A Model System for Studying the DNMT3A Hotspot Mutation (DNMT3A<sup>R882</sup>) Demonstrates a Causal Relationship between Its Dominant-Negative Effect and Leukemogenesis

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Abstract

Mutation of DNA methyltransferase 3A at arginine 882 (DNMT3A<sup>R882mut</sup>) is prevalent in hematologic cancers and disorders. Recently, DNMT3A<sup>R882mut</sup> has been shown to have hypomorphic, dominant-negative, and/or gain-of-function effects on DNA methylation under different biological contexts. However, the causal role for such a multifaceted effect of DNMT3A<sup>R882mut</sup> in leukemogenesis remains undetermined. Here, we report TF-1 leukemia cells as a robust system useful for modeling the DNMT3A<sup>R882mut</sup>-dependent transformation and for dissecting the cause–effect relationship between multifaceted activities of DNMT3A<sup>R882mut</sup> and leukemic transformation. Ectopic expression of DNMT3A<sup>R882mut</sup> and not wild-type DNMT3A promoted TF-1 cell transformation characterized by cytokine-independent growth, and induces CpG hypomethylation predominantly at enhancers. This effect was dose dependent, acted synergistically with the isocitrate dehydrogenase 1 (IDH1) mutation, and resembled what was seen in human leukemia patients carrying DNMT3A<sup>R882mut</sup>. The transformation- and hypomethylation-inducing capacities of DNMT3A<sup>R882mut</sup> relied on a motif involved in heterodimerization, whereas its various chromatin-binding domains were dispensable. Mutation of the heterodimerization motif that interferes with DNMT3A<sup>R882mut</sup> binding to endogenous wild-type DNMT proteins partially reversed the CpG hypomethylation phenotype caused by DNMT3A<sup>R862mut</sup>, thus supporting a dominant-negative mechanism in cells. In mice, bromodomain inhibition repressed gene-activation events downstream of DNMT3A<sup>R882mut</sup>-induced CpG hypomethylation, thereby suppressing leukemogenesis mediated by DNMT3A<sup>R882mut</sup>. Collectively, this study reports a model system useful for studying DNMT3A<sup>R882mut</sup> shows a requirement of the dominant-negative effect by DNMT3A<sup>R882mut</sup> for leukemogenesis, and describes an attractive strategy for the treatment of leukemias carrying DNMT3A<sup>R882mut</sup>.

Significance: These findings highlight a model system to study the functional impact of a hotspot mutation of DNMT3A at R882 in leukemia.

Introduction

Aberration of the epigenomic state is commonly utilized by tumors to alter gene-expression programs and to gain growth advantage (1, 2). Sequencing of primary cancer samples has identified recurrent mutations of genes involved in epigenomic regulation (2, 3). In particular, somatic mutation of DNA methyltransferase 3A (DNMT3Amut) was detected in a wide range of blood cancers including 20% to 30% of acute myeloid leukemia (AML; refs. 3–7), as well as elderly individuals with clonal hematopoiesis (8–11).

DNMT3A forms a complex with accessory cofactors, serving as one of the major de novo DNA methyltransferases (12–14). DNMT3A (Supplementary Fig. S1A) harbors various motifs, which include a N-terminal domain (NTD) shown to interact with transcription factors (7), a Pro–Trp–Pro motif domain shown to engage methylated histone H3 lysine 36 (H3K36me; ref. 15), an ATRX–DNMT3–DNMT3L (ADD) domain known to bind specifically to the unmodified histone H3 lysine 4 (H3K4me0; refs. 14, 16), and a C-terminal catalytic domain that methylates cytosine bases, especially those in the CpG dinucleotides (12–14). Cellular contexts such as interacting partners and chromatin states are crucial for exquisite modulation of DNMT3A’s genomic targeting and enzymatic functions. For
example, DNMT3A adopts an auto-inhibitory conformation due to interaction between its ADD and methyltransferase domains, and such self-inhibition is released upon engagement of ADD to histone tails with H3K4me0 (14). The methyltransferase domain, which binds DNA using specified protein motifs (12), also contains crucial interfaces for forming DNMT dimers, tetramers, and/or oligomers to regulate the methylation activities (13, 14, 17–22).

