Biallelic \textit{TET2} mutation sensitizes to 5’-azacitidine in acute myeloid leukemia

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\textit{JCI Insight}. 2022. https://doi.org/10.1172/jci.insight.150368.

Graphical abstract
Biallelic TET2 mutation sensitizes to 5’-azacitidine in acute myeloid leukemia

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Conflicts of interest

MM is employed by the Munich Leukemia Laboratory and TH is part owner of the Munich Leukemia Laboratory. The remaining authors declare no competing financial interests or conflicts of interest.
Abstract

Precision medicine can significantly improve outcomes for cancer patients, but implementation requires comprehensive characterization of tumor cells to identify therapeutically exploitable vulnerabilities. Here we describe somatic biallelic TET2 mutations in an elderly patient with acute myeloid leukemia (AML) that was chemoresistant to anthracycline and cytarabine (Ara-C), but acutely sensitive to 5’-azacitidine (5’-Aza) hypomethylating monotherapy resulting in long-term morphological remission. Given the role of TET2 as a regulator of genomic methylation, we hypothesized that mutant TET2 allele dosage affects response to 5’-Aza. Using an isogenic cell model system and an orthotopic mouse xenograft, we demonstrate that biallelic TET2 mutations confer sensitivity to 5’-Aza compared to cells with monoallelic mutation. Our data argue in favor of using hypomethylating agents for chemoresistant disease or as first line therapy in patients with biallelic TET2-mutated AML and demonstrate the importance of considering mutant allele dosage in the implementation of precision medicine for cancer patients.
Introduction

Acute myeloid leukemia (AML) is the exemplar of how interrogation of the somatic genome has facilitated understanding of disease pathogenesis and led to the development of novel therapies and stratified treatment approaches for some disease sub-groups (1, 2). For example, the outcome of t(15;17)-positive acute promyelocytic leukemia has been revolutionized by the introduction of differentiation chemotherapy targeted against the promyelocytic leukemia-retinoic acid receptor-α fusion oncoprotein that defines this sub-group of AML (3, 4). Despite this success, therapeutic options for the majority of AML patients are limited and outcome remains very poor, with a 5-year overall survival (OS) of just 15% (5). Subsequent refinement of the AML genomic landscape has revealed a plethora of somatically mutated genes, including TET2 which has been associated with poor outcome following treatment with standard anthracycline and nucleoside analogue-based chemotherapy (1, 6).

Given the ineffectiveness of standard chemotherapy for many patients and the resulting poor outcome it is essential to fully understand how somatic genetics can be utilized to identify vulnerabilities that can be therapeutically exploited using existing treatments and the expanding catalogue of new agents. To this end, we present data that biallelic TET2 mutations in AML confer sensitivity to hypomethylating chemotherapy. The TET2 enzyme catalyzes DNA demethylation by converting 5’-methylcytosine to 5’-hydroxymethylcytosine, and loss or attenuation of TET2 function leads to a somatically acquired global genomic hypermethylation and transcriptional and phenotypic re-programming underpinning the development of a leukemia phenotype (7). As such, it is mechanistically plausible that hypomethylating chemotherapy could be particularly effective in TET2 null AML. These data serve as a paradigm for clinical
diagnostics incorporating comprehensive genomic analyses to implement first line therapies with a higher likelihood of response in patients with AML.
Results

AML index case with biallelic TET2 mutations

We describe a 76 year old male who presented with AML characterized by a t(4;12) translocation but no other cytogenetic abnormalities (46,XY,t(4;12)(q27;q13)[12]/46,XY[10]) as identified by G-banding and confirmed by spectral karyotyping (Figure 1A). Standard 3+7 induction chemotherapy with daunorubicin and cytarabine (Ara-C) gave rise to a reduction in white blood cell count (Figure 1B). However, the patient became pancytopenic and developed acute septicemia requiring intensive care, intravenous antibiotics and vasopressor therapy. Following recovery, blasts persisted in the bone marrow (BM) (29% at day 30; Figure 1C) indicating chemoresistant disease. The patient was subsequently treated with single agent 5’-azacitidine (5’-Aza) monthly as palliation (Figure 1B), which unexpectedly resulted in prolonged complete morphological remission (CR) characterized by restoration of normal morphology (Figure 1C). The patient remained in CR for 24 months prior to emergence of relapsed AML. Relapsed disease was treated with subcutaneous Ara-C and Sorafenib but was unresponsive to chemotherapy and the patient died 28 months after first diagnosis. Autopsy revealed subtotal AML BM infiltration and multiple extramedullary AML sites, including lymph nodes and numerous parenchymatous organs (Figure S1).

In order to investigate the molecular basis underlying the prolonged response to 5’-Aza in this patient we performed exome sequencing, interphase FISH and single nucleotide polymorphism (SNP) array analysis of BM at AML presentation, during morphological remission and at relapse. SNP array analysis demonstrated that the major cell clone at presentation was characterized by a focal 1.1Mb deletion encompassing the TET2, CXXC4 and PPA2 genes (Figure 1D). Interphase FISH on diagnostic BM demonstrated that approximately 95% of cells
carried the \textit{TET2} deletion (Figure 1E). Additionally, the retained \textit{TET2} allele harbored a nonsense base substitution mutation in exon 3 affecting codon 939 (c.2815C>T, Q939*; Figure S2) which was detected in 96% of cells. The presentation AML was also characterized by a heterozygous \textit{NPM1} mutation (c.863_864insTCTG; Figure S3) in all cells with \textit{TET2} deletion. Integration of SNP array, exome and FISH data was used to infer tumor phylogeny which indicated that disease pathogenesis was initiated by the \textit{TET2} nonsense mutation with subsequent deletion of the second \textit{TET2} allele, followed by acquisition of the \textit{NPM1} mutation (Figure 1F). Both the \textit{TET2} gene deletion and base substitution mutation were also present at high levels in the remission BM, despite this appearing morphologically normal (Figures 1C, 1F and S2). Although not discernible in the remission BM, the \textit{NPM1} mutation was presumed to have persisted at levels below detection given that it was a prominent feature of relapse disease, in addition to the \textit{TET2} gene deletion and \textit{TET2} base substitution (Figure 1F and Figures S2-S3). The relapse was also characterized by a heterozygous \textit{FLT3} internal tandem duplication (c.1780_1800dupTTCAGAGAATATGAATATGAT; Figure S4) in 80% of cells, which was not discernible in diagnostic or remission samples (Figure 1F and Figure S4).

