

# IGFBP7 activates retinoid acid–induced responses in acute myeloid leukemia stem and progenitor cells

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## Key Points

- rhIGFBP7 activates ATRA-driven elimination of leukemia stem/progenitor cells in non-APL AML with high RARA expression.
- rhIGFBP7 treatment of non-APL AML cells reduces GFI1 expression, leading to increased sensitivity to ATRA.

Treatment of acute promyelocytic leukemia (APL) with all-trans retinoic acid (ATRA) in combination with low doses of arsenic trioxide or chemotherapy leads to exceptionally high cure rates (>90%). ATRA forces APL cells into differentiation and cell death. Unfortunately, ATRA-based therapy has not been effective among any other acute myeloid leukemia (AML) subtype, and long-term survival rates remain unacceptably low; only 30% of AML patients survive 5 years after diagnosis. Here, we identified insulin-like growth factor binding protein 7 (IGFBP7) as part of ATRA-induced responses in APL cells. Most importantly, we observed that addition of recombinant human IGFBP7 (rhIGFBP7) increased ATRA-driven responses in a subset of non-APL AML samples: those with high RARA expression. In nonpromyelocytic AML, rhIGFBP7 treatment induced a transcriptional program that sensitized AML cells for ATRA-induced differentiation, cell death, and inhibition of leukemic stem/progenitor cell survival. Furthermore, the engraftment of primary AML in mice was significantly reduced following treatment with the combination of rhIGFBP7 and ATRA. Mechanistically, we showed that the synergism of ATRA and rhIGFBP7 is due, at least in part, to reduction of the transcription factor GFI1. Together, these results suggest a potential clinical utility of IGFBP7 and ATRA combination treatment to eliminate primary AML (leukemic stem/progenitor) cells and reduce relapse in AML patients.

## Introduction

Acute myeloid leukemia (AML) is a devastating disease that is characterized by transcriptional dysregulation that results in a block in differentiation and increased malignant self-renewal. Next to genetic aberrancies, epigenetic modifications and transcriptional regulators play an essential role in the generation of diversity in the transcriptional landscape of AML cells related to their potential to respond to therapy. Chemotherapy has been the standard treatment for AML patients for decades, unfortunately with low success rates. The 5-year overall survival rate for AML patients is <40%,<sup>1</sup> and these cure rates will not significantly improve unless efficient and well-tolerated alternative treatment strategies are developed.

Currently, acute promyelocytic leukemia (APL) is the only AML subtype that is successfully treated with all-trans retinoic acid (ATRA) and low doses of arsenic trioxide or chemotherapy, resulting in cure rates >90%.<sup>2,3</sup> Expression of the fusion protein PML-RAR $\alpha$  in APL cells serves as a dominant negative inhibitor for retinoic acid receptor (RAR) signaling. Addition of ATRA induces degradation of the PML-RAR $\alpha$  fusion protein, leading to dissociation of corepressors and histone deacetylases (HDACs), allowing epigenetic changes and reactivation of transcription facilitating neutrophil differentiation and

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cell death.<sup>4-7</sup> Several studies implied that the mere induction of differentiation is insufficient to cure APL, and the success of ATRA therapy for APL is likely due, in part, to activation of p53 by ATRA-induced degradation of the PML-RAR $\alpha$  fusion protein.<sup>8,9</sup> Although the success of ATRA-based therapy has been demonstrated for APL patients, it has not proven effective for patients with other AML subtypes.<sup>10-17</sup> To unlock the therapeutic potential of ATRA-based therapy for non-APL AML patients, identification of novel therapeutic strategies regulating gene-expression programs associated with ATRA susceptibility are key.

Recently, several *in vitro* studies showed that ATRA can drive leukemia cells into differentiation and/or apoptosis in a subset of AML patients with a mutation in NPM1<sup>18,19</sup> or IDH1.<sup>20</sup> We found that AML with overexpression of EVI-1 is susceptible to ATRA-induced differentiation and cell death.<sup>21</sup> Moreover, several studies suggest that resistance to ATRA therapy in non-APL AML is due to epigenetic and transcriptional deregulation.<sup>22-24</sup> The lysine-specific demethylase LSD1 (KDM1A) is overexpressed in several tumors and is required for maintenance of AML.<sup>24,25</sup> Inhibition of the nonenzymatic activities of LSD1 by tranylcypromine (TCP) activates the ATRA-driven differentiation pathway in non-APL AML cells. Interestingly, the histone 3 lysine 4 demethylase activity of LSD1 was not involved in this activation.<sup>24,26,27</sup> LSD1 interacts with growth factor independent 1 (GFI1), and the activity of LSD1 is dependent on this interaction.<sup>27-29</sup> Multiple distinct LSD1 inhibitors disrupt the interaction of LSD1 with GFI1, destabilizing GFI1 on chromatin and abrogating its repressive activity. The subsequent activation of enhancers following disruption of the GFI1 repressor complex is believed to be dependent on the presence of SPI1 and CEBP $\alpha$  at GFI1-bound loci.<sup>29</sup> Furthermore, the lysine acetyltransferase GCN5 contributes to ATRA resistance in non-APL AML via aberrant acetylation of histone 3 lysine 9 and maintenance of leukemic stem cell (LSC)-associated genes. Inhibition of GCN5 also unlocks an ATRA-driven therapeutic response.<sup>23</sup> Inhibition of LSD1 and GCN5 induce sensitivity to ATRA only in part of the non-APL AML cases.

We previously demonstrated that insulin-like growth factor binding protein 7 (IGFBP7), a tumor suppressor and negative regulator of IGF1 receptor (IGF1R) activity, induces differentiation and apoptosis in primary AML cells and sensitizes AML cells for chemotherapy-induced cell death.<sup>30,31</sup> Because IGF1R inhibitors can eliminate therapy-resistant cancer cells by modifying their chromatin state,<sup>32</sup> and *IGFBP7* is highly expressed in APL,<sup>33</sup> we hypothesized that *IGFBP7* may be part of the differentiation pathway in APL and may have the capacity to modify the epigenetic state of non-APL AML cells, resulting in susceptibility to ATRA-induced differentiation and cell death. Here, we show that increasing *IGFBP7* expression or treatment of non-APL AML cells with recombinant human *IGFBP7* (rhIGFBP7) could promote ATRA-induced responses, including differentiation, cell death, and inhibition of leukemic stem/progenitor cell survival. We also show that combination treatment with rhIGFBP7 and ATRA significantly reduced the engraftment of primary AML cells *in vivo*.

## Methods

### Cell lines and primary cells

HL60, KG1a, THP1, and HEK293T cells were purchased from the American Type Culture Collection; NB4 and OCI-AML3 cells were

from the German Collection of Microorganisms and Cell Cultures. Human AML material was obtained from patients hospitalized at Amsterdam UMC, location VUmc at the time of diagnosis, according to HOVON AML protocols. Normal bone marrow (NBM) was obtained from otherwise healthy patients undergoing cardiothoracic surgery. Informed consent was obtained for every patient sample used, procedures were approved by the ethical committee of Amsterdam UMC, and all experiments were conducted in accordance with the Declaration of Helsinki. Culture conditions are described in supplemental Methods.

### Lentiviral transduction

The lentiviral plasmid expressing human *IGFBP7* (*IGFBP7-OE*) was generated as described previously.<sup>30</sup> For *IGFBP7* knockdown, pLKO1 short hairpin RNA #1 (shRNA#1; TRCN0000077943) and shRNA#2 (TRCN0000077945) were used. The lentiviral plasmid pLenti6.2/V5-DEST expressing GFI1-YFP (*GFI1-OE*), as well as the plasmids to knockdown GFI1, *GFI1-shRNA65-pLKO.1-Venus*, and *GFI1-shRNA68-pLKO.1-Venus*, were a gift from B. A. van der Reijden.

AML cells with enhanced or decreased levels of *IGFBP7* or *GFI1* were generated using lentiviral transduction. A total of 12  $\mu$ g of plasmid DNA mix (ie, 1 of the above-described vectors, 5  $\mu$ g of pRRE, 3  $\mu$ g of pMD2G, and 2.5  $\mu$ g of pRSV-REV) was mixed with 150 mM calcium chloride in HEPES-buffered saline and added to HEK293T cells seeded in medium containing 25  $\mu$ M chloroquine. After 72 to 96 hours, medium was harvested. Retronectin (Takara Bio)-coated plates were incubated with virus and centrifuged at 3000 rpm and 32°C for 2 hours. AML cells were transduced in the presence of 8  $\mu$ g/mL polybrene (Sigma-Aldrich). For *IGFBP7* overexpression and knockdown, NB4 cells were selected with 1  $\mu$ g/mL puromycin (Sigma-Aldrich) for 7 days. For *GFI1* overexpression, HL60 cells were selected with 6  $\mu$ g/mL blasticidin (Invitrogen) for 21 days, or primary AML cells were transduced in Iscove modified Dulbecco medium with 500 nM StemRegenin 1 (STEMCELL Technologies), 50 ng/mL Flt-3 ligand, 20 ng/mL interleukin-3, 100 ng/mL stem cell factor, and 20 ng/mL granulocyte colony-stimulating factor. For *GFI1* knockdown, HL60 cells were transduced and subsequently Venus<sup>+</sup> cells were sorted using a FACSAria II flow cytometer (BD Biosciences).

### Immunostaining and flow cytometry analysis

AML cells were treated with ATRA (Sigma-Aldrich), rhIGFBP7, TCP (Selleckchem), or combinations for 72 to 120 hours (cell lines) or for 7 days (primary samples) before analysis. At least  $2 \times 10^5$  cells were harvested and washed in phosphate-buffered saline (PBS) with 0.1% human serum albumin. Samples were incubated with monoclonal antibody combinations consisting of anti-human 7AAD-PerCP (1:10), CD11b-PE (D12; 1:50) or CD11b-APC (MoP9; 1:50) or CD11b-FITC (Bear1; 1:10), CD45-HV500c (2D1; 1:20), CD33-PC7 (1:20), CD34-BV421 (581; 1:20), CD38-APC (HB7; 1:50), CD15-FITC (HI98; 1:50), CD56-PE (MY31; 1:20), CD3-FITC (SK7; 1:50), CD19-APC-H7 (SJ25C1; 1:10), or anti-mouse CD45-PerCP (30-F11; 1:50) (all from BD Biosciences) for 30 minutes and washed once with PBS and 1% human serum albumin. Flow-Count Fluorospheres (Beckman Coulter) were added, according to the manufacturer's instructions, directly before analysis using an LSRFortessa flow cytometer (BD Biosciences). Data analysis was performed using FACSDiva software (BD Biosciences).

