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The tumor suppressor MIR139 is silenced by POLR2M to promote AML oncogenesis

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MIR139 is a tumor suppressor and is commonly silenced in acute myeloid leukemia (AML). However, the tumor-suppressing activities of miR-139 and molecular mechanisms of MIR139-silencing remain largely unknown. Here, we studied the poorly prognostic MLL-AF9 fusion protein-expressing AML. We show that MLL-AF9 expression in hematopoietic precursors caused epigenetic silencing of MIR139, whereas overexpression of MIR139 inhibited in vitro and in vivo AML outgrowth. We identified novel miR-139 targets that mediate the tumor-suppressing activities of miR-139 in MLL-AF9 AML. We revealed that two enhancer regions control MIR139 expression and found that the polycomb repressive complex 2 (PRC2) downstream of MLL-AF9 epigenetically silenced MIR139 in AML. Finally, a genome-wide CRISPR-Cas9 knockout screen revealed RNA Polymerase 2 Subunit M (POLR2M) as a novel MIR139-regulatory factor. Our findings elucidate the molecular control of tumor suppressor MIR139 and reveal a role for POLR2M in the MIR139-silencing mechanism, downstream of MLL-AF9 and PRC2 in AML. In addition, we confirmed these findings in human AML cell lines with different oncogenic aberrations, suggesting that this is a more common oncogenic mechanism in AML. Our results may pave the way for new targeted therapy in AML.

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INTRODUCTION

Mixed-lineage leukemia (MLL) or Lysine Methyltransferase 2A (KMT2A) rearrangements occur in 10% of adult acute leukemias and in 35% of pediatric cases [1, 2]. MLL rearrangements cause in-frame fusions with >60 different genes of which ENL, AF4, AF9, AF10, EML, and ELL are most common [3, 4]. MLL-fusion proteins cause heterogeneous leukemia of lymphoid or myeloid origin and are associated with therapy resistance and poor prognosis [3, 5]. MLL-fusion proteins retain the DNA-binding domain of MLL and deregulate target genes that are normally controlled by MLL [6]. The most common MLL-rearrangement in acute myeloid leukemia (AML) is caused by the t(9;11)(p22q23) fusing MLL to the A9 gene in-frame and occurs in 30% of MLL-rearranged AMLs [3, 5]. Continuous expression of MLL-AF9 is critical for the survival of AML cells, which suggests that genes deregulated by MLL-AF9 are potential targets for therapy [7, 8].

MicroRNAs (miRNAs) are small non-coding RNAs of 19–22 nt in length and post-transcriptionally regulate mRNAs involved in critical cellular functions [9]. MiRNAs are commonly deregulated in cancer [10, 11]. For instance, the expression of MIR139, encoding miR-139-5p and miR-139-3p, is downregulated and has been identified as a tumor suppressor in AML [12–14]. The MIR139 gene is located on chromosome-11 in intron-1 of PDE2A [15]. However, the molecular mechanism of MIR139 silencing in AML is largely unknown. Previously, we have found that mir-139-3p expression is induced by DNA interstrand-crosslinks [12]. There is evidence that mir-139-5p controls the translation machinery through downregulation of Elf4g2 [13, 14].

Polycomb-group (PcG) proteins have been implicated in silencing tumor suppressor genes in various cancers, including MLL-AF9 AML [16–19]. One class of PcG proteins, polycomb repressive complex 2 (PRC2), consists of the methyltransferase EZH2 or EZH1, EED, SUZ12, and RBBP4 [20]. EZH2 or EZH1 can hypermethylate K27 on Histone-H3, which marks silenced genes [21]. PRC2 contributes to MLL-AF9 pathogenesis and is a target for therapy [22, 23]. However, an additional unknown activity mediated by PRC2 that regulates transcriptional repression, other
Fig. 1 MLL-AF9 downregulates Mir139 expression. A Flow cytometric analysis of surface marker expression of MLL-AF9 or MLL-AF9-Mir139KO cells stained for myeloid lineage markers CD11b, CD16/32, c-Kit, GR-1 and lymphoid lineage marker CD3. B Heatmap showing the differential gene expression profiles of WT HSPCs (WT), Mir139KO HSPCs (KO), and MLL-AF9 cells (MA9). C The number of colony-forming units (CFUs) per 5000 empty vector control (−) and MLL-AF9 (MA9) transduced WT and Mir139KO HSPCs are shown. Total number of colonies (50 – 250 cells/colony) and large colonies (>250 cells/colony) are presented. Micrographs depict representative colonies. Scale bar indicates 100 µm. The two-tailed unpaired student’s t test was used for the statistical analysis. D Expression of mir-139-5p and mir-139-3p in MA9 cells and MLL-AF9-Mir139KO relative to snRNA U6 and EV transduced HSPCs (EV) as determined by miRNA qPCR is shown. The two-tailed unpaired student’s t test with Welch’s correction was used for the statistical analysis. The presented data are representative of two experiments. E Expression of Pde2a in MA9 cells and WT HSPCs in fragments per kilobase and per million mapped fragments (FPKM) as determined by RNA-seq is shown. The Wald’s test with Benjamin–Hochberg correction was used for the statistical analysis. All graphs show mean ± SEM.
than blocking the accessibility of chromatin remodeling proteins, cannot be ruled out [24].

