A novel network pharmacology approach for leukaemia differentiation therapy using Mogrify®

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Abstract

Acute myeloid leukaemia (AML) is a rapidly fatal blood cancer which is characterised by the accumulation of immature myeloid cells in the blood and bone marrow as a result of blocked differentiation. Methods which identify master transcriptional regulators of AML subtype-specific leukaemia cell states and their combinations could be critical for discovering novel differentiation-inducing therapies. In this proof-of-concept study, we demonstrate a novel utility of the Mogrify® algorithm in identifying combinations of transcription factors (TFs) and drugs which recapitulate granulocytic differentiation of the NB4 acute promyelocytic leukaemia (APL) cell line, using two different approaches. In the first approach, Connectivity Map (CMAP) analysis of these TFs and their target networks outperformed standard approaches, retrieving ATRA as the top hit. We identify dimaprit and mebendazole as a drug combination which induces myeloid differentiation. In the second approach, we show that genetic manipulation of specific Mogrify®-identified TFs (MYC and IRF1) leads to co-operative induction of APL differentiation, as does pharmacological targeting of these TFs using currently available compounds. We also show that loss of IRF1 blunts ATRA-mediated differentiation, and that MYC represses IRF1 expression through recruitment of PML-RARα, the driver fusion onco-protein in APL, to the IRF1 promoter. Finally, we demonstrate that these drug combinations can also induce differentiation of primary patient-derived APL cells, and highlight the potential of targeting MYC and IRF1 in high-risk APL. Thus, these results suggest that Mogrify® could be used for drug discovery or repositioning in leukaemia differentiation therapy for other subtypes of leukaemia or cancers.

Introduction

Acute myeloid leukaemia is a blood cancer characterized by blocked differentiation and increased proliferation of immature myeloid or lymphoid blasts in the bone marrow and peripheral blood. The differentiation block and increased self-renewal are consequences of dysregulated gene expression control, due in turn to the altered activity or expression of transcriptional or epigenetic regulators, as well as signaling pathway components [1]. However, targeting aberrant signaling can be problematic due to mutations in pathway components or bypass mechanisms, which allow for therapy evasion and resistance [2]. An alternative approach is the identification of drugs which can reverse the aberrant activity of transcriptional regulators underlying the differentiation process.

Transcription factors (TFs) orchestrate the control of gene expression by interacting directly with *cis*-acting DNA sequences, recruiting cofactors as well as epigenetic factors, and in turn initiating or repressing gene expression [3]. TFs can act as potent modifiers of cell fates, including the reprogramming or trans-differentiation of normal somatic, or even leukaemic, cells when over-expressed [4-8]. In leukaemia, a number of subtype-specific, oncogenic TFs not previously implicated in lineage determination have also been identified by various methods to block differentiation or maintain self-renewal programmes [9-11]. Although CRISPR screening remains a popular tool, it does not efficiently utilize limited amounts of patient-derived primary cells, and can require significant effort and time to establish. While integrated genomic approaches have enabled the construction of subtype-associated TF networks [12, 13], systematic and efficient methods for prioritising TF targets or their combinations for therapeutic intervention are still lacking. We previously developed Mogrify®, a computational method which requires only input gene expression data to predict TFs or their combinations which can mediate reprogramming or transdifferentiations between normal somatic cell types [14]. TFs for mediating cellular

conversions are ranked based on their degree of regulatory influence over genes which are highly expressed in a target cell type of interest, while excluding TFs which maintain the identity of the starting cell type. In addition, Mogrify® can be used to identify combinations of ranked TFs with minimal redundancy in gene regulation, thereby maximizing the probability of accomplishing the desired lineage transition. However, it is currently unknown if Mogrify® can be used to identify TFs or their combinations that can induce leukaemia cell differentiation.

In this proof-of-concept study, we show that Mogrify® predicts TFs which regulate the granulocytic differentiation of the acute promyelocytic leukaemia (APL) cell line NB4 upon *all-trans* retinoic acid (ATRA) treatment, leading to the identification of combinations of differentiation-inducing drugs. We chose this disease model since the process is both thoroughly characterised and robust [15-17]. Mechanistically, ATRA binds to the driver fusion oncoprotein PML-RARα in NB4 cells, changing the repertoire of transcriptional cofactors with which it interacts, thus yielding activation of differentiation genes [18, 19]. Eventually, ATRA induces PML-RARα degradation by proteasomal or caspase-dependent mechanisms.

Using Mogrify®, we identified combinations of TFs which exert a significant regulatory influence over direct and early responses to ATRA-mediated differentiation. Connectivity Map (CMAP) [20] analysis using these TF combinations and their networks recovered dimaprit and mebendazole as a drug combination which drives TF targets involved in myeloid maturation. As an alternative approach, we show that combined targeting of two specific TFs (MYC and IRF1) genetically or pharmacologically induces differentiation in NB4 cells, and identify a mechanism by which MYC suppresses IRF1 expression to prevent differentiation. Finally, we demonstrate that these identified drug combinations promote the differentiation of patient-derived APL blasts, and highlight the potential of targeting MYC and IRF1 for the differentiation therapy of high-risk APL.

Results

RNA-Seq captures biologically relevant ATRA-induced transcriptional changes as input to Mogrify®

We treated NB4 cells with 1μM ATRA over a period of 5 days to induce granulocytic differentiation (Fig. 1a), as assessed by Wright staining and the nitroblue tetrazolium (NBT) assay, which indicated a continuous increase in reactive oxygen species (ROS) production (Fig. 1b, top and bottom panels). We performed qPCR validation for genes previously reported to be up- or down-regulated by ATRA in NB4 cells, including early (4h: *CEBPB, GATA2*), intermediate (12-24h: *UBA7, VIM*) or late (72h: *ICAM1, MPO*) response genes (Fig. 1c).

We then carried out RNA sequencing (RNA-Seq) on five time-points: 4h, 12h, 24h, 72h and 120h after ATRA treatment, as well as corresponding DMSO controls. We verified that the normalised RNA-Seq counts for the genes of interest were consistent with the qPCR measurements (Fig. S1 and S2). Principal component analysis (PCA) of the top 10% most variable genes across time (Fig. 1d) revealed that after only 4 hours of ATRA treatment there was a clear distinction between the ATRA and DMSO-treated cells, with a further drastic change at 72h. Following this, we identified differentially expressed (DE) genes at each time-point (Fig. 1e), which revealed that the most widespread changes occur from time=0 to time=4h and from time=24 to time=72h post-treatment, consistent with the PCA.

Gene Ontology (GO) enrichment analysis of the DE genes revealed that the up-regulated genes detected at early time-points (between 4h and 24h) were enriched for inflammatory and immune system processes (Fig. S4, Tables S1-S4), whilst at later time-points we identified a shift towards myeloid differentiation, cell cycle arrest, and apoptosis-specific processes. These observations

are in agreement with existing literature [15-17], suggesting that our time series captures the important biological features of ATRA-induced differentiation.

Mogrify® detects known and novel transcription factors involved in myeloid cell differentiation

We next set out to identify TFs and their regulatory gene networks which are induced or repressed by ATRA treatment at each time-point. To this end, we supplemented the Mogrify® algorithm [14] with a three-step filtering process to identify TFs which exert a significant transcriptional influence on ATRA-regulated target genes, in a time-point specific manner (Fig. 2a). In the first step (Filter 1), we retained only those TFs which regulate part of the network uniquely (i.e., removing TFs which are redundant to each other) (Fig. 2b). We identified a wave-like pattern of transcriptional changes induced by the filtered TFs, one at 4h and another at 72h, consistent with the observed transcriptional and phenotypic changes (Fig. 1b and 1c), and other reports on ATRA-treated leukaemic cells [15, 17]. At the early time-points, CEBPβ, ID1, GFI1B, GATA2, and MYC mediate the majority of transcriptional changes, whereas in later time-points STAT1, FOXC1, MEF2C, EGR2, and EGR3 seem more prominent (Fig. 2b). Notably, most of these identified TFs are known to be involved in neutrophil lineage commitment [21], which is characteristic of ATRA-induced differentiation.

To further refine the predicted TFs, we applied our second filter (Filter 2). This filter identifies TFs whose expression is strongly correlated with those of their target genes across time by calculating a 'c-score' (Fig. 2c, Fig. S5-S8, Table S5 and Supplementary Methods), and excludes TFs with weak or no correlations (based on a c-score p-value threshold of 0.05). Visualisation of the TF-driven gene networks that passed filter one and two revealed that the TFs' expression and number of targets change substantially across time, revealing critical dynamic responses in the gene

expression programs during the differentiation process (Fig. 2d and S9, where nodes represent the TFs).

In our third filter, we established combination scores which reflect the number of targets regulated by TF combinations of various sizes (*n*-TFs, where *n*=1,2..), expressed as a fraction of the targets of those TFs that passed filter one at each time-point. We then kept the highest scoring combination of TFs for each combination size (Fig. 2e). For instance (Fig. 2f, left panel), when up- and down-regulated TFs at t=4h were considered separately, the combination score almost reached its maximum when combining three TFs, and showed only marginal increases when more TFs were included. We therefore kept 3 TFs in the TF combinations throughout our analysis. Similar observations were made for up- and down-regulated TFs at t=72h (Table S6). GO enrichment analysis of the TF regulatory networks revealed enrichment for neutrophil migration and chemotaxis, macrophage differentiation and ribosomal small/large subunit biogenesis, processes that are intrinsic to myelopoiesis (Fig. 2g, Fig. S10-S11, Tables S7 and S8), suggesting specific and functional specialisation of these TF networks.