## Liquid culture and colony-forming unit assays

After treatment of NB4 cells with ATRA for 4 days or primary samples with ATRA, rhIGFBP7, TCP, or combinations for 7 days (progenitor assay) and 4 weeks (long-term liquid culture assay) in CellGro-SCGM medium (CellGenix) with 50 ng/mL Flt-3 ligand, 20 ng/mL interleukin-3, and 100 ng/mL human stem cell factor (all from PeproTech), 1 to  $5 \times 10^4$  cells were transferred to MethoCult without erythropoietin (STEMCELL Technologies) and incubated in a humidified atmosphere at 37°C and 5% CO<sub>2</sub>. After 10 to 14 days, colonies were quantified using an Axiovert 25 bright-field microscope (Zeiss).

## Patient-derived xenograft AML mouse model

NOD/SCID/IL2ry<sup>null</sup> mice (NSG) were purchased from The Jackson Laboratory. All mice were maintained in a specific pathogen-free facility at the Amsterdam Animal Research Center of VU University in accordance with protocols (DEC-HEMA12-02 and DEC-HEMA13-02) approved by Centrale Commissie Dierproeven. Female and male mice, 6 to 9 weeks of age, were irradiated with 200 cGy 1 day prior to injection of cells. Primary AML cells were thawed, incubated with anti-CD3 MicroBeads (Miltenyi Biotec), and depleted of T cells using MACS columns (Miltenyi Biotec), according to the manufacturer's protocol. AML cells were intravenously injected into the tail vein of the mice. Mice were treated with ATRA and/or rhIGFBP7, and their bone marrow was analyzed for the presence of human cells using a FACSCanto flow cytometer (BD Biosciences). Further details are provided in the supplemental Methods.

## Immunoblotting

Cells were lysed in 1% NP40 lysis buffer containing 50 mM Tris (pH 7.5), 150 mM NaCl, 5 mM EDTA (pH 8.0), protease inhibitors (cOmplete Mini protease inhibitor cocktail; Roche), and phosphatase inhibitors (PhosSTOP; Sigma-Aldrich). Equal amounts of protein were loaded and separated on 4% to 16% Mini-PROTEAN TGX Precast Gels (sodium dodecyl sulfate polyacrylamide gel electrophoresis) (Bio-Rad) and transferred to polyvinylidene difluoride membranes (Millipore). Membranes were blocked for 45 minutes in 2.5% (weight-to-volume ratio, %) nonfat dry milk powder (Nutricia) and 2.5% (weight-to-volume ratio, %) bovine serum albumin (Millipore) in PBS/0.1% Tween 20 (PBS/T; Thermo Fisher Scientific), and incubated with mouse anti-IGFBP7 (1:500, MAB1334; R&D Systems), mouse anti-GFI1 (clone B9, 1:500, sc-376949; Santa Cruz Biotechnology), or mouse anti-β-actin (clone C4, 1:5000, MAB1504; Sigma-Aldrich) for ≥2 hours at room temperature (RT). After washing with bovine serum albumin in PBS/0.1% Tween 20, membranes were incubated with goat anti-mouse immunoglobulins/horseradish peroxidase (1:1000, P0447; Dako) for 1 hour at RT. Membranes were developed with enhanced chemiluminescence (GE Healthcare) and imaged using a UVITEC imaging system (Clever Scientific).

## May-Grünwald-Giemsa staining

After treatment of AML cells with ATRA, rhIGFBP7, or the combination for 72 hours,  $5 \times 10^4$  cells were seeded on an object glass, using a Shandon Cytospin centrifuge (Thermo Fisher Scientific), dried overnight at RT, and stained using a Aerospray Hematology Pro Slide Stainer (ELITechGroup) with May-Grünwald-Giemsa staining. Cytospin images were acquired at 0.5 μm per pixel resolution using a Vectra Polaris Imaging System (Akoya Biosciences) in bright-field mode. Images were analyzed using QuPath Software (University of Edinburgh).

## Data quantification and statistical analysis

Statistical analyses were performed using GraphPad Prism 8.0. Results are reported as mean ± standard error of the mean (SEM) or standard deviation (SD). Statistical significance between 2 measurements was determined using 2-tailed (un)paired Student *t* tests. To compare multiple groups, 1- or 2-way analysis of variance (ANOVA) with a post hoc Tukey's, Dunnett, or Bonferroni multiple-comparison test were used. To measure the degree of association, Spearman's rank correlation was used.

Detailed methodology used for RNA isolation and quantitative reverse transcriptase polymerase chain reaction (qRT-PCR), molecular diagnostics and cytogenetic analysis, production of rhIGFBP7, and RNA sequencing is described in supplemental Methods.

