The megakaryocytic transcription factor ARID3A suppresses leukemia pathogenesis

Oriol Alejo-Valle1, Karoline Weigert1, Raj Bhayadia1, Michelle Ng1, Stephan Emmrich2, Christoph Beyer1, Konstantin Schuschel1, Christian Ihling3, Andrea Sinz3, Marius Flasinski4, Hasan Issa1, Eniko Regenyi1,5, Maurice Labuhn6, Dirk Reinhardt7, Marie-Laure Yaspo5, Dirk Heckl1*, Jan-Henning Klusmann1*

1Pediatric Hematology and Oncology, Martin-Luther-University Halle-Wittenberg, 06120 Halle, Germany
2Department of Biology, University of Rochester, Rochester NY 14627, USA
3Department of Pharmaceutical Chemistry and Bioanalytics, Institute of Pharmacy, Martin-Luther-University Halle-Wittenberg, 06099 Halle, Germany
4Department of Psychiatry, Psychosomatic Medicine and Psychotherapy, Hospital Tauberbischofsheim, 97941 Tauberbischofsheim, Germany
5Max Planck Institute for Molecular Genetics, 14195 Berlin, Germany.
6Institute for Experimental Virology, TWINCORE, Center for Experimental and Clinical Infection Research, 30625 Hannover, Germany
7Pediatric Hematology and Oncology, Pediatrics III, University Hospital Essen, 45122 Essen, Germany.

* Joint last authors
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Corresponding authors: Jan-Henning Klusmann (Jan-Henning.Klusmann@uk-halle.de), Tel.: +493455572388, Fax: +493455572389; Dirk Heckl (Dirk.Heckl@uk-halle.de), Tel.: +493455577273.
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Graphical abstract

Normal fetal hematopoiesis (normal miR-125b)

GATA1 ARID3A

miR-125b

SMAD2/3

Ery  Mega

apoptosis
cell cycle arrest

fetal hematopoietic stem cell

erythrocytes

megakaryocytes

GATA1s + trisomy 21 (high miR-125b)

GATA1s ARID3A

miR-125b

Ery  Mega

apoptosis
cell cycle arrest

Megakaryocytic/erythroid leukemia
ARID3A suppresses leukemogenesis

Key points

- Repression of megakaryocytic transcription factor ARID3A by miR-125b synergizes with GATA1s to induce leukemia
- Restoring ARID3A expression relieves megakaryocytic differentiation arrest in megakaryoblastic leukemia

Abstract

Given the plasticity of hematopoietic stem/progenitor cells, multiple routes of differentiation must be blocked during acute myeloid leukemia pathogenesis – the molecular basis of which is incompletely understood. Here we report that post-transcriptional repression of transcription factor ARID3A by miR-125b is a key event in megakaryoblastic leukemia (AMKL) pathogenesis. AMKL is frequently associated with trisomy 21 and GATA1 mutations (GATA1s), and children with Down syndrome are at a high risk of developing this disease. We show that chromosome 21-encoded miR-125b synergizes with Gata1s to drive leukemogenesis in this context. Leveraging forward and reverse genetics, we uncover Arid3a as the main miR-125b target underlying this synergy. We demonstrate that during normal hematopoiesis this transcription factor promotes megakaryocytic differentiation in concert with GATA1 and mediates TGFβ-induced apoptosis and cell cycle arrest in complex with SMAD2/3. While Gata1s mutations perturb erythroid differentiation and induce hyperproliferation of megakaryocytic progenitors, intact ARID3A expression assures their megakaryocytic differentiation and growth restriction. Upon knockdown, these tumor suppressive functions are revoked, causing a dual megakaryocytic/erythroid differentiation blockade and subsequently AMKL. Inversely, restoring ARID3A expression relieves the megakaryocytic differentiation arrest in AMKL patient-derived xenografts. This work illustrates how mutations in lineage-determining transcription factors and perturbation of post-transcriptional gene regulation interplay to block multiple routes of hematopoietic differentiation and cause leukemia. Surmounting this differentiation blockade in megakaryoblastic leukemia by restoring the tumor suppressor ARID3A represents a promising strategy for treating this lethal pediatric disease.
Introduction

Acute myeloid leukemia (AML) is a hematologic malignancy characterized by the uncontrolled proliferation of immature progenitor cells, which are blocked from differentiating towards normal blood cells. Given the plasticity of hematopoietic stem/progenitor cells (HSPCs), multiple routes of differentiation must be blocked during AML pathogenesis – the molecular basis of which is incompletely understood. In particular, the role of perturbed post-transcriptional gene regulation in leukemic differentiation arrest remains unknown. Understanding how these altered processes lead to leukemic transformation is crucial for developing strategies to overcome the differentiation blockade in AML and for designing related therapeutic concepts, as has been successfully applied in acute promyelocytic leukemia.

Acute megakaryoblastic leukemia (AMKL) is an aggressive subtype of AML. Acquired trisomy 21 is a common feature of AMKL and children with Down syndrome are at a high risk of developing this disease. Megakaryoblastic leukemia of Down syndrome (ML-DS) is characterized by mutations in the hematopoietic transcription factor GATA1, which occur in fetal hematopoietic stem/progenitor cells (HSPCs) and cause the exclusive expression of a N-terminal truncated protein known as GATA1s – hereafter referred to as GATA1s mutations. Noteworthy, ML-DS pathogenesis is a stepwise clonal progression evolving from a transient form of leukemia called transient abnormal myelopoiesis (TAM), which occurs in approximately 30% of neonates with Down syndrome. Today it is widely accepted that the interplay between trisomy 21, fetal origin and the mutated GATA1s is necessary and sufficient to cause TAM, as none of these elements lead to a TAM-like phenotype alone, but yet no additional events/factors are required. During fetal development, GATA1s induces hyperproliferation of human and murine megakaryocytic progenitor cells, but leaves their capacity to differentiate into normal megakaryocytes intact. Meanwhile, Gata1s causes anemia in murine fetuses and interferes with normal erythroid differentiation, and GATA1s mutations are seen in patients with Diamond-Blackfan anemia. These data suggest that both the erythroid and megakaryocytic differentiation paths are perturbed by the interplay between GATA1s and trisomy 21, and that this combination causes TAM and subsequently ML-DS. Despite the apparent simplicity of this model, the factors on chromosome 21 that underlie this synergy remain enigmatic. Identifying the oncogenic factors on chromosome 21 will also be pertinent to deciphering its role in perturbed differentiation and leukemogenesis beyond ML-DS.