Identification of pharmacological inducers of APL differentiation

We initially sought to build a computational framework which directly identifies drugs which mimic the key TF-induced transcriptional changes that occur during ATRA treatment of NB4 cells (Fig 3a). To this aim, we queried the Connectivity Map (CMAP) repository of small molecule-induced transcriptional signatures [20], using the statistically significant CMAP (ssCMAP) algorithm [22], with each TF-driven network or the combined networks of the best 3-TF combinations from t=4 or 72h as the input (Tables S5 and S6). Briefly, a compound in CMAP received a positive connectivity score to an individual TF or combination of TFs, if it induced gene expression changes in TF targets in a similar direction and magnitude to that which occurred under ATRA treatment. Following correction for statistical significance, connectivity scores to the TFs or their

combinations were summed for each compound, yielding an "E-score" according to which the compounds were ranked. Thus, a more positive E-score indicated that the compound was more likely to perturb the networks of these TFs in a manner concordant with ATRA treatment. (see Supplementary text).

Based on our computed E-scores, we found that the Mogrify® networks at t=4h identified either ATRA (tretinoin) or isotretinoin among the top predictions, which had strong positive connectivities across the individual TF networks or their combinations. (Fig. 3b, Table S9). These predictions were derived from the reference transcriptional signatures of HL-60 myeloid leukaemia cells in CMAP, which were more likely to be concordant with changes induced by ATRA in APL cells. In contrast, using the set of DE genes as input, ATRA or isotretinoin were more lowly ranked among the drug predictions, with the highest rank being 20 (rank of 1 is considered the best) (Fig. 3b). In addition, the ATRA and isotretinoin connectivity z-scores were higher when the TF-driven networks, compared to the total set of DE genes, was used as the input to CMAP (Fig. 3c). Therefore, using the Mogrify®-predicted TF-networks provided a more specific identification of ATRA from the CMAP repository. The results of the drug predictions and the retrieval of ATRA were consistent when other methods were used to interrogate CMAP (Table S11 and Supplementary Text). We conducted the same analysis using the TF-driven networks (or DE genes) identified at 72h (Table S10), however, this did not retrieve ATRA or isotretinoin as top-ranked drugs. This implies that the early 4h TF-driven networks are more informative in capturing the instigating transcriptional changes induced by ATRA, while later time-points may be more reflective of downstream events. Therefore, we chose to focus further analysis on compounds identified at 4h.

Among the top 15 candidate instances (ranked by E-score) with positive connectivity to the TF networks at t=4h (Fig. 3d), we filtered for those with at least one unique target expressed in NB4

cells at baseline (t=0h), using information from the STITCH database [23] of drug and protein-coding-gene interactions (Fig. S12 and Supplementary Text). In summary, we selected seven drugs to be experimentally validated: Prestwick-983 (dimaprit), quinpirole, colchicine, mebendazole, podophyllotoxin, dinoprost (PGF2α) and isoproterenol.

Experimental validation of CMAP-predicted drugs

To evaluate our chosen list of compounds for differentiation-inducing ability, we treated NB4 cells for 72h with individual CMAP drugs at doses up to 100μM (for dimaprit, quinpirole or dinoprost), or not exceeding their IC50s (for the microtubule inhibitors including mebendazole, colchicine and podophyllotoxin), based on prior cell viability assays (Fig. S13). Unexpectedly, these compounds induced minimal differentiation, as determined by flow cytometric analysis of CD11b, a marker of granulocytic maturation (Fig. 4a). However, all of these compounds enhanced differentiation induced by a suboptimal dose of ATRA (3nM). These results initially suggested that in APL cells, a certain threshold of perturbation with specific targeting of PML-RARα as the centrepiece is required for initiation of the differentiation process. We further validated the differentiation-enhancing effect of two drugs from our screen (dimaprit, a cyclic-AMP/cAMP inducer [24] and mebendazole, a microtubule inhibitor [25]) using Wright's staining and the NBT assay (Fig. S14a and S14b). Notably, we excluded isoproterenol from our screen due to autofluorescence observed in the absence of CD11b-APC antibody.

Given these findings, we inferred that mutations in PML-RARα which confer a loss of ATRA binding, and inhibition of the fusion oncoprotein, would prevent dimaprit and mebendazole-induced differentiation. The NB4-MR2 cell line exhibits partial ATRA resistance owing to aberrant corepressor recruitment by PML-RARα [26, 27], but retains a wild-type PML-RARα sequence and ATRA binding [28, 29]. Conversely, NB4-LR2 cells possess a truncated form of PML-RARα due to a nonsense mutation leading to the elimination of amino acid residues important for ATRA

binding [30]. We thus anticipated that dimaprit and mebendazole would overcome the differentiation defect in MR2 cells in response to ATRA, but not in LR2. Our results (Fig. 4b and 4c) are in keeping with this hypothesis, indicating that ATRA-mediated PML-RARα inhibition facilitates the differentiating-inducing abilities of dimaprit and mebendazole.

We next explored the possibility that combinations of non-RA compounds identified in our screen could induce differentiation, bypassing the requirement for direct PML-RARα inhibition. Based on the connectivity heatmap (Fig. 3d), dimaprit and mebendazole appeared to exhibit complementary positive connections against different TFs (such as to GATA2 and TGIF1 for dimaprit but not mebendazole, and vice versa for ELF4 and MYC). Therefore, we speculated that combining these two compounds might induce a pattern of connectivity similar to that of ATRA/isotretinoin. Treatment of NB4 cells for 72h with the combination of dimaprit and mebendazole resulted in higher CD11b expression compared to individual drugs, and more closely approached the level of differentiation obtained with ATRA treatment (0.01 or 1μM) (Fig. 4d and 4e). Wright staining of drug-treated cells revealed chromosome condensation in mebendazole-treated cells suggestive of mitotic arrest (Fig. 4f), and a moderate amount of cell death based on Annexin-V flow cytometry was observed (Fig. 4g). However, morphological changes indicative of granulocytic differentiation were observed under treatment with dimaprit or the drug combination (Fig. 4f).

To validate if dimaprit and mebendazole were perturbing Mogrify®-identified TF targets involved in differentiation, we identified a collection of genes which were targets of at least one TF and fulfilled the following criteria: a) (log2FC) > 2 and b) (ssCMAP score) > 18,000, indicating similar extent and direction of regulation by both dimaprit/mebendazole and ATRA. qPCR analysis revealed that upon 24h of drug treatment, the expression of these genes was enhanced by the combination of dimaprit and mebendazole compared to either drug alone, consistent with the ATRA-induced upregulation of these genes (Fig. 4h). However, these increases in gene

253 expression were less evident at early (4h) or late (72h) timepoints compared to ATRA treatment
254 (Fig. S14c), suggesting direct but relatively transient effects. Western blot analysis of drug-treated
255 cells showed variable induction of granulopoietic TFs identified by Mogrify® including IRF1 and
256 CEBPβ/ε [31, 32]. Notably, the combination of the 2 compounds resulted in more significant
257 downregulation of Mogrify®-identified TFs known to block differentiation (especially MYC and
258 TGIF1) [33, 34], compared to either drug alone (Fig. 4i). In all, these data indicate that dimaprit
259 and mebendazole exert combinatorial effects on differentiation, either by inducing TF targets
260 involved in myeloid maturation, or inhibiting the expression of oncogenic TFs.
261
262 **MYC and IRF1 regulate the differentiation status of NB4 cells**
263 As an alternative approach, we reasoned that by identifying a minimal set of 2 or 3 TFs which
264 regulate differentiation, it would be possible to infer drug combinations which specifically target
265 these TFs or mechanisms by which they operate. We performed Gene Set Enrichment Analysis
266 (GSEA) [35] using publicly available datasets of genes directly bound by and positively regulated
267 by TFs identified by Mogrify® at t=4h (Filter 3, Fig. 2f) [36-39] (Supplementary Methods). Among
268 our comparisons, we observed that genes which were positively regulated by IRF1 and MYC were
269 strongly induced and suppressed by ATRA, respectively, and that these enrichments were
270 maintained throughout the differentiation time-course (Fig. 5a and S15a). Conversely, we found
271 no consistent pattern of enrichment for GATA2 positively-regulated genes. In support of a role for
272 IRF1 (an interferon-inducible TF) and MYC, GSEA analysis using the MSigDB collection of
273 hallmark gene sets [40] revealed that ATRA-induced transcriptional changes showed clear
274 positive and negative enrichments for interferon alpha/gamma signalling and MYC targets,
275 respectively (Fig. S15b). In addition, we observed that the expression of several Mogrify®-
276 identified myeloid TFs (IRF1, ELF4, CEBPβ/ε) were all consistently de-repressed by shRNA-
277 mediated knockdown of MYC, but not GATA2 (Fig. S16). Taken together, these observations led
278 us to prioritize targeting of IRF1 and MYC for subsequent differentiation assays.

We next investigated if knockdown (KD) of MYC combined with over-expression (OE) of IRF1 would induce differentiation of NB4 cells. Lenti- and retroviral transduction experiments revealed that shRNA-mediated KD of MYC led to de-repression of endogenous IRF1 protein (Fig. 5b), consistent with the qPCR data. KD of MYC induced differentiation, and this was further enhanced by concurrent OE of IRF1 (Fig. 5c and 5d). Wright staining showed granulocytic differentiation initiated by MYC KD (with or without IRF1) (Fig. 5e). We observed that the IRF1-mediated enhancement of differentiation was more pronounced for shMYC#1 compared to #3, which could be due to stronger effects of sh#3 on cellular viability (Fig. 5f).

