DNA Hydroxymethylation Profiling Reveals that WT1 Mutations Result in Loss of TET2 Function in Acute Myeloid Leukemia

Highlights

WT1 mutations anticorrelate with TET2 and IDH1/IDH2 mutations in AML

WT1 mutant AMLs have decreased global and locus-specific 5hmC levels

Changes in Wt1 expression levels result in changes in 5hmC levels

WT1 binds TET2 and TET3, providing a link between WT1 and TET enzymatic function

In Brief

Mutational studies in patients with acute myeloid leukemia (AML) have identified recurrent mutations in TET2 and IDH1/IDH2, and these mutations result in a reduction in 5-hydroxymethylcytosine (5hmC) levels. Rampal et al. demonstrate that WT1 mutations anticorrelate with TET2 and IDH1/IDH2 mutations, and WT1 mutant AMLs have decreased 5hmC levels, consistent with reduced TET2 function.

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Authors

Raajit Rampal, Altuna Alkalin, ..., Maria E. Figueroa, Ross L. Levine

Correspondence

chm2042@med.cornell.edu (C.E.M.), lgodley@medicine.bsd.uchicago.edu (L.A.G.), amm2014@med.cornell.edu (A.M.), marfigue@med.umich.edu (M.E.F.), leviner@mskcc.org (R.L.L.)
DNA Hydroxymethylation Profiling Reveals that WT1 Mutations Result in Loss of TET2 Function in Acute Myeloid Leukemia

Raajit Rampal,1,2,22 Altuna Alkalin,16,19,22 Jozef Madzo,3,22 Aparna Vasanthakumar,3,22 Elodie Pronier,1 Jay Patel,1 Yushan Li,1 Jihae Ahn,1 Omar Abdel-Wahab,1,2 Alan Shih,1,2 Chao Lu,5 Jihae Ahn,1 Omar Abdel-Wahab,1,2 Alan Shih,1,2 Chuan He,8 Francois Fuks,15 Martin S. Tallman,2 Adolfo Ferrando,6 Stephen Nimer,11 Elisabeth Paietta,10 Craig B. Thompson,9 Jonathan D. Licht,12 Christopher E. Mason,16,20,21,* Lucy A. Godley,3,13,* Ari Melnick,4,* Maria E. Figueroa,4,18,* and Ross L. Levine1,2,*

1Human Oncology and Pathogenesis Program, Memorial Sloan-Kettering Cancer Center, New York, NY 10065, USA
2Leukemia Service, Department of Medicine, Memorial Sloan-Kettering Cancer Center, New York, NY 10065, USA
3Section of Hematology/Oncology, Department of Medicine, The University of Chicago, Chicago, IL 60637, USA
4Department of Hematology/Oncology, Weill Cornell Medical College, New York, NY 10065, USA
5Cancer Biology and Genetics Program, Memorial Sloan-Kettering Cancer Center, New York, NY 10065, USA
6Institute for Cancer Genetics, Columbia University Medical Center, New York, NY 10032, USA
7Department of Pathology, New York University Cancer Institute, New York, NY 10016, USA
8Laboratory of Cancer Epigenetics, Faculty of Medicine, Université Libre de Bruxelles, 1070 Brussels, Belgium
9Institute for Biophysical Dynamics, The University of Chicago, Chicago, IL 60637, USA
10The Feil Family Brain and Mind Research Institute, Weill Cornell Graduate School of Medical Sciences, New York, NY 10065, USA
11Robert H. Lurie Comprehensive Cancer Center, Northwestern University, Chicago, IL 60611, USA
12Laboratory of Cancer Epigenetics, Faculty of Medicine, Université Libre de Bruxelles, 1070 Brussels, Belgium
13Department of Pathology, University of Michigan, Ann Arbor, MI 48109, USA
14Department of Medicine, Sylvester Comprehensive Cancer Center, Miami, FL 33136, USA
15Department of Immunology and Microbial Pathogenesis, Weill Cornell Graduate School of Medical Sciences, New York, NY 10065, USA
16Department of Physiology and Biophysics, Weill Cornell Medical College, New York, NY 10065, USA
17Promega Corporation, Madison, WI 53703, USA
18Department of Pathology, University of Michigan, Ann Arbor, MI 48109, USA
19Berlin Institute for Medical Systems Biology, Max Delbrück Centre for Molecular Medicine, Robert-Rössle-Straße 10, 13125 Berlin, Germany
20The HRH Prince Alwaleed Bin Talal Bin Abdulaziz Alsaud Institute for Computational Biomedicine, Weill Cornell Graduate School of Medical Sciences, New York, NY 10065, USA
21The Feil Family Brain and Mind Research Institute, Weill Cornell Graduate School of Medical Sciences, New York, NY 10065, USA
22Co-first author

*Correspondence: chm2042@med.cornell.edu (C.E.M.), lgodley@medicine.bsd.uchicago.edu (L.A.G.), amm2014@med.cornell.edu (A.M.), marfigue@med.umich.edu (M.E.F.), leviner@mskcc.org (R.L.L.)

