2f interaction domains are critical for c/ebp alpha-mediated
778 induction of granulopoietic development of hematopoietic cells. Blood 102: 3163-
779 71.
780 83. Pan Z, Hetherington CJ, Zhang DE (1999) Ccaat/enhancer-binding protein activates
781 the cd14 promoter and mediates transforming growth factor beta signaling in mono-
782 cyte development. J Biol Chem 274: 23242-8.
783 84. Harris TE, Albrecht JH, Nakanishi M, Darlington GJ (2001) Ccaat/enhancer-binding
784 protein-alpha cooperates with p21 to inhibit cyclin-dependent kinase-2 activity and
785 induces growth arrest independent of dna binding. J Biol Chem 276: 29200-9.
786 85. Bauvois B, Durant L, Laboureau J, Barthélémy E, Rouillard D, et al. (1999) Upreg-
787 ulation of cd38 gene expression in leukemic b cells by interferon types i and ii. J
788 Interferon Cytokine Res 19: 1059-66.

86. Passioura T, Dolnikov A, Shen S, Symonds G (2005) N-ras-induced growth suppression of myeloid cells is mediated by irf-1. Cancer Res 65: 797-804.
87. Dahl R, Iyer SR, Owens KS, Cuylear DD, Simon MC (2007) The transcriptional repressor gfi-1 antagonizes pu.1 activity through protein-protein interaction. J Biol Chem 282: 6473-83.
88. Duan Z, Horwitz M (2003) Targets of the transcriptional repressor oncoprotein gfi-1. Proc Natl Acad Sci U S A 100: 5932-7.
89. Chen H, Zhang P, Radomska HS, Hetherington CJ, Zhang DE, et al. (1996) Octamer binding factors and their coactivator can activate the murine pu.1 (spi-1) promoter. J Biol Chem 271: 15743-52.
90. Behre G, Whitmarsh AJ, Coghlan MP, Hoang T, Carpenter CL, et al. (1999) c-jun is a jnk-independent coactivator of the pu.1 transcription factor. J Biol Chem 274: 4939-46.
91. Kardassis D, Papakosta P, Pardali K, Moustakas A (1999) c-jun transactivates the promoter of the human p21(waf1/cip1) gene by acting as a superactivator of the ubiquitous transcription factor sp1. J Biol Chem 274: 29572-81.
92. Johnson DG, Ohtani K, Nevins JR (1994) Autoregulatory control of e2f1 expression in response to positive and negative regulators of cell cycle progression. Genes Dev 8: 1514-25.
93. Fu M, Zhang J, Lin Y, Zhu X, Ehrengruber MU, et al. (2002) Early growth response factor-1 is a critical transcriptional mediator of peroxisome proliferator-activated receptor-gamma 1 gene expression in human aortic smooth muscle cells. J Biol Chem 277: 26808-14.
94. Mak KS, Funnell APW, Pearson RCM, Crossley M (2011) Pu.1 and haematopoietic cell fate: Dosage matters. Int J Cell Biol 2011: 808524.
95. Chen F, Wang Q, Wang X, Studzinski GP (2004) Up-regulation of egr1 by 1,25-

dihydroxyvitamin d3 contributes to increased expression of p35 activator of cyclin-dependent kinase 5 and consequent onset of the terminal phase of hl60 cell differentiation. Cancer Res 64: 5425-33.

96. Suh J, Jeon YJ, Kim HM, Kang JS, Kaminski NE, et al. (2002) Aryl hydrocarbon receptor-dependent inhibition of ap-1 activity by 2,3,7,8-tetrachlorodibenzo-p-dioxin in activated b cells. Toxicol Appl Pharmacol 181: 116-23.
97. Shen M, Bunaciu RP, Congleton J, Jensen HA, Sayam LG, et al. (2011) Interferon regulatory factor-1 binds c-cbl, enhances mitogen activated protein kinase signaling and promotes retinoic acid-induced differentiation of hl-60 human myelomonoblastic leukemia cells. Leuk Lymphoma 52: 2372-9.
98. Bunaciu RP, Yen A (2011) Activation of the aryl hydrocarbon receptor ahr promotes retinoic acid-induced differentiation of myeloblastic leukemia cells by restricting expression of the stem cell transcription factor oct4. Cancer Res 71: 2371-80.
99. Jackson DA, Pombo A, Iborra F (2000) The balance sheet for transcription: an analysis of nuclear rna metabolism in mammalian cells. FASEB J 14: 242-54.
100. Zhao ZW, Roy R, Gebhardt JCM, Suter DM, Chapman AR, et al. (2014) Spatial organization of rna polymerase ii inside a mammalian cell nucleus revealed by reflected light-sheet superresolution microscopy. Proc Natl Acad Sci U S A 111: 681-6.
101. Freitas R, Merkle R (2004) Kinematic Self-Replicating Machines. Oxford University Press.
102. Yang E, van Nimwegen E, Zavolan M, Rajewsky N, Schroeder M, et al. (2003) Decay rates of human mrnas: correlation with functional characteristics and sequence attributes. Genome Res 13: 1863-72.
103. Doherty MK, Hammond DE, Clague MJ, Gaskell SJ, Beynon RJ (2009) Turnover of the human proteome: determination of protein intracellular stability by dynamic silac. J Proteome Res 8: 104-12.