To determine if IRF1 was important for ATRA-induced differentiation, we generated IRF1 knockout (KO) NB4 cell lines through CRISPR/Cas9-mediated gene editing and clonal isolation (Fig. 5g). Flow cytometry analyses revealed that KO of IRF1 attenuated the differentiation response to low-dose (3nM), but not high-dose (1μM) ATRA (Fig. 5h, 5i and S17a). KO of IRF1 did not result in reciprocal upregulation of MYC (Fig. S17b). Although MYC expression was slightly decreased in 2 of 3 IRF1 KO lines relative to a non-targeting (NT) control, no significant differences in CD11b expression were observed (Fig. S17c). When we further silenced MYC expression in these lines with shRNAs, we observed that KO of IRF1 did not prevent MYC knockdown-induced differentiation, but rather enhanced it in some instances (Fig. S17d and S17e). Taken together, these results suggest that loss of IRF1 blunts the sensitivity of NB4 cells to ATRA-induced differentiation, whereas other factors may drive the differentiation response to silencing of MYC.

In support of a role for MYC in interfering with IRF1, we observed that forced MYC OE could prevent the ATRA-mediated induction of IRF1, thus counteracting the expression changes normally induced by ATRA (Fig. 5j). This was in turn associated with a suppression of ATRA-induced differentiation (Fig. 5k), consistent with previous reports [41]. Thus, these results suggest

that MYC limits sensitivity to ATRA-mediated differentiation in NB4 cells, in part, by suppressing the induction of IRF1.

MYC prevents expression of IRF1 and its target genes

We next investigated if MYC directly suppresses IRF1 gene transcription, and whether PML-RARα was also involved in this process. Analysis of publicly available MYC [42] and PML-RARα [43] ChIP-Seq data in NB4 cells revealed that both factors were co-bound at the promoters of the IRF1 and adjacent IRF1-AS1 genes, as well as multiple regions proximal or distal to IRF1 (Fig. 6a). In-house ChIP- and ATAC-Seq analyses showed that these regions underwent increases in H3K27ac, and mild increases in chromatin accessibility upon ATRA treatment. These observations were in line with ATRA-mediated transcriptional activation of IRF1, and a putative role for these non-promoter regions as distal regulatory enhancers. Analysis of predicted chromatin interactions in NB4 cells (EnhancerAtlas) [44] as well as previously reported PML-RARα ChIA-PET data [45] , indicated a possible regulatory interaction between the putative distal enhancer designated E4 ("E4"), and the IRF1 promoter ("Pr").

Given the observed co-binding of MYC and PML-RARα, we asked if MYC was involved in recruiting PML-RARα to the IRF1 Pr or E4. KD of MYC with shRNAs led to ~50% reduction in MYC binding at Pr, but this was accompanied by a drastic reduction in bound PML-RARα. Similar observations were made for E4, although the effects were more modest (Fig. 6b).

We then interrogated the activation status of Pr and E4 in response to ATRA treatment or MYC KD using luciferase reporter assays. We cloned genomic regions corresponding to the IRF1 Pr or E4, as well as a E4-Pr fusion (to simulate a long-range chromatin interaction) into empty pGL4(luc2) reporter constructs (Fig. 6c) and transfected these into NB4 cells, followed by treatment with 1μM ATRA for 24h. ATRA treatment led to increases in Pr activity over untreated

cells, as expected. While E4 had no activity of its own, the fusion of E4 to Pr led to slight reduction in reporter activity in untreated cells, indicating suppression of the IRF1 promoter. Upon ATRA treatment, this suppressive effect was reversed, yielding a signal above that obtained with Pr alone (Fig. 6d). This suggests that under ATRA-induced differentiation, the E4-Pr interaction switches from being suppressive to activating, triggering expression of IRF1.

To more specifically query the role of MYC in regulating E4/Pr activity, we co-transfected the abovementioned constructs with siRNAs against MYC into NB4 cells (Fig. 6e), followed by a luciferase reporter assay 24h later. KD of MYC resulted in increases in IRF1 Pr activity, but reporter activity remained low when E4 was fused to Pr (E4-Pr) (Fig. 6f). We speculate that other repressive factors than MYC may be binding at E4, and the removal of their repressive activity may require administration of ATRA.

Finally, KD of MYC led to reduced expression of MYC target genes (based on leading edge analysis of our GSEA, Fig. 5a) involved in metabolic processes, cellular growth and proliferation. Conversely, IRF1 and its target genes (with anti-proliferative, pro-differentiation or immune functions) were up-regulated (Fig. 6g), indicating that inhibition of MYC activates the expression of IRF1 target genes.

Thus, our results suggest that ATRA-mediated induction of IRF1 expression involves long-range interactions between transcriptionally active E4 and Pr. However, suppression of MYC activates the IRF1 Pr directly, possibly involving the dissociation of PML-RARα and recruitment of *cis*-acting factors which facilitate transcription (Fig. 6h).

MYC inhibitor IZCZ-3 co-operates with IFNγ to induce differentiation

Next, we asked if it was possible to pharmacologically recapitulate our combination genetic experiment (MYC KD and IRF1 OE) for differentiation therapy. Treatment of NB4 cells with the MYC inhibitor IZCZ-3 (which prevents MYC transcription) [46] and IFNγ (which activates IRF1 expression [47]) induced protein-level changes in MYC and IRF1 according to the expected directions (Fig. 7a). In addition, the combination of both compounds resulted in greater differentiation compared to either alone, following 72h of treatment (Fig. 7b and 7c). These observations were validated with Wright stain and NBT assay, in which a small population of ROS-producing cells (~5%) appeared exclusively under the combination, indicating functional maturation (Fig. 7d). Cell viability measurements indicated minimal changes relative to untreated controls at 72h (Fig. 7e). Thus, small-molecule inhibition of MYC and activation of IRF1 could "mimic" the differentiated phenotypes obtained by genetic manipulation of these TFs.

Drug combinations induce differentiation in primary APL cells

To investigate the clinical applicability of our identified drug combinations, we treated bone marrow-derived mononuclear cells (MNCs) from 2 APL patients with these drugs for 72h in the presence of cytokines, alone or in combination with low-dose ATRA (5nM). We observed that either forskolin (a cAMP inducer) or mebendazole could enhance differentiation induced by low-dose ATRA, and this effect was more pronounced when all three compounds were combined. (Fig. 8a and 8b). We used forskolin (a cell permeable activator of adenylate cyclase [48]) in place of dimaprit given the high doses of dimaprit used in our NB4 experiments which may limit its clinical application. Furthermore, previous reports showed that dimaprit may induce only limited or transient increases in intracellular cAMP, due to histamine receptor desensitization [49-51]. Similar observations were made when IZCZ-3 and IFNγ were combined with ATRA (Fig. 8c and 8d).

While the use of ATRA and arsenic trioxide (ATO) cures most patients with APL [18, 19], a minority still experience early death and/or incomplete remissions before or during induction therapy owing to haemorrhagic complications [52-54]. In this regard, high-risk APL (WBC count >10 X 10^9/L at diagnosis) has to be shown to be prognostic for treatment failure and poorer overall/disease-free survival compared to standard (intermediate and low) risk, especially for regimens involving ATRA and chemotherapy [55, 56].

Given that high MYC and low IRF1 expression enforces an undifferentiated state even in the presence of ATRA in NB4 (Fig. 5h and 5i), we explored if this would also translate into adverse clinical outcomes. We analyzed recently published RNA-Seq data of 323 APL patients who had been classified into two groups (revised high or standard risk) which demonstrated differences in survival outcomes (individual survival data were unfortunately not available) [57]. We did not observe statistically significant differences in *MYC*, *IRF1* or *MYC*:*IRF1* expression ratios between high and standard risk groups (Fig. S18a and S18b). However, GSEA analysis revealed that IRF1 target genes [36] were significantly enriched in standard vs. high risk groups, whereas MYC target genes [37] were enriched in high risk APL, but this did not attain statistical significance (Fig. S18c and S18d). These data could be suggestive of an imbalance of MYC transcriptional activity at the expense of IRF1 in high-risk APL, and that a MYCi/IFNγ/ATRA combination may be useful for tilting cell states in favour of differentiation.

Discussion

In this study, we show that the Mogrify® algorithm performs *de novo* identification of TFs which exert significant regulatory influence over the ATRA-induced differentiation of NB4 APL cells, analogous to the manner in which it has been previously used to identify factors for normal cellular interconversions. Furthermore, we identified differentiation-inducing drugs, using CMAP to query the networks of TF combinations, or by specifically targeting MYC and IRF1.