Here, we have performed a comprehensive analysis of the tumor suppressor MIR139. We show that MIR139 is a critical tumor suppressor that is silenced by MLL-AF9 in AML. Furthermore, we have identified MIR139 targets that mediate its tumor suppressor activity in AML. Our study has elucidated the molecular silencing mechanism of MIR139 and provides insight into its tumor suppressor activity, offering potential targets of intervention for the poorly prognostic MLL-AF9 AML.
Fig. 2 MiR-139 upregulation and repression of miR-139 targets eliminates MLL-AF9 AML in vitro and inhibits leukemogenesis in mice.

A Expression levels of mir-139-5p and mir-139-3p in MLL-AF9-i139 clones (n = 3), treated with DOX (5 µg/mL, (+) relative to snRNA U6 and mock-treated (−) cells, as determined by miRNA qPCR in triplicate, are shown. The two-tailed unpaired student's t test was used for the statistical analysis.
B The number of colony-forming units (CFU) of MLL-AF9-ieGFP and MLL-AF9-i139 (n = 3) HSPC clones treated with doxycycline (DOX; 5 µg/mL, (+)) or mock-treated (−) as control per 8000 cells plated are shown. Data represent the average of three independent experiments. The two-tailed paired student's t test was used for the statistical analysis.

METHODS

Generation of knockout mice
C57BL/6 Mir139-deficient mice (Mir139KO) were generated using the CRISPR-Cas9 double-nicking strategy employed by the Cas9D10A mutant and 4 sgRNAs (Mir139OsgRNAs plus, Mir139KosgRNAs minus, Mir139-KOgRNAs plus, and Mir139KosgRNAs minus) flanking the Mir139 gene as described [25]. Enhancer 1 KO mice (E1KO) and E2KO mice were generated by zygote injection with TrueCut Cas9 Protein v2 (A36499, Thermo Fisher Scientific) and sgRNAs E1KO-5p, E1KO-3p, E2KO-5p, and E2KO-3p (Supplementary Table 1). Heterozygous or homozygous offspring were crossbred for at least three generations to reduce CRISPR-Cas9-induced off-target effects. All primer and sgRNA sequences are listed in Supplementary Table 1. All animal experiments were approved by the Instantie voor Dierenwelzijn (IVD) and the Erasmus Medical Center Animal Welfare and Ethics Committee.

 Colony assays, inhibitor experiments, cell culture, and patient samples
For colony assays, 5000 MLL-AF9 cells were plated in MethoCult (M3231, StemCell Technologies), supplemented with IL-3 (1:1000 supplement), IL-6 (10 ng/mL), granulocyte-macrophage colony-stimulating factor (GM-CSF) (10 ng/mL), and stem cell factor (SCF) (20 ng/mL), penicillin/streptomycin (100 U/mL) and neomycin 500 µg/mL and expanded for 7 days. DOT1L inhibitor SGC0946 (SML1107, Sigma Aldrich) or UNC1999 (SML0778, Sigma Aldrich) were dissolved in dimethyl sulfoxide (DMSO) and added to the medium at indicated concentrations. Human MLL-AF9 patients were diagnosed according to WHO criteria by cytomorphology, cytogenetics, molecular diagnostics and flow cytometry immunophenotyping. Mononuclear cells were isolated by Ficoll-Paque (density 1.077 g/mL) from peripheral blood or bone marrow. All included patients were positive for the t(9;11)(p22;q23) translocation and samples used for this study contained at least 84% AML blasts as defined by flow cytometry [26]. The use of human MLL-AF9 patient samples was approved by the Erasmus MC Ethics Committee (METC 2016-606). Human MLL-AF9 AML cell lines THP-1, Molm-13, and other AML cell lines MV4-11, HL-60, HEL, and U937 were cultured in Roswell Park Memorial Institute-1640 supplemented with Pen-Strep (100 U/mL), fetal bovine serum (FBS) (10%), and L-Glut (2 mM). AML cell line Kasumi-1 was cultured as above with 20% FBS and AML cell line TF-1 was cultured as above supplemented with human granulocyte-macrophage colony-stimulating factor (5 ng/mL, 300-03-3B, Peprotech) and sodium pyruvate (1%). The MLL-AF9 patient samples were cultured in Iscove's Modified Dulbecco's Medium, supplemented with FBS (20%), Pen-Strep (100 U/mL), IL-3 (10 ng/mL, 200-03, Peprotech), IL-6 (10 ng/mL, 200-06, Peprotech), TPO (50 ng/mL, 300-18, Peprotech), FLT-3L (50 ng/mL, 300-09, Peprotech), and SCF (50 ng/mL, 300-07, Peprotech).

Statistical analyses
Data are presented as mean ± SEM, unless otherwise specified in the figure legends. Data were compared with the two-tailed independent samples Student's t test, unless otherwise specified in the figure legends.

