miR-22 has a potent anti-tumour role with therapeutic potential in acute myeloid leukaemia

Xi Jiang1,2, Chao Hu1,2,3, Stephen Arnovitz2, Jason Bugno4, Miao Yu5, Zhixiang Zuo1,2,6, Ping Chen2, Hao Huang2, Bryan Ulrich2, Sandeep Gurbuxani7, Hengyou Weng1,2, Jennifer Strong1, Yungui Wang1,2,3, Yuanyuan Li2, Justin Salat2, Shenglai Li2, Abdel G. Elkahloun8, Yang Yang4, Mary Beth Neilly2, Richard A. Larson2, Michelle M. Le Beau2, Tobias Herold9, Stefan K. Bohlander10, Paul P. Liu8, Jiawang Zhang11, Zejuan Li2,12, Chuan He5, Jie Jin3, Seungpyo Hong4,13 & Jianjun Chen1,2

MicroRNAs are subject to precise regulation and have key roles in tumorigenesis. In contrast to the oncogenic role of miR-22 reported in myelodysplastic syndrome (MDS) and breast cancer, here we show that miR-22 is an essential anti-tumour gatekeeper in de novo acute myeloid leukaemia (AML) where it is significantly downregulated. Forced expression of miR-22 significantly suppresses leukaemic cell viability and growth in vitro, and substantially inhibits leukaemia development and maintenance in vivo. Mechanistically, miR-22 targets multiple oncogenes, including CRTC1, FLT3 and MYCBP, and thus represses the CREB and MYC pathways. The downregulation of miR-22 in AML is caused by TET1/GFI1/EZH2/SIN3A-mediated epigenetic repression and/or DNA copy-number loss. Furthermore, nanoparticles carrying miR-22 oligos significantly inhibit leukaemia progression in vivo. Together, our study uncovers a TET1/GFI1/EZH2/SIN3A/miR-22/CREB-MYC signalling circuit and thereby provides insights into epigenetic/genetic mechanisms underlying the pathogenesis of AML, and also highlights the clinical potential of miR-22-based AML therapy.
As one of the most common and fatal forms of hematopoietic malignancies, acute myeloid leukaemia (AML) is frequently associated with diverse chromosome translocations (for example t(11q23)/MLL-rearrangements, t(15;17)/PML-RARA and t(8;21)/AML1-ETO) and molecular abnormalities (for example, internal tandem duplications of FLT3 (FLT3-ITD) and mutations in nucleophosmin (NPM1c)\textsuperscript{1}). Despite intensive chemotherapies, the majority of patients with AML fail to survive longer than 5 years\textsuperscript{2}. Thus, development of effective therapeutic strategies based on a better understanding of the molecular mechanisms underlying the pathogenesis of AML is urgently needed.

MicroRNAs (miRNAs) are a class of small, non-coding RNAs that post-transcriptionally regulate gene expression\textsuperscript{3}. Individual miRNAs may play distinct roles in cancers originating from different tissues or even from different lineages of hematopoietic cells\textsuperscript{4}. It is unclear whether a single miRNA can play distinct roles between malignancies originating from the same hematopoietic lineage, such as de novo AML and myelodysplastic syndrome (MDS). Although around 30% of MDS cases transform to AML, the genetic and epigenetic landscape of MDS or MDS-derived AML are largely different from those of de novo AML\textsuperscript{5,6}. MDS and MDS-derived AML are more responsive to hypomethylating agents than de novo AML\textsuperscript{7}. The molecular mechanisms underlying the distinct pathogenesis and drug response between MDS (or MDS-derived AML) and de novo AML remain unclear.

The ten-eleven translocation (Tet1/2/3) proteins play critical transcriptional regulatory roles in normal developmental processes as activators or repressors\textsuperscript{8-10}. In contrast to the frequent loss-of-function mutations and tumour-suppressor role of TET2 observed in hematopoietic malignancies\textsuperscript{11-13}, we recently reported that TET1 plays an essential oncogenic role in MLL-rearranged AML where it activates expression of homeobox genes\textsuperscript{14}. However, it is unknown whether TET1 can also function as a transcriptional repressor in cancer. Moreover, Tet1-mediated regulation of miRNA expression has rarely been studied\textsuperscript{10}.

In the present study, we demonstrate that miR-22, an oncogenic miRNA reported in breast cancer and MDS\textsuperscript{15,16}, is significantly downregulated in most cases of de novo AML due to TET1/GFI1/EZH2/SIN3A-mediated epigenetic repression and/or DNA copy-number loss. miR-22 functions as an essential anti-tumour gatekeeper in various AML and holds great therapeutic potential to treat AML.

**Results**

The **downregulation of miR-22 in de novo AML**. Through Exiqon miRNA array profiling, we previously identified a set of miRNAs, such as miR-150, miR-148a, miR-29a, miR-29b, miR-184, miR-342, miR-423 and miR-22, which are significantly downregulated in AML compared with normal controls\textsuperscript{17}. Here we showed that among all the above miRNAs, miR-150 and especially miR-22 exhibited the most significant and consistent inhibitory effect on MLL-AF9-induced cell immortalization in colony-forming/replating assays (CFA) (Supplementary Fig. 1a). In contrast to the reported upregulation of miR-22 in MDS\textsuperscript{16}, our original microarray data\textsuperscript{17} (Fig. 1a,b) and new quantitative PCR-independent validation data (Supplementary Fig. 1b) demonstrated a significant and global downregulation of miR-22 in de novo AML relative to normal controls. Notably, miR-22 is significantly downregulated in AML samples (P<0.05) compared with all three sub-populations of normal control cells, that is, normal CD34\textsuperscript{+} hematopoietic stem/progenitor cells (HSPCs), CD34\textsuperscript{+} myeloid progenitor cells, or mononuclear cells (MNCs) (Fig. 1a). Expression of miR-22 is significantly downregulated in all or the majority of individual subsets of AML samples than in the normal CD34\textsuperscript{+} or CD34\textsuperscript{+} cell samples (Fig. 1b).

To rule out the possibility that the inhibitory effect of miR-22 shown in Supplementary Fig. 1a was due to a non-specific effect of our miR-22 construct, we included the MSCV-PIG-miR-22 construct from Song et al\textsuperscript{18} in a repeated CFA. Both miR-22 constructs dramatically inhibited MLL-AF9-induced colony formation (Fig. 1c). As the 'seed' sequences at the 5’ end of individual miRNAs are essential for the miRNA-target binding\textsuperscript{18}, we also mutated the 6-bases 'seed' sequence of miR-22 and found that the miR-22 mutant did not inhibit colony formation anymore (Fig. 1c). In human AML cells, forced expression of miR-22, but not miR-22 mutant, significantly inhibited cell viability and growth/proliferation, while promoting apoptosis (Supplementary Fig. 1d).

Furthermore, as miR-22 is globally downregulated in all major types of AML (Fig. 1b), we also investigated the role of miR-22 in colony formation induced by other oncogenic fusion genes, including MLL-AF10/t(10;11), PML-RARA/t(15;17) and AML1-ETO\textsubscript{9a}/t(8;21) (ref. 19). As expected, forced expression of miR-22 significantly inhibited colony formation induced by all individual oncogenic fusions; conversely, miR-22 knockout\textsuperscript{20} significantly enhanced colony forming (Fig. 1d). These results suggest that miR-22 likely plays a broad anti-tumour role in AML.

In accordance with the potential anti-tumour function of miR-22 in AML, miR-22 was expressed at a significantly higher level (P<0.05) in human normal CD34\textsuperscript{+} myeloid progenitor cells than in more immature CD34\textsuperscript{+} HSPCs or MNCs (a mixed population containing both primitive progenitors and committed cells) (Fig. 1a,b), implying that miR-22 is upregulated during normal myelopoiesis. Similarly, we showed that miR-22 was also expressed at a significantly higher level in mouse normal bone marrow (BM) myeloid (Gr-1\textsuperscript{+}/Mac-1\textsuperscript{+}) cells, relative to lineage negative (Lin\textsuperscript{-}) progenitor cells, long-term hematopoietic stem cells (LT-HSCs), short-term HSCs (ST-HSCs), and committed progenitors (CPs) (Supplementary Fig. 1e), further suggesting that miR-22 is upregulated in normal myelopoiesis.

The **anti-tumour effect of miR-22 in the pathogenesis of AML**. Through bone marrow transplantation (BMT) assays, we showed that forced expression of miR-22 (but not miR-22 mutant) dramatically blocked MLL-AF9 (MA9)-mediated leukaemogenesis in primary BMT recipient mice, with a more potent inhibitory effect than miR-150 (Fig. 1e; Supplementary Fig. 2a). All MA9 + miR-22 mice exhibited normal morphologies in peripheral blood (PB), BM, spleen and liver tissues (Fig. 1d), with a substantially reduced c-Kit\textsuperscript{+} blast cell population in BM (Supplementary Fig. 2b). Forced expression of miR-22 also almost completely inhibited leukaemogenesis induced by MLL-AF10 (Fig. 1g; Supplementary Fig. 2a). Conversely, miR-22 knockout significantly promoted AML1-ETO\textsubscript{9a} (AF9a)-induced AML (Fig. 1h). Thus, the repression of miR-22 is critical for the development of primary AML. Notably, forced expression of miR-22 in MLL-AFP and MLL-AF10 leukaemia mouse models caused only a 2–3-fold increase in miR-22 expression level (Supplementary Fig. 2a), in a degree comparable to the difference in miR-22 expression levels between human AML samples and normal controls (Fig. 1a), suggesting that a 2–3-fold change in miR-22 expression level appears to be able to exert significant physiological or pathological effects.

