DNA hydroxymethylation profiling reveals that WT1 mutations result in loss of TET2 function in acute myeloid leukemia

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Summary

Somatic mutations in IDH1/2 and TET2 result in impaired TET2 mediated conversion of 5-methylcytosine (5-mC) to 5-hydroxymethylcytosine (5-hmC). The observation that WT1 inactivating mutations anti-correlate with TET2/IDH1/2 mutations in AML led us to hypothesize that WT1 mutations may impact TET2 function. WT1 mutant acute myeloid leukemia (AML) patients have reduced 5-hmC levels similar to TET2/IDH1/2-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 5-hmC, and WT1 silencing reduced 5-hmC 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 novel role for WT1 in regulating DNA hydroxymethylation and suggest that TET2 IDH1/2, and WT1 mutations define a novel AML subtype defined by dysregulated DNA hydroxymethylation.

Keywords
Epigenetics; AML; hydroxymethylation; WT1; TET2

Introduction

Gene discovery studies in human cancers have identified novel mutations that inform new mechanisms of malignant transformation. Recurrent somatic mutations in epigenetic regulators comprise 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; Filippakopoulos et al., 2010; Zuber et al., 2011).

One class of mutations found in AML and in other malignancies affects the conversion of 5-methylcytosine (5-mC) to 5-hydroxymethylcytosine (5-hmC), mediated by the TET family of enzymes. These include mutations in TET2 and IDH1/2. Mutational profiling of 398
patients with de novo AML demonstrated that TET2 and IDH1/2 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 5-hmC and a reciprocal increase in 5-mC (Ko et al., 2010). A similar effect is caused by aberrant production of the oncometabolite 2-hydroxyglutarate (2-HG) by gain of function IDH1/2 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 5-hmC. Of note, mutations or altered expression of IDH1/2 and TET genes likewise result in altered 5-hmC content in glioblastomas and melanomas (Lian et al., 2012). Yet, it has been shown that not all AML cases with low levels of 5-hmC harbor somatic mutations in TET2 and IDH1/2 (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 5-hmC mapping to be performed in normal tissues and in embryonic stem cells. These studies showed that 5-hmC is commonly localized to gene regulatory elements, including promoters, gene bodies, and enhancers (Stroud et al., 2011). However, to date, genome-wide localization of 5-hmC has not been reported in human malignancies, and the impact of TET2 and IDH1/2 mutations and/or other mutations on 5-hmC 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 5-hmC, 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 IDH1/2 and TET2. Here we show that WT1 mutations are significantly reduced in frequency in patients with TET2/IDH1/2 mutations in AML, and that WT1 mutant AML is characterized by altered DNA methylation and global reductions in 5-hmC similar to that observed in TET2/IDH1/2 mutant AML. Furthermore, we demonstrate that alterations in WT1 levels directly regulate 5-hmC 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 IDH1/2 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 IDH1/2 mutations. Mutations in the WT1 gene were mutually exclusive of IDH1/2 mutations (Patel et al., 2012), and negatively correlated with TET2 mutations (Figure 1A, Figure S1A). 28/313 (9%) of TET2/IDH-wild-type patients had somatic WT1 mutations, whereas 2/85 (2%) TET2/IDH1/2-mutant patients had co-occurring WT1 mutations.

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We observed a similar inverse relationship between WT1 mutations and TET/IDH1/2 mutations in the AML samples analyzed by TCGA (Table S1B). Analysis of combined data from the ECOG1900 study and the AML TCGA dataset confirmed a significant anti-correlation between WT1 mutations and TET2/IDH1/2 mutations (p=0.0164, Fisher's Exact test, Table S1C, Figure S1B). These data suggested a shared functional role for WT1, TET2, and IDH1/2 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 methods) 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/IDH1/2 mutations and identified 124 DMRs, 68% (n=84) of which were hypermethylated in WT1-mutant AML patients (Figure 1C). TET2-mutant and IDH1/2-mutant AML patients were also characterized by hypermethylation compared to AML1-ETO-positive AML (Figure S1C and S1D). Comparative analysis of the three hypermethylation profiles revealed a near-complete overlap of TET2 and WT1 hypermethylated loci within the IDH1/2 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 IDH1/2 mutations are characterized by global reductions in 5-hmC in primary AML samples**

