

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.

1 Introduction

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

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

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

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

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

71 **Results**

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

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

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

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

123 signals were maintained following ATRA withdraw (Fig. 3B, gray). On the other hand,
124 BLR1 deletion removed the ability of the circuit to maintain a sustained MAPK response
125 following the withdraw of ATRA (Fig. 3B, blue). Lastly, washout experiments in which
126 cells were exposed to $1\mu\text{M}$ ATRA for 24 hr, and then transferred to fresh media with-
127 out ATRA, confirmed the persistence of the self sustaining activated state for up to 144
128 hr (Fig. 3C). Thus, these experiments confirmed that reinforcing positive feedback likely
129 drives the ATRA-induced differentiation program. Next, we analyzed the ATRA-induced
130 downstream gene expression program following signalsome and cRaf activation.

131 The signal integration and phenotype modules described ATRA-induced gene expres-
132 sion events in wild-type HL-60 cells (Fig. 4). The signal initiation module produced two
133 outputs, activated Trigger and cRaf-pS621 which drove the expression of ATRA-induced
134 transcription factors, which then in turn activated the phenotypic program. In particular,
135 Trigger, which is a surrogate for the RAR α /RXR transcriptional complex, regulated the ex-
136 pression of the transcription factors CCATT/enhancer binding protein α (C/EBP α), PU.1,
137 and EGR1. In turn, these transcription factors, in combination with cRaf-pS621, regulated
138 the expression of downstream phenotypic markers such as CD38, CD11b or P47Phox.
139 We assembled the connectivity of the signal integration and phenotypic programs driven
140 by Trigger and cRaf-pS621 from literature (Table 1). We estimated the parameters which
141 appeared in the control laws regulating these programs from steady-state and dynamic
142 measurements of transcription factor and phenotypic marker expression following the ad-
143 dition of ATRA (22–25). However, the bulk of the remaining model parameters were taken
144 from directly from literature (26) and were not estimated in this study (see materials and
145 methods). The model simulations captured the time dependent expression of CD38 and
146 CD11b following the addition ATRA (Fig. 4A), and the steady-state for signal integration
147 and phenotypic markers (Fig. 4B). Lastly, we used the *predicted* values of the P21 and
148 E2F protein abundance to estimate a blackbox model of ATRA-induced G0 arrest (Fig.

149 5). The phenotype module predicted P21 expression significantly increased and E2F ex-
150 pression decreased, in response to ATRA exposure (Fig. 5A). We then used the ratio of
151 these values in a polynomial model to calculate the fraction of HL-60 cells in G0 arrest
152 following the addition of ATRA (Fig. 5B). The third-order polynomial model captured the
153 trend in measured G0-arrest values as a function of time, and was robust to uncertainty
154 in the measured data (Fig. 5B, gray). Taken together, the output of the signal integra-
155 tion and phenotypic modules was consistent with time-series and steady-state measure-
156 ments, thereby validating the assumed molecular connectivity. Moreover, outputs from
157 the phenotype module described the trend in ATRA-induced G0 cell cycle arrest. Next,
158 we explored which nodes and interactions between nodes in the signal integration module
159 most influenced the system response.

160 The composition of the signalingosome, and the kinase ultimately responsible for medi-
161 ating ATRA-induced Raf activation is currently unknown. To explore this question, we
162 conducted immunoprecipitation and subsequent Western blotting to identify physical in-
163 teractions between Raf and 19 putative interaction partners. A panel of 19 possible Raf
164 interaction partners (kinases, GTPases, scaffolding proteins etc) was constructed based
165 upon known signaling pathways. We did not consider the most likely binding partner, the
166 small GTPase RAS, as previous studies have ruled it out in MAPK activation in HL-60 cells
167 (20, 27). Total Raf was used as a bait protein for the immunoprecipitation studies. Interro-
168 gation of the Raf interactome suggested Vav1 was involved with ATRA-induced initiation
169 of MAPK activity (Fig. 7). Western blot analysis using total Raf and pS621 Raf specific
170 antibodies confirmed the presence of the bait protein, total and phosphorylated forms, in
171 the immunoprecipitate (Fig. 7A). Of the 19 proteins sampled, Vav1, Src, CK2, Akt, and
172 14-3-3 precipitated with Raf, suggesting a direct physical interaction was possible. How-
173 ever, only the associations between Raf and Vav1 and Raf and Src were ATRA-inducible
174 (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

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

223 **Discussion**

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

241 We conducted immunoprecipitation studies and identified a limited number of ATRA-
242 dependent and -independent Raf interaction partners. While we were unable to detect
243 the association of Raf with common kinases and GTPases such as PKC, PKA, p38, Rac
244 and Rho, we did establish potential interactions between Raf and key partners such as
245 Vav1, Src, Akt, CK2 and 14-3-3. All of these partners are known to be associated with Raf
246 activation or function. Src is known to bind Raf through an SH2 domain, and this associ-
247 ation has been shown to be dependent of the serine phosphorylation of Raf (34). Thus,
248 an ATRA inducible Src/Raf association may be a result of ATRA-induced Raf phospho-

249 phosphorylation at S259 or S621. We also identified an interaction between Raf and the Ser/Thr
250 kinases Akt and CK2. Akt can phosphorylate Raf at S259, as demonstrated by studies
251 in a human breast cancer line (35). CK2 can also phosphorylate Raf, although the lit-
252 erature has traditionally focused on S338 and not S621 or S259(36). However, neither
253 of these kinase interactions were ATRA-inducible, suggesting their association with Raf
254 alone was not associated with ATRA-induced Raf phosphorylation. The adapter protein
255 14-3-3 was also constitutively associated with Raf. The interaction between Raf and 14-
256 3-3 has been associated with both S621 and S259 phosphorylation and activity (37).
257 Additionally, the association of Raf with 14-3-3 not only stabilized S621 phosphorylation,
258 but also reversed the S621 phosphorylation from inhibitory to activating (38). Finally, we
259 found that Vav1/Raf association correlated with Raf activity, was ATRA-inducible and de-
260 creased after treatment with GW5074. The presence of Vav1 in Raf/Grb2 complexes has
261 been shown to correlate with increased Raf activity in mast cells (31). Furthermore, stud-
262 ies on Vav1 knockout mice demonstrated that the loss of Vav1 resulted in deficiencies of
263 ERK signaling for both T-cells as well as neutrophils (32, 33). Interestingly, while an in-
264 tegrin ligand-induced ROS response was blocked in Vav1 knockout neutrophils, TPA was
265 able to bypass the Vav1 requirement and stimulate both ERK phosphorylation and ROS
266 induction (33). In this study, the TPA-induced ROS response was dependent upon Raf
267 kinase activity, and was mitigated by the addition of GW5074. It is possible that Vav1 is
268 downstream of various integrin receptors but upstream of Raf in terms of inducible ROS
269 responses. Vav1 has also been shown to associate with a Cbl-Slp76-CD38 complex in an
270 ATRA-dependent manner; furthermore, transfection of HL-60 cells with Cbl mutants that
271 fail to bind CD38, yet still bind Slp76 and Vav1, prevents ATRA-induced MAPK activation
272 (13). The literature suggest a variety of possible receptor-signaling pathways, which in-
273 volve Vav1, for MAPK activation; moreover, given the ATRA-inducible association Vav1
274 may play a direct role in Raf activation.

275 We hypothesized that Vav1 is a member of an ATRA-inducible complex which propels
276 sustained MAPK activation, arrest and differentiation. Initially, ATRA-induced Vav1 ex-
277 pression drives increased association between Vav1 and Raf. This increased interaction
278 facilitates phosphorylation and activation of Raf by pre-bound Akt and/or CK2 at S621
279 or perhaps S259. Constitutively bound 14-3-3 may also stabilize the S621 phosphory-
280 lation, modulate the activity and/or up-regulate autophosphorylation. Activated Raf can
281 then drive ERK activation, which in turn closes the positive feedback loop by activating
282 Raf transcription factors, e.g. Sp1 and/or STAT1 (39–42). We tested this working hy-
283 pothesis using mathematical modeling. The model recapitulated both ATRA time-course
284 data as well as the GW5074 inhibitor effects. This suggested the proposed Raf-Vav1
285 architecture was at least consistent with the experimental studies. Further, analysis of
286 the Raf-Vav1 model identified bistability in ppERK levels. Thus, two possible MAPK ac-
287 tivation branches were possible for experimentally testable ATRA values. The analysis
288 also suggested the ATRA-induced Raf-Vav1 architecture could be locked into a sustained
289 signaling mode (high ppERK) even in the absence of a ATRA signal. This locked-in prop-
290 erty could give rise to an ATRA-induction memory. We validated the treatment memory
291 property predicted by the Raf-Vav1 circuit experimentally using ATRA-washout experi-
292 ments. ERK phosphorylation levels remained high for more then 96 hr after ATRA was
293 removed. Previous studies demonstrated that HL-60 cells possessed an inheritable mem-
294 ory of ATRA stimulus (43). Although the active state was self-sustaining, the inactive state
295 demonstrated considerable robustness to perturbation. For example, we found that 50x
296 overexpression of Raf was required to reliably lock MAPK into the activated state, while
297 small perturbations had almost no effect on ppERK levels over the entire ensemble. CD38
298 expression correlated with the ppERK, suggesting its involvement in the signaling com-
299 plex. Our computational and experimental results showed that positive feedback, through
300 ERK-dependent Raf expression, could sustain MAPK signaling through many division cy-

301 cles. Such molecular mechanisms could underly aspects of cellular memory associated
302 to consecutive ATRA treatments.