RESULTS

MLL-AF9 downregulates Mir139 expression
We generated a murine MLL-AF9 model by transduction of bone marrow (BM) HSPCs with MLL-AF9-expressing retroviruses. MLL-AF9 leukemia cells were positive for c-kit, GR-1, CD16/CD32, and CD11b and negative for CD3 expression, suggesting a myeloid progenitor origin of the leukemia cells (Fig. 1A). As expected from previous studies [27], DOT1L-inhibitors diminished cell survival of MLL-AF9 cells (Supplementary Fig. 1A). To validate our model, we performed proteomics and observed differential protein expression in MLL-AF9 cells (Supplementary Fig. 1B, Supplementary Table 2). The most upregulated protein was 15-hydroxyprostaglandin dehydrogenase (HPGD), suggesting a role for the HPGD pathway in MLL-AF9 leukemogenesis (Supplementary Fig. 1B). We performed Ingenuity Pathway Analysis of differentially expressed proteins and found that pathways involved in Cell Death and Survival (P < 2.74e-3), Hematological System Development and Function (P < 2.60e-5), and Hematological Diseases (P < 2.60e-5) were deregulated (Supplementary Fig. 1C). Transcriptomics and Gene Set Enrichment Analyses showed enrichment of MLL-AF9 targets [28] in the upregulated fraction of genes in MLL-AF9 cells compared to WT HSPCs (Supplementary Fig. 1D). In contrast, myeloid differentiation factors such as Cebpa, Csf1r, Csf3r, and Id2 were downregulated (Supplementary Fig. 1E). In concordance with previous studies [22, 29], we found enrichment of PRC1 and PRC2 targets, class-I (EZH2-controlled) and class-II (EZH2-independent) PcG target genes in the set of downregulated genes in MLL-AF9 cells compared to WT HSPCs (Supplementary Fig. 1F).

Mir139 is downregulated in AML patients including cases with MLL rearrangements and in THP-1 cells that express MLL-AF9 compared with normal HSPCs and myeloid cells including monocytes, macrophages, and neutrophils [13]. We therefore asked whether genomic Mir139 deletion collaborates with MLL-AF9 in AML outgrowth. Mir139KO mice developed normally and had expected HSPC counts and hematopoietic cell types in peripheral blood and BM [25]. Next, we asked whether Mir139 influences genes that have potential effects on pathways other than those that are critical for hematopoiesis. We found that WT and Mir139KO HSPCs were indistinguishable at the transcriptome and proteome level, including Pde2a host gene expression (Fig. 1B, Supplementary Fig. 2A, Supplementary Table 3). These data suggest that Mir139 is expressed at too low levels in normal HSPCs to sufficiently downregulate its targets to detectable levels. In contrast, genomic deletion of Mir139 enhanced primary and secondary colony-forming capacity (more colonies) and proliferation (larger colonies) of MLL-AF9 cells (Fig. 1C). Strikingly, we observed a >60% reduction of miR-
139-3p and miR-139-5p expression in MLL-AF9 cells, suggesting that MLL-AF9 silences Mir139 (Fig. 1D). Pde2a expression in MLL-AF9 was unchanged, indicating that Mir139 and the host gene are not co-regulated (Fig. 1E). Together, these results indicate that Mir139 is a target of MLL-AF9.

**MIR-139 upregulation and repression of miR-139 targets eliminate MLL-AF9 AML**

To determine effects of miR-139 expression on MLL-AF9 cell survival, we generated (DOX)-inducible miR-139-expressing MLL-AF9 clones (MLL-AF9-i139). DOX-treatment of MLL-AF9-i139 cells

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**C.J. Stavast et al.**

Leukemia (2022) 36:687 – 700

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**SPRINGER NATURE**
induced miR-139-5p (up to 130 Fold) and miR-139-3p (up to 100 Fold) expression (Fig. 2A). DOX-treated MLL-AF9-i139 cells lost their colony-forming capacity, whereas DOX-inducible eGFP expressing MLL-AF9 control cells (MLL-AF9-iEGFP) expanded as expected (Fig. 2B). DOX-mediated induction of miR-139 expression caused cell death and apoptosis of MLL-AF9-i139 cells as indicated by Annexin-V positivity and loss of reaping capacity (Fig. 2C, D). In addition, induction of miR-139 expression in MLL-AF9-i139 cells cultured in expansion medium without GM-CSF, stimulating mainly leukemia stem cell (LSC) expansion, completely blocked colony outgrowth (Fig. 2D). Similarly, MLL-AF9-i139 cells generated from hematopoietic stem cells (Lin–Sca-1+ c-Kit+, LSK/LSK-MLL-AF9-i139) showed loss of cells upon miR-139 induction (Supplementary Fig. 2B). Together, our results suggest that miR-139 expression also eliminates LSCs.