Given IDH1/2 mutations or silencing of TET2 leads to reduced 5-hmC 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 5-hmC 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 5-hmC when compared to AML patients wild-type for WT1, TET2 or IDH1/2 (p=0.016 T-test Figure 1E). Similarly, the reduction in 5-hmC levels was comparable in extent in WT1-mutant AML patients compared to IDH1/2-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 5-mC in WT1 mutant AML, suggesting that WT1 mutant AML is primarily characterized by alterations in 5-hmC leading to site-specific effects on DNA methylation rather than a global increase in 5-mC.

**5-hmC mapping reveals similar alterations in 5-hmC localization in WT1, TET2, and IDH1/2 mutant AML**

In order to determine the impact of IDH1/2, WT1 and TET2 mutations on the distribution of epigenetic marks throughout the genome more precisely, we examined 5-mC and 5-hmC localization in primary AML specimens with next-generation sequencing. Once again, specimens with AML1-ETO translocation, which are all wild-type for IDH1/2, 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 5-hmC (hMe-Seal, see methods (Song et al., 2011)). We identified areas of 5-hmC enrichment for each sample using ChiPseeqer (Giannopoulou & Elemento, 2011). The average number of peaks identified in AML1-ETO specimens was 192,066, for TET2-mut 114,865, for IDH1/2 70,622, and for WT1-mut 60,258. The average number of 5-hmC 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 5-hmC and Figure S2A for overall changes in 5-hmC loss). These data are consistent with the global reduction in 5-hmC observed by mass spectrometry (Figure 1E). We calculated pair-wise comparisons of peaks of 5-hmC enrichment in IDH1/2, WT1 and TET2 mutant AML against 5-hmC sites identified in control AML patients. All three AML subtypes (IDH1/2, WT1 and TET2) displayed a significant reduction in 5-hmC peaks across the entire genome vs. controls, with a smaller proportion (between 1% and 5%) of regions presenting with gains in 5-hmC (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 5-mC. ERRBS assayed 1,433,193 CpGs across all AML subtypes. Pair-wise differential methylation comparisons performed on these ERRBS profiles revealed that 5-mC levels increased genome-wide in IDH1/2, WT1 and TET2 compared to control AML patients; specifically 4 to 6.5% of CpGs were methylated, in IDH1/2, TET2 and WT1 mutations (see Figure 2B). This contrasts with the reduced levels of 5-hmC in these samples. These observations, specifically the loss of 5-hmC and gain of 5-mC in WT1-mutant, TET2-mutant and IDH1/2-mutant AML patients, also holds when we compared 5-hmC and 5-mC levels against normal bone marrows (NMBs) (See Figure S2). Moreover, AML1-ETO AMLs showed no significant difference in the average total number of 5-hmC peaks when compared to NMBs, indicating that the reduction of 5-hmC peaks is specific to those AMLs with disruption of TET2, IDH1/2 or WT1 (Figure S2B).

**Aberrant 5-hmC distribution in WT1, TET2, and IDH1/2 mutant AML occurs predominantly at enhancers and distal regulatory elements**

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

**Differential 5-hmC more strongly correlates with differential gene expression than differential 5-mC in AML with WT1, TET2, and IDH1/2 mutations**