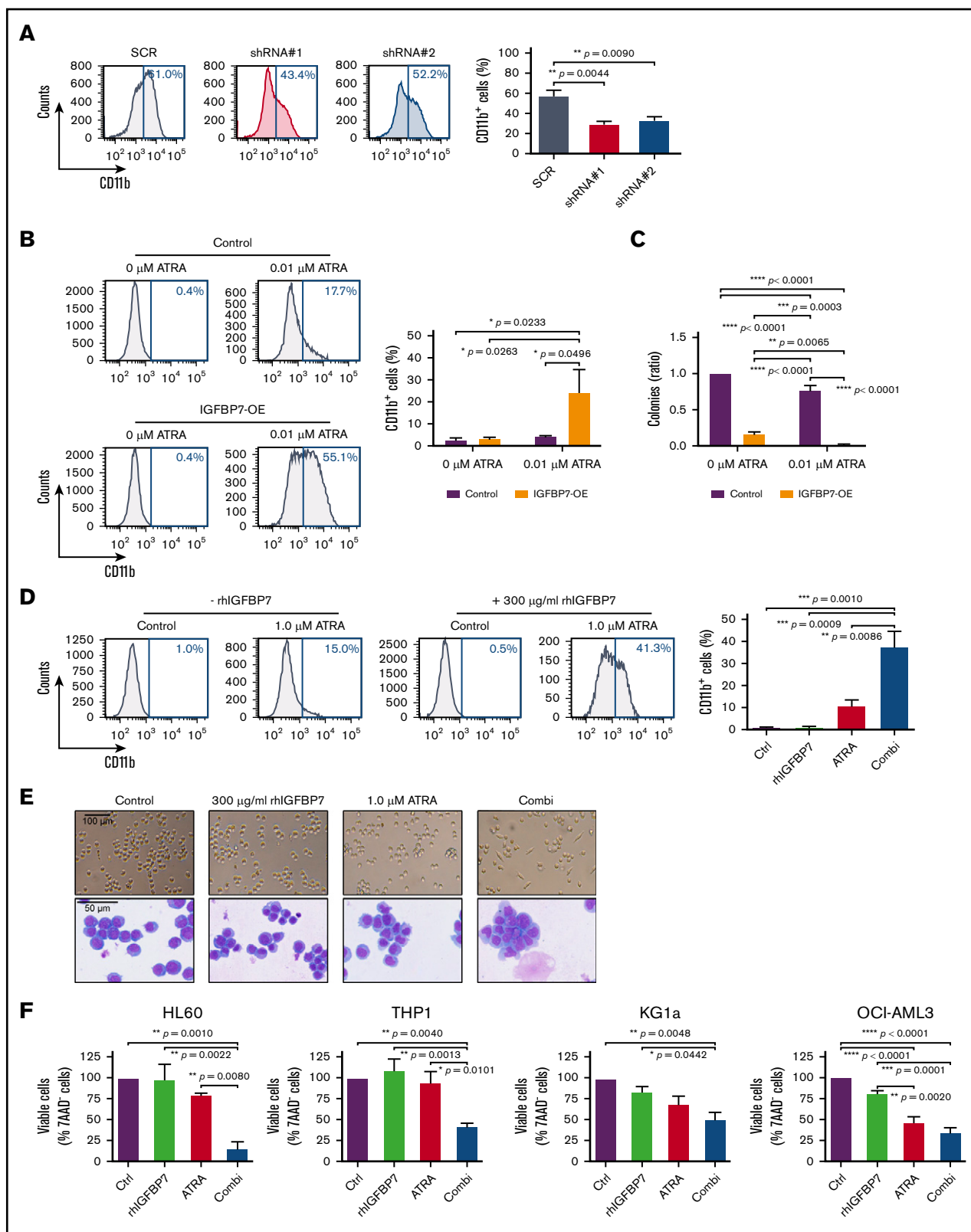
## Results

### Enhanced IGFBP7 expression sensitizes APL cells to physiological concentrations of ATRA

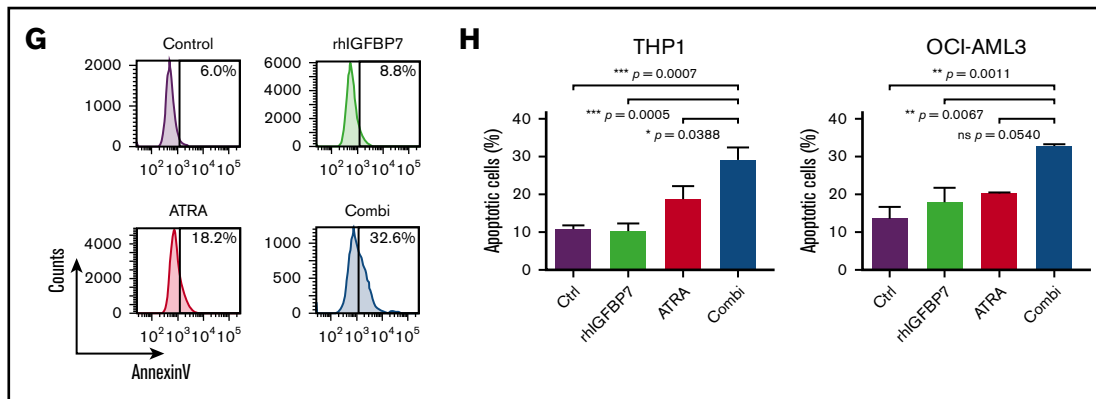
Pharmacological doses of ATRA promote myeloid differentiation of APL cells.<sup>34</sup> To determine whether IGFBP7 is part of the ATRA-induced responses in APL, we generated APL cells (NB4) with decreased *IGFBP7* using lentiviral transduction of 2 independent shRNAs (supplemental Figure 1A-B). Downregulation of *IGFBP7* in NB4 cells resulted in a significant reduction in the membrane expression of the myeloid differentiation marker CD11b after treatment with high concentrations of ATRA (0.5 μM) (Figure 1A), suggesting that *IGFBP7* is part of the signaling pathway crucial for ATRA-induced differentiation in APL cells. Increasing the expression of *IGFBP7* alone (supplemental Figure 1C-D) did not lead to induction of differentiation in NB4 cells; however, incubation of the cells with a low dose of ATRA (0.01 μM) in the presence of enhanced *IGFBP7* significantly increased the number of CD11b-expressing cells (5.7-fold) (Figure 1B). Overexpression of *IGFBP7* in NB4 cells resulted in reduced clonogenic capacity of the cells and could significantly enhance the ATRA-driven decrease in clonogenic potential (Figure 1C). Thus, low doses of ATRA induced acquisition of CD11b and a reduction in the clonogenic capacity of NB4 cells with enhanced *IGFBP7* levels, suggesting that *IGFBP7* could potentiate the ability of ATRA to induce terminal differentiation in APL cells.

### rhIGFBP7 potentiates ATRA-induced differentiation, inhibition of proliferation, and apoptosis in non-APL AML cells

To explore therapeutic strategies that enhance the effects of ATRA in AML, we investigated whether addition of rhIGFBP7 could activate ATRA-driven responses in AML cells from subtypes other than APL. Incubation of the non-APL cell line HL60 with ATRA alone resulted in a small increase in the fraction of HL60 cells expressing membrane CD11b. Treatment with rhIGFBP7 alone did not significantly upregulate CD11b (Figure 1D). However, incubation with the combination of ATRA and rhIGFBP7 resulted in a significant increase in the fraction of cells expressing CD11b (Figure 1D), implicating that rhIGFBP7 sensitized HL60 cells to ATRA-induced differentiation. In addition to induction of CD11b membrane expression, the cells show morphological changes typical of terminal myeloid differentiation, such as elongated cells and nuclear lobulation, after treatment with the combination of



**Figure 1. Enhanced IGFBP7 expression sensitizes APL cells to physiological concentrations of ATRA, and rhIGFBP7 potentiates ATRA-induced differentiation, inhibition of proliferation, and apoptosis in non-APL AML cells.** For all experiments, AML cells were cultured in low serum condition and stimulated with PBS (Control/Ctrl), 300  $\mu\text{g}/\text{mL}$  rhIGFBP7, 1.0  $\mu\text{M}$  ATRA, or the combination (Combi) for 96 hours unless stated otherwise. Differentiation (membrane CD11b expression), proliferation, and apoptosis were measured using flow cytometry, and data are plotted as mean  $\pm$  SEM. (A) Percentage of differentiation in NB4 cells, transduced



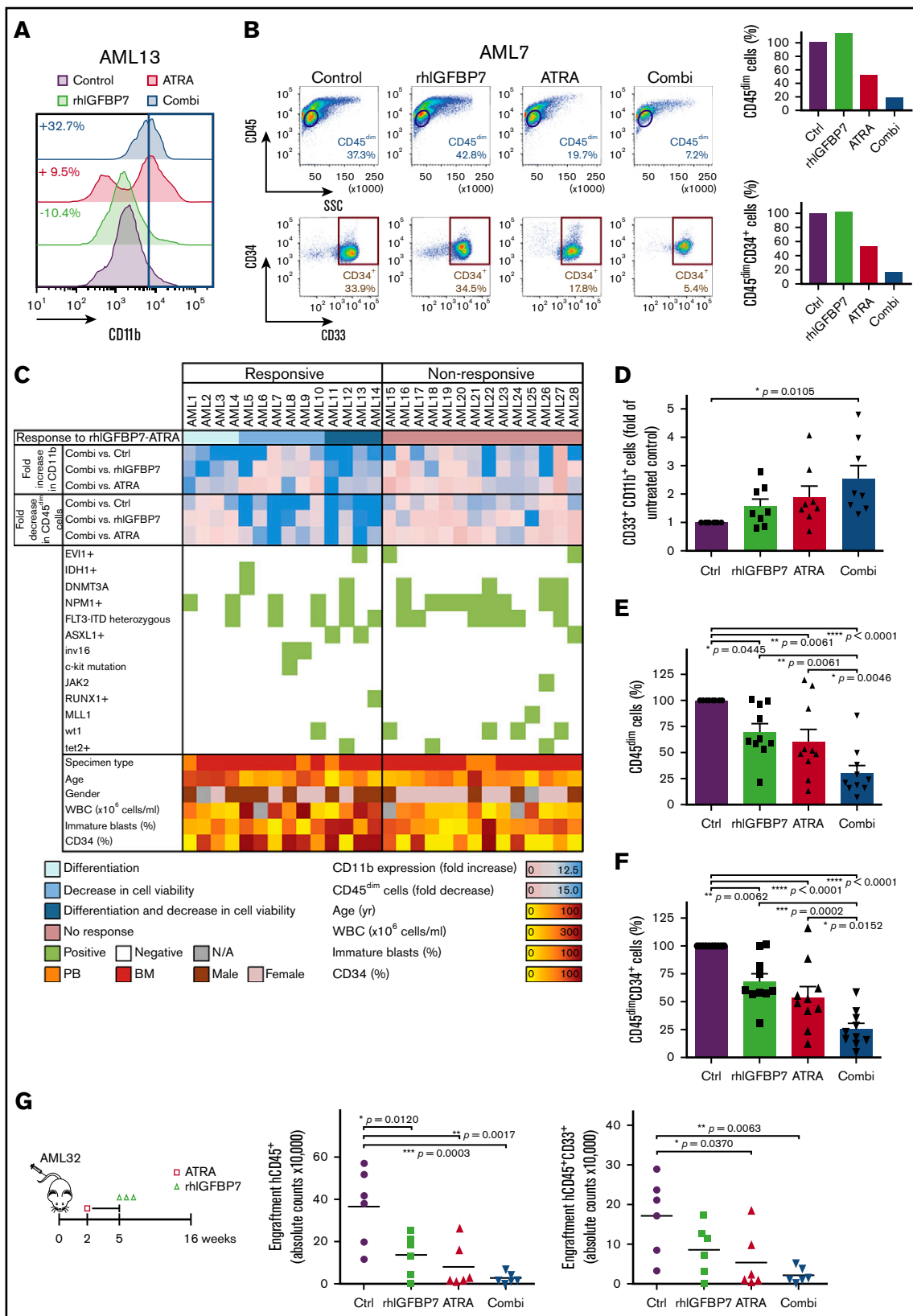
**Figure 1. (Continued)** with IGFBP7 shRNAs (#1 and #2) or scrambled (SCR) shRNA, after stimulation with 0.5  $\mu$ M ATRA. *P* values were determined using 1-way ANOVA with post hoc Dunnett multiple-comparison test. (B-C) NB4 cells, transduced with control or IGFBP7-expressing (IGFBP7-OE) vectors, were stimulated with 0.01  $\mu$ M ATRA. (B) Percentage increase in CD11b expression. (C) CFU assay (in duplicate), normalized against untreated control. (D) CD11b expression in HL60 cells, quantified relative to flow count beads. (E) Morphology of HL60 cells after stimulation for 72 hours, analyzed using a bright-field microscope (upper panels) and by May-Grünwald-Giemsa staining (lower panels). (F) Viability of AML cell lines after stimulation for 72 hours (HL60 and OCI-AML3 cells) or 120 hours (THP1 and KG1a cells) with rhIGFBP7, ATRA, or the combination, normalized against untreated control. (G) AnnexinV<sup>+</sup> THP1 cells after stimulation for 120 hours. (H) Percentage of apoptotic (AnnexinV<sup>+</sup> and 7AAD<sup>+</sup>) THP1 cells (left panel) or OCI-AML3 cells (right panel) after stimulation for 120 hours, quantified relative to flow count beads. Graphs are representative of  $\geq 3$  independent experiments. \**P* < .05, \*\**P* < .01, \*\*\**P* < .001, \*\*\*\**P* < .0001, 1- or 2-way ANOVA with post hoc Tukey's multiple-comparison test, unless stated otherwise.

ATRA and rhIGFBP7 (Figure 1E). Induction of differentiation was accompanied by a significant reduction in the number of viable HL60 cells after incubation with the combination, whereas the single treatments had little or no effect (Figure 1F, far left panel). In the non-APL AML cell line THP1, containing a MLL-AF9 translocation, ATRA monotherapy induced membrane CD11b expression but addition of rhIGFBP7 did not enhance this effect (data not shown). In the cell lines THP1, KG1a, overexpressing Myc and containing a p53 and PTEN mutation, and OCI-AML3, containing a NPM1 and DNMT3a mutation, incubation with the combination of rhIGFBP7 and ATRA resulted in a significant reduction in viable cells compared with the single treatments (Figure 1F). Moreover, in agreement with previous findings,<sup>18,19</sup> ATRA monotherapy reduced the number of viable OCI-AML3 cells, although to a lesser extent than the combination did. Treatment with rhIGFBP7 alone did not result in a reduction in cell viability in any of the 3 cell lines. The reduction in viable THP1 and OCI-AML3 cells after combination treatment was due to induction of apoptosis, as indicated by a significant enhanced binding of Annexin V (example in Figure 1G) and the presence of Annexin V<sup>+</sup> and 7-AAD<sup>+</sup> cells (Figure 1H). Together, these results indicate that ATRA-driven responses in non-APL AML cells belonging to various molecular subgroups can be activated by treatment with rhIGFBP7.