These data demonstrate that 5'-Aza treatment almost completely eliminated the \textit{TET2/NPM1}-mutated clone dominant at disease presentation. Although also reduced by 5'-Aza treatment, ancestral AML cells carrying biallelic \textit{TET2} mutations but negative for the \textit{NPM1} mutation retained viability and presumably re-acquired the ability to differentiate and recapitulate normal hematopoiesis rendering a cytomorphological remission. Based on these observations, we hypothesized that mutant \textit{TET2} allele dosage could affect cellular response and sensitivity to 5'-Aza.
Biallelic TET2 mutations result in a hypermethylation phenotype in AML cells and confer sensitivity to 5’-Aza hypomethylating chemotherapy in vitro and in vivo

In order to test whether biallelic TET2 mutations sensitize AML cells to 5’-Aza we used CRISPR-Cas9 gene editing to completely inactivate TET2 in the HEL AML cell line. HEL cells, derived from a 30-year old male with erythroleukemia, have a complex hypotriploid karyotype with 60-64 chromosomes (Table S1) and are reported to carry a monoallelic TET2 gene deletion(8). Consistently, high density SNP array analysis demonstrated that HEL cells carry a large deletion and concomitant loss of heterozygosity (LOH) affecting the majority of the long arm of chromosome 4 which includes the TET2 gene (Figure 2A). Following transduction of HEL cells with CRISPR-Cas9 directed to TET2, Sanger sequencing revealed that the retained TET2 allele was mutated in several independent clones. In particular, a 4bp deletion in exon 6 (Figure S5) was frequently observed. Regardless of the underlying mutation, HEL clones from independent CRISPR-Cas9 transductions were consistently null for TET2 protein expression (Figure 2B). Biallelic TET2 mutations and consequent complete loss of TET2 protein expression did not affect proliferation kinetics of HEL clones in liquid media (Figure 2C) nor cloning efficiency (CE) in soft agar (Figure 2D).

Analysis on human methylation (450K) arrays revealed the acquisition of an overall hypermethylation genotype in TET2 null HEL clones with biallelic TET2 mutations (HEL TET2 biallelic clones) compared to parental HEL clones with a monoallelic TET2 deletion (referred to as HEL TET2 monoallelic clones) (Figure 2E). Specifically, 7,960 of 410,811 probes (1.9%) were significantly hypermethylated (Log2FC ≥ 2) and 4,584 probes (1.1%) were significantly hypomethylated (Log2FC ≤ -2) in HEL TET2 biallelic clones (n=4) compared to HEL TET2 monoallelic clones (n=2) (Figure 2E and Table S2). Differentially methylated probes mapped to
loci throughout the genome (Table S2). Unsupervised hierarchical clustering demonstrated clustering of HEL clones based on TET2 mutation status (Figure 2F).

When treated with 5’-Aza in vitro, HEL TET2 biallelic clones had significantly lower CE (P = 0.003) and proliferation in liquid culture (P < 0.001) compared to isogenic parental HEL TET2 monoallelic clones (Figure 3A and 3B). In contrast, TET2 mutation load did not affect CE or cell proliferation following treatment with Ara-C or daunorubicin (Figure 3A and 3B). TET2 mutation status did not affect apoptosis induction in response to 5’-Aza, as measured by the induction of cleaved PARP (Figure 3C) or the induction of cells with sub-G1 DNA content (Figure S6), suggesting that complete loss of TET2 expression does not sensitize cells to 5’-Aza-induced apoptosis.

Having demonstrated that HEL cells null for TET2 protein expression were sensitive to growth inhibition by 5’-Aza we sought to test the hypothesis in a second AML cell line mutant for TET2. SKM1 cells have a monoallelic TET2 mutation (c.4253_4254insTT, p.1419fsX30) (9), but are phenotypically null for TET2 protein expression (Figure 3D), despite having an intact wild-type TET2 allele. Nevertheless, consistent with the HEL cell data, TET2 null SKM1 cells are acutely sensitive to 5’-Aza (Figure 3D). We also quantified TET2 protein levels in a panel of 9 additional AML cells lines and determined sensitivity to 5’-Aza (Figure 3D). Strikingly, there was significant correlation between TET2 protein levels and 5’-Aza IC90 (R² = 0.77, P = 0.0008) and IC50 (R² = 0.88, P < 0.0001) (Figure 3D). THP1 cells had the highest TET2 protein expression and were the most resistant to 5’-Aza. In contrast, SKM1 cells had the lowest TET2 protein expression and were the most sensitive to 5’-Aza.

We next sought to determine whether biallelic TET2 mutations sensitize AML cells to 5’-Aza in vivo in an orthotopic xenograft mouse model. We used a competitive engraftment approach in
which HEL cell clones with monoallelic and biallelic $TET2$ mutation were co-transplanted into mice and an allele-specific qPCR assay for the WT and CRISPR-Cas9-modified $TET2$ alleles was subsequently utilized to determine preferential engraftment and/or elimination of either cell clone, with or without treatment with $5'$-Aza. Specifically, following prior validation of the qPCR assay (Figure S7), HEL $TET2$ monoallelic and HEL $TET2$ biallelic cell clones were co-injected intrafemorally (IF) in a 1:1 ratio into $Rag2^{-/-}Il2rg^{-/-}$ mice (day 0; Figure 4A). Physical symptoms associated with the proliferation of AML cells (including weight loss, limited mobility and growth of leg tumors in some animals) became apparent approximately 4 weeks post-IF injection. On day 28 mice began once daily treatment with 5 mg/kg $5'$-Aza (or vehicle only as control (VC)) for a total of 5 days and were then euthanized 3 days later (day 35 after engraftment and day 8 after the initiation of treatment) for sample collection and qPCR analysis (Figure 4A). Tissue samples were collected from six $5'$-Aza-treated and nine VC-treated mice. We were able to consistently amplify human $TET2$ DNA in tissue obtained from injected femurs, as well as non-injected femurs, spleens and other organs showing evidence of AML infiltration, yielding a total of 55 individual samples (32 from VC-treated mice and 23 from $5'$-Aza-treated mice). There was no overall mean preferential amplification of either the intact WT or CRISPR-modified $TET2$ allele in all 32 tissue samples from VC-treated mice (median inverse $\log_2 [\Delta Ct] = 0.81$; Figure 4B), although the CRISPR-modified $TET2$ allele slightly dominated in spleen samples ($P = 0.047$; Figure S8) suggesting preferential engraftment of TET2 null cells specifically in this tissue. Conversely, in $5'$-Aza-treated mice the WT $TET2$ allele was dominant in 19 of 23 (83%) tissue samples (median inverse $\log_2 [\Delta Ct] = 16.81$; Figure 4B), demonstrating significant negative selection against TET2 null cells ($P = 3.6 \times 10^{-4}$) as a result of $5'$-Aza treatment. There was also significant negative selection of TET2 null cells specifically in the BM
of 5’-Aza-treated mice (median inverse Log₂ [ΔCt] = 2.09 and 79.34 for VC- and 5’-Aza-treated mice, respectively; P = 0.014) with 2 (of a total of 12 femurs from 5’-Aza-treated mice) completely negative for the CRISPR-modified TET2 allele (Figure 4B).