The distinct localization patterns of 5-mC and 5-hmC raised the question of whether these marks can function independently to coordinate gene expression. We used gene expression profiling to compare TET2-mut, IDH1/2-mut, WT1-mut against AML-1ETO and identify the top 500 up-regulated and the top 500 down-regulated genes in the same AML samples (see methods for details). We then examined the relationship between changes in gene expression with changes in 5-mC and 5-hmC 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 - -0.4, Pearson’s R Test p-values between 0.02 and 0001 in the different AML subsets). By contrast, 5-hmC 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^{-14}) in the different AML subsets (Figure 3A bottom) than the correlation observed with 5-mC levels. 5-hmC 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, 5-mC 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 IDH1/2-mutant and TET2-mutant AML, 5-hmC levels at enhancers performed better than 5-mC present at promoters at predicting gene expression, judging from AUC (area under receiver operator curves). The AUC shows the performance of the classifier where AUC of 1 will indicate a perfect model, whereas a random model will have an AUC of 0.5 (Figure 3B). For each model, we measured the AUC using 10-fold cross-validation, which gives a
distribution of AUC values for each model that is generated by training and testing models with randomized subsets of the whole dataset. In WT1 mutant AML, differential 5-mC and differential 5-hmC occupancy independently predicted gene expression equally well (similar AUC values), but a model with combined 5-hmC and 5-mC attributes increased classification performance judging by mean AUC values from cross-validation models (Figure 3B). When comparing the AUC from the different models, the performance of 5-hmC+5-mC and 5-hmC models were significantly better at predicting gene expression (pairwise T-test P-values between $10^{-9}$ and $10^{-8}$). Our findings are consistent with 5-hmC functioning as an independent epigenetic mark that is linked to potential distal regulation, and suggests that 5-hmC has additional functions independent of its role of an intermediate step to DNA demethylation at gene promoters (Yu et al., 2012).

Site-specific 5-hmC alterations in TET2/WT1 mutant AMLs comprise a subset of the alterations seen in IDH1/2 mutant AML

The data presented above suggest a potential unifying link between IDH1/2, TET2, and WT1-mutant AMLs. We therefore assessed site-specific alterations in 5-hmC in IDH1/2, TET2 and WT1 mutant AMLs. IDH1/2-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 5-hmC 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 IDH1/2-mutant AML (Figure 4A and B and Figure S3C). We observed highly significant overlap of differential 5-hmC peaks lost in WT1-mutant AML and TET2-mutant AML (Figure 4B) (Hypergeometric test p-value value < $10^{-133}$). In a manner analogous to the findings for 5-hmC, the hypermethylated sites identified in WT1/TET2 mutant AML were a subset of those found in IDH1/2 mutant cases (Figure 4C and D). 44% of peaks of promoter hypermethylation identified in TET2 mutant AML and 65% of those of WT1 mutant specimens overlap with peaks of 5-mC in IDH1/2 mutant AML (Hypergeometric test p-value value < $10^{-133}$). Collectively these data suggest that a core set of deregulated and presumably silenced genes might represent a unifying pathway in IDH1/2, TET2, and WT1-mutant AML.

While WT1 is a sequence specific transcription factor, the mechanisms by which TET2 is recruited to specific loci to convert 5-mC to 5-hmC has not been delineated. To define candidate transcription factors (TFs) that might be important for TET2 action we examined regions of differential 5-hmC modification for the presence of specific DNA motifs characteristic of known TFs. This motif analysis revealed an over-representation of ETS motifs with GGAA core sequence (Figure S4A and S4B) in regions with 5-hmC enrichment. Notably, we observed that regions with loss of 5-hmC 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. (Wang et al., 1993). Consistent with these data, we observed co-localization of WT1 and TET2 at specific loci with 5-hmC enrichment, including SHANK1 (Figure S4C). We also observed WT1 occupancy at regions with differential 5-hmC which are not bound by TET2, suggesting that other factors including other TET proteins might co-localize with WT1 at other gene regulatory elements (Figure
S4D). We also curated ChIP-seq experiments to identify myeloid lineage specific transcription factors that were enriched at regions with differential 5-hmC 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 5-hmC, but not in hypo-5-hmC regions, suggesting these transcription factors bind to regions with increased 5-hmC, but are not enriched at sites with reduced 5-hmC in IDH1/2, TET2, and WT1-mutant AML.