Next, we asked whether miR-139 expression inhibits MLL-AF9 leukemia development in mice. Mice transplanted with MLL-AF9-i139 cells developed AML with a short latency of 3 weeks. However, DOX-mediated miR-139 induction significantly delayed leukemia development of transplanted mice (Fig. 2E). Mice had malignant MLL-AF9-i139 cells in BM (Supplementary Fig. 2C), blood, and spleen (data not shown). Leukemia cells from mice that were treated with DOX showed an average miR-139-5p and miR-139-3p overexpression induction of eightfold and sevenfold, respectively (Fig. 2F), which suggests that this level of upregulation of escaping MLL-AF9-i139 cells is not sufficient to eliminate MLL-AF9-i139 cells. In full agreement, secondary transplantation experiments with these AML cells show no difference in latency between DOX-treated and non-treated controls (data not shown). These data indicate the tumor-suppressing activities of miR-139 in MLL-AF9 AML.

To gain mechanistic insight, we performed mRNA sequencing of DOX-treated MLL-AF9-i139 and untreated clones and found that a subset of genes were differentially expressed between these populations (Supplementary Fig. 2D, Supplementary Table 4). We established that genes that inhibit cell cycle progressions, such as Btg2, Cdkn1a, and Cdkn2d, and pro-apoptotic genes such as Bmf, Dusp1, Rnasel, Trp53inp1, Ypel1, and Zc3h12a were upregulated in DOX-treated MLL-AF9-i139 cells (Supplementary Table 4, Supplementary Fig. 2F). Furthermore, we noted that predicted miR-139 targets such as Eif4g2, Ptprt and Hpgd were downregulated in miR-139 expressing MLL-AF9 cells (Fig. 2G, Supplementary Table 4).

Next we wanted to validate miR-139 targets that mediate its tumor suppressor activity in MLL-AF9 cells. We performed a genome-wide CRISPR-KO screen to identify genes critical for MLL-AF9 survival or proliferation (Supplementary Fig. 2E). After 14 days, cells with sgRNAs that target such genes will be lost. Confirming the effectivity of our sgRNA screen, we found that sgRNAs targeting Men1 (six sgRNAs), a crucial co-factor for MLL-AF9 [30], the PRC2 subunits Eed (three out of six sgRNAs) and Rbpb4 (five out of six sgRNAs), Dot1l (five out of six sgRNAs) and Mllt3 (four out of six sgRNAs) were lost (Fig. 2H, Supplementary Table 5). We found that MLL-AF9 cells with sgRNAs targeting Ptprt (three out of six) Hpgd (five out of six) and Eif4g2 (five out of six) were also lost, suggesting that expression of our identified miR-139-targets is essential for cell survival (Fig. 2H, Supplementary Table 5). Validation experiments with single sgRNAs targeting Hpgd, Eif4g2, and Ptprt in colony-forming unit assays confirmed these results and showed that the depletion of these miR-139 target genes could, at least in part, phenocopy miR-139 expression in MLL-AF9 cells (Supplementary Fig. 2G). Furthermore, luciferase-based miRNA target validation assays confirmed that Hpgd and, Eif4g2 and Ptprt are direct targets of miR-139 (Supplementary Fig. 2H). Taken together, these data show that miR-139 expression and the downregulation of miR-139 targets inhibit MLL-AF9 leukemia.

**miR139 is epigenetically silenced by PRC2 in MLL-AF9 cells**

We next investigated how miR139 expression is regulated. miR139 is epigenetically silenced in AML, including the THP-1 cell line that expresses MLL-AF9 [13]. We hypothesized that miR139 silencing by histone modifications is critical for MLL-AF9 AML. To test this, we treated MLL-AF9 and MLL-AF9-Mir139KO cells with Trichostatin-A (TSA) or valproic acid (VPA), which are inhibitors of class I and II histone deacetylases. We found that MLL-AF9 cells treated with either TSA or VPA, were significantly reduced in their colony-forming capacity compared with MLL-AF9-Mir139KO cells (Supplementary Fig. 3A). These results suggest a role for MiR139 epigenetic silencing in MLL-AF9 AML expansion and survival.

