

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

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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 the ATRA-induced HL-60 differentiation which de-
73 scribed signaling and gene expression events following the addition of ATRA (Fig. 1).
74 The model connectivity was developed from literature and the studies presented here.
75 We decomposed the ATRA program into three modules; a signal initiation module that
76 was responsible for sensing and transforming the ATRA signal into activated cRaf-S621
77 and the ATRA-RXR/RAR (Trigger) signals (Fig. 1A); a signal integration module that con-
78 trolled the expression of upstream transcription factors given cRaf-S621 and activated
79 Trigger (Fig. 1B); and a phenotype module which encoded the expression of functional
80 differentiation markers (Fig. 1C).

81 The signal initiation module recapitulated sustained signalsome/MAPK activation fol-
82 lowing exposure to $1\mu\text{M}$ ATRA (Fig. 2A-B). An ensemble of effective model parameters
83 was estimated by minimizing the difference between simulations and time-series mea-
84 surements of BLR1 mRNA and cRaf-S621 following the addition of $1\mu\text{M}$ ATRA. We fo-
85 cused on the S621 phosphorylation site of cRaf since enhanced phosphorylation at this
86 site is a defining characteristic of sustained MAPK activation in HL-60. The effective
87 model captured both ATRA-induced BLR1 expression (Fig. 2A) and sustained phospho-
88 rylation of cRaf-pS621 (Fig. 2B) in a growing population of HL-60 cells. However, the
89 effective model failed to capture the decline of BLR1 message after 48 hr of ATRA expo-
90 sure. Next, we tested the response of the signalsome/MAPK signaling module to different
91 ATRA dosages.

92 The signalsome/MAPK signaling model was bistable with respect to ATRA induction
93 (Fig. 2C-D). Nullcline analysis predicted two stable steady-states and a single unstable
94 state when ATRA was present below a critical threshold (Fig. 2C). In the lower stable
95 state, neither the signalsome nor cRaf-pS621 were present (thus, the differentiation pro-
96 gram was deactivated). However, at the high stable state, both the signalsome and cRaf-

97 pS621 were present, allowing for sustained activation and differentiation. Interestingly,
98 when ATRA was above a critical threshold, only the activated state was accessible (Fig.
99 2D). To test these findings, we first identified the ATRA threshold. We exposed HL-60 cells
100 to different ATRA concentrations for 72 hr (Fig. 2E). Morphological changes associated
101 with differentiation were visible for ATRA $\geq 0.25 \mu\text{M}$, suggesting the critical ATRA thresh-
102 old was near this concentration. Next, we conducted washout ATRA washout experiments
103 to determine if activated cells remained activated even in the absence of ATRA. HL-60
104 cells locked into an activated state remained activated following ATRA withdraw (Fig. 3).
105 Sustained activation resulted from reinforcing feedback between the signalsome and the
106 MAPK pathway. Thus, following activation, if we inhibited or removed elements from the
107 effective circuit we expected the siganlsome and MAPK signals to decay. We simulated
108 ATRA induced activation in the presence of kinase inhibitors, and without key circuit ele-
109 ments. Consistent with experimental results using multiple MAPK inhibitors, ATRA activa-
110 tion in the presence of MAPK inhibitors lowered the steady-state value of signalsome (Fig.
111 3A). In the presence of BLR1, the signalsome and cRaf-pS621 signals were maintained
112 following ATRA withdraw (Fig. 3B, blue). On the other hand, BLR1 deletion removed
113 the ability of the circuit to maintain a sustained MAPK response following the withdraw
114 of ATRA (Fig. 3B, gray). Lastly, washout experiments in which cells were exposed to
115 $1 \mu\text{M}$ ATRA for 24 hr, and then transferred to fresh media without ATRA, confirmed the
116 persistence of the self sustaining activated state for up to 144 hr (Fig. 3C). Thus, these
117 experiments and simulations confirmed that reinforcing positive feedback likely drives the
118 ATRA-induced differentiation program. Next, we analyzed the ATRA-induced downstream
119 gene expression program following signalsome and cRaf activation.

120 The reduced order gene expression model described signal integration and ATRA-
121 induced gene expression events in wild-type HL-60 cells (Fig. 4). The signalsome-MAPK
122 model produced two outputs, Trigger and cRaf-S621 which drove the downstream dif-

123 ferentiation program. In particular, Trigger, which is a surrogate for the RAR α /RXR tran-
124 scriptional complex, regulated the expression of the transcription factors CCATT/enhancer
125 binding protein α (C/EBP α), PU.1, and EGR1. In turn, these transcription factors, in com-
126 bination with cRaf-S621, regulated the expression of downstream phenotypic markers
127 such as CD38, CD11b or P47Phox. We assembled the connectivity of the signal integra-
128 tion program driven by Trigger, and the phenotypic program from literature (supplemental
129 materials). We estimated the parameters of the signal integration and phenotype pro-
130 grams from previous studies which contained both steady-state and dynamic measure-
131 ments of transcription factor and phenotypic marker expression following the addition of
132 ATRA [REFHERE]. The model simulations captured the time dependent expression of
133 both CD38 and CD11b following the addition ATRA (Fig. 4A), and steady-state values for
134 upstream members of the signal integration unit (Fig. 4B).

135 The composition of the siganlsome, and the kinase ultimately responsible for medi-
136 ating ATRA-induced Raf activation is currently unknown. To explore this question, we
137 conducted immunoprecipitation and subsequent Western blotting to identify physical in-
138 teractions between Raf and 19 putative interaction partners. A panel of 19 possible Raf
139 interaction partners (kinases, GTPases, scaffolding proteins etc) was constructed based
140 upon known signaling pathways. We did not consider the most likely binding partner, the
141 small GTPase RAS, as previous studies have ruled it out in MAPK activation in HL-60 cells
142 (20, 22). Total Raf was used as a bait protein for the immunoprecipitation studies. Interro-
143 gation of the Raf interactome suggested Vav1 was involved with ATRA-induced initiation
144 of MAPK activity (Fig. 5). Western blot analysis using total Raf and pS621 Raf specific
145 antibodies confirmed the presence of the bait protein, total and phosphorylated forms, in
146 the immunoprecipitate (Fig. 5A). Of the 19 proteins sampled, Vav1, Src, CK2, Akt, and
147 14-3-3 precipitated with Raf, suggesting a direct physical interaction was possible. How-
148 ever, only the associations between Raf and Vav1 and Raf and Src were ATRA-inducible