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SUMMARY

Somatic mutations in IDH1/IDH2 and TET2 result in impaired TET2-mediated conversion of 5-methylcytosine (5mC) to 5-hydroxymethylcytosine (5hmC). The observation that WT1 inactivating mutations anticorrelate with TET2/IDH1/IDH2 mutations in acute myeloid leukemia (AML) led us to hypothesize that WT1 mutations may impact TET2 function. WT1 mutant AML patients have reduced 5hmC levels similar to TET2/IDH1/IDH2 mutant AML. These mutations are characterized by convergent, site-specific alterations in DNA hydroxymethylation, which drive differential gene expression more than alterations in DNA promoter methylation. WT1 overexpression increases global levels of 5hmC, and WT1 silencing reduced 5hmC levels. WT1 physically interacts with TET2 and TET3, and WT1 loss of function results in a similar hematopoietic differentiation phenotype as observed with TET2 deficiency. These data provide a role for WT1 in regulating DNA hydroxymethylation and suggest that TET2 IDH1/IDH2 and WT1 mutations define an AML subtype defined by dysregulated DNA hydroxymethylation.

INTRODUCTION

Gene discovery studies in human cancers have identified novel mutations that inform new mechanisms of malignant transformation. Recurrent somatic mutations in epigenetic regulators compose an emerging class of disease alleles. Mutations in epigenetic modifiers have been observed in the majority of patients...
with acute myeloid leukemia (AML), including mutations in DNA methyltransferases (Ley et al., 2010; Yan et al., 2011), chromatin modifying enzymes (Ernst et al., 2010), and histone methyltransferase readers (Wang et al., 2009). Notably, mutations in epigenetic modifiers and epigenetic signatures have been found to have prognostic and biologic relevance in AML (Bullinger et al., 2010; Figueroa et al., 2010b; Patel et al., 2012) and have led to the development of epigenetic therapies, in the context of clinical trials, for molecularly defined AML subsets (Bernt et al., 2011; Daigle et al., 2011; Dawson et al., 2011; Filippapopoulos et al., 2010; Zuber et al., 2011).

One class of mutations found in AML and in other malignancies affects the conversion of 5-methylcytosine (5mC) to 5-hydroxymethylcytosine (5hmC), mediated by the TET family of enzymes. These include mutations in TET2 and IDH1/IDH2. Mutational profiling of 398 patients with de novo AML demonstrated that TET2 and IDH1/IDH2 mutations were mutually exclusive and featured extensive promoter hypermethylation (Figueroa et al., 2010a; Patel et al., 2012). TET2 has been implicated in mediating demethylation of DNA with hydroxymethylation as an intermediate step in this process, TET2 loss of function results in reduction of genomic 5hmC and a reciprocal increase in 5mC (Ko et al., 2010). A similar effect is caused by aberrant production of the oncometabolite 2-hydroxyglutarate (2-HG) by gain-of-function IDH1/IDH2 mutations, which result in inhibition of TET enzyme catalytic functions (Figueroa et al., 2010a). Hence, these mutations define a class of AMLs with reduced genome-wide 5hmC. Notably, mutations or altered expression of IDH1/IDH2 and TET genes likewise result in altered 5hmC content in glioblastomas and melanomas (Lian et al., 2012). Yet, it has been shown that not all AML cases with low levels of 5hmC harbor somatic mutations in TET2 and IDH1/IDH2 (Konstandin et al., 2011). Hence, there are likely additional somatic mutations that can lead to direct or indirect alterations in TET enzyme function.

Recent technologic developments have enabled 5hmC mapping to be performed in normal tissues and in embryonic stem cells. These studies showed that 5hmC is commonly localized to gene regulatory elements, including promoters, gene bodies, and enhancers (Stroud et al., 2011). However, to date, genome-wide localization of 5hmC has not been reported in human malignancies, and the impact of TET2 and IDH1/IDH2 mutations and/or other mutations on 5hmC distribution has not been investigated. Cytosine methylation studies have often showed a weak inverse correlation between alterations in promoter DNA methylation and differential gene expression (Bell et al., 2011; Kulis et al., 2012), raising the possibility that other epigenetic modifications, such as 5hmC, may be more tightly linked with transcriptional changes.

In this study, we examined the mutational status, gene expression profiles, and cytosine methylation profiles of a cohort of 398 AML patients for novel mutations that might functionally overlap with IDH1/IDH2 and TET2. Here, we show that WT1 mutations are significantly reduced in frequency in patients with TET2/IDH1/IDH2 mutant AML, and that WT1 mutant AML is characterized by altered DNA methylation and global reductions in 5hmC similar to that observed in TET2/IDH1/IDH2 mutant AML. Furthermore, we demonstrate that alterations in WT1 levels directly regulate 5hmC levels, which is due to an interaction between TET2/TET3 and WT1.

RESULTS

WT1 Mutations Are Inversely Correlated with IDH/TET2 Mutations in AML and Display Overlapping Promoter Hypermethylation Signatures

We recently performed mutational profiling of 398 AML patients and noted that TET2 and IDH1/IDH2 mutations were mutually exclusive (Figueroa et al., 2010a; Patel et al., 2012). We next investigated the same patient cohort for other mutations inversely correlated with TET2 and IDH1/IDH2 mutations. Mutations in the WT1 gene were mutually exclusive of IDH1/IDH2 mutations (Patel et al., 2012) and negatively correlated with TET2 mutations (Figure 1A; Figure S1A). Twenty-eight of 313 (9%) of TET2/IDH wild-type patients had somatic WT1 mutations, whereas two of 5% (2%) TET2/IDH1/IDH2 mutant patients had co-occurring WT1 mutations (p = 0.026, Fisher’s exact test, Table S1). We observed a similar inverse relationship between WT1 mutations and TET/IDH1/IDH2 mutations in the AML samples analyzed by TCGA (Table S2). Analysis of combined data from the ECOG1900 study and the AML TCGA data set confirmed a significant anticorrelation between WT1 mutations and TET2/IDH1/IDH2 mutations (p = 0.0164, Fisher’s exact test, Table S3; Figure S1B). These data suggested a shared functional role for WT1, TET2, and IDH1/IDH2 mutations in AML.

