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## RNA-binding protein IGF2BP1 maintains leukemia stem cell properties by regulating *HOXB4*, *MYB*, and *ALDH1A1*.

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### Abstract

Insulin-like growth factor 2 mRNA-binding protein 1 (IGF2BP1) is an oncofetal protein expressed in various cancers including leukemia. In this study, we assessed the role of *IGF2BP1* in orchestrating leukemia stem cell properties. Tumor-initiating potential, sensitivity to chemotherapeutic agents and expression of cancer stem cell markers were assessed in a panel of myeloid, B-, and T-cell leukemia cell lines using gain- and loss-of-function systems, cross-linking immunoprecipitation (CLIP), and photoactivatable ribonucleoside-enhanced crosslinking and immunoprecipitation (PAR-CLIP) techniques. Here we report that genetic or chemical inhibition of *IGF2BP1* decreases leukemia cells' tumorigenicity, promotes myeloid differentiation, increases leukemia cell death, and sensitizes leukemia cells to chemotherapeutic drugs. *IGF2BP1* affects proliferation and tumorigenic potential of leukemia cells through critical regulators of self-renewal *HOXB4* and *MYB* and through regulation of expression of the aldehyde dehydrogenase,

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Data availability

Data used for bioinformatics analysis were deposited in NCBI Gene Expression Omnibus (GEO) repository and can be accessed through the series accession numbers GSE138704 and GSE138063.

Conflict of interest

The authors declare no conflicts of interest.

*ALDH1A1*. Our data indicate that IGF2BP1 maintains leukemia stem cell properties by regulating multiple pathways of stemness through transcriptional and metabolic factors.

## Keywords

IGF2BP1; cancer stem cells; leukemia; HOXB4; MYB; ALDH

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## Introduction

Leukemia, a wide spectrum of diseases with altered proliferation and differentiation capacity of myeloid or lymphoid blood progenitors, is the most frequent type of cancer in children and one of the most common in adults<sup>1</sup>. Despite the significant progress in treatments and improved survival outcomes, cancer resistance to chemotherapeutics is the major cause of disease relapse. The 5-year recurrence estimate for acute myeloid and lymphoblastic leukemia (AML, ALL) is 20–30% in children and 70% in adults<sup>2,3</sup>. The incidence of molecular recurrence in treatment-free patients with chronic myeloid leukemia (CML) is 30% at 3 years<sup>4</sup>.

The relapse of leukemia and other cancers is associated with tumor heterogeneity and the presence of groups of cells that possess stem cell properties, such as self-renewal, differentiation block, resistance to apoptosis, drug efflux and detoxification that make them resistant to therapies. The ability of human leukemia-initiating or leukemia stem cells (LICs/LSCs) to regenerate the disease is assessed by leukemia engraftment into immunodeficient mice and expression of hallmark stem cell markers such as ALDH1A, ABC transporters, CD34, DNMT3A and others<sup>5–7</sup>.

Insulin-like growth factor 2 mRNA-binding protein 1 (IGF2BP1), also known as CRD-BP, IMP1, ZBP1 or VICKZ1, is an oncofetal RNA-binding protein that plays a major role in RNA transport, translation, and stability<sup>8</sup>. IGF2BP1 belongs to the IGF2BP family that consists of three members: IGF2BP1, IGF2BP2, and IGF2BP3, with 56% overall sequence homology. Among the three paralogs, IGF2BP1 and IGF2BP3 display greater resemblance in structure and expression patterns. They function mostly during embryogenesis, display low or no expression in adult tissues, and are often reactivated in cancer.

While the tumorigenic and metastatic properties of IGF2BPs have been intensively investigated in solid tumors, less is known about their role in leukemia<sup>9</sup>. The overexpression of IGF2BP1 and 3 was linked to certain types of B-cell acute lymphoblastic leukemia (B-ALL) with *ETV-RUNX1* and *MLL* rearrangements, respectively<sup>10</sup>. In addition, IGF2BP1 was shown to mediate tumorigenic functions of LIN28B in AML cells<sup>11</sup>.

Given the physiological role of IGF2BP1 in stem cell maintenance and development<sup>8</sup>, we sought to investigate the impact of IGF2BP1 expression on LSC properties. To this end, we assessed the role of IGF2BP1 in leukemia cells with respect to their capacity to engraft, differentiate, and respond to differentiating or cytotoxic agents.

We found that IGF2BP1 regulates the LSC phenotype affecting leukemia engraftment upon xenotransplantation, differentiation capacity in response to all-trans retinoic acid (ATRA), and induction of cell death by various drugs. We identified a number of novel IGF2BP1 targets with known functions in regulating hematopoietic stem cells (HSC) self-renewal and showed that *HOXB4*, *MYB*, and *ALDH1A1* mediate the IGF2BP1-dependent LSC phenotype. The results of this study delineate novel mechanisms of IGF2BP1-mediated regulation of leukemogenesis.

## Methods

For the detailed description of materials and methods, please refer to the supplemental information.

### Cell lines selection and characterization of leukemia stem cell phenotype

The thirteen human leukemia cell lines with different histological and genetic backgrounds randomly chosen for this study are listed in Supplementary Table 1. SKNO1, TANOUE, REH, and MOLT16 were purchased from Leibniz Institute DSMZ, Germany. Other cell lines were obtained from ATCC (Manassas, VA). For leukemia stem cell phenotype characterization, cell lines were assessed for leukemia initiating capacity in NSG mice, colony forming cell (CFC) potential, and expression of stem cell markers. The antibodies used for flow cytometry and western blotting are listed in Supplementary Table 2.

### Gain- and loss-of-function systems

The lentivirus constructs for constitutive and doxycycline-inducible expression of short-hairpin (sh) RNAs are listed in Supplementary Table 3. The constitutive expression of shIGF2BP1 (short-hairpin sequence 1 (SH1)), shIGF2BP3 and shControl were obtained from Sigma (St. Louis, MO). The doxycycline-inducible shIGF2BP1 (sequences 2 and 3 (SH2 and SH3)) and scrambled shControl were obtained from GE Dharmacon (Lafayette, CO). Plasmids and lentiviral vectors for doxycycline-inducible and constitutive protein overexpression are listed in Supplementary Table 4.