To examine whether the maintenance of AML is also dependent on the repression of miR-22, we performed secondary BMT assays. Forced expression of miR-22 remarkably inhibited progression of MLL-AF9-, AE9a- or FLT3-ITD/NPM1c\textsuperscript{+} induced AML in secondary recipient mice (Fig. 2a–d), resulting in largely normal morphologies in PB, BM, spleen and liver tissues (Fig. 2b; Supplementary Fig. 2c). Collectively, our findings demonstrate that miR-22 is a pivotal anti-tumour gatekeeper in both development and maintenance of various AML.

ARTICLE NATURE COMMUNICATIONS | DOI: 10.1038/ncomms11452 | www.nature.com/naturecommunications
Identification of critical target genes of miR-22 in AML. To identify potential targets of miR-22 in AML, we performed a series of data analysis. Analysis of In-house_81S (ref. 21) and TCGA_177S (ref. 22) data sets revealed a total of 999 genes exhibiting significant inverse correlations with miR-22 in expression. Of them, 137 genes, including 21 potential targets of miR-22 as predicted by TargetScan18 (Supplementary Table 1), were significantly upregulated in both human and mouse AML compared with normal controls as detected in two additional in-house data sets14,23. Among the 21 potential targets, CRTC1,
ETV6 and FLT3 are known oncogenes\(^{24-29}\). We then focused on these three genes, along with MYCBP that encodes the MYC-binding protein and is an experimentally validated target of miR-22 (ref. 30) although due to a technical issue it was not shown in the 21-gene list (Supplementary Table 1), for further studies.

As expected, all four genes were significantly downregulated in expression by ectopic expression of miR-22 in human MONO-MAC-6/t(9;11) cells (Fig. 3a). The coincidence of downregulation of those genes and upregulation of miR-22 was also observed in mouse MLL-ENL-ER\(\text{tm}\) cells, a leukaemic cell line with an inducible MLL-ENL derivative\(^3\), when MLL-ENL was depleted by 4-hydroxy-tamoxifen (4-OHT) withdrawal (Fig. 3b; Supplementary Fig. 3a). While MLL-AF9 remarkably promoted expression of those four genes in mouse BM progenitor cells, co-expressed miR-22 reversed the upregulation (Fig. 3c). In leukaemia BM blast cells of mice with MLL-AF9-induced AML, the expression of Crtc1, Flt3 and Mycbp, but not Etv6, was significantly downregulated by co-expressed miR-22 (but not by miR-22 mutant) (Fig. 3d). Because miR-22-mediated downregulation of Etv6 could be observed only in the in vitro models (Fig. 3a–c), but not in the in vivo model (Fig. 3d), which was probably due to the difference between in vitro and in vivo environments, we decided to focus on the three target genes (that is, Crtc1, Flt3 and Mycbp) that showed consistent patterns between in vitro and in vivo for further studies. The repression of Crtc1, Flt3 and Mycbp was also found in leukaemia BM cells of mice with AE9a or FLT3-ITD/NPM1c\(^{+}\)-induced AML (Fig. 3e,f). As Mycbp is already a known target of miR-22 (ref. 30), here we further confirmed that FLT3 and CRTC1 are also direct targets of miR-22 (Fig. 3g,h). The downregulation of CRTC1, FLT3 and MYCBP by miR-22 at the protein level was confirmed in both human and mouse leukaemic cells (Supplementary Fig. 3b,c). Overexpression of miR-22 had no significant influence on the level of leukaemia fusion genes (Supplementary Fig. 3d).

Co-expression of the coding region (CDS) of each of the three target genes (that is, CRTC1, FLT3 and MYCBP) largely reversed the effects of miR-22 on cell viability, apoptosis and proliferation (Fig. 4a–e). More importantly, in vivo BMT assays showed that co-expressing CRTC1, FLT3 or MYCBP largely rescued the inhibitory effect of miR-22 on leukemogenesis (Fig. 4f,g; Supplementary Fig. 3e). Our data thus suggest that CRTC1, FLT3 and MYCBP are functionally important targets of miR-22 in AML.
miR-22 represses both CREB and MYC signalling pathways. CRTC1, a CREB-regulated transcription coactivator, facilitates CREB in regulating transcription of its targets, in both normal and malignant hematopoiesis24–26. CDK6, HOXA7 and RGS2 are known direct targets of CREB that are either positively (CDK6 and HOXA7) or negatively (RGS2) regulated by CREB24–26. In both In-house_81S (ref. 21) and TCGA_177S (ref. 22) data sets, CDK6 and HOXA7 inversely, while RGS2 positively, correlated with miR-22 in expression (Supplementary Table 2; Supplementary Fig. 3f). In leukaemic BM cells from primary and secondary BMT recipients, overexpression of miR-22 (but not miR-22 mutant) significantly downregulated expression of Cd6 and Hoxa7, while upregulating Rgs2, which could be reversed by co-expressing CRTC1 (Supplementary Fig. 3g,h).

These results suggest that miR-22 represses the CREB signalling pathway in AML by targeting CRTC1.

MYCBP, a MYC-binding protein, is essential for MYC-mediated gene regulation30. FLT3 is an upstream regulator of MYC37. In leukaemic BM cells, forced expression of miR-22, but not miR-22 mutant, significantly repressed expression of MYC downstream oncogenic targets Bmi1, Fasn and Hmga1 (refs 36–38); the repression could be reversed by co-expressing MYCBP or FLT3 (Supplementary Fig. 3i,j). Those three genes all showed significant inverse correlations with miR-22 in expression in human AML (Supplementary Table 2; Supplementary Fig. 3f). The miR-22-induced repression of Bmi1, Cdk6 and Hmga1 at the protein level was also observed (Supplementary Fig. 3c).

Figure 3 | miR-22 targets multiple oncogenes. (a) Downregulation of CRTC1, FLT3, MYCBP and ETV6 by forced expression of miR-22 in MONOMAC-6 cells. Expression of these genes was detected 48h post transfection of MSCV-PIG (Ctrl) or MSCV-PIG-miR-22 (miR-22). (b) Crtc1, Flt3, Mycbp and Etv6 levels in MLL-ENL-ERtm cells after withdrawal of 4-OHT for 0, 7 or 10 days. (c) Expression levels of Crtc1, Flt3, Mycbp and Etv6 in mouse BM progenitor cells retrovirally transduced with MSCV-PIG-MLL-ENL (Ctrl), MSCV-PIG-miR-22+MSCV-neo (miR-22), MSCV-PIG + MSCV-neo-MLL-AF9 (MLL-AF9) or MSCV-PIG-miR-22 +MSCV-neo-MLL-AF9 (MLL-AF9 + miR-22). (d) Expression levels of Crtc1, Flt3, Mycbp and Etv6 in BM blast cells of leukaemic mice transplanted with MLL-AF9, MLL-AF9 + miR-22 or MLL-AF9 + miR-22mut primary leukaemic cells. (e) Expression levels of Crtc1, Flt3 and Mycbp in BM blast cells of leukaemic mice transplanted with MSCV-PIG or MSCV-PIG-miR-22-retrovirally transduced AE9a (e) or FLT3-ITD/NPM1c+ (f) primary leukaemic cells. (g) Putative miR-22 target sites and mutants in the 3′UTRs of CRTC1 (upper panel) and FLT3 (lower panel). (h) Effects of miR-22 on luciferase activity of the reporter gene bearing wild type or mutant 3′UTRs of CRTC1 or FLT3 in HEK293T cells. The mean ± s.d. values from three replicates are shown. *P<0.05, t-test.
miR-22 transduced with MSCVneo. Leukemogenesis mediated by miR-22. The secondary recipients were transplanted with BM blast cells of the primary mice. Here we found that 18% (9/50) of the AML samples reported in various types of leukemia, lymphoma and solid cancers including AML. Deletions of human chromosome 17 band p13.3, where miR-22 is located, have been frequently reported in various types of leukemia, lymphoma and solid tumors. Here we found that 18% (9/50) of the AML samples showed deletions (mostly hemizygous) of the miR-22 gene locus (Supplementary Fig. 4a,b). Similarly, in analysis of three publicly available AML data sets, we found that 7–9% of the AML cases carried loss of one or even two alleles of the miR-22 locus (Supplementary Fig. 4c). Therefore, DNA copy-number loss in miR-22 gene locus does exist in AML cases.