Many TFs identified at an early stage of differentiation (4h) (Fig. 2b) such as IRF1, CEBPβ/ε, MYC, MYB and GATA2 [ref. 31-33, 58] have been directly implicated in the activation or blockage of normal myelopoiesis. We chose to focus on early-acting TFs (4h), as they may be initiators of the transcriptional response to ATRA, rather than secondary or tertiary changes [20]. CMAP analysis of the TF networks successfully recovered ATRA and its isomer isotretinoin (13-*cis* RA) as being among the top hits. Isotretinoin, as with ATRA, has been described to induce the functional maturation of NB4 cells [59]. We also identified other non-retinoid compounds including dimaprit (a cAMP agonist) and mebendazole (a microtubule inhibitor) (Fig. 3d). These two compounds were previously identified as potent inducers of differentiation in non-APL AML cells such as HL60 using CMAP or other gene signature-based approaches [60, 61]. In NB4 cells, we observed that administering these two compounds concurrently could circumvent the requirement for direct PML-RARα inhibition by ATRA, and induce differentiation (Fig. 4d and 4e).

Mechanistically, dimaprit and mebendazole enhanced the expression of TF targets associated with myeloid maturation (Fig. 4h), and silenced oncogenic TFs (GATA2, MYC and TGIF1) associated with blocking differentiation (Fig. 4i). Previous studies have shown that cAMP agonists enhance ATRA-induced differentiation [62, 63], and trigger activation of protein kinase A (PKA), which regulates the transcription or stability of PML-RARα [64] and MYC [65, 66]. Meanwhile, mebendazole has been shown to promote the proteasomal degradation of c-MYB by inhibiting its association with the heat shock protein 70 (HSP70) chaperone system, thereby reducing the growth and viability of AML cell lines [67]. However, this study did not assess the effect on differentiation. Future investigations will seek to clarify how these two compounds function together to silence TFs which block differentiation in APL cells, and potentially lead to their repurposing as agents for differentiation therapy.

In our second approach, we used GSEA to narrow down on MYC and IRF1 as a putative combination of TF targets for differentiation therapy (Fig. 5a). Previous evidence suggests that IRF1 is required for the expression of myeloid TFs such as CEBPα, ε and PU.1 [31, 68]. Our results (Fig. 5g-5i, S17a) indicate that loss of IRF1 prevents differentiation induced by low, but not high-dose ATRA, suggesting that stronger inhibition of PML-RARα leads to de-repression of additional factors involved in myeloid maturation. In addition, IRF1 KO did not prevent MYC knockdown-induced differentiation (Fig. S17d and S17e). Recent evidence has implicated MYC in suppressing MIZ1-mediated trans-activation of CEBPα and δ [69, 70], thereby maintaining the self-renewal and undifferentiated state of AML leukaemia stem cells (LSCs) [70]. It is thus possible that other myeloid TFs are also activated by knockdown of MYC, compensating for the loss of IRF1. Nevertheless, our experiments suggest that Mogrify® identifies TFs which influence differentiation of leukaemic cells, and suggest future complementation with other readouts of functional importance to highlight additional driving factors.

Our genetic experiments confirmed that MYC suppresses IRF1 induction during ATRA treatment. (Fig. 5j and 5k), thus possibly impairing differentiation. ChIP-qPCR and luciferase reporter assays suggested that MYC functions primarily to suppress activity at the IRF1 promoter, possibly through recruitment of PML-RARα (Fig. 6a-6f). These observations add to the existing evidence that PML-RARα is recruited by other TFs such as PU.1 or GFI1 to mediate transcriptional activation or repression in APL cells [43, 71]. Nevertheless, both MYC and PML-RARα are known to interact with co-factors and epigenetic enzymes mediating transcriptional repression (such as HDACs, MIZ1 and G9a) [18, 72], and it remains to be determined if these are present at the IRF1 promoter as well.

Lastly, our study led to the identification of drug combinations (forskolin/mebendazole or IZCZ-3/IFNγ) which enhance ATRA-induced differentiation of NB4 cells (Fig. 7a-7d) and/or primary APL

MNCs (Fig. 8a-8d), in line with previous reports that MYC inhibitors or IFNγ can initiate or promote differentiation [73, 74]. GSEA analysis using publicly available gene expression data [57] suggested an enrichment of MYC and IRF1-driven transcriptional programmes in high and standard-risk patients, respectively (Fig. S18c and S18d). Nevertheless, additional clinical data will be necessary to more firmly support the utility of targeting these two TFs in high risk patients or those with suboptimal ATRA responses.

In all, our study has demonstrated that Mogrify® identifies combinations of TFs and drugs which induce leukaemia differentiation. We anticipate that these approaches will be useful for addressing biological questions relevant to cell state changes in cancer including phenotypic plasticity, heterogeneity in drug responses and disease-phase transitions.

Materials and Methods

Cell culture and drug treatments

NB4, NB4-LR2 and NB4-MR2 cell lines were a gift from Prof. Eric C.W. So, King's College London, UK. All cell lines were maintained in RPMI 1640 (Nacalai Tesque, Kyoto, Japan) supplemented with 10% FBS (Biowest, MO, USA), 100U/mL penicillin-streptomycin and 2mM L-glutamine (Gibco, Thermo Fisher Scientific, MA, USA) in a 37°C humidified incubator with 5% CO_2. The NB4 cell lines were authenticated with a short tandem repeat (STR) genotyping service from 1st base (Singapore). Mycoplasma contamination in these lines was initially detected through the use of genus-specific PCR primers, eliminated through BM cyclin (Roche) treatment for 2 weeks, and validated to have no influence on early experimental data (refer to Fig. S19).

Frozen stocks of APL patient MNCs were obtained from the Singapore General Hospital (SGH) Department of Haematology with approval from the SingHealth Centralised Institutional Review Committee (CIRB 2008-072). APL MNCs were cultured in Stemspan SFEM-II (Stemcell

Technologies, Vancouver, Canada) supplemented with 50ng/mL hFLT3L, 50ng/mL hSCF, 10ng/mL hIL3, 10ng/mL hIL6 and 50ng/mL hG-CSF (Peprotech, NJ, USA) in the presence of 100U/mL penicillin-streptomycin (Gibco). Following overnight recovery, dead cells were removed with Dead Cell Removal Kit (Miltenyi Biotec, Germany) and the MNCs were thereafter seeded for drug treatments ($0.1X10^6$/mL). All drugs used in CMAP analysis were purchased from Sigma-Aldrich (MO, USA), except for colchicine (Little Pharmaceutical Suppliers, Singapore and kindly provided by Dr. Than Hein, Singapore General Hospital). IZCZ-3 (MedChemExpress, NJ, USA), forskolin (Abcam, Cambridge, UK) and IFNγ (Peprotech) were reconstituted according to datasheet instructions. For 5-day experiments, fresh media with drugs was added on the 2nd or 3rd day.

RNA-Seq, Mogrify® pipeline, ssCMAP, CMAP GSEA and STITCH

Details of the above are described in Supplementary Methods.

Wright staining and NBT reduction assay

Wright staining was performed by resuspending NB4 cells at 0.5 x 10^5 cells in 100µL PBS and centrifugation onto microscope glass slides with a Cytospin™ 4 Cytocentrifuge (Thermo Fisher Scientific) at 500rpm for 5 mins with low acceleration. Dried cytospots were incubated with Wright stain (Sigma-Aldrich) with an equal volume of buffered water, pH 6.8 (Millipore, MA, USA) for 5 mins, rinsed with deionized water and dried fully before mounting with DPX (Sigma-Aldrich). The NBT assay was performed as previously described [8], and for quantification at least 100 cells per cytospot were evaluated for ROS production. Brightfield images were captured at 40X magnification with an Olympus IX71 inverted microscope.

Quantitative Real-Time PCR (qRT-PCR)

RNEasy Mini Kit (Qiagen, Germany) and Superscript™ VILO™ cDNA Synthesis Kit (Thermo Fisher Scientific) were used for RNA extraction and reverse transcription to cDNA, respectively. qPCR was performed in triplicate using CFX384 Touch Real-Time PCR Detection System (Bio-Rad, CA, USA) with iQ SYBR® Green Supermix using primers listed in Table S12. Relative mRNA expression was determined by normalization to *TBP* followed by the relevant experimental controls (ΔΔCt method).

Western Blotting

Cells were lysed in RIPA buffer as previously described [75] (for whole cell extracts) or processed with NE-PER™ Nuclear and Cytoplasmic Extraction Reagents (Thermo Fisher Scientific) for preparation of nuclear lysates. Details of antibodies for Western blotting are listed in Table S13. Blot images were obtained with ChemiDoc™ Touch Imaging System (Bio-Rad) and densitometry performed with ImageJ Software.

CellTiter-Glo® Cell Viability Assay

CellTiter-Glo® Luminescent Assay (Promega, WI, USA) was performed based on the kit instructions, using a seeding density of 5,000 cells/well for all drug treatments in triplicate.

GSEA of TF target genes

Details of the above are described in Supplementary Methods.

Cloning of plasmid constructs

MSCV-Puro-IRES-GFP (MPIG) and MSCV-IRES-tdTomato (MIT) retroviral constructs were obtained from Addgene. MSCV-Puro-IRES-tdTomato (MPIT) was derived by replacement of IRES-GFP sequence in MPIG with a NsiI-IRES-tdTom fragment from MIT. MPIG-MYC and MPIT-IRF1 were derived by cloning the respective ORFs between XhoI and EcoRI restriction sites in

the plasmid backbone. pLVX-shRNA2-ZsGreen1-blasti lentiviral vector was derived by insertion of a XhoI-SV40 promoter-blasti-XbaI fragment into pLVX-shRNA2-ZsGreen1 (Takara Bio, Japan). shRNA sequences obtained from the Mission® shRNA (Sigma-Aldrich) or SMARTvector collection (Dharmacon, CO, USA) against MYC or GATA2 (listed in Table S14) were cloned between BamHI and EcoRI sites in the pLVX backbone. sgRNA sequences for IRF1 KO (Table S15) were cloned into LentiCRISPR v2 [76] (Addgene) according to the provided protocol. For luciferase reporter constructs, the following regions were amplified from NB4 genomic DNA with Primestar GXL DNA Polymerase (Takara Bio), and cloned between KpnI and XhoI sites in the pGL4.10[luc2] (Promega) multiple cloning site: Human IRF1 promoter, Pr (-743 to +1021bp relative to transcriptional start site/TSS), E4 (1.88kb fragment) and E4-Pr (a KpnI-E4-EcoRI fragment was pre-ligated to EcoRI-Pr-XhoI, and the fusion was re-amplified and cloned into pGL4). Primers used for cloning are listed in Table S18.