### Chemical compounds

For chemical compounds used in this study, please refer to Supplementary Table 5.

### Gene expression analysis

Quantitative PCR (qPCR) reactions were assembled with at least two technical replicates, and at least three biological replicates were performed for each experiment. qPCR data are presented as a mean value of biological replicates ( $n$ ) and the error bars indicating standard error of mean ( $\pm$  SEM). The primers are listed in Supplementary Table 6. RNA sequencing analysis of 697 (EU3) ALDH<sup>+</sup> subpopulations with and without IGF2BP1 knockdown was performed from four biological replicates ( $n=4$ ). Each biological replicate represents *de novo* selection of ALDH<sup>+</sup> cells and independent doxycycline treatments.

### **In vivo experiments**

Non-obese diabetic/severe combined immunodeficient gamma (NSG) mice were obtained from Jackson Laboratory. For the engraftment experiments,  $1 \times 10^3 - 1 \times 10^6$  cells were injected into tail veins of non-irradiated 6–10 week-old female mice in 100  $\mu$ L of DPBS per mouse. No blinding or randomization was applied to mice experiments. Routinely, each *in vivo* experiment was performed with three technical replicates (three mice per group) and independently repeated two to three times for each cell line. The biological replicates were conducted with the *de novo* transduced, puromycin or GFP selected cells, and with the efficient IGF2BP1 knockdown verified by western blot.

### **Data deposition**

Gene expression profiling data by high throughput sequencing of 697 (EU3) ALDH<sup>+</sup> cells with IGF2BP1 knockdown were deposited to NCBI Gene Expression Omnibus (GEO) and can be accessed through GEO series number GSE138704. The PAR CLIP data of IGF2BPs RNA targets in K562 CML can be accessed through GEO series number GSE138063. The hyperlinks to submissions are provided in the supplemental methods.

### **Statistical Analysis**

Data were analyzed using GraphPad Prism (GraphPad Software Inc., San Diego, CA, USA) and R programming language version 3.4.4 (R Foundation, Vienna, Austria). A log-rank (Mantel-Cox) test was used to determine p values in Kaplan-Meier survival curves comparison. For two-group analysis, two-sample Student's or Welch's t-tests were used. All tests were two-sided, and values with \*P < 0.05, \*\*P < 0.01, \*\*\*P < 0.001 were considered statistically significant.

## **Results**

### **The role of IGF2BP1 in leukemia cell proliferation and tumorigenesis.**

The role of IGF2BP1 expression in LSC properties was examined in multiple leukemia cell lines of diverse histological and genetic backgrounds (Supplementary Table 1), using *in vitro* and *in vivo* assays. The expression of IGF2BP1 and its two paralogs was analyzed together with each cell lines' tumorigenicity, colony-forming cell (CFC) potential, the presence of CD34<sup>+</sup>CD38<sup>-</sup> subpopulation, and expression of aldehyde dehydrogenase (ALDH) (Fig. 1a, Supplementary Fig. 1a–e, Supplementary Table 2). Among the 13 tested cell lines, the least colony-forming and non-tumor-initiating cells had low levels of IGF2BP1 and IGF2BP3 (KASUMI1 and MOLT16) or expressed mostly IGF2BP2 protein (SKNO1 and KASUMI6) (Fig. 1a, Supplementary Fig. 1a, 1b). Although activation of CD34 expression was seen upon xenotransplantation (Supplementary Fig. 1c), only KASUMI1 cells expressed a well-defined CD34<sup>+</sup>CD38<sup>-</sup> subpopulation in culture (Supplementary Fig. 1d), but the cells were slow-growing and did not engraft. ALDH<sup>+</sup> subpopulations were detected by flow cytometry in three of the cell lines: K562, 697(EU3), and MOLT4 (Supplementary Fig. 1e). Based on the cumulative screening results, the leukemia cell lines were stratified for LSC-relevant phenotypes (Supplementary Fig. 1f). Based on endogenous levels of IGF2BP1, we established stable and inducible IGF2BP1 loss- and gain-of-function systems where high

IGF2BP1 expressers were used for knockdown and low IGF2BP1 expressers for overexpression (Supplementary Fig. 2a, 4a, Supplementary Tables 3, 4). Three cell lines, K562, 697(EU3) and HL60/S4, scored highest for LSCs properties and had high levels of IGF2BP1. IGF2BP1 knockdown reduced colony-forming capacity in most cell lines with high levels of IGF2BP1 expression (Fig. 1b, Supplementary Fig. 2b, 2c). Similarly, in leukemia cells with higher endogenous levels of IGF2BP1 paralog, IGF2BP3, inhibition of IGF2BP3 reduced colony formation (Supplementary Fig. 2d). Decreased cell numbers in CFCs were associated with reduced staining of the proliferation marker Ki67 (Supplementary Fig. 2e). BrdU staining of 697(EU3) showed statistically significant reduction of cells replicating their DNA (Fig. 1c, Supplementary Fig. 2f). Lower levels of IGF2BP1 expression are associated with better survival in patients with AML (Supplementary Fig. 2g). IGF2BP1 downregulation also resulted in the reduced tumorigenicity of various subtypes of leukemia as measured by survival after leukemia cell injection into healthy NSG mice (Fig. 1d, Supplementary Fig. 2h). These data suggest that IGF2BP1, expressed at varied levels in leukemia cells of different histological subtypes, is important for maintaining leukemogenesis.