Taken together, these data demonstrate that TET2 null cells are sensitive to the hypomethylating agent 5’-Aza both in vitro and in vivo, consistent with the response to 5’-Aza observed in the index AML patient.

We next investigated whether knockdown or knockout of TET2 affects sensitivity to 5’-azacitidine in AML cell lines derived from primary AML that was not TET2 mutant. shRNA-mediated knockdown of TET2 in THP1 AML cells (10, 11) conferred sensitivity to the growth inhibitory effects of 5-Aza (P=0.005), although the phenotype was relatively weak (Figure S9). In contrast, CRISPR-mediated knockout of TET2 in KG1 AML cells (11) conferred resistance to the growth inhibitory effects of 5-Aza (P<0.0001; Figure S9). It should be noted that neither of these cell models are null for TET2 and both retain some residual protein expression (Figure S9), which is possibly due to incomplete shRNA-mediated knockdown in THP1 cells and incomplete CRISPR-targeting in KG1 cell populations, respectively.

**Gene expression analysis identifies downregulation of small nuclear ribonucleoprotein complex components and ABCB1 drug efflux in cells with biallelic TET2 mutations**

RNA sequencing analysis was performed to identify differentially expressed genes and potential mechanisms responsible for 5’-Aza sensitivity in HEL TET2 biallelic cell clones. Using unsupervised hierarchical clustering of transcript data, HEL cell clones clustered broadly by TET2 genotype (Figure 5A), suggesting that complete loss of TET2 protein significantly impacted on transcription. Differential expression analysis identified 695 significantly differentially expressed transcripts (Padj < 0.05; |Log₂FC| ≥ 0.3) in HEL TET2 biallelic clones.
compared to HEL TET2 monoallelic clones (Figure 5B; Table S3). Gene ontology analysis identified several significantly affected cellular components (Table S4), of which the spliceosomal small nuclear ribonucleoprotein (snRNP) complex (GO:0097525), was the most significantly affected (Padj = 8.7 x 10^{-4}). In differential expressional analysis, the 12 RNA genes and 1 protein coding gene (LSM8) which make up this complex were all significantly downregulated in TET2 null cells (Figure 5C; Table S5). Likewise, spliceosomal tri-snRNP complex assembly (GO:0000244) was identified as the most significantly affected biological process (Padj = 3.4 x 10^{-4}; Table S6). Downregulation of protein expression in TET2 null cells was confirmed for LSM8, consistent with transcript expression data (Figure 5D). Significant differences in expression were also identified for other genes that could potentially affect cellular response to 5’-Aza (Table S3). These included ABCB1 (MDR1) (12), encoding a member of the ATP-binding cassette (ABC) family of drug transporters, which was down-regulated in TET2 null cells at the transcript (Padj = 8.1 x 10^{-4}) and protein level (Figure 5D).

In order to further investigate the role of ABCB1 as a determinant of sensitivity to 5’-Aza, HEL AML cell clones were treated with ABCB1 inhibitors verapamil or tariquidar. When used in combination with 5’-Aza, both agents sensitized HEL AML cells to the growth inhibitory effects of 5’-Aza. Moreover, co-treatment with either ABCB1 inhibitor and 5’-Aza was synergistic in HEL cell clones with monoallelic TET2 mutation, which express high levels of ABCB1, but not in HEL cell clones with biallelic TET2 mutation, which express low levels of ABCB1 (Figures 6A and 6B; Figures S10 and S11). We next cloned HEL AML cells in soft agar supplemented with 10 μM 5’-Aza. Colonies that survived 5’-Aza exposure were expanded and all shown to be resistant to 5’-Aza (Figure 6C). Regardless of TET2 mutant allele dosage, eight of nine 5’-Aza resistant HEL cell clones had up-regulated ABCB1 protein expression relative to their respective
parental cells from which they were derived (P=0.0069, Figures 6D and 6E). Taken together, these data demonstrate a role for ABCB1 as a determinant of sensitivity to 5’-Aza.

**Biallelic TET2 alterations in AML patients with cytogenetically discernible chromosome 4 aberrations**

The *TET2* locus can be somatically affected via numerous mechanisms, including point mutations as well as gains and losses of material, although how these give rise to biallelic *TET2* mutation remains unclear. In order to investigate we screened the Study Alliance Leukemia (SAL) biobank for AML patients presenting with a cytogenetically discernible aberration affecting chromosome 4; a population likely to be enriched for structural *TET2* alterations. However, because *TET2* base substitutions are reported with high frequency in cytogenetically normal AML (1) our approach of selecting cases with chromosome 4 abnormalities does not inform on the overall frequency of alterations in AML. Thirty cases recruited to the SAL biobank had a chromosome 4 aberration visible cytogenetically and had sufficient material for SNP array analysis and sequencing (Table S7).

Gains affecting *TET2* were discernible by SNP array in 6 patients (all with trisomy 4 visible cytogenetically), which included two cases with homozygosity affecting most of the long arm of chromosome 4 (Figure 7A). One of these two patients (UPN25) also had a *TET2* base substitution (c.4133G>A, p.Cys1378Tyr) carried by almost 100% of the cells (Figure 7A and Figure S12) and as such has biallelic mutations affecting *TET2*. Six cases had loss of genetic material affecting *TET2* discernible from SNP array data, which included three cases with large deletions (UPN09, UPN10 and UPN18) and two cases with a focal deletion in one allele and a nonsense mutation in the other allele (index case (UPN01) and UPN30)) (Figure 7A and Figure S12). The sixth case (UPN28) had trisomy 4 but with a focal 585Kb deletion affecting the entire
TET2 gene, resulting in copy number reduction (< 2 copies) and loss of heterozygosity (Figure 7A and Figure S12). A further 6 cases had copy number alterations (5 with loss, 1 with gain) on chromosome 4 which did not affect the TET2 locus (Figure 7A) and the remaining 12 cases had no evidence of TET2 base substitution or gain/loss of material on chromosome 4. Although two of these 12 cases had trisomy 4 (UPN26 and UPN29) and one case had monosomy 4 (UPN13) visible cytogenetically, these aneuploidies were present in a minor sub-clonal population (Table S7), which explains why they were not visible in the SNP array data. These data demonstrate that TET2 alterations are complex, often involving gains or losses of material in combination with base substitution mutations.