**WT1 directly regulates 5-hmC levels in hematopoietic cells**

The overlap in regions of 5-hmC 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 5-hmC levels in M15 murine mesonephron cells, which express high levels of Wt1 (Larsson et al., 1995). Knockdown of Wt1 in M15 cells significantly decreased 5-hmC levels in M15 cells (p<0.01, T-test)(Figure 5A,B). Similarly, in primary murine bone marrow (BM) cells, silencing of Wt1 by shRNA (Vicent et al., 2010) (Figure S5A, S5B) significantly reduced 5-hmC 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 5-hmC 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 5-hmC, and if AML-associated WT1 mutations abrogated the ability of WT1 to impact 5-hmC. 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 5-hmC levels compared to cells expressing a control vector or WT1-mutant (p <0.05 for either comparison) (Figure 5D). WT1 overexpression did not alter the expression of TET1, TET2 or TET3 (Figure S5E). In addition, shRNA mediated knockdown of WT1 in primary human CD34+ cells did not result in changes in TET1, TET2, or TET3 expression levels, and WT1 silencing or WT1 overexpression in K562 did not alter TET2 protein expression (Figure S5F-H). Expression analysis of the ECOG1900 cohort data demonstrated TET1, TET2, and TET3 mRNA were expressed at similar levels in WT1 mutant AML patient samples compared to WT1 wild-type AML cases, and WT1 expression was not altered in TET2 mutant versus TET2 wildtype cases, nor in IDH1/2 mutant versus IDH1/2 wildtype cases (Figure S6A-C). We observed no changes in WT1 expression in TET2 mutant AML patients in the TCGA data set (data not shown). In contrast to mutant IDH1/2 alleles, expression of
WT1+/+ or WT1-mutant proteins did not impact 2-HG levels in hematopoietic cells (Figure S6D). Taken together, these data suggest alterations in WT1 expression do not regulate DNA hydroxymethylation by altering TET enzyme expression or by altering IDH1/2 enzymatic function.

**WT1 forms a complex with TET2 in hematopoietic cells**

Given the effects of WT1 on 5-hmC levels and the inverse correlation between WT1 and TET2 mutations in AML, we hypothesized that WT1 might modulate TET2 function through direct interaction. Co-immunoprecipitation 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 co-immunoprecipitation 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 non-specific association of highly expressed nuclear proteins (Figure S6G). Co-immunoprecipitation 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). Co-immunoprecipitation 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 showed 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, erythrocyte, macrophage, megakaryocyte) similar to that observed with Tet2 silencing (Figure 6B). Given the observations that WT1 expression can modulate 5-hmC 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;
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 5-hmC 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 5-hmC 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 2HG) (Lu et al., 2012), which inhibits the activity of all alpha-ketoglutarate-dependent TET enzymes, inhibited the ability of WT1 to alter 5-hmC levels consistent with a TET-family dependent effect of WT1 (Figure 7A). Consistent with these data, co-immunoprecipitation studies demonstrated WT1 directly interacts with TET3, but not TET1 (Figure 7B). We next sought to determine if TET3 could modulate WT1-mediated effects on hematopoiesis in the absence of TET2. We co-expressed 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. (Figure 7C, Figure 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 IDH1/2 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 5-hmC abundance. Our genetic, epigenetic, and biochemical data indicate that TET2, IDH1/2, 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 5-hmC localization in AML patients with and without WT1, TET2 and IDH1/2 mutations. We observed differential 5-hmC localization at enhancers, gene bodies, and distal regulatory elements and differential 5-mC localization at intronic regions near transcription start sites in IDH1/2, TET2 and WT1 mutant AMLs. Moreover, we observed a strong, positive correlation between 5-hmC changes and gene expression as compared to a weaker inverse correlation with 5-mC. These data suggest that 5-hmC has distinct effects on gene regulation independent of its role as an intermediate step to DNA demethylation, and also indicate that 5-hmC may regulate enhancers/chromatin conformation, histone state, and/or transcription factor binding. Subsequent studies using base-pair resolution mapping of 5-hmC and other recently described DNA modifications, combined with mapping other cis/trans-acting elements will help elucidate the complex roles of 5-hmC and other DNA modifications on gene regulation in different cellular contexts.

Our 5-hmC profiling data in AML samples with IDH1/2, WT1 and TET2 mutations reveal site-specific loss of 5-hmC in AMLs with impaired TET function, which is most widespread in IDH1/2 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 5-hmC modification are more substantial than in cases with mutations which 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 5-hmC loss, the majority of the loci with altered 5-hmC in AMLs with WT1 and TET2 mutations represent a subset of the loci with differential 5-hmC seen in patients with IDH1/2 mutations. Subsequent functional studies are needed to determine if the “core” set of loci with altered 5-hmC are universally altered in all AML patients with IDH1/2, WT1 and TET2 mutations and how they precisely contribute to leukemic transformation.