Expression of miR-22 is epigenetically repressed in AML. It was reported that TET2 is repressed by miR-22 as its direct target in

Figure 4 | Multiple oncogenes are functionally important targets of miR-22 in AML. (a,b) Relative viability (a) and apoptosis (b) levels of MONOMAC-6 cells transfected with MSCV-PIG-CRTC1, -FLT3 or -MYCBP alone, or together with MSCVneo-miR-22. Values were detected 48 h post transfection. (c–e) Rescue effects of CRTC1 (c), FLT3 (d) and MYCBP (e) on the inhibition of MONOMAC-6 growth mediated by miR-22. Cell counts at the indicated time points are shown. Mean ± s.d. values are shown. *P < 0.05, t-test. (f) In vivo rescue effects of CRTC1, FLT3 and MYCBP on the inhibition of MLL-AF9-induced leukemogenesis mediated by miR-22. The secondary recipients were transplanted with BM blast cells of the primary MLL-AF9 leukaemic mice retrovirally transduced with MSCVneo + MSCV-PIG (MA9-AML + Ctrl; n = 7), MSCVneo-miR-22 + MSCV-PIG (MA9-AML + miR-22; n = 10), MSCVneo-miR-22 + MSCV-PIG-CRTC1 (MA9-AML + miR-22 + CRTC1; n = 5), MSCVneo-miR-22 + MSCV-PIG-FLT3 (MA9-AML + miR-22 + FLT3; n = 6) or MSCVneo-miR-22 + MSCV-PIG-MYCBP (MA9-AML + miR-22 + MYCBP; n = 6). Kaplan-Meier curves for all the five groups of transplanted mice are shown. MA9-AML + Ctrl versus MA9-AML + miR-22, P < 0.001 (log-rank test); MA9-AML + Ctrl versus any other groups, P > 0.05 (log-rank test). (g) Wright–Giemsa stained PB and BM, and H&E stained spleen and liver of the secondary leukaemic mice.
breast cancer and MDS\textsuperscript{15,16}. Here we analysed the expression patterns of TET1/2/3 and miR-22 in three independent AML patient data sets\textsuperscript{21,22,44} (Supplementary Table 3). To our surprise, we found that TET2 (and likely also TET3) exhibited a positive correlation, whereas only TET1 exhibited a negative correlation, with miR-22 in expression in AML (Supplementary Table 3; Fig. 5a). The primary, precursor and mature miR-22 levels were all significantly downregulated by MLL-AF9, MLL-AF10, AE9a and FLT3-ITD/NPM1c\textsuperscript{+} in colony-forming cells, while Tet1 (but not Tet2 or Tet3) was upregulated (Fig. 5b). Conversely, in \textit{MLL-ENL-ERtm} cells\textsuperscript{31}, Tet1, but not Tet2 or Tet3, was downregulated when miR-22 was upregulated after withdrawal of 4-OHT (Fig. 5c). Thus, Tet1, instead of Tet2, exhibited an inverse correlation with miR-22 in expression in both human and mouse leukemic cells. Tet1 also exhibits an inverse correlation with miR-22 in expression during mouse normal myeloid differentiation (Supplementary Fig. 5a). Furthermore, as miR-22 and TET1 were expressed at a significantly higher and lower level, respectively, in human normal CD34\textsuperscript{+} myeloid progenitor cells than in CD34\textsuperscript{+} HSPCs or MNCs (see Fig. 1a and ref. 14), the inverse expression correlation between miR-22 and TET1 likely also existed in human normal hematopoietic cells.

However, forced expression of miR-22 caused no noticeable changes in Tet1 expression in MLL-AF9, AE9a or FLT3-ITD/NPM1c\textsuperscript{+} colony-forming cells (Fig. 5d). Similarly, neither miR-22 knockout nor overexpression resulted in any significant changes of Tet1/TET1 expression (Fig. 5e,f). In contrast, Tet1 knockout remarkably increased the levels of pri-, pre- and mature miR-22 (Fig. 5g). Thus, our data suggest that miR-22 is a downstream target of and negatively regulated by Tet1, and that there is no negative feedback of miR-22 on Tet1 expression.

Tet1 has been shown to cooperate with Polycomb repressive complex 2 (PRC2) components and cofactors, such as Ezh2 and Sin3a, to repress transcription of their co-target genes in mouse embryonic stem cells\textsuperscript{8,9}. Our luciferase reporter assay showed that forced expression of Tet1 significantly repressed the transcriptional activity controlled by the miR-22 promoter\textsuperscript{45}, suggesting that miR-22 is a direct repressed target of Tet1 (Fig. 6a). In an all-\textit{trans} retinoic acid (ATRA)-induced THP-1/t(9;11) monocytic differentiation model\textsuperscript{46}, we showed...
ATRA treatment showed no significant effects on gene with the opposite orientation. 

inhibits the transcription of miR-22, but not its neighbouring (Supplementary Fig. 5b), suggesting that TET1 specifically ATRA on the expression level of gene loci (Fig. 6d). We also tested the potential influence of is located closely (within 500 bp) but oppositely to the miR-22 region of miR-22 gene locus. Note: miR-22 is coded within the second exon of a long non-coding RNA (MIR22HG), which represents the primary transcript of miR-22. Lower panels: enrichment of MLL-N terminal (for both wild-type MLL and MLL-fusion proteins), MLL-C terminal (for wild-type MLL), TET1, EZH2, SIN3A and miR-22 in THP-1 cells 72 h post transfection with siTET1, siEZH2, siSIN3A were significantly downregulated, accompanied and/or SIN3A and miR-22 in THP-1 cells 72 h post treatment with 1 μM ATRA or DMSO control. (c) Co-immunoprecipitation assay showing the binding of endogenous GFI1 and TET1 in THP1 cells. (d) ChIP-qPCR analyses of the promoter region of miR-22 in THP-1 cells 72 h post treatment with 1 μM ATRA or DMSO. Upper panel: PCR site on the CpG-enriched region of miR-22 gene locus. Note: miR-22 is coded within the second exon of a long non-coding RNA (MIR22HG), which represents the primary transcript of miR-22. Lower panels: enrichment of MLL-N terminal (for both wild-type MLL and MLL-fusion proteins), MLL-C terminal (for wild-type MLL), TET1, EZH2, SIN3A, GFI1, H3K27me3, H3K4me3 or RNA pol II at miR-22 promoter region. (e) Expression levels of TET1, EZH2, SIN3A and miR-22 in GFI1 knockdown cells. (f) ChIP-qPCR analyses of the promoter region of miR-22 in THP-1 cells transduced with GFI1 shRNA or control shRNA. Enrichment of GFI1, TET1, EZH2 and SIN3A are shown. (g) Effects of knockdown of TET1, EZH2 and/or SIN3A on miR-22 expression. The expression level of miR-22 was detected in THP-1 cells 72 h post transfection with siRNAs targeting TET1, EZH2 and/or SIN3A. Mean ± s.d. values are shown. *P < 0.05; **P < 0.01 (t-test). (h) Schematic model of the regulatory pathway involving miR-22 in AML and ATRA treatment.

that on treatment with ATRA, TET1 (but not TET2 or TET3), EZH2 and SIN3A were significantly downregulated, accompanied by the upregulation of miR-22 (Fig. 6b). WDR81 is the gene that is located closely (within 500 bp) but oppositely to the miR-22 gene loci (Fig. 6d). We also tested the potential influence of ATRA on the expression level of WDR81 in the same model. ATRA treatment showed no significant effects on WDR81 level (Supplementary Fig. 5b), suggesting that TET1 specifically inhibits the transcription of miR-22, but not its neighbouring gene with the opposite orientation.

While miR-22 expression level had a more than fivefold increase on ATRA treatment, the degrees of decrease in expression levels of TET1, EZH2 and SIN3A are relatively mild (though statistically significant) (Fig. 6b). To identify additional transcription factor(s) that is (are) more responsive to ATRA treatment and can facilitate TET1 binding to miR-22 promoter region, we searched for transcription factors that have evolutionarily conserved binding sites within the CpG island of miR-22 locus. Among a set of such transcription factors (including GFI1, STAT, PAX4, HMX1 and SRF), only GFI1 exhibited a significant

Figure 6 | TET1 mediates epigenetic repression of miR-22 transcription. (a) TET1 targets miR-22 promoter region (−1,100/+55 bp), as detected by luciferase reporter assay 48 h post transfection in HEK293T cells. (b) Expression of TET1/2/3, EZH2, SIN3A, GFI1 and miR-22 in THP-1 cells 72 h post treatment with 1 μM ATRA or DMSO control. (c) Co-immunoprecipitation assay showing the binding of endogenous GFI1 and TET1 in THP1 cells. (d) ChIP-qPCR analyses of the promoter region of miR-22 in THP-1 cells 72 h post treatment with 1 μM ATRA or DMSO. Upper panel: PCR site on the CpG-enriched region of miR-22 gene locus. Note: miR-22 is coded within the second exon of a long non-coding RNA (MIR22HG), which represents the primary transcript of miR-22. Lower panels: enrichment of MLL-N terminal (for both wild-type MLL and MLL-fusion proteins), MLL-C terminal (for wild-type MLL), TET1, EZH2, SIN3A, GFI1, H3K27me3, H3K4me3 or RNA pol II at miR-22 promoter region. (e) Expression levels of TET1, EZH2, SIN3A and miR-22 in GFI1 knockdown cells. (f) ChIP-qPCR analyses of the promoter region of miR-22 in THP-1 cells transduced with GFI1 shRNA or control shRNA. Enrichment of GFI1, TET1, EZH2 and SIN3A are shown. (g) Effects of knockdown of TET1, EZH2 and/or SIN3A on miR-22 expression. The expression level of miR-22 was detected in THP-1 cells 72 h post transfection with siRNAs targeting TET1, EZH2 and/or SIN3A. Mean ± s.d. values are shown. *P < 0.05; **P < 0.01 (t-test). (h) Schematic model of the regulatory pathway involving miR-22 in AML and ATRA treatment.
or miR-22 mutant nanoparticles. GFI1 for further studies. Notably, we found that ATRA treatment substantially reduced the transcription level of GFI1 to the loci of many of its target genes, for example, IL-6R, JAK3, E2F6 and so on. Thus, we chose GFI1 for further studies.