Next, we investigated the epigenetic landscape of MiR139 by reanalyzing publicly available Chip-seq data sets from mouse MLL-AF9 cells and WT HSPCs [29, 31, 32]. MiR139 is located in intron-1 of the Pde2a host gene with the same orientation, downstream of the highly conserved putative Pde2a promoter (P). As expected, H3K4me3 peaks overlapped with P and H3K27Ac peaks overlapped with enhancer region-1 (E1) and E2 (Fig. 3A). In these genomic regions we noted two POL-II peaks: one located at the first exon of Pde2a and one on E2, in intron-1 of Pde2a (Fig. 3A). We found H3K27me3 levels, which are associated with epigenetic silencing, at P, E1, and E2 in MLL-AF9 cells (Fig. 3A). MLL-AF9 cells treated with UNC1999, a specific inhibitor of the methyltransferases EZH1 and EZH2 [29], showed decreased H3K27me3 and increased H3K27Ac levels compared to UNC2400 control inhibitor-treated cells. In addition, Chip-seq data revealed SUZ12 binding to P, E1, and E2, which was decreased in cells treated with UNC1999 (Fig. 3A).
Aberrant enhancer DNA methylation is acquired during malignant transformation [33]. PRC2, a chromatin-modifying enzyme that catalyzes histone-H3 lysine 27 trimethylation, is known to have a critical role in MLL-AF9 pathogenesis [22, 23] and prefers binding to methylated DNA via AEBP2 [34]. To address the role of PRC2 in context of MIR139, we determined the CpG methylation status of human MLL-AF9 cell lines Molm-13 and THP-1. We observed that a small percentage of CpGs present in the MIR139 promoter region of Molm-13 (22.73%) and THP-1 (6.36%) are methylated (Fig. 3B). Although there is a lower density of CpGs in the enhancer regions close to MIR139, we detected an increased percentage of methylated CpGs in E1 (67%) and E2 (79%) in Molm-13 cells and THP-1 cells, respectively (Fig. 3B).
To experimentally validate the relevance of PRC2-mediated silencing of Mir139, we treated MLL-AF9 cells with UNC1999 and determined cell survival compared with MLL-AF9-Mir139KO cells. UNC1999 activity was confirmed by reduced H3K27me3 levels at the P, E1, and E2 (Fig. 3C). We observed a decreased viability of MLL-AF9 cells compared with MLL-AF9-Mir139KO cells, strongly suggesting that UNC1999 treatment activates miR-139 expression (Fig. 3D). Indeed, loss of cell viability coincided with up to 25-fold increased levels of miR-139-5p and up to 20-fold increased miR-139-3p expression (Fig. 3E). Notably, treatment of MLL-AF9 cells with UNC2400 did not upregulate miR-139 and sustained cell viability (Supplementary Fig. 3B, C). Expression of miR-139-5p was not induced when WT HSPCs were treated with UNC1999 (Supplementary Fig. 3D). Cdkn2a is a known target of PRC2 in MLL-AF9 AML [22, 23]. In agreement, we observed a three to sixfold upregulation of Cdkn2a (Supplementary Fig. 3E). The expression of Pde2a was unaffected by UNC1999 (Supplementary Fig. 3F). This mechanism is conserved between mouse and human, because comparable results were obtained with Molm-13 (Fig. 3F, G), THP-1 (Supplementary Fig. 3G, H) and primary MLL-AF9 patient samples (Fig. 3H, I). In addition, UNC1999 treatment of a panel of AML cell lines caused decreased cell viability and increased miR-139 expression (Supplementary Fig. 3I, J). This suggests that PRC2-mediated Mir139 silencing may not be exclusive for MLL-AF9 AML. Taken together, these data indicate that MLL-AF9 silences Mir139 by a PRC2-mediated mechanism.

E1 and E2 control the expression of Mir139

To further investigate the role of the P, E1, and E2 in Mir139 expression, we deleted these genomic regions in mice (Fig. 4A). We were unable to generate mice with genomic deletion of the Pde2a promoter, which overlaps with exon-1, suggesting that a Pde2a KO is embryonically lethal. In contrast, E1 and E2 deletion mutant mice were viable and developed normally. Under physiological conditions, deletion of either E1 or E2 caused a >60% reduction of miR-139-5p expression, a 40–50% reduction of miR-139-3p expression in WT HSPCs and a 70–80% reduction of Pde2a expression (Fig. 4B, C). Next, we investigated whether E1 and E2 are involved in Mir139 silencing in MLL-AF9 cells. Therefore, we generated MLL-AF9-E1KO and MLL-AF9-E2KO cells. Similar to MLL-AF9 cells, these cells were positive for GR-1, CD11b, CD16/32, c-Kit, and negative for CD3 (Fig. 4D). Next, we treated these cells with UNC1999 and noted reduced cell death of MLL-AF9-E1KO and MLL-AF9-E2KO cells compared to MLL-AF9 cells, suggesting that the mir-139 induction is affected (Fig. 4E). Indeed, we found that upon UNC1999 treatment, mir-139-5p and mir-139-3p were not upregulated in MLL-AF9-E1KO cells (Fig. 4F). Notably, Pde2a expression was not affected in UNC1999-treated MLL-AF9-E1KO cells (Fig. 4G). MLL-AF9-E2KO cells showed a significantly reduced mir-139-5p and mir-139-3p induction, which coincided with slightly reduced Pde2a levels with 5 μM UNC1999 (Fig. 4F, G). Together, these data indicate that both E1 and E2 regions control Mir139 expression in AML.