### **Inhibition of IGF2BP1 sensitizes leukemia cells to chemotherapeutics.**

The acute myeloblastic leukemia cell line HL60/S4 is a good model to study the differentiation of myeloid leukemia cell upon retinoid acid treatment targeting the nuclear retinoic acid receptors (RARs)<sup>12, 13</sup>. Downregulation of IGF2BP1 stimulated spontaneous differentiation of HL60/S4, producing ~20% CD11b<sup>+</sup> cells in DMSO treatments, and further potentiated ATRA-induced differentiation (Supplementary Fig. 3a, and Supplementary Table 5). IGF2BP1 knockdown sensitized various leukemia cell lines to treatments with doxorubicin, cytarabine, and cyclophosphamide measured by the staining with cell-impermeant DNA-binding dye 7-Amino-Actinomycin D (7-AAD) (Supplementary Fig. 3b). Treatment with the recently identified small molecule inhibitor of IGF2BP1 BTYNB<sup>14</sup> was effective in inducing cytotoxicity in AML, B-, and T-cell leukemia cells, and significantly increased the effect of doxorubicin in these assays (Supplementary Fig. 3c–e). Conversely, the ectopic expression of IGF2BP1 in MOLT16, characterized by very low or undetectable endogenous expression of IGF2BP proteins, reduced cellular death and increased resistance to doxorubicin compared to control (Supplementary Fig. 3f). The elevated levels of IGF2BP1 expression in leukemia cells were also associated with a modest increase in amount of Ki67<sup>+</sup> cells (Supplementary Fig. 4b). IGF2BP1-dependent changes in leukemia cell proliferation, tumorigenicity, and sensitivity to drugs are likely not associated with changes in expression of fusion proteins since no statistically significant differences were detected in the mRNA levels of AML1/ETO, MLL/AF4, and TCF/PBX1 measured by qPCR in both loss- and gain-of function systems (Supplementary Fig. 4c, Supplementary Table 6).

### **IGF2BP1 supports leukemia stem cell phenotype.**

To investigate the role of IGF2BP1 in LSC gene expression, we separated ALDH-enriched (ALDH<sup>+</sup>) and depleted (ALDH<sup>-</sup>) subpopulations of 697(EU3) cells that scored highly for LSC properties, including ALDH expression. As expected ALDH<sup>+</sup> 697(EU3) cells expressed higher levels of *ALDH1A1* and *2*, *ABC* transporters, and some LSC markers associated with acute leukemia<sup>7</sup> (Supplementary Fig. 5a). They also displayed higher

tumorigenic potential than ALDH<sup>-</sup> cells (Supplementary Fig. 5b). Importantly, the ALDH<sup>+</sup> population expressed higher mRNA and protein levels of IGF2BP1 compared to ALDH<sup>-</sup> cells (Fig. 2a). Ectopic expression of IGF2BP1 in MOLT16 cells with low endogenous IGF2BPs expression, resulted in elevated levels of *ABCB1* transporter, *CD34* and *MMRN1* mRNAs (Fig. 2b). IGF2BP1 knockdown in ALDH<sup>+</sup> cells led to a two-fold reduction of their proliferative potential, making them equally or less tumorigenic than 697(EU3) ALDH<sup>-</sup> cells (Fig. 2c, Supplementary Fig. 5c). The anti-proliferative effect of IGF2BP1 downregulation in ALDH<sup>-</sup> cells was half as effective when compared to ALDH<sup>+</sup> cells (Supplementary Fig. 5d) indicating that IGF2BP1's regulatory potential is higher in the ALDH-enriched cells. No difference in cell proliferation nor tumorigenicity was observed in cells expressing shControl (Supplementary Fig. 5e). Together these data suggest that IGF2BP1 is an important regulator of the leukemia stem cell phenotype.

### Identification of IGF2BP1 targets responsible for IGF2BP1-mediated phenotypes in LSCs.

Gene expression profiling of *IGF2BP1* knockdown in ALDH-enriched cell populations revealed downregulation of 1,234 genes (\*p<0.05, FDR<0.3). The Gene Ontology enrichment analysis for inhibited mRNAs (GO confidence interval 98%, FDR<0.05) indicates negative regulation of somatic stem cell division, cellular metabolic processes, and translation (Fig. 3a). Multiple genes involved in drug metabolic processes, including *ALDH1A1*, *ALDH1A3*, *ALDH2*, and *LDHA*, were downregulated in *IGF2BP1* knockdown cells. Downregulated stem cell factors include the key regulators of hematopoietic stem cell division *HOXB4* and *MYB* and previously characterized IGF2BP1 mRNA targets *c-MYC*, *H19*, and *LEF1*<sup>15-17</sup> (Fig. 3a). The cross-linking and immunoprecipitation analysis (CLIP) in 697(EU3) and other cells with the highest LSC properties, resulted in 10- to 120-fold enrichment of the selected mRNAs, including *HOXB4*, *ALDH1A1*, and *MYB*, compared to control immunoprecipitation with IgG (Fig. 3b, IgG is set at zero). The endogenous protein levels of *ALDH1A1*, *HOXB4*, and *MYB* were reduced upon shIGF2BP1 knockdown in these cells (Fig. 3c).

To determine IGF2BP1 direct binding mRNA targets, we performed photoactivatable ribonucleoside-enhanced crosslinking and immunoprecipitation (PAR-CLIP) analysis in K562 CML. The GO analysis of biological processes indicated enrichment with regulators of cell division, protein modification and ubiquitination, cellular metabolic processes, and regulation of cellular transport (Supplementary Fig. 6a). KEGG pathway analysis demonstrated enrichment with regulators of proteolysis, DNA replication and repair, and programmed cell death (Supplementary Fig. 6a). In line with the RNA sequencing and CLIP analysis, PAR-CLIP identified *MYB* and *ALDH1A1* among the mRNAs bound by IGF2BP1 (Fig. 4a). *HOXB4* mRNA, significantly enriched in CLIP, displayed a stronger binding with IGF2BP3 rather than IGF2BP1 in PAR-CLIP, probably due to technical limitations of the method. However, a number of other homeobox (*HOX*)-encoding mRNAs also bound to IGF2BP1, such as *HOXB2* and *HOXB9* (Supplementary Fig. 6b). CLIP analysis confirmed the enrichment with *HOXB9* mRNA compared to *CEBPA* mRNA, which did not indicate binding either with IGF2BP1 or IGF2BP3 in PAR-CLIP (Supplementary Fig. 6c, d).