### rhIGFBP7 activates ATRA-driven responses in primary non-APL AML cells

To elucidate whether rhIGFBP7 could activate ATRA-induced responses in human non-APL AML patient cells, we cultured primary AML cells from 28 non-APL AML patients at diagnosis with rhIGFBP7, ATRA, or the combination. Induction of differentiation was investigated by measuring membrane CD11b expression in the CD33<sup>+</sup> myeloid blast population (examples in Figure 2A; supplemental Figure 2A), and cell viability was quantified by measuring the number of viable CD45<sup>dim</sup> blasts and immature CD45<sup>dim</sup>CD34<sup>+</sup> leukemia

cells (example in Figure 2B), using flow cytometry. Responsive AML cases were defined as showing a >5% increase in CD11b-expressing CD33<sup>+</sup> blasts and/or a >5% reduction in survival of CD45<sup>dim</sup> blasts after treatment with the combination of rhIGFBP7 and ATRA compared with the single treatments (Figure 2C; supplemental Figure 2B; supplemental Tables 1 and 2). In 8 of the 14 responsive cases, we observed a significant increase in CD11b in the myeloid CD33<sup>+</sup> blasts after treatment with the combination (Figure 2D; supplemental Figure 2C). In 10 of the 14 responders, the combination of rhIGFBP7 and ATRA resulted in a significant reduction in the number of viable AML CD45<sup>dim</sup> blasts (example in Figure 2B, upper panels; Figure 2E; supplemental Figure 2D) and CD45<sup>dim</sup>CD34<sup>+</sup> immature leukemic cells (example in Figure 2B, lower panels; Figure 2F; supplemental Figure 2E) compared with the single treatments. In 3 of the 14 responders there was even enhanced induction of >35% of cell death in the blasts after the combination compared with the single treatments. We also measured induction of the myeloid differentiation marker CD14 in 17 of the 28 primary AML cases after incubation with rhIGFBP7, ATRA, or the combination; however, an increase in CD14 was not observed after combination therapy (supplemental Figure 2F). Notably, some responsive cases (AML5-10) showed a marked reduction in leukemic blast viability but very little induction of differentiation after rhIGFBP7 and ATRA (Figure 2C; supplemental Table 2). To determine whether differentiation happens prior to cell death after combination treatment, we determined the induction of differentiation by measuring membrane myeloid differentiation markers CD11b, CD14, CD15, and CD86, as well as the reduction in cell viability over time in 2 AML cases (AML11 and AML12) that showed both differentiation and reduced cell viability after the combination. We did not observe an induction of these myeloid markers at day 3 or day 5 after treatment with the combination (data not shown), whereas CD11b was clearly upregulated and cell viability was reduced at day 7 (Figure 2C-E), suggesting that, in these cases, the combination directly promoted cell death and the differentiation route is likely parallel to the one inducing cell death, similar to what was observed for LSD1 inhibition.<sup>35</sup> Together, these



**Figure 2.** rhIGFBP7 activates ATRA-driven responses in primary non-APL AML cells. For all ex vivo experiments, cells were stimulated with PBS (Control/Ctrl), 100  $\mu$ g/mL rhIGFBP7, 0.5  $\mu$ M ATRA, or the combination (Combi) for 7 days. Percentages of CD33<sup>+</sup>CD11b<sup>+</sup>, viable CD45<sup>dim</sup> and CD45<sup>dim</sup>CD34<sup>+</sup> cells were measured using flow cytometry, quantified relative to flow count beads, normalized against untreated control cells, and plotted as mean  $\pm$  SEM. Patient sample characteristics are summarized

results indicate that primary non-APL AML cases belonging to a variety of molecular subclasses can be sensitized to ATRA-driven responses by rhIGFBP7.

Comparing clinical features, molecular aberrancies, cytogenetics, and the associated French-American-British classification between responsive and nonresponsive cases revealed that, although not significant, samples with a higher percentage of CD34-expressing blasts at diagnosis (mean, 58.0% for responders and 29.7% for nonresponders) and elevated levels of white blood cells (WBCs; mean,  $146.1 \times 10^6$  cells per milliliter in responders vs  $79.4 \times 10^6$  cells per milliliter in nonresponders) respond in general better to the combination treatment than those with lower CD34 or lower WBCs (Figure 2C; supplemental Figure 2G; supplemental Table 1). More specifically, AML cases showing a reduction in cell viability after the combination have a significantly higher percentage of CD34-expressing blasts at diagnosis (mean, 68.4%) and elevated levels of WBCs (mean,  $178.6 \times 10^6$  cells per milliliter) compared with nonresponders (supplemental Figure 2H). We also observed an increase in CD38 expression after ATRA treatment in 85% of the tested AML cases (supplemental Figure 2I), which was previously shown to be caused by ATRA-induced transcriptional activation of CD38.<sup>36</sup>

To investigate whether treatment with rhIGFBP7 can add to the ATRA-induced reduction in AML burden in vivo, primary AML cells from an ATRA-sensitive EVI-1<sup>+</sup> patient<sup>21</sup> were transplanted into NSG mice. Two weeks later, ATRA (release of 10 mg over 21 days) or placebo pellets were implanted in the neck of the mice, followed by 3 treatments with 12 mg/kg rhIGFBP7 or PBS (on days 1 through 3). Analysis of the bone marrow at week 16 revealed that the combination was better at reducing engraftment of human (h) CD45<sup>dim</sup> leukemia cells ( $P = .0003$ ) and hCD45<sup>dim</sup>CD33<sup>+</sup> myeloid leukemia cells ( $P = .0063$ ) compared to ATRA or rhIGFBP7 alone (Figure 2G; supplemental Figure 2J), indicating the potential for rhIGFBP7 to enhance ATRA-driven in vivo depletion of leukemic burden.

### Enhanced *IGFBP7* expression or treatment with rhIGFBP7 induces sensitivity to ATRA in primary AML stem and progenitor cells

To determine whether an increase in *IGFBP7* levels can be a strategy to reduce self-renewal capacity and/or survival of primary AML cells upon ATRA treatment, we overexpressed *IGFBP7* in 2 primary AML cases using lentiviral transduction. Immature CD45<sup>dim</sup> blasts and CD45<sup>high</sup> mature blast cells were efficiently transduced, whereas the lymphocytes were not (supplemental Figure 3A). First, we investigated the effect of enhanced *IGFBP7* on survival of clonogenic

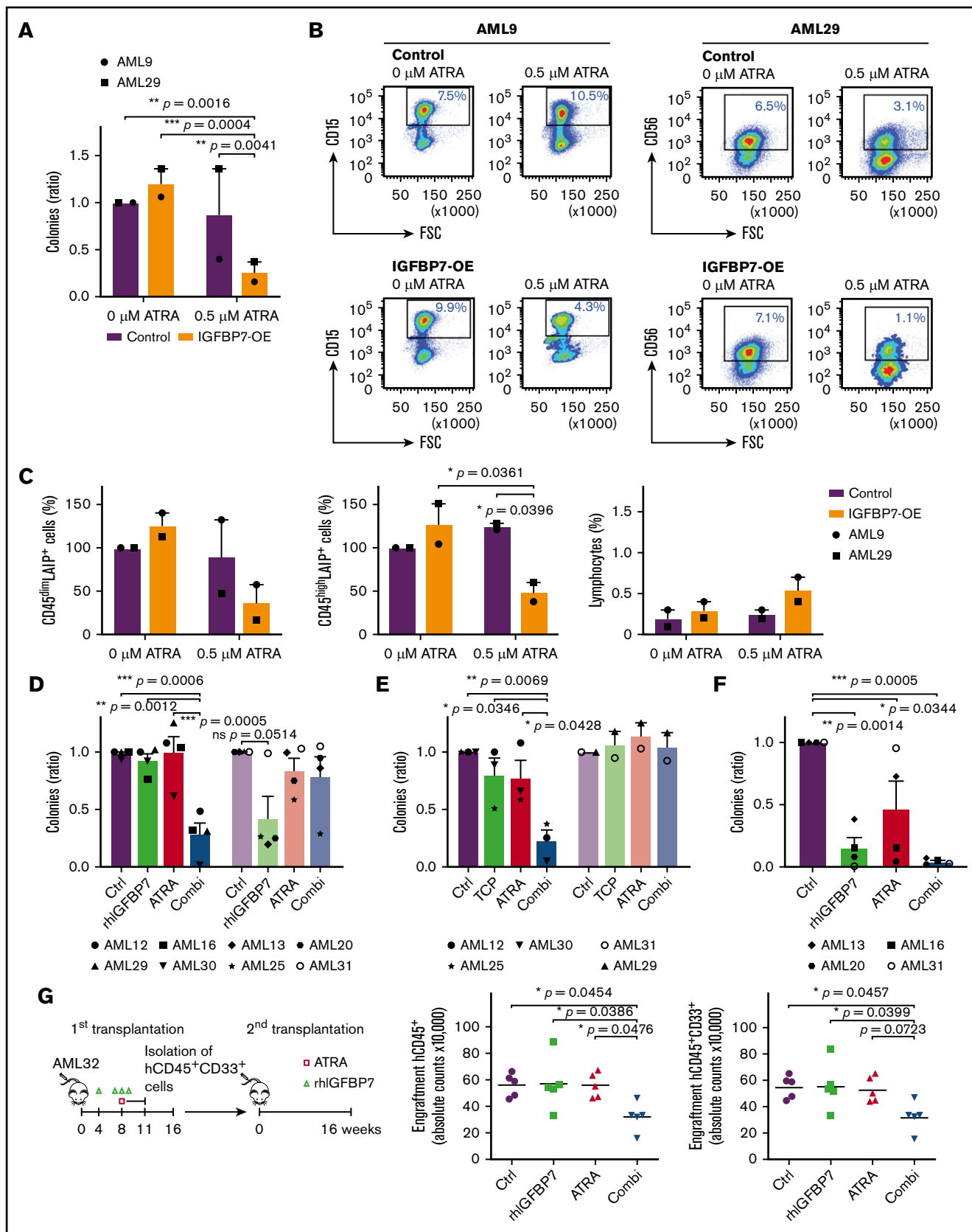
leukemia progenitor cells by short liquid culturing and a colony-forming unit (CFU) assay. Although ATRA monotherapy resulted in a reduced number of colonies in 1 case, ATRA treatment of AML cells with enhanced *IGFBP7* significantly reduced the colony-forming capacity in both samples (Figure 3A; supplemental Figure 3B), suggesting that *IGFBP7* potentiates ATRA-induced elimination of leukemic progenitors. The leukemic phenotype of these cells was confirmed by detection of a leukemia-associated immunophenotype (LAIP)<sup>37</sup> on the CD45<sup>dim</sup> blasts derived from the colonies (Figure 3B). Flow cytometric analysis of AML cells derived from these colonies also showed that CD45<sup>dim</sup> and CD45<sup>high</sup> leukemic cells with enhanced *IGFBP7* were less viable after treatment with ATRA compared with control cells, whereas the lymphocytes were unaffected (Figure 3C; supplemental Figure 3C), again indicating that *IGFBP7* potentiates susceptibility for ATRA-induced cell death in leukemic blasts.