Chromosome 21 harbors the phylogenetically conserved miR-99a~125b cluster, which encodes the miRNAs let-7c, miR-99a and miR-125b. All three are transcribed simultaneously as a polycistron and are highly expressed in TAM, ML-DS and non-DS AMKL. MiR-125b was first described as an oncogenic miRNA in ML-DS and other types of AML. However, the role of the miRNA tricistron in ML-DS pathogenesis and its function in perturbed differentiation remained an open question. Here we
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resolved the interplay between GATA1s-mutations and the miR-99a~125b cluster and propose a mechanism through which the dual differentiation arrest is achieved in ML-DS and AMKL. By systematically dissecting the miR-125b targetome – the dominant oncogenic member of the cluster in this context – we establish ARID3A as a transcriptional activator that promotes megakaryocytic differentiation in concert with GATA1. These insights will advance our knowledge of the complex regulation of normal hematopoiesis and our understanding of how post-transcriptional regulators of gene expression interact with known oncogenic drivers during leukemic differentiation blockade, as well as during the initiation and progression of cancer.

Methods

Reagents and Resources
Supplemental Table S1 contains a list of all relevant reagents.

Patient samples
Pediatric AML samples were collected from patients enrolled in AML Berlin-Frankfurt-Münster treatment protocols for children and adolescents. Written informed consent was obtained from all patients and custodians in accordance with the Declaration of Helsinki and local laws and regulations, and the study was approved by the institutional review board of each participating center. For details, see Supplemental Table S2.

Animal studies
All experimental procedures involving mice were performed in accordance with protocols approved by the local authorities (Landesverwaltungsamt Niedersachsen/Sachsen-Anhalt). B6J.129(Cg)-Gt(ROSA)26Sor1(CAG-cas9*,-EGFP/Fedar)J (Jackson Laboratory, RRID: IMSR_JAX:026179), C57BL/6J mice (Charles River, RRID: IMSR_JAX:000664) and C129S4-Rag2<sup>TM1.1Flv</sup>Csf1<sup>TM1(CSF1)Flv</sup>Csf2<sup>II3<sup>TM1(CSF2,IL3)Flv</sup>Thpo<sup>TM1.1(7PO)Flv</sup>Il2rg<sup>TM1.1Flv</sup>Tg(SIRPA)1Flv/J (MISTRG) mice (Regeneron Pharmaceuticals) were maintained in a specific pathogen free environment in individual ventilated cages and fed with autoclaved food and water at Martin-Luther-University Halle-Wittenberg. Syngeneic transplantation and patient-derived xenograft protocols have been described previously.

Statistical analysis
All statistical analyses were carried out using GraphPad Prism 8. Data were analyzed by Student t test (two-tailed). Survival was measured according to the Kaplan–Meier method and analyzed by log-rank (Mantel–Cox) test. P<0.05 was considered significant. All statistical tests and sample numbers are disclosed in the respective figure legends/Supplemental tables.
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Data availability

RNA-Seq gene expression data have been deposited in NCBI’s Gene Expression Omnibus and are accessible through GEO Series accession numbers: GSE169738, GSE169739 and GSE169740. Data regarding shRNA-positive screen is accessible through European Genome-phenome Archive accession number: PRJEB43922. Mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD025027. Other remaining data are available within the Article and Supplemental Files, or from the authors upon request.

Additional and detailed methods can be found in Supplemental Methods.

Results

miR-125b cooperates with Gata1s to induce megakaryoblastic leukemia

We previously generated a murine model of Gata1s-driven preleukemia, in which we introduce the Gata1s mutation in the fetal context using the CRISPR-Cas9 system. The generated Gata1s fetal liver cells (FLCs) show a hyperproliferative phenotype but unperturbed terminal megakaryocytic differentiation in vitro. Importantly, upon transplantation into syngeneic recipients (C57Bl/6J), Gata1s-FLCs become abundant in the peripheral blood and then disappear over time. Thus, our murine preleukemic model constitutes a suitable platform to study and identify GATA1s-cooperating events, and we leveraged this system in combination with fluorescence-based lentiviral barcoding to dissect the role of miR-99a, let-7c and miR-125b – the members of the miR-99a~125b tricistron – in TAM/ML-DS pathogenesis. Each of the miRNAs was associated with a reporter fluorescent protein (dTomato, GFP and mTagBF2, respectively), which enabled monitoring and competition between all possible miRNA permutations (Figure 1A and Supplemental Figure 1A). This experimental setup revealed major synergy between Gata1s and miR-125b – but no other miRNA alone or in combination – leading to a 131-fold expansion of FLCs (Figure 1A and Supplemental Figure 1B-C). MiR-125b further enhanced the Gata1s-induced expansion of CD117+ stem/progenitor cells, including CD117+CD41+ megakaryocytic progenitors (Figure 1B and Supplemental Figure 1D). Correspondingly, the fraction of mature CD41+CD42d+ megakaryocytes was reduced, implying perturbed megakaryocytic differentiation, while erythroid differentiation was enhanced (Figure 1B). In methylcellulose-based colony forming unit (CFU) assays with thrombopoietin (Thpo) as the only cytokine, Gata1s-miR-125b FLCs were able to generate megakaryocyte-like CFUs with replating potential, in contrast to Gata1s control cells (Figure 1C-D). Complete cytokine conditions led to the formation of fewer but larger CFUs – including colonies with blast-like features – in Gata1s-miR-125b FLCs (Figure 1C and Supplemental Figure 1E-F), with a relative increase of erythroid colonies (CFU-E/ BFU-E; Figure 1E).
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Most importantly, upon transplantation into syngeneic C57BL6/J recipients, miR-125b-transduced Gata1s-FLCs caused high penetrance (90%) megakaryoblastic leukemia after a latency of 39 days (median disease-free survival, Figure 1F-H). Leukemic blasts engrafted and induced leukemia in secondary recipients with 100% penetrance, underlining the leukemic potential of the Gata1s-miR-125b combination (Supplemental Figure 1G-H).