**Biallelic TET2 alterations in AML patients treated with 5’-Aza**

Having described a single patient with biallelic TET2 mutation (index case UPN01) who responded very well to 5’-Aza we sought to determine whether biallelic TET2 mutation was also associated with a favorable response in other patients treated with 5’-Aza. AML patients over the age of 65 years were recruited to the PETHEMA FLUGAZA phase 3 clinical trial and were randomized to receive either 5’-Aza or low-dose Ara-C plus fludarabine (FLUGA) (13). Fifty patients had a TET2 mutation identified by targeted sequencing which included 6 patients with a mutant allele frequency >85% indicative of biallelic TET2 mutation (3 patients were randomized to each arm of the trial) (Table S8). None of the three patients with biallelic TET2 mutation randomized to the FLUGA arm (UPN68, UPN73, UPN78) achieved CR and all had relatively short OS (111, 45 and 17 days) (Figure 7B, Table S8). In contrast, 2 of the 3 patients with biallelic TET2 mutation randomized to the 5’-Aza arm achieved CR (UPN31 and UPN33) and had prolonged OS (767 and 579 days) (Figure 7B, Table S8). The third patient treated with 5’-Aza (UPN47) failed to achieve CR and died (day 62) after cycle 1 with progressive disease.
(Figure 7B, Table S8). Furthermore, all three patients with biallelic TET2 mutation treated with 5’-Aza had European LeukemiaNet (ELN) adverse risk AML and an Eastern Cooperative Oncology Group (ECOG) performance score of 3. Also, one of the patients with biallelic TET2 mutation who responded to 5’-Aza (UPN33) had AML that was TP53 mutant (Figure 7B; Table S8).

In summary, we have identified 3 patients with biallelic TET2 mutation who had a favorable response to single-agent 5’-Aza, including the index case (UPN01) who had disease resistant to standard daunorubicin and Ara-C remission-induction chemotherapy.
Discussion

Up to 30% of AML patients present with a somatically acquired TET2 mutation (14-20), with biallelic mutations representing a minority of all TET2-mutated AML cases (20-22). The prognostic effect of TET2 mutation in AML treated with anthracycline and nucleoside analogue-based regimens remains controversial (16, 18, 19), although meta-analyses suggest an association with poor prognosis (23, 24). As such, there is an urgent clinical need to identify novel therapeutic approaches to improve outcome of TET2-mutated AML. Some studies have reported an association between TET2 mutation and favorable outcome of myelodysplastic syndrome (MDS) following treatment with hypomethylating chemotherapy such as 5’-Aza (25-29), although other studies have not replicated these findings (30). Our data demonstrate that single-agent 5’-Aza treatment of AML harboring biallelic TET2 mutations can give rise to long term CR, including disease otherwise refractory to standard 3+7 induction chemotherapy with daunorubicin and Ara-C and also in patients with adverse risk disease or poor performance status. Furthermore, using an isogenic model system, we demonstrate that biallelic TET2 mutations confer cellular hypersensitivity to 5’-Aza in vitro, as well as significant negative selection when competitively xenografted with monoallelic TET2-mutated cells into the bone marrow of mice.

It should be noted that our data were primarily generated using HEL AML cells, which were derived from primary AML with monoallelic TET2 mutation. We also observed acute sensitivity to 5’-Aza in TET2 null SKM1 cells, which were also derived from TET2 mutant primary AML. We also investigated 5’-Aza sensitivity in AML cell lines not derived from TET2-mutant disease and although we observed sensitivity in THP-1 cells with TET2-knockdown we did not see the same phenotype in KG-1 cells with TET2 knockout, which were relatively resistant to 5’-Aza.
compared to TET2 wild-type KG-1 cells. These data suggest that complete loss of TET2 expression might be required for sensitivity to 5'-Aza or that sensitivity is modified by other somatic mutations. For example, HEL and SKM1 cells are wild-type for TP53 whereas THP-1 and KG-1 are both mutant for TP53, which is associated with poor outcome in AML (31, 32).

Although we report one patient with TET2 biallelic-mutant TP53-mutant AML who responded well to 5'-Aza (UPN33) all other patients with biallelic TET2 mutation were wild-type for TP53. As such, further work is warranted to understand the impact of TP53 and other somatic mutations in determining response to 5'-Aza in TET2 null AML.

An effect of mutant TET2 gene dosage on response to therapy is perhaps not surprising given the evidence demonstrating that mutant allele dose also affects disease development. Specifically, monoallelic (Tet2+/−) and biallelic Tet2 deletions (Tet2−/−) both result in myeloid malignancy in animal models, but the latency and OS are significantly shorter in Tet2 null animals (33, 34). Furthermore, Tet2 null (Tet2−/−) mice with myeloid disease also have more pronounced splenomegaly compared to heterozygous (Tet2+/−) littermates (33), and splenomegaly (and extramedullary disease) is a general feature of myeloid disease developing in Tet2 knockout mouse models (10, 34). Consistent with this, young healthy mice null for Tet2 have elevated extramedullary hematopoiesis in the spleen, which develops into splenomegaly concomitant with the onset of myeloid dysplasia (35). These observations are consistent with our data demonstrating a significant competitive advantage of TET2 null human cells to populate the spleen of engrafted animals and also that the index patient (UPN01) reported herein presented with splenomegaly and extramedullary disease. Taken together, these data suggest that TET2 loss could predispose to myeloid disease characterized by splenomegaly and extramedullary
disease in general, which is mutant TET2 gene dosage-dependent, although investigation in large patient cohorts is warranted.

Our data suggest that complete loss of TET2 renders cells more sensitive to the anti-proliferative effects of 5'-Aza, rather than enhancing susceptibility to drug-induced apoptosis, consistent with the observed negative selection against cells with biallelic TET2 mutation observed in vivo. Despite this, 5'-Aza treatment rarely resulted in the complete elimination of TET2 null cells in mice, consistent with data from the index patient in whom 5'-Aza-induced morphological remission was characterized by the persistence of cells with biallelic TET2 mutations. Mutation persistence in morphological remission has been reported for several leukemia driver genes, including those characteristic of age-associated clonal hematopoiesis such as TET2, DNMT3A, SRSF2, RUNX1 and ASXL1 (36-39). Likewise, persistence of Tet2-mutated cells has also been reported in animal models treated with 5'-Aza (40). Targeting two different epigenetic layers in monoallelic TET2 mutated AML with 5'-Aza and LSD1 inhibition has been demonstrated to be effective in primary AML cells ex vivo (41). However, a model analyzing responsiveness of biallelic TET2 mutated AML to 5'-Aza has not been reported thus far.