We further showed that GFI1 is a binding partner of TET1 in both THP1 and HEK-293T cells (Fig. 6c; Supplementary Fig. 5a). ATRA treatment remarkably reduced the binding of GFI1, TET1, EZH2 and SIN3A, but not that of MLL protein, to the miR-22 promoter region (Fig. 6d). H3K27me3 modifications and RNA polymerase II (RNA pol II) occupancy were significantly decreased and increased, respectively, while H3K4Me3 modifications showed no significant change (Fig. 6d). Noticeably, the enrichment of GFI1 to this region was diminished by ATRA to a greater degree than that of TET1, EZH2 or SIN3A (Fig. 6d), suggesting that GFI1 might be the primary effector of ATRA treatment in regulating miR-22 expression. Consistently, knockdown of GFI1 resulted in a dramatic increase in miR-22 expression (> 4 fold; Fig. 6e), associated with a significant decrease in the binding of TET1, EZH2, SIN3A and GFI1 itself to the miR-22 promoter region (Fig. 6f). Knockdown of expression of TET1, EZH2 or SIN3A resulted in a 2–3-fold increase in miR-22 expression (Fig. 6g), with no effects on GFI1 expression (Supplementary Fig. 5d); only their combinational knockdown could cause a similar level of increase in miR-22 expression (Fig. 6g; Supplementary Fig. 5e) to that induced by GFI1 knockdown (Fig. 6e). As expected, the expression level of WDR81 was not changed on knockdown of GFI1, TET1, EZH2 or SIN3A (Supplementary Fig. 5f,g). The above data suggest that GFI1, TET1, EZH2 and SIN3A are all involved in transcriptional repression of miR-22 expression; and when treated with ATRA, GFI1 likely functions as the primary effector that facilitates the binding of TET1/EZH2/SIN3A complex to the miR-22 promoter region.

As TET1 is a methylcytosine dioxygenase, we conducted bisulfite sequencing analysis to investigate whether TET1 affects the methylation status of the miR-22 promoter. The analysis showed that the miR-22 promoter was hypomethylated in AML cells, no matter with ATRA treatment or not (Supplementary Fig. 5h). The hypomethylated status of the miR-22 promoter region in various AML was confirmed by analysing the TCGA_194S data set with DNA methylation information.
The methylcytosine dioxygenase activity.

The miR-22-associated regulatory circuit in AML. The above data suggest that repression of miR-22 in AML is attributed to both DNA copy-number loss and especially TET1-mediated transcriptional suppression. Interestingly, among the nine AML samples with DNA copy-number loss of miR-22 locus (Supplementary Fig. 4a,b), the AML samples with both copy-number loss and TET1 overexpression generally exhibited a more significant repression of miR-22 expression than those with copy-number loss alone (Supplementary Fig. 5k). Thus, those two mechanisms are not mutually exclusive and can have synergistic effect on reducing miR-22 expression.

Collectively, our studies revealed a previously unappreciated genetic/epigenetic regulatory circuit in AML (Fig. 6h). In this circuit, oncogenic fusion genes or gene mutants (for example, MLL-fusions, AE9a and FLT3-ITD(NPM1c+)) function as the ‘drivers’. They promote the expression of TET1, which in turn, through recruiting polycomb cofactors such as EZH2 and SIN3A, represses the transcription of miR-22 by increasing H3K27me3 and decreasing RNA Pol II binding at the miR-22 promoter. When AML cells are treated with ATRA, ATRA substantially diminishes the enrichment of GFI1, a binding partner of TET1, at the miR-22 promoter, and thereby inhibits the recruitment of the TET1/EZH2/SIN3A complex to this region. In addition, miR-22 can also be compromised in its function by genetic mechanism(s) such as DNA copy-number loss in a portion (7–18%) of the AML cases. The inactivation of miR-22 results in the de-repression of its critical oncogenic targets such as CRTC1, MYCBP and FLT3, and thereby the activation of both CREB and MYC signalling pathways, leading to cell transformation and leukemogenesis.

Restoration of miR-22 expression and function to treat AML. To investigate the therapeutic potential of restoration of miR-22 expression/function in treating AML, we employed amine-terminated, generation 7 (G7) poly(amideamine) (PAMAM) nanoparticles carrying miR-22 oligos significantly delayed AML progression in both MLL-AF9 and AE9a-induced secondary leukaemic recipients (Fig. 7a,b). Notably, at least 40% of the treated mice seemed to be completely cured by the miR-22 nanoparticles as the pathological morphologies in PB, BM, spleen and liver tissues all became normal (Fig. 7d; Supplementary Fig. 6c). In contrast, the miR-22 mutant nanoparticles exhibited no significant therapeutic effect (Fig. 7a,b,d; Supplementary Fig. 6c). As expected, miR-22 oligos, but not miR-22 mutant oligos, significantly inhibited expression of its critical targets (that is, Crtc1, Flt3 and MycBP) in BM cells of the treated mice (Supplementary Fig. 6d). The miR-22-nanoparticles showed no noticeable effects on blood cell lineages (Supplementary Table 4).

We then tested the miR-22 nanoparticles in a xeno-transplantation model. Similarly, the nanoparticles carrying miR-22 oligos, but not miR-22 mutant, significantly delayed AML progression induced by human MV4;11/t(4;11) cells (Fig. 7c). The miR-22-nanoparticle administration also resulted in less aggressive leukaemic pathological phenotypes in the recipient mice (Supplementary Fig. 6e). Thus, our studies demonstrated the therapeutic potential of using miR-22-based nanoparticles to treat AML.

Discussion

It remains poorly understood how TET proteins mediate gene regulation in cancer. Here we show that in de novo AML, it is TET1, but not TET2 (a reported direct target of miR-22 in MDS and breast cancer15,19), that inversely correlates with miR-22 in expression and negatively regulates miR-22 at the transcriptional level. Likely together with GFI1, TET1 recruits polycomb cofactors (for example, EZH2/SIN3A) to the miR-22 promoter, leading to a significant increase in H3K27me3 occupancy and decrease in RNA pol II occupancy at that region, and thereby resulting in miR-22 repression in AML cells; such a repression can be abrogated by ATRA treatment. Thus, our study uncovers a novel epigenetic regulation mechanism in leukaemia involving the cooperation between TET1/GFI1 and polycomb factors.

Besides GFI1, it was reported that LSD1 is also a binding partner of TET1 (ref. 50). Interestingly, LSD1 is known as a common binding partner shared by TET1 and GFI1, and mediates the effect of GFI1 on hematopoietic differentiation71,72. Thus, it is possible that LSD1 might also participate in the transcriptional repression of miR-22 as a component of the GFI1/TET1 repression complex.

We previously reported that TET1 cooperates with MLL fusions in positively regulating their oncogenic co-targets in MLL-rearranged AML.14 Here we show that TET1 can also function as a transcriptional repressor (of a miRNA) in cancer. The requirement of TET1-mediated regulation on expression of its positive (for example, HOXA/MEIS1/PBX3)14 or negative (for example, miR-22) downstream effectors in leukemogenesis likely explains the rareness of TET1 mutations in AML33, and highlights its potent oncogenic role in leukaemia.

The aberrant activation of both CREB and MYC signalling pathways has been shown in AML24–26,54,55, but the underlying molecular mechanisms remain elusive. Our data suggest that the activation of these two signalling pathways in AML can be attributed, at least in part, to the repression of miR-22, which in turn, results in the de-repression of CRTCI (CREB pathway), FLT3 and MYCBP (MYC pathway), and leads to the upregulation of oncogenic downstream targets (for example, CDK6, HOXA7, BMI1, FASN and HMGAI) and downregulation of tumour-suppressor downstream targets (for example, RGS2).

In summary, we uncover a TET1/GFI1/EZH2/SIN3A–miR-22–CREB-MYC signalling circuit in de novo AML, in which miR-22 functions as a pivotal anti-tumour gate-keeper, distinct from its oncogenic role reported in MDS or MDS-derived AML.16 Thus, our study together with the study of Song et al.16 highlight the complexity and functional importance of miR-22-associated gene regulation and signalling pathways in hematopoietic malignancies, and may provide novel insights into the genetic/epigenetic differences between de novo AML and MDS.

Our findings also highlight the possibility of using miR-22-based therapy to treat AML patients. Our proof-of-concept studies demonstrate that the nanoparticles carrying miR-22 oligos significantly inhibit AML progression and prolong survival of leukaemic mice in both BMT and xeno-transplantation models. Notably, miRNA-based nanoparticles have already entered clinical trials.56 It would be important, in the future, to further test the combination of miR-22-carrying nanoparticles (or small-molecule compounds that can induce endogenous expression of miR-22) with standard chemotherapy agents (cytotoxic arabinoside and anthracycline), or with the emerging small molecule inhibitors against MYC and/or CREB pathway effectors, to achieve optimal anti-leukaemia effect with minimal side effects. Overall, our results suggest that restoration of miR-22 expression/function (for example, using miR-22-carrying nanoparticles or small-molecule compounds) holds great therapeutic potential to treat AML, especially those resistant to current therapies.
RNA extraction and quantitative RT–PCR

APC-B220 (RA3-6B2) staining. All fluorescent antibodies used were purchased from BD Biosciences, San Jose, CA. TaqMan qPCR assay was performed to validate the differential expression of snoRNA202, miR-122, and GAPDH (described previously57).