POLL2M silences Mir139 in MLL-AF9 cells

To identify novel factors that are involved in transcriptional silencing of Mir139 in MLL-AF9 cells, we performed a genome-wide CRISPR-Cas9 screen [35]. We used MLL-AF9-Mir139KO cells as a reference, which allowed for screening of factors that specifically silence Mir139 transcription. We expected that inactivation of genes encoding for factors responsible for Mir139 silencing would upregulate mir-139 expression and induce apoptosis in MLL-AF9 cells, but not in MLL-AF9-Mir139KO cells (Fig. 5A). We selected all sgRNAs with 10 or more reads in MLL-AF9 cells and MLL-AF9-Mir139KO cells (MLL-AF9-WT + MLL-AF9-Mir139KO ≥ 10) after 14 days of incubation (Fig. 5B). We reasoned that a higher number of sgRNAs lost per gene will increase the chance that these genes are relevant for Mir139 regulation. According to our analysis of control sgRNAs, the chance that a loss of five sgRNAs or more per gene is an artifact is >0.0016%. Out of the genes shown in Fig. 5B, we identified only 16 genes targeted by 5–6 sgRNAs that were significantly reduced by ≥ 10 fold in MLL-AF9 cells compared with MLL-AF9-Mir139KO cells (Supplementary Fig. 4A, Supplementary Table 6). Among these 16 genes, RNA Polymerase 2 Subunit M (Polr2m), involved in stable transcriptional pausing of POL II [36–38], was the only negative transcriptional regulator and was selected for further validation (Fig. 5C). CRISPR-Cas9-mediated KO of Polr2m reduced colony outgrowth and cell viability of MLL-AF9 cells compared with MLL-AF9-139-KO cells (Fig. 5D). In agreement, POLR2M depletion reduced the viability of Molm-13 cells and primary MLL-AF9 cells from patients (Fig. 5E). Furthermore, sgRNA-mediated targeting of POLR2M caused an increase of pri-miR-139 and mature miR-139 expression in Molm-13 cells and in primary MLL-AF9 patient samples compared with control sgRNA treated cells (Fig. 5F, Supplementary Fig. 4B, C). In these experiments, Pde2a expression was not affected by Polr2m depletion (Supplementary Fig. 4D). These results were confirmed in various human AML cell lines with different oncogenic aberrations (Fig. 5G, Supplementary Fig. 4D). Collectively, these data suggest that POLR2M is a silencing factor of Mir139 downstream of PRC2, an oncogenic mechanism that may not be exclusive for MLL-AF9 AML.

POLL2M binds to E1, E2, and the transcriptional start site of Mir139

Because POLR2M binds to POL II to mediate stable transcriptional pausing [36–38], we hypothesized that POLR2M is bound at the POL-II interaction sites within the Mir139 locus. We used publicly available data sets to determine enrichment of H3K4me3, H3K27Ac, H3K4me1, and POL-II binding on E1, E2, and P regions (Supplementary Fig. 5). In our analysis, we included a previously identified Mir139 transcriptional start site (TSS) [15] downstream...
of E2 (Fig. 6A). ChIP-qPCR experiments with chromatin isolated from Molm-13 cells showed significant enrichment of POL-II at P, E1, and E2 regions and the TSS downstream of E2 (Fig. 6B). We observed POLR2M interactions with E1, E2, and the TSS downstream of E2, whereas this was not observed at P (Fig. 6C).

Treatment of Molm-13 cells with UNC1999 abrogated POLR2M binding to E1 and E2, but not to the TSS downstream of E2 (Fig. 6D). In UNC1999-treated Molm-13 cells we found strong induction of transcripts originating from the TSS, which was minor at E1 or E2 (Fig. 6E). These data suggest that E1 and E2 in
UNC1999-treated cells, strongly induces the transcription by direct interaction with the downstream TSS of MIR139 (Supplementary Fig. 5B). Taken together, these data indicate that POLR2M-binding to E1 and E2 silences the transcription of MIR139 at the TSS downstream of PRC2 in MLL-AF9 AML, which is also illustrated in our model (Fig. 7).

**DISCUSSION**

Our study has revealed key aspects of the molecular regulation of the tumor suppressor MIR139 in MLL-AF9 leukemia. We describe that the expression of miR-139 in MLL-AF9 cells causes apoptosis, abolished colony formation in vitro, and inhibited MLL-AF9 leukemia in vivo. Besides the previously published target Elif4g2 [13], we identified Hpgd and Ptprt as novel mir-139-controlled targets of which depletion in MLL-AF9 AML at least in part phenocopied mir-139 expression. Our data suggest a mechanism of MIR139 silencing in MLL-AF9 AML in which PRC2 activity and recruitment of POLR2M at the E1, E2 inhibit MIR139 expression to promote oncogenesis (Fig. 7).

There is emerging evidence that MIR139 is a tumor suppressor in various types of solid cancer [15, 39] and leukemia [12–14]. Elif4g2 is a target of mir-139-5p in AML [13], which is confirmed in our study. The RNA-binding protein Elav1 is commonly found as a target for mir-139-3p in solid cancer [39] and upon ICL-induced stress in HSPCs [12]. In the present study however, Elav1 is not differentially expressed between HSPCs and MLL-AF9 cells and when mir-139 expression is induced in MLL-AF9 cells. This suggests that mir-139 dependent target regulation is cell type- and context-dependent, which has been previously observed for other miRNAs and cell types [40–42].