We then evaluated whether the addition of rhIGFBP7 protein to primary AML cells could potentiate ATRA-driven reduction in clonogenic leukemia progenitor cells. Although 3 of the 8 cases responded to rhIGFBP7 monotherapy, the combination of rhIGFBP7 and ATRA significantly reduced the numbers of colonies in 4 of the 8 cases, while the single treatments did not (Figure 3D; supplemental Figure 3D). Incubation of primary AML cells with TCP, an inhibitor of LSD1, in combination with ATRA significantly reduced the clonogenic capacity of leukemia progenitors in 3 of the 5 tested cases (Figure 3E; supplemental Figure 3D). Interestingly, 2 AML samples responded to the combination of ATRA with rhIGFBP7 and ATRA with TCP (AML12 and AML30), whereas 2 other cases only responded to 1 of the combinations (ATRA + rhIGFBP7 [AML29] or ATRA + TCP [AML25]) (Figure 3D-E; supplemental Figure 3D), suggesting that rhIGFBP7 and TCP activate ATRA-driven depletion of leukemic progenitors in a distinct panel of AML cases.

Next, we evaluated whether the combination of rhIGFBP7 and ATRA could reduce the survival of clonogenic long-term surviving leukemia (stem) cells. Although there was already a significant reduction in clonogenic long-term surviving leukemia cells after incubation with the single treatments, the combination led to an even larger reduction in colony number (to nearly 0), in 3 of the 4 tested cases (Figure 3F; supplemental Figure 3E).

To study whether rhIGFBP7 could sensitize LSCs for ATRA-induced cell death in vivo, we equalized the number of human AML cells derived from first transplanted mice that were treated with rhIGFBP7 or ATRA monotherapy or pretreated with rhIGFBP7 and subsequently treated with the combination of rhIGFBP7 and ATRA and retransplanted the cells into secondary recipients (Figure 3G, left panel). The single treatments did not have an effect on leukemic engraftment; however,

**Figure 2. (continued)** in supplemental Table 1. (A) CD11b membrane expression on CD33<sup>+</sup> blasts relative to the untreated control sample in AML13. (B) Percentage of viable CD45<sup>dim</sup> cells (blue; upper panels) and CD45<sup>dim</sup>CD34<sup>+</sup> cells (red; lower panels) in AML7. (C) Heat map of clinical and genetic features of 28 primary AML samples. Responsive AML cases were defined as >5% increase in CD11b-expressing myeloid CD33<sup>+</sup> blasts and/or >5% reduction in CD45<sup>dim</sup> blast survival upon ATRA-rhIGFBP7 combination treatment compared with the single treatments. The fold increase in CD11b and fold decrease in CD45<sup>dim</sup> cells represent the ratio of CD11b-expressing CD33<sup>+</sup> blasts and the ratio of reduction in CD45<sup>dim</sup> blast survival following combination treatment relative to untreated control or single therapies. Percentages are shown in supplemental Table 2. (D) Induction of differentiation in 8 primary AML samples responsive to rhIGFBP7-ATRA. Percentage of viable CD45<sup>dim</sup> cells (E) and CD45<sup>dim</sup>CD34<sup>+</sup> cells (F) in 10 primary AML samples responding to rhIGFBP7-ATRA. (G) Schematic overview of the experiment (left panel). After injection of T-cell-depleted primary AML cells, NSG mice were treated with ATRA (10 mg, 21-day-release pellet) in week 2 and/or rhIGFBP7 (12 mg/kg) in week 5 (days 1-3). At week 16, the bone marrow cells of the mice were analyzed for the presence of hCD45<sup>+</sup> cells (middle panel) and myeloid hCD45<sup>+</sup>CD33<sup>+</sup> cells (right panel) using flow cytometry. \* $P < .05$ , \*\* $P < .01$ , \*\*\* $P < .001$ , \*\*\*\* $P < .0001$ , 1-way ANOVA with post hoc Tukey's multiple-comparison test. BM, bone marrow; N/A, not available; PB, peripheral blood.



**Figure 3. Enhanced IGFBP7 expression or treatment with rhIGFBP7 induces sensitivity to ATRA in primary AML stem and progenitor cells.** For all ex vivo experiments, cells were incubated with PBS (Control/Ctrl), 100 μg/mL rhIGFBP7, 0.5 μM ATRA, or the combination (Combi). For CFU progenitor and long-term liquid culture



combining rhIGFBP7 with ATRA resulted in significantly fewer hCD45<sup>+</sup> and hCD45<sup>+</sup>CD33<sup>+</sup> leukemia cells in the bone marrow of the secondary recipients (Figure 3G; supplemental Figure 3F), indicating that treatment with rhIGFBP7 enhanced ATRA-induced reduction in LSC activity *in vivo*.

### rhIGFBP7 and ATRA combination treatment does not affect healthy bone marrow cells

Therapeutic approaches targeting LSCs should spare normal healthy hematopoietic stem cells (HSCs) to restore normal hematopoiesis after therapy. Hence, bone marrow cells derived from 4 healthy donors were incubated with rhIGFBP7, ATRA, or the combination, and cell viability was assessed using flow cytometry. The total number of viable cells was not reduced after the single treatments or after rhIGFBP7 and ATRA combination treatment (Figure 4A). The total numbers of CD45<sup>dim</sup> cells (Figure 4B), myeloid CD45<sup>dim</sup>CD33<sup>+</sup> cells (Figure 4C; supplemental Figure 4A), CD34<sup>+</sup> cells (Figure 4D; supplemental Figure 4B), and T and B cells (Figure 4E-F; supplemental Figure 4C) were not affected or were only mildly affected. Incubation with rhIGFBP7 and ATRA monotherapy or the combination resulted in a small increase in differentiated CD11b<sup>+</sup> myeloid cells in 1 of the 2 tested NBM samples (supplemental Figure 4D). Moreover, the colony-forming capacity of normal progenitor cells was not significantly affected after rhIGFBP7 and ATRA combination treatment (Figure 4G; supplemental Figure 4E).