149 (Fig. 5). Furthermore, the Vav1 and Src associations were correlated with pS621 Raf
150 abundance in the precipitate. Others proteins e.g., CK2, Akt and 14-3-3, generally bound
151 Raf regardless of phosphorylation status or ATRA treatment. The remaining 14 proteins
152 were expressed in whole cell lysate (Fig. 5B), but were not detectable in the precipitate
153 of Raf IP. Treatment with the Raf kinase inhibitor GW5074 following ATRA exposure re-
154 duced the association of both Vav1 with Raf and Src with Raf (Fig. 5), although the signal
155 intensity for Src was notably weak. However, GW5074 did not influence the association
156 of CK2 or 14-3-3 with Raf, further demonstrating their independence from Raf phospho-
157 rylation. Interestingly, the Raf-Akt interaction qualitatively increased following treatment
158 with GW5074; however, it remained unaffected by treatment with ATRA. Src family ki-
159 nases are known to be important in myeloid differentiation (23) and their role in HL-60
160 differentiation has been investigated elsewhere (11). Given the existing work and variable
161 reproducibility in the context of the Raf immunoprecipitate, we did not investigate the role
162 of Src further in this study. Taken together, the immunoprecipitation and GW5074 results
163 implicated Vav1 association to be correlated with Raf activation following ATRA-treatment.
164 Previous studies demonstrated that a Vav1-Slp76-Cbl-CD38 complex plays an important
165 role in ATRA-induced MAPK activation and differentiation of HL-60 cells (13). Here we
166 did not observe direct interaction of Raf with Cbl or Slp76; however, this complex could
167 be involved upstream.

168 Next, we considered the effect of the Raf kinase inhibitor GW5074 on functional mark-
169 ers of ATRA-induced growth arrest and differentiation. Inhibition of Raf kinase activity
170 modulated MAPK activation and differentiation markers following ATRA exposure (Fig.
171 5D-F). ATRA treatment alone statistically significantly increased the G1/G0 percentage
172 over the untreated control, while GW5074 alone had a negligible effect on the cell cycle
173 distribution (Fig. 5D). Surprisingly, the combination of GW5074 and ATRA statistically
174 significantly increased the G1/G0 population ($82 \pm 1\%$) compared with ATRA alone (61

¹⁷⁵ \pm 0.5%). Increased G1/G0 arrest following the combined treatment with GW5074 and
¹⁷⁶ ATRA was unexpected, as the combination of ATRA and the MEK inhibitor (PD98059) has
¹⁷⁷ been shown previously to decrease ATRA-induced growth arrest (8). However, growth ar-
¹⁷⁸ rest is not the sole indication of functional differentiation. Expression of the cell surface
¹⁷⁹ marker CD11b has also been shown to coincide with HL-60 cells myeloid differentiation
¹⁸⁰ (24). We measured CD11b expression, for the various treatment groups, using immuno-
¹⁸¹ fluorescence flow cytometry 48 hr post-treatment. As with G1/G0 arrest, ATRA alone
¹⁸² increased CD11b expression over the untreated control, while GW5074 further enhanced
¹⁸³ ATRA-induced CD11b expression (Fig. 5E). GW5074 alone had no statistically significant
¹⁸⁴ effect on CD11b expression, compared with the untreated control. Lastly, the inducible re-
¹⁸⁵ active oxygen species (ROS) response was used as a functional marker of differentiated
¹⁸⁶ neutrophils (16). We measured the ROS response induced by the phorbol ester 12-O-
¹⁸⁷ tetradecanoylphorbol-13-acetate (TPA) using flow cytometry. Untreated cells showed no
¹⁸⁸ discernible TPA response, with only $7.0 \pm 3.0\%$ ROS induction (Fig. 5F). Cells treated
¹⁸⁹ with ATRA had a significantly increased TPA response, $53 \pm 7\%$ ROS induction 48 hr
¹⁹⁰ post-treatment. Treatment with both ATRA and GW5074 statistically significantly reduced
¹⁹¹ ROS induction ($22 \pm 0.6\%$) compared to ATRA alone. Interestingly, Western blot analy-
¹⁹² sis did not detect a GW5074 effect on ATRA-induced expression of p47phox, a required
¹⁹³ upstream component of the ROS response (Fig. 5F, bottom). Thus, the inhibitory effect
¹⁹⁴ of GW5074 on inducible ROS might occur downstream of p47phox expression. How-
¹⁹⁵ ever, the ROS producing complex is MAPK dependent, therefore it is also possible that
¹⁹⁶ GW5074 inhibited ROS production by interfering with MAPK activation (in which case the
¹⁹⁷ p47Phox marker might not accurately reflect phenotypic conversion and differentiation).

198 **Discussion**

199 In this study, we presented an effective model of ATRA-inducible differentiation of HL-60
200 cells which encoded positive feedback between the ATRA-inducible signalsome complex
201 and the MAPK pathway. Despite its simplicity, the model captured key features of the
202 ATRA induced differentiation such as sustained MAPK activation, and bistability with re-
203 spect to ATRA exposure. We also reported a new ATRA-inducible component of the
204 signalsome, Vav1. Vav1 is a guanine nucleotide exchange factor for Rho family GTPases
205 that activate pathways leading to actin cytoskeletal rearrangements and transcriptional al-
206 terations (25). The Vav1/Raf association correlated with Raf activity, was ATRA-inducible
207 and decreased after treatment with GW5074. The presence of Vav1 in Raf/Grb2 com-
208 plexes has been shown to correlate with increased Raf activity in mast cells (26). Fur-
209 thermore, studies on Vav1 knockout mice demonstrated that the loss of Vav1 resulted
210 in deficiencies of ERK signaling for both T-cells as well as neutrophils (27, 28). While its
211 function in the signalsome is unclear, Vav1 has been shown to associate with a Cbl-Slp76-
212 CD38 complex in an ATRA-dependent manner; furthermore, transfection of HL-60 cells
213 with Cbl mutants that fail to bind CD38, yet still bind Slp76 and Vav1, prevented ATRA-
214 induced MAPK activation (13). Thus, interaction of Cbl-Slp76-Vav1 and CD38 appears to
215 be required for transmission of the ATRA signal by the signalsome.