These results from the murine Gata1s preleukemic model concur with our previous study\(^\text{21}\), and confirm miR-125b as the sole driver of the miR-99a~125b tricistron’s synergy with Gata1s in FLCs and the ensuing ML-DS-like megakaryocytic leukemia in vivo.

shRNA-based positive selection screening in combination with RNA-sequencing identifies miR-125b targets that synergize with Gata1s

To identify downstream targets of miR-125b that mediate its synergy with Gata1s, we performed an in vitro short hairpin RNA (shRNA)-based positive selection screen individually probing 220 predicted miR-125b targets that are downregulated by miR-125b in HSPCs (Figure 2A and Supplemental Table 3)\(^\text{20,26}\). Next generation sequencing (NGS)-based shRNA quantification 4 days and 30 days post-transduction followed by MAGeCK analysis\(^\text{37}\) revealed a continuous significant enrichment of shRNAs targeting 13 candidate genes (Figure 2A, Supplemental Figure 2A and Supplemental Table 4). We complemented the data obtained from the shRNA screening with gene expression profiling (RNA-seq) after switching miR-125b expression on and off using a doxycycline-inducible lentiviral expression system (Figure 2B). We showed that sustained miR-125b expression, e.g. continuous addition of doxycycline, is required for the enhanced proliferation of Gata1s-FLCs and related transcriptional changes, such as the induction of ML-DS and stem cell gene expression signatures\(^\text{28}\) as well as of MYC target genes\(^\text{29}\) (Figure 2B, Supplemental Figure 2B-C and Supplemental Table 5). Hence, candidate targets of miR-125b should be downregulated upon miR-125b induction (doxycycline addition) and become re-expressed upon miR-125b release (doxycycline withdrawal).

Only one candidate followed this expression pattern, in addition to being significantly enriched in the shRNA screen: the transcription factor Arid3a (Figure 2C). This convergence from two complementary approaches implicates Arid3a as the primary player in the synergy between miR-125b with Gata1s.

Arid3a knockdown mimics the miR-125b phenotype in Gata1s-FLCs

Next, we sought to validate Arid3a as the primary target of miR-125b in this context. We confirmed repression of ARID3A protein as well as direct targeting of the Arid3a/ARID3A 3’UTR by miR-125b (Supplemental Figure 3A-D). Knockdown of Arid3a in Gata1s-FLCs using two efficient shRNAs (shArid3a #2 and shArid3a #3, Supplemental Figure 3A and Supplemental Table 6) mimicked the miR-125b pro-proliferative phenotype and megakaryocytic differentiation arrest (Figure 3A-B). In CFU assays (Thpo only), Arid3a knockdown resulted in the formation of megakaryocyte-like CFUs with high
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replacing potential (Figure 3C-D and Supplemental Figure 3E), while in complete cytokine conditions, knockdown of Arid3a led to a relative increase in erythroid colonies (CFU-E/ BFU-E) and colony size (Supplemental Figure 3F-H). Inversely, restoring Arid3a levels in Gata1s-miR-125b FLCs via cDNA overexpression reestablished megakaryocytic differentiation while impeding miR-125b-driven proliferation (Figure 3E-F) and CFU formation (Thpo only, Figure 3G-H). Most importantly, knockdown of Arid3a in Gata1s-FLCs was sufficient to recapitulate the miR-125b-induced leukemic phenotype in vivo, leading to the development of CD117⁺CD41⁺ megakaryoblastic leukemia with a short latency of 71 days (median disease-free survival, p=0.02), accompanied by marked splenomegaly (Figure 3I-K).

In summary, these results strongly implicate Arid3a as the main target of miR-125b that drives the synergy with Gata1s in leukemogenesis in vitro and in vivo.

ARID3A promotes terminal megakaryocytic differentiation

ARID3A has been extensively studied in the B-cell lineage – also in the context of miR-125b – where it upregulates IgH transcription in activated B-cells. More recent reports have highlighted its role in myelopoiesis and erythropoiesis. Gene expression analysis in stringently sorted blood cells (R.B. and J.-H.K, unpublished data) showed that ARID3A expression is elevated in megakaryocytes compared to erythroid cells (Figure 4A), indicating that ARID3A also acts as a regulator of megakaryopoiesis. Accordingly, shRNA-mediated knockdown of Arid3a greatly enhanced the colony-forming and replating capacity of wild type (WT) FLCs with Thpo as the only cytokine and impaired terminal megakaryocytic differentiation in the liquid culture (Figure 4B-C). Inversely, Arid3a overexpression promoted megakaryocytic differentiation at the expense of erythroid differentiation (Figure 4D), which was also evident in CFU assays (Figure 4E and Supplemental Figure 4A). To verify the effects of Arid3a on megakaryopoiesis and erythropoiesis in vivo, we transplanted shRNA-transduced FLCs into lethally irradiated syngeneic recipients. After 16 weeks, we observed a marked increase in the percentage of CD71⁺Ter-119⁺ early and CD71 Ter-119⁺ late erythroblasts as well as of CD41⁺CD42d⁻ immature megakaryocytic cells in the bone marrow of the recipient mice upon Arid3a knockdown, while the percentage of CD41⁺CD42d⁺ mature megakaryocytes was not significantly changed (Figure 4F). Other lineages were not affected (Supplemental Figure 4B).