PRC2-mediated repression of tumor suppressor genes, including Cdkn2a (p16[INK4A]), Cdkn2b (p15[INK4B]), Gata2 and Egr1, is a critical event in MLL-AF9 AML [22, 23, 43]. We provide evidence that MIR139 is a tumor suppressor that is targeted by PRC2 downstream of MLL-AF9. First, MLL-AF9 expression in HSPCs caused strong repression of MIR139 via PRC2. This is a critical step in oncogenic transformation as we demonstrated that mir-139 induction in MLL-AF9 cells caused apoptosis, loss of colony-forming capacity and inhibition of leukemogenesis in mice. We found that escaping AML cells in the DOX-treated mice have a mir-139 upregulation of only less than eightfold due to unknown reasons, in contrast to the 50–100-fold upregulation as found in vitro experiments. Apparently, an eightfold induction of mir-139 is not sufficient to cause apoptosis in the transplanted AML cells, allowing this aberrant sub-clone to give rise to full-blown leukemia. Second, genetic MIR139 deletion partly rescued the oncolytic effect of PRC2 inhibition in MLL-AF9 cells. Notably, the enhanced expansion capacity of MLL-AF9-Mir139KO cells compared with MLL-AF9 cells indicates that complete loss of Mir139 is a selective advantage for leukemia outgrowth in vitro. These results explain clinical data showing that AML patients with the lowest mir-139 expression levels have a poor prognosis [13]. Third, inhibition of methyltransferases EZH1 and EZH2 by UNC1999 greatly induced mir-139 expression in MLL-AF9 AML patient samples and other subtypes of AML, demonstrating direct silencing of MIR139 by PRC2 in AML, which is in agreement with previous findings in solid cancers [44, 45]. This suggests that the PRC2-mediated MIR139 silencing is a general mechanism in cancer and may be a key event in oncogenesis. Fourth, we identified Eif4g2, Ptprt, and Hpgd as mir-139 targets, which are involved in MLL-AF9 cell survival. Derepression of these three genes by mutation, disruption of miRNA-mediated regulation or genomic aberrations are frequently found in different types of cancer [13, 46–50]. Increased expression of Eif4g2, caused by MIR139 silencing, enhances protein synthesis [46], which is essential for the proliferation of AML cells [13]. Ptprt controls JAK/STAT signaling by dephosphorylation of STAT3, which is frequently dysregulated in human cancer, including AML [51, 52]. The expression and phosphorylation of STAT3 beta (STAT3β), which is an isoform of STAT3 which has tumor-suppressive activities in MLL-AF9 AML [53]. Therefore, as a result of MIR139 silencing, STAT3β phosphorylation is potentially decreased, thereby contributing to MLL-AF9 oncogenesis. HPGD is an enzyme that converts PGE2 to 15-keto-PGE2 and has been associated with epithelial-to-mesenchymal transition and poor survival of patients with breast cancer and lung cancer [46, 54]. Furthermore, our proteomic data demonstrate HPGD as the most upregulated protein in MLL-AF9 cells compared with WT HSPCs, suggesting that PGE2 conversion by HPGD contributes to MLL-AF9 oncogenesis. At last, we further demonstrate that mir-139 expression in MLL-AF9-MI139 cells induces loss of colony-forming capacity and apoptosis. Our gene expression data show that genes that inhibit cell cycle progression, such as Btg2 [55], Cdkn1a [56], and Cdkn2a [57], and genes that promote apoptosis such as Bmf [58], Dusp1 [59], Rrasel [60], Trp53inp [61], Ype3 [62], and Zcsh12a [63] are upregulated upon mir-139 expression and may explain this phenotype. Taken together, we conclude that MIR139 is silenced via PRC2 and that this silencing mechanism induces different pathways via mir-139 targets that contribute to cell survival and MLL-AF9 leukemogenesis.

Our data indicate that MIR139 silencing is independent of the transcriptional regulation of Pde2a. This may be explained by the high percentage of methylated CpGs within E1 and E2 in MLL-AF9 cells, whereas the upstream promoter region is relatively less methylated. PRC2 prefers binding to methylated DNA via AEIBP2 [34]. Accordingly, CpGs in the genomic regions overlapping with the POL-II binding sites in E1 and E2 were methylated in Molm-13 and THP-1 cells. In addition, we found that both enhancers are essential for PRC2-mediated MIR139 silencing in MLL-AF9 cells.
Thus, CpG methylation at these enhancers may be involved in the selective repression of Mir139, leaving the Pde2a host gene expression intact.

We found that Pde2a expression and intron-1 splicing did not result in mir-139 expression in MLL-AF9 cells. These data suggest that in MLL-AF9 cells pri-mir-139 is degraded. We did not observe mir-139 induction upon PRC2 inhibition in MLL-AF9-E1KO cells and a reduced mir-139 induction in MLL-AF9-E2KO, suggesting that factors involved in mir-139 stability and processing may be recruited to the enhancers. Enhancer regions may play critical roles in the transcriptional regulation of miRNAs in cancer [64]. A recent study showed that DROSHA and DGCR8 interact with super-enhancer regions and boost miRNA production [65]. In agreement with these studies, our data suggest a mechanism in which the RNA-processing machinery is directly coupled to enhancer-mediated transcription of MIR139. This interaction may