DNMT3Amut is primarily heterozygous in AMLs and shows a mutational hotspot at the Arg882 residue (DNMT3AArg882mut), which accounts for 50% to 60% of identified DNMT3Amut in AMLs (2, 3, 7, 23). Because of prevalence and clinical relevance of DNMT3AArg882mut in blood cancer and clonal hematopoiesis, considerable progress was made in understanding the mechanisms by which DNMT3AArg882mut mediates transformation. DNMT3AArg882mut is detected in hematopoietic stem/progenitor cells (HSPC) of apparently healthy elderly individuals, supporting its role as a preleukemic founder mutation that provides initial selective advantage of mutant HSPC clones (8–11). We and others have shown that a cooperating genetic lesion is required for DNMT3AR882mut or Dnmt3a loss to induce fully-blown leukemias in mice (24–28). Biochemically, partial loss-of-function, dominant-negative, and gain-of-function effects have all been associated with DNMT3AR882mut. In 2018, DNMT3AR882mut is a hypomorphic allele and purified DNMT3AR882wt enzymes reduced methyltransferase activity on CpG substrates in vitro (4, 12, 29, 30). Particularly, the structure of the DNMT3A–DNMT3L–CpG complexes was recently solved, which revealed that the residue R882 forms interactions with both DNA substrates and a so-called "Target Recognition Domain" loop, a DNMT3A motif critically involved in engaging CpG dinucleotides (12). Furthermore, the dominant-negative effect was proposed for DNMT3AR882mut (29, 31). Here, DNMT3AR882mut associates with wild-type DNMT3A and DNMT3B, presumably interfering with the formation, stability, DNA-engaging, and/or DNA-methylating activity of the whole complex. The combined hypomorphic and dominant-negative effects of DNMT3AR882mut may explain focal CpG hypomethylation seen in leukemias harboring DNMT3AR882mut. On the other hand, recent studies reported an altered substrate preference of DNMT3AR882mut towards CpG sites with specific flanking sequence, which is termed as the gain-of-function effect of DNMT3AR882mut (32). Theoretically, these above effects of DNMT3AR882mut are not mutually exclusive, indicating that DNMT3AR882 mutations cause redistribution of DNA methylation in the cancer genome. However, it remains elusive by what effect(s) DNMT3AR882mut contributes to transformation.

This study aims to determine a causal relationship between the multifaceted effect of DNMT3AR882mut and leukemic transformation. Previously, the systems reported for studying the leukemia-promoting functions of DNMT3AR882mut used retrovirus-based or knock-in expression of DNMT3AR882mut, often in combination with other mutations, in HSPCs (24–28), which represents a challenge to perform cause–effect relationship studies of DNMT3AR882mut. We here report a leukemia cell line, TF-1 cells, as a straightforward, robust cell model for assaying the DNMT3AR882mut-dependent leukemic transformation and, more importantly, for dissecting the molecular basis by which DNMT3AR882mut mediates transformation. We show that enforced expression of DNMT3AR882mut alone, but not DNMT3AWT, is sufficient to induce TF-1 cell transformation characterized by cytokine-independent growth and arrested cell differentiation. In TF-1 cells, DNMT3AR882mut induces hypomethylation of CpG sites that significantly overlap those found in primary AMLs with DNMT3AArg882mut. With this model system, we also demonstrated the dosage effect of DNMT3AR882mut on transformation, as well as cooperativity between DNMT3AR882mut and IDH1 mutation, the 2 lesions coexisting in human leukemias. Importantly, this system has allowed us to systematically analyze dependency of various functional motifs within DNMT3AR882mut during transformation. Here we identified a heterodimerization domain (also known as DNMT3A’s heterotetramer or FF interface; refs. 13, 18–21), and not its catalytic and chromatin-binding domains, to be essential for TF-1 cell transformation. The mutation of the heterodimerization interface interfered with interaction of DNMT3AR882mut with endogenous wild-type DNMT proteins and partially reversed CpG hypomethylation caused by DNMT3AR882mut supporting a dominant-negative mechanism that underlies transformation. Finally, we found the bromodomain inhibitor suppressed the gene-activation programs associated with DNMT3AR882mut-induced hypomethylation. Collectively, this study describes a model system for studying the DNMT3AR882mut-related leukemogenesis, demonstrates a cause–effect relationship between the dominant-negative effect and DNMT3AR882mut-mediated transformation, and provides an attractive therapeutic means for treatment of leukemias carrying DNMT3AR882mut.

Materials and Methods

Cell lines

The human erythroleukemic cell line TF-1 (ATCC #CRL-2003) is cultured in the RPMI1640 medium (Invitrogen) containing 10% of FBS and 2 ng/ml of recombinant human GM-CSF (R&D Systems) as described (12). Authentication of identities of parental and derived cell lines was ensured by the Tissue Culture Facility affiliated to UNC Lineberger Comprehensive Cancer Center with the genetic signature profiling and fingerprinting analysis. Every month, a routine examination of cell lines in culture for any possible mycoplasma contamination was performed using commercially available detection kits (Lonza). Cells with less than 10% of passages were used in the study.

Coimmunoprecipitation

Coimmunoprecipitation was carried out as previously described (33).