To verify the relevance of our findings to human hematopoiesis, we tested human CD34⁺ HSPCs. Under conditions promoting megakaryocytic differentiation, ARID3A overexpression strongly promoted terminal differentiation, as determined by the presence of CD41⁺CD61⁺CD42b⁺ cells (Figure 4G). Inversely, under conditions promoting erythroid differentiation, ARID3A caused a 60% reduction in mature CD71⁺CD235a⁺ erythroid cells (Figure 4G), and in CFU assays, we observed a decrease in CFU/BFU-E (Figure 4H and Supplemental Figure 4C). In contrast, shRNA-mediated knockdown of
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ARID3A impaired megakaryocytic differentiation (Figure 4I, Supplemental Figure 4D and Supplemental Table 6).

These data reveal a previously unknown role for ARID3A in the regulation of megakaryocytic differentiation, and confirm previous reports suggesting its negative impact on erythroid differentiation32.

ARID3A acts in concert with GATA1 to activate megakaryocytic transcriptional programs

To define the function of ARID3A during megakaryopoiesis and leukemogenesis, we proceeded with the molecular characterization of this transcription factor. To elucidate whether the observed effects were mediated by its activating or repressing function35,36, we fused the KRAB inhibitor domain37 and the VP64 activator38 to the N-terminus of ARID3A (Supplemental Figure 5A). Lentiviral Arid3a expression reduced the proliferation of Gata1s-FLCs cells – an effect that was further enhanced by the addition of VP64 and abrogated by the addition of the KRAB (Supplemental Figure 5B), suggesting that ARID3A acts mainly through transcriptional activation in this context. These data were supported by gene expression profiling on pre-leukemic Gata1s-FLCs, as well as on Gata1s-FLCs after doxycycline-induced knockdown (shArid3a) or overexpression of Arid3a (Figure 5A and Supplemental Figure 5C-E). Differentially expressed genes occupied by ARID3A39 were mostly upregulated upon Arid3a overexpression and downregulated upon Arid3a knockdown (89% and 63.4% respectively), including several megakaryocytic genes such as Meis1 and Slc2a340,41 (Supplemental Figure 5F-H).

Next, we determined in FLCs the gene expression changes induced by the Gata1s mutation and the additive effects of Arid3a repression. As previously described15,42, gene set enrichment analysis (GSEA)43 revealed a marked reduction of erythroid genes and concurrent activation of pro-proliferative genes, including MYC and E2F targets, induced by the Gata1s mutation (Figure 5A-B and Supplemental Table 7). On this background, knockdown of Arid3a impaired the expression of megakaryocytic genes, which was accompanied by a further amplification of pro-proliferative gene expression and induction of a ML-DS gene expression signature, whereas the opposite was true upon Arid3a overexpression (Figure 5B and Supplemental Table 7). We note that these gene expression changes were highly reminiscent of miR125b overexpression (Supplemental Figure 2B).

Interestingly, GSEA analysis also indicated that Arid3a knockdown enhances the Gata1s gene expression signature. Genes that were downregulated upon loss of full-length GATA1 due to the Gata1s mutation were further repressed by Arid3a knockdown and re-induced by Arid3a overexpression (Figure 5C), suggesting that ARID3A maintains expression of these genes at the preleukemic stage. In accordance with this, two-thirds of ARID3A-occupied genes are also bound by GATA139, including several genes involved in megakaryocytic differentiation such as THBS1, ITGB3 (CD61) and PLEK44-46 (Figure 5D-F). Importantly, these genes show similar expression patterns in miR-
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125b- and shArid3a-transduced Gata1s-FLCs (Supplemental Figure 5G), but do not possess miR-125b binding sites in their 3’UTRs as assessed by TargetScan26, suggesting that they are indirectly regulated via miR-125b-mediated knockdown of Arid3a. Co-immunoprecipitation (Co-IP) followed by western blot excluded a direct interaction between ARID3A and GATA1 (data not shown), rather suggesting a functional interaction at the promoter level.

Altogether, these data establish ARID3A as a transcriptional activator that promotes megakaryocytic differentiation in concert with GATA1 and that its repression is a key event in megakaryoblastic leukemia (AMKL) pathogenesis.

ARID3A interacts with SMAD2/3 and promotes TGFβ pathway activation

To further elucidate the tumor suppressive function of ARID3A in the context of ML-DS, we probed the ARID3A protein interaction network through Co-IP of ARID3A in the ML-DS cell line CMK, followed by liquid chromatography with tandem mass spectrometry (LC-MS/MS) (Figure 6A). Thirty-seven proteins were significantly enriched, including previously described ARID3A interactors such as ARID3B and PML (Figure 6B and Supplemental Table 8)27,48. Interestingly, one of the top interaction partners was SMAD2, a downstream effector of the TGFβ pathway. To confirm the LC-MS/MS results, we performed western blots on ARID3A Co-IP samples, and showed a direct interaction between ARID3A and SMAD2 or its paralog SMAD3 (Figure 6C).