Preparation of AML and MDS samples.

The primary AML and MDS samples were stored in liquid nitrogen until used. Blasts and mononuclear cells were purified by use of NycoPrep 1.077A (Axis- Shield, Oslo, Norway) according to the manufacturer’s manual.

Human normal hematopoietic control cell samples.

The MNC normal control samples were isolated from normal BM cells purchased from AllCells, LLC (Emeryville, CA) by use of NycoPrep 1.077A (Axis- Shield, Oslo, Norway) according to the manufacturer’s manual.

Mouse normal BM cell population sorting.

As described previously23, wild-type C57BL/6 mice were used for the sorting. All laboratory mice were maintained in the animal facility at the University of Chicago and the University of Cincinnati. All experiments on mice in our research protocol were approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Chicago and the University of Cincinnati.

The long-term hematopoietic stem cells (LT-HSCs; Lin− / Sca1+ / c-Kit+ / Flk2−, LSKF−), short-term HSCs (ST-HSCs; Lin− / Sca1+ / c-Kit+ / Flk2−, LSKF−), and the committed progenitors (CP, Lin− / Sca1+ / c-Kit+ / Flk2−, lineage−) were enriched by lineage− cell depletion (EasySep Mouse Hematopoietic Progenitor Cell Enrichment Kit; StemCell Technologies, Vancouver, BC), and purified by FACS Aria flow cytometer (BD Biosciences, San Jose, CA) sorting after 20 μl per test fluorescein isothiocyanate-conjugated lineage (FITC-Lin) cocktail (including FITC-CD3 (17A2), FITC-B220 (RA3-6B2), FITC-CD11b (M1/70), FITC-TER-119 (TERR-119), FITC-Gr1 (RB6-8C5), 5 μg ml−1 phycoerythrin (PE)-Sca1 (D7), 1.5 μg ml−1 APC-c-Kit (ACK2) and 20 μg ml−1 PE-Cy5.5-Flk2 (A2F10) staining. Then, Gr1+ Mac1+ myeloid cells and B220+ B cells were sorted from BM cells after 2.5 μg ml−1 FITC-Gr1 (RB6-8C5), 2.5 μg ml−1 PE-Mac1 (M1/70) and 5 μg ml−1 APC-B220 (RA3-6B2) staining. All fluorescent antibodies used were purchased from eBioScience (San Diego, CA).

RNA extraction and quantitative RT–PCR.

Total RNA was extracted with the mirNeasy extraction kit (Qiagen, Valencia, CA, USA) and was used as template to synthesize complementary DNA for quantitative reverse transcription PCR (qRT–PCR) analysis in a 7900HT real-time PCR system (Applied Biosystems, Foster City, CA). TaqMan qPCR assay was performed to validate the differential expression patterns of snoRNA202 using commercial kits from Applied Biosystems (Cat. no. 4429775). Sequences for the controls are: sno202: 5′-GGCTGTACGACTGATGAAAGTACTTTTGAACCCTTTTCCATCTGATG-3′; snoRNA202: 5′-TGATGAAAGTACTTTTGAACCCTTTTCCATCTGATG-3′. For the quantification of snoRNA202, snoGAPDH, and snoGFI1, snoRNA202 was used as endogenous controls for qPCR of mRNA and mirRNA, respectively. Each sample was run in triplicate. qPCR primers are available on request. For determining the miR-22 DNA locus copy number, TaqMan qPCR assay was used as described previously57.

microRNA microarray and exon array assays.

As described previously17,23, our miRNA expression profiling assay of 85 (including 10 t(8;21), 9 inv(16), 9 t(15;17), 10 MLL-rearranged, 11 (8, +8), 29 normal karyotype and 7 others) AML samples, and 15 normal BM samples were performed using Exiqon microRNA microarray and exon array assays of the 177 AML cases were collectively referred to as TCGA_177S. One hundred ninety-four adult de novo AML cases (including 7 t(8;21), 11 inv(16), 16 t(15;17), 9 MLL-rearranged, 3 t(9;22), 22 complex, 75 normal karyotype and 34 others) were analysed by use of Affymetrix Human Genome U133Plus2.0 GeneChips (n = 140) or Affymetrix Human Genome U133A and B (n = 422) GeneChips. RMA method28 was used for data normalization. The AML samples were collected by the German AMLCG study group. Part of the microarray data have been reported previously44. The GEO ID of the entire data set is GSE73642.

TCGA data sets.

The Cancer Genome Atlas (TCGA) AML database22 includes miRNA gene expression profiling data of 183 adult de novo AML cases (that is, TCGA, 1835; including 7 t(8;21), 11 inv(16), 17 t(15;17), 3 MLL-rearranged, 3 t(9;22), 15 complex, 78 normal karyotype and 36 others), which were generated by use of Affymetrix Human Genome U133Plus2.0 GeneChips. Among the 183 AML cases, 177 (including 7 t(8;21), 11 inv(16), 16 t(15;17), 9 MLL-rearranged, 3 t(9;22), 22 complex, 75 normal karyotype and 34 others) also have microRNA expression profiles as detected by Illumina GA miRNASeq platform, and the miRNA/miRNA profiles of the 177 AML cases were collectively referred to as TCGA_177S.

Cell culture and transfection.

These experiments were conducted as described previously17,23 with some modifications. THP-1, KOCL-48 and MV4;11 cells were grown in RPMI medium 1640 (Invitrogen, Carlsbad, CA) containing 10% FBS, 1% HEPS and 1% penicillin-streptomycin. MONOMAC-6 cells were maintained in RPMI 1640 supplemented with 10% FBS, 1% HEPS, 2 mM l-glutamine, 100 × Non-Essential Amino Acid, 1 mM sodium pyruvate, 9 μg ml−1 insulin and 1% penicillin-streptomycin. Plasmids or siRNAs were transfected into MONOMAC-6 cells with Cell Line Nucleofector Kit V following program T-037, and THP-1 and KOCL-48 cells following program U-001, using the Amaxa Nucleofector Technology (Amaxa Systems, Berlin, Germany). Experiments were performed 48 h after transfection.

For the ATRA-treatment study, THP-1 cells were seeded at a concentration of 0.4 × 10⁶ ml⁻¹ and treated with ATRA (1 μmol l⁻¹) or vehicle control (DMSO, 0.001%) for 72 h before cells were collected for RNA analysis or chromatin immunoprecipitation (ChIP) assays.

The MLL-ENL-ERm cell line was kept in RPMI 1640 supplemented with interleukin 3 (IL-3), IL-6 and granulocyte-macrophage colony-stimulating factor (GM-CSF), 10 ng ml⁻¹; stem cell factor (SCF) 100 ng ml⁻¹; 10% FBS and 1% penicillin-streptomycin. 4-OHT (Sigma-Aldrich, St Louis, MO) was added at a 10 nM final concentration as a 1-mM stock solution in ethanol. Cells were collected for experiments at the indicated days after drug withdrawal.

The MEF and HEK-293T cells were kept in DMEM (Invitrogen, Carlsbad, CA) containing 10% FBS, 1% HEPS and 1% penicillin-streptomycin. HEK-293T cells were transfected with Qagen Effectene Transfection Kit (Qiagen) following the manufacturer’s protocol. All the cell lines were mycoplasma negative.

Lentivirus production and infection.

All the plasmid for packaging lentivirus, including pMD2.G, pMDLg/pRRE and pRSV-Rev, were purchased from Addgene (Cambridge, MA). First, 0.5 μg pMD2.G, 0.3 μg pMDLg/pRRE, 0.7 μg pRSV-Rev and 1.5 μg shRNA constructs, that is shGFI1 and control (purchased from GE Dharmacon, Pittsburgh, PA) were co-transfected into HEK-293T cells in 60 mm cell culture dish with Effectene Transfection Reagent (Qiagen). The lentivirus particles were harvested at 48 and 72 h after transfection and concentrated with PEG–It Virus Precipitation Solution (SBI). Finally, the lentivirus particles were

Among the above 100 human AML samples, 81 samples (that is, the in-house _R1S_ including 29 t(8;21), 26 inv(16) and 26 t(15;17) AML) have been also included in the Exiqon microRNA array assay22. The microarray data set of those 81 AML samples has been deposited in GEO database under the accession code GSE27370.

Affymetrix gene arrays of mouse samples.

As described previously17, a total of 15 mouse BM samples including 6 primary (including three each of negative control and MLL-AFP) and 9 secondary (including three negative control and six MLL-AFP) obtained from the in vivo mouse BM reconstitution assays were analysed by use of Affymetrix GeneChip Mouse Gene 1.0 ST Array (Affymetrix). The RNA quality control, cDNA amplification, hybridization and image scan were conducted in the Functional Genomics Facility of the University of Chicago. RNA was used for the data normalization with Partek Genomics Suite (Partek Inc.). The microarray data set of those 15 mouse AML samples has been deposited at GEO database (GSE34185).
directly added into leukemic cells and these cells were washed with PBS 24–48 h after infection.

Viability and proliferation assays. These experiments were conducted as described previously27,28 with some modifications. For apoptosis and viability assays, 48 h after transfection, cells were collected and seeded with requested concentration. Cell apoptosis and viability were assessed using ApoLive-Glo Multiplex Assay Kit (Promega, Madison, WI) following the corresponding manufacturer's manuals. For cell proliferation assays, per million cells were electrotransfected with 1.5 µg plasmid. Twenty-four hours after transfection, cells were seeded in 96-well plates at the concentration of 10,000 cells per well. Cell numbers were counted at the indicating days.