216 We conducted immunoprecipitation studies and identified a limited number of ATRA-
217 dependent and -independent Raf interaction partners. While we were unable to detect
218 the association of Raf with common kinases and GTPases such as PKC, PKA, p38, Rac
219 and Rho, we did establish potential interactions between Raf and key partners such as
220 Vav1, Src, Akt, CK2 and 14-3-3. All of these partners are known to be associated with Raf
221 activation or function. Src is known to bind Raf through an SH2 domain, and this associ-
222 ation has been shown to be dependent of the serine phosphorylation of Raf (29). Thus,
223 an ATRA inducible Src/Raf association may be a result of ATRA-induced Raf phospho-

224 phosphorylation at S259 or S621. We also identified an interaction between Raf and the Ser/Thr
225 kinases Akt and CK2. Akt can phosphorylate Raf at S259, as demonstrated by studies
226 in a human breast cancer line (30). CK2 can also phosphorylate Raf, although the lit-
227 erature has traditionally focused on S338 and not S621 or S259(31). However, neither
228 of these kinase interactions were ATRA-inducible, suggesting their association with Raf
229 alone was not associated with ATRA-induced Raf phosphorylation. The adapter protein
230 14-3-3 was also constitutively associated with Raf. The interaction between Raf and 14-
231 3-3 has been associated with both S621 and S259 phosphorylation and activity (32).
232 Additionally, the association of Raf with 14-3-3 not only stabilized S621 phosphorylation,
233 but also reversed the S621 phosphorylation from inhibitory to activating (33). Finally, we
234 found that Vav1/Raf association correlated with Raf activity, was ATRA-inducible and de-
235 creased after treatment with GW5074. The presence of Vav1 in Raf/Grb2 complexes has
236 been shown to correlate with increased Raf activity in mast cells (26). Furthermore, stud-
237 ies on Vav1 knockout mice demonstrated that the loss of Vav1 resulted in deficiencies of
238 ERK signaling for both T-cells as well as neutrophils (27, 28). Interestingly, while an in-
239 tegrin ligand-induced ROS response was blocked in Vav1 knockout neutrophils, TPA was
240 able to bypass the Vav1 requirement and stimulate both ERK phosphorylation and ROS
241 induction (28). In this study, the TPA-induced ROS response was dependent upon Raf
242 kinase activity, and was mitigated by the addition of GW5074. It is possible that Vav1 is
243 downstream of various integrin receptors but upstream of Raf in terms of inducible ROS
244 responses. Vav1 has also been shown to associate with a Cbl-Slp76-CD38 complex in an
245 ATRA-dependent manner; furthermore, transfection of HL-60 cells with Cbl mutants that
246 fail to bind CD38, yet still bind Slp76 and Vav1, prevents ATRA-induced MAPK activation
247 (13). The literature suggest a variety of possible receptor-signaling pathways, which in-
248 volve Vav1, for MAPK activation; moreover, given the ATRA-inducible association Vav1
249 may play a direct role in Raf activation.

250 We hypothesized that Vav1 is a member of an ATRA-inducible complex which propels
251 sustained MAPK activation, arrest and differentiation. Initially, ATRA-induced Vav1 ex-
252 pression drives increased association between Vav1 and Raf. This increased interaction
253 facilitates phosphorylation and activation of Raf by pre-bound Akt and/or CK2 at S621
254 or perhaps S259. Constitutively bound 14-3-3 may also stabilize the S621 phosphory-
255 lation, modulate the activity and/or up-regulate autophosphorylation. Activated Raf can
256 then drive ERK activation, which in turn closes the positive feedback loop by activating
257 Raf transcription factors, e.g. Sp1 and/or STAT1 (34–37). We tested this working hy-
258 pothesis using mathematical modeling. The model recapitulated both ATRA time-course
259 data as well as the GW5074 inhibitor effects. This suggested the proposed Raf-Vav1
260 architecture was at least consistent with the experimental studies. Further, analysis of
261 the Raf-Vav1 model identified bistability in ppERK levels. Thus, two possible MAPK ac-
262 tivation branches were possible for experimentally testable ATRA values. The analysis
263 also suggested the ATRA-induced Raf-Vav1 architecture could be locked into a sustained
264 signaling mode (high ppERK) even in the absence of a ATRA signal. This locked-in prop-
265 erty could give rise to an ATRA-induction memory. We validated the treatment memory
266 property predicted by the Raf-Vav1 circuit experimentally using ATRA-washout experi-
267 ments. ERK phosphorylation levels remained high for more then 96 hr after ATRA was
268 removed. Previous studies demonstrated that HL-60 cells possessed an inheritable mem-
269 ory of ATRA stimulus (38). Although the active state was self-sustaining, the inactive state
270 demonstrated considerable robustness to perturbation. For example, we found that 50x
271 overexpression of Raf was required to reliably lock MAPK into the activated state, while
272 small perturbations had almost no effect on ppERK levels over the entire ensemble. CD38
273 expression correlated with the ppERK, suggesting its involvement in the signaling com-
274 plex. Our computational and experimental results showed that positive feedback, through
275 ERK-dependent Raf expression, could sustain MAPK signaling through many division cy-

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

278 Several engineered, or naturally occurring systems involved in cell fate decisions incor-
279 porate positive feedback and bistability (39). One of the most well studied cell fate circuits
280 is the Mos mitogen-activated protein kinase cascade in *Xenopus* oocytes. This cascade
281 is activated when oocytes are induced by the steroid hormone progesterone (40). The
282 MEK-dependent activation of p42 MAPK stimulates the accumulation of the Mos onco-
283 protein, which in turn activates MEK, thereby closing the feedback loop. This is similar to
284 the differentiation circuit presented here; ATRA drives signalsome which activates MAPK,
285 cell-cycle arrest, differentiation and signalsome. Thus, while HL-60 and *Xenopus* oocytes
286 are vastly different biological models, they share similar cell fate decision architectures.
287 Other unrelated cell fate decisions such as programmed cell death have also been sug-
288 gested to be bistable (41). Still more biochemical networks important to human health,
289 for example the human coagulation or complement cascades, also feature strong positive
290 feedback elements (42). Thus, while positive feedback is sometimes not desirable in man-
291 made systems, it may be at the core of a diverse variety of cell fate programs and other
292 networks important to human health.

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

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

312 **Materials and Methods**

313 *Effective ATRA differentiation model.* ATRA induced signaling events were modeled us-
 314 ing saturation kinetics within an ordinary differential equation (ODE) framework:

$$\frac{1}{\tau_i} \frac{dx_i}{dt} = \sum_{j=1}^{\mathcal{R}} \sigma_{ij} r_j(\mathbf{x}, \epsilon, \mathbf{k}) - (\mu + k_d) x_i \quad i = 1, 2, \dots, \mathcal{M} \quad (1)$$

315 The quantity x_i denotes concentration of signaling species i , while \mathcal{R} and \mathcal{M} denote the
 316 number of signaling reactions and signaling species in the model, respectively. The quan-
 317 tity τ_i denotes a time scale parameter for species i which captures un-modeled effects; in
 318 the current study $\tau_i = 1$ for all species. The quantity $r_j(\mathbf{x}, \epsilon, \mathbf{k})$ denotes the rate of pro-
 319 cess j . Typically, process j is a non-linear function of biochemical and enzyme species
 320 abundance, as well as unknown model parameters \mathbf{k} ($\mathcal{K} \times 1$). The quantity σ_{ij} denotes the
 321 stoichiometric coefficient for species i in reaction j . If $\sigma_{ij} > 0$, species i is produced by
 322 reaction j . Conversely, if $\sigma_{ij} < 0$, species i is consumed by reaction j , while $\sigma_{ij} = 0$ indi-
 323 cates species i is not connected with reaction j . Lastly, μ denotes the specific growth rate,
 324 and k_d denotes the rate constant controlling cell death. Species balances were subject to
 325 the initial conditions $\mathbf{x}(t_o) = \mathbf{x}_o$.