Lenti/Retrovirus Preparation and Infection

Lentiviral preparation and shRNA transduction with pLVX vector was performed as previously described [75]. MSCV retroviral infection of NB4 cells was performed by concentrating viral supernatants 20-fold with Amicon® Ultra-15 Centrifugal Filter Units (Millipore), followed by two rounds of spinoculation 24h apart in the presence of 8μg/mL polybrene. tdTom or GFP-positive cells were isolated with a BD FACSAria® II sorter (Duke-NUS Flow Cytometry Facility, Singapore) at 3 days post-infection and/or selected with 0.3μg/mL puromycin for 72h. For TF combination experiments, cells were expanded thereafter prior to re-infection with pLVX-shRNA2-ZsGreen1.

Generation of IRF1 KO cells by CRISPR-Cas9

NB4 cells were spin-infected once with LentiCRISPR v2 plasmid encoding control or IRF1 sgRNAs (Table S15), followed by puromycin selection (as above). Cells were re-plated at low density (1,200/60mm dish) in Methocult H4230 (Stemcell Technologies, Vancouver, Canada) with

puromycin and without added cytokines. Following 10-12 days of culture, well-isolated individual colonies were picked and expanded in liquid medium. Genomic DNA was isolated with DNEasy Blood and Tissue Kit (Qiagen, Germany) and the sgRNA targeted regions were PCR-amplified (using primers listed in Table S16), gel-extracted and subjected to Sanger sequencing (1st Base, Singapore). Details of genomic indels in individual clones are listed in Table S17. For analysis of MYC knockdown, control and IRF1 KO lines were re-transduced with pLVX-shRNA2-ZsGreen1-blasti followed by 48h of blasticidin selection (10µg/mL) prior to analysis.

Flow Cytometry

For assessment of differentiation, cells were washed once in PBS and incubated with human FcR Blocking Reagent (Miltenyi Biotec) at 4ºC for 10 mins, followed by 1µg/mL CD11b-APC antibody or isotype control (Rat IgG2b-APC, Miltenyi Biotec) at 4ºC for 15 mins. Cells were washed again and resuspended in PBS containing DAPI at 1µg/mL prior to analysis using a BD LSRFortessa™ analyzer (BD Biosciences, NJ, USA). The data was processed using FlowJo v10.5.3 software (Flowjo, OR, USA). Cell viability analysis was performed using Annexin V-FITC/-AAD Kit (Beckman Coulter, CA, USA), or a combination of Annexin-V eFluor450 (eBioscience, Thermo Fisher Scientific) and Zombie NIR™ Fixable Viability Dye (Biolegend, CA, USA) with compensation in the case of tdTom/GFP-positive cells.

ChIP-qPCR, ChIP-Seq and ATAC-Seq

ChIP-qPCR was performed as previously described [77], using 10 X 10^6 NB4 cells per antibody pulldown and SONICS Vibracell sonicator (Sonics and Materials, Inc., CT, USA) for chromatin shearing. Details of ChIP-Seq, as well as antibodies used for IP are listed in Supplementary Methods and Table S13 respectively. Fast-ATAC-Seq was performed on 50,000 control or ATRA-treated NB4 cells per replicate as previously described [78], with the assistance of the Duke-NUS Genome Biology Facility (Duke-NUS, Singapore).

589

590 **Luciferase Reporter Assays**

591 $1X10^6$ NB4 cells were transfected with 1µg of each pGL4.10[luc2] construct, 50ng pRL-SV40, and
592 /or DsiRNAs (Integrated DNA Technologies, IA, USA) at final concentration of 25nM in media
593 using the Amaxa Nucleofector™ 2b device and Cell Line Nucleofector kit V (Lonza, Switzerland)
594 based on protocols provided. The DsiRNA sequences used are listed in Table S14. Cells were
595 subsequently cultured in 3mL RPMI for the indicated durations, with or without ATRA addition at
596 24h post-transfection. Thereafter, firefly and renilla luciferase signals were quantified with the
597 Dual Luciferase Reporter Assay System (Promega) on the TECAN Infinite M200 system (Tecan,
598 Switzerland). Firefly luciferase signals were normalized to those of renilla luciferase for each
599 sample to determine the relative luciferase activity.

600

601 **Statistics**

602 Statistical analyses for experimental data were performed with Graphpad Prism 9. Two-tailed
603 unpaired t-test was applied on three biological replicates per treatment, or on data from three
604 independent experiments (for the flow cytometry screen in Fig. 4a). The sample size of n=3 is
605 commonly used for *in vitro* experiments. The significance of the results was calculated, with *$p \leq$
606 *0.05, **$p \leq 0.01$,***$p \leq 0.001$, and ****$p \leq 0.0001$*. Error bars indicate mean ± SD.

607

608 **Data availability**

609 The raw and processed data were submitted to NCBI GEO. The accession number will be
610 available upon publication.

611

612 **Code availability**

613 The source code of our pipeline will be available upon publication. Source code for the Mogrify®
614 algorithm is not available.

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Author contributions

Wet lab experiments were performed by LLM and PS. RNA-Seq was planned and designed by KLL. RNA-Seq data QC, analysis, Gene Set Functional Enrichments, querying of CMAP, drug ranking, and network analysis were performed by EGC. ChIP data was analysed by BJC. KLL provided guidance on TF GSEA analyses. EGC and LLM prepared the manuscript and generated the figures with input from co-authors. KLL, OJLR, EP, and STO designed the study, after conceptualisation by OJLR, EP, and STO. ES and TKF provided the NB4/MR2/LR2 cell lines. GCW provided primary human APL samples from the Singapore General Hospital Haematology Repository.

Competing Interests

OJLR is a co-inventor of the patent (WO/2017/106932) and is a co-founder, shareholder and director of Mogrify Ltd, a cell therapy company. All other authors declare no competing interest.

641 **References**

642 1 Khwaja A, Bjorkholm M, Gale RE, Levine RL, Jordan CT, Ehninger G *et al.* Acute myeloid
643 leukaemia. *Nat Rev Dis Primers* 2016; **2**: 16010.

644 2 Gonda TJ, Ramsay RG. Directly targeting transcriptional dysregulation in cancer. *Nat Rev*
645 *Cancer* 2015; **15**: 686–694.

646 3 Bradner JE, Hnisz D, Young RA. Transcriptional Addiction in Cancer. *Cell* 2017; **168**: 629–
647 643.

648 4 Takahashi K, Yamanaka S. Induction of pluripotent stem cells from mouse embryonic and
649 adult fibroblast cultures by defined factors. *Cell* 2006; **126**: 663–676.

650 5 Takahashi K, Tanabe K, Ohnuki M, Narita M, Ichisaka T, Tomoda K *et al.* Induction of
651 pluripotent stem cells from adult human fibroblasts by defined factors. *Cell* 2007; **131**: 861–
652 872.

653 6 Chao MP, Gentles AJ, Chatterjee S, Lan F, Reinisch A, Corces MR *et al.* Human AML-
654 iPSCs Reacquire Leukemic Properties after Differentiation and Model Clonal Variation of
655 Disease. *Cell Stem Cell* 2017; **20**: 329–344.e7.

656 7 Kotini AG, Chang C-J, Chow A, Yuan H, Ho T-C, Wang T *et al.* Stage-Specific Human
657 Induced Pluripotent Stem Cells Map the Progression of Myeloid Transformation to
658 Transplantable Leukemia. *Cell Stem Cell* 2017; **20**: 315–328.e7.

659 8 McClellan JS, Dove C, Gentles AJ, Ryan CE, Majeti R. Reprogramming of primary human
660 Philadelphia chromosome-positive B cell acute lymphoblastic leukemia cells into
661 nonleukemic macrophages. *Proc Natl Acad Sci U S A* 2015; **112**: 4074–4079.

662 9 Park S-M, Cho H, Thornton AM, Barlowe TS, Chou T, Chhangawala S *et al.* IKZF2 Drives

Leukemia Stem Cell Self-Renewal and Inhibits Myeloid Differentiation. *Cell Stem Cell* 2019; **24**: 153–165.e7.

10 Wang E, Zhou H, Nadorp B, Cayanan G, Chen X, Yeaton AH *et al.* Surface antigen-guided CRISPR screens identify regulators of myeloid leukemia differentiation. *Cell Stem Cell* 2021; **28**: 718–731.e6.

11 Cao Z, Budinich KA, Huang H, Ren D, Lu B, Zhang Z *et al.* ZMYND8-regulated IRF8 transcription axis is an acute myeloid leukemia dependency. *Mol Cell* 2021; **81**: 3604–3622.e10.