Using promoter DNA methylation microarrays (Figueroa et al., 2010a), we analyzed the DNA methylation profiles of 30 WT1 mutant AML samples compared to 11 normal CD34+ bone marrow cells and identified 653 differentially methylated regions (DMRs, see Experimental Procedures) in WT1 mutant AML patients. The vast majority of the DMRs were aberrantly hypermethylated (Figure 1B). Next, we compared WT1 mutant AML samples to a cohort of 29 AML1-ETO AMLs wild-type for WT1/ TET2/IDH1/IDH2 mutations and identified 124 DMRs, 68% (n = 84) of which were hypermethylated in WT1 mutant AML patients (Figure 1C). TET2 mutant and IDH1/IDH2 mutant AML patients were also characterized by hypermethylation compared to AML1/ETO-positive AML (Figures S1C and S1D). Comparative analysis of the three hypermethylation profiles revealed a near-complete overlap of TET2 and WT1 hypermethylated loci within the IDH1/IDH2 hypermethylation signature, and a highly significant overlap between the TET2 and WT1 mutant signatures (Fisher’s exact test, p value < 0.001 for all comparisons) (Figure 1D), consistent with convergent, site-specific effects on DNA methylation.

WT1, TET2, and IDH1/IDH2 Mutations Are Characterized by Global Reductions in 5hmC in Primary AML Samples

Given that IDH1/IDH2 mutations or silencing of TET2 leads to reduced 5hmC levels in hematopoietic cells (Figueroa et al., 2010a; Ko et al., 2010), we hypothesized that AML patients with WT1 mutations would also be characterized by reduced 5hmC due to reduced TET enzymatic function. Liquid chromatography-electron spray ionization-tandem mass spectrometry (LC-ESI-MS/MS) revealed that WT1 mutant AML patients had significantly reduced 5hmC when compared to AML patients.
Figure 1. WT1 Mutations Are Inversely Correlated with TET2/IDH1/IDH2 Mutations and Display Similar Global Methylation Profile

(A) Circos representation of targeted mutational data from 398 AML patients. Co-occurrence of mutations is represented by lines connecting genes. The width of connecting lines represents frequency of mutations. TET2 and IDH mutations are combined in this analysis. IDH mutations are designated by orange ribbons, TET2 mutations by yellow ribbons, and WT1 mutations by blue ribbons.

(B) Promoter methylation signatures in WT1 mutant AML versus normal bone marrow (NBM).

(C) Comparison of promoter methylation signatures in WT1 mutant AML and AML1-ETO AML.

(D) Overlap of hypermethylated loci in WT1 mutant AML compared with those previously identified in TET2 and IDH1/IDH2 mutant AMLs.

(E) 5-methylcytosine (5mC, left) and 5-hydroxymethylcytosine (5hmC, right) levels in AML samples from patients with or without WT1, TET2, or IDH1/IDH2 mutations. 5mC and 5hmC levels were determined by liquid chromatography–electron spray ionization tandem mass spectrometry (LC-ESI-MS/MS). Error bars represent SEM.
Figure 2. Convergent, Site-Specific Alterations in DNA Hydroxymethylation in AML Patients with TET2, IDH1/IDH2, and WT1 Mutations

(A) KIRREL locus demonstrating depletion of 5hmC marks in AML patients with TET2, WT1, and IDH1/IDH2 mutations.

(B) Percentages of differential 5hmC regions and 5mC bases. Bar plot on the left demonstrates percentages of hypo- or hyper-5hmC regions out of all canonical peaks in WT1, TET2, and IDH1/IDH2 mutants compared to AML1-ETO patients. Bar plot on the right demonstrates the percentages of hypo- and hyper-methylated CpGs out of all covered CpGs in WT1, TET2, and IDH1/IDH2 mutants compared to AML1-ETO patients. Differentially methylated CpGs that overlap with differential 5hmC regions are removed from the analysis.
wild-type for WT1, TET2, or IDH1/IDH2 (p = 0.016 t test, Figure 1E). Similarly, the reduction in 5mC levels was comparable in extent in WT1 mutant AML patients compared to IDH1/IDH2 mutant and TET2 mutant AML patient samples. This finding was confirmed by dot-blot analysis (Song et al., 2011) (Figure S1E). Although global cytosine methylation abundance was significantly increased in IDH1, IDH2, and TET2 mutant patients, there was no significant increase of 5mC in WT1 mutant AML, suggesting that WT1 mutant AML is primarily characterized by alterations in 5mC leading to site-specific effects on DNA methylation rather than a global increase in 5mC.

5mC Mapping Reveals Similar Alterations in 5hmC Localization in WT1, TET2, and IDH1/IDH2 Mutant AML