DNA methylation array and data analysis

Genomic DNA was extracted using the DNeasy Blood & Tissue Kit (Qiagen), followed by bisulfite-conversion using the EZ DNA Methylation-Gold Kit (Zymo Research). DNA methylation profiling was performed by the UNC Genomics Core using the Illumina HumanMethylation450 BeadChip (Illumina) according to the manufacturer’s instructions. Methylation data were then subject to background subtraction and control normalization by executing preprocessillumina in the R “minfi” package (34). Differentially methylated CpGs were identified using dmpFinder in a categorical mode. Methylation changes were considered significant at a q-value of less than 0.05 and a β value difference of more than 0.1. Hierarchical clustering analysis, scatter plots, and density plots were generated in R using “pheatmap,” and “ggplot2” packages as described previously (12).
Chromatin immunoprecipitation followed by deep sequencing

Collectively, we show that DNMT3AR882mut induces cytokine-independent growth (Fig. 1E, bottom; Supplementary Fig. S1F), we found that these DNMT3AR882mut induces transformation of TF-1 cells and that maintenance of this effect relationship studies of DNMT3A

Results

DNMT3AR882mut induces transformation of TF-1 cells characterized by cytokine-independent growth

To delineate the molecular underpinnings of DNMT3A R882mut-mediated leukemogenesis, we sought to identify a robust, straightforward cell transformation system that allows cause–effect relationship studies of DNMT3A R882mut. We chose to use TF-1 cells, for this leukemia line normally relies on survival-supporting cytokines to sustain cell proliferation but displays cytokine-independent growth upon acquisition of certain leukemia-related alterations such as IDH2 or TET2 mutation (38, 39). To test whether DNMT3AR882mut has transformation activities in this model, we stably expressed comparable levels of DNMT3AWT or a prevalent form of DNMT3AR882mut, Arg882His (DNMT3AR882H), into TF-1 cells (Fig. 1A insert; Supplementary Fig. S1A and S1B). In the presence of survival cytokines, TF-1 cells with either DNMT3A form showed the same rates of proliferation (Fig. 1A); however, under cytokine-poor conditions, only TF-1 cells with DNMT3AR882H, not vehicle or DNMT3AWT, demonstrated robust growth (Fig. 1B). Relative to controls, TF-1 cells with DNMT3AR882H also showed a mild but reproducible differentiation arrest in response to differentiation signals (Supplementary Fig. S1C and S1D). As some DNMT3A mutations found in hematologic cancer are combating ones such as frame-shifting, we asked whether TF-1 cell transformation can be recapitulated by DNMT3A loss. Indeed, knockdown of DNMT3A by independent shRNAs, and not mock, resulted in cytokine-independent proliferation (Supplementary Fig. S1E; Fig. 1C). Similar phenotypes were observed with other DNMT3A R882mut variants (Arg882Cys and Arg882Ser; Fig. 1D). Next, we ask whether such a transformation phenotype requires continuous expression of DNMT3A R882H. Here, a reversible expression system, which carries a pair of LoxP sites flanking the transgene (Fig. 1E, top), was used to generate cytokine-independent TF-1 lines with DNMT3AR882H. After cre-mediated deletion of DNMT3AR882mut (Fig. 1E, bottom; Supplementary Fig. S1F), we found that these cells no longer sustained cytokine-independent growth (Fig. 1F). Collectively, we show that DNMT3AR882mut induces cytokine-independent growth of TF-1 cells and that maintenance of this phenotype relies on continuous presence of DNMT3AR882mut.

DNMT3AR882mut induces a CpG hypomethylation phenotype in TF-1 cells, resembling what was seen in AML patients with DNMT3AR882mut

Human AMLs carrying DNMT3AR882mut exhibit focal DNA hypomethylation (29), a phenotype also observed in murine leukemias with DNMT3AR882mut (24). To investigate effect of DNMT3AR882mut on DNA methylation in TF-1 cells, we performed methylome profiling with Illumina Infinium HumanMethylation450 BeadChip (450K array). Out of a total of 485,512 CpG sites, we identified 6,995 (1.44%) as differentially methylated CpG probes (DMP) among TF-1 lines with DNMT3AR882mut relative to vector-expressing controls (Fig. 2A; Supplementary Table S1). Among the identified DMPs, almost all (6,956; 99.47%) showed hypomethylation and only 37 (0.53%) displayed hypermethylation (Fig. 2A and B). In contrast, the majority of DMPs in TF-1 cells expressing DNMT3AWT, relative to control, showed the increased DNA methylation (Supplementary Fig. S2A). Similar patterns of focal CpG hypomethylation were seen with DNMT3AR882C and DNMT3AR882S (Supplementary Fig. S2B). To delineate the genomic feature of DNMT3AR882mut-associated DMPs in TF-1 cells, we related the identified DMPs to 15 chromatin modifications of K562 leukemia cells (40) and found that DNMT3AR882H-associated DMPs with hypomethylation occurred preferentially at gene enhancers, and not promoters or heterochromatin (Fig. 2C). These results suggest enhancer as genomic regions predominantly affected by DNMT3AR882mut in TF-1 cells, which is consistent with previous findings seen in murine leukemias harboring DNMT3AR882H or DNMT3A knockout (24, 41, 42).