303 Several engineered, or naturally occurring systems involved in cell fate decisions incor-
304 porate positive feedback and bistability (44). One of the most well studied cell fate circuits
305 is the Mos mitogen-activated protein kinase cascade in *Xenopus* oocytes. This cascade
306 is activated when oocytes are induced by the steroid hormone progesterone (45). The
307 MEK-dependent activation of p42 MAPK stimulates the accumulation of the Mos onco-
308 protein, which in turn activates MEK, thereby closing the feedback loop. This is similar to
309 the differentiation circuit presented here; ATRA drives signalsome which activates MAPK,
310 cell-cycle arrest, differentiation and signalsome. Thus, while HL-60 and *Xenopus* oocytes
311 are vastly different biological models, they share similar cell fate decision architectures.
312 Other unrelated cell fate decisions such as programmed cell death have also been sug-
313 gested to be bistable (46). Still more biochemical networks important to human health,
314 for example the human coagulation or complement cascades, also feature strong positive
315 feedback elements (47). Thus, while positive feedback is sometimes not desirable in man-
316 made systems, it may be at the core of a diverse variety of cell fate programs and other
317 networks important to human health.

318 Model performance was impressive given its limited size. However, there were several
319 issues to explore further. First, there was likely missing connectivity in the effective differ-
320 entiation circuit. Decreasing BLR1 expression with simultaneously sustained cRaf-pS261
321 activation was not captured by the current network architecture. This suggested that
322 signalsome, once activated, had a long lifetime as decreased BLR1 expression did not
323 impact cRaf-pS261 abundance. We could model this by separating signalsome formation
324 into an inactive precursor pool that is transformed to a long-lived activated signalsome by
325 MAPK activation. We should also explore adding additional downstream biological mod-
326 ules to this skeleton model, for example the upregulation of reactive oxygen markers such

327 as p47Phox or cell cycle arrest components to capture the switch from an actively prolif-
328 erating population to a population in G0-arrest. Next, the choice of max/min integration
329 rules or the particular form of the transfer functions could also be explored. Integration
330 rules other than max/min could be used, such as the mean or the product, assuming the
331 range of the transfer functions is always $f \in [0, 1]$. Alternative integration rules might
332 have different properties which could influence model identification or performance. For
333 example, a mean integration rule would be differentiable, allowing derivative-based opti-
334 mization approaches to be used. The form of the transfer function could also be explored.
335 We choose hill-like functions because of their prominence in the systems and synthetic
336 biology community. However, many other transfer functions are possible.

337 **Materials and Methods**

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

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

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

$$g(p_1, \dots, p_{\mathcal{G}}, \kappa) = 0 \quad (3)$$

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

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

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

355 $V_T^{max} = k_T(R_1)$. The $(L_{T,o}/L_{T,j})$ term denotes the ratio of transcription read lengths; $L_{T,o}$
 356 represents a characteristic gene length, while $L_{T,j}$ denotes the length of gene j . Thus,
 357 the ratio $(L_{T,o}/L_{T,j})$ is a gene specific correction to the characteristic transcription rate
 358 V_T^{max} . The degradation rate constants were defined as $\theta_{m,j}$ and $\theta_{p,j}$ denote characteristic
 359 degradation constants for mRNA and protein, respectively. Lastly, the λ_j term denotes the
 360 constitutive rate of expression of gene j .

361 The gene expression control term $0 \leq u_j \leq 1$ depended upon the combination of fac-
 362 tors which influenced the expression of gene j . If the expression of gene j was influenced
 363 by $1, \dots, m$ factors, we modeled this relationship as $u_j = \mathcal{I}_j(f_{1j}(\cdot), \dots, f_{mj}(\cdot))$ where
 364 $0 \leq f_{ij}(\cdot) \leq 1$ denotes a regulatory transfer function quantifying the influence of factor i
 365 on the expression of gene j , and $\mathcal{I}_j(\cdot)$ denotes an integration rule which combines the
 366 individual regulatory inputs for gene j into a single control term. In this study, the integra-
 367 tion rule governing gene expression was the weighted fraction of promoter configurations
 368 that resulted in gene expression (48):

$$u_j = \frac{W_{R_{1,j}} + \sum_n W_{nj} f_{nj}}{1 + W_{R_{1,j}} + \sum_d W_{dj} f_{dj}} \quad (5)$$

369 The numerator, the weighted sum (with weights W_{nj}) of promoter configurations leading to
 370 gene expression, was normalized by all possible promoter configurations. The likelihood
 371 of each configuration was quantified by the transfer function f_{nj} (which we modeled using
 372 hill like functions), while the lead term in the numerator $W_{R_{1,j}}$ denotes the weight of con-
 373 stitutive expression for gene j . Given this formulation, the rate of constitutive expression
 374 was then given by:

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

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

376 lation rate was modeled as:

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

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

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

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

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

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

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

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

401 The generation rate was written as the product of a kinetic term ($\bar{r}_{+,i}$) and a control term
 402 (v_i). The control terms $0 \leq v_j \leq 1$ depended upon the combination of factors which in-
 403 fluenced rate process j . If rate j was influenced by $1, \dots, m$ factors, we modeled this
 404 relationship as $v_j = \mathcal{I}_j(f_{1j}(\cdot), \dots, f_{mj}(\cdot))$ where $0 \leq f_{ij}(\cdot) \leq 1$ denotes a regulatory
 405 transfer function quantifying the influence of factor i on rate j . The function $\mathcal{I}_j(\cdot)$ is an
 406 integration rule which maps the output of regulatory transfer functions into a control vari-
 407 able. In this study, we used $\mathcal{I}_j \in \{\min, \max\}$ and hill transfer functions (49). If a process
 408 had no modifying factors, $v_j = 1$. The kinetic rate of cRaf-pS621 generation $\bar{r}_{+,cRaf}$ was
 409 modeled as:

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

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

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

413 where I denotes the abundance of the MAPK inhibitor, and K_D denotes the inhibitor
414 affinity.

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

427 Signaling and gene expression model parameters were estimated by minimizing the
428 squared difference between simulations and experimental protein data set j . We mea-
429 sured the squared difference in the scale, fold change and shape for protein j :

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

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

436 following ATRA addition. The second term quantified the difference in the *fold-change* of
 437 protein j as a function of time. The terms $\hat{\mathcal{M}}_{ij}$ and \hat{y}_{ij} denote the scaled experimental
 438 observations and simulation outputs (fold-change; protein normalized by control value
 439 directly before ATRA addition) at time i from protein j , where T_j denoted the number of
 440 time points for data set j . Lastly, the third term of the objective function measured the
 441 difference in the *shape* of the measured and simulated protein levels. The scaled value
 442 $0 \leq \mathcal{M}'_{ij} \leq 1$ was given by:

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

443 where $\mathcal{M}'_{ij} = 0$ and $\mathcal{M}'_{ij} = 1$ describe the lowest (highest) intensity bands. A similar
 444 scaling was used for the simulation output. We minimized the total model residual $\sum_j E_j$
 445 using a heuristic direct-search optimization procedure, subject to box constraints on the
 446 parameter values, starting from a random initial parameter guess. Each downhill step was
 447 archived and used for ensemble calculations. The optimization procedure (a covariance
 448 matrix adaptation evolution strategy) has been reported previously (50).

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

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

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

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

455 The parameters a_0, \dots, a_3 were estimated directly from cell-cycle measurements (biologi-
456 cal replicates) using least-squares.

457 *Availability of model code.* The signaling and gene expression model equations, and the
458 parameter estimation procedure, were implemented in the Julia programming language.
459 The model equations were solved using the ODE23s routine of the ODE package (51). The
460 model code and parameter ensemble is freely available under an MIT software license
461 and can be downloaded from <http://www.varnerlab.org>.

462 *Cell culture and treatment* Human myeloblastic leukemia cells (HL-60 cells) were grown
463 in a humidified atmosphere of 5% CO₂ at 37°C and maintained in RPMI 1640 from Gibco
464 (Carlsbad, CA) supplemented with 5% heat inactivated fetal bovine serum from Hyclone
465 (Logan, UT) and 1× antibiotic/antimicotic (Gibco, Carlsbad, CA). Cells were cultured in
466 constant exponential growth (52). Experimental cultures were initiated at 0.1×10^6 cells/mL
467 24 hr prior to ATRA treatment; if indicated, cells were also treated with GW5074 (2 μ M) 18
468 hr before ATRA treatment. For the cell culture washout experiments, cells were treated
469 with ATRA for 24 hr, washed 3x with prewarmed serum supplemented culture medium
470 to remove ATRA, and reseeded in ATRA-free media as described. Western blot analysis
471 was performed at incremental time points after removal of ATRA.

472 *Chemicals* All-Trans Retinoic Acid (ATRA) from Sigma-Aldrich (St. Louis, MO) was dis-
473 solved in 100% ethanol with a stock concentration of 5mM, and used at a final concen-
474 tration of 1 μ M (unless otherwise noted). The cRaf inhibitor GW5074 from Sigma-Aldrich
475 (St. Louis, MO) was dissolved in DMSO with a stock concentration of 10mM, and used
476 at a final concentration of 2 μ M. HL-60 cells were treated with 2 μ M GW5074 with or with-
477 out ATRA (1 μ M) at 0 hr. This GW5074 dosage had a negligible effect on the cell cycle
478 distribution, compared to ATRA treatment alone.