Our data demonstrate that cells with monoallelic and biallelic TET2 mutations have significantly different genomic methylation profiles, and although we observed a genome-wide shift towards hypermethylation in cells with biallelic TET2 mutation, the effect was relatively modest and there were also large numbers of CpG sites that became hypomethylated. Consistent with this, we also noted up-regulated transcript levels for numerous genes. As such, it seems unlikely that global genomic DNA methylation and concomitant global loss of expression is responsible for the observed sensitivity to 5'-Aza. Rather, the prevailing evidence suggests that the underlying mechanism conferring sensitivity to 5'-Aza is gene/pathway specific, and our investigations
identified significant down-regulation of spliceosomal small nuclear ribonucleoprotein (snRNP) complex components in cells with biallelic TET2 mutations. The snRNP pathway has previously been implicated as a determinant of cellular sensitivity to 5’-Aza (42), although the underlying mechanisms remain to be fully deciphered. We also show that ABCB1 is down-regulated in AML cells with biallelic TET2 mutation and that inhibition of this efflux transporter sensitizes to the growth inhibitory effects of 5’-Aza. Inhibition of ABCB1 leads to increased intracellular accumulation of 5’-Aza in SKM1 AML cells (43, 44), providing further evidence that ABCB1 is involved in 5’-Aza efflux. Consistent with our data, treatment with a combination of 5’-Aza and erlotinib, which antagonizes ABCB1, is synergistically cytotoxic in several AML cell lines, including SKM1, MOLM-13, HL-60 and MV4-11 (44). We also demonstrate significant up-regulation of ABCB1 protein expression in 5’-Aza-resistant HEL cell clones. Messingerova and colleagues (45) also reported up-regulation of ABCB1 protein in 5’-Aza-resistant clones developed from SKM1 and MOLM-13 AML cell lines.

Mutations in other genes operating in the TET2 hydroxymethylation pathway are also reported in AML, including IDH1, IDH2 and WT1. Mutations in IDH1 and IDH2 inhibit TET2 function (and TET1 and TET3) via production of 2-hydroxyglutarate (46). WT1 mutations are also reported in AML (47) and drive leukemogenesis via inhibition of TET2. As such, loss of WT1, IDH1 or IDH2 partially phenocopies loss of TET2 function and could sensitize AML cells to the inhibitory effects of 5’-Aza. In support of this notion, 5’-Aza as a single agent and particularly in combination with either Ivosidenib (IDH-inhibitor) or Venetoclax (BCL-2 inhibitor) has efficacy in IDH-mutated AML (48, 49). It will therefore be important to determine whether mutations in other members of the hydroxymethylation pathway confer sensitivity to 5’-Aza in AML, as we report here for biallelic TET2 mutation. TET2 mutations have also been reported in up to 28% of
MDS and MPN (15, 20, 50) and up to 50% of angioimmunoblastic T-cell lymphoma (AITL), where they are associated with poor response to anthracycline-based chemotherapy (51). However, there is evidence of sensitivity to 5'-Aza in TET2-mutated AITL cases (52), with prolonged CR reported in one case with double (presumed biallelic) mutation (51).

Models for reliably predicting response to 5’-Aza in AML would be of clinical benefit. Our study suggests that TET2 mutational profiling or TET2 protein expression analysis could potentially identify a subgroup of patients with disease that was null for protein expression and acutely sensitive to hypomethylating therapy, suggesting an alternative first line therapy for frail AML patients or salvage therapy for patients with chemoresistant disease. There is potential value in advocating TET2 mutational or protein expression profiling in elderly patients with AML, where disease is more likely to have evolved from TET2 clonal hematopoiesis and therefore likely to be enriched for AML with biallelic TET2 mutations and null for expression (20). Indeed, clinical studies in elderly AML have already documented excellent responses to 5’-Aza in some patients (53), although the impact of TET2 status would need to be confirmed in prospective studies in all age groups. Likewise, there is a case to be made for implementing TET2 mutational or expression profiling in AML patients with extramedullary disease, and particularly splenomegaly, given our data linking biallelic TET2 mutation with colonization of the spleen in conjunction with data from mouse models showing a proclivity of Tet2 mutation to drive extramedullary hematopoiesis and myeloid disease.

In summary, the prevailing evidence argues in favor of investigating mutant TET2 allele dosage and TET2 protein expression as a determinant of sensitivity to 5’-Aza in large prospective studies of AML and other hematological conditions characterized by TET2 loss of function. However, comprehensive TET2 mutational profiling that includes both sequence and copy
number analysis would be required to identify patients with potentially complex alterations affecting the TET2 locus. Furthermore, TET2 expression profiling could identify patients with disease that is null/low for protein expression regardless of gene mutation status, and who might also benefit from 5’-Aza treatment.
Methods

Patients

AML patients with an abnormal chromosome 4 (UPN01-UPN30) were recruited to the Study Alliance Leukemia AML registry biobank in Dresden (Germany) (institutional review board (IRB) number EK98032010).

*TET2* mutant patients over 65 years of age with newly diagnosed *TET2* mutant AML (UPN31-80) were enrolled in the Programa para el Estudio de la Terapeutica en Hemopatias Malignas (PETHEMA) phase 3 FLUGAZA clinical trial (NCT02319135), as previously described (13).

**BM morphological assessment of the AML index case (UPN01)**

For morphological analyses at AML presentation and during follow up, smears were prepared from BM aspirates, stained with Giemsa and visualized according to routine diagnostic protocols.

**Cytogenetic analyses of UPN01**

G-banding analysis of metaphase chromosomes from short-term cultures established from presentation BM aspirate was performed using well-established techniques. Interphase FISH was performed using the XL *TET2* kit (Metasystems, Germany). Spectral karyotyping (SKY) was performed using the SKYPaint probe mixture kit (Applied Spectral Imaging, Israel) according to the manufacturer’s protocol, with the exception that the hybridization time was extended from two to three days.

**AML cell lines and culture**
HEL, THP-1, HL-60, AML2, Kasumi, MV4-11, AML3, U937, SKM1 and NB4 AML cell lines were obtained from DSMZ (Braunschweig, Germany). THP-1 shRNA-mediated TET2 knockdown cells (and parental cells) were obtained from Steven Altschuler and Lani Wu at the University of California San Francisco, USA. KG-1 AML cells were a kind gift from Ross Levine at the Memorial Sloan Kettering Cancer Center, New York City, USA. All AML cell lines were maintained in complete medium (CM) [RPMI1640 with 10% FBS and 50µg/ml penicillin/streptomycin] at 37°C in a humidified 5% CO2 incubator. The identity of AML cell lines was confirmed by short tandem repeat profiling (NewGene, Newcastle University, UK) and cell cultures were regularly tested for mycoplasma using a MycoAlert kit (Lonza, Slough, UK).