In order to determine the impact of IDH1/IDH2, WT1, and TET2 mutations on the distribution of epigenetic marks throughout the genome more precisely, we examined 5mC and 5mC localization in primary AML specimens with next-generation sequencing. Once again, specimens with AML1-ETO translocation, which are all wild-type for IDH1/IDH2, TET2, and WT1, were profiled as a control AML cohort. We used a selective chemical labeling approach followed by streptavidin capture and sequencing to map the abundance and distribution of 5mC (nMe-Seal, see Experimental Procedures) (Song et al., 2011). We identified areas of 5mC enrichment for each sample with ChiPseeqer (Giannopoulou and Elemento, 2011). The average number of peaks identified in AML1-ETO specimens was 192,066, for TET2-mut 114,865, for IDH1/IDH2 70,622, and for WT1-mut 60,258. The average number of 5hmC peaks called per sample was significantly lower in AML patients with TET2, WT1, IDH1, or IDH2 mutations compared to control AMLs (t test p values between 0.0005 and 0.003 for all comparisons; see Figure 2A as an example for regions of loss of 5hmC and Figure S2A for overall changes in 5hmC loss). These data are consistent with the global reduction in 5hmC observed by mass spectrometry (Figure 1E). We calculated pairwise comparisons of peaks of 5hmC enrichment in IDH1/IDH2, WT1, and TET2 mutant AML against 5mC sites identified in control AML patients. All three AML subtypes (IDH1/IDH2, WT1, and TET2) displayed a significant reduction in 5hmC peaks across the entire genome versus controls, with a smaller proportion (between 1% and 5%) of regions presenting with gains in 5mC (Figure 2B). We then performed DNA methylation bisulfite sequencing by enhanced reduced representation bisulfite sequencing (ERRBS) on the same patients to map the distribution of 5mC. ERRBS assayed 1,433,193 CpGs across all AML subtypes. Pairwise methylation differences were compared on these ERRBS data sets to map the distribution of 5mC. ERRBS profiles revealed that 5mC increased genome-wide in IDH1/IDH2, WT1, and TET2 compared to control AML patients; specifically, 4%–6.5% of CpGs were methylated, in IDH1/IDH2, TET2, and WT1 mutations (see Figure 2B). These contrasts with the reduced levels of 5hmC in these samples.

These observations, specifically the loss of 5hmC and gain of 5mC in WT1 mutant, TET2 mutant, and IDH1/IDH2 mutant AML patients, also hold when we compared 5hmC and 5mC levels against normal bone marrows (NBMs; see Figure S2). Moreover, AML1-ETO AMLs showed no significant difference in the average total number of 5hmC peaks when compared to NBMs, indicating that the reduction of 5mC peaks is specific to those AMLs with disruption of TET2, IDH1/IDH2, or WT1 (Figure S2B).

Aberrant 5mC Distribution in WT1, TET2, and IDH1/IDH2 Mutant AML Occurs Predominantly at Enhancers and Distal Regulatory Elements

Next, we sought to determine whether IDH1/IDH2, WT1, and TET2 somatic mutations affect not only the abundance but also the genomic distribution pattern of 5hmC. We first examined 5hmC peak profiles in patient specimens through unsupervised analyses using hierarchical clustering and multidimensional scaling (MDS), which can be thought of as 2D representations of pairwise distance between samples. Hierarchical clustering and MDS results show the relationship between different samples based on their 5mC and 5mC profile similarities. IDH1 and IDH2 mutant AMLs exhibited the most significant difference in 5hmC and clustered furthest away from the control AMLs (Figures S2C and S2D). WT1 and TET2 mutant AML patients clustered closer to each other and localized in between IDH1/IDH2 and control AML patients in the first dimension of the multidimensional scaling. These findings suggest the underlying alterations in 5mC patterning in TET2 and WT1 mutant AMLs are less widespread across the genome than in AML specimens carrying IDH1/IDH2 mutations. Given that 2-HG is predicted to inhibit the function of all three TET enzymes (Xu et al., 2011), these data are consistent with more profound pan-TET enzyme inhibition in IDH1/IDH2 mutant AML. Regardless of AML subtype, 5mC peaks were most commonly (52%–59%) located within gene bodies and somewhat less commonly in intergenic regions (37%–44.2% across subtypes). Less than 5% of 5mC peaks were found at promoter regions (Figure 2C). Most regions with differential 5mC enrichment in IDH1/IDH2, WT1, and TET2 AMLs were located at a significant distance from transcription start sites (median distance between 31 and 44 kb). By contrast, differentially methylated loci were closer to the TSS of known genes, suggesting that the perturbation of 5mC and 5mC patterns in IDH1/IDH2, WT1, and TET2 AMLs can occur at distinct genomic regions (Figure 2D). Most regions with differential 5mC enrichment were located outside of CpG islands and CpG island shores (87%–89%). Yet, about half of the differential 5mC peaks were located at enhancer regions as defined by the ENCODE project (see the Experimental Procedures) (43%–53%) (Figures 2C and 2D), suggesting differential 5mC localization at enhancers may contribute to aberrant gene expression in leukemia.
Differential 5hmC More Strongly Correlates with Differential Gene Expression than Differential 5mC in AML with WT1, TET2, and IDH1/IDH2 Mutations

The distinct localization patterns of 5mC and 5hmC raised the question of whether these marks can function independently to coordinate gene expression. We used gene expression profiling to compare TET2-mut, IDH1/IDH2-mut, and WT1-mut against AML-ETO and identify the top 500 upregulated and the top 500 downregulated genes in the same AML samples (see the Experimental Procedures for details). We then examined the relationship between changes in gene expression with changes in 5mC and 5hmC abundance in each leukemia subtype (IDH1, IDH2, TET2, and WT1). As expected, differential cytosine methylation at promoters was negatively correlated with gene expression (Figure 3A, top) but with a relatively low correlation coefficient (r = -0.348 to -0.4, Pearson’s R test p values between 0.02 and 0001 in the different AML subsets). By contrast, 5hmC changes in gene body and distal regulatory regions had a positive correlation with gene expression and showed a much stronger and more significant correlation (r = 0.52-0.75, Pearson’s R test p value between 10^-9 and 10^-15) in the different AML subsets (Figure 3A, bottom) than the correlation observed with 5mC levels. 5hmC changes were strongly correlated with differential expression regardless of genomic location, including first introns (r = 0.75, Pearson’s R test p values < 0.0001), distal regions (r = 0.69, Pearson’s R test p value < 0.0001), gene bodies (r = 0.67, Pearson’s R test p values < 0.0001), and promoter region (r = 0.61, Pearson’s R test p values < 0.0001) (Figure S3A). By contrast, 5mC changes were most strongly correlated with gene expression when present near TSS and on first intron but less strongly correlated with gene expression when present at other genomic locations investigated (CpG island shores and gene body; Figure S3B).