479 *Immunoprecipitation and western blotting* Approximately 1.2×10^7 cells were lysed using
480 $400\mu\text{L}$ of M-Per lysis buffer from Thermo Scientific (Waltham, MA). Lysates were cleared
481 by centrifugation at $16,950 \times g$ in a micro-centrifuge for 20 min at 4°C . Lysates were
482 pre-cleared using $100\mu\text{L}$ protein A/G Plus agarose beads from Santa Cruz Biotechnology
483 (Santa Cruz, CA) by inverting overnight at 4°C . Beads were cleared by centrifugation and
484 total protein concentration was determined by a BCA assay (Thermo Scientific, Waltham,
485 MA). Immunoprecipitations were setup by bringing lysate to a concentration of 1g/L in a
486 total volume of $300\mu\text{L}$ (M-Per buffer was used for dilution). The anti-Raf antibody was
487 added at $3\mu\text{L}$. A negative control with no bait protein was also used to exclude the di-
488 rect interaction of proteins with the A/G beads. After 1 hr of inversion at 4°C , $20\mu\text{L}$ of
489 agarose beads was added and samples were left to invert overnight at 4°C . Samples
490 were then washed three times with M-Per buffer by centrifugation. Finally proteins were
491 eluted from agarose beads using a laemmli loading buffer. Eluted proteins were resolved
492 by SDS-PAGE and Western blotting. Total lysate samples were normalized by total protein
493 concentration ($20\mu\text{g}$ per sample) and resolved by SDS-PAGE and Western blotting. Sec-
494 ondary HRP bound antibody was used for visualization. All antibodies were purchased
495 from Cell Signaling (Boston, MA) with the exception of α -p621 Raf which was purchased
496 from Biosource/Invitrogen (Carlsbad, CA), and α -CK2 from BD Biosciences (San Jose,
497 CA).

498 *Morphology assessment* Untreated and ATRA-treated HL-60 cells were collected after
499 72 hr and cytocentrifuged for 3 min at 700 rpm onto glass slides. Slides were air-dried
500 and stained with Wright's stain. Slide images were captured at 40X (Leica DM LB 100T
501 microscope, Leica Microsystems).

502 **Competing interests**

503 The authors declare that they have no competing interests.

504 **Author's contributions**

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

510 **Acknowledgements**

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

513 **Funding**

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

521 **References**

- 522 1. Bushue N, Wan YJY (2010) Retinoid pathway and cancer therapeutics. *Adv Drug
523 Deliv Rev* 62: 1285-98.
- 524 2. Tang XH, Gudas LJ (2011) Retinoids, retinoic acid receptors, and cancer. *Annu Rev
525 Pathol* 6: 345-64.
- 526 3. Cheung FSG, Lovicu FJ, Reichardt JKV (2012) Current progress in using vitamin d
527 and its analogs for cancer prevention and treatment. *Expert Rev Anticancer Ther*
528 12: 811-37.
- 529 4. Nilsson B (1984) Probable in vivo induction of differentiation by retinoic acid of
530 promyelocytes in acute promyelocytic leukaemia. *Br J Haematol* 57: 365-71.
- 531 5. Warrell RP Jr (1993) Retinoid resistance in acute promyelocytic leukemia: new
532 mechanisms, strategies, and implications. *Blood* 82: 1949-53.
- 533 6. Freemantle SJ, Spinella MJ, Dmitrovsky E (2003) Retinoids in cancer therapy and
534 chemoprevention: promise meets resistance. *Oncogene* 22: 7305-15.
- 535 7. Breitman TR, Selonick SE, Collins SJ (1980) Induction of differentiation of the hu-
536 man promyelocytic leukemia cell line (HL-60) by retinoic acid. *Proc Natl Acad Sci U
537 S A* 77: 2936–2940.
- 538 8. Yen A, Roberson MS, Varvayanis S, Lee AT (1998) Retinoic acid induced
539 mitogen-activated protein (MAP)/extracellular signal-regulated kinase (ERK) kinase-
540 dependent MAP kinase activation needed to elicit HL-60 cell differentiation and
541 growth arrest. *Cancer Res* 58: 3163–3172.
- 542 9. Hong HY, Varvayanis S, Yen A (2001) Retinoic acid causes MEK-dependent RAF
543 phosphorylation through RARalpha plus RXR activation in HL-60 cells. *Differentia-
544 tion* 68: 55–66.
- 545 10. Mangelsdorf DJ, Ong ES, Dyck JA, Evans RM (1990) Nuclear receptor that identifies
546 a novel retinoic acid response pathway. *Nature* 345: 224–229.

- 547 11. Congleton J, MacDonald R, Yen A (2012) Src inhibitors, PP2 and dasatinib, increase
548 retinoic acid-induced association of Lyn and c-Raf (S259) and enhance MAPK-
549 dependent differentiation of myeloid leukemia cells. Leukemia 26: 1180-8.
- 550 12. Shen M, Bunaci R, Congleton J, Jensen H, Sayam L, et al. (2011) Interferon regu-
551 latory factor-1 binds c-Cbl, enhances mitogen activated protein kinase signaling and
552 promotes retinoic acid-induced differentiation of HL-60 human myelo-monoblastic
553 leukemia cells. Leuk Lymphoma 52: 2372-9.
- 554 13. Shen M, Yen A (2009) c-Cbl tyrosine kinase-binding domain mutant G306E abol-
555 ishes the interaction of c-Cbl with CD38 and fails to promote retinoic acid-induced
556 cell differentiation and G0 arrest. J Biol Chem 284: 25664–25677.
- 557 14. Yen A, Varvayanis S, Smith J, Lamkin T (2006) Retinoic acid induces expression of
558 SLP-76: expression with c-FMS enhances ERK activation and retinoic acid-induced
559 differentiation/G0 arrest of HL-60 cells. Eur J Cell Biol 85: 117–132.
- 560 15. Marchisio M, Bertagnolo V, Colamussi ML, Capitani S, Neri LM (1998) Phos-
561 phatidylinositol 3-kinase in HL-60 nuclei is bound to the nuclear matrix and increases
562 during granulocytic differentiation. Biochem Biophys Res Commun 253: 346-51.
- 563 16. Congleton J, Jiang H, Malavasi F, Lin H, Yen A (2011) ATRA-induced HL-60 myeloid
564 leukemia cell differentiation depends on the CD38 cytosolic tail needed for mem-
565 brane localization, but CD38 enzymatic activity is unnecessary. Exp Cell Res 317:
566 910–919.
- 567 17. Wang J, Yen A (2004) A novel retinoic acid-responsive element regulates retinoic
568 acid induced BLR1 expression. Mol Cell Biol 24: 2423 - 2443.
- 569 18. Yen A (1990) HL-60 cells as a model of growth and differentiation: the significance
570 of variant cells. Hematology Review 4: 5-46.
- 571 19. Yang T, Xiong Q, Enslen H, Davis R, Chow CW (2002) Phosphorylation of NFATc4
572 by p38 mitogen-activated protein kinases. Mol Cell Biol 22: 3892–3904.

- 573 20. Wang J, Yen A (2008) A MAPK-positive Feedback Mechanism for BLR1 Signaling
574 Propels Retinoic Acid-triggered Differentiation and Cell Cycle Arrest. *J Biol Chem*
575 283: 4375–4386.
- 576 21. Tasseff R, Nayak S, Song S, Yen A, Varner J (2011) Modeling and analysis of retinoic
577 acid induced differentiation of uncommitted precursor cells. *Integr Biol* 3: 578 - 591.
- 578 22. Jensen HA, Styskal LE, Tasseff R, Bunaciu RP, Congleton J, et al. (2013) The
579 src-family kinase inhibitor pp2 rescues inducible differentiation events in emergent
580 retinoic acid-resistant myeloblastic leukemia cells. *PLoS One* 8: e58621.
- 581 23. Jensen HA, Bunaciu RP, Ibabao CN, Myers R, Varner JD, et al. (2014) Retinoic acid
582 therapy resistance progresses from unilineage to bilineage in hl-60 leukemic blasts.
583 *PLoS One* 9: e98929.
- 584 24. Jensen HA, Bunaciu RP, Varner JD, Yen A (2015) Gw5074 and pp2 kinase inhibitors
585 implicate nontraditional c-raf and lyn function as drivers of retinoic acid-induced mat-
586 uration. *Cell Signal* 27: 1666-75.
- 587 25. Jensen HA, Yourish HB, Bunaciu RP, Varner JD, Yen A (2015) Induced myelomono-
588 cytic differentiation in leukemia cells is accompanied by noncanonical transcription
589 factor expression. *FEBS Open Bio* 5: 789-800.
- 590 26. Milo R, Jorgensen P, Moran U, Weber G, Springer M (2010) Bionumbers—the
591 database of key numbers in molecular and cell biology. *Nucleic Acids Res* 38: D750-
592 3.
- 593 27. Katagiri K, Hattori S, Nakamura S, Yamamoto T, Yoshida T, et al. (1994) Activation
594 of ras and formation of gap complex during tpa-induced monocytic differentiation of
595 hl-60 cells. *Blood* 84: 1780–1789.
- 596 28. Miranda MB, Johnson DE (2007) Signal transduction pathways that contribute to
597 myeloid differentiation. *Leukemia* 21: 1363–1377.
- 598 29. Hickstein DD, Back AL, Collins SJ (1989) Regulation of expression of the cd11b and