12 Assi SA, Imperato MR, Coleman DJL, Pickin A, Potluri S, Ptasinska A *et al.* Subtype-specific regulatory network rewiring in acute myeloid leukemia. *Nat Genet* 2019; **51**: 151–162.

13 Yun H, Narayan N, Vohra S, Giotopoulos G, Mupo A, Madrigal P *et al.* Mutational synergy during leukemia induction remodels chromatin accessibility, histone modifications and three-dimensional DNA topology to alter gene expression. *Nat Genet* 2021; **53**: 1443–1455.

14 Rackham OJL, Firas J, Fang H, Oates ME, Holmes ML, Knaupp AS *et al.* A predictive computational framework for direct reprogramming between human cell types. *Nat Genet* 2016; **48**: 331–335.

15 Liu TX, Zhang JW, Tao J, Zhang RB, Zhang QH, Zhao CJ *et al.* Gene expression networks underlying retinoic acid-induced differentiation of acute promyelocytic leukemia cells. *Blood* 2000; **96**: 1496–1504.

16 Yang L, Zhao H, Li S-W, Ahrens K, Collins C, Eckenrode S *et al.* Gene expression profiling during all-trans retinoic acid-induced cell differentiation of acute promyelocytic leukemia cells. *J Mol Diagn* 2003; **5**: 212–221.

17 Zheng P-Z, Wang K-K, Zhang Q-Y, Huang Q-H, Du Y-Z, Zhang Q-H *et al.* Systems analysis of transcriptome and proteome in retinoic acid/arsenic trioxide-induced cell differentiation/apoptosis of promyelocytic leukemia. *Proc Natl Acad Sci U S A* 2005; **102**: 7653–7658.

18 de Thé H, Pandolfi PP, Chen Z. Acute Promyelocytic Leukemia: A Paradigm for Oncoprotein-Targeted Cure. *Cancer Cell* 2017; **32**: 552–560.

19 de Thé H. Differentiation therapy revisited. *Nat Rev Cancer* 2018; **18**: 117–127.

20 Lamb J. The Connectivity Map: Using Gene-Expression Signatures to Connect Small Molecules, Genes, and Disease. *Science* 2006; **313**: 1929–1935.

21 Paul F, Arkin Y 'ara, Giladi A, Jaitin DA, Kenigsberg E, Keren-Shaul H *et al.* Transcriptional Heterogeneity and Lineage Commitment in Myeloid Progenitors. *Cell* 2016; **164**: 325.

22 Zhang S-D, Gant TW. A simple and robust method for connecting small-molecule drugs using gene-expression signatures. *BMC Bioinformatics* 2008; **9**: 258.

23 Kuhn M, Szklarczyk D, Franceschini A, von Mering C, Jensen LJ, Bork P. STITCH 3: zooming in on protein-chemical interactions. *Nucleic Acids Res* 2012; **40**: D876–D880.

24 Kalinyak KA, Sawutz DG, Lampkin BC, Johnson CL, Whitsett JA. Effects of dimaprit on growth and differentiation of human promyelocytic cell line, HL-60. *Life Sci* 1985; **36**: 1909–1916.

25 Pantziarka P, Bouche G, Meheus L, Sukhatme V, Sukhatme VP. Repurposing Drugs in Oncology (ReDO)-mebendazole as an anti-cancer agent. *Ecancermedicalscience* 2014; **8**: 443.

26 McNamara S, Wang H, Hanna N, Miller WH Jr. Topoisomerase IIbeta negatively modulates

retinoic acid receptor alpha function: a novel mechanism of retinoic acid resistance. *Mol Cell Biol* 2008; **28**: 2066–2077.

27 Nichol JN, Galbraith MD, Kleinman CL, Espinosa JM, Miller WH Jr. NPM and BRG1 Mediate Transcriptional Resistance to Retinoic Acid in Acute Promyelocytic Leukemia. *Cell Rep* 2016; **14**: 2938–2949.

28 Rosenauer A, Raelson JV, Nervi C, Eydoux P, DeBlasio A, Miller WH Jr. Alterations in expression, binding to ligand and DNA, and transcriptional activity of rearranged and wild-type retinoid receptors in retinoid-resistant acute promyelocytic leukemia cell lines. *Blood* 1996; **88**: 2671–2682.

29 Shao W, Benedetti L, Lamph WW, Nervi C, Miller WH Jr. A retinoid-resistant acute promyelocytic leukemia subclone expresses a dominant negative PML-RAR alpha mutation. *Blood* 1997; **89**: 4282–4289.

30 Duprez E, Benoit G, Flexor M, Lillehaug JR, Lanotte M. A mutated PML/RARA found in the retinoid maturation resistant NB4 subclone, NB4-R2, blocks RARA and wild-type PML/RARA transcriptional activities. *Leukemia* 2000; **14**: 255–261.

31 Testa U, Stellacci E, Pelosi E, Sestili P, Venditti M, Orsatti R *et al.* Impaired myelopoiesis in mice devoid of interferon regulatory factor 1. *Leukemia* 2004; **18**: 1864–1871.

32 Fiedler K, Brunner C. The role of transcription factors in the guidance of granulopoiesis. *Am J Blood Res* 2012; **2**: 57–65.

33 Leon J, Ferrandiz N, Acosta JC, Delgado MD. Inhibition of cell differentiation: a critical mechanism for MYC-mediated carcinogenesis? *Cell Cycle* 2009; **8**: 1148–1157.

34 Prunier C, Zhang M-Z, Kumar S, Levy L, Ferrigno O, Tzivion G *et al.* Disruption of the

730 PHRF1 Tumor Suppressor Network by PML-RARα Drives Acute Promyelocytic Leukemia
731 Pathogenesis. *Cell Rep* 2015; **10**: 883–890.

732 35 Subramanian A, Tamayo P, Mootha VK, Mukherjee S, Ebert BL, Gillette MA *et al.* Gene set
733 enrichment analysis: a knowledge-based approach for interpreting genome-wide
734 expression profiles. *Proc Natl Acad Sci U S A* 2005; **102**: 15545–15550.

735 36 Shi L, Perin JC, Leipzig J, Zhang Z, Sullivan KE. Genome-wide analysis of interferon
736 regulatory factor I binding in primary human monocytes. *Gene* 2011; **487**: 21–28.

737 37 Zeller KI, Zhao X, Lee CWH, Chiu KP, Yao F, Yustein JT *et al.* Global mapping of c-Myc
738 binding sites and target gene networks in human B cells. *Proc Natl Acad Sci U S A* 2006;
739 **103**: 17834–17839.

740 38 Fujiwara T, O'Geen H, Keles S, Blahnik K, Linnemann AK, Kang Y-A *et al.* Discovering
741 hematopoietic mechanisms through genome-wide analysis of GATA factor chromatin
742 occupancy. *Mol Cell* 2009; **36**: 667–681.

743 39 Lan X, Witt H, Katsumura K, Ye Z, Wang Q, Bresnick EH *et al.* Integration of Hi-C and
744 ChIP-seq data reveals distinct types of chromatin linkages. *Nucleic Acids Res* 2012; **40**:
745 7690–7704.

746 40 Liberzon A, Birger C, Thorvaldsdóttir H, Ghandi M, Mesirov JP, Tamayo P. The Molecular
747 Signatures Database Hallmark Gene Set Collection. *Cell Systems* 2015; **1**: 417–425.

748 41 Pan X-N, Chen J-J, Wang L-X, Xiao R-Z, Liu L-L, Fang Z-G *et al.* Inhibition of c-Myc
749 overcomes cytotoxic drug resistance in acute myeloid leukemia cells by promoting
750 differentiation. *PLoS One* 2014; **9**: e105381.

751 42 Mei S, Qin Q, Wu Q, Sun H, Zheng R, Zang C *et al.* Cistrome Data Browser: a data portal

for ChIP-Seq and chromatin accessibility data in human and mouse. *Nucleic Acids Res* 2017; **45**: D658–D662.

43 Tan Y, Wang X, Song H, Zhang Y, Zhang R, Li S *et al.* A PML/RARα direct target atlas redefines transcriptional deregulation in acute promyelocytic leukemia. *Blood* 2021; **137**: 1503–1516.

44 Gao T, He B, Liu S, Zhu H, Tan K, Qian J. EnhancerAtlas: a resource for enhancer annotation and analysis in 105 human cell/tissue types. *Bioinformatics* 2016; **32**: 3543–3551.

45 Wang P, Tang Z, Lee B, Zhu JJ, Cai L, Szalaj P *et al.* Chromatin topology reorganization and transcription repression by PML-RARα in acute promyeloid leukemia. *Genome Biol* 2020; **21**: 110.

46 Hu M-H, Wang Y-Q, Yu Z-Y, Hu L-N, Ou T-M, Chen S-B *et al.* Discovery of a New Four-Leaf Clover-Like Ligand as a Potent c-MYC Transcription Inhibitor Specifically Targeting the Promoter G-Quadruplex. *J Med Chem* 2018; **61**: 2447–2459.

47 Michalska A, Blaszczyk K, Wesoly J, Bluyssen HAR. A Positive Feedback Amplifier Circuit That Regulates Interferon (IFN)-Stimulated Gene Expression and Controls Type I and Type II IFN Responses. *Front Immunol* 2018; **9**: 1135.

48 Sapio L, Gallo M, Illiano M, Chiosi E, Naviglio D, Spina A *et al.* The Natural cAMP Elevating Compound Forskolin in Cancer Therapy: Is It Time? *J Cell Physiol* 2017; **232**: 922–927.