Plasmid construction. The home-prepared expression vector of miR-22, that is MSCV-PiG-miR-22, was amplified by PCR using primers: forward: 5’-GGCTCGAGTGCAGCTCCCGGCCT-3’ and reverse: 5’-GGGGAATTCCTACTCACTTACAC-3’. and was subsequently cloned into the XhoI and EcoRI sites of the retrovirus vector MSCV-PiG (that is, MSCV-puro-IRES-GFP vector; bearing GFP gene), a kind gift from Drs Gregory Hannon, Scott Hammond and Lin He (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY). The other miR-22 construct, the MSCV-PiG-miR-22 expression vector, was a gift from Dr Pier P. Pandolfini (Harvard Medical School, Boston, MA). The MSCV-neo-PIG plasmid was kindly provided by Dr Scott Armstrong (Harvard Medical School, Boston, MA). The MSCV-FL'I3-CDs plasmid was a gift from Dr Michael Cleary (Stanford University, Stanford, CA). The p1005-Cri1 plasmid was provided by Dr Sheena Josselyn (The Hospital for Sick Children, Toronto, Canada) and sub-cloned into MSCV-PiG vector. The MSCV-PiG-MYCP plasmid was PCR-amplified using primers: forward: 5’-GCGAGTACGAGTGGACACAG-3’ and reverse: 5’-CGCGGATCCATTATCACCGCC-3’. The 3’UTR constructs of CRC1 and FL'I3 containing putative binding sites for miR-22 were amplified by PCR from human normal bone marrow mononuclear cells using the primers below: CRC1-3’UTR: forward: 5’-GCCTTACCGTACCGCACTTGG-3’ and reverse: 5’-GCCGACATTCTATCCAGCAAG-3’. The 3’UTR constructs of CRC1 and FL'I3. The miR-22 promoter region (~ 1100 bp; as was identified by Bar et al.49) was PCR-amplified using primers: forward: 5’-AATAATGACGTGCAAG-3’ and reverse: 5’-AATAATGATATCCTTTAGCTGGGTC-3’ and then cloned into pMiR-REPORT Luciferase miRNA Expression Reporter Vector (Ambion, Austin, TX). Site mutations were induced by PCR based on the sequence shown above. 35 bp downstream of the binding site of miR-22 for 3’UTR of CRC1 and FL'I3. The miR-22 promoter region (~ 1100 bp; as was identified by Bar et al.49) was PCR-amplified using primers: forward: 5’- GGCCGCCACTAGTAGGAACAATTTAGTTTTAAG-3’ and reverse: 5’-GCCGCCACTAGTAGGAACAATTTAGTTTTAAG-3’. The 3’UTR constructs of CRC1 and FL'I3 containing putative binding sites for miR-22 were amplified by PCR from human normal bone marrow mononuclear cells using the primers below: CRC1-3’UTR: forward: 5’-GCCTTACCGTACCGCACTTGG-3’ and reverse: 5’-GCCGACATTCTATCCAGCAAG-3’. The 3’UTR constructs of CRC1 and FL'I3. The miR-22 promoter region (~ 1100 bp; as was identified by Bar et al.49) was PCR-amplified using primers: forward: 5’- AATAATGACGTGCAAG-3’ and reverse: 5’-AATAATGATATCCTTTAGCTGGGTC-3’ and then cloned into the pGL4.15 Luciferase Reporter Vector (Promega). The MSCV-TetI construct was as described previously48, 49, and all the above insertions were confirmed by DNA sequencing.

Chromatin immuno precipitation. ChIP assay was performed as described previously14,17, with SABiosciences Corporation’s ChampionChIP One-Day kit (Qiagen, Frederick, MD) following the manufacturer’s protocol, with some modifications. Briefly, pellets of 5 x 10³ cells were treated with fresh fixing buffer (1% formaldehyde) for 10 min at 37°C to crosslink DNA and proteins. The reaction was terminated by the addition of stop buffer and incubated at room temperature for 5 min. After cell lysis, the cross-linked chromatin was sonicated to an average size of ~ 500 bp and was immunoprecipitated with antibodies against TET1, GFI1 (Santa Cruz Biotechnology Inc., Santa Cruz, CA), the N’-terminal portion of MLL (MLL-N), the C’-terminal of MLL (MLL-C), H3K27Me3, H3K4Me3, RNA polymerase II, EZH2, SIN3A or IgG (Abcam, Cambridge, MA). Purified ChIP DNA was amplified by real-time qPCR using specific primers targeting the CpG-enriched upstream region of human miR-22: forward: 5’-GTTGTTGAGCTGTGATG-3’; reverse: 5’-CGCTCCACCTCTTTATATAA-3’; and mouse miR-22: forward: 5’-TGATGGGCGGCGGATGA-3’; reverse: 5’-CAAGAGGCTTGGCAATGAA-3’. Bisulfite sequencing. TPH-1 cells were treated with 1 µM ATRA or DMSO control for 72 h. Genomic DNA was extracted thereafter. One microgram of genomic DNA was then applied to MethylCode Bisulfite Conversion Kit (Invitrogen) following the manufacturers’ instructions. After bisulfite conversion, 3 µl of purified converted DNA was PCR amplified using ZymoTaQ DNA polymerase (Zymo Research, Irvine, CA) following the manufacturers’ instructions. The PCR products were purified using PCR purification kits (Qiagen) and sent for sequencing. Primers applied in the PCR assays: miR-22 promoter: forward: 5’-TTTGTGTATTGTGTGTGTGTGT-3’; reverse: 5’-ACAACCCCCCCTTCTATTAAAATG-3’; SLc041a. forward: 5’-TGTGGTGTGTGTGTGTGTGTGTGTGTGTGT-3’; reverse: 5’-AAAAATACCAAAATACCATCCTCC-3’.

Luciferase reporter and mutagenesis assays. Luciferase reporter and mutagenesis assays were conducted as described previously27,28 with some modifications. Briefly, for transfection, HEK-293T cells were plated in 96-well plates at a concentration of 6,000 cells per well in triplicate for each condition. For the miR-22 targeting CRC1 and FL'T3 experiments, after overnight incubation, cells were transfected with 20 ng of the pMiR-REPORT bearing the CRC1 or FL'T3 UTR or the 3’UTRs with miR-22 binding site mutations, and 25 ng of MSCV-miR-22 or an empty MSCV vector using Effective Transfection Reagent (Qiagen) according to the manufacturer’s protocol. pMiR-REPORT Beta-galactosidase Reporter Control Vector (Ambion) (1 ng) was co-transfected for transfection efficiency control in all transfections. Cells were lysed and firefly luciferase and β-galactosidase activities were detected using Dual-Light Combined Reporter Gene Assay System (Applied Biosystems, Foster City, CA) 48 h post transfection. Firefly luciferase activity was normalized to β-galactosidase activity for each transfected well. For the Tet1 targeting miR-22 study, HEK-293T cells were transfected with 20 ng MSCV-Tet1 construct and/or 20 ng pGL4.15-miR-22 promoter. The succeeding luciferase reporter assay was conducted according to the manufacturer’s protocol (Promega). Each experiment was performed in triplicate and repeated three times.

Packaging of retroviral vectors and CFA assays. Those experiments were conducted as described previously27,28 with some modifications. Briefly, retroviral vectors were co-transfected with pL-Ceco packaging vectors (IMGENEX, San Diego, CA) into HEK-293T cells using Lipofectamine 2000 (Invitrogen) following the manufacturers’ instructions. For cell proliferation assays, per million cells were electrotransfected with 1.5 µg plasmid. Twenty-four hours after transfection, cells were collected and seeded with requested concentration. After bisulfite conversion, 3 µl of purified converted DNA was PCR amplified using ZymoTaQ DNA polymerase (Zymo Research, Irvine, CA) following the manufacturers’ instructions. The PCR products were purified using PCR purification kits (Qiagen) and sent for sequencing. Primers applied in the PCR assays: miR-22 promoter: forward: 5’-TTTGTGTATTGTGTGTGTGTGT-3’; reverse: 5’-ACAACCCCCCCTTCTATTAAAATG-3’; SLc041a. forward: 5’-TGTGGTGTGTGTGTGTGTGTGTGTGTGTGT-3’; reverse: 5’-AAAAATACCAAAATACCATCCTCC-3’.
streptomycin, supplemented with a supply of SCF, IL-3 and IL-8. The cells were selected with both puroycin and G418 for 7 days before transplantation. After that, 1.5 x 10^6 donor cells plus a radioprotective dose of whole bone marrow cells (1 x 10^6; freshly collected from a B6.SJL (CD45.1) mouse) were injected into per lethally irradiated (960 rads) 8-10-week-old B6.SJL recipient mouse. For primary leukaemic mice (CD45.1) shown in Fig. C (CD45.2) BM progenitor cells or miR-22^-/- BM progenitor cells were retrovirotransduced with MSCV-PIG-AE9a. The cells were selected with puroycin for 7 days before transplantation. After that, 1.5 x 10^6 donor cells plus a radioprotective dose of whole bone marrow cells (1 x 10^6; freshly collected from a B6.SJL (CD45.1) mouse) were injected into per lethally irradiated (960 rads) 8-10-week-old B6.SJL recipient mouse.