- 599 cd18 subunits of the neutrophil adherence receptor during human myeloid differen-
600 tiation. *J Biol Chem* 264: 21812–21817.
- 601 30. Hornstein I, Alcover A, Katzav S (2004) Vav proteins, masters of the world of cy-
602 toskeleton organization. *Cell Signal* 16: 1-11.
- 603 31. Song JS, Gomez J, Stancato LF, Rivera J (1996) Association of a p95 vav-containing
604 signaling complex with the fcepsilonlonri gamma chain in the rbl-2h3 mast cell line.
605 evidence for a constitutive in vivo association of vav with grb2, raf-1, and erk2 in an
606 active complex. *J Biol Chem* 271: 26962–26970.
- 607 32. Costello PS, Walters AE, Mee PJ, Turner M, Reynolds LF, et al. (1999) The rho-
608 family gtp exchange factor vav is a critical transducer of t cell receptor signals to the
609 calcium, erk, and nf-kappab pathways. *Proc Natl Acad Sci U S A* 96: 3035–3040.
- 610 33. Graham D, Robertson C, Bautista J, Mascarenhas F, Diacovo M, et al. (2007)
611 Neutrophil-mediated oxidative burst and host defense are controlled by a Vav-
612 PLCgamma2 signaling axis in mice. *J Clin Invest* 117: 3445–3452.
- 613 34. Cleghon V, Morrison DK (1994) Raf-1 interacts with fyn and src in a non-
614 phosphotyrosine-dependent manner. *J Biol Chem* 269: 17749–17755.
- 615 35. Zimmermann S, Moelling K (1999) Phosphorylation and regulation of raf by akt (pro-
616 tein kinase b). *Science* 286: 1741–1744.
- 617 36. Ritt DA, Zhou M, Conrads TP, Veenstra TD, Copeland TD, et al. (2007) Ck2 is a
618 component of the ksr1 scaffold complex that contributes to raf kinase activation.
619 *Curr Biol* 17: 179–184.
- 620 37. Hekman M, Wiese S, Metz R, Albert S, Troppmair J, et al. (2004) Dynamic changes
621 in c-raf phosphorylation and 14-3-3 protein binding in response to growth factor stim-
622 ulation: differential roles of 14-3-3 protein binding sites. *J Biol Chem* 279: 14074–
623 14086.
- 624 38. Dhillon AS, Yip YY, Grindlay GJ, Pakay JL, Dangers M, et al. (2009) The c-terminus

- 625 of raf-1 acts as a 14-3-3-dependent activation switch. *Cell Signal* 21: 1645–1651.
- 626 39. Kim HS, Lim IK (2009) Phosphorylated extracellular signal-regulated protein kinases
627 1 and 2 phosphorylate sp1 on serine 59 and regulate cellular senescence via tran-
628 scription of p21sdi1/cip1/waf1. *J Biol Chem* 284: 15475–15486.
- 629 40. Milanini-Mongiat J, Pouyss?gur J, Pag? G (2002) Identification of two sp1 phos-
630 phorylation sites for p42/p44 mitogen-activated protein kinases: their implication in
631 vascular endothelial growth factor gene transcription. *J Biol Chem* 277: 20631–
632 20639.
- 633 41. Zhang Y, Cho YY, Petersen BL, Zhu F, Dong Z (2004) Evidence of stat1 phosphory-
634 lation modulated by mapks, mek1 and msk1. *Carcinogenesis* 25: 1165–1175.
- 635 42. Li Z, Theus MH, Wei L (2006) Role of erk 1/2 signaling in neuronal differentiation of
636 cultured embryonic stem cells. *Dev Growth Differ* 48: 513–523.
- 637 43. Yen A, Reece SL, Albright KL (1984) Dependence of hl-60 myeloid cell differentiation
638 on continuous and split retinoic acid exposures: precommitment memory associated
639 with altered nuclear structure. *J Cell Physiol* 118: 277–286.
- 640 44. Ferrell J (2002) Self-perpetuating states in signal transduction: positive feedback,
641 double-negative feedback and bistability. *Curr Opin Cell Biol* 14: 140-8.
- 642 45. Xiong W, Ferrell J (2003) A positive-feedback-based bistable 'memory module' that
643 governs a cell fate decision. *Nature* 426: 460-5.
- 644 46. Bagci EZ, Vodovotz Y, Billiar TR, Ermentrout GB, Bahar I (2006) Bistability in apop-
645 tosis: roles of bax, bcl-2, and mitochondrial permeability transition pores. *Biophys J*
646 90: 1546-59.
- 647 47. Luan D, Zai M, Varner JD (2007) Computationally derived points of fragility of a
648 human cascade are consistent with current therapeutic strategies. *PLoS Comput
649 Biol* 3: e142.
- 650 48. Moon TS, Lou C, Tamsir A, Stanton BC, Voigt CA (2012) Genetic programs con-

- 651 stuctured from layered logic gates in single cells. *Nature* 491: 249-53.
- 652 49. Wayman JA, Sagar A, Varner JD (2015) Dynamic modeling of cell-free biochemical
653 networks using effective kinetic models. *Processes* 3: 138.
- 654 50. Igel C, Hansen N, Roth S (2007) Covariance matrix adaptation for multi-objective
655 optimization. *Evol Comput* 15: 1-28.
- 656 51. Bezanson J, Edelman A, Karpinski S, Shah VB (2014) Julia: A fresh approach to
657 numerical computing. *CoRR* abs/1411.1607.
- 658 52. Brooks SC, Kazmer S, Levin AA, Yen A (1996) Myeloid differentiation and retinoblas-
659 toma phosphorylation changes in HL-60 cells induced by retinoic acid receptor- and
660 retinoid X receptor-selective retinoic acid analogs. *Blood* 87: 227-237.
- 661 53. Rishi AK, Gerald TM, Shao ZM, Li XS, Baumann RG, et al. (1996) Regulation of the
662 human retinoic acid receptor alpha gene in the estrogen receptor negative human
663 breast carcinoma cell lines skbr-3 and mda-mb-435. *Cancer Res* 56: 5246-52.
- 664 54. Mueller BU, Pabst T, Fos J, Petkovic V, Fey MF, et al. (2006) Atra resolves the dif-
665 ferentiation block in t(15;17) acute myeloid leukemia by restoring pu.1 expression.
666 *Blood* 107: 3330-8.
- 667 55. Friedman AD (2007) Transcriptional control of granulocyte and monocyte develop-
668 ment. *Oncogene* 26: 6816-28.
- 669 56. Luo XM, Ross AC (2006) Retinoic acid exerts dual regulatory actions on the ex-
670 pression and nuclear localization of interferon regulatory factor-1. *Exp Biol Med*
671 (Maywood) 231: 619-31.
- 672 57. Sylvester I, Schöler HR (1994) Regulation of the oct-4 gene by nuclear receptors.
673 *Nucleic Acids Res* 22: 901-11.
- 674 58. Drach J, McQueen T, Engel H, Andreeff M, Robertson KA, et al. (1994) Retinoic
675 acid-induced expression of cd38 antigen in myeloid cells is mediated through
676 retinoic acid receptor-alpha. *Cancer Res* 54: 1746-52.

- 677 59. Liu M, Iavarone A, Freedman LP (1996) Transcriptional activation of the human
678 p21(waf1/cip1) gene by retinoic acid receptor. correlation with retinoid induction of
679 u937 cell differentiation. J Biol Chem 271: 31723-8.
- 680 60. Bunaciu RP, Yen A (2013) 6-formylindolo (3,2-b)carbazole (ficz) enhances retinoic
681 acid (ra)-induced differentiation of hl-60 myeloblastic leukemia cells. Mol Cancer 12:
682 39.
- 683 61. Balmer JE, Blomhoff R (2002) Gene expression regulation by retinoic acid. J Lipid
684 Res 43: 1773-808.
- 685 62. Rosen ED, Hsu CH, Wang X, Sakai S, Freeman MW, et al. (2002) C/ebpalpha in-
686 duces adipogenesis through ppargamma: a unified pathway. Genes Dev 16: 22-6.
- 687 63. Varley CL, Bacon EJ, Holder JC, Southgate J (2009) Foxa1 and irf-1 intermediary
688 transcriptional regulators of ppargamma-induced urothelial cytodifferentiation. Cell
689 Death Differ 16: 103-14.
- 690 64. Bruemmer D, Yin F, Liu J, Berger JP, Sakai T, et al. (2003) Regulation of the growth
691 arrest and dna damage-inducible gene 45 (gadd45) by peroxisome proliferator-
692 activated receptor gamma in vascular smooth muscle cells. Circ Res 93: e38-47.
- 693 65. Delerive P, De Bosscher K, Besnard S, Vanden Berghe W, Peters JM, et al. (1999)
694 Peroxisome proliferator-activated receptor alpha negatively regulates the vascular
695 inflammatory gene response by negative cross-talk with transcription factors nf-
696 kappaB and ap-1. J Biol Chem 274: 32048-54.
- 697 66. Altinok S, Xu M, Spiegelman BM (1997) Ppargamma induces cell cycle withdrawal:
698 inhibition of e2f/dp dna-binding activity via down-regulation of pp2a. Genes Dev 11:
699 1987-98.
- 700 67. Fei J, Cook C, Gillespie M, Yu B, Fullen K, et al. (2011) Atherogenic ω -6 lipids mod-
701 ulate ppar- egr-1 crosstalk in vascular cells. PPAR Res 2011: 753917.
- 702 68. Song EK, Lee YR, Kim YR, Yeom JH, Yoo CH, et al. (2012) Naadp mediates insulin-