### Forced expression of stem cell genes *ALDH1A1*, *HOXB4* and *MYB* rescues IGF2BP1 loss-of-function phenotype.

The ectopic expression of *ALDH1A1*, *HOXB4* and *MYB* in K562 cells with shIGF2BP1 knockdown reversed the anti-proliferative effect of IGF2BP1 inhibition with varied efficiency (Fig. 5a, Supplementary Fig. 7a). The Ki67 staining intensity notably increased upon *HOXB4* and *MYB* overexpression compared with a control vector overexpression (Fig. 5a, upper panel). Forced expression of *ALDH1A1*, *HOXB4* and *MYB* in K562 cells expressing scrambled shRNAs (shControl) did not have any effect on K562 cell proliferation (Fig. 5a, lower panel). Forced expression of either *ALDH1A1* or *HOXB4* completely rescued the colony forming capacity of 697(EU3) and HL60/S4 cells (Fig. 5b, c; Supplementary Fig. 7b, 7c). The overexpression of *HOXB4* in the HL60/S4 cell line with IGF2BP1 knockdown, restored the morphology of the colony forming units and enhanced the HL60/S4 resistance to ATRA-mediated differentiation (Fig. 5c). The overexpression of either *HOXB4* or *MYB* partially rescued colony forming capacity of K562 cells expressing shIGF2BP1 and significantly improved survival of K562 cells (Supplementary Fig. 7d–f).

## Discussion

IGF2BP1 expression is elevated in a variety of human cancers, including melanoma, breast, colon, lung, ovarian, and hepatocellular tumors, and its high expression is associated with metastasis and poor prognosis<sup>18–22</sup>.

Whereas the oncogenic role of IGF2BPs has been reported for a number of solid tumors, the role of IGF2BP1 and its paralogs in normal and aberrant hematopoiesis is not well understood. Several reports indicate association of IGF2BPs with leukemia driven by specific chromosomal rearrangements: IGF2BP1 is associated with ETV6/RUNX1C translocation<sup>23–25</sup>, while IGF2BP3 is commonly overexpressed in B-ALL with MLL rearrangements<sup>10</sup>. It has been shown that overexpression of IGF2BP3 in normal murine progenitors directs hematopoiesis towards the myeloid lineage<sup>10</sup>, and orchestrates a fetal-like B cell development program in collaboration with LIN28B<sup>26</sup>. Another study describes the shift of definitive erythropoiesis to a fetal-like state upon IGF2BP1 expression<sup>27</sup>. Therefore, IGF2BPs may have a distinct role in stem and progenitor lineage specification and the development of myeloid, B-, or T-cell leukemia. In addition, there is controversy concerning the role of IGF2BP1 in blood cells as an oncogene or tumor, suppressor with one report indicating that downregulation of IGF2BP1 increases proliferation of K562<sup>28</sup>.

Our study showed that IGF2BP1 expression is associated with LSC properties that can be acquired regardless of the histological and genetic subtype of advanced leukemia. The hallmarks of human LSCs are their engraftment potential upon xenotransplantation and a colony forming capacity *in vitro*. Among 13 tested cell lines, the least colony-forming and non-tumor-initiating leukemia cells had low levels of IGF2BP1 and IGF2BP3 or express mostly IGF2BP2 protein. IGF2BP1 enrichment was detected in fast engrafting ALDH<sup>+</sup> population of leukemia cells. IGF2BP1 knockdown significantly delayed leukemia development in mice and reduced leukemia cell proliferation by more than 30%. RNA sequencing and PAR-CLIP analysis identified a hematopoiesis-specific factor of self-

renewal, HOXB4, and the proto-oncogene, MYB, that can both rescue the anti-proliferative phenotype upon IGF2BP1 withdrawal.

Homeobox B4 (HOXB4) protein plays an important role in the self-renewal of normal HSCs and, unlike other HOX proteins, does not possess strong leukemogenic capacity<sup>29</sup>. This associates HOXB4 expression with a favorable prognosis in de novo acute leukemia<sup>30</sup>. However, a stem cell therapy with HOXB4-expressing vectors led to a high incidence of leukemia in large animals<sup>31</sup>. The overexpression of HOXB4 in CD34<sup>+</sup> cells of dogs and monkeys had contributed to leukemia development in those animals, while the knockdown of HOXB4 induced leukemia cells differentiation<sup>31</sup>. A recent study on the K562/ADM chemo resistant line provides evidence that downregulation of HOXB4 increases sensitivity to drugs and decreases drug efflux<sup>32</sup>.

Our PAR-CLIP analysis revealed IGF2BP1 binding sites within mRNAs of multiple HOXB genes. A recent study in synthetic modeling of T-cell leukemia showed that HOXB genes are critical for leukemia initiation and maintenance, providing growth advantage in both pre-leukemia cells and established clones<sup>33</sup>.

The MYB proto-oncogene encodes a nuclear transcription factor with an essential role in the proliferation, lineage commitment, and differentiation of hematopoietic progenitor cells<sup>34</sup>. Genomic duplications and high levels of MYB expression, often in cooperation with NOTCH1 mutations, are associated with T-cell acute lymphoblastic leukemia pathogenesis, increased leukemia cell proliferation, viability, and differentiation block<sup>35, 36</sup>. MYB is responsible for maintaining an aberrant self-renewal program that is essential for the initiation and progression of MLL-rearranged AML<sup>37, 38</sup>. Our data demonstrate the importance of IGF2BP1-mediated post-transcriptional regulation of MYB in leukemia cell proliferation.