To determine clinical relevance of our above finding, we compared DNMT3AR882mut-associated DMPs found in TF-1 cells to those based on the TCGA methylome studies of patients with AML (3). Here, we found a significant overlap between hypomethylated DMPs identified from TF-1 cells and human AMLs with DNMT3AR882mut (Fig. 2D; Supplementary Table S1), as exemplified by DMPs at SH3TC2, FOXK2, and GP9 (Fig. 2E). In addition, principal component analysis (PCA) of DNMT3AR882mut-induced hypomethylated DMPs showed that the methylation pattern of TF-1 cells with DNMT3AR882mut resembled patients with DNMT3AR882mut more than those with DNMT3AWT (Supplementary Fig. S2C). Moreover, in TF-1 lines with reversible DNMT3AR882mut expression (Fig. 1E and F), we found that hypomethylation at the examined region relies on continuous presence of DNMT3AR882H (Fig. 2F; cre vs. cre–). Together, we show that DNMT3AR882H-expressing TF-1 cells carry methylation alterations shared by human AMLs, lending support for using TF-1 cells as a model to study DNMT3AR882mut.

DNMT3AR882H and IDH mutations cooperate to promote TF-1 cell transformation

In the clinic, DNMT3A and IDH1/2 mutations frequently cooccur (3) and genetic interaction between DNMT3AR882H and IDH1R132H was verified in mice (42). To query whether TF-1 cells is also suitable for studying synergy between IDH1 and DNMT3A mutations, we established TF-1 lines with expression of DNMT3AR882H, IDH1R132H, or their combination (Fig. 3A). TF-1 cells expressing either mutation showed considerable proliferation postremoval of survival cytokines; however, significantly more growth under cytokine-poor conditions was seen with cells carrying both mutations (Fig. 3B). Methylome analysis revealed that DNMT3AR882H alone and IDH1R132H alone predominantly...
induced hypo- and hypermethylation, respectively (Fig. 3C), with the commonly affected sites accounting for merely nearly 5% to 10% (Fig. 3D). These suggest that DNMT3A^{R882H} and IDH1^{R132H} target different genomic regions to induce methylation changes. Moreover, a large majority of CpGs affected by either mutation alone were no longer affected in TF-1 cells with dual mutations, whereas new events of hyper- and hypomethylation at additional CpG sites were gained by these cells (Fig. 3E). This phenomenon of "epigenetic antagonism" is similar to what was seen in AMLs carrying dual mutations of DNMT3A^{R882H} and IDH2^{R140Q} (42). Together, our results support TF-1 cells as a model useful for studying synergistic effect of DNMT3A and IDH1 mutations.

Figure 1. R882-mutated DNMT3A (DNMT3A^{R882mut}), and not the wild-type one (DNMT3A^{WT}), induces transformation of TF-1 cells characterized by cytokine-independent growth. A and B, Proliferation of TF-1 cells with stable transduction of the indicated gene in the presence (A) and absence (B) of GM-CSF. Inset in A, anti-Myc immunoblotting of tagged DNMT3A. C, Proliferation of TF-1 cells posttransduction of shRNA vector (control) or DNMT3A-targeted shRNAs under the GM-CSF–depleted culture condition. D, Proliferation of TF-1 cells with stable expression of vector, DNMT3A^{WT}, or DNMT3A^{R882mut} (DNMT3A^{R882C/S}) upon GM-CSF removal. Inset, anti-Myc immunoblotting of tagged DNMT3A. E, A vector LEGO-iG2 (top) that allows the cre-mediated depletion and thus reversible expression of DNMT3A^{R882H} as verified by immunoblotting (bottom). F, Proliferation of the indicated TF-1 lines upon GM-CSF removal after depletion of DNMT3A^{R882H} under the cytokine-rich condition.
Enrichment of DNMT3AR882H-induced hypomethylated DMPs at genomic features annotated by the ENCODE K562 ChromHMM (40).

as a control, we used a cytokine-independent outgrowth assay to correlate relative methylation of DMPs defined in A among independent TF-1 lines with stable transduction of DNMT3AR882H or DNMT3AWT, or DNMT3AR882mut (RH, RC, or RS; top) or among human AMLs with DNMT3AWT or DNMT3Amut at the R882 or non-R822 residue (bottom).