326 Signaling rate processes were written as the product of a kinetic term (\bar{r}_j) and a control
 327 term (v_j) in the HL-60 model. The rate of an enzyme catalyzed process was modeled
 328 using saturation kinetics:

$$\bar{r}_j = k_j \epsilon_i \prod_{s \in m_j^-} \left(\frac{x_s}{K_{js} + x_s} \right) \quad (2)$$

329 where k_j denotes the catalytic rate constant for reaction j , ϵ_i denotes the abundance of the
 330 enzyme catalyzing reaction j , and K_{js} denotes the saturation constant for species s and
 331 $s \in m_j$ denotes the set of *reactants* for reaction j . The control terms $0 \leq v_j \leq 1$ depended
 332 upon the combination of factors which influenced rate process j . For each rate, we used

333 a rule-based approach to select from competing control factors. If rate j was influenced
 334 by $1, \dots, m$ factors, we modeled this relationship as $v_j = \mathcal{I}_j(f_{1j}(\cdot), \dots, f_{mj}(\cdot))$ where
 335 $0 \leq f_{ij}(\cdot) \leq 1$ denotes a regulatory transfer function quantifying the influence of factor i
 336 on rate j . The function $\mathcal{I}_j(\cdot)$ is an integration rule which maps the output of regulatory
 337 transfer functions into a control variable. In this study, we used $\mathcal{I}_j \in \{\min, \max\}$ and hill
 338 transfer functions (43). If a process had no modifying factors, $v_j = 1$.

339 The HL-60 model described both signal transduction and gene expression events fol-
 340 lowing the addition of ATRA. The output of the signal transduction model was the input to
 341 the gene expression model. For each gene $j = 1, 2, \dots, \mathcal{G}$, we modeled both the mRNA
 342 (m_j) and protein (p_j):

$$\frac{dm_j}{dt} = r_{T,j} - (\mu + \theta_{m,j}) m_j + \lambda_j \quad (3)$$

$$\frac{dp_j}{dt} = r_{X,j} - (\mu + \theta_{p,j}) p_j \quad (4)$$

343 The terms $r_{T,j}$ and $r_{X,j}$ denote the specific rates of transcription, and translation while
 344 the terms $\theta_{m,j}$ and $\theta_{p,j}$ denote first-order degradation constants for mRNA and protein,
 345 respectively. The specific transcription rate was modeled as the product of a kinetic term
 346 $\bar{r}_{T,j}$ and a control term u_j which described how the abundance of transcription factors, or
 347 other regulators influenced the expression of gene j . The kinetic rate of transcription was
 348 modeled as:

$$\bar{r}_{T,j} = V_T^{\max} \left(\frac{L_{T,o}}{L_{T,j}} \right) \left(\frac{G_j}{K_T + G_j} \right) \quad (5)$$

349 where the maximum gene expression rate V_T^{\max} was defined as the product of a char-
 350 acteristic transcription rate constant (k_T) and the abundance of RNA polymerase (R_1),
 351 $V_T^{\max} = k_T (R_1)$. The $(L_{T,o}/L_{T,j})$ term denotes the ratio of transcription read lengths,
 352 where $L_{T,o}$ is a characteristic gene length, and $L_{T,j}$ denotes the length of gene j . Thus,
 353 the $(L_{T,o}/L_{T,j})$ term is gene specific correction to the characteristic transcription rate. The

354 degradation rate constants were defined as $\theta_{m,j}$ and $\theta_{p,j}$ denote characteristic degradation
 355 constants for mRNA and protein, respectively.

356 The gene expression control term $0 \leq u_j \leq 1$ depended upon the combination of
 357 factors which influenced rate process j . If the expression of gene j was influenced
 358 by $1, \dots, m$ factors, we modeled this relationship as $u_j = \mathcal{I}_j(f_{1j}(\cdot), \dots, f_{mj}(\cdot))$ where
 359 $0 \leq f_{ij}(\cdot) \leq 1$ denotes a regulatory transfer function quantifying the influence of factor
 360 i on the expression of gene j , and $\mathcal{I}_j(\cdot)$ denotes an integration rule. In this study, the
 361 integration rule governing gene expression was the weighted fraction of promoter config-
 362 urations resulting in gene expression. Thus, the control variable u_j took the form:

$$u_j = \frac{W_{R_{1,j}} + \sum_n W_{nj} f_{nj}}{1 + W_{R_{1,j}} + \sum_d W_{dj} f_{dj}} \quad (6)$$

363 where the numerator, the weighted sum (with weights W_{nj}) of promoter configurations
 364 leading to gene expression, was normalized by all possible promoter configurations. The
 365 likelihood of each configuration was quantified by the transfer function f_{nj} (which we mod-
 366 eled using hill like functions), while the lead term in the numerator $W_{R_{1,j}}$ denotes the
 367 weight of constitutive expression for gene j . If a gene expression process had no modify-
 368 ing factors, $u_j = 1$. Lastly, the specific translation rate was modeled as:

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

369 where V_X^{\max} denotes a characteristic maximum translation rate estimated from literature,
 370 and K_X denotes a translation saturation constant. The characteristic maximum translation
 371 rate was defined as the product of a characteristic translation rate constant (k_X) and the
 372 Ribosome abundance (R_2), $V_X^{\max} = k_X (R_2)$.

373 In this study, we estimated the W_{ij} parameters, and the parameters in the trans-

fer functions f_{dj} from gene expression data sets. On the other hand, we estimated
 $k_T, k_X, \theta_{m,j}, \theta_{p,j}, R_1$ and R_2 using estimates of transcription and translation rates, the half-life of a typical mRNA and protein, and a typical value for the copies per cell of RNA polymerase and ribosomes from literature (44). 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 parameters were based directly upon literature values, and were not adjusted during model identification. The values used for the characteristic transcription/translation parameters, degradation constants and macromolecular copy number are given in the supplemental materials along with the specific formulas required to calculate all derived constants.