Next, we sought to determine which of these two epigenetic marks could more accurately predict changes in gene expression. We used a machine-learning model for predicting differentially expressed genes using differential methylation and hydroxymethylation. In IDH1/IDH2 mutant and TET2 mutant AML, 5hmC levels at enhancers performed better than 5mC present at promoters at predicting gene expression, judging from the performance of the classifier where AUC of 5hmC + 5mC and 5hmC models were significantly better at predicting gene expression (pairwise t test p values between 10^-9 and 10^-5). Our findings are consistent with 5hmC functioning as an independent epigenetic mark that is linked to potential distal regulation, and suggests that 5hmC has additional functions independent of its role as an intermediate step to DNA demethylation at gene promoters (Yu et al., 2012).

Site-Specific 5hmC Alterations in TET2/WT1 Mutant AMLs Compose a Subset of the Alterations Seen in IDH1/IDH2 Mutant AML

The data presented above suggest a potential unifying link between IDH1/IDH2, TET2, and WT1 mutant AMLs. We therefore assessed site-specific alterations in 5hmC in IDH1/IDH2, TET2, and WT1 mutant AMLs. IDH1/IDH2 mutant AMLs displayed the greatest number of hydroxymethylation peaks lost (n = 20,286) compared to control AML specimens (AML1-ETO AMLs). By contrast, TET2 mutant and WT1 mutant AML samples had fewer 5hmC peaks lost (n = 5,030 and 5,484, respectively). However, 68% of the peaks lost in WT1 mutant specimens and 81% of those lost in TET2 mutant AML overlapped with those lost in IDH1/IDH2 mutant AML (Figures 4A and 4B; Figure S3C). We observed highly significant overlap of differential 5hmC peaks lost in WT1 mutant AML and TET2 mutant AML (Figure 4B; hypergeometric test p value <10^-133). In a manner analogous to the findings for 5hmC, the hypermethylated sites identified in WT1/TET2 mutant AML were a subset of those found in IDH1/IDH2 mutant cases (Figures 4C and 4D). 44% of peaks of promoter hypermethylation identified in TET2 mutant AML and 65% of those of WT1 mutant specimens overlap with peaks of 5mC in IDH1/IDH2 mutant AML (hypergeometric test p value <10^-133). Collectively these data suggest that a core set of deregulated and presumably silenced genes might represent a unifying pathway in IDH1/IDH2, TET2, and WT1 mutant AML.

Although WT1 is a sequence-specific transcription factor, the mechanisms by which TET2 is recruited to specific loci to convert 5mC to 5hmC have not been delineated. To define candidate transcription factors (TFs) that might be important for TET2 action, we examined regions of differential 5hmC modification for the presence of specific DNA motifs characteristic of known TFs. This motif analysis revealed an overrepresentation of ETS motifs with GGAA core sequence (Figures S4A and S4B) in regions with 5hmC enrichment. Notably, we observed that regions with loss of 5hmC peaks in WT1 mutant AML cases were enriched for a AGG[AC]AGG (CCT[TG]CCT) motif that is analogous to a WT1 binding motif reported by Wang et al. (1993). Consistent with these data, we observed colocalization of WT1 and TET2 at specific loci with 5hmC enrichment, including SHANK1 (Figure S4C). We also observed WT1 occupancy at regions with differential 5hmC, which are not bound by TET2, suggesting that other factors including other TET proteins might colocalize with WT1 at other gene regulatory elements (Figure S4D). We also curated chromatin immunoprecipitation sequencing experiments to identify myocardial lineage specific transcription factors that were enriched at regions with differential 5hmC in AML cells. This showed that ETS factors like FLI1, ERG, and their binding partners RUNX1 and CEBPA/B (Figure S4E) were enriched in regions of increased 5hmC, but not in hypo-5hmC regions, suggesting these transcription factors bind to regions with increased 5hmC, but are not enriched at sites with reduced 5hmC in IDH1/IDH2, TET2, and WT1 mutant AML.
WT1 Directly Regulates 5hmC Levels in Hematopoietic Cells

The overlap in regions of 5hmC lost when TET2 and WT1 were mutated in AML and the inverse association between WT1 and TET2 mutations in AML suggested a potential functional interaction between these two proteins, and that WT1 might play a direct role in regulating TET-mediated hydroxymethylation. Previous studies have shown that AML-associated WT1 mutations result in premature stop codons or are targeted by nonsense-mediated decay (Abbas et al., 2010), which results in loss of WT1 protein expression. We therefore investigated the effects of WT1 loss of function on 5hmC levels in M15 murine mesonephron cells, which express high levels of Wt1 (Larsson et al., 1995). Knockdown of Wt1 in M15 cells significantly decreased 5hmC levels in M15 cells (p < 0.01, t test) (Figures 5A and 5B). Similarly, in primary murine bone marrow (BM) cells, silencing of Wt1 by small hairpin RNA (shRNA) (Vicent et al., 2010) (Figures S5A and S5B) significantly reduced 5hmC compared to cells expressing an empty vector (Figure 5C) (p < 0.01, t test). Similar effects were observed in primary murine BM cells transduced with
Tet2 shRNA (Figure S5C). Perturbations in WT1 did not significantly alter proliferation (Figure S5D). These convergent data suggest that reductions of 5hmC levels in AML could be a direct result of loss of WT1 function in AML.