In accordance with ARID3A’s previously described role in TGFβ signaling50, activation of TGFβ pathway genes correlated with Arid3a levels in Gata1s-FLCs (Supplemental Figure 6A). Moreover, we observed considerable co-occupancy of genomic loci by ARID3A and SMAD2/3 (29% of ARID3A occupied loci; 1815/6238) (Figure 6D), and expression of the co-bound genes – including several genes involved in cell cycle arrest and apoptosis such as Pmaip1 (Noxa), Ripk1 and Pkrab251–53 – correlated with Arid3a expression (Figure 6E-G and Supplemental Figure 6B-C). In line with these transcriptional changes, ectopic expression of Arid3a not only induced megakaryocytic differentiation (Figure 3B) but also led to rapid induction of apoptosis in Gata1s-FLCs overexpressing miR-125b, accompanied by a decrease in S-phase cells compared to the Luc control (Supplemental Figure 6D-E).

In summary, these results confirm the tumor suppressive role of ARID3A, positioning it as a mediator of TGFβ-induced cell cycle arrest and apoptosis in complex with SMAD2/3.

Restoring ARID3A expression re-establishes normal differentiation of leukemic blasts

Gene expression data from AML patient samples show reduced ARID3A expression in TAM, ML-DS and non-DS AMKL compared to other AML subtypes and CD34+ HSPCs (Figure 7A). We therefore considered whether restoring ARID3A expression could be applied towards overcoming the leukemic phenotype and/or differentiation block in AMKL. Indeed, doxycycline-induced ARID3A expression in
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the ML-DS cell line CMK (Supplemental Figure 7A) led to growth arrest and a massive expansion of mature CD41+CD61+CD42b+ megakaryocytes, accompanied by induction of megakaryocytic genes as well as repression of the ML-DS expression signature and oncogenic programs (Supplemental Figure 7B-E and Supplemental Table 8). Corroborating its role as mediator of TGFβ signaling, we observed increased apoptosis and cell cycle arrest upon ARID3A expression (Supplemental Figure 7F-G), as well as reduced leukemic growth in other AMKL cell lines (Supplemental Figure 7H).

We next restored ARID3A expression in ML-DS and non-DS AMKL patient samples that were expanded in mouse xenografts (see Supplemental Table 2 for patient characteristics). Except for one non-DS AMKL sample harboring a KMT2A mutation, we observed a decrease in leukemic growth in vitro and induction of megakaryocytic differentiation upon doxycycline-induced ARID3A expression (Figure 7B-C). Lastly, we evaluated the effect of ARID3A re-expression on leukemic growth in vivo through a fluorescence-based competitive transplantation assay using two ML-DS and one AMKL patient-derived xenografts (PDX; Figure 7D). In all three cases, at the experimental endpoint, ARID3A-transduced leukemic blasts were significantly diminished in the bone marrow of the transplanted mice (Figure 7E), underlining the role of ARID3A as a miR-125b targeted novel tumor suppressor in ML-DS and AMKL. To assess the role of ARID3A in AML more globally, we analyzed the impact of ARID3A expression on the prognosis of AML across all subtypes and age groups. Kaplan-Meier analysis demonstrated that low ARID3A expression was significantly associated with lower event-free survival and overall survival in a pediatric and adult cohort (Figure 7G and Supplemental Figure 8A-D), therefore identifying ARID3A expression as a prognostic factor. In line with a putative role as tumor suppressor in AML, ARID3A restoration exerted tumor suppressive effects in a panel of five myeloid leukemia cell lines in vitro and two PDXs in vitro and in vivo, representing AML subtypes with low ARID3A expression (Supplemental Figure 8E-K).

Discussion

Here, we delineated the molecular basis of the differentiation blockade in AMKL. Using ML-DS – characterized by the interplay of trisomy 21 and GATA1s mutations – as a genetically simple model, we demonstrate that miR-125b increases proliferation and blocks terminal megakaryocytic differentiation in Gata1s-FLCs, thereby leading to leukemia in vivo, and that miR-125b-mediated downregulation of Arid3a underlies this phenotype. While the contribution of other miR-125b targets cannot be fully excluded, our comprehensive experimental data using complementary methods and different models strongly suggest Arid3a as the main target of miR-125b in this context. We further uncover novel functions for ARID3A in promoting megakaryocytic differentiation in concert with GATA1 and mediating TGFβ-induced cell cycle arrest and apoptosis in complex with SMAD2/3, indicating a tumor suppressive function for ARID3A in this context. Inversely, we propose that the dual
hit of ARID3A repression and loss of full-length GATA1, and the resulting perturbation of erythropoiesis and megakaryopoiesis, is key to the pathogenesis of ML-DS. In this model, Arid3a downregulation blocks the otherwise unperturbed megakaryocytic differentiation of hyperproliferative Gata1s megakaryocytic progenitors, arresting them in an undetermined differentiation state and thereby causing leukemia. Reinstating ARID3A expression relieved megakaryocytic differentiation arrest in ML-DS and non-DS AMKL patient-derived xenografts, opening the way for new therapeutic concepts. As miR-125b is also upregulated in non-DS AMKL, which frequently presents with oncogene-mediated functional perturbation of GATA1 and acquired trisomy 21\(^5\), these findings are also more broadly applicable beyond the scope of TAM/ML-DS. This is underlined by our finding that ARID3A expression is a prognostic factor in AML across subtypes and age groups, which may be accounted to ARID3A’s function in controlling TGFβ signaling.