Estimation of signaling and gene expression model parameters. Signal and gene expression model parameters were estimated by minimizing the squared difference between simulations and experimental data set j :

$$E_j(\mathbf{k}) = \sum_{i=1}^{\mathcal{T}_j} \left(\hat{\mathcal{M}}_{ij} - \hat{y}_{ij}(\mathbf{k}) \right)^2 + \left(\frac{\mathcal{M}'_{ij} - \max y_{ij}}{\mathcal{M}'_{ij}} \right)^2 \quad (8)$$

The terms $\hat{\mathcal{M}}_{ij}$ and \hat{y}_{ij} denote scaled experimental observations and simulation outputs at time i from training set j , where \mathcal{T}_j denoted the number of time points for data set j . The first term in Eqn. (8) quantified the relative simulation error. We used immunoblot intensity measurements for model training. Thus, we trained the model on the *relative* change between bands within each data set. Suppose we have the intensity of species x at time $\{t_1, t_2, \dots, t_n\}$ in condition j . The scaled value $0 \leq \hat{\mathcal{M}}_{ij} \leq 1$ is given by:

$$\hat{\mathcal{M}}_{ij} = \left(\mathcal{M}_{ij} - \min_i \mathcal{M}_{ij} \right) / \left(\max_i \mathcal{M}_{ij} - \min_i \mathcal{M}_{ij} \right) \quad (9)$$

395 where $\hat{M}_{ij} = 0$ and $\hat{M}_{ij} = 1$ describe the lowest (highest) intensity bands. A similar
396 scaling was used for the simulation output. The second term in the objective function
397 ensured a realistic concentration scale was estimated by the model. We set the highest
398 intensity band to $M'_{ij} = 10$ [AU] for all simulations. We minimized the total model residual
399 $\sum_j E_j$ using heuristic optimization starting from a random initial parameter guess.

400 The signaling and gene expression model equations were implemented in Julia and
401 solved using the CVODE routine of the Sundials package (45, 46). The model code and
402 parameter ensemble is freely available under an MIT software license and can be down-
403 loaded from <http://www.varnerlab.org>.

404 *Cell culture and treatment* Human myeloblastic leukemia cells (HL-60 cells) were grown
405 in a humidified atmosphere of 5% CO₂ at 37°C and maintained in RPMI 1640 from Gibco
406 (Carlsbad, CA) supplemented with 5% heat inactivated fetal bovine serum from Hyclone
407 (Logan, UT) and 1× antibiotic/antimicotic (Gibco, Carlsbad, CA). Cells were cultured in
408 constant exponential growth (47). Experimental cultures were initiated at 0.1×10^6 cells/mL
409 24 hr prior to ATRA treatment; if indicated, cells were also treated with GW5074 (2 μ M) 18
410 hr before ATRA treatment. For the cell culture washout experiments, cells were treated
411 with ATRA for 24 hr, washed 3x with prewarmed serum supplemented culture medium
412 to remove ATRA, and reseeded in ATRA-free media as described. Western blot analysis
413 was performed at incremental time points after removal of ATRA.

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

421 *Immunoprecipitation and western blotting* Approximately 1.2×10^7 cells were lysed using
422 $400\mu\text{L}$ of M-Per lysis buffer from Thermo Scientific (Waltham, MA). Lysates were cleared
423 by centrifugation at $16,950 \times g$ in a micro-centrifuge for 20 min at 4°C . Lysates were
424 pre-cleared using $100\mu\text{L}$ protein A/G Plus agarose beads from Santa Cruz Biotechnology
425 (Santa Cruz, CA) by inverting overnight at 4°C . Beads were cleared by centrifugation and
426 total protein concentration was determined by a BCA assay (Thermo Scientific, Waltham,
427 MA). Immunoprecipitations were setup by bringing lysate to a concentration of 1g/L in a
428 total volume of $300\mu\text{L}$ (M-Per buffer was used for dilution). The anti-Raf antibody was
429 added at $3\mu\text{L}$. A negative control with no bait protein was also used to exclude the di-
430 rect interaction of proteins with the A/G beads. After 1 hr of inversion at 4°C , $20\mu\text{L}$ of
431 agarose beads was added and samples were left to invert overnight at 4°C . Samples
432 were then washed three times with M-Per buffer by centrifugation. Finally proteins were
433 eluted from agarose beads using a laemmli loading buffer. Eluted proteins were resolved
434 by SDS-PAGE and Western blotting. Total lysate samples were normalized by total protein
435 concentration ($20\mu\text{g}$ per sample) and resolved by SDS-PAGE and Western blotting. Sec-
436 ondary HRP bound antibody was used for visualization. All antibodies were purchased
437 from Cell Signaling (Boston, MA) with the exception of α -p621 Raf which was purchased
438 from Biosource/Invitrogen (Carlsbad, CA), and α -CK2 from BD Biosciences (San Jose,
439 CA).

440 *Morphology assessment* Untreated and ATRA-treated HL-60 cells were collected after
441 72 hr and cytocentrifuged for 3 min at 700 rpm onto glass slides. Slides were air-dried
442 and stained with Wright's stain. Slide images were captured at 40X (Leica DM LB 100T
443 microscope, Leica Microsystems).

⁴⁴⁴ **Competing interests**

⁴⁴⁵ The authors declare that they have no competing interests.

⁴⁴⁶ **Author's contributions**

⁴⁴⁷ J.V and A.Y directed the study. R.T, H.J and J.C conducted the cell culture measurements. J.V and W.D developed the reduced order HL-60 models and the parameter ensemble. W.D analyzed the model ensemble, and generated figures for the manuscript.

⁴⁵⁰ The manuscript was prepared and edited for publication by W.D, A.Y and J.V.