- 703 stimulated glucose uptake and insulin sensitization by ppar γ in adipocytes. Cell Rep
704 2: 1607-19.
- 705 69. Szanto A, Nagy L (2005) Retinoids potentiate peroxisome proliferator-activated re-
706 ceptor gamma action in differentiation, gene expression, and lipid metabolic pro-
707 cesses in developing myeloid cells. Mol Pharmacol 67: 1935-43.
- 708 70. Han S, Sidell N, Fisher PB, Roman J (2004) Up-regulation of p21 gene expres-
709 sion by peroxisome proliferator-activated receptor gamma in human lung carcinoma
710 cells. Clin Cancer Res 10: 1911-9.
- 711 71. Von Knethen A, Brüne B (2002) Activation of peroxisome proliferator-activated re-
712 ceptor gamma by nitric oxide in monocytes/macrophages down-regulates p47phox
713 and attenuates the respiratory burst. J Immunol 169: 2619-26.
- 714 72. Dispirito JR, Fang B, Wang F, Lazar MA (2013) Pruning of the adipocyte peroxisome
715 proliferator-activated receptor γ cistrome by hematopoietic master regulator pu.1.
716 Mol Cell Biol 33: 3354-64.
- 717 73. Chen H, Ray-Gallet D, Zhang P, Hetherington CJ, Gonzalez DA, et al. (1995) Pu.1
718 (spi-1) autoregulates its expression in myeloid cells. Oncogene 11: 1549-60.
- 719 74. Steidl U, Rosenbauer F, Verhaak RGW, Gu X, Ebralidze A, et al. (2006) Essential
720 role of jun family transcription factors in pu.1 knockdown-induced leukemic stem
721 cells. Nat Genet 38: 1269-77.
- 722 75. Laslo P, Spooner CJ, Warmflash A, Lancki DW, Lee HJ, et al. (2006) Multilineage
723 transcriptional priming and determination of alternate hematopoietic cell fates. Cell
724 126: 755-66.
- 725 76. Pahl HL, Scheibe RJ, Zhang DE, Chen HM, Galson DL, et al. (1993) The proto-
726 oncogene pu.1 regulates expression of the myeloid-specific cd11b promoter. J Biol
727 Chem 268: 5014-20.
- 728 77. Yuki H, Ueno S, Tatetsu H, Niiro H, Iino T, et al. (2013) Pu.1 is a potent tumor

- 729 suppressor in classical hodgkin lymphoma cells. Blood 121: 962-70.
- 730 78. Li SL, Schlegel W, Valente AJ, Clark RA (1999) Critical flanking sequences of pu.1
731 binding sites in myeloid-specific promoters. J Biol Chem 274: 32453-60.
- 732 79. Dahl R, Walsh JC, Lancki D, Laslo P, Iyer SR, et al. (2003) Regulation of macrophage
733 and neutrophil cell fates by the pu.1:c/ebpalpha ratio and granulocyte colony-
734 stimulating factor. Nat Immunol 4: 1029-36.
- 735 80. Timchenko N, Wilson DR, Taylor LR, Abdelsayed S, Wilde M, et al. (1995) Autoreg-
736 ulation of the human c/ebp alpha gene by stimulation of upstream stimulatory factor
737 binding. Mol Cell Biol 15: 1192-202.
- 738 81. Lidonnici MR, Audia A, Soliera AR, Prisco M, Ferrari-Amorotti G, et al. (2010) Ex-
739 pression of the transcriptional repressor gfi-1 is regulated by c/ebpalpha and is
740 involved in its proliferation and colony formation-inhibitory effects in p210bcr/abl-
741 expressing cells. Cancer Res 70: 7949-59.
- 742 82. D'Alo' F, Johansen LM, Nelson EA, Radomska HS, Evans EK, et al. (2003) The
743 amino terminal and e2f interaction domains are critical for c/ebp alpha-mediated
744 induction of granulopoietic development of hematopoietic cells. Blood 102: 3163-
745 71.
- 746 83. Pan Z, Hetherington CJ, Zhang DE (1999) Ccaat/enhancer-binding protein activates
747 the cd14 promoter and mediates transforming growth factor beta signaling in mono-
748 cyte development. J Biol Chem 274: 23242-8.
- 749 84. Harris TE, Albrecht JH, Nakanishi M, Darlington GJ (2001) Ccaat/enhancer-binding
750 protein-alpha cooperates with p21 to inhibit cyclin-dependent kinase-2 activity and
751 induces growth arrest independent of dna binding. J Biol Chem 276: 29200-9.
- 752 85. Bauvois B, Durant L, Laboureau J, Barthélémy E, Rouillard D, et al. (1999) Upreg-
753 ulation of cd38 gene expression in leukemic b cells by interferon types i and ii. J
754 Interferon Cytokine Res 19: 1059-66.

- 755 86. Passioura T, Dolnikov A, Shen S, Symonds G (2005) N-ras-induced growth suppression
756 of myeloid cells is mediated by irf-1. *Cancer Res* 65: 797-804.
- 757 87. Dahl R, Iyer SR, Owens KS, Cuylear DD, Simon MC (2007) The transcriptional
758 repressor gfi-1 antagonizes pu.1 activity through protein-protein interaction. *J Biol
759 Chem* 282: 6473-83.
- 760 88. Duan Z, Horwitz M (2003) Targets of the transcriptional repressor oncoprotein gfi-1.
761 *Proc Natl Acad Sci U S A* 100: 5932-7.
- 762 89. Chen H, Zhang P, Radomska HS, Hetherington CJ, Zhang DE, et al. (1996) Octamer
763 binding factors and their coactivator can activate the murine pu.1 (spi-1) promoter.
764 *J Biol Chem* 271: 15743-52.
- 765 90. Behre G, Whitmarsh AJ, Coghlan MP, Hoang T, Carpenter CL, et al. (1999) c-jun
766 is a jnk-independent coactivator of the pu.1 transcription factor. *J Biol Chem* 274:
767 4939-46.
- 768 91. Kardassis D, Papakosta P, Pardali K, Moustakas A (1999) c-jun transactivates the
769 promoter of the human p21(waf1/cip1) gene by acting as a superactivator of the
770 ubiquitous transcription factor sp1. *J Biol Chem* 274: 29572-81.
- 771 92. Johnson DG, Ohtani K, Nevins JR (1994) Autoregulatory control of e2f1 expression
772 in response to positive and negative regulators of cell cycle progression. *Genes Dev*
773 8: 1514-25.
- 774 93. Fu M, Zhang J, Lin Y, Zhu X, Ehrengruber MU, et al. (2002) Early growth response
775 factor-1 is a critical transcriptional mediator of peroxisome proliferator-activated
776 receptor-gamma 1 gene expression in human aortic smooth muscle cells. *J Biol
777 Chem* 277: 26808-14.
- 778 94. Mak KS, Funnell APW, Pearson RCM, Crossley M (2011) Pu.1 and haematopoietic
779 cell fate: Dosage matters. *Int J Cell Biol* 2011: 808524.
- 780 95. Chen F, Wang Q, Wang X, Studzinski GP (2004) Up-regulation of egr1 by 1,25-

- 781 dihydroxyvitamin d3 contributes to increased expression of p35 activator of cyclin-
782 dependent kinase 5 and consequent onset of the terminal phase of hl60 cell differ-
783 entiation. *Cancer Res* 64: 5425-33.
- 784 96. Suh J, Jeon YJ, Kim HM, Kang JS, Kaminski NE, et al. (2002) Aryl hydrocarbon
785 receptor-dependent inhibition of ap-1 activity by 2,3,7,8-tetrachlorodibenzo-p-dioxin
786 in activated b cells. *Toxicol Appl Pharmacol* 181: 116-23.
- 787 97. Shen M, Bunaciu RP, Congleton J, Jensen HA, Sayam LG, et al. (2011) Inter-
788 feron regulatory factor-1 binds c-cbl, enhances mitogen activated protein kinase
789 signaling and promotes retinoic acid-induced differentiation of hl-60 human myelo-
790 monoblastic leukemia cells. *Leuk Lymphoma* 52: 2372-9.
- 791 98. Bunaciu RP, Yen A (2011) Activation of the aryl hydrocarbon receptor ahr promotes
792 retinoic acid-induced differentiation of myeloblastic leukemia cells by restricting ex-
793 pression of the stem cell transcription factor oct4. *Cancer Res* 71: 2371-80.
- 794 99. Jackson DA, Pombo A, Iborra F (2000) The balance sheet for transcription: an anal-
795 ysis of nuclear rna metabolism in mammalian cells. *FASEB J* 14: 242-54.
- 796 100. Zhao ZW, Roy R, Gebhardt JCM, Suter DM, Chapman AR, et al. (2014) Spatial orga-
797 nization of rna polymerase ii inside a mammalian cell nucleus revealed by reflected
798 light-sheet superresolution microscopy. *Proc Natl Acad Sci U S A* 111: 681-6.
- 799 101. Freitas R, Merkle R (2004) Kinematic Self-Replicating Machines. Oxford University
800 Press.
- 801 102. Yang E, van Nimwegen E, Zavolan M, Rajewsky N, Schroeder M, et al. (2003) De-
802 cay rates of human mrnas: correlation with functional characteristics and sequence
803 attributes. *Genome Res* 13: 1863-72.
- 804 103. Doherty MK, Hammond DE, Clague MJ, Gaskell SJ, Beynon RJ (2009) Turnover
805 of the human proteome: determination of protein intracellular stability by dynamic
806 silac. *J Proteome Res* 8: 104-12.

- 807 104. Darzacq X, Shav-Tal Y, de Turris V, Brody Y, Shenoy SM, et al. (2007) In vivo dy-
808 namics of rna polymerase ii transcription. Nat Struct Mol Biol 14: 796-806.
- 809 105. Boström K, Wettsten M, Borén J, Bondjers G, Wiklund O, et al. (1986) Pulse-chase
810 studies of the synthesis and intracellular transport of apolipoprotein b-100 in hep g2
811 cells. J Biol Chem 261: 13800-6.
- 812 106. Meyers R, editor (2004) Encyclopedia of Molecular Cell Biology and Molecular
813 Medicine, Volume 1, 2nd Edition. ISBN: 978-3-527-30543-8. Wiley-Blackwell.
- 814 107. Rosenbluth MJ, Lam WA, Fletcher DA (2006) Force microscopy of nonadherent
815 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.