Previous studies have shown that overexpression of wild-type WT1 can contribute to malignant transformation in AML (Nishida et al., 2006), lung cancer (Oji et al., 2002; Vicent et al., 2010), and in Wilms tumor cases without WT1 mutations (Kim et al., 2008).

We therefore evaluated whether WT1 overexpression could lead to increases in 5hmC, and if AML-associated WT1 mutations abrogated the ability of WT1 to impact 5hmC. The most commonly expressed WT1 isoform (isoform D) contains exon 5 (17AA+) and a KTS site between exons 3 and 4 (Haber et al., 1991), hereafter referred to as WT1+/+. We first expressed wild-type WT1+/+ and a WT1+/+ construct with a known AML truncation mutant in exon 7 (WT1 mutant) in 32D myeloid cells. WT1+/+ expression significantly increased 5hmC levels compared to cells expressing a control vector or WT1 mutant (p < 0.05 for either comparison) (Figure 5D).

WT1 Forms a Complex with TET2 in Hematopoietic Cells

Given the effects of WT1 on 5hmC levels and the inverse correlation between WT1 and TET2 mutations in AML, we hypothesized that WT1 might modulate TET2 function through direct interaction. Coimmunoprecipitation experiments in 293T cells revealed that WT1 interacts with TET2 (Figure 5E; Figure S6E). This interaction was not abrogated by ethidium bromide exposure (Figure S6F), consistent with a DNA-binding-independent interaction. We next did coimmunoprecipitation studies to determine the domain(s) of WT1 that are required for interaction with TET2. The different isoforms of WT1 also interact with TET2, suggesting the KTS domain is dispensable for TET2 interaction (Figure S6F). Deletion of the zinc-finger domain abrogated binding of WT1 to TET2, whereas truncation of the N-terminal region did not alter TET2 binding (Figure 5F). We did not observe interaction of WT1 or TET2 with HDAC6, suggesting the interaction between WT1 and TET2 is not due to nonspecific association of highly expressed nuclear proteins (Figure S6G). Coimmunoprecipitation
studies revealed interaction of endogenous TET2 and WT1, in HEL and Nomo-1 cells, confirming endogenous WT1 and TET2 can directly interact in hematopoietic cells (Figure 6G). As a control, we did not observe any association between TET2 and WT1 in AML14 cells, which do not express detectable levels of TET2 protein (Figure 6G). Coimmunoprecipitation was also performed using buffer with increasing NaCl concentrations, which did not result in abrogation of the interaction between WT1 and TET2 (Figure S6H).

**WT1 Loss Leads to Impaired Hematopoietic Differentiation Similar to that Observed with TET2 Loss**

We and others have shown that loss of TET2 expression leads to expansion of c-Kit positive cells in vitro and in vivo (Li et al., 2011; Moran-Crusio et al., 2011; Quivoron et al., 2011). WT1 silencing in primary hematopoietic cells using two independent hairpins led to a similar increase in c-kit expression (Figure 6A; Figure S7A) (p < 0.05 t test). Furthermore, WT1 silencing in primary murine BM cells led to expansion of the lineage-negative, Sca-positive, Kit-positive stem/progenitor population to a similar extent as observed with Tet2 downregulation (Figure S7B). Previous studies have revealed a role for TET2 in myelomonocytic fate commitment (Ko et al., 2010). WT1 silencing led to an increase in the population of CFU-GEMM (colony forming unit-granulocyte, erythrocye, macrophage, megakaryocyte) similar to that observed with Tet2 silencing (Figure 6B). Given the observations that WT1 expression can modulate 5hmC levels, and that WT1 downregulation in hematopoietic cells can recapitulate phenotypes associated with Tet2 downregulation, we examined the transcriptional profile of primary murine BM cells transduced with vector or hairpins targeting Tet2 or WT1. We found a significant overlap between differentially expressed genes in primary murine BM cells transduced with shRNA targeting Tet2 or WT1, when compared with vector-transduced cells (hypergeometric test p < 10^-50, Figure S7C; Table S4). Collectively, these data indicate that reduced WT1 expression has similar effects on hematopoietic differentiation as observed with Tet2 attenuation.

**WT1 Expression Rescues the Effects of TET2 Loss through Interactions with TET3 In Vivo**

We next determined whether overexpression of WT1 could attenuate the effects of Tet2 loss. Expression of WT1+/+, but not a WT1 mutation observed in AML patients, significantly reduced colony growth in Tet2-deficient cells at primary and secondary plating (p < 0.01, t test) (Figure 6C; Figure S7D). Mass spectrometric analysis revealed that expression of WT1+/+, but not WT1 mutant increased 5hmC levels in Tet2 KO cells (Figure 6D). Accordingly, overexpression of wild-type, but not mutant, WT1 reduced c-Kit expression, consistent with restored hematopoietic differentiation (Figure 6E). In order to assess whether loss of WT1 produced an additive phenotype in conjunction with Tet2 loss, shRNA targeting WT1 was transduced into Tet2 KO cells and plated in methylcellulose. No increase in colony formation was noted with concomitant Tet2/WT1 loss (Figure S7E). By contrast, expression of wild-type WT1, but not mutant WT1, abrogated the ability of Tet2 knockout cells to reconstitute hematopoiesis in vivo (Figure 6F).