In addition to the self-renewal factors, HOXB4 and MYB, we found that IGF2BP1 post-transcriptionally regulates the expression of the ubiquitous, hallmark stem cell marker ALDH1A1. The aldehyde dehydrogenase (ALDH) superfamily consists of 19 NAD(P)-dependent enzymes that can oxidize a structurally diverse group of endogenous and exogenous aldehyde substrates<sup>39</sup>. High levels of ALDH1A1 activity protect normal and leukemia hematopoietic stem cells from the toxicity of the alkylating agents<sup>40</sup>. In our study, downregulation of IGF2BP1 in ALDH-expressing 697(EU3) and K562 cells sensitized them to chemotherapeutic-induced cell death. In addition to cellular detoxification functions, ALDH expression is associated with cell proliferation and cancer aggressiveness<sup>41–44</sup>. ALDH1A1 overexpression in IGF2BP1-depleted 697(EU3) cells fully restored their clonogenic potential indicating an important role for ALDH1A1 in mediating the effects of IGF2BP1 in leukemia cell proliferation and survival.

IGF2BP1 expression in LSCs is in line with the recent report that the stem cell factor LIN28B, which is often upregulated in advanced forms of blood cancer, promotes leukemia aggressiveness at least partially via interaction with IGF2BP1<sup>11</sup>. It is also plausible that IGF2BP1 functions in LSCs as a reader of m<sup>6</sup>A-methylated RNA. Members of the IGF2BP family were recently shown to recognize the consensus GG(m<sup>6</sup>A)C sequence, promoting the



stability and storage of their target mRNAs in an m<sup>6</sup>A-dependent manner in HepG2 cells and human embryonic stem cells (hESCs)<sup>45</sup>.

Our study provides further evidence that IGF2BP1 maintains tumorigenicity and drug resistance of leukemia cells by enhancing critical transcriptional and metabolic regulators. For the first time, we have found that ALDH1A1, HOXB4, and MYB are directly regulated by IGF2BP1 at the post-transcriptional level.

Given the fact that IGF2BP1 is often upregulated in various types of malignancies and not expressed in most normal tissues, IGF2BP1 could become an important target for anti-cancer therapy. Developing clinically relevant IGF2BP1 inhibitors will help sensitize leukemia cells to programmed cell death and induce differentiation by targeting stem cell pathways that are common for various types of advanced cancers.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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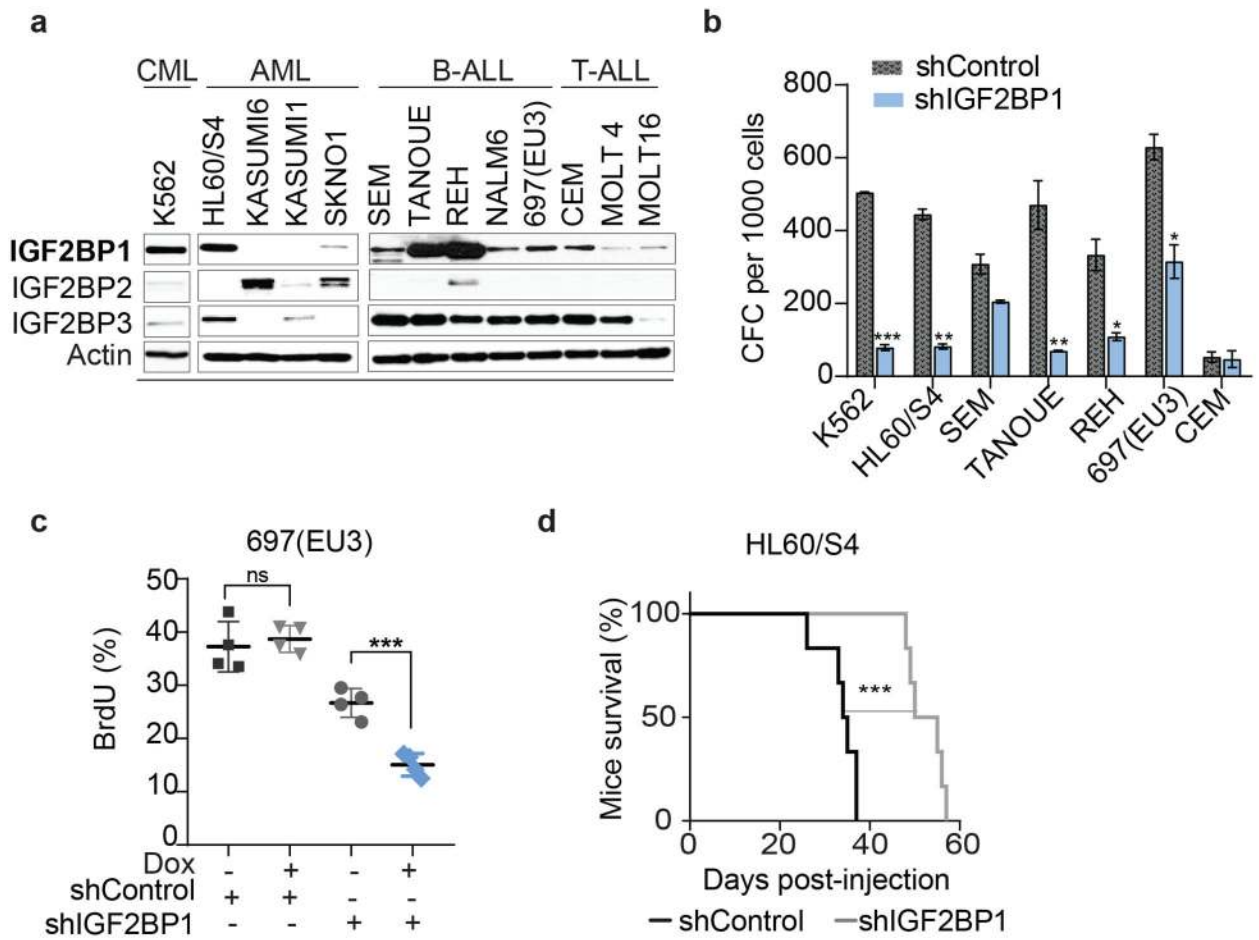
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**Key points:**