**CRISPR-Cas9 TET2-targeting**

CRISPR-Cas9 TET2-targeted HEL cells were generated using a one vector system (pLV-U6-gRNA/EF1a-puro-2A-Cas9-2A-GFP in lentiviral particles (Sigma-Aldrich, Dorset, UK)) with the sgRNA sequence 5’GTTTGGTGCGGGAGCGAGC3’ targeting TET2 exon 6 (TET2 transcript ID ENST00000540549.5). Lentiviral particles were incubated with HEL cells (at MOI of 2) in CM supplemented with 8µg/ml hexadimethrine bromide and centrifuged at 800 x g for 30 min at 32°C. Transduced cells were selected by culture in CM supplemented with 2µg/ml puromycin then subsequently cloned by plating in soft agar [CM supplemented with 0.2% agarose]. DNA was extracted from cell clones using a QIAamp DNA micro kit (Qiagen, Manchester, UK) and TET2 mutation was confirmed by Sanger sequencing of exon 4 as described below (Table S9). Control cell clones (with monoallelic TET2 mutation) were derived from parental HEL cells transduced with virus carrying an empty vector. All of the clones used in experiments were generated independently and there is some heterogeneity in expression.
profiles and phenotype. Moreover, it should be noted that several independent cell clones were generated for each TET2 genotype and not every clone was used in every experiment.

**Nucleic acid preparation**

DNA was extracted from BM mononuclear cells (BMMNCs), peripheral blood (PB) or methanol:acetic acid-fixed cells using an appropriate Qiagen kit or from saliva using an Oragene kit (DNA Genotek, Ottawa, Canada).

**SNP array genotyping**

SNP array genotyping was performed on DNA from BMMNCs using the OmniExpressExome (v1.4) platform and analyzed using GenomeStudio 2.0.3 (Illumina, San Diego, CA) with genotype, minor (‘B’) allele frequency (B / A + B) and logR ratio at each locus calculated using standard parameters (GenCall Threshold 0.15). SNP coordinates are based on human genome build 37. Regions of copy number loss were identified manually based on interrogation of LogR ratios and B allele frequencies.

**Whole exome sequencing**

Exome capture (using Agilent SureSelect Protocol v1.2), library preparation and sequencing of pooled DNA samples (from PB or saliva) was carried out by Oxford Gene Technology (Oxfordshire, UK) on the Illumina HiSeq2000 platform. Reads were mapped to human genome build 37 (hg19) using the Burrows-Wheeler Aligner MEM package (54) and local realignment of mapped reads around potential insertion/deletion (indel) sites was carried out using Genome Analysis Toolkit (55) (GATK; v1.6). Duplicate reads were marked using Picard (v1.98) and excluded from analysis. SNPs and indels were called using GATK HaplotypeCaller, with SNP novelty determined against dbSNP release 135. Variants were annotated with gene data from
Ensembl. A read depth of at least 20x was achieved for a minimum of 95.61% of on-target regions.

**RNA sequencing and differential gene expression analysis**

Total RNA was extracted using the RNeasy micro kit (Qiagen) and quantified using a Qubit 2.0 Fluorometer with Qubit RNA BR assay kit (Thermo Fisher Scientific, MA, USA). Quality control, library preparation and sequencing on the NextSeq 550 platform (Illumina) was performed by Edinburgh Clinical Research Facility (Edinburgh, UK).

Sequencing reads were mapped to human genome build 37 (hg19) and annotated using STAR aligner (56). Aligned reads were summarized over gene features using the Rsubread package (57) (using featureCounts function) in R (v3.5.1). Read counts were normalized by expressing as CPM. Gene level differential expression analysis was performed on normalized read counts using DESeq2 (version 1.16.1) (58). Resulting $P$-values were adjusted to control for the false discovery rate (FDR; 5%) (59) and significantly differentially expressed genes were defined as those with FDR-adjusted $P$ value < 0.05 and $|\log_2 FC| \geq 0.3$.

**Illumina 450k arrays and differential methylation analysis**

DNA was extracted from HEL cell clones using a DNA Mini Kit (Qiagen) and sent for processing and hybridization to Infinium® HumanMethylation450 Beadchips (Illumina) by Eurofins Genomics (Galten, Denmark). Data processing and analysis was performed according to an established workflow (60). Specifically, raw intensity data (IDAT) files containing methylated (M) and unmethylated (U) intensity measurements were imported into R and the minfi Bioconductor package (61) was used to calculate detection p-values ($detP$), normalize data (using the preprocessFunnorm function) and generate $\beta$ ($\beta = M/(M+U+100)$) and M (M =
log$_2$(M/U)) values for individual CpG probes. Poorly performing probes ($detP < 0.01$) and those interrogating SNPs were removed, leaving 410,811 probes in the final dataset. The limma Bioconductor package (62) was used to identify significantly differentially methylated probes based on $TET2$ mutation status (monoallelic vs biallelic) using M values. Resulting $P$-values were adjusted to control for FDR (5%) (59) and significantly differentially methylated CpGs were defined as those with FDR-adjusted $P$-value < 0.05 and $|\text{Log}_2\text{FC}| \geq 2$. Unsupervised hierarchical clustering based on M values was performed in R with scaling by standard deviation.

**$TET2$, $NPM1$ and $FLT3$ mutation analysis**

Whole gene $TET2$ mutation analysis of the SAL abnormal chromosome 4 cases was performed by the MLL Munich Leukemia Laboratory (Munich, Germany) on DNA from fixed BMMNCs via the generation of 27 exon-specific amplicons using the FastStart High Fidelity PCR system kit (Roche Applied Science, Penzberg, Germany) as previously described (63, 64). Mutation of $TET2$ exon 3 in the index AML case (UPN01), as well as $NPM1$ exon 11 and $FLT3$ exon 14, was confirmed by Sanger sequencing. PCR reactions consisted of 0.5 units ThermoPrime Taq DNA polymerase with 1x ReddyMix PCR buffer (Thermo Fisher Scientific), 1.5mM MgCl$_2$, 10pmol primers, 0.2mM (each) dNTPs (Invitrogen Life Technologies, Paisley, UK) and 100ng template DNA in a total volume of 20μl. Primer sequences and thermal cycling conditions for individual amplicons are shown in Table S9. PCR products were purified using the QIAquick® PCR Purification kit (Qiagen) and sequenced using the indicated primers (Table S9) by Source BioScience (Nottingham, UK). Mutation in $TET2$ was determined on the PETHEMA-FLUGAZA AML clinical trial patients from whole exome sequencing, as previously described (13).
Western Immunoblotting