Effect of DNMT3AR882mut on CpG hypomethylation and TF-1 cell transformation is dosage dependent

DNMT3AR882mut in inducing CpG hypomethylation and TF-1 cell transformation is dosage dependent

Previously, it was shown that DNMT3AR882mut is not a null mutant and shows a mildly or partially reduced methyltransferase activity as assayed in vitro (4, 12, 20, 29, 30). Moreover, recent studies reported that, relative to DNMT3AWT, DNMT3AR882mut exhibits the increased methylation activity in vitro towards certain CpG substrates with a specific flanking sequence, implicating a gain-of-function action (32). However, to what extent gain-of-function effect of DNMT3AR882mut contributes to transformation remains undefined. First, we found that, in TF-1 cells with DNMT3AR882mut, the induced hypermethylation only represents a minor event (Fig. 2A). This is reminiscent of what was observed in murine DNMT3AR882H-expressing HSPCs with a more comprehensive enhanced reduced representation bisulfite sequencing (eRRBS) platform where hypomethylation accounted for 80.8% of detected changes (24). Next, we closely examined this published eRRBS dataset and focused on sites with hypermethylation and direct binding of DNMT3AR882H (24). Here, we did not observe significant preference towards CpG-flanking sequences (Supplementary Fig. S4A and S4B). Next, to test whether the enzymatic activity retained in DNMT3AR882mut is essential for transformation, we introduced an enzymatic-dead mutation, P709V/C710D or E756A (12, 15), into DNMT3AR882H, followed by TF-1 cell transduction (Fig. 5A). Under cytokine-poor culture conditions, neither of enzymatic-dead mutations interfered with the transforming capacity of DNMT3AR882H (Fig. 5B). These observations support that the remaining catalytic
activity harbored within DNMT3A\textsuperscript{R882mut} is not essential for transformation.

Effect of DNMT3A\textsuperscript{R882H} on TF-1 leukemia cell transformation relies on its heterodimerization interface and not chromatin-binding motifs

To dissect the basis underlying DNMT3A\textsuperscript{R882mut}-mediated transformation, we systematically mutated its functional motifs (Fig. 5C) that were known to mediate chromatin or protein–protein interactions (19). First, we found that, unlike deletion of the NTD or PWWP domains, deletion of ADD severely affected protein stability of DNMT3A\textsuperscript{R882H} (Fig. 5D). Thus, we also used point mutations of the motif that did not affect stability, D333A (D329A in mouse Dnmt3a) and D529A/D531A shown to disrupt the H3K36me3- and H3K4me0-binding of PWWP and ADD, respectively (Fig. 5E and F; refs. 14, 15). Relative to DNMT3A\textsuperscript{R882H}, all derived deletion or double mutants affecting the NTD, PWWP, or ADD domain showed comparable abilities to sustain cytokine-free proliferation in TF-1 cells (Fig. 5G and H).

DNMT3L is not expressed in AML (29) and DNMT3A may form high-order protein complexes with itself (17, 18, 20, 22, 29, 32, 43) or DNMT3B (31, 44, 45). DNMT3A\textsuperscript{R882mut} was proposed to act in a dominant-negative manner by "hijacking" and interfering with functions of wild-type DNMT3A/B proteins via a heterodimerization interface (29, 31). However, the causal role for such an effect in leukemic transformation has not been formally tested. To this end, we introduced into DNMT3A\textsuperscript{R882H} a mutation previously shown to disrupt the heterodimerization-interface-mediated interaction, i.e. F732A or Y735A (Fig. 5E; Supplementary Fig. S4C; refs. 18, 20). After transduction into TF-1 cells, the DNMT3A\textsuperscript{R882H}/Y735A double mutant showed comparable protein stability (Fig. 5F) whereas DNMT3A\textsuperscript{R882H}/F732A was unstable and excluded from subsequent analyses (Supplementary Fig. S4D).

We found that, relative to DNMT3A\textsuperscript{R882H}, DNMT3A\textsuperscript{R882H}/Y735A displayed significantly reduced abilities to sustain cytokine-free growth of TF-1 cells (Fig. 5H, orange).