Our work on miR-125b and Gata1s represents a rare example of dissecting the specific interaction between a dysregulated miRNA and a known oncogene in the process of transformation. It was previously shown that GATA1s mutations perturb erythroid differentiation but leaves megakaryocytic differentiation unaffected\(^3\, 4\, 11\, 12\). Accordingly, GATA1s occupancy at a number of erythroid specific genes is reduced but unaltered at megakaryocytic genes\(^3\, 42\, 60\). Since the erythroid and megakaryocytic lineages share a common progenitor, GATA1s has been speculated to bias differentiation away from the erythroid lineage and towards megakaryocytes, while failing to control their proliferation\(^21\). Here, we find that high levels of miR-125b – as seen in TAM and ML-DS – block this megakaryocytic route favored by Gata1s cells. Instead, the combination of Gata1s and miR-125b results in an accumulation of hyperproliferative megakaryocytic progenitors with erythroid features, which remain in an undetermined differentiation state and ultimately lead to leukemia \textit{in vivo}. We note that the synergy between Gata1s and miR-125b alone in ML-DS was unexpected, as we previously showed that concerted action from all three members of the miR-99a~125b tricistron was required to enhance self-renewal and megakaryocytic differentiation in adult HSCs\(^20\). However, the current findings corroborate our previous study\(^21\) as well as others\(^61\, 62\) describing miR-125b as an oncogenic miRNA in AML and ALL, and highlight the importance of developmental and cellular context in studying oncogenes and their partners – a phenomenon that has been described for other oncogenes in AMKL\(^5\).

Our results suggest an unexpected role for ARID3A in the regulation of megakaryopoiesis and megakaryocyte/erythroid cell fate decision, where high levels direct cells towards megakaryocytic and low levels towards erythroid fate. Our comprehensive molecular characterization of ARID3A further provides a mechanistic explanation for the synergy between miR-125b and GATA1s at the genetic level: ARID3A occupies megakaryocytic genes, inducing their transcriptional activation, and the majority of ARID3A regulated genes are also co-occupied by GATA1, suggesting that ARID3A acts as a GATA1 co-regulator during megakaryopoiesis. This co-regulation is sufficient to maintain megakaryocytic
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Differentiation even in GATA1s mutated cells. Upon knockdown of ARID3A, this concerted transcriptional activation of megakaryocytic genes is lost, leading to a megakaryocytic differentiation arrest. Hence, together, ARID3A knockdown and GATA1s mutations block both functions of full-length GATA1, i.e. induction of erythroid and megakaryocytic differentiation. Since the respective abilities of GATA1 and ARID3A to control the proliferation of hematopoietic cells are similarly lost\(^6\), this combination results in drastic perturbation of normal hematopoiesis and development of megakaryoblastic leukemia with erythroid features in vivo, a hallmark of ML-DS\(^6\).\(^6\). Using a proteomic approach, we found that the tumor suppressive functions of ARID3A are mediated in complex with SMAD2/3 – downstream effectors of the TGFβ signaling pathway, which induce apoptosis as well as cell cycle arrest in megakaryocytic progenitor cells\(^6\).\(^6\). Thus, our current work adds the downregulation of ARID3A as another mechanism of miR-99a~125b-mediated control of TGFβ-signaling in hematopoietic cells\(^2\).\(^0\).

Overall, our works helps explain the synergy between trisomy 21 and GATA1 mutations in leukemogenesis, through the functional dissection of a chromosome 21-encoded gene that is highly expressed in TAM/ML-DS. Although other factors on chromosome 21 – such as CHAF1B\(^1\)\(^3\) and RUNX1\(^1\)\(^5\) – might be relevant for leukemogenesis, these findings can extend to non-DS AMKL with acquired trisomy 21 and functional GATA1 perturbation. In addition, we exemplify a general framework that can be used to interrogate oncogene-miRNA interactions in cancer and provide a basis for developing refined treatment approaches centered on ARID3A.
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Author contributions

O.A.V. performed experiments, analyzed the data and wrote the manuscript. K.W., M.N., S.E., C.B., M.F., H.I. and R.B. performed experiments, analyzed the data and revised the manuscript. M.L. performed experiments. E.R. and K.S. analyzed and interpreted the data and revised the manuscript. M.L.Y. supervised data analysis, interpreted the data and revised the manuscript. D.R. provided patient material and/or data and revised the manuscript. D.H. and J.H.K. designed the study, analyzed and interpreted the data, wrote the manuscript and academically drove the project.

Disclosure of conflicts of interest

D.R. has advisory roles for Celgene Corporation, Novartis, Bluebird Bio, Janssen, and receives research funding from CLS Behring and Roche. J.H.K. has advisory roles for Bluebird Bio, Novartis, Roche and Jazz Pharmaceuticals.

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Figures and figure legends

Figure 1. miR-125b cooperates with Gata1s to induce leukemia in vivo.
(A) Schematic of in vitro and in vivo setup for modeling synergy between Gata1s and the members of miR-99a~125b tricistrons (left). Percentage of Gata1s-FLCs transduced with different miRNA permutations (marked by dTomato [miR-125b], mTagBFP2 [let-7c] and GFP [miR-99a]) normalized to day 0 (n=4, paired t-test) (right).
(B) Bar graph showing the percentage of stem/progenitor cells (CD117+), mature megakaryocytes (MK, CD41+CD42d+) and erythroid cells (ERY, CD71+Ter-119+) after 6 days of differentiation. Cells shown are Gata1s-FLCs transduced with miR-125b or miR-ctrl (n=3, paired t-test).
(C) Representative methylcellulose-based CFU assays from one of n=4 independent experiments. Depicted are Gata1s-FLCs transduced with miR-125b or miR-ctrl in complete or low (Thpo 20ng/mL) cytokine conditions.
(D) Bar graph showing the number of megakaryocytic colonies after serial replating of miR-125b Gata1s-FLCs in methylcellulose-based media with low (Thpo 20ng/mL) cytokine conditions compared to miR-ctrl Gata1s-FLCs.
(E) Classification of colonies after plating miR-125b or miR-ctrl Gata1s-FLCs in methylcellulose-based CFU assays under complete cytokine conditions (paired t-test). CFU-G/M: granulocytic (CFU-G), monocytic (CFU-M) and granulocytic/monocytic (CFU-GM); CFU/BFU-E: erythroid.
(F-H) Kaplan-Meier survival curve of recipients transplanted with Gata1s-FLCs overexpressing miR-125b or miR-ctrl (n=10, log-rank test) (F), spleen weight (unpaired t-test) (G), and representative flow cytometry plots of bone marrow (BM)-derived leukemic cells in the diseased mice (H). Gata1s-FLC-derived blasts overexpressing miR-125b are highlighted in red. Average percentage of miR-125b+ blasts belonging to each immunophenotype are indicated in the corresponding gate (H).
Data are presented as mean ± SD.
Figure 2. shRNA-based positive selection screening in combination with RNA-sequencing identifies miR-125b targets that synergize with Gata1s
(A) Schematic of shRNA positive selection screen, where a pool of shRNAs directed against miR-125b target genes mimics the effect of miR-125b (left). Dot plot showing significantly enriched shRNA-targeted genes (right). Arid3a highlighted in green.
(B) Percentage of Gata1s-FLCs expressing doxycycline-regulated miR-125b or miR-ctrl upon addition or removal of doxycycline (500ng/mL; n=4). Data presented as mean ± SD.
(C) Venn diagram of differentially expressed genes upon miR-125b modulation, overlapped with the results of the shRNA positive selection screen (from (A)).
**Figure 3. Arid3a knockdown mimics the miR-125b phenotype in Gata1s-FLCs**