Cellular proteins were extracted using Phosphosafe reagent (Millipore Ltd, Watford, UK) and quantified by Pierce BCA assay (Thermo Fisher Scientific). Proteins were separated using Novex® NUPAGE 3-8% tris-acetate gels (Invitrogen Life Technologies), transferred to nitrocellulose membranes and immunoblotted according to routine techniques. Antibodies used were TET2 (Mab-179-050; Diagenode, NJ, USA), ABCB1 (G-1; Santa Cruz Biotechnology, Dallas, Texas), LSM8 (F-8; Santa Cruz Biotechnology), α-tubulin (T9026; Sigma-Aldrich), cleaved PARP (mAB #9541; Cell Signaling, Danvers, Massachusetts) and GAPDH (0411; Santa Cruz). HRP-conjugated secondary antibodies were from Agilent Technologies (CA, USA) and included Goat Anti-Mouse immunoglobulins/HRP (P044701-2) and Goat Anti-Rabbit immunoglobulins/HRP (P044801-2). Protein quantification was performed on immunoblots using the Fuji LAS-300 Image Analyser System (Raytek, Sheffield UK).

Cell proliferation, drug sensitivity and cloning efficiency assays

Cytotoxic agents were purchased from Sigma-Aldrich. Ara-C was reconstituted in DMSO and daunorubicin or 5-Aza in dH2O and aliquots were prepared and stored at -80°C. Stocks were diluted in CM immediately prior to use in cytotoxicity assays.

To compare cell proliferation between parental and CRISPR-Cas9-mutated HEL clones, exponentially growing cells were seeded at low density (2x10^4 cells ml^-1) in CM and counted using a hemocytometer at regular intervals up to 192 hours post-seeding. Cell growth at each timepoint was calculated relative to initial seeding density. Two-way ANOVA was used to test for significant differences in relative cell growth based on TET2 mutation status.
For drug sensitivity experiments, cells were incubated in CM supplemented with appropriate concentrations of cytotoxic agent (5’-Aza, daunorubicin or Ara-C) or relevant vehicle control (VC) for 96 hours, after which viable cells were identified by trypan blue dye exclusion and counted using a hemocytometer. Survival fractions were determined at each drug concentration relative to VC-treated controls. Two-way ANOVA was used to test for significant differences in drug sensitivity based on TET2 mutation status. Inhibition of proliferation in drug-treated cultures was compared to VC-treated cultures and used to calculate the IC50 and IC90 values in GraphPad Prism (PRISM 6.0.2, Graphpad Software).

For determination of CE, exponentially growing cells were seeded in soft agar [CM supplemented with 0.2% agarose] supplemented with cytotoxic agent (5’-Aza, daunorubicin or Ara-C) or VC. Macroscopically visible colonies were counted on day 30 and CE was calculated relative to number of cells initially seeded. Student’s t-tests (2-tailed) were used to identify significant differences in CE based on TET2 mutation status.

In order to determine the effect of ABCB1 inhibition on 5’-Aza sensitivity, cells were incubated in CM supplemented with increasing doses of 5’-Aza and an ABCB1 inhibitor (Verapamil or Tariquidar) or VC. After 96 hours of incubation, viable cells were identified using CellTiter-Glo Luminescent Cell Viability Assay (Promega) and the surviving fraction was determined at each drug concentration relative to VC-treated controls. The resulting dose-response matrix was used to calculate drug synergy using SynergyFinder 2.0. The student’s t-test was used to identify significant differences in synergy scores based on TET2 mutation status.

All assays were performed in triplicate at a minimum and means ± SD were calculated.

**Generation of 5’-Aza-resistant clones**
Exponentially growing HEL AML cells were seeded in soft agar [CM supplemented with 0.2% agarose] supplemented with 10 μM 5’-Aza (which corresponds to 90%-95% cytotoxicity). Colonies were picked after 28 days and were subsequently expanded and maintained in CM supplemented with 5’-Aza in order to establish putative 5’-Aza-resistant clones. Following expansion each cell clone was tested for sensitivity to 5’-Aza along with the parental cell line from which the 5’-Aza-resistant clone was developed. Student’s t-test was performed to test for significant differences in IC50 values between 5’-Aza-resistant clones and parental cells.

For determination of ABCB1 protein expression in 5’-Aza-resistant clones, western immunoblotting was performed and the resulting ABCB1 band intensities in 5’-Aza-resistant clones were normalized to the ABCB1 band intensity in the respective parental cells. Student’s t-test was performed to test for significant differences in ABCB1 protein expression between 5’-aza-resistant clones and parental cells.

Flow cytometry for cell cycle analysis

HEL AML cells were treated with 5’-Aza and sampled at 24 and 48 hours by fixation in 70% ethanol. Fixed cells were incubated in 50 μg per mL propidium iodide, 20 μg per mL RNase in PBS for 30 minutes in the dark. Cellular DNA content was determined using a FACSCanto II flow cytometer (BD, New Jersey, USA) with gating to exclude cell debris and doublets. Ten thousand events were acquired for each sample and the resulting data was analysed using FCS Express 7 software (De Novo Software, California, USA).

In vivo mouse model

Eight week-old male and female Rag2<sup>−/−</sup>Il2rg<sup>−/−</sup> (129xBalb/c) mice (65) were used for in vivo investigations in accordance with UK Home Office Project License PPL60/4552. For IF
injection (on day 0), mice were anaesthetized and 5x10^5 cells (HEL TET2 monoallelic and HEL TET2 biallelic mixed in a 1:1 ratio) in 20µl CM were injected through the knee into the marrow cavity of the right femur. Mice were monitored for signs of disease progression and were euthanized if any tumors reached a diameter of 15mm, or prior to this point if any signs of animal suffering were observed (including but not limited to sustained weight loss of 15% of normal weight and reduced mobility). 5’-Aza was dissolved in sterile water and was administered via intraperitoneal (IP) injection once daily starting on day 28 for 5 days at 5mg/kg. Control mice received sterile water only via IP administration. Mice were euthanized on day 35 and tissues (BM from right and left femurs, PB, spleen and any detected tumors) were harvested during post-mortem investigation. Genomic DNA was extracted using either a DNA Mini kit or DNA Micro kit (Qiagen) as required, according to sample volume.