The observation that wild-type WT1, rescued 5hmC levels, and abrogated the phenotype of TET2-deficient cells suggested the possibility that WT1 might also regulate the activity of the other TET enzymes. Expression of WT1+/+ in the presence of 1-octyl-D-2-hydroxyglutarate (octyl-2HG, a cell permeable form of 2-HG) (Lu et al., 2012), which inhibits the activity of all alpha-ketoglutarate-dependent TET enzymes, inhibited the ability of WT1 to alter 5hmC levels consistent with a TET-family-dependent effect of WT1 (Figure 7A). Consistent with these data, coimmunoprecipitation studies demonstrated WT1 directly interacts with TET3, but not WT1 (Figure 7B). We next sought to determine if TET3 could modulate WT1-mediated effects on hematopoiesis in the absence of TET2. We coexpressed WT1 with two different validated shRNA constructs against TET3 in Tet2-deficient BM cells. When Tet3 was silenced in Tet2−/− marrow, WT1 could no longer suppress hematopoietic colony formation, demonstrating that Tet3 can act as a WT1 effector in the absence of Tet2 (Figures 7C and S7F). These data indicate WT1 is able to interact with Tet2 and TET3, and that WT1 overexpression can rescue the effects of TET2 loss in a TET3-dependent manner.

**DISCUSSION**

Here, we report that WT1 mutations are inversely correlated with TET2 and IDH1/IDH2 mutations in AML, and that WT1 mutant AML samples are characterized by significantly marked reductions in global and site-specific DNA hydroxymethylation. We show that WT1 interacts with TET2 and TET3, and that alterations in WT1 expression regulate 5hmC abundance. Our genetic, epigenetic, and biochemical data indicate that TET2, IDH1/IDH2, and WT1 mutant AMLs are characterized by disordered DNA hydroxymethylation potentially representing a convergent mechanism of leukemic transformation involving disordered DNA hydroxymethylation. These data also suggest that, in addition to its role as a sequence-specific transcription factor, WT1 may act as a cofactor for TET enzymes recruiting or stimulating their activity at specific sites in the genome.

We also employed next-generation sequencing methodologies to map 5hmC localization in AML patients with and without WT1, TET2, and IDH1/IDH2 mutations. We observed differential 5hmC localization at enhancers, gene bodies, and distal regulatory elements and differential 5mC localization at intronic regions near transcription start sites in IDH1/IDH2, TET2, and WT1 mutant AMLs. Moreover, we observed a strong, positive correlation between 5hmC changes and gene expression as compared to a weaker inverse correlation with 5mC. These data suggest that 5hmC has distinct effects on gene regulation independent of its role as an intermediate step to DNA demethylation, and also indicate that 5hmC may regulate enhancers/chromatin conformation, histone state, and/or transcription factor binding. Subsequent studies using base-pair resolution mapping of 5hmC and other recently described DNA modifications, combined with mapping other cis/trans-acting elements will help elucidate the complex roles of 5hmC and other DNA modifications on gene regulation in different cellular contexts.

Our 5hmC profiling data in AML samples with IDH1/IDH2, WT1, and TET2 mutations reveal site-specific loss of 5hmC in AMLs with impaired TET function, which is most widespread in
Figure 5. WT1 Complexes with TET2 and Alterations in Wt1 Levels Result in Changes in 5hmC Levels

(A) Western blot analysis of Wt1 silencing in mouse mesonephron cells (M15 cells) using vector or shRNA targeting Wt1 (all constructs contained a puromycin resistance marker). Analysis was carried out after puromycin selection.

(B) 5hmC levels were measured by LC-MS from samples of Mouse mesonephron cells (M15) transfected with vector or Wt1-targeted shRNA (both with a puromycin resistance marker) following puromycin selection and confirmation of knockdown.

(C) 5hmC levels were measured by LC-MS from samples of murine whole bone marrow transduced with either vector or Wt1-targeted shRNA.

(legend continued on next page)
IDH1/IDH2 mutant AML. Given that IDH mutations lead to chemical inhibition of all three TET enzymes, it is not surprising that the impact of IDH mutations on global and site specific 5mC modification are more substantial than in cases with mutations that affect a single TET enzyme (i.e., in TET2 mutant AML) or which impact TET2/TET3 but not TET1 (WT1). Consistent with the convergent mechanism of 5mC loss, the majority of the loci with altered 5mC in AMLs with WT1 and TET2 mutations represent a subset of the loci with differential 5mC seen in patients with IDH1/IDH2 mutations. Subsequent functional studies are needed to determine if the “core” set of loci with altered 5mC are universally altered in all AML patients with WT1 overexpression. WT1 overexpression has been shown to contribute to leukemogenesis (Hosen et al., 2007). As such, WT1 can function as an oncogene and tumor suppressor in AML. WT1 has previously been demonstrated to interact with TET2 and TET3. WT1 loss led to marked reductions in 5mC levels and a defect in hematopoietic differentiation, a phenotype similar to that observed with loss of TET2. Taken together, these results suggest that the hydroxymethylation pathway may be affected by mutations not previously implicated in epigenetic regulation. We hypothesize there are additional disease alleles that induce transformation through perturbations in TET enzyme function in different malignant contexts.