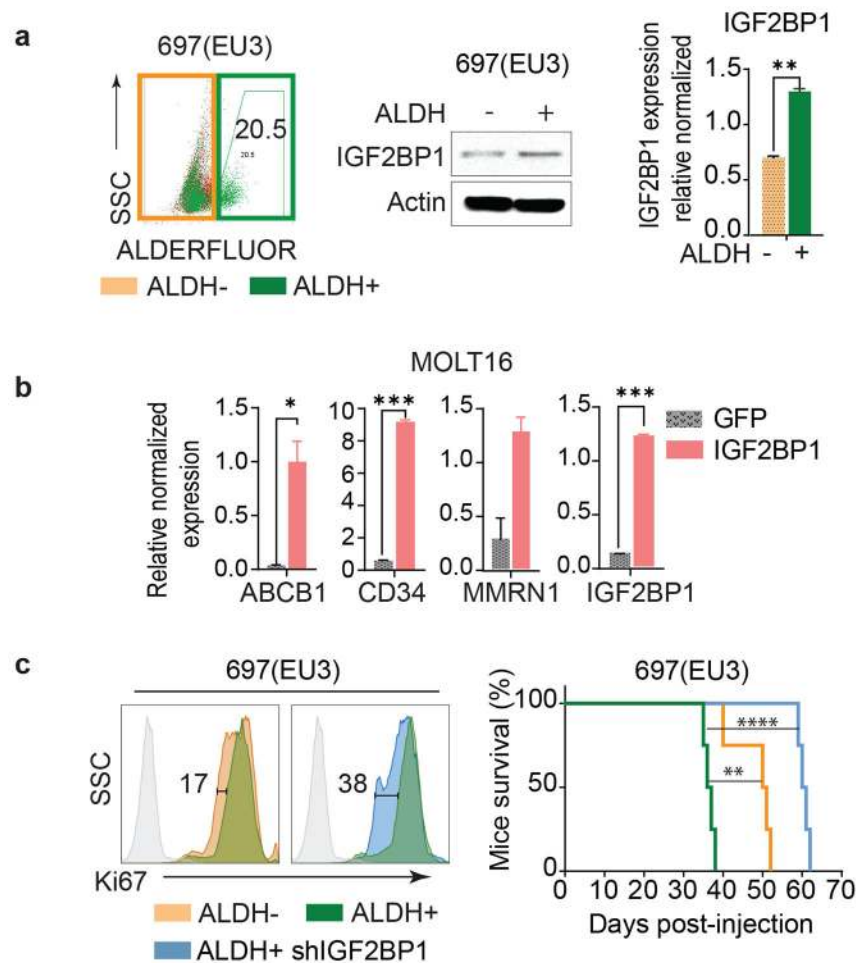
- IGF2BP1 promotes leukemia stem cell phenotype by regulating *HOXB4*, *MYB* and *ALDH1A1*.
- Inhibition of IGF2BP1 reduces leukemia tumorigenicity, and enhances leukemia cell death and differentiation.



**Figure 1. IGF2BP1 loss-of-function reduces leukemia cell growth and tumorigenesis.**

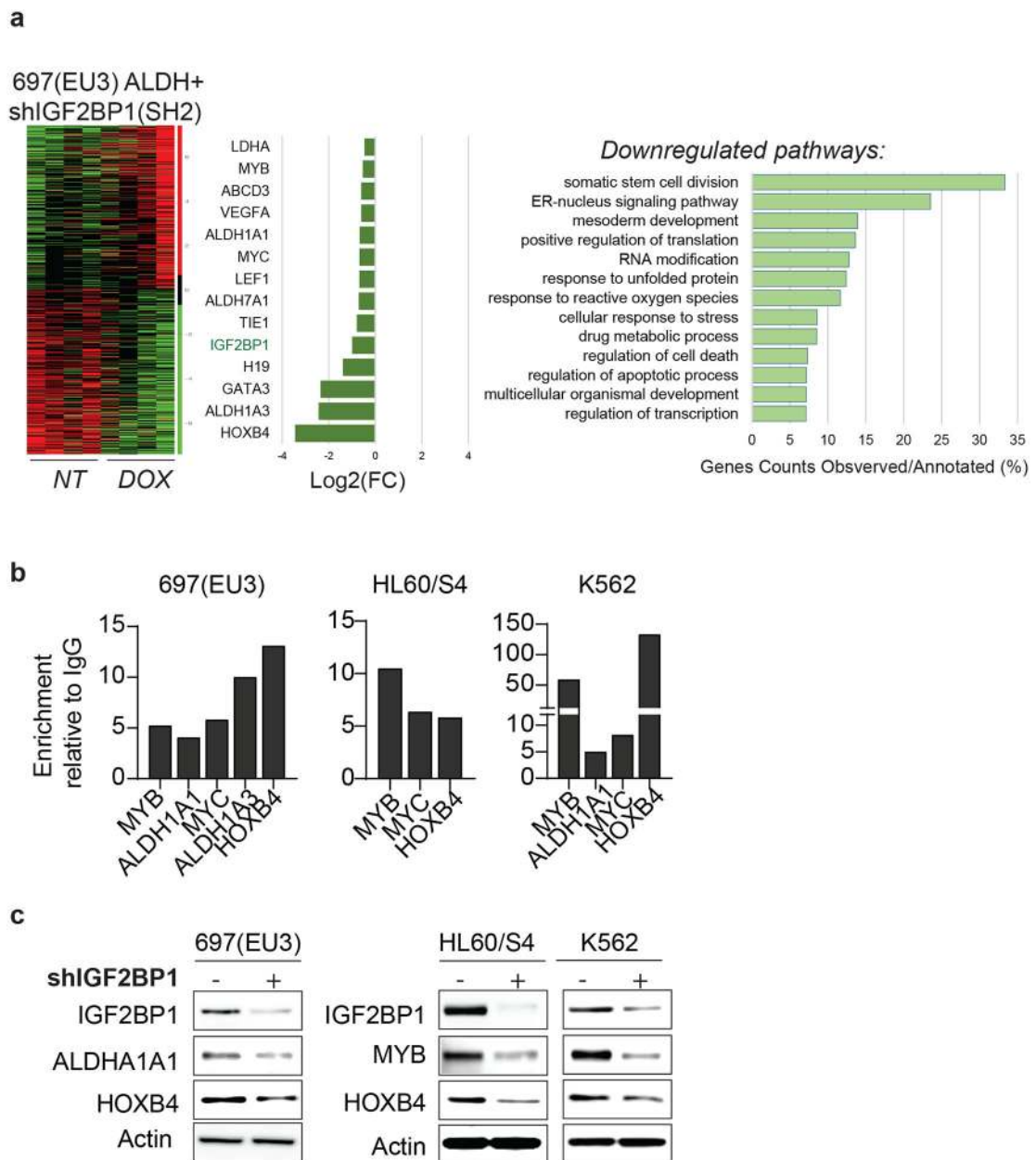
**a**, Western blot analysis of IGF2BP1, 2, and 3 endogenous expression in leukemia cells (30  $\mu$ g of total protein per sample); **b**, Colony-forming cell (CFC) assay for shIGF2BP1 (SH1) and shControl expressing cells, (t-test,  $n=3$ ,  $\pm$  SEM); **c**, Quantitative analysis of BrdU<sup>+</sup> cells in S-phase of a cell cycle, 697(EU3) with doxycycline-induced shIGF2BP1 (SH2) (t-test, \*\*\* $P=0.0005$ ,  $n=4$ ) and shControl (t-test,  $P=0.60$ ,  $n=4$ , non-significant (ns)); **d**, Kaplan-Meier survival analysis of mice transplanted with HL60/S4 cells with and without IGF2BP1 knockdown (Mantel-Cox test, \*\*\* $P=0.0008$ ,  $n=6$ ).