816

817

Effector	Effect	Target	Source
RAR α	+	RAR α	(53)
	+	PU.1	(54)
	+	C/EBP α	(55)
	+	IRF-1	(56)
	-	Oct4	(57)
	+	CD38	(58)
	+	p21	(59)
	+	AhR	(60)
	+	EGR1	(61)
PPAR γ	+	C/EBP α	(62)
	+	IRF-1	(63)
	+	Oct1	(64)
	-	AP-1	(65)
	-	E2F	(66)
	-	EGR1	(67)
	+	CD38	(68)
	+	CD14	(69)
	+	p21	(70)
	-	p47phox	(71)
PU.1	-	PPAR γ	(72)
	+	PU.1	(73)
	+	AP-1	(74)
	+	EGR1	(75)
	+	CD11b	(76)
	+	p21	(77)
	+	p47phox	(78)
C/EBP α	+	PPAR γ	(62)
	+	PU.1	(79)
	+	C/EBP α	(80)
	+	Gfi-1	(81)
	-	E2F	(82)
	+	CD14	(83)

	+	p21	(84)
IRF-1	+	CD38	(85)
	+	p21	(86)
	-	PU.1	(87)
	-	C/EBP α	(88)
	-	E2F	(88)
	-	EGR1	(75)
	-	p21	(88)
Oct1	+	PU.1	(89)
AP-1	-	PPAR γ	(65)
	+	PU.1	(90)
	+	p21	(91)
E2F	+	E2F	(92)
EGR1	+	PPAR γ	(93)
	-	Gfi-1	(94)
	+	CD14	(95)
AhR	+	AP-1	(96)
	+	IRF-1	(97)
	-	Oct4	(98)
	-	PU.1	

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	1×10^6	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
820	t_d	HL-60 doubling time	19.5	hr
	μ	growth rate	0.035	hr^{-1}
	k_d	death rate	0.10μ	hr^{-1}
	e_T	elongation rate RNA polymerase	6	nt/s
	e_X	elongation rate Ribosome	5	aa/s
	$L_{T,o}$	characteristic gene length	15,000	nt
	$L_{X,o}$	characteristic transcript length	5,000	nt
	k_T	characteristic transcription rate	1.44	hr^{-1}
	k_X	characteristic translation rate	3.60	hr^{-1}
	D	Diameter of an HL-60 cell	12.4	μm^3
	f_C	cytoplasmic fraction	0.51	dimensionless

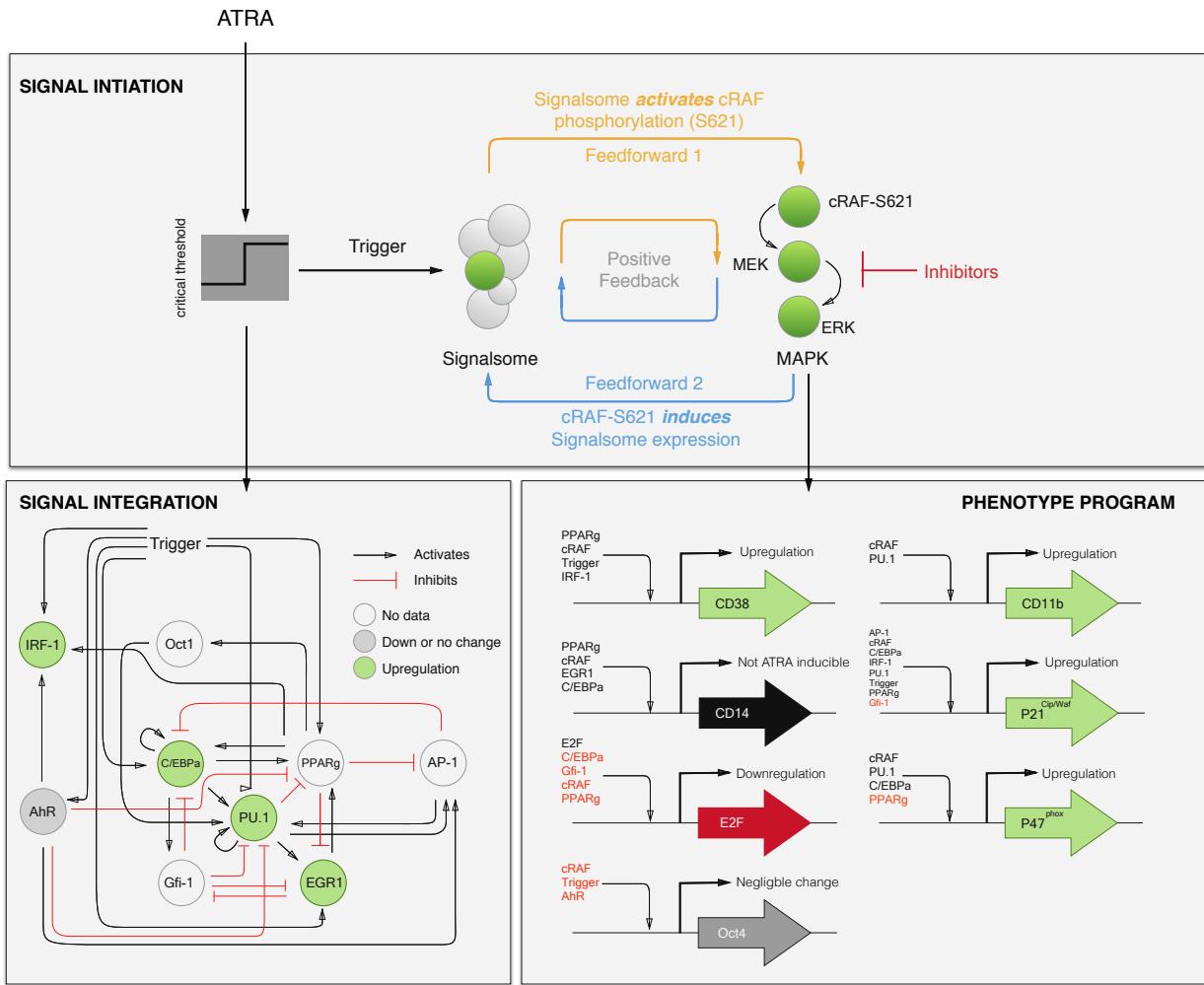


Fig. 1: Schematic of the effective ATRA differentiation circuit. Above a critical threshold, ATRA activates an upstream Trigger, which induces signalsome complex formation. Signalsome activates the mitogen-activated protein kinase (MAPK) cascade which in turn drives the differentiation program and signalsome 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.

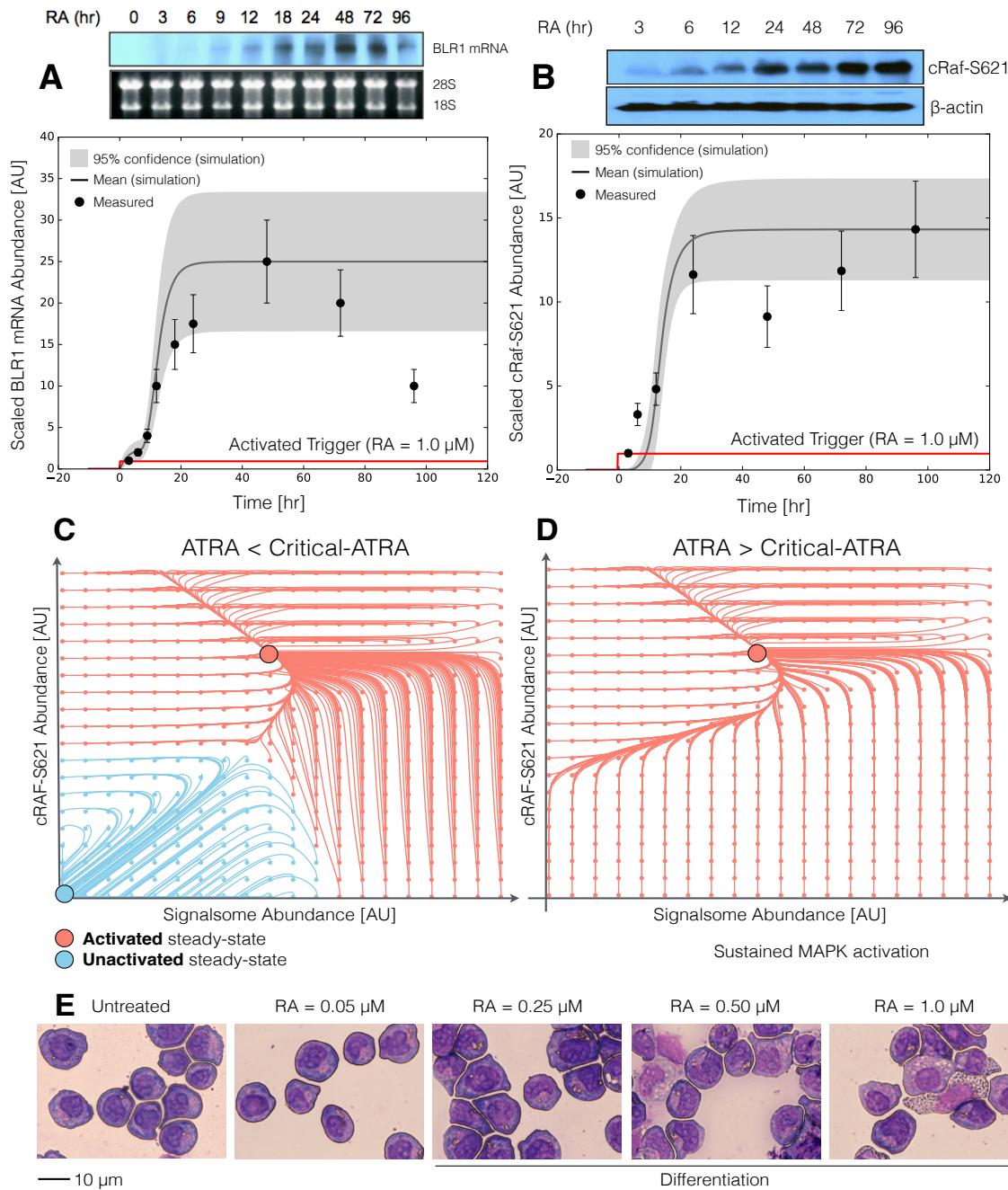


Fig. 2: Model analysis for ATRA-induced HL-60 differentiation. A: BLR1 mRNA versus time following exposure to $1\mu\text{M}$ ATRA at $t = 0$ hr. B: cRaf-pS621 versus time following exposure to $1\mu\text{M}$ ATRA at $t = 0$ hr. Points denote experimental measurements, solid lines denote the mean model 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).