### rhIGFBP7 induces susceptibility to ATRA by reducing *GFI1* expression

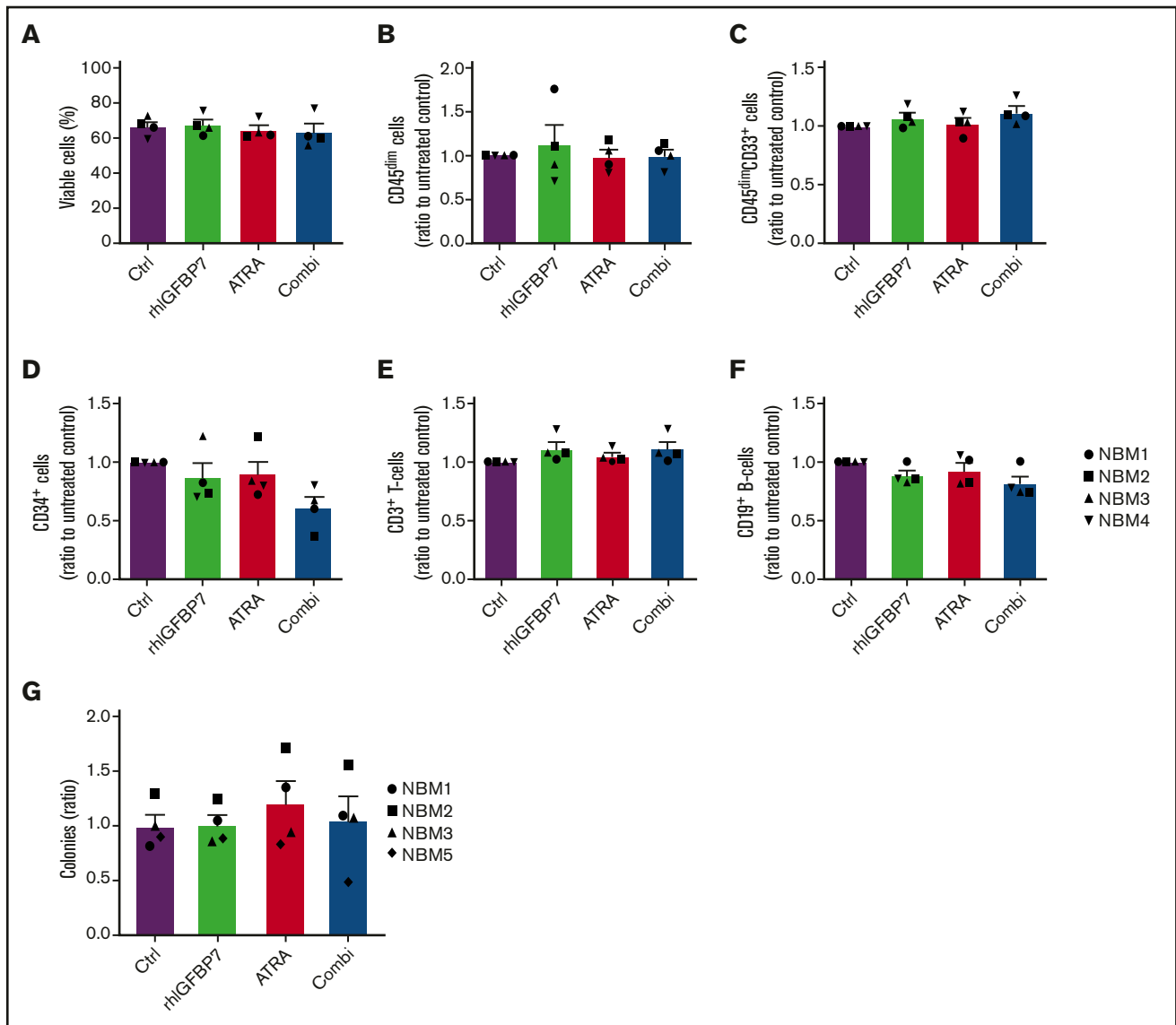
To elucidate the potential mechanisms through which rhIGFBP7 is enhancing sensitivity to ATRA in primary AML cells, we incubated 3 AML samples with rhIGFBP7 and measured the change in gene expression using RNA sequencing (Figure 5A). Using gene set enrichment analysis, we demonstrated previously that rhIGFBP7 treatment resulted in reversal of expression of LSC genes and induction of differentiation toward the monocytic lineage.<sup>31</sup> Pathway analysis, using DAVID, of the top upregulated and downregulated genes after treatment of the 3 samples with rhIGFBP7 revealed enhanced expression of components involved in regulation of differentiation and cell death, as well as factors involved in epigenetic mechanisms, including regulation of transcription and transcription factor activity (Figure 5B). Moreover, the identified transcriptomes revealed several modulated genes after rhIGFBP7 treatment in all 3 cases, including downregulation of *GFI1* (Figure 5A; supplemental Table 3). *GFI1* is a transcriptional repressor<sup>38-40</sup> that is involved in the self-renewal capacity of HSCs and multilineage potential of progenitors.<sup>41,42</sup>

Deletion of *GFI1* results in a block in differentiation, leading to loss of neutrophils and accumulation of immature myelomonocytic cells.<sup>43</sup> qRT-PCR confirmed that rhIGFBP7 reduces *GFI1* in primary AML (Figure 5C).

To elucidate whether reduced expression of *GFI1* is mechanistically involved in rhIGFBP7-induced sensitivity to ATRA, we downregulated *GFI1* in HL60 cells using 2 independent shRNAs (supplemental Figure 5A-B). Reduced expression of *GFI1* led to a significant increase in the induction of differentiation after treatment with ATRA (Figure 5D; supplemental Figure 5C), indicating that low levels of *GFI1* contribute to susceptibility to ATRA-induced differentiation. To investigate whether high *GFI1* inhibits rhIGFBP7-induced ATRA responses, we overexpressed *GFI1* in HL60 cells (supplemental Figure 5D-E). Elevated levels of *GFI1* significantly blocked differentiation induced by combination treatment with rhIGFBP7 and ATRA (Figure 5E; supplemental Figure 5F), suggesting that maintaining the repressive function of *GFI1* is involved, at least in part, in resistance to ATRA. To examine whether this also holds true for primary AML, we increased *GFI1* expression in 2 primary AML cases (supplemental Figure 5G-H). Similar to in HL60 cells, enhanced *GFI1* blocked the induction of membrane CD11b expression on CD45<sup>dim</sup> blasts after rhIGFBP7 and ATRA treatment (example in Figure 5F-G; supplemental Figure 5I). Interestingly, enhancing *GFI1* expression did not affect TCP-induced susceptibility to ATRA-driven differentiation in HL60 cells (Figure 5H; supplemental Figure 5J), suggesting that increased sensitivity to ATRA after LSD1 inhibition is independent of *GFI1* levels. Together, these results demonstrated that rhIGFBP7 can potentiate the ability of ATRA to induce differentiation in non-APL AML cells by reducing the transcriptional repressor *GFI1*.

Previously, we showed that a substantial proportion of EVI-1<sup>+</sup> AML cases respond to ATRA by induction of differentiation and decreased clonogenic capacity of myeloid blasts.<sup>21</sup> Because we showed here that low *GFI1* increased the response to ATRA, we determined *GFI1* expression in EVI-1<sup>+</sup> and EVI-1<sup>-</sup> cases using a publicly available data set<sup>44</sup> (supplemental Figure 5K) and a panel of cases from the Amsterdam UMC, location VUmc (Figure 6A). *GFI1* was expressed at significantly lower levels in EVI-1<sup>+</sup> cases compared with EVI-1<sup>-</sup> cases, suggesting that the sensitivity to ATRA in this group of patients might be correlated with low *GFI1*. Because the presence of a super-enhancer at the *RARA* gene locus and high *RARA* messenger RNA levels have been linked to sensitivity to retinoids in a subset of non-APL AML patients,<sup>45,46</sup> we also evaluated expression of *RARA* in these cases. Notably, *RARA* levels were not significantly different between EVI-1<sup>+</sup> and EVI-1<sup>-</sup> AML patients (supplemental Figure 5L),

**Figure 3. (continued)** (stem cell) assays, samples (in duplicate) were incubated for 7 days or 4 weeks, respectively, and normalized against untreated controls. Data are plotted as mean  $\pm$  SEM, unless stated otherwise. Patient sample characteristics are summarized in supplemental Table 1. (A-C) Primary AML samples were lentivirally transduced with control or IGFBP7-OE vectors. (A) CFU progenitor assays of primary AML samples, plotted as mean  $\pm$  SD. (B) CFU plates, containing all colonies, were harvested, and flow cytometric analysis of AML cells derived from the colonies was performed, showing leukemic CD45<sup>dim</sup> blasts in the transduced cell population for AML9 (LAIP = CD15; left panels) and AML29 (LAIP = CD56; right panels). (C) Percentage of CD45<sup>dim</sup>LAIP<sup>+</sup> blasts (left panel) and CD45<sup>high</sup>LAIP<sup>+</sup> blasts (middle panel) derived from the colonies, quantified, and normalized against untreated controls. Percentage of lymphocytes (right panel), measured using flow cytometry and plotted as mean  $\pm$  SD. (D-E) CFU progenitor assays of primary AML samples, with AML cases not responding to combination therapies shown in the right lighter bars. Samples were incubated with rhIGFBP7 and ATRA (D) or with TCP (10  $\mu$ M) and ATRA (E). (F) CFU stem cell assay (long-term liquid culture) of primary AML samples incubated with rhIGFBP7 and ATRA. (G) Schematic overview of the experiment (left panel). After injection of T-cell-depleted primary AML cells, NSG mice were treated with rhIGFBP7 (10 mg/kg) in week 4 (day 1) and week 8 (day 1-3) and/or ATRA (10 mg, 21-day-release pellet) in week 8 (day 3). Subsequently, equal numbers of human myeloid hCD45<sup>+</sup>CD33<sup>+</sup> cells derived from the first transplant were injected into secondary recipients. At week 16, bone marrow cells from the mice were analyzed for the presence of hCD45<sup>+</sup> cells (middle panel) and myeloid hCD45<sup>+</sup>CD33<sup>+</sup> cells (right panel). \**P* < .05, \*\**P* < .01, \*\*\**P* < .001, 1- or 2-way ANOVA with post hoc Tukey's multiple-comparison test, unless stated otherwise.