(A) Percentage of transduced (sh-ctrl or shArid3a #2 or #3) Gata1s-FLCs compared to day 0 (n=4, paired t-test).

(B) Bar graph showing the percentage of stem/progenitor cells (CD117+), mature megakaryocytes (MK, CD41+CD42d+) and erythroid cells (ERY, CD71+Ter-119+) in transduced (sh-ctrl (black) or shArid3a #2 or #3 (dark or light green, respectively)) Gata1s-FLC after 6 days of differentiation. *= p<0.05; ** = p<0.01; n=3 (paired t-test).

(C-D) Bar graph showing the number of megakaryocytic colonies after serial replating of transduced (sh-ctrl (black) or shArid3a #2 or #3 (dark or light green, respectively)) Gata1s-FLCs in methylcellulose-based media with low concentrations of Thpo (20ng/mL); serial replating number given on x-Axis (C). Representative image of one of n=3 independent experiments (D).

(E) Percentage of transduced (Arid3a (green) or LUC cDNA (black)) miR-125b-Gata1s-FLCs normalized to day 0 (n=4, paired t-test).

(F) Percentage of stem/progenitor cells (CD117+), mature megakaryocytes (MK, CD41+CD42d+) and erythroid cells (ERY, CD71+Ter-119+) in transduced (Arid3a (green) or LUC cDNA (black)) miR-125b-Gata1s-FLCs after 6 days of differentiation (n=3, paired t-test).

(G and H) Bar graph depicting the number of colonies after plating transduced (Arid3a or LUC cDNA) miR-125b Gata1s-FLCs in methylcellulose-based media with low Thpo conditions (20ng/mL) (paired t-test) (G). Representative image of one of n=3 independent experiments (H).

(I-K) Kaplan-Meier survival curve of C57BL-6J recipients upon transplantation of transduced (sh-ctrl or shArid3a) Gata1s-FLCs (log-rank test) (I), spleen weights (unpaired t-test) (J), and representative flow cytometry plots of bone marrow (BM)-derived leukemic cells from the diseased mice. shArid3a-expressing Gata1s-FLC-derived blasts are highlighted in green. Average percentage of shArid3a+ blasts belonging to each immunophenotype is indicated in the corresponding gate (K).

All data are presented as mean ± SD.
Figure 4. ARID3A promotes terminal megakaryocytic differentiation.

(A) ARID3A expression (FPKM) in megakaryocytic (CD41⁺CD61⁺) and erythroid cells (CD71⁺) derived from human CD34⁺ HSPCs after 7 days of differentiation in megakaryocytic- or erythroid-promoting culture conditions, respectively (unpaired t-test).

(B) Bar graph showing the number of colonies after replating transduced (sh-ctrl, miR-125b or shArid3a) murine FLCs in low Thpo methylcellulose-based CFU-assays (n=6, paired t-test).

(C-D) Ratio of terminal megakaryocytic (MK, CD41⁺CD42d⁺) and erythroid cells (ERY, CD71⁺Ter-119⁺) in shArid3a- versus sh-ctrl-transduced murine FLCs (C) and Arid3a- versus LUC-transduced FLCs (D) after 6 and 4 days of differentiation, respectively (n=3, paired t-test).

(E) Classification of colonies after plating transduced (Arid3a or LUC cDNA) murine FLCs in methylcellulose-based CFU assays under complete cytokine conditions (n=3, paired t-test). CFU-G/M: granulocytic (CFU-G), monocyctic (CFU-M) and granulocytic/monocytic (CFU-GFM); CFU/BFU-E: erythroid.

(F) Bar graph showing the percentage of mature megakaryocytes (Mature MK, CD41⁺CD42d⁺), immature megakaryocytes (Immature MK, CD41⁺CD42⁺), early erythroblasts (ProE I, CD71⁺Ter-119⁺) and late erythroblasts (ProE II, CD71⁺Ter-119⁺) in the BM of mice transplanted with Lin⁻ FLCs transduced with sh-ctrl (black) or shArid3a (green). shRNA⁺ cells shown. n=5 (unpaired t-test).

(G) Percentage of differentiated cells after transducing human CD34⁺ HSPCs with ARID3a cDNA, normalized to HSPCs transduced with LUC. (Left) Percentage of mature megakaryocytes (CD41⁺CD61⁺/CD42⁺) after 11 days of differentiation in media promoting megakaryocytic differentiation. (Right) Percentage of mature erythroid cells (CD71⁺CD235a⁺) after 7 days of differentiation in media promoting erythroid differentiation. (n=6, paired t-test).