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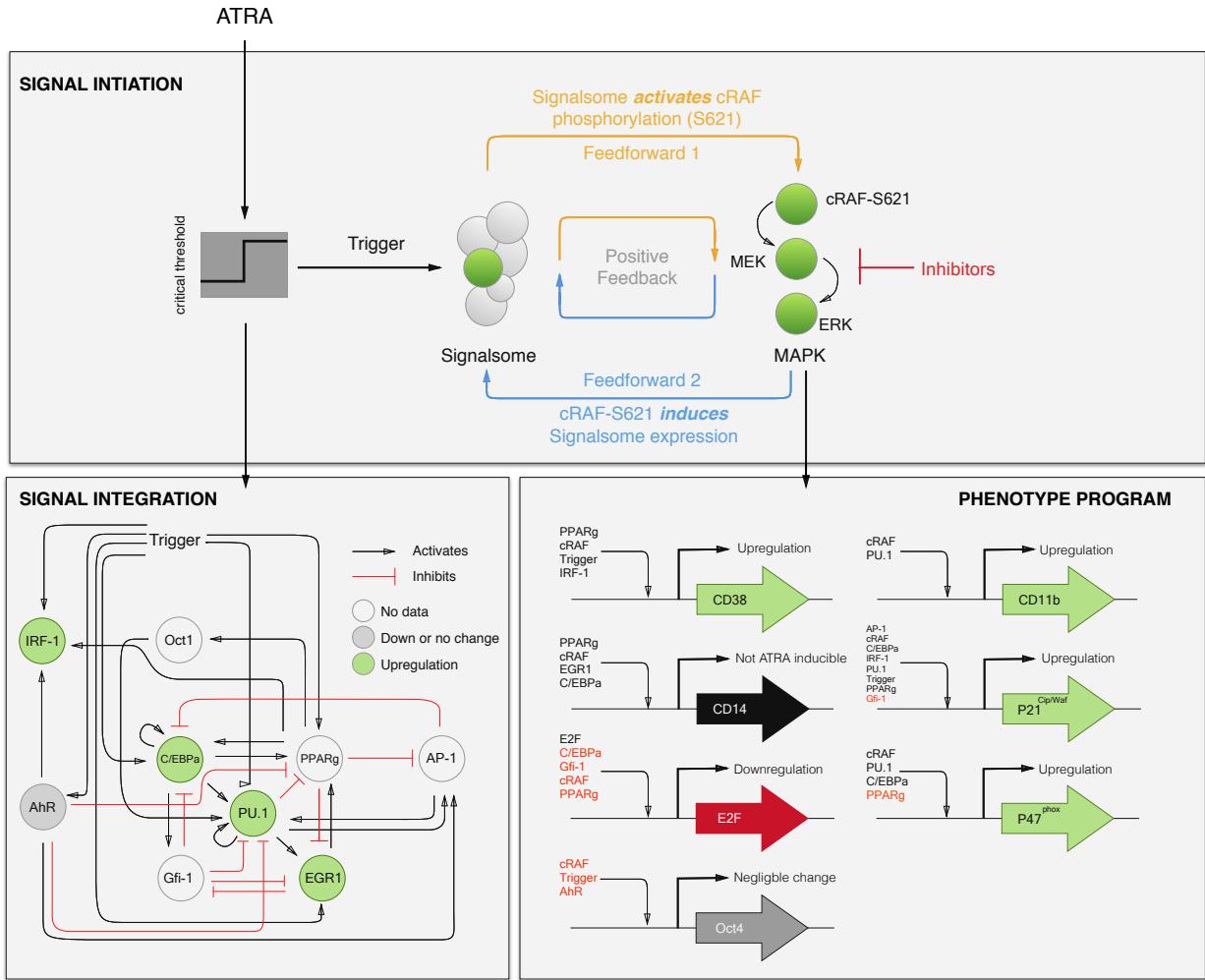


Fig. 1: Schematic of the effective ATRA differentiation circuit. Above a critical threshold, ATRA activates an upstream Trigger, which induces signalsome complex formation. Signalsome activates the mitogen-activated protein kinase (MAPK) cascade which in turn drives the differentiation program and signalsome formation. Both Trigger and activated cRaf-S621 drive a phenotype gene expression program responsible for differentiation. Trigger activates the expression of a series of transcription factors which in combination with cRaf-S621 result in phenotypic change.