To gain mechanistic insights, we further assessed interactions of DNMT3A\textsuperscript{R882H}/F732A with endogenous DNMTs, and observed that, relative to DNMT3A\textsuperscript{R882H}, DNMT3A\textsuperscript{R882H}/Y735A is defective in forming efficient interactions with wild-type DNMT3A and DNMT3B in cells (Fig. 6A), which is in agreement with in vitro analysis of mutations affecting this heterodimerization or FF interface (18, 20). Next, we examined methylation changes induced by the double mutant forms of DNMT3A\textsuperscript{R882H}. Consistent with transformation results, deletion or point mutation of PWWP and ADD did not interfere with the ability of DNMT3A\textsuperscript{R882H} to induce hypomethylation (Fig. 6B). In contrast,
methyltransferase, DNMT3A R882H. This further indicates that targeting events downstream of the induced CpG hypomethylation represents a therapeutic strategy. Our prior study of DNMT3A R882H–dependent murine AML models has revealed a marked increase of histone acetylation at gene-regulatory regions carrying DNMT3A R882H–induced CpG hypomethylation (24). Histone acetylation “readers” such as BRD4 engage acetylated histones, potentiating gene activation. Using ChIP-seq performed with DNMT3A R882H–dependent murine AML cells, we found H3K27ac and BRD4 bound at the DNMT3A R882H–associated target signature genes that we previously defined (24), which include leukemia-related “stemness” and prosurvival oncogenes such as Mni1, Mycn, and Bcl2 (Fig. 7A). Next, we queried whether BRD4 inhibition has therapeutic effect. We first examined sensitivity to I-BET151, a BRD4 inhibitor, using murine AML lines we previously established with dual DNMT3A R882H and RAS mutations (24). A strong growth inhibition effect of I-BET151 was observed, with IC50 calculated at ~100 nmol/L (Fig. 7B). Importantly, transcriptome analysis showed that BRD4 blockade resulted in downregulation of DNMT3A R882H–associated target signature genes (Fig. 7C and D; ref. 24). As well, gene ontology (GO) analysis showed that I-BET151 caused profound changes, including upregulation of apoptosis-related genes and downregulation of cell-cycle progression genes (Supplementary Fig. S6A). Gene set enrichment analysis (GSEA) further revealed that genes related to MYC targets, stemness, proliferation, DNA replication, and cancer-associated signatures were all suppressed by I-BET151 (Fig. 7E–G; Supplementary Fig. S6B).

Next, we evaluated in vivo therapeutic effect of I-BET151 on murine AMLs induced by dual DNMT3A R882H and RAS mutations. Our treatment regimen included daily subcutaneous injections of I-BET151 4 days after tumor cell injection, followed by withdraw of drug 17 days later. As shown in Figure 8A, 6 days after I-BET151 treatment, tumor size was significantly reduced in the group treated with 25 mg/kg I-BET151 (P < .05), whereas tumor growth was not affected by 10 mg/kg I-BET151. Moreover, the effect of I-BET151 was further confirmed by body weight change, as seen in Figure 8B (P < .05). Our findings indicate that targeted therapy with I-BET151 represents a promise therapeutic approach for treatment of AML patients with DNMT3A R882H mutations.
Figure 5.
Effect of DNMT3A<sup>R882H</sup> on TF-1 cell transformation is independent of its catalytic function but requires its heterodimerization interface. 
A, Immunoblot showing the level of the indicated Myc-tagged DNMT3A mutant posttransduction into TF-1 cells. B, Proliferation of TF-1 cells expressing the indicated DNMT3A mutant upon cytokine removal. C–F, Schematic representation of different truncation (C) or point mutation (E) of Myc-tagged DNMT3A<sup>R882H</sup>, with anti-Myc immunobLOTS (D and F) showing their protein levels posttransduction into TF-1 cells. EV, empty vector. G and H, Proliferation of TF-1 cells expressing the indicated truncation (G) or point mutant form (H) of DNMT3A<sup>R882H</sup> upon cytokine removal.
mutations. Compared with mock, I-BET151 treatment significantly prolonged survival of leukemic mice (Fig. 7H). Compared with mock, I-BET151 treatment also significantly delayed development of AML phenotypes such as splenomegaly, elevated counts of white blood cells and reduce counts of red blood cells (Fig. 7I and J; Supplementary Fig. S4C). Following I-BET151 treatment, less AML blasts were also observed in the bone marrow (Fig. 7J, bottom). Given that I-BET151 inhibits the DNMT3A R882H-related gene-expression programs, we next ask whether inhibition of RAS signaling has additional therapeutic effect in this model. To this end, we used trametinib, an FDA-approved inhibitor of MEK (49). Indeed, while dosing with trametinib or I-BET151 alone considerably delayed AML progression as measured by animal survival and bioluminescence imaging, their combinational treatment had more significant AML-inhibitory effect in vivo (Fig. 7K and L).

Collectively, bromodomain inhibition not only represses gene-expression programs related to DNMT3A R882H-induced hypomethylation but, importantly, suppresses development of the DNMT3A R882H-related murine AMLs in vivo, either alone or in combination with MEK inhibitors.