(H) Classification of colonies after plating ARID3A- or LUC-expressing human CD34⁺ HSPCs in methylcellulose-based CFU assays under complete cytokine conditions (n=6, unpaired t-test). CFU-
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G/M: granulocytic (CFU-G), monocytic (CFU-M) and granulocytic/monocytic (CFU-GFM); CFU/BFU-E: erythroid.

(I) Percentage of terminally differentiated cells after transducing human CD34+ HSPCs with shRNAs targeting ARID3A, normalized to sh-ctrl. (Left) Percentage of mature megakaryocytes (CD41+CD61+CD42+) after 11 days of differentiation in media promoting megakaryocytic differentiation. (Right) Percentage of mature erythroid cells (CD71+CD235a+) after 7 days of differentiation in media promoting erythroid differentiation (n=3, paired t-test).

Data are presented as mean ± SD.
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Figure 5. ARID3A acts in concert with GATA1 to activate megakaryocytic transcriptional programs

(A) Modelling the molecular mechanism of TAM to ML-DS transition via sequential Gata1s and Arid3a-repression: Gata1s-FLCs were expanded for three weeks and then transduced with either Arid3a cDNA, shArid3a or their respective controls. RNA samples were obtained from each of the 4 different conditions and subjected to RNA-Sequencing-based gene expression analysis.

(B) Bar graphs showing normalized enrichment scores from up- or downregulated gene sets involved in hematopoietic differentiation, cell proliferation and ML-DS progression. Gata1s-FLCs were compared against WT FLCs (top); Arid3a and shArid3a Gata1s-FLCs were compared against their respective Gata1s-FLCs controls (LUC or sh-ctrl) after doxycycline induction. *= p<0.05; ** = p<0.01; ***=p<0.001.

(C) GSEA enrichment plots showing genes downregulated by the Gata1s mutation in FLCs, and their response to Arid3a modulation in Gata1s-FLCs.

(D) Venn diagram of ARID3A and GATA1 ChIP-seq peaks in K562 cells from the ENCODE datasets.

(E) Volcano plot showing differential expression of genes bound by both ARID3A and GATA1 in shArid3a Gata1s-FLCs. Genes involved in megakaryocytic differentiation are highlighted in green; significantly downregulated and upregulated genes in blue and red, respectively.

(F) IGV snapshots of megakaryocytic genes co-regulated by ARID3A and GATA1 (K562 cells, ENCODE datasets). Scale (y-axis) represents fold enrichment of normalized reads compared to the IgG control. Scale and chromosome location are shown (top).
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Figure 6. ARID3A interacts with SMAD2/3 and promotes TGFβ pathway activation

(A) Experimental design for isolating ARID3A-containing protein complexes from CMK cells.

(B) Volcano plot showing enriched proteins in LC-MS/MS after ARID3A pulldown compared to the IgG control. Significantly-enriched proteins highlighted in red; ARID3A in green, SMAD2 in blue.

(C) Western blot showing binding of SMAD2 and SMAD3 to co-immunoprecipitated ARID3A.

(D) Venn diagram of ARID3A and SMAD2 and SMAD3 ChIP-seq peaks in K562 cells from the ENCODE datasets.

(E) Volcano plot showing differential expression of genes bound by both ARID3A and GATA1 upon Arid3a knockdown in Gata1s-FLCs. Genes involved in apoptosis and cell cycle arrest are highlighted in green; significantly downregulated and upregulated genes in blue and red, respectively.

(F) GSEA enrichment plots showing genes bound by ARID3A and SMAD2/3 (from D), and their response to Arid3a modulation in Gata1s-FLCs.

(G) IGV snapshots of genes involved in apoptosis and cell cycle arrest that are co-bound and regulated by the ARID3A/SMAD2/3 protein complex (K562 cells, ENCODE datasets). Scale (y-axis) represents fold enrichment of normalized reads compared to the IgG control. Scale and chromosome location are shown (top).
Figure 7. Restoring ARID3A expression re-establishes normal differentiation of leukemic blasts

(A) ARID3A expression (FPKM) in sorted pediatric AML blasts of different subtypes and CD34+ HSPCs. Others: inv(16), t(8;21), MLL-rearranged, t(10;11) and t(9;11) (unpaired t-test).

(B-C) AMKL (n=2) and ML-DS (n=3) PDXs were transduced with doxycycline-inducible ARID3A or LUC cDNA vectors. (B) Bar graph showing the percentage of ARID3A+ cells after 12 days of induction with doxycycline, normalized to the LUC control. (C) Bar graph showing the percentage of ARID3A+ terminally differentiated megakaryocytic cells (CD41+CD61+CD42+) after 8 days of induction with doxycycline, normalized to the LUC control. * = p<0.05; ** = p<0.01 (n=3 per PDX, paired t-test).

(D) Experimental design for evaluating whether ARID3A restores normal differentiation in vivo. Leukemic blasts were transduced with ARID3A (GFP+) or a LUC control (GFP+) and mixed 1:1 with LUC control-transduced blasts (dTomato+), before transplantation into sub-lethally irradiated recipient mice.

(E) Ratio of GFP+ to dTomato+ cells in input cells (IP), and in the BM of mice sacrificed 4-5 weeks after transplantation (n=5, unpaired t-test).

(F) Probability of event-free survival in 258 NCI-TARGET pediatric AML patients with high (green; > 12.0 normalized reads; cutoff determined via maximally selected rank statistics) or low ARID3A expression (black; ≤ 12.0 normalized reads).

(G) Probability of event-free survival in 171 TCGA adult AML patients with high (green; > 12.3 normalized reads; cutoff determined via maximally selected rank statistics) or low ARID3A expression (black; ≤ 12.3 normalized reads).

(A-E) All data are presented as mean ± SD.