In the majority of AML patients, WT1 is not mutated and in fact is overexpressed. 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 several different proteins, including p53 (Zhan et al., 1998). Furthermore, through protein-protein interactions, WT1 can suppress the activity of the TCF transcription factor and Wnt pathway targets (Kim et al., 2009). However the role of these specific functionalities in WT1-mediated hematopoietic transformation is not known. Here we demonstrate a direct role for WT1 in regulating 5-hmC placement in hematopoietic cells through interaction with TET2 and TET3. WT1 loss led to marked reductions in 5-hmC 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 which induce transformation through perturbations in TET enzyme function in different malignant contexts.
Experimental Procedures

Patient Samples—398 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 was available for 325/398 cases. IRB 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 (Alameda, CA). 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 5-hmC levels assessed by LC/MS was performed using two-sided T-test.

Constructs—Human WT1 isoform (both containing and not containing a 17 amino acid region within exon 5 as well as the KTS region of the c-terminal) cDNA was cloned into Migr1 (Addgene). WT1-MUTANT (containing a 17 amino acid region within exon 5) cDNA was cloned into Migr1. TET2 cDNA was subcloned into pCMV6-ENTRY (Origene) with a C-terminal FLAG tag and myc tag. TET1 and TET3 cDNA was synthesized and subcloned into HaloTag vector pFN21A (Promega).

Cell Culture and Transfection—GP2-293T cells were cultured as previously described (Marubayashi et al., 2010). Transfection was performed with X-treme 9 transfection reagent (Roche). 32D cells were cultured in RPMI-1640 medium (Invitrogen) supplemented with 10% fetal bovine serum and 1 ng/ml recombinant IL-3 (BD Biosciences, 554579).

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 Supplemental Methods for description of protocol used.

shRNA knockdown

TET2 shRNA was produced as previously described (Figueroa et al., 2010a). WT1 shRNA was produced using previously described and validated target sequence (Vicent et al., 2010) and inserted into a pSIREN vector (Clontech). Wt1 ShRNA in a pLKO-Puromycin vector was generated by the Broad Institute RNAi Consortium (Cambridge, Mass). 21nt shRNAs targeting mouse Tet3 were designed and cloned into the LMP retroviral vector. (Dow et al., 2012). ShRNA sequences are provided in supplemental methods. All shRNA experiments were carried out using three biologic replicates and three technical replicates for each condition.
Western Blot and Co-Immunoprecipitation

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 150mM NaCl, 20mM Tris, 5mM 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 (F1804) and Novus Biologicals (NBPI-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 (GTX124207) 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 Pharmigen) was utilized for c-KIT staining. 5-hmC staining, reagents utilized, and analysis were performed as described (Figueroa et al., 2010a). Staining with cleaved-caspase3 was used for apoptosis studies. Staining with DAPI was used for cell cycle analysis.

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 (GC-MS)