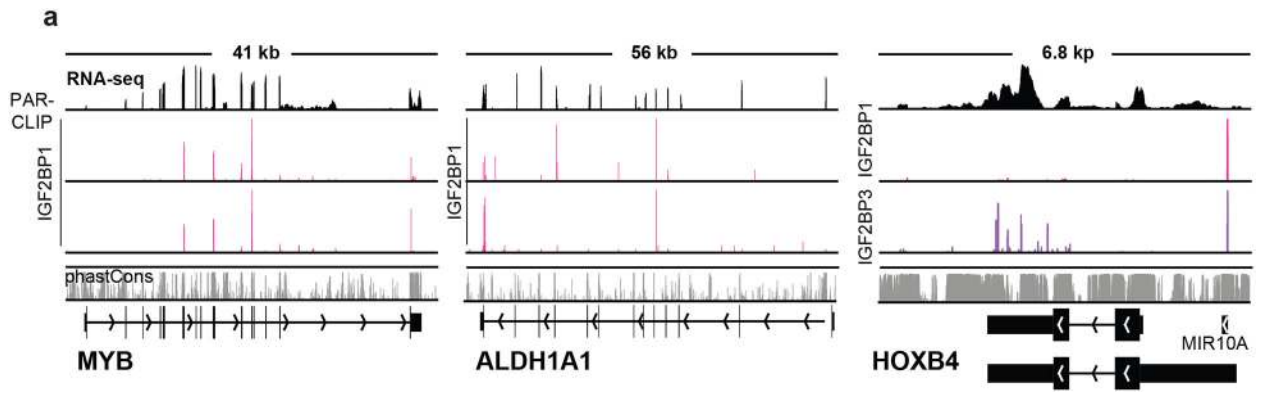
**Figure 2. IGF2BP1 expression supports leukemia cancer stem cell properties.**

**a**, Schematic representation of ALDH<sup>-</sup> (depleted) and ALDH<sup>+</sup> (enriched, 20.5%) sorted subpopulations of 697(EU3) (left); endogenous levels of IGF2BP1 mRNA and protein in 697(EU3) ALDH<sup>-</sup> and ALDH<sup>+</sup> cells (t-test, \*\*P<0.01, *n*=3, ± SEM); **b**, Expression of selected leukemia stem cell markers in MOLT16 with overexpression of IGF2BP1 and GFP control (t-test, \*P<0.05, \*\*\*P<0.001, *n*=3, ± SEM); **c**, Representative flow cytometric analysis of Ki67 staining of 697(EU3) ALDH<sup>-</sup> and ALDH<sup>+</sup> subpopulations, and ALDH<sup>+</sup> cells with IGF2BP1 knockdown (SH2), and Kaplan-Meier survival analysis of mice injected with  $1 \times 10^3$  of those cells (Mantel-Cox test, \*\*P=0.0067, \*\*\*\*P<0.0001, *n*=4).