49 Sawutz DG, Kalinyak K, Whitsett JA, Johnson CL. Histamine H2 receptor desensitization in HL-60 human promyelocytic leukemia cells. *J Pharmacol Exp Ther* 1984; **231**: 1–7.

50 Shayo C, Davio C, Brodsky A, Mladovan AG, Legnazzi BL, Rivera E *et al.* Histamine

modulates the expression of c-fos through cyclic AMP production via the H2 receptor in the human promonocytic cell line U937. *Mol Pharmacol* 1997; **51**: 983–990.

51 Brodsky A, Davio C, Shayo C, Lemos Legnazzi B, Barbosa M, Lardo M *et al.* Forskolin induces U937 cell line differentiation as a result of a sustained cAMP elevation. *Eur J Pharmacol* 1998; **350**: 121–127.

52 Norsworthy KJ, Altman JK. Optimal treatment strategies for high-risk acute promyelocytic leukemia. *Curr Opin Hematol* 2016; **23**: 127–136.

53 Testa U, Lo-Coco F. Prognostic factors in acute promyelocytic leukemia: strategies to define high-risk patients. *Ann Hematol* 2016; **95**: 673–680.

54 Dos Santos GA, Kats L, Pandolfi PP. Synergy against PML-RARa: targeting transcription, proteolysis, differentiation, and self-renewal in acute promyelocytic leukemia. *J Exp Med* 2013; **210**: 2793–2802.

55 Ravandi F, Estey E, Jones D, Faderl S, O'Brien S, Fiorentino J *et al.* Effective treatment of acute promyelocytic leukemia with all-trans-retinoic acid, arsenic trioxide, and gemtuzumab ozogamicin. *J Clin Oncol* 2009; **27**: 504–510.

56 Lucena-Araujo AR, Coelho-Silva JL, Pereira-Martins DA, Silveira DR, Koury LC, Melo RAM *et al.* Combining gene mutation with gene expression analysis improves outcome prediction in acute promyelocytic leukemia. *Blood* 2019; **134**: 951–959.

57 Lin X, Qiao N, Shen Y, Fang H, Xue Q, Cui B *et al.* Integration of Genomic and Transcriptomic Markers Improves the Prognosis Prediction of Acute Promyelocytic Leukemia. *Clin Cancer Res* 2021; **27**: 3683–3694.

58 Vicente C, Conchillo A, García-Sánchez MA, Odero MD. The role of the GATA2

transcription factor in normal and malignant hematopoiesis. *Crit Rev Oncol Hematol* 2012; **82**: 1–17.

59 Chen A, Licht JD, Wu Y, Hellinger N, Scher W, Waxman S. Retinoic acid is required for and potentiates differentiation of acute promyelocytic leukemia cells by nonretinoid agents. *Blood* 1994; **84**: 2122–2129.

60 Stegmaier K, Ross KN, Colavito SA, O'Malley S, Stockwell BR, Golub TR. Gene expression-based high-throughput screening(GE-HTS) and application to leukemia differentiation. *Nat Genet* 2004; **36**: 257–263.

61 Li Y, Thomas D, Deutzmann A, Majeti R, Felsher DW, Dill DL. Mebendazole for Differentiation Therapy of Acute Myeloid Leukemia Identified by a Lineage Maturation Index. *Sci Rep* 2019; **9**: 16775.

62 Quenech'Du N, Ruchaud S, Khelef N, Guiso N, Lanotte M. A sustained increase in the endogenous level of cAMP reduces the retinoid concentration required for APL cell maturation to near physiological levels. *Leukemia* 1998; **12**: 1829–1833.

63 Zhao Q, Tao J, Zhu Q, Jia P-M, Dou A-X, Li X *et al.* Rapid induction of cAMP/PKA pathway during retinoic acid-induced acute promyelocytic leukemia cell differentiation. *Leukemia* 2004; **18**: 285–292.

64 Nasr R, Guillemin M-C, Ferhi O, Soilihi H, Peres L, Berthier C *et al.* Eradication of acute promyelocytic leukemia-initiating cells through PML-RARA degradation. *Nat Med* 2008; **14**: 1333–1342.

65 Padmanabhan A, Li X, Bieberich CJ. Protein kinase A regulates MYC protein through transcriptional and post-translational mechanisms in a catalytic subunit isoform-specific manner. *J Biol Chem* 2013; **288**: 14158–14169.

66 Liu Q, Nguyen E, Døskeland S, Ségal-Bendirdjian É. cAMP-Dependent Protein Kinase A (PKA)-Mediated c-Myc Degradation Is Dependent on the Relative Proportion of PKA-I and PKA-II Isozymes. *Mol Pharmacol* 2015; **88**: 469–476.

67 Walf-Vorderwülbecke V, Pearce K, Brooks T, Hubank M, van den Heuvel-Eibrink MM, Zwaan CM *et al.* Targeting acute myeloid leukemia by drug-induced c-MYB degradation. *Leukemia* 2018; **32**: 882–889.

68 Coccia EM, Stellacci E, Valtieri M, Masella B, Feccia T, Marziali G *et al.* Ectopic expression of interferon regulatory factor-1 potentiates granulocytic differentiation. *Biochem J* 2001; **360**: 285–294.

69 Si J, Yu X, Zhang Y, DeWille JW. Myc interacts with Max and Miz1 to repress C/EBPδ promoter activity and gene expression. *Mol Cancer* 2010; **9:** 92.

70 Zhang L, Li J, Xu H, Shao X, Fu L, Hou Y *et al.* Myc-Miz1 signaling promotes self-renewal of leukemia stem cells by repressing Cebpα and Cebpδ. *Blood* 2020; **135**: 1133–1145.

71 Wang K, Wang P, Shi J, Zhu X, He M, Jia X *et al.* PML/RARα Targets Promoter Regions Containing PU.1 Consensus and RARE Half Sites in Acute Promyelocytic Leukemia. *Cancer Cell* 2010; **17**: 186–197.

72 Lourenco C, Resetca D, Redel C, Lin P, MacDonald AS, Ciaccio R *et al.* MYC protein interactors in gene transcription and cancer. *Nat Rev Cancer* 2021; **21**: 579–591.

73 Huang M-J, Cheng Y-C, Liu C-R, Lin S, Liu HE. A small-molecule c-Myc inhibitor, 10058-F4, induces cell-cycle arrest, apoptosis, and myeloid differentiation of human acute myeloid leukemia. *Exp Hematol* 2006; **34**: 1480–1489.

74 Nason-Burchenal K, Gandini D, Botto M, Allopenna J, Seale JR, Cross NC *et al.* Interferon

augments PML and PML/RAR alpha expression in normal myeloid and acute promyelocytic cells and cooperates with all-trans retinoic acid to induce maturation of a retinoid-resistant promyelocytic cell line. *Blood* 1996; **88**: 3926–3936.

75 Ng KP, Manjeri A, Lee LM, Chan ZE, Tan CY, Tan QD *et al.* The arginase inhibitor Nω-hydroxy-nor-arginine (nor-NOHA) induces apoptosis in leukemic cells specifically under hypoxic conditions but CRISPR/Cas9 excludes arginase 2 (ARG2) as the functional target. *PLoS One* 2018; **13**: e0205254.

76 Sanjana NE, Shalem O, Zhang F. Improved vectors and genome-wide libraries for CRISPR screening. *Nat Methods* 2014; **11**: 783–784.

77 Shyamsunder P, Shanmugasundaram M, Mayakonda A, Dakle P, Teoh WW, Han L *et al.* Identification of a novel enhancer of CEBPE essential for granulocytic differentiation. *Blood* 2019; **133**: 2507–2517.

78 Corces MR, Buenrostro JD, Wu B, Greenside PG, Chan SM, Koenig JL *et al.* Lineage-specific and single-cell chromatin accessibility charts human hematopoiesis and leukemia evolution. *Nat Genet* 2016; **48**: 1193–1203.

79 Franceschini A, Szklarczyk D, Frankild S, Kuhn M, Simonovic M, Roth A *et al.* STRING v9.1: protein-protein interaction networks, with increased coverage and integration. *Nucleic Acids Res* 2013; **41**: D808–D815.

Figure Legends

Figure 1: Schema of the experimental design and data analysis of ATRA-induced differentiation of NB4 cells.

(a) NB4 cells were treated with 1μM ATRA or DMSO control at five time-points and subjected to RNA-Seq.

(b) Wright's stain and NBT assay of NB4 cells treated with ATRA over 120h. Scale bar, 25μm.

(c) qRT-PCR validation of genes known to be up-regulated (left panels) or down-regulated (right panels) by ATRA treatment in NB4 cells. Error bars correspond to standard deviations for n = 3 biological replicates.

(d) PCA plot of the top 10% most variable genes.

(e) Barchart shows the number of differentially expressed coding and non-coding genes between ATRA and DMSO-treated samples calculated based on HUGO gene IDs. Fig. S3 shows the intersections of gene numbers among different time-points. For a full list of DE genes at each time-point, their log2 fold changes and their adjusted p-values please see Tables S1 and S2.

Figure 2: Filtering of the TF regulatory networks

(a) Summary of the pipeline and filters for each time-point.

(b) Heatmaps of the network coverage regulated by the Mogrify®-selected TFs after applying filter one. The early (4h) and late (72h) wave-like patterns of regulation are evident.