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**Fig. 6 POLR2M binds to E1, E2, and the TSS.** A Schematic representation of the PDE2A locus (chr11:72,605,000–72,644,500) with the indicated promoter (P, yellow) and enhancer (E1 or E2, blue) regions and transcriptional start sites (arrows). The first two exons of PDE2A are depicted with a black horizontal bar. The MIR139 encoding sequence is depicted in red. The location of the primer sets with the respective numbers used for the experiments in this figure is depicted in gray. B, C Fold enrichment of POL-II (yellow) and POLR2M (orange) on the depicted loci relative to IgG (white) as determined by ChIP-qPCR in Molm-13 cells are shown. Data are representative of three experiments. D Same as B and C, except that Molm-13 cells were treated with indicated concentrations of UNC1999 (different shades of orange). Data are representative of two independent experiments. E Schematic overview of the MIR139 locus. The primer sets to detect transcripts coming from E1 (green), E2 (red), and TSS (blue) are shown. The relative expression of transcripts in UNC1999-treated Molm-13-treated cells is shown below. The two-tailed unpaired student’s t test with Welch’s correction was used for statistical analysis in B–E. Data are representative of three experiments. All graphs are depicted as mean ± SEM.
occur under cellular stress conditions resulting in increased miR-139 expression and cell death to prevent oncogenic transformation. POL-II is the key factor responsible for the transcription of miRNAs [66]. Although stable paused transcription seems a logical downstream consequence of PcG-mediated gene silencing [67], the factors involved in the molecular mechanism of PcG-mediated transcriptional pausing remained unknown. We found that POLR2M is recruited in a PRC2-dependent fashion to E1 and E2 and silences MIR139 expression. POLR2M interacts with the POL-II complex to pause transcription [38, 68, 69]. Promoter-proximal pausing of POL-II at TSSs has been correlated with H3K27me3 and promoter proximal pausing remained unknown. We found that POLR2M is recruited in a PRC2-dependent fashion to E1 and E2 and silences MIR139 expression. POLR2M interacts with the POL-II complex to pause transcription [38, 68, 69]. Promoter-proximal pausing of POL-II at TSSs has been correlated with H3K27me3 and PcG-silenced genes [70–72]. Paused POL-II complexes have also been found at enhancer regions [73]. Together, our study indicates that POLR2M interacts with E1 and E2 and silences MIR139 expression in MLL-AF9.

Our data demonstrate in different ways that POLR2M is a direct silencer of MIR139 in AML. First, POLR2M depletion in MLL-AF9 patient samples and human AML cell lines induced the expression of pri-miR-139. Although the levels of the mature miR-139-5p and miR-139-3p are low compared with experiments with UNC1999-mediated repression of PRC2, this could be explained by the fact that the CRISPR-Cas9-mediated depletion of POLR2M by lentiviral transduction is an asynchronous process. This causes a heterogeneous induction of MIR139 expression and subsequent cell death, thereby underestimating miR-139 levels. Second, POLR2M-depleted MLL-AF9 cells were lost in our CRISPR-Cas9 screen and validation experiments, whereas POLR2M-depleted MLL-AF9-MIR139KO cells survived, indicating that POLR2M-controlled survival is specifically mediated by the silencing of MIR139. Third, we showed that POLR2M is bound to the E1 and E2 regions and TSS of MIR139. Depletion of POLR2M at these loci was observed as a result of UNC1999-mediated inhibition of PRC2 and coincides with induction of miR-139 expression and cell death.

In conclusion, we have identified key molecular mechanisms that control tumor suppressor MIR139 in MLL-AF9 leukemia and report functionally relevant targets of miR-139-mediated cell death of MLL-AF9 cells. Furthermore, our findings reveal a PRC2-dependent POLR2M-mediated silencing mechanism of the MIR139 tumor suppressor in MLL-AF9 leukemia. The specific interaction of POLR2M with the POL-II complex is essential for transcriptional inhibition [74]. Therefore, POLR2M would be interesting for therapeutic targeting, for instance by molecularly interfering with the interaction with POL-II, thereby activating MIR139 expression and eliminating AML. Together, our findings highlight the importance of POLR2M-mediated silencing of MIR139 in MLL-AF9 AML.

**DATA AVAILABILITY**

The NGS data generated in this paper is available from the GEO database with the following identifiers. GSE160404: RNA-seq of WT HSPCs, MIR139KO HSPCs and MLL-AF9 cells; GSE160405: RNA-seq of MLL-AF9 i139 cells treated with and without DOX; GSE160403: DNA-seq of MLL-AF9 WT and MLL-AF9 MIR139KO cells transduced with LentCRISPRv2 GeCKOv2 sgRNA library. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the identifier PXD022488.

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AUTHOR CONTRIBUTIONS

C.J.S., P.D.K., and S.J.E. wrote the manuscript. S.J.E. designed the project. C.J.S., I.V.Z., A.O., and A.V.H.B. performed most of the experiments. G.M.C.J. and P.A.V.V. performed the proteomic analysis. E.B. performed next-generation sequencing (NGS). I.G.L. performed the bioinformatics analysis of the RNA-sequence data. C.J.S., C.S., and J.S.A.V. performed CRISPR-KO screens. R.W.W.B. and W.F.J. performed CRISPR library sequencing and bioinformatic analysis. A.M. generated the E1KO and E2KO mouse models. V.H.J.vd V. wrote the METC protocol and collected human MLL-AF9 patient samples. E.K., M.B., and M.A.J. performed the mouse leukemia and MLL-AF9 stem cell experiments.

COMPETING INTERESTS

The authors declare no competing interests.

ADDITIONAL INFORMATION

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