**Discussion**

DNMT3A<sub>mut</sub> is prevalent in patients with hematologic malignancies and disorders. It is proposed that DNMT3A<sub>mut</sub> acts as a founder mutation shaping the course of leukemia evolution and progression. The literature has documented that DNMT3A<sub>R882Hmut</sub> has hypomorphic, dominant-negative, and/or gain-of-function effects under different biological contexts. However, it remains undefined by what effects DNMT3A<sub>R882Hmut</sub> mediates leukemogenesis. We started the investigations first by reporting TF1 cells as a robust system useful for studying DNMT3A<sub>mut</sub>-related leukemic phenotypes, which then allowed us to next examine activities underlying DNMT3A<sub>R882Hmut</sub>-induced transformation. Based on the mechanistic studies, we further show that BRD4 inhibition reverses gene-expression programs downstream of disease-relevant effect of DNMT3A<sub>R882Hmut</sub>, thus suggesting a potential means of therapeutics.

Compared with the previously described in vivo and primary cell systems for studying DNMT3A<sub>R882Hmut</sub> (24–28), TF-1 cells is straightforward and suitable for cause–effect relationship studies to delineate the role for multifaceted effects of DNMT3A<sub>R882Hmut</sub> during leukemic transformation. Via a systematic interrogation of various motifs in DNMT3A<sub>R882Hmut</sub> with this system, we show that the transformation-promoting function of DNMT3A<sub>R882Hmut</sub> is independent of its residual catalytic activity (as assayed by double mutants carrying an enzyme-dead mutation), suggesting that CpG methylation gains at certain specific sequence contexts, as proposed in a gain-of-function model (32), is unlikely to contribute to leukemogenesis. In contrast, mutating a heterodimerization interface not only interfered with physical associations of DNMT3A<sub>R882Hmut</sub> to endogenous wild-type DNMT3A/B proteins but also largely abolished the ability of DNMT3A<sub>R882Hmut</sub> in inducing CpG hypomethylation and TF-1 cell transformation. These findings support a causal relationship between the dominant-negative effect of DNMT3A<sub>R882Hmut</sub> and

**Figure 6.**

CpG hypomethylation-inducing effect of DNMT3A<sub>R882Hmut</sub> requires its heterodimerization interface. A, Coimmunoprecipitation with anti-Myc antibodies detects interaction of the Myc-tagged DNMT3A<sub>R882Hmut</sub> (middle) or DNMT3A<sub>R882Hmut/C333A</sub> (right) with endogenous wild-type DNMT3B or DNMT3A proteins. B, Summary of the total number of hypomethylated DMPs induced by the indicated variant forms of DNMT3A<sub>R882Hmut</sub> relative to vector control (as shown in Fig. 2A; with p value decrease more than 0.1). C, RT-qPCR detecting expression of LMO2 in TF-1 lines with stable transduction of empty vector (EV) or the indicated DNMT3A forms at day 12 post-withdrawal of GM-CSF. ***, P < 0.01; ****, P < 0.001.
pediatric cancer markers (either vehicle (transplanted with the DNMT3AR882H/NRASG12D AML cells and treated with either vehicle or I-BET151 for 10 days. Dashed line, IC50).

trametinib (1 mg/kg daily for 10 days), or I-BET151 alone (50 mg/kg daily for 10 days), or their combination. Significance was tested by log-rank test. I and J, White blood cell counts (WBC), representative images of spleens (J, top; scale bar, 1 cm) and Wright-Giemsa staining of bone marrow cells (J, bottom; scale bar, 10 μm) from mice transplanted with the DNMT3AR882H/NRASG12D AML cells and then treated with either vehicle (n = 8) or I-BET151 (n = 6; 50 mg/kg daily for 10 days). Significance was tested by log-rank test.

Figure 7.
BRD4 blockade is efficient in treatment of murine AML established by combinational DNMT3A{R882H} and NRAS{G12D} mutations. A, IGV track views of BRD4 and H3K27ac ChIP-seq signals at the indicated gene in murine AML cells established by coexpressed DNMT3A{R882H} and NRAS{G12D} (termed as murine DNMT3A{R882H}/NRAS{G12D} AML lines; ref. 24). B, Relative proliferation of the DNMT3A{R882H}/NRAS{G12D} AML lines posttreatment with I-BET151 relative to mock. Dashed line, IC50. GSEA showing that the DNMT3A signature genes were repressed by I-BET151. Mouse_Gene_2.0_ST arrays were performed using the Red, replicate. E-G, GSEA shows downregulation of the indicated gene sets related to MYC targets (E), stem cell (F), or pediatric cancer markers (G) posttreatment with I-BET151.