104. Darzacq X, Shav-Tal Y, de Turris V, Brody Y, Shenoy SM, et al. (2007) In vivo dynamics of rna polymerase ii transcription. Nat Struct Mol Biol 14: 796-806.

105. Boström K, Wettesten M, Borén J, Bondjers G, Wiklund O, et al. (1986) Pulse-chase studies of the synthesis and intracellular transport of apolipoprotein b-100 in hep g2 cells. J Biol Chem 261: 13800-6.

106. Meyers R, editor (2004) Encyclopedia of Molecular Cell Biology and Molecular Medicine, Volume 1, 2nd Edition. ISBN: 978-3-527-30543-8. Wiley-Blackwell.

107. Rosenbluth MJ, Lam WA, Fletcher DA (2006) Force microscopy of nonadherent cells: a comparison of leukemia cell deformability. Biophys J 90: 2994-3003.

**Table 1:** Myelomonocytic transcription factor connectivity used in the signal integration and phenotype modules.

| Effector | Effect | Target | Source |
|---|---|---|---|
| RAR$\alpha$ | + | RAR$\alpha$ | (54) |
| | + | PU.1 | (55) |
| | + | C/EBP$\alpha$ | (56) |
| | + | IRF-1 | (57) |
| | - | Oct4 | (58) |
| | + | CD38 | (59) |
| | + | p21 | (60) |
| | + | AhR | (61) |
| | + | EGR1 | (62) |
| PPAR$\gamma$ | + | C/EBP$\alpha$ | (63) |
| | + | IRF-1 | (64) |
| | + | Oct1 | (65) |
| | - | AP-1 | (66) |
| | - | E2F | (67) |
| | - | EGR1 | (68) |
| | + | CD38 | (69) |
| | + | CD14 | (70) |
| | + | p21 | (71) |
| | - | p47phox | (72) |
| PU.1 | - | PPAR$\gamma$ | (73) |
| | + | PU.1 | (74) |
| | + | AP-1 | (75) |
| | + | EGR1 | (45) |
| | + | CD11b | (76) |
| | + | p21 | (77) |
| | + | p47phox | (78) |
| C/EBP$\alpha$ | + | PPAR$\gamma$ | (63) |
| | + | PU.1 | (79) |
| | + | C/EBP$\alpha$ | (80) |
| | + | Gfi-1 | (81) |
| | - | E2F | (82) |
| | + | CD14 | (83) |

| | | | |
|---|---|---|---|
| | + | p21 | (84) |
| IRF-1 | + | CD38 | (85) |
| | + | p21 | (86) |
| | - | PU.1 | (87) |
| | - | C/EBP$\alpha$ | (88) |
| | - | E2F | (88) |
| | - | EGR1 | (45) |
| | - | p21 | (88) |
| Oct1 | + | PU.1 | (89) |
| AP-1 | - | PPAR$\gamma$ | (66) |
| | + | PU.1 | (90) |
| | + | p21 | (91) |
| E2F | + | E2F | (92) |
| EGR1 | + | PPAR$\gamma$ | (93) |
| | - | Gfi-1 | (94) |
| | + | CD14 | (95) |
| AhR | + | AP-1 | (96) |
| | + | IRF-1 | (97) |
| | - | Oct4 | (98) |
| | - | PU.1 | |

853 **Table 2:** Characteristic model parameters estimated from literature.

| Symbol | Description | Value | Units | Source |
|---|---|---|---|---|
| $R_1$ | RNA polymerase abundance | 75,000 | copies/cell | (99, 100) |
| $R_2$ | Ribosome abundance | 1 x $10^6$ | copies/cell | (101) |
| $G_i$ | Characteristic gene abundance | 2 | copies/cell | this study |
| $K_X$ | Saturation constant transcription | 4,600 | copies/cell | this study |
| $K_T$ | Saturation constant translation | 100,000 | copies/cell | this study |
| | | | | |
| $t_{1/2,m}$ | characteristic mRNA half-life (transcription factor) | 2 | hr | (102) |
| $t_{1/2,p}$ | characteristic protein half-life | 10 | hr | (103) |
| $\theta_{m,j}$ | characteristic mRNA degradation constant | 0.34 | hr$^{-1}$ | derived |
| $\theta_{p,j}$ | characteristic protein degradation constant | 0.07 | hr$^{-1}$ | derived |
| | | | | |
| $t_d$ | HL-60 doubling time | 19.5 | hr | this study |
| $\mu$ | growth rate | 0.035 | hr$^{-1}$ | derived |
| $k_d$ | death rate | 0.10$\mu$ | hr$^{-1}$ | derived |
| | | | | |
| $e_T$ | elongation rate RNA polymerase | 6 | nt/s | (104) |
| $e_X$ | elongation rate Ribosome | 5 | aa/s | (105) |
| $L_{T,o}$ | characteristic gene length | 15,000 | nt | (106) |
| $L_{X,o}$ | characteristic transcript length | 5,000 | nt | derived |
| $k_T$ | characteristic transcription rate | 1.44 | hr$^{-1}$ | derived |
| $k_X$ | characteristic translation rate | 3.60 | hr$^{-1}$ | derived |
| | | | | |
| $D$ | Diameter of an HL-60 cell | 12.4 | $\mu$m$^3$ | (107) |
| $f_C$ | cytoplasmic fraction | 0.51 | dimensionless | (107) |