**EXPERIMENTAL PROCEDURES**

**Patient Samples**

Three hundred ninety-eight AML samples were obtained at diagnosis from patients enrolled in the E1900 clinical trial (Fernandez et al., 2009). DNA methylation microarrays using the HELP assay was available for 383/398 cases studied for mutational profiling, and gene expression data were available for 325/398 cases. Institutional review board approval was obtained at Weill Cornell Medical College and at Memorial Sloan-Kettering Cancer Center. Eleven human CD34+ bone marrow samples were provided by the Stem Cell and Xenograft Core Facility of the University of Pennsylvania or purchased from AllCells. These studies were performed in accordance with the Helsinki protocols, and all patients provided informed consent.

**Statistical Analysis**

Statistical analysis of mutational frequencies was performed using Fisher’s exact test. Statistical analysis of colony-forming assays, gene expression levels, c-Kit expression, and 5hmC levels assessed by LC/MS was performed using two-sided t test.

**Liquid Chromatography-Electron Spray Ionization-Tandem Mass Spectrometry**

DNA hydrolysis and LC-MS analysis of 5-methylcytosine and 5-hydroxymethylcytosine was performed as described previously (Vasanthakumar et al., 2013). Please see the Supplemental Experimental Procedures for description of protocol used.

**Western Blot and Coimmunoprecipitation**

Cell lysis, immunoprecipitation, and western blot analysis was performed as previously described (Marubayashi et al., 2010). Cell lysis and immunoprecipitation was carried out in buffer containing 150 mM NaCl, 20 mM Tris, 5 mM EDTA, 1% Triton X-100, and 10% glycerol (with addition of protease arrest, phosphatase inhibitor cocktail II, 1 mM phenylmethylsulfonyl fluoride, and 0.02 mM phenylarsine oxide in PBS). Washes were carried out in either PBS or lysis buffer. Anti-FLAG antibodies were purchased from Sigma-Aldrich (F1804) and Novus Biologicals (NB1P-06712). Anti-TET2 antibody was generated as described below. Anti-actin antibody utilized was purchased from Calbiochem (CP01). Anti-WT1 antibodies used for western blot were purchased from Upstate (05-753) and Abcam (ab28428). Anti-TET1 (GTX1214207) and anti-TET3 (GTX121453) antibodies were purchased from GeneTex.

**Flow Cytometry**

Flow cytometry studies were performed as previously described (Figueroa et al., 2010a). c-Kit coupled to APC (BD Pharmingen) was utilized for c-KIT staining. ShmC staining, reagents utilized, and analysis were performed as described (Figueroa et al., 2010a). Staining with cleaved caspase-3 was used for apoptosis studies. Staining with DAPI was used for cell-cycle analysis.

(D) 5hmC levels were measured by LC-MS from samples of 32D cells transduced with WT1 isofom D or a WT1 truncation mutant (**p < 0.01, t test). No statistically significant difference was observed between 32D cells transduced with migR1 and 32D cells transduced with WT1 mutant. Error bars represent SEM. (E) IP was carried out with anti-FLAG antibody on lysate from GP2/293T-overexpressing vector, TET2-Halo, or both WT1-TET-FLAG isoform D and TET2-Halo. IP was also carried out with an equal amount of rat immunoglobulin G (IgG) on lysate from GP2/293T cells overexpressing both WT1-TET-FLAG and TET2-Halo. (F) IP performed on lysate from GP2/293T cells overexpressing both full-length or truncated forms of WT1-HA and TET2-Halo. Control IP performed with rat IgG. (G) IP performed on lysate of Human leukemia cell lines using an anti-TET2 antibody, IP, immunoprecipitation; IB, immunoblot.
Figure 6. Wt1 Silencing Phenocopies Tet2 Silencing

(A) Murine bone marrow was transduced with a vector or with shRNA (all constructs containing IRES-GFP) targeting Tet2 or Wt1. GFP-positive cells were selected by flow cytometry. GFP-positive cells were maintained in liquid culture and analyzed by flow for c-KIT expression.

(B) GFP-positive cells were plated in methylcellulose and assessed for colony morphology.

(C) Whole bone marrow extracted from a Tet2 knockout mouse was transduced with vector, WT1 isoform D, or a WT1 truncation mutant GFP-positive cells were selected by flow cytometry. Cells were plated in methylcellulose and colony formation was assessed (**p < 0.01 t test).

(D) Cells derived from first methylcellulose plating were analyzed for 5hmC levels by LC-MS (**p < 0.05 t test).

(E) GFP-positive cells from initial transduction were also maintained in liquid culture for 3 days and analyzed for c-KIT expression.

(F) Whole bone marrow from Tet2KO mice was transduced with vector, WT1 isoform D, or WT1 mutant. Cells were then injected into lethally irradiated wild-type recipient mice. GFP percentage was assessed from peripheral blood of mice at time points indicated. Error bars represent SEM.
Murine In Vitro Assays

Methylcellulose assays were carried out as previously described (Figueroa et al., 2010a). Animal care was in strict compliance with Memorial Sloan-Kettering Cancer Center, the National Academy of Sciences Guide for the Care and Use of Laboratory Animals, and the Association for Assessment and Accreditation of Laboratory Animal Care guidelines. All methylcellulose assays were carried out with three biologic replicates and four technical replicates per condition.

Gas Chromatography-Mass Spectrometry

Intracellular 2HG metabolite levels were assayed by GC-MS as previously described (Lu et al., 2012).

ACCESSION NUMBERS

All ERRBS and hme-Seal data have been deposited to the NCBI Gene Expression Omnibus under the accession numbers GSE52945 and GSE37454.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, seven figures, and four tables and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2014.11.004.

AUTHOR CONTRIBUTIONS


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