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

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Based on the observation that WT1 mutations are inversely correlated with TET2 and IDH1/2 mutations in AML, we found that AML patients with WT1 mutations had reduced 5-hmC levels consistent with a defect in TET2 enzymatic function. Genome-wide 5-hmC-sequencing revealed similar changes in 5-hmC occupancy in patients with WT1, TET2, and IDH1/2 mutations, consistent with a convergent mechanism of epigenetic remodeling. We showed that WT1 modulates 5-mC through direct interactions with TET2 and TET3. These data provide a novel mechanism for the role of WT1 mutations in AML and provide evidence that there are additional somatic mutations that directly modulate TET2 function in human malignancies.
Figure 1.
WT1 mutations are inversely correlated with TET2/IDH1/2 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 IDH1/2 mutant AMLs (E) 5-methylcytosine (5-mC, left panel) and 5-hydroxymethylcytosine (5-hmC, right panel) levels in AML samples from patients with or without WT1, TET2, or IDH1/2 mutations. 5-mC and 5-hmC 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, IDH1/2, and WT1 mutations. (A) KIRREL locus demonstrating depletion of 5-hmC marks in AML patients with TET2, WT1, and IDH1/2 mutations. (B) Percentages of differential 5-hmC regions and 5-mC bases. Bar plot on the left demonstrates percentages of hypo- or hyper-5-hmC regions out of all canonical peaks in WT1, TET2, and IDH1/2 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 IDH1/2 mutants compared to AML1-ETO patients. Differentially methylated CpGs that overlap with differential 5-hmC regions are removed from the analysis. (C) Genomic locations of differentially hydroxymethylated regions and DMCs. The first row shows percentages of differentially hydroxymethylated regions overlapping with gene annotation, CpG island annotation and enhancer annotation. The second row shows the percentage of DMCs overlapping with the aforementioned annotation categories. (D) Distances to nearest TSS for differentially DHMRs and DMCs for IDH1/2, TET2, and WT1 mutants. All comparisons are against AML1-ETO patients.
Figure 3.
Correlation of gene expression with DNA methylation and hydroxymethylation. (A) Scatterplots and correlations of differential gene expression and average methylation difference on CpG islands near TSS for IDH-mut vs. AML1-ETO, TET2-mut vs. AML1-ETO and WT1-mut vs. AML1-ETO (top row). Scatterplots and correlations of differential gene expression and average adjusted fold changes of 5-hmC canonical peaks for IDH-mut vs. AML1-ETO, TET2-mut vs. AML1-ETO and WT1-mut vs. AML1-ETO (bottom row). (B) Mean AUC (area under receiver operator curve) for gene expression classification models based on differential 5-mC and 5-hmC attributes for IDH-mut vs. AML1-ETO, TET2-mut vs. AML1-ETO and WT1-mut vs. AML1-ETO. Classification models are based on differential 5-hmC attributes and/or differential 5-mC attributes aiming to predict up-regulated and down-regulated genes. Error bars represent SD of AUC of the cross-validation models.
Figure 4.
Site-specific 5-hydroxymethylcytosine alterations in WT1 and TET2 mutant AML comprise a subset of the alterations in IDH1/2 mutant AML. (A) Bar plots showing number of hypo-DHMRs per subtype compared to AML1-ETO. For each subtype, the number of hypo-DHMRs that do not overlap with hypo-DHMRs of IDH-mut are color-coded. (B) Venn diagram showing the number hypo-DHMRs for each subtype and their overlap. (C) Bar plots showing number of hyper-DMCs per subtype compared to AML1-ETO. For each subtype, the number of hyper-DMCs that do not overlap with hyper-DMCs of IDH-mut are color-coded. (D) Venn diagram showing the number hyper-DMCs for each subtype and their overlap.
Figure 5.
WT1 complexes with TET2 and alterations in Wt1 levels result in changes in 5-hydroxymethylcytosine 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) 5-hmC 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) 5-hmC levels were measured by LC-MS from samples of murine whole bone marrow transduced with either vector or Wt1-targeted shRNA (D) 5-hmC levels were measured by LC-MS from samples of 32D cells transduced with WT1 isoform 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-FLAG isoform D and TET2-Halo. IP was also carried using an equal amount of Rat IgG on lysate from GP2/293T cells overexpressing both WT1-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 5-hmC levels by LC-MS (** p<0.05 T-test) (E) GFP-positive cells from initial transduction were also maintained in liquid culture for three 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 wildtype recipient mice. GFP percentage was assessed from peripheral blood of mice at time points indicated. Error bars represent SEM.
WT1 binds to TET3 (A) GP2/293T cells were transfected with vector or WT1 isoform D. Cells were grown in the presence of DMSO or 2HG. 5-hmC levels were subsequently analyzed by LC-MS. (B) GP2/293T cells were transfected with a WT1-FLAG construct along with TET3 or TET1 construct. IP was carried out with either an anti-FLAG antibody or an equivalent amount of Rat IgG. (C) Tet2-deficient BM cells were transduced with empty vector or two different shRNAs targeting Tet3 (all with IRES GFP). GFP positive cells were sorted for, and then transduced with a WT1 construct (with puromycin resistance marker), followed by puromycin selection. Cells were plated in methycellulose, and colonies were counted. Comparison of post-WT1 selection samples demonstrated statistically significant increase in colonies in cells transduced with Tet3 shRNA (** p<0.01). Error bars represent SEM.