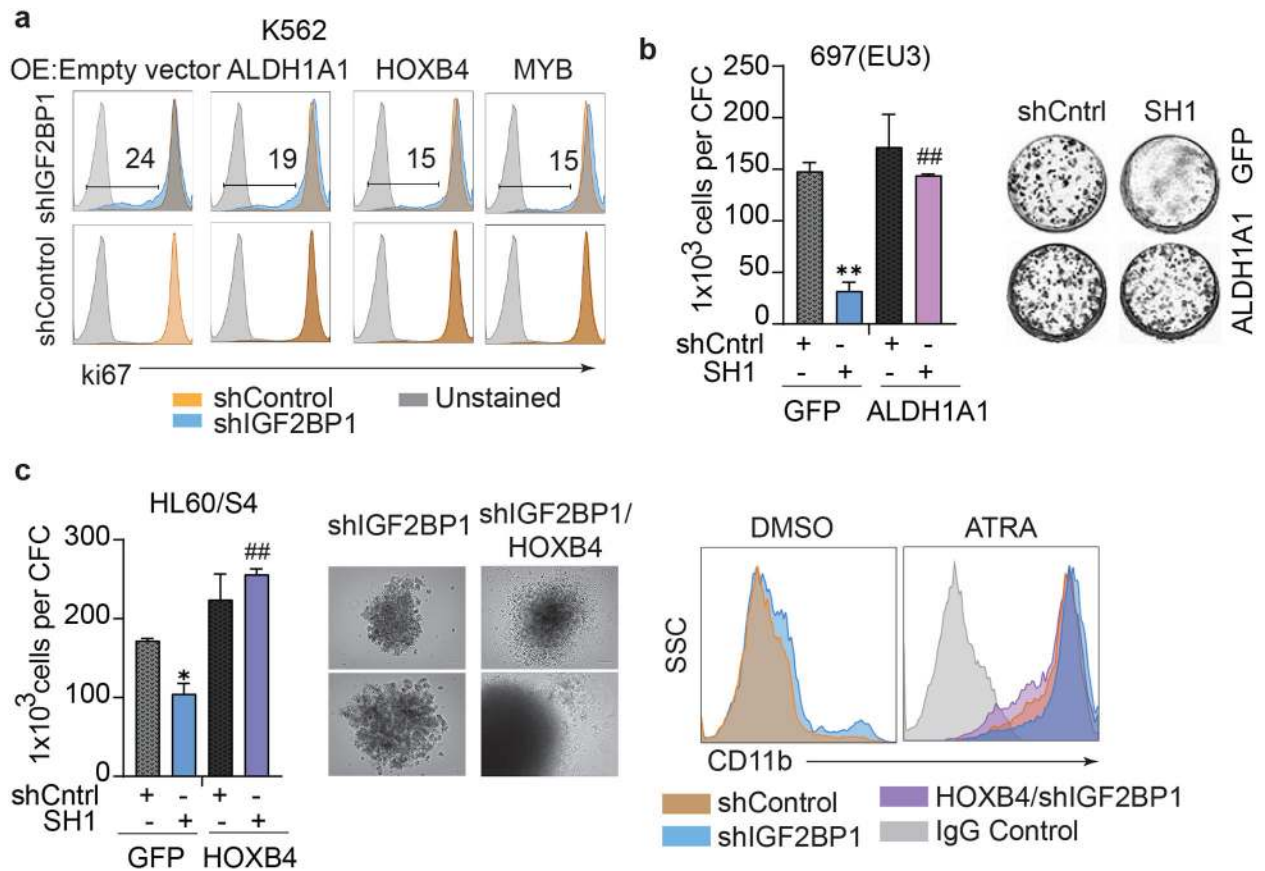
**Figure 3. IGF2BP1-dependent changes in gene expression profile of ALDH-enriched 697(EU3) leukemia cells.**

**a**, The analysis of differentially expressed genes in ALDH<sup>+</sup> subpopulation of 697(EU3) leukemia cells with shIGF2BP1(SH2) knockdown: a heatmap, a list of selected downregulated genes, and Gene Ontology (GO) enrichment analysis for downregulated genes upon IGF2BP1 knockdown; **b**, qPCR analysis of selected gene targets in the cross-linked immunoprecipitations with anti-IGF2BP1 antibodies relative to anti-IgG, (IgG set at zero). **c**, Western blot analysis of endogenous ALDH1A1, HOXB4, and MYB expression in 697(EU3), HL60/S4, and K562 cells with and without IGF2BP1 knockdown.



**Figure 4. PAR-CLIP analysis of K562 cells.**

**a**, The representative photographs of the screenshots of two independent PAR-CLIP experiments illustrating the amplification (vertical red bars) in IGF2BP1-mRNA-binding sites of *MYB*, *ALDH1A1*, *HOXB4*, binding *HOXB4*-IGF2BP3 (lower panel, blue bars);



**Figure 5. Ectopic expression of stem cell genes rescues IGF2BP1 loss-of-function phenotype in leukemia.**

**a**, Ki67 staining of K562 cells co-expressing shIGF2BP1(SH1) or shControl together with the indicated constructs (over expression (OE): empty control vector, *ALDH1A1*, *HOXB4*, *MYB*). **b**, Co-expression of shIGF2BP1(SH1) or shControl with *ALDH1A1* in 697(EU3) cells: quantitative assessment and phase contrast photographs of CFC assay of 697(EU3) cells expressing shIGF2BP1(SH1) and shControl in combination with *GFP* (t-test for shCntrl-*GFP* and SH1-*GFP*: \*\* $P < 0.01$ ,  $n = 3$ ,  $\pm$  SEM) or *ALDH1A1* (t-test for SH1-*GFP* and SH1-*ALDH1A1*: ## $P < 0.01$ ,  $n = 3$ ,  $\pm$  SEM), day 14. **c**, Co-expression of shIGF2BP1(SH1) or shControl with *HOXB4* in HL60/S4 cells: CFC assay of HL60/S4 cells expressing shIGF2BP1(SH1) and shControl in combination with *GFP* (t-test for shCntrl-*GFP* and SH1-*GFP*: \* $P < 0.05$ ,  $n = 3$ ,  $\pm$  SEM) or *HOXB4* (t-test for SH1-*GFP* and SH1-*HOXB4*: ## $P < 0.01$ ,  $n = 3$ ,  $\pm$  SEM), day 14; phase contrast photograph of CFC colonies expressing only shIGF2BP1 (left), and *HOXB4*-coexpressing (right), day 14; flow cytometric analysis of CD11b expression in HL60/S4 cells transduced with indicated constructs and treated with DMSO or all-trans retinoic acid (ATRA, 1 $\mu$ M, 4 days).