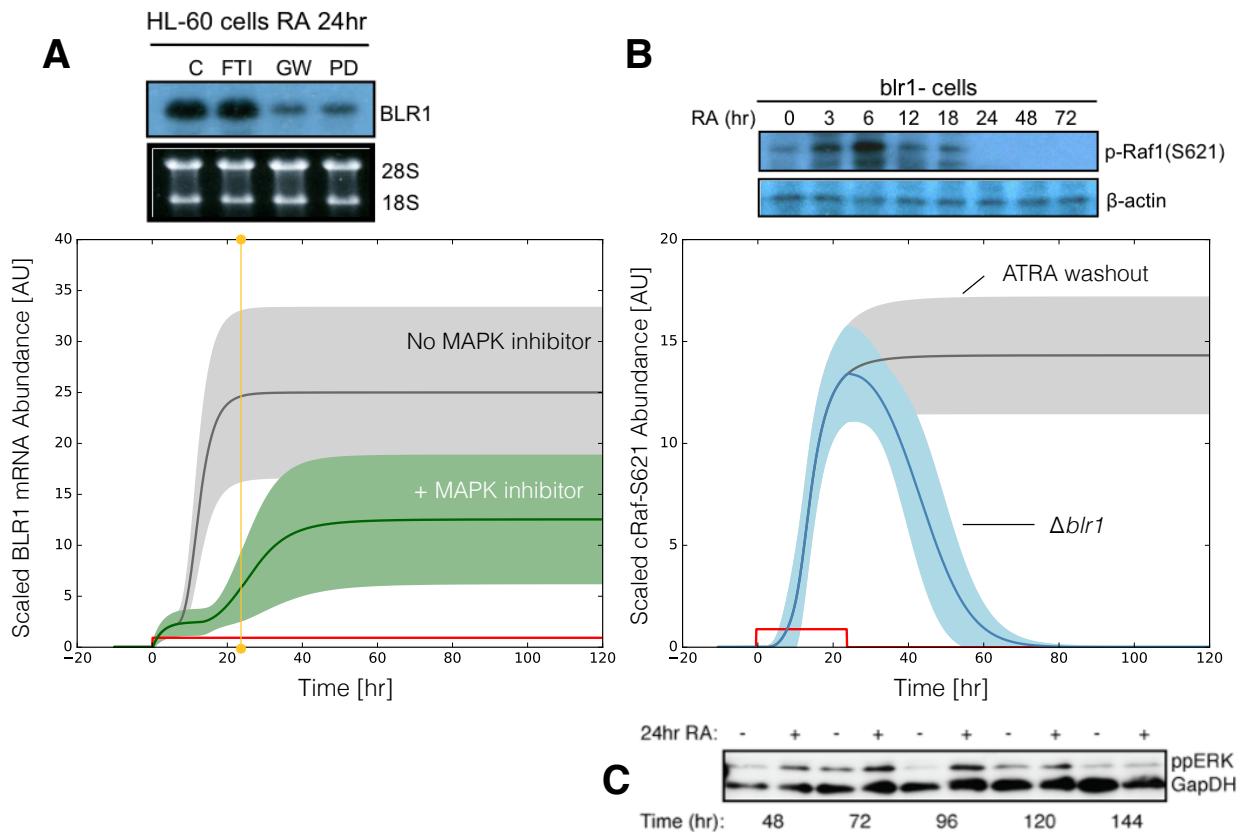


Fig. 3: Model simulation following exposure to $1\mu\text{M}$ ATRA. A: BLR1 mRNA versus time with and without MAPK inhibitor. B: cRaf-pS621 versus time following pulsed exposure to $1\mu\text{M}$ ATRA with and without BLR1. Solid lines denote the mean model performance, while shaded regions denote the 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.

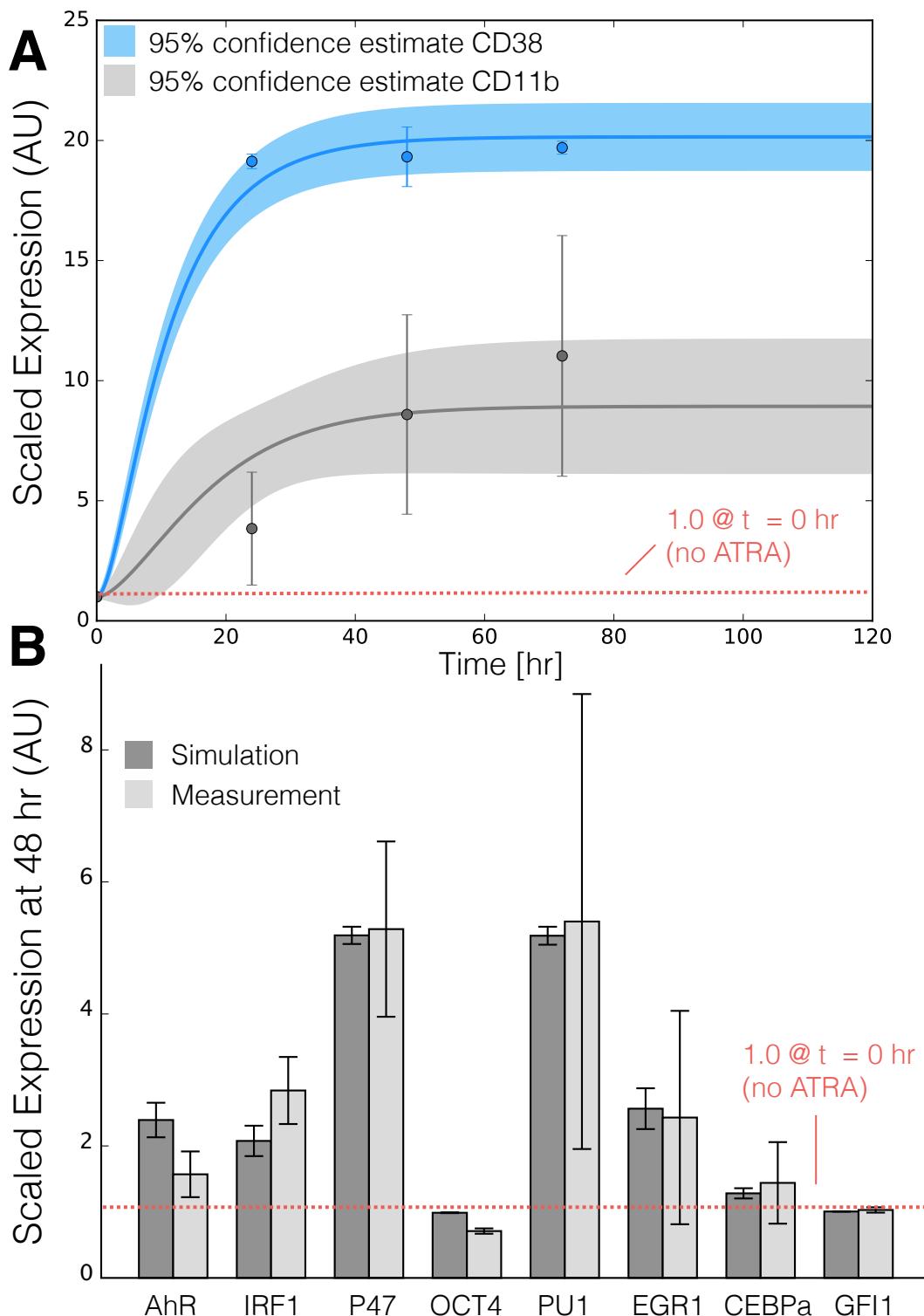


Fig. 4: Model simulation of the HL-60 gene expression program following exposure to $1\mu\text{M}$ ATRA at $t = 0$ hr. A: CD38 and CD11b expression versus time following ATRA exposure at time $t = 0$ hr. B: Gene expression at $t = 48$ hr following ATRA exposure. Experimental data in panels A and B were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

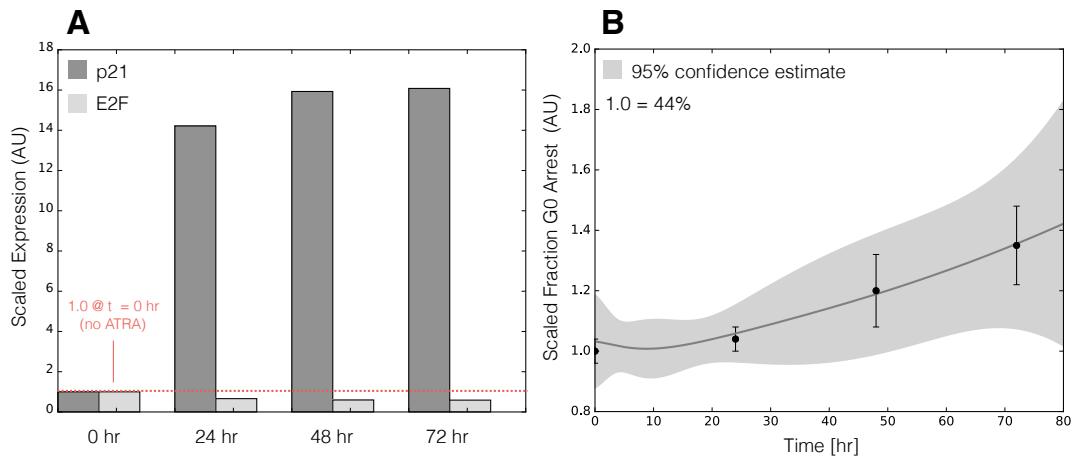


Fig. 5: Model simulation of HL-60 cell-cycle arrest following exposure to $1\mu\text{M}$ ATRA at $t = 0$ hr. A: Predicted P21 and E2F expression levels for the best parameter set following ATRA exposure at time $t = 0$ hr. B: Estimated fraction of HL-60 cells in G0 arrest following ATRA exposure at time $t = 0$ 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).