854

**Fig. 1:** Schematic of the effective ATRA differentiation circuit. Above a critical threshold, ATRA activates an upstream Trigger, which induces signalsome complex formation. Signalsome activates the mitogen-activated protein kinase (MAPK) cascade which in turn drives the differentiation program and signalsome formation. Both Trigger and activated cRaf-pS621 drive a phenotype gene expression program responsible for differentiation. Trigger activates the expression of a series of transcription factors which in combination with cRaf-pS621 result in phenotypic change.

**Fig. 2:** Model analysis for ATRA-induced HL-60 differentiation. A: BLR1 mRNA versus time following exposure to 1$\mu$M ATRA at t = 0 hr. B: cRaf-pS621 versus time following exposure to 1$\mu$M ATRA at t = 0 hr. Points denote experimental measurements, solid lines denote the mean model performance. Shaded regions denote the 99% confidence interval calculated over the parameter ensemble. C: Signalsome and cRaf-pS621 nullclines for ATRA below the critical threshold. The model had two stable steady states and a single unstable state in this regime. D: Signalsome and cRaf-pS621 nullclines for ATRA above the critical threshold. In this regime the model had only a single stable steady state. E: Morphology of HL-60 as a function of ATRA concentration (t = 72 hr).

**Fig. 3:** Model simulation following exposure to 1$\mu$M ATRA. A: BLR1 mRNA versus time with and without MAPK inhibitor. B: cRaf-pS621 versus time following pulsed exposure to 1$\mu$M ATRA with and without BLR1. Solid lines denote the mean model performance, while shaded regions denote the 99% confidence interval calculated over the parameter ensemble. C: Western blot analysis of phosphorylated ERK1/2 in ATRA washout experiments. Experimental data in panels A and B were reproduced from Wang and Yen (20), data in panel C is reported in this study.

**Fig. 4:** Model simulation of the HL-60 gene expression program following exposure to 1$\mu$M ATRA at t = 0 hr. A: CD38 and CD11b expression versus time following ATRA exposure at time t = 0 hr. B: Gene expression at t = 48 hr following ATRA exposure. Experimental data in panels A and B were reproduced from Jensen et al. (25).

**Fig. 5:** Model simulation of HL-60 cell-cycle arrest following exposure to 1$\mu$M ATRA at t = 0 hr. A: Predicted p21 and E2F expression levels for the best parameter set following ATRA exposure at time t = 0 hr. B: Estimated fraction of HL-60 cells in G0 arrest following ATRA exposure at time t = 0 hr. The gray region denotes the 95% confidence estimate of the polynomial model. Experimental data in panel B was reproduced from Jensen et al. (25).

**Fig. 6:** Robustness of the HL-60 differentiation program following exposure to 1$\mu$M ATRA at t = 0 hr. A: Singular value decomposition of the system response ($l^2$-norm between the perturbed and nominal state) following pairwise gene knockout simulations using the best fit parameter set. The percentage at the top of each column describes the fraction of the variance in the system state captured by the node combinations in the rows. B: Singular value decomposition of the system response ($l^2$-norm between the perturbed and nominal state) following the pairwise removal of connections from the PU.1 and AP1 nodes.

**Fig. 7:** Investigation of a panel of possible Raf interaction partners in the presence and absence of ATRA. A: Species identified to precipitate out with Raf: first column shows Western blot analysis on total Raf immunoprecipitation with and without 24 hr ATRA treatment and the second on total lysate. B: The expression of species considered that did not precipitate out with Raf at levels detectable by Western blot analysis on total lysate. C: Effect of the Raf inhibitor GW5074 on Raf interactions as determined by Western blot analysis of total Raf immunoprecipitation. The Authors note the the signal associated with Src was found to be weak. D: Cell Cycle distribution as determined by flow cytometry indicated arrest induced by ATRA, which was increased by the addition of GW5074. E: Expression of the cell surface marker CD11b as determined by flow cytometry indicated increased expression induced by ATRA, which was enhanced by the addition of GW5074. F: Inducible reactive oxygen species (ROS) as determined by DCF flow cytometry. The functional differentiation response of ATRA treated cells was mitigated by GW5074.