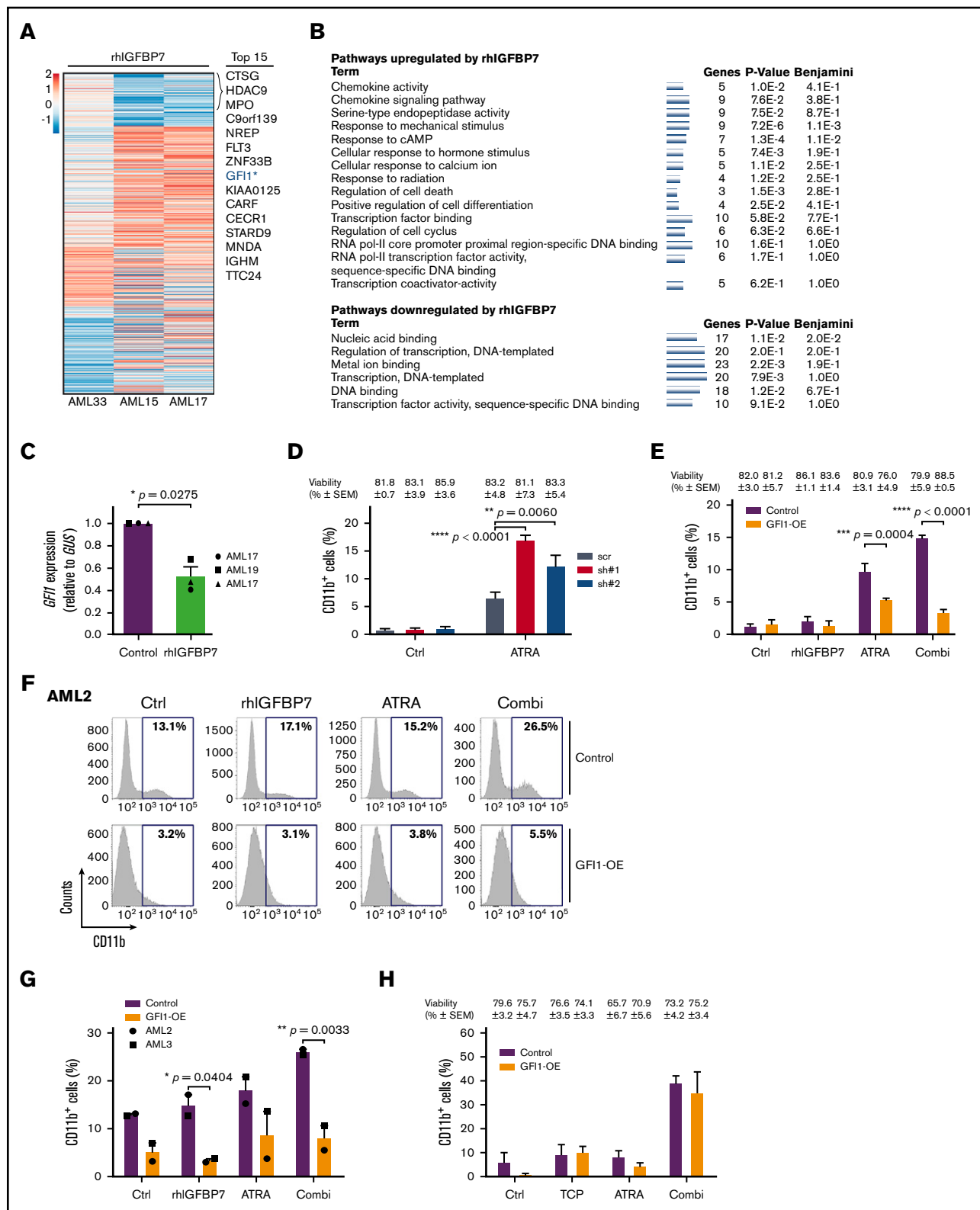
**Figure 4. rhIGFBP7 and ATRA combination treatment does not affect healthy NBM cells.** NBM cells derived from healthy donors were incubated with PBS (Ctrl), 100  $\mu$ g/mL rhIGFBP7, 0.5  $\mu$ M ATRA, or the combination (Combi) for 5 to 7 days. The effects of the treatments were normalized against untreated control cells and plotted as mean  $\pm$  SEM. NBM samples were analyzed for the percentage of viable cells (A), CD45<sup>dim</sup> cells (B), myeloid CD45<sup>dim</sup>CD33<sup>+</sup> cells (C), CD45<sup>dim</sup>CD34<sup>+</sup> cells (D), CD3<sup>+</sup> T cells (E), and CD19<sup>+</sup> B cells (F) using flow cytometry and quantified relative to flow count beads. (G) CFU progenitor assays of NBM samples (in duplicate) after 7 days of treatment.

implicating that low *GFI1*, and not high *RARA*, is associated with susceptibility to ATRA in AML with overexpression of *EVI-1*.

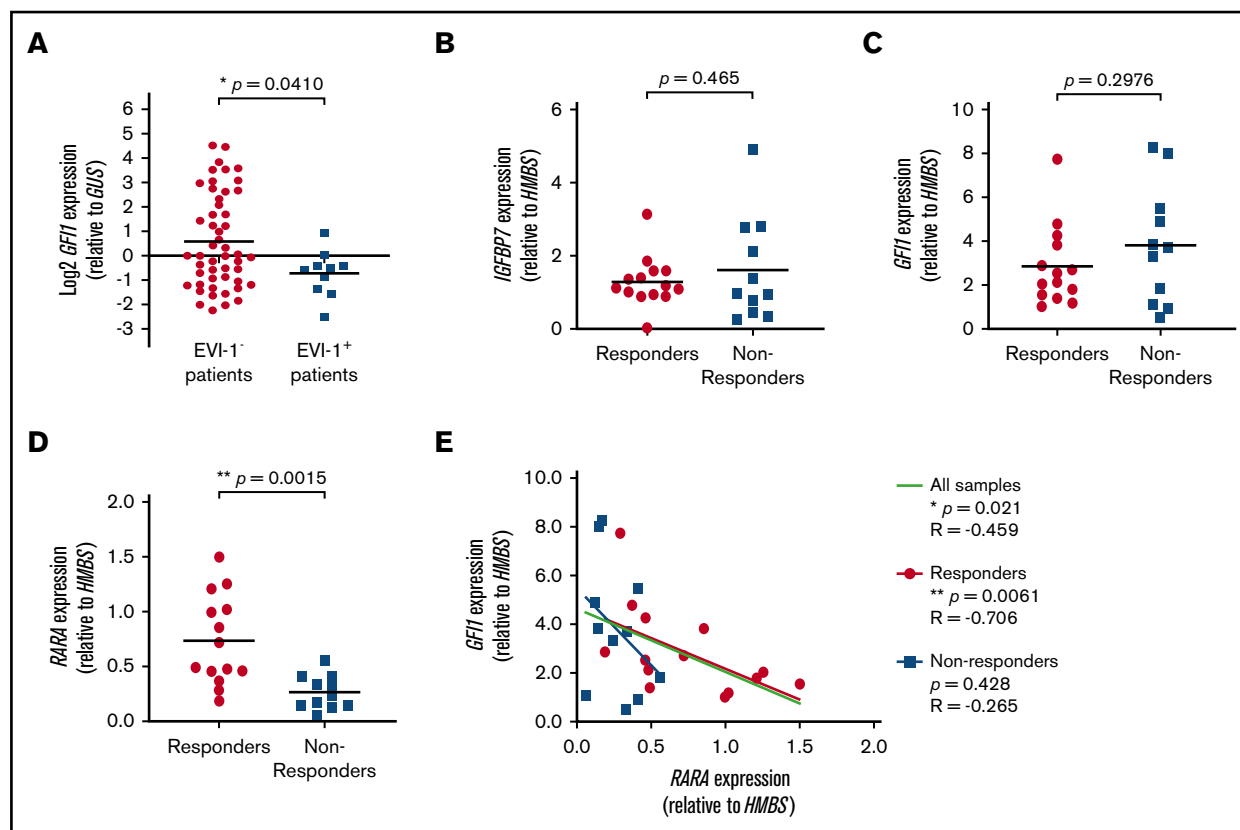
### High *RARA* expression is a biomarker predicting sensitivity for rhIGFBP7-induced activation of the ATRA response in primary AML

To investigate whether *IGFBP7*, *GFI1*, or *RARA* levels in AML cell lines and primary AML at diagnosis are predictive for the increase in ATRA sensitivity after rhIGFBP7, we measured their expression in AML cell lines (supplemental Figure 5M-O) and AML samples responsive or nonresponsive to rhIGFBP7-induced ATRA sensitivity (Figure 6B-D). We did not observe a correlation between the expression levels of *IGFBP7* and *GFI1* and response to ATRA,

rhIGFBP7, or the combination in the cell lines. In OCI-AML3 cells, high *RARA* levels might explain their sensitivity to ATRA monotherapy. In primary AML, we did not observe a significant difference in *IGFBP7* and *GFI1* expression between responders and nonresponders; however, a significantly higher level of *RARA* was observed in the responders. This result suggests that an elevated level of *RARA* is an indicator for sensitivity to rhIGFBP7-induced ATRA responses in primary AML. Notably, there was a significant inverse correlation between *GFI1* and *RARA* expression in AML cases responsive to rhIGFBP7-induced ATRA sensitivity (Figure 6E; for responders,  $R = -0.706$  and  $P = .0061$ ; for nonresponders,  $R = -0.265$  and  $P = .428$ ). Thus, our results indicate that rhIGFBP7 reduces *GFI1* levels, likely resulting in reduced *GFI1* complexes at the chromatin, activation of *GFI1* target gene transcription, and an ATRA-susceptible



**Figure 5. rhIGFBP7 induces susceptibility to ATRA by reducing *GF11* expression.** AML cells were stimulated with 100  $\mu\text{g}/\text{mL}$  rhIGFBP7, 0.5  $\mu\text{M}$  ATRA, 1.0  $\mu\text{M}$  TCP, or the combination (Combi) for 4 days (HL60 cells) or 7 days (primary AML cells). Induction of differentiation (membrane CD11b expression) and cell viability were measured using flow cytometry and quantified relative to flow count beads. Data are representative of  $\geq 3$  independent experiments, plotted as mean  $\pm$  SEM. Patient sample characteristics are summarized in supplemental Table 1. (A) Heat map of the top 111 differentially expressed genes in CD45<sup>dim</sup> cells from 3 primary AML samples after treatment with rhIGFBP7 for 48 hours. Genes were selected based on their log<sub>2</sub>-fold change (FC) in rhIGFBP7-stimulated samples compared with untreated controls (cutoff



**Figure 6. High RARA expression is a biomarker predicting sensitivity for rhIGFBP7-induced ATRA susceptibility in primary AML.** Gene expression levels for *GF11* and *RARA* were measured by qRT-PCR (in triplicate). Patient sample characteristics of responders and nonresponders to rhIGFBP7 and ATRA combination treatment are summarized in supplemental Table 1. (A) Log<sub>2</sub>-transformed *GF11* gene expression in EVI-1<sup>-</sup> (n = 50) and EVI-1<sup>+</sup> (n = 10) AML samples at diagnosis, measured relative to *GUS* expression. The relative *GF11* expression of 1 of the EVI-1<sup>+</sup> AML samples was set at 1. *IGFBP7* (B), *GF11* (C), and *RARA* (D) expression in responders and non-responders to rhIGFBP7 and ATRA combination treatment, relative to *HMBS* expression, as measured by qRT-PCR. (E) *GF11* and *RARA* gene expression levels in responders and nonresponders to the combination of rhIGFBP7 and ATRA, relative to *HMBS*, as by qRT-PCR. Spearman's correlation coefficients were calculated. \**P* < .05, \*\**P* < .01, Student *t* test, unless stated otherwise.

transcriptional program. This effect of rhIGFBP7 might be especially present in non-APL AML cases that have high *RARA* levels in combination with relatively low *GF11*.