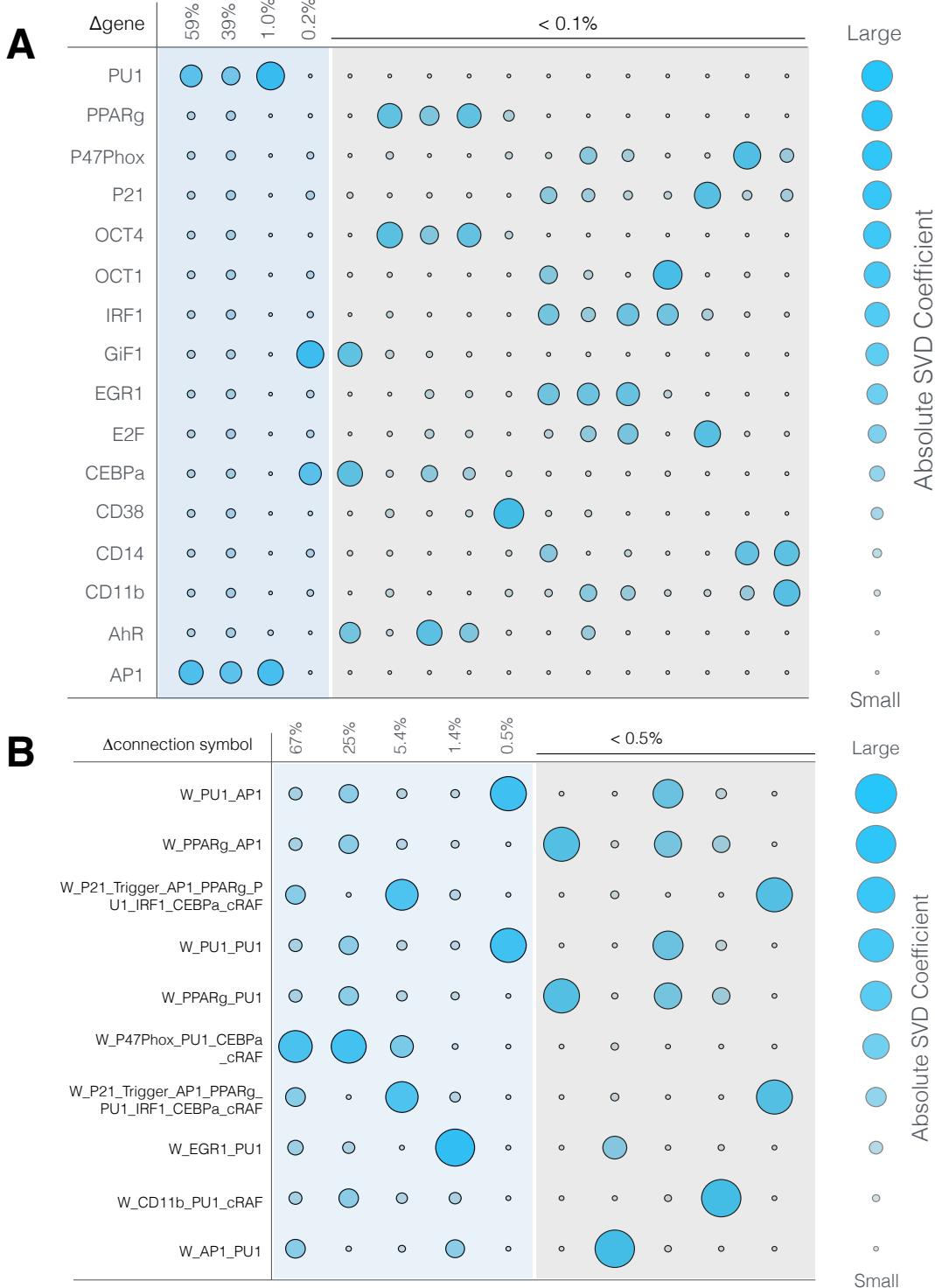


Fig. 6: Robustness of the HL-60 differentiation program following exposure to $1\mu\text{M}$ ATRA at $t = 0$ hr. A: Singular value decomposition of the system response (l^2 -norm between the perturbed and nominal state) following pairwise gene knockout simulations using the best fit parameter set. The 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.

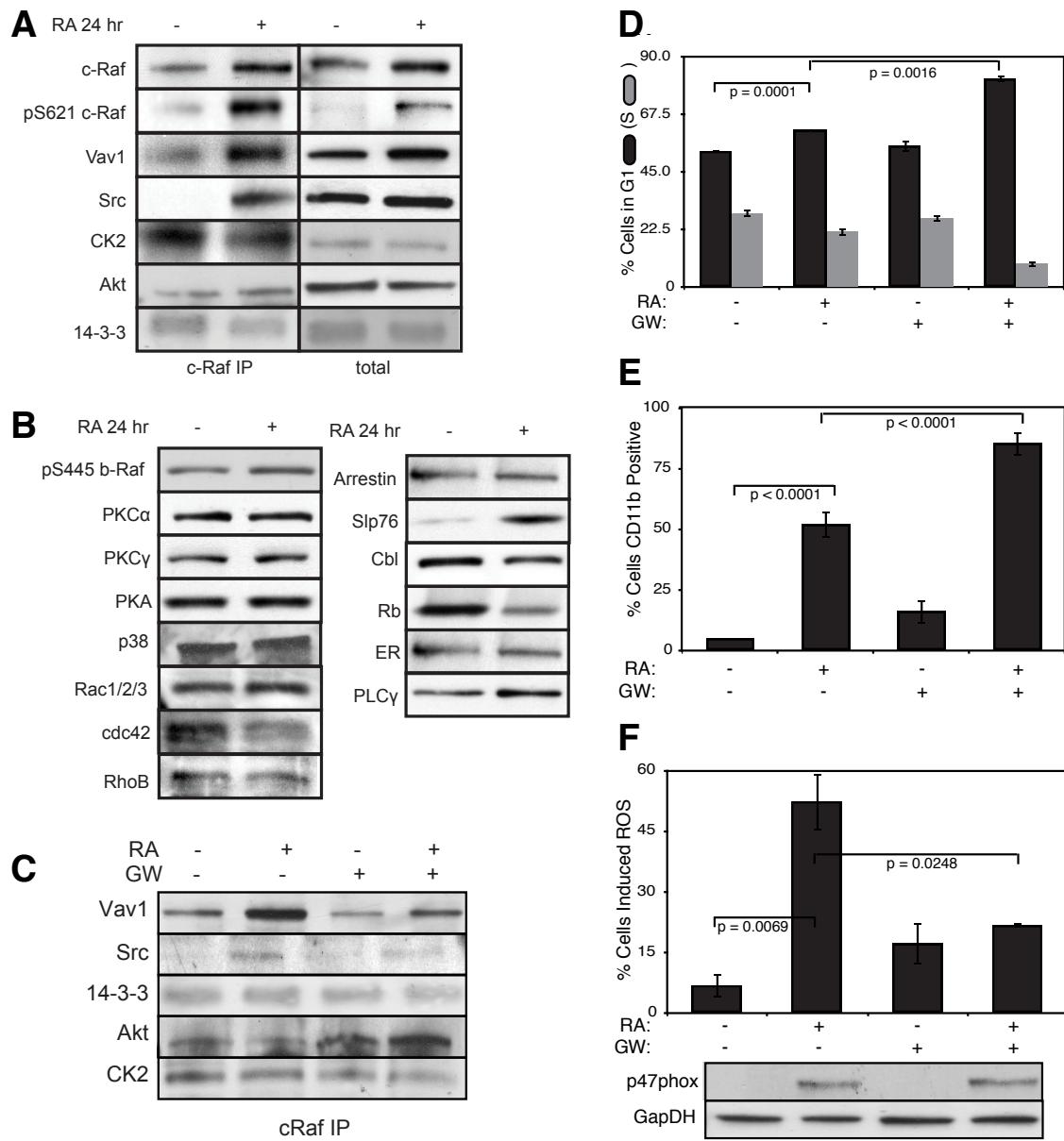


Fig. 7: Investigation of a panel of possible Raf interaction partners in the presence and absence of ATRA. A: Species identified to precipitate out with Raf: first column shows Western blot analysis on total Raf immunoprecipitation with and without 24 hr ATRA treatment and the second on total lysate. 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 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.