White blood cell counts (WBC), representative images of spleens (J, top; scale bar, 1 cm) and Wright-Giemsa staining of bone marrow cells (J, bottom; scale bar, 10 μm) from mice transplanted with the DNMT3A{R882H}/NRAS{G12D} AML cells and then treated with either vehicle (n = 8) or I-BET151 (n = 6; 50 mg/kg daily for 10 days). Significance was tested by log-rank test. I and J, White blood cell counts (WBC), representative images of spleens (J, top; scale bar, 1 cm) and Wright-Giemsa staining of bone marrow cells (J, bottom; scale bar, 10 μm) from mice transplanted with the DNMT3A{R882H}/NRAS{G12D} AML cells and then treated with either vehicle (n = 8) or I-BET151 (n = 6; 50 mg/kg daily for 10 days). Significance was tested by log-rank test. I and J, White blood cell counts (WBC), representative images of spleens (J, top; scale bar, 1 cm) and Wright-Giemsa staining of bone marrow cells (J, bottom; scale bar, 10 μm) from mice transplanted with the DNMT3A{R882H}/NRAS{G12D} AML cells and then treated with either vehicle (n = 8) or I-BET151 (n = 6; 50 mg/kg daily for 10 days). Significance was tested by log-rank test.
those seen in leukemia patients with DNMT3A R882mut, suggesting a clinical relevance of findings from this model. Furthermore, there is a dose-dependent effect by DNMT3A R882mut on CpG hypomethylation and transformation (cytokine-free proliferation) in TF-1 cells, which provides an explanation for age-related increase in incidence of clonal hematopoiesis seen among elderly individuals. We observed synergy between DNMT3A R882mut and IDH1 mutation in TF-1 cells, as well as a phenomenon of "epigenetic antagonism" that resembles what was seen in human AML cells carrying dual DNMT3A R882mut and IDH2 mutations (42). Future investigation, however, is warranted to delineate interplays between DNMT3A R882mut and coexisting IDH mutations for inducing epigenomic alterations in both DNA methylation (including 5mC and 5hmC) and histone modifications. Finally, missense mutations at non-R882 residues account for nearly 40% to 50% of DNMT3A-mut in blood cancer and disorders. Considering many mutant forms for such a non-R882 category of DNMT3A-mut, TF-1 cells shall provide a simple yet robust system for studying their biological effects as shown recently with several DNMT3A-mut known to affect DNMT3A's DNA binding (12). Together, TF-1 cells represent a straightforward, robust, and disease-relevant model system for investigating numerous unsolved issues relating DNMT3A-mut.

This study not only demonstrated a requirement for the dominant-negative effect of DNMT3A R882mut (most likely to act in concert with a hypomorphic nature of DNMT3A R882mut) during leukemic transformation but also explored potential therapeutic utilities. For the latter, our mechanistic studies suggest that intervention shall be developed for targets and events associated with DNMT3A R882mut-induced CpG hypomethylation. Here, we first used a transgene-reversible system to show that DNMT3A R882mut-induced TF-1 cell transformation relies on its continuous expression; as well, we find CpG hypomethylation correlated to transcriptional activation of LMO2, a leukemia-related oncogene, thereby providing a glimpse of how induced CpG hypomethylation influences gene expression in TF-1 cells. It is consistent to previous reports showing that sites with DNMT3A R882mut-induced CpG hypomethylation are enriched in cis-regulatory elements such as enhancers and display increased histone acetylation, which then recruits "reader" protein complexes to potentiate gene activation (24, 50). Besides DOT1L complexes (24, 50), we here show that BRD4, another histone acetylation "reader," also binds to DNMT3A R882H-associated gene targets (notably Mnt1, Mycn, and Bcl2) in murine AMLs and that pharmacologic inhibition of BRD4 suppressed activation of DNMT3A R882H-related gene signatures. BRD4 inhibitors efficiently suppressed activation in vitro and in vivo growth of murine AMLs carrying dual DNMT3A R882mut and RAS mutations. Note that DNA methylation potentially regulates numerous biological processes including gene transcription, genome stability, chromatin architecture and looping, mRNA splicing, and silencing of repetitive DNA elements (46, 47). Determination of putative effects of DNMT3A R882H-related CpG hypomethylation on these crucial processes requires future investigation.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Conception and design: R. Lu, G.G. Wang
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Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): R. Lu, J. Wang, Z. Ren, J. Yin, Y. Wang, L. Cai, G.G. Wang
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): R. Lu, J. Wang, Z. Ren, J. Yin, Y. Wang, G.G. Wang
Writing, review, and/or revision of the manuscript: R. Lu, G.G. Wang
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): R. Lu, Z. Ren, Y. Wang, L. Cai, G.G. Wang
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A Model System for Studying the DNMT3A Hotspot Mutation (DNMT3A R882) Demonstrates a Causal Relationship between Its Dominant-Negative Effect and Leukemogenesis

Rui Lu, Jun Wang, Zhihong Ren, et al.


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