For secondary BMT assays shown in Fig. 2a,c, leukemic BM cells isolated from the primary leukaemic mice bearing MLL-AF9, AML1-ETO (A9a) or FLT3-ITD/NPM1^-/- were retrovirally transduced with MSCV-PIG- MSCV-neo (as control) (that is, MA9-AML + CLL), MSCV-neo-miR22 (that is, MA9-AML + miR-22, AE9a-AML + miR-22, or FLT3-ITD/NPM1^-/- + miR-22). Similarly, retrovirotransduced cells were plated into methylcellulose medium supplied with puroycin and G418 (for selection) and a set of cytokines to form colonies. Seven days later, the colony cells were collected and washed, and then were transplanted into sub-lethally irradiated (480 rads) 8-10-week-old C57BL/6 (CD45.2) secondary recipient mice via tail vein injection, with the dosage of 1.5 x 10^5 donor cells per recipient mouse.

Preparation of Cys5.5-labelled G7 PAMAM dendrimers. G7 PAMAM dendrimers obtained from Sigma-Aldrich were purified and fluorescence labelled using an N-hydroxysuccinimide ester of cyanine5.5 (NHS-Cy5.5) (Lumiprobe Corporation, Hallandale Beach, FL), as has been previously reported46. In brief, G7 PAMAM dendrimers (38.7 mg, 332 nmol) were dissolved in 2 ml ddH2O, to which 0.5 mg/kg -NHS-Cy5.5 was added dropwise, and the reaction allowed to proceed under vigorous stirring for 24 h at room temperature. Excess NHS-C5.5 was removed using an Amicon Ultra-15 Centrifugal Filter Unit (MWCO 10,000, Millipore, Billerica, MA) at 4,000 r.p.m. and 4°C for 20 min and washing with ddH2O 10 times. Remaining product was re-dissolved in ddH2O and hypochlorite (0.2% final concentration) was added to the following for 1 h. The dendrimers were lyophilized, resulting in G7-Cy5.5-NH2. All products were characterized by 1H NMR (300 MHz) for purity.

For xeno-BMT, the recipient mouse was injected with PBS control, or miR-22^-/- miR-22 mutant RNA oligos formulated with G7-NH2 nanocarriers, i.e., every other day, until the PBS-treated group all died of leukaemia. The maintenance and monitoring of mice. C57BL/6 (CD45.2), B6.SJL (CD45.1) mice were purchased from the Jackson Lab (Bar Harbor, ME, USA) or Harlan Laboratories, Inc (Indianapolis, IN, USA). NSGS (NSG-SGM3) immunodeficient mice49 and miR-22^-/- (ref. 20) mice were purchased from the Jackson Lab and were bred and maintained in house. Both male and female mice were used for the experiments. All laboratory mice were maintained in the animal facility at the University of Chicago and the University of Cincinnati. The maintenance, monitoring and end-point treatment of mice were conducted as described previously47,48,49.

Western blotting. Western blotting was conducted as described previously14,17,23 with some modifications. Briefly, transiently transducted MONOMAC-6 cells were collected and lysed with RIPA buffer (Thermo Scientific, Rockford, IL). Proteins from the lysate were fractionated by electrophoresis through 4–15% polyacrylamide gels (Bio-rad, Hercules, CA) and transferred to polyvinylidene fluoride membranes using tris-glycine transfer buffer (Thermo Scientific). Blots were incubated with IRDye 800CW-conjugated or 700CW-conjugated antibody and infrared fluorescence images were obtained with the Odyssey infrared imaging system (Li-Cor Bioscience, Lincoln, NE). 100-200 ng ml^-1 anti-CRT1C1, anti-FLT3, anti-MYCP, anti-BMI1, anti-CD-K6, anti-FGK1 (Santa Cruz Biotechnology Inc.), anti-STAT3 (Abcam) and anti-GAPDH (Thermo Scientific) antibodies were used to detect corresponding proteins. Original scans can be found as Supplementary Figs 7–9.

**Target gene prediction.** Putative targets of miR-22 was predicted by TargetScan (http://www.targetscan.org).45

**DNA copy-number analysis of miR-22 gene locus in human AML.** The copy-number data of AML from The Cancer Genome Atlas (TCGA) project were downloaded from Broad Firehose’s analyses runs. The putative copy-number calls were determined using GISTIC 2.0 (ref. 65). The latest GISTIC analyses data were obtained using the following shell command: 'firehose_get -o 'GISTIC' analyses latest LAML'. The files of Affymetrix SNP 6.0 data for GSE21107 (ref. 66) and GSE23452 (ref. 67) were downloaded from NCBI GEO. The raw data were preprocessed using PennCNV68. Then ASCAT69 was used to obtain the copy-number alterations. The putative copy-number calls were determined using GISTIC 2.0 as described above.

**Software and statistical analyses.** The miRNA and gene/exon array data analyses were conducted using the Bioconductor software suite (Partek Inc.), TIGR Multiarray Viewer software package (TMeV version 4.6; TIGR, Rockville, MA)70 and/or Bioconductor R packages. The miRNA-gene expression correlation was analysed by use of Partek Genomics Suite (Partek Inc.). The t-test, Kaplan–Meier method and log-rank testand so on were performed with WebPageTools (TIGR, Rockville, MA), GraphPad Prism version 5.00 (GraphPad Software, San Diego, CA) and/or Partek (Genomics Suite, Partek Inc.). The P values <0.05 were considered as statistically significant. Significance analysis of microarrays, embedded in the TMEV package (TIGR, Rockville, MA), was used to identify the genes that are significantly (q <0.05; false discovery rate, FDR <0.05) dysregulated in the MLL-AF9-mediated mouse leukaemia samples or human AML samples relative to the normal controls. Pearson correlation was used in the analysis of the correlation between miR-22 and candidate genes in expression. The list of transcription factors that have evolutionarily conserved binding sites within the miR-22 promoter region (that is, the adjacent upstream CpG island) was obtained by searching UCSC Genome Browser (https://genome.ucsc.edu/cgi-bin/hgTcNc/dgh?hg190:position=chr17:1614689-1623188&hgsid=467668777_3TvT3r4a0ZIt7FDAaA1AaYAr2B6).

**Data availability.** Data referenced in this study are available in The Gene Expression Omnibus. The Affymetrix exon array data and the microarray data are available under accession codes GSE34184 and GSE30285. Additional exon array data are available under accession code GSE27370. The mouse microarray data is available under accession code GSE34185. The AML samples collected by the German AMLCG study group are available under accession code GSE37642. The miRNA gene expression data of 183 adult de novo AML cases is available from the TCGA.

**References.**

1. Zeisig, B. B., Kulasekararaj, A. G., Mufti, G. J. & So, C. W. Snapshot: acute myeloid leukemia. Cancer Cell 22, 698–498.e1 (2012).
2. Estey, E. & Dohner, H. Acute myeloid leukemia. Lancet 368, 1894–1907 (2006).
3. Grimwade, D. & Mruzek, K. Diagnostic and prognostic value of cytogenetics in acute myeloid leukemia. Hematol. Oncol. Clin. North Am. 25, 1135–1161 vii (2011).
4. Chen, J., Odenike, O. & Rowley, J. D. Leukaemogenesis: more than mutant MLL-AF9 genomes. Dual functions of Tet1 in transcriptional regulation in mouse leukemia. Blood 116, 389–391 (2011).
5. Williams, K. et al. 1 miR-22 or miR-22 mutant RNA oligos formulated with G7-NH2 nanocarriers, i.e., every other day, until the PBS-treated group all died of leukaemia.
27. Haferlach, C.

14. Song, S. J.

28. Armstrong, S. A.

29. Xiong, J., Du, Q. & Liang, Z. Tumor-suppressive microRNA-22 inhibits the

16. Song, J. S.

29. Xiong, J., Du, Q. & Liang, Z. Tumor-suppressive microRNA-22 inhibits the

12. Ko, M. et al. Impaired hydroxylation of 5-methylcytosine in myeloid cancers with

15. Song, J. S. et al. Oncogenic microRNA-miR-22 targets the TET2 tumor suppressor to promote hematopoietic stem cell self-renewal and transformation. Cell Stem Cell 13, 87–101 (2013).

17. Jiang, X. et al. Blockade of miR-150 maturation by MLL-fusion/MYC/ENL-28 is required for MLL-associated leukemia. Cancer Cell 22, 524–532 (2012).

18. Lewis, B. P., Burge, C. B. & Bartel, D. P. Conserved seed pairing, often flanked by adenosines, indicates that thousands of human genes are microRNA targets. Cell 120, 15–20 (2005).

19. Yan, M. et al. A previously unidentified alternatively spliced isoform of 5(8)21 transcript promotes leukemogenesis. Nat. Med. 12, 945–949 (2006).

20. Gurha, P. et al. Targeted deletion of microRNA-22 promotes stress-induced cardiac dilation and contractile dysfunction. Circulation 125, 2751–2761 (2012).

21. He, L. et al. Changes in TET, Cpr, and Huang, H., Hurst, L. D. & Chen, J. Young intragene mirRNAs are less coexpressed with host genes than old ones: implications of miRNA-host gene coevolution. Nucleic Acids Res. 40, 4002–4012 (2012).