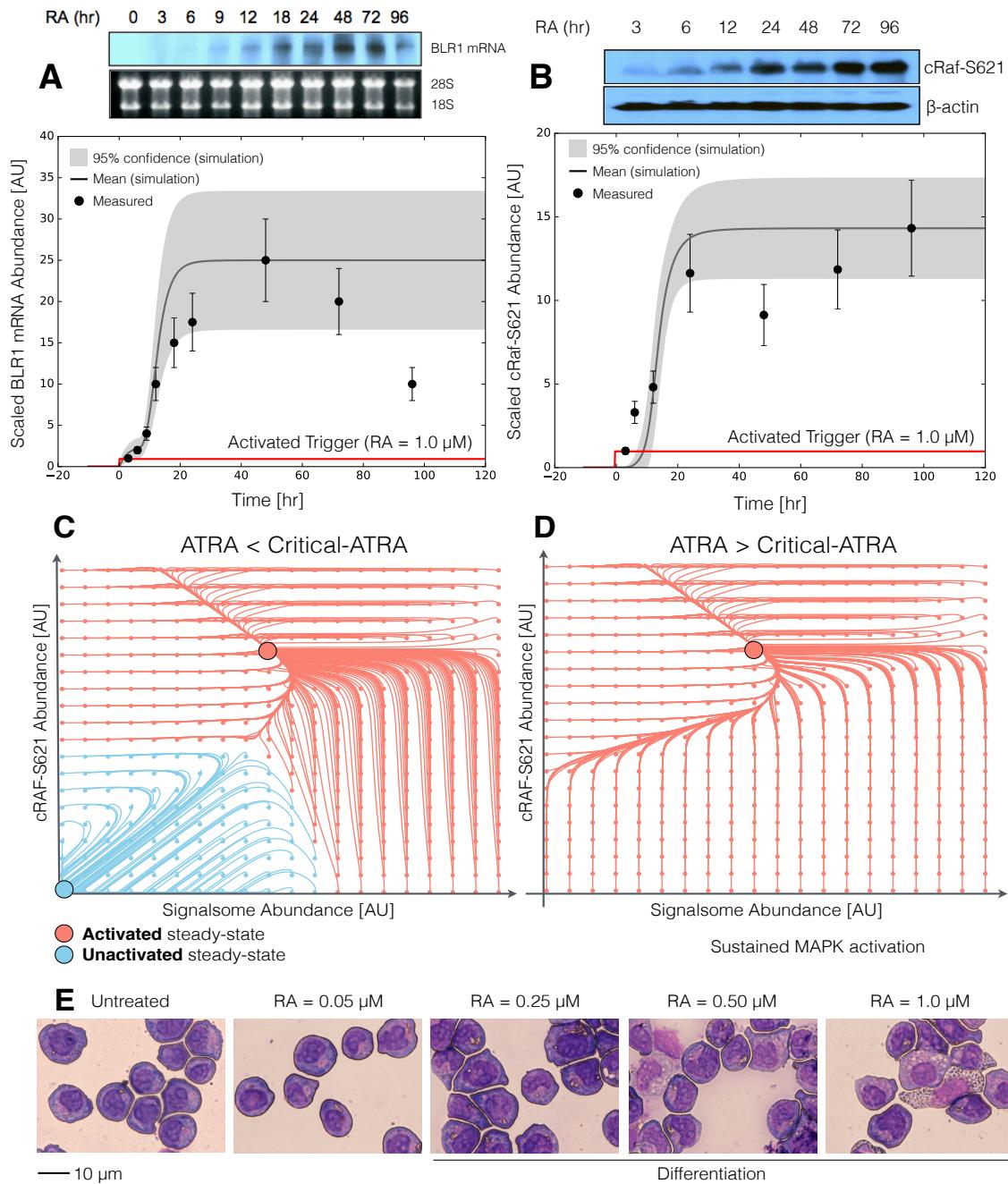


Fig. 2: Model analysis for ATRA-induced HL-60 differentiation. A: BLR1 mRNA versus time following exposure to $1\mu\text{M}$ ATRA at $t = 0$ hr. B: cRaf-pS621 versus time following exposure to $1\mu\text{M}$ ATRA at $t = 0$ hr. Points denote experimental measurements, solid lines denote the mean model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

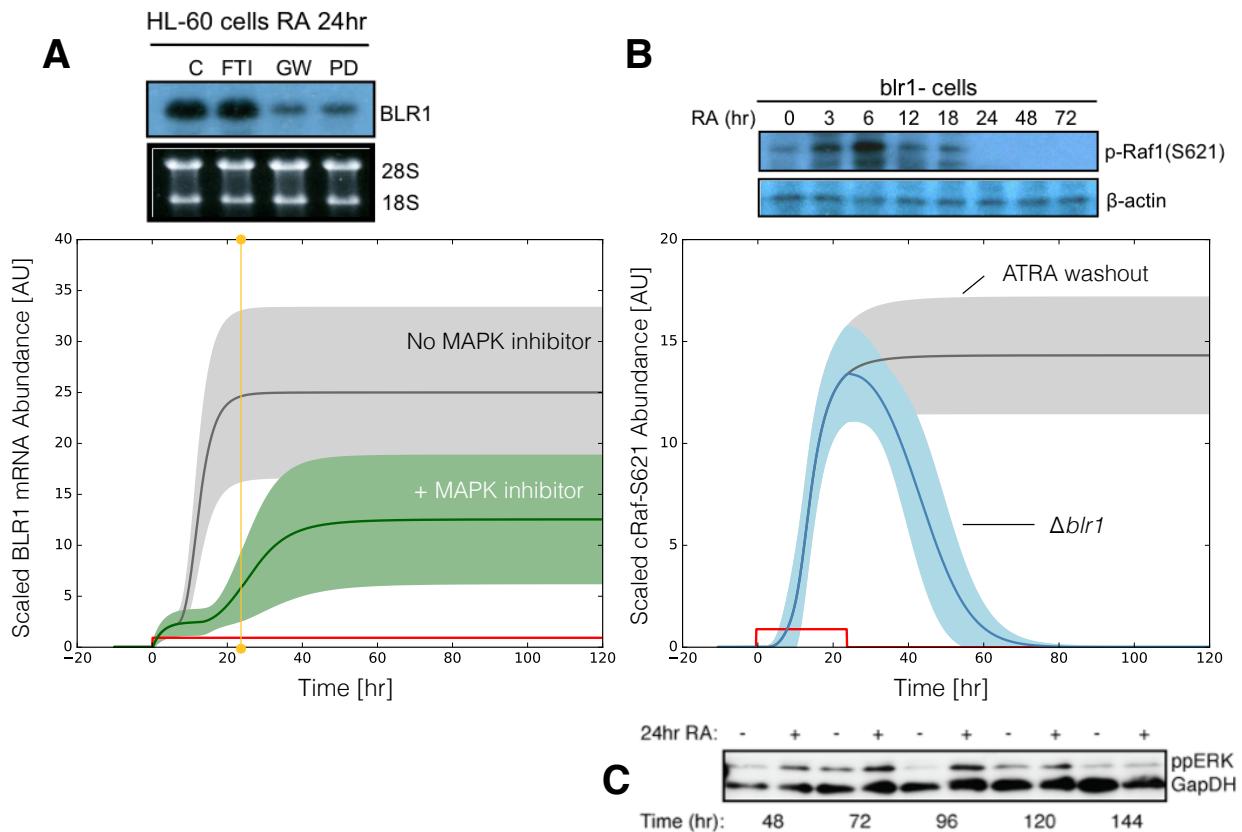


Fig. 3: Model simulation following exposure to $1\mu\text{M}$ ATRA. A: BLR1 mRNA versus time with and without MAPK inhibitor. B: cRaf-pS621 versus time following pulsed exposure to $1\mu\text{M}$ ATRA with and without BLR1. Solid lines denote the mean model performance, while shaded regions denote the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