## Discussion

Defective differentiation of normal hematopoietic cells, resulting in accumulation of immature myeloid cells, is a general feature of AML.<sup>1</sup> Over the past decades, AML patients have been treated with

standard induction chemotherapy, consisting of cytarabine and an anthracycline, followed in some cases by a stem cell transplantation. Unfortunately, despite high complete remission rates, many AML patients still relapse with these treatment protocols.<sup>47</sup> APL is the only AML subtype that is highly curable by an alternative treatment consisting of ATRA and arsenic trioxide.<sup>2</sup> ATRA-based treatment has not been very effective for any other AML subtype. However, preclinical studies suggest that a subset of non-APL AML cases are sensitive to ATRA-induced differentiation and cell death,<sup>18-21</sup> and

**Figure 5. (continued)** value = log<sub>2</sub>FC < -0.5 and > +0.5; *P* < .01). The top 15 downregulated genes are listed. \*Position of *GF11*. (B) Pathway analysis of the top upregulated and downregulated genes in response to rhIGFBP7 treatment for 48 hours. The minimum number of overrepresented genes was set to 3, and the Kyoto Encyclopedia of Genes and Genomes pathway and Gene Ontology process were used to perform functional pathway analysis using DAVID (v6.8). (C) *GF11* expression (in triplicate) in primary AML samples after treatment with rhIGFBP7, measured relative to *GUS* expression using qRT-PCR, and normalized against control sample. *P* values were determined using a paired Student *t* test. (D) HL60 cells were lentivirally transduced with *GF11*-shRNAs (sh#1 and sh#2) or scrambled (scr) shRNA. The percentage of (Venus<sup>+</sup>)CD11b<sup>+</sup> HL60 cells was measured after 4 days of ATRA treatment, and the percentage of viable cells (blue) for each condition is listed above the graph. *P* values were determined using 2-way ANOVA with a post hoc Tukey's multiple-comparison test. (E-H) AML cells were lentivirally transduced with control-YFP (Control) or *GF11*-YFP (*GF11*-OE)-expressing vectors. (E) Percentage of CD11b<sup>+</sup> cells after treatment of HL60 cells. The percentage of viable cells (blue) for each condition is listed above the graph. Example of flow cytometric analysis of AML2 (F) and percentage of membrane CD11b in the transduced (YFP<sup>+</sup>)CD45<sup>dim</sup> cell population of 2 primary AML samples, measured 14 days posttransduction (G). (H) Percentage of CD11b<sup>+</sup> cells after treatment of HL60 cells with TCP, ATRA, or the combination (Combi). The percentage of viable cells (blue) for each condition is listed above the graph. \**P* < .05, \*\**P* < .01, \*\*\**P* < .001, \*\*\*\**P* < .0001, 2-way ANOVA with post hoc Bonferroni multiple-comparison test, unless stated otherwise.

inhibition of the epigenetic modifiers LSD1 and GCN5 can enhance sensitivity to ATRA in AML.<sup>23,24</sup> Based on these studies, it is believed that AML cells may require a particular transcriptional state to be responsive to ATRA or that transcriptional reprogramming may enhance ATRA sensitivity.

We previously demonstrated that IGFBP7, a tumor suppressor and negative regulator of IGF1R activity, induces differentiation and/or cell death, as well as sensitizes primary AML cells to chemotherapy-induced leukemia cell death.<sup>30,31</sup> IGFBP7 is upregulated in APL,<sup>33,48</sup> and we showed in this study that IGFBP7 plays a role in sensitivity to retinoic acids in APL. In APL cells, susceptibility to ATRA is dependent on expression, function, and localization of several coactivators, transcription factors, and epigenetic modifiers. The majority of PML-RAR $\alpha$  DNA binding sites in APL cells are in close proximity to consensus binding sites for the transcription factors PU.1, ETS, and AP1, indicating that PML-RAR $\alpha$  cooperates with these factors to generate an ATRA-susceptible transcriptional program.<sup>49</sup> Moreover, PML-RAR $\alpha$  interacts with proteins that are important for normal hematopoietic differentiation, such as GFI1, HOXA9A, MEIS1, and RUNX1.<sup>48,50</sup> GFI1 is a target gene of the PML-RAR $\alpha$  fusion protein and is necessary for the maintenance of APL cells.<sup>51</sup> Combined treatment using LSD1 inhibitors and retinoic acids allows differentiation and growth arrest of APL cells without affecting the stability of PML-RAR $\alpha$  and its recruitment to chromatin.<sup>27</sup>

In primary non-APL, rhIGFBP7 treatment reduced *GFI1* and led to increased sensitivity for ATRA-induced responses. GFI1 is a transcriptional repressor interacting with the REST corepressor 1 (CoREST) complex and LSD1 to regulate gene expression and inhibit p53 activity, and depletion of LSD1 enhances transcription of GFI1 targets.<sup>52,53</sup> We observed that rhIGFBP7 treatment of AML cells activated a gene expression program associated with regulation of cell death pathways, including activation of p53, and reduced *GFI1*, thereby likely leading to GFI1 transcriptional target activation and induction of an ATRA-susceptible transcriptional program. Previously, GFI1 has been identified as a p53 target gene controlling the quiescence of HSCs,<sup>54</sup> and inhibition of GFI1 induced p53-dependent proapoptotic responses and blocked the expansion of leukemia cells.<sup>55</sup> Inhibitors of LSD1 disrupt the interaction between LSD1 with GFI1, thereby targeting the GFI1/CoREST complex for disruption and release from the chromatin.<sup>27,29</sup> Because disruption of this complex is required for leukemia cells to differentiate,<sup>29</sup> rhIGFBP7 is expected to reduce GFI1 levels, leading to fewer GFI1 complexes on the chromatin and enhanced sensitivity for ATRA-induced differentiation. Pharmacological inhibition of LSD1 mimics depletion of *GFI1*,<sup>27</sup> and activation of ATRA-driven responses by rhIGFBP7 might mimic LSD1 inhibition by reducing *GFI1*. LSD1 interacts with the chromatin, in different cell types, through distinct complexes of epigenetic modifiers and transcription factors,<sup>56</sup> and inhibition of LSD1 was demonstrated to interfere with GFI1-mediated repression of PU.1 target gene expression.<sup>41</sup> Sensitivity to ATRA after LSD1 inhibition or rhIGFBP7 may require different and similar proteins at the chromatin sites of the target genes, explaining the variation in response profiles in the different AML cases.

In general, AML cases responding to rhIGFBP7-ATRA therapy have greater numbers of CD34-expressing blasts and elevated WBCs. Because these cases contain more immature leukemia cells, these cells may have specific protein complexes at the chromatin of myeloid

differentiation genes required for induction of ATRA responses after lowering *GFI1*. Notably, the transcriptome of low *GFI1*-expressing human leukemia cells have a resemblance to the transcriptome of LSCs,<sup>41</sup> and reduced *GFI1* marks AML cases with an adverse prognosis.<sup>41,57</sup> Consistent with these findings, we demonstrated that low *GFI1* levels are a characteristic of the subgroup of EVI-1<sup>+</sup> AML cases. EVI-1<sup>+</sup> AML samples are sensitive to ATRA-driven responses,<sup>21</sup> have a stem cell phenotype,<sup>58,59</sup> and have reduced *GFI1* levels, suggesting that low levels of *GFI1*, or the change in the transcriptome associated with downregulation of *GFI1*, in the context of an immature AML phenotype may convert cells to an ATRA-susceptible state. AML with low *GFI1* has a poor response to chemotherapy,<sup>41,57</sup> but it may have an efficient response to ATRA-based treatment. Epigenetic therapies, such as inhibition of HDACs, have become more prevalent for AML patients who are not eligible for standard chemotherapy. However, patients with low *GFI1* in their blasts are often resistant to HDAC inhibitors,<sup>41</sup> and ATRA-based therapies may be a successful treatment for these patients. Moreover, AML cells that contain a strong super-enhancer at the RARA gene locus, and consequently have high *RARA* expression, are more sensitive to retinoic acid-induced treatment.<sup>45</sup> We observed that responders to rhIGFBP7 and ATRA combination therapy have elevated *RARA*, as well as a significant inverse correlation between *GFI1* and *RARA* expression.

The combination of rhIGFBP7 and ATRA, as well as both monotherapies, did not induce differentiation or cell death in normal hematopoietic cells and HSCs. This result is consistent with previous data showing that inhibition of the retinoic acid receptor results in expansion of normal CD34<sup>+</sup>CD38<sup>-</sup> cells and enhances the engraftment potential of normal hematopoietic repopulating cells.<sup>60</sup> Moreover, treatment with ATRA antagonized stress-induced activation of HSCs, whereas induction of differentiation or cell death was not affected in HSCs.<sup>61</sup>

Together, our results demonstrated that IGFBP7 is part of ATRA-driven responses in APL cells. We showed that treatment of primary AML cells with rhIGFBP7 can enhance ATRA-induced differentiation, can reduce AML cell viability, and, importantly, can increase ATRA-driven reduction in AML progenitor and stem cells. These observations suggest that treatment of AML patients with a combination of rhIGFBP7 and ATRA might prove to be an effective therapy in a subset of non-APL AML patients to prevent relapse and improve patient survival, which has to be confirmed in further clinical studies. Moreover, in light of the ongoing clinical trials evaluating the effects of LSD1 inhibition in combination with ATRA, it might be of interest to add rhIGFBP7 to this combination therapy to enhance successful induction of differentiation and/or cell death in a large group of AML patients.

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## Authorship

Contribution: N.v.G., H.J.M.P.V., A.R., M.-L.T., E.V., E.D., F.B., F.D., and F.L.K. conducted the experiments; N.v.G., H.J.M.P.V., and L.S. designed experiments; J.J.W.M.J. and G.J.O. provided AML patient samples; R.X.M. performed data analysis; N.v.G. and L.S. wrote the manuscript; and H.J.M.P.V. and G.J.O. revised the manuscript.

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