22. Ley, T. J. et al. Genomic and epigenomic landscapes of adult de novo acute

23. Li, Z. et al. miR-196b directly targets both HOXA9/MEIS1 oncogenes and FAS tumor suppressor in MLL-rearranged leukemia. Nat. Commun. 2, 689 (2012).

24. Wang, Z. et al. GSK-3 promotes conditional association of CREB and its

25. Cheng, J. C. et al. CREB is a critical regulator of normal hematopoiesis and leukemogenesis. Blood 111, 1182–1192 (2008).

26. Sandoval, S., Pigazzi, M. & Sakamoto, K. M. CREB: a key regulator of normal and neoplastic hematopoiesis. Adv. Hematol. 2009, 634292 (2009).

27. Haeflacher, C. et al. ETV6 rearrangements are recurrent in myeloid malignancies and are frequently associated with other genetic events. Genes Chromosomes Cancer 51, 328–337 (2012).

28. Armstrong, S. A. et al. Inhibition of FLT3 in MLL. Validation of a therapeutic target identified 2011 in an expression based classification. Cancer Cell 3, 173–183 (2003).

29. Takahashi, S. Downstream molecular pathways of FLT3 in the pathogenesis of acute

30. Piek, T. et al. Requirement for Cdk6 in MLL-rearranged acute

31. Smith, L. et al.Functional crosstalk between Bmi1 and MLL/Hoxa9 axis in establishment of normal hematopoietic and leukemic stem cells. Cell Stem Cell 8, 649–662 (2011).

32. Placke, T. et al. The HMG-I oncogene causes highly penetrant, aggressive lymphoid malignancy in transgenic mice and is overexpressed in human leukemia. Cancer Res. 64, 3371–3375 (2004).

33. Jacoby, M. A. & Walter, M. J. Detection of copy number alterations in acute

34. Ninomiya, S. et al. Integrated analysis of gene copy number, copy neutral LOH, and microRNA profiles in adult acute lymphoblastic leukemia. Cytogetet. Genome Res. 136, 246–255 (2012).

35. Konishi, H. et al. Detailed characterization of a homozygously deleted region corresponding to a candidate tumor suppressor locus at distal 17p13.3 in human lung cancer. Oncogene 22, 1892–1905 (2003).

36. Sankar, M. et al. Identification of a commonly deleted region at 17p13.3 in leukemia and lymphoma associated with 17p abnormality. Leukemia 12, 510–516 (1998).

37. Chattopadhyay, P. et al. Loss of heterozygosity of a locus on 17p13.3, independent of p53, is associated with higher grades of astrocytic tumours. Oncogene 15, 871–874 (1997).

38. Li, Z. et al. Identification of a 24-gene prognostic signature that improves the European LeukemiaNet risk classification of acute myeloid leukemia: an international collaborative study. J. Clin. Oncol. 31, 1172–1181 (2013).

39. Bar, N. & Dukstein, R. miR-22 forms a regulatory loop in PTEN/AKT pathway and modulates signaling kinetics. PLoS ONE 5, e10859 (2010).

40. Drach, J., Lopez-Rodriguez, G., McQueen, T., Andreeff, M. & Mehta, K. Induction of differentiation in myeloid leukemia cell lines and acute promyelocytic leukemia cells by liposomal all-trans-retinoic acid. Cancer Res. 63, 2100–2104 (1993).

41. Duan, Z. & Horwitz, M. Targets of the transcriptional repressor oncoprotein Gfi-1. Proc. Natl Acad. Sci. USA 100, 5932–5937 (2003).

42. Pack, D. W., Hoffman, A. S., Pun, S. & Stayton, P. S. Design and development of polymers for gene delivery. Nat. Rev. Drug Discov. 4, 581–593 (2005).

43. Wunderlich, M. et al. AML xenograft efficiency is significantly improved in NOD/SCID-IL2Rγ mice constitutively expressing human SCF, GM-CSF and IL-3. Leukemia 24, 1785–1807 (2010).

44. Cartron, P. F., Nadaud, A., Lepape, F., Lalier, L., Gardie, B. & Valfette, F. M. Identification of TET1 partners that control its DNA-methylating function. Cancer Cell 4, 235–241 (2013).

45. Saleque, S., Kim, J., Roone, H. M. & Orkin, S. H. Epigenetic regulation of hematopoietic differentiation by Gfi-1 and Gfi-1b is mediated by the cofactors COREST and LSD1. Mol. Cell 27, 562–572 (2006).

46. Thambirajah, R. A. et al. Gfi1 proteins orchestrate the emergence of haematopoietic

47. Abdel-Wahab, O. et al. Genetic characterization of TET1, TET2, and TET3 alterations in myeloid malignancies. Blood 114, 144–147 (2009).

48. Zubler, J. et al. RNAi screen identifies Brd4 as a therapeutic target in acute

49. Huang, M. J., Cheng, Y. C., Liu, C. R., Lin, S. & Liu, H. H. A small-molecule c-Myc inhibitor, 1008-F4, induces cell-cycle arrest, apoptosis, and myeloid differentiation of human acute myeloid leukemia. Exp. Hematol. 34, 1480–1489 (2006).

50. Ling, H., Fabri, M. & Calin, G. A. MicroRNAs and other non-coding RNAs as targets for anticancer drug development. Nat. Rev. Drug Discov. 12, 847–865 (2013).

51. Li, Z. et al. Distinct microRNA expression profiles in acute myeloid leukemia with common translocations. Proc. Natl Acad. Sci. USA 105, 15535–15540 (2008).

52. Irizarry, R. A. et al. Summary of Affymetrix GeneChip probe level data. Nucleic Acids Res. 31, e15 (2003).

53. Poliseno, L. et al. Identification of the miR-10b–25 microRNA cluster as a proto- oncogenic PTEN-targeting intron that cooperates with its host gene MCM7 in transformation. Sci. Signal. 3, ra29 (2010).

54. Krivtsov, A. V. et al. Transformation from committed progenitor to leukemia stem cell initiated by MLL-AF9. Nature 494, 818–822 (2006).

55. Lemieux, T. et al. The HMG-I oncogene causes highly penetrant, aggressive lymphoid malignancy in transgenic mice and is overexpressed in human leukemia. Cancer Res. 61, 2107–2114 (2001).

56. Smith, L. et al. Functional crosstalk between Bmi1 and MLL/Hoxa9 axis in establishment of normal hematopoietic and leukemic stem cells. Cell Stem Cell 8, 649–662 (2011).

57. Wu, X. et al. Tet and TDG mediate DNA demethylation essential for mesenchymal-to-epithelial transition in somatic cell reprogramming. Cell Stem Cell 14, 512–522 (2014).

58. Delhommeau, F. et al. Mutation in TET2 in myeloid cancers. N. Engl. J. Med. 360, 2289–2301 (2009).

59. Ko, M. et al. Impaired hydroxylation of 5-methylcytosine in myeloid cancers with

60. Fang, J. et al. Identification of a 24-gene prognostic signature that improves the European LeukemiaNet risk classification of acute myeloid leukemia: an international collaborative study. J. Clin. Oncol. 31, 1172–1181 (2013).

61. Bar, N. & Dukstein, R. miR-22 forms a regulatory loop in PTEN/AKT pathway and modulates signaling kinetics. PLoS ONE 5, e10859 (2010).
Acknowledgements

Special thanks to the late Dr Janet Rowley for her long-term support. We also thank Drs Pier P. Pandolfi, Sheena Josselyn, Dong Er Zhang, Scott Armstrong, Michael Cleary, James Mulloy, Robert Slany, Lin He and Michael Thirman for providing mouse models, retroviral constructs or cell lines, and the German AMLCG study group for AML microarray data. This work was supported by Leukemia & Lymphoma Society (LLS) Translational Research Grant (J.C.), the National Institutes of Health (NIH) R01 Grants CA178454, CA182528 and CA127277 (J.C.), American Cancer Society (ACS) Research Scholar grant (J.C.), The University of Chicago Committee on Cancer Biology (CCB) Fellowship Program (X.J.), LLS Special Fellowship (Z.L.), Gabrielle’s Angel Foundation for Cancer Research (J.C., Z.L., X.J. and H.H.) and Intramural Research Program of National Human Genome Research Institute, NIH (A.G.E. and P.P.L.). C.H. is an investigator of the Howard Hughes Medical Institute.

Author contributions

X.J. and J.C. conceived the project and designed the research. X.J., S.A., J.B., M.Y., Z.Z., P.C., H.H., B.U., C.H., S.G., H.W., Y.W., Y.L., J.S., S.L., A.G.E., Y.Y., J.S., M.R.N., Z.L. and J.C. performed experiments and/or data analyses; X.J., H.H., A.G.E., R.A.L., M.M.L.L., T.H., S.K.R., P.P.L., J.J., J.Z., Z.L., C.H., S.H. and J.C. contributed reagents/analytic tools and/or grant support; X.J. and J.C. wrote the paper. All authors discussed the results and commented on the manuscript.

Additional information

Accession codes: The microarray data have been deposited in the Gene Expression Omnibus (GEO) database under the accession code GSE34184, GSE30285, GSE27370, GSE34185, and GSE37642.

Supplementary Information accompanies this paper at http://www.nature.com/naturecommunications

Competing financial interests: The authors declare no competing financial interests.

How to cite this article: Jiang, X. et al. miR-22 has a potent anti-tumour role with therapeutic potential in acute myeloid leukaemia. Nat. Commun. 7:11452 doi: 10.1038/ncomms11452 (2016).