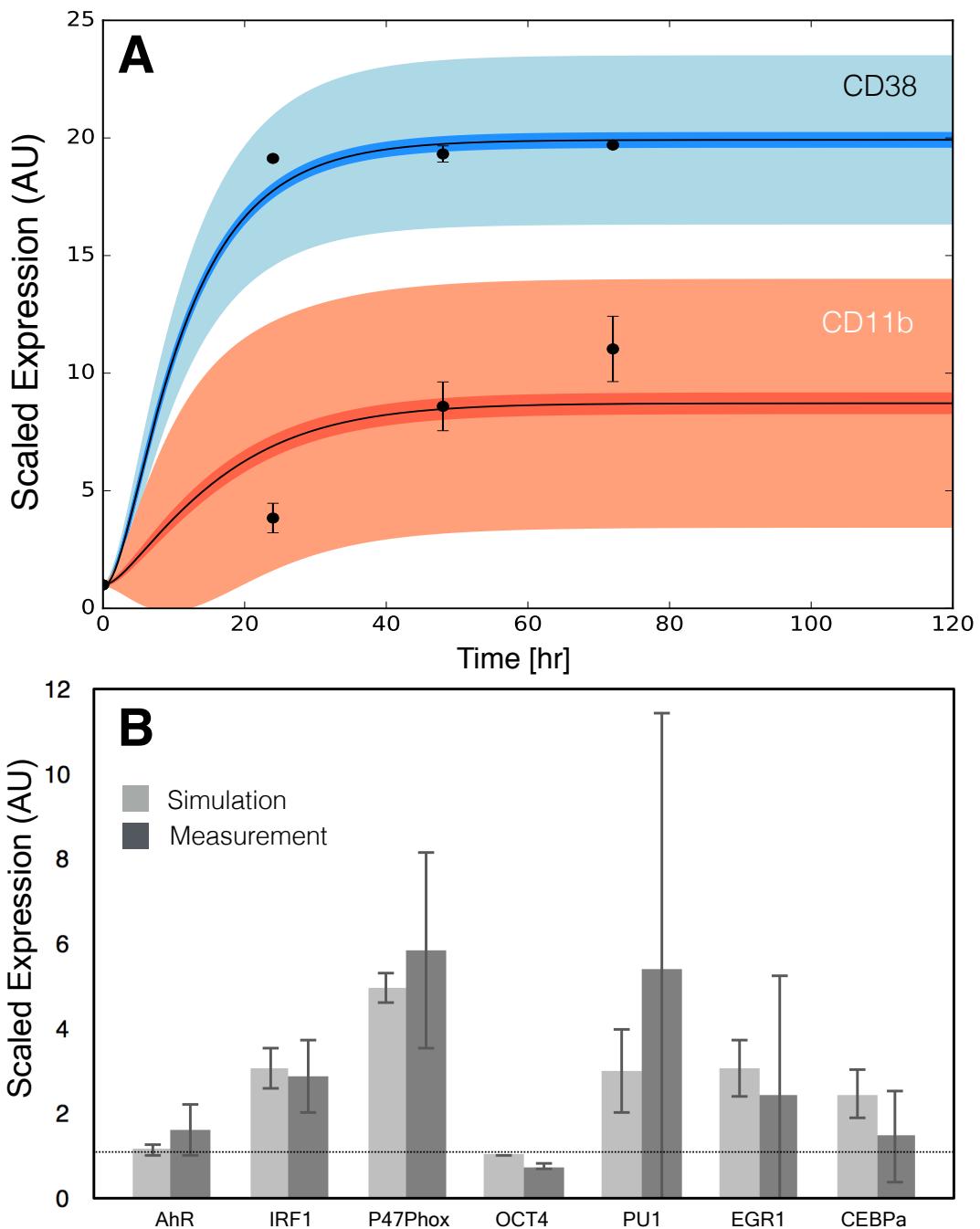


Fig. 4: Model simulation of the HL-60 gene expression program following exposure to $1\mu\text{M}$ ATRA at $t = 0$ hr. A: CD38 and CD11b expression versus time following ATRA exposure at time $t = 0$ hr. B: Gene expression at $t = 48$ hr following ATRA exposure. Experimental data in panels A and B were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

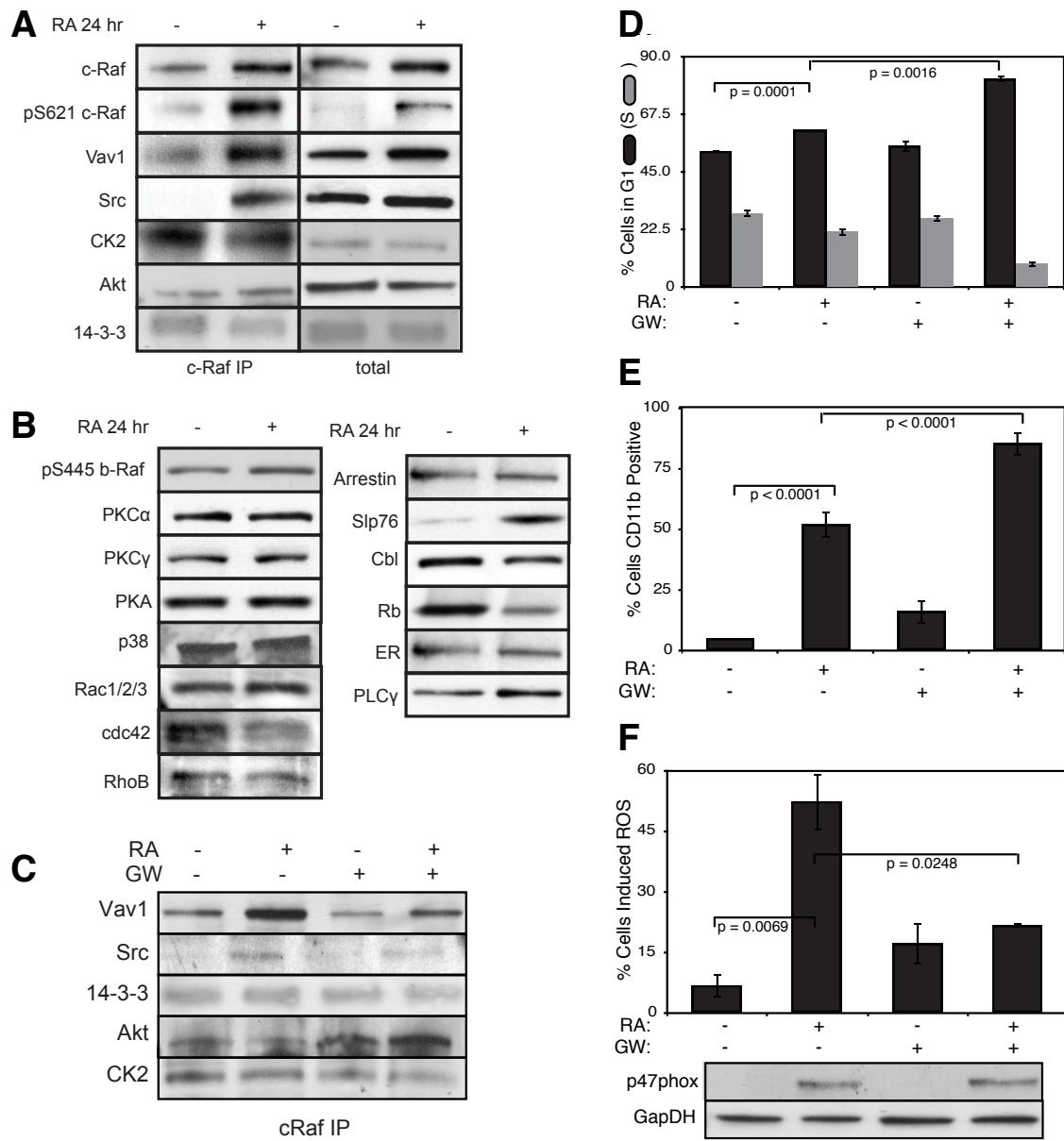


Fig. 5: Investigation of a panel of possible Raf interaction partners in the presence and absence of ATRA. A: Species identified to precipitate out with Raf: first column shows Western blot analysis on total Raf immunoprecipitation with and without 24 hr ATRA treatment and the second on total lysate. 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.