(c) Filter 2: Time-wise correlation of expression of selected TFs with those of their targets. IRF1 is a good example of TF-target correlation pattern, as shown in the top histogram. The c-score for IRF1 is 2.745 and is very significant compared to the null distribution. The distribution of c-scores for all TFs is shown in the bottom histogram plot, with IRF1 as indicated.

(d) Network of the selected TFs after the application of filter two at 4h. The nodes represent the TFs and their sizes are proportional to the $\log_2$ c-score, as calculated by filter two. The node colour

indicates the extent of up- or down-regulation of the TF gene expression in ATRA-treated NB4 cells. The edges represent connections between the node TFs according to the STRING database [79].

(e) A graphical depiction of filter three (see Supplementary Methods) with three hypothetical TFs.

(f) (Left) Line graph shows the best scores for different numbers of TFs in the combination. Scores represent up and downregulated TFs for 4h. Red circle highlights the Filter 3 score obtained with 3 TFs in the combination. (Right) Bar chart shows the scores of the top ten 3-TF combinations for the up and down-regulated TFs at 4h.

(g) Selected GO biological processes and their statistical significance for each TF network of the TF combination, resulting from the application of all three filters at 4h.

Figure 3: CMAP-based identification of drugs which induce transcriptional changes recapitulating changes in TF networks under ATRA treatment.

(a) Schematic of the network pharmacology framework. DE genes between ATRA and DMSO-treated NB4 cells were calculated for five time-points using this pipeline. Driver transcription factors (TFs) and their gene regulatory networks were detected using the Mogrify® algorithm. This corresponds to the combination of TFs resulting from filter 3 (Fig. 2e). CMAP was queried using these gene regulatory networks. The output of ssCMAP was a ranked list of drugs, from most similar to least similar to ATRA.

(b) The ranks of the two ATRA and one isotretinoin cell-line level instances when we ran ssCMAP using different input signatures.

(c) The z-scores of the connectivity for the two ATRA and one isotretinoin instances, as calculated by ssCMAP, for different input signatures.

(d) (Left) Table showing drug instances ranked by absolute E-Score (the sum of each drug's connectivity scores across all TFs or the respective combinations, see Supplementary Methods).

(Right) Heatmap depicting connectivity (red: positive; blue: negative) between each drug and the respective TF.

Figure 4: Experimental validation of the detected drugs

(a) CD11b flow cytometry assay of NB4 cells treated with CMAP positive connectivity drugs colchicine (Colc), dimaprit (Dima), dinoprost (Dino), mebendazole (meb), podophyllotoxin (Podo) or quinpirole (Quin) alone or in combination with a suboptimal dose of ATRA (3nM) for 72h. The statistical comparisons are made between each CMAP drug concentration and the respective baseline treatment.

(b) CD11b flow cytometry analysis of NB4-MR2 cells treated for 5 days with dimaprit or mebendazole, alone or in combination with ATRA.

(c) Same as (b), but for NB4-LR2 cells.

(d) CD11b flow cytometry data for NB4 cells treated with the indicated concentrations of dimaprit, mebendazole or the combination of both for 72h. ATRA (0.01 and 1μM) are shown for comparison.

(e) Flow cytometry plot for data in (d).

(f) Wright staining of drug-treated NB4 cells; scale bar, 20μm.

(g) Annexin V-based flow cytometry analysis of cell viability in drug-treated NB4 cells. The statistical comparisons are made between each treatment and DMSO control.

(h) qPCR analysis of TF target genes with high ssCMAP ranks (see main text) in response to drug treatments for 24h. Data for ATRA treatment was obtained from our RNA-seq experiment (t=24h). Extended bar chart (below) for genes with moderate upregulation.

(i) Western blot analysis in nuclear extracts demonstrating up and down-regulation of Mogrify®-identified TFs in response to the indicated drug treatments for 24h in NB4 cells, similar to ATRA treatment.

Error bars represent mean ± SD of 3 biological replicates, except for (h), qPCR technical replicates. *$p \leq 0.05$, **$p \leq 0.01$,***$p \leq 0.001$, and ****$p \leq 0.0001$ respectively by unpaired two-tailed *t*-test.

Figure 5: MYC and IRF1 influence differentiation in NB4 cells

(a) GSEA of IRF1, MYC, or GATA2 directly-bound and positively-regulated gene sets derived from published studies (see Supplementary Methods), against ATRA-mediated gene expression changes relative to DMSO in NB4 cells for t=4h (gene lists). NES, Normalized Enrichment Score; FDR, False Discovery Rate.

(b) Western blot of MYC and IRF1 expression at Day 3 post-infection with MYC shRNAs in EV or IRF1-overexpressing NB4 cells.

(c) CD11b flow cytometry data for cells Day 6 post-infection.

(d) Representative flow cytometry plot for data in (c).

(e) Wright stain of NB4 cells at Day 6. Black arrowheads indicate differentiated cells.

(f) Cell viability analysis by Annexin-V flow cytometry at Day 6. The statistical comparisons are made between each treatment and shLuc/EV control.

(g) Western blot demonstrating loss of IRF1 protein expression in KO lines, in response to treatment with 3nM ATRA for 72h (see also Fig. S17a for 1μM ATRA).

(h) CD11b flow cytometry analysis of non-targeting control (sg-NT) or IRF1 KO lines treated for 72h with DMSO or indicated doses of ATRA.

(i) Representative flow cytometry plot for data in (h).

(j) Western blot showing MYC-induced repression of IRF1 protein levels with 24h ATRA treatment, in MYC-overexpressing NB4 cells.

(k) CD11b flow cytometry data showing that enforced MYC expression leads to reduction in ATRA-mediated differentiation (72h).

Error bars represent mean ± SD of 3 biological replicates. Statistical analysis was performed by unpaired two-tailed *t*-test.

Figure 6: IRF1 transcription is regulated by MYC and ATRA treatment

(a) Genome view of the IRF1 gene region (right of plot) as well as the adjacent antisense transcript region (IRF1-AS1, left). ChIP-Seq tracks for MYC (Cistrome DB) [42] and PML-RARα [43] in NB4 cells are shown, as well as our H3K27ac, ATAC-seq and RNA-seq data in DMSO or ATRA-treated NB4 cells. Also shown are two tracks consisting of predicted enhancer-promoter interactions from EnhancerAtlas [44] (top of figure) and PML-RARα ChIA-PET data from Wang *et al.* [45] (bottom), respectively. Grey regions indicate a putative chromatin interaction between IRF1 Pr and E4. Pr, promoter. E4, putative distal enhancer of IRF1.

(b) ChIP-qPCR analysis of MYC and PML-RARα binding at the IRF1 Pr and E4 in NB4 cells upon shRNA-mediated knockdown of MYC.

(c) Schematic of luciferase reporter constructs consisting of IRF1 Pr, E4 or the fusion of both (E4-Pr).

(d) Luciferase assay results for NB4 cells transfected with the indicated constructs, followed by addition of ATRA 24h post-transfection and further cultured for 24h prior to readout.

(e) Western blot of MYC expression in NB4 cells at 24h post-transfection with anti-MYC siRNAs.

(f) Luciferase assay results of NB4 cells co-transfected with constructs from (c) and MYC siRNAs, followed by readout 24h post-transfection.

(g) qPCR of MYC (left) and IRF1 (right) target gene expression in NB4 cells in response to shRNA-mediated knockdown of MYC.

(h) A model of MYC-mediated silencing of IRF1 expression in NB4 cells. In this model, PML-RARα and other co-repressors (“Rep”) are recruited to the IRF1 promoter (Pr) and distal enhancer E4 in a MYC-dependent manner, thereby preventing IRF1 expression. Upon ATRA treatment or

MYC inhibition, these repressive complexes dissociate, possibly facilitating recruitment of as-yet identified co-activators ("Act") which stimulate IRF1 gene transcription.

Error bars represent mean ± SD of 3 biological replicates, except for (g), qPCR technical replicates. Statistical analysis was performed by unpaired two-tailed *t*-test.

Figure 7: Combined targeting of MYC and IRF1 induces differentiation in NB4 cells

(a) Western blot analysis of IRF1 and MYC protein levels in response to 24h treatment with IZCZ-3, IFNγ or the combination of both.

(b) Flow cytometry analysis for CD11b under the indicated treatments for 72h.

(c) Representative flow cytometry plot for data in (b).

(d) Wright stain and NBT assay for 72h-treated cells. Cells in black boxes (top panel) undergoing granulocytic differentiation are magnified above figure; black arrows (bottom panel) indicate NBT positive cells under drug combination.

(e) Corresponding cell viability analysis by flow cytometry (Annexin V). The statistical comparisons are made between each treatment and DMSO control.

Error bars represent mean ± SD of 3 biological replicates. Statistical analysis was performed by unpaired two-tailed *t*-test.

Figure 8: Drug combinations induce differentiation in primary APL MNCs

(a) Flow cytometry analysis of CD11b expression in Patient (P) 748 (left) or 74 (right) MNCs treated with forskolin (FSK) and Meb combinations for 72h in the presence of myeloid cytokines.

(b) Representative flow cytometry plot of CD11b expression for P748 for results in (a).

(c) IZCZ-3 and IFNγ combinations in the same two patients.

(d) Representative flow cytometry plot for P748 for results in (c).

Error bars represent mean ± SD of 3 biological replicates. Statistical analysis was performed by unpaired two-tailed *t*-test.