**TET2 allele-specific qPCR assay**

A custom TaqMan SNP Genotyping assay (Applied Biosystems, CA, USA) was designed using probes that differentiate between intact WT TET2 sequence (HEL TET2 monoallelic clones) and TET2 sequence with a 4bp deletion generated by CRISPR-Cas9 targeting (HEL TET2 biallelic clones) in samples collected from mice. qPCR reactions were setup in triplicate and consisted of genomic DNA (50ng), primers (forward: 5’-GTGAAGAGAGTACTGTGTTTGGT-3’, reverse: 5’-ACAATCAGACTGACAGCCTCACA-3’), fluorescent allele-specific probes (WT TET2: 5’-CCAGCTCGCTCCCG-3’-VIC, 4bp deleted TET2: 5’-TGGCCAGCTCCCG-3’-FAM) and TaqMan SNP genotyping mastermix (Applied Biosystems) according to manufacturer’s recommended volumes. Controls were prepared using a 1:1 mix of DNA extracted from HEL TET2 monoallelic and HEL TET2 biallelic cells. Thermal cycling (50°C 2 min, 95°C 10 min, followed by 40 cycles of 95°C 15 sec, 60°C 1 min) was performed using a 7300 Real Time PCR
System (Applied Biosystems). Detected fluorescence for the two probes was converted to Ct values using SDS version 1.4 software (Applied Biosystems) and a sample was considered positive if Ct was 38 or higher for either allele. In samples where only one allele was amplified in all replicates (due to complete domination of one cell population in the sample), a Ct value of 38 was assigned to the non-amplified allele such that the sample could be included in the analysis (such samples are indicated on relevant figures). For all samples, adjusted ΔCt values (difference between WT TET2 allele Ct and 4bp deleted TET2 allele Ct, adjusted by subtracting ΔCt calculated from the control DNA with 1:1 allelic ratio) were converted to inverse Log₂ values, such that a value of 1 indicated a 1:1 ratio between the two alleles (and hence the two cell populations in the sample). Inverse Log₂ [ΔCt] values were compared between 5’-Aza-treated and VC-treated mice for each tissue type using the Mann-Whitney test.

Statistics

All statistical tests were performed using GraphPad Prism, or R in the case of large-scale array data analysis. Specific tests and corrections applied, as well as details of experimental replicates and summary statistics are given above and in relevant figure legends. For all analyses, a P value ≤ 0.05 was considered statistically significant unless otherwise stated.

Study approval

For human studies, approval was received from institutional review boards and/or ethics committees at all sites and written informed consent was received from all participants prior to inclusion. In addition, written informed consent was provided for pictures appearing in the manuscript.
For animal studies, experimental procedures were approved by the Animal Welfare Ethical Review Body at Newcastle University and the UK Home Office and were performed in compliance with the UK Animals (Scientific Procedures) Act 1986 and its associated Codes of Practice.

**Data Availability**

Genome-wide methylation data described in Figure 2e and 2f has been deposited at the Gene Expression Omnibus with accession numbers GSE217940 and GSE218228. RNA-seq gene expression data described in Figure 5a, 5b and 5c has been deposited at the Gene Expression Omnibus with accession numbers GSE218227 and GSE218228.
Author contributions

FS, SEF, DN and W-YL designed experiments, generated data, analyzed data and wrote the manuscript. HB, CE, HLB, BM, LR, DK, CD, DA, RP, E-NS, CP, MF, TR, AA, MW, HA, CR, LW, GLJ, TM, GHJ, HJM, JF, KO, MM, AR, ZB, EB, OH, TH, SV, BA, RAD, SA, LW, FP, PM, JM-L and MB generated/collated data/reagents and/or advised on data analysis. JMA designed experiments, generated data, analyzed data, directed the research and wrote the manuscript. FS and JMA also conceived of the project and secured funding. All authors contributed to the final version of the manuscript.

FS, SEF, DN and W-YL are listed as co-first authors of the manuscript.
Acknowledgments

The authors wish to acknowledge Professor Jörg Kotzerke (Technical University of Dresden, Germany) for providing PET-CT images (Figure S1), Professor Ross Levine (Memorial Sloan Kettering Cancer Center, New York City, United States of America) for providing TET2 knockdown THP-1 AML cells and Dr. Helena Jampor (Technical University of Dresden, Germany) for technical support. This work was funded by a specialist programme grant from Blood Cancer UK (#13044 to JMA). This work was also supported in part by grants from the German Consortium for Translational Cancer Research (DKTK) Dresden (to FS) and the J.G.W. Patterson Foundation (#30015.088.085/PA/IXS to JMA). We also acknowledge funding from Cancer Research UK [C355/A26819] and FC AECC and AIRC under the Accelerator Award Program.
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Figure 1. Biallelic TET2 mutation in a patient with 5’-Aza-sensitive AML. (A) Identification of the t(4;12) translocation by G-banding (top) and spectral karyotyping (bottom) in leukemic blasts from the AML index patient (UPN01). Translocated chromosomes 4 and 12 are arrowed. (B) Hemoglobin, platelet and white blood cell (WBC) counts from diagnosis to relapse of UPN01. Grey shading indicates period of induction chemotherapy with daunorubicin and Ara-C (DA). Initiations of palliative 5’-Aza treatment cycles are arrowed. (C) Giemsa-stained BM smears at day 30 (left) (after failed induction chemotherapy) and day 180 (right) (during 5’-Aza-induced remission) in UPN01. Images are 500x magnification. (D) High density array copy number profiles of chromosome 4 from leukemic blasts of UPN01 at AML presentation, during CR and at relapse. Points represent individual SNPs, aligned relative to their position on chromosome 4 (indicated by the ideogram). Copy number is measured as Log R ratio, with 0 indicating diploid SNPs and positive and negative values indicating gain and loss, respectively. B allele frequency represents the ratio of the two alleles of each SNP such that 0.5 indicates allele heterozygosity and 0 and 1 indicate homozygosity. Inset shows expanded view of the boxed region. Green bar above plots highlights focal deletion within 4q24 encompassing TET2, CXCC4 and PPA2 (locations indicated by bars below plots). (E) FISH on leukemic blasts from UPN01 showing TET2 (red) deletion in a metaphase cell (left of image) and two interphase cells (right of image). A probe binding within 4q12 (green) was used as reference. (F) Fishplot derived from sequencing analysis of leukemic blasts from UPN01 showing temporal acquisition of a TET2 point mutation, TET2 deletion, a NPM1 insertion mutation and FLT3 internal tandem duplication (ITD). Dashed lines represent timepoints at which blasts were analyzed. PRES; disease presentation, CR; complete remission, REL; relapse.
Figure 2. Complete loss of TET2 expression confers a hypermethylation phenotype.

(A) High density array copy number profile of chromosome 4 from HEL cells showing large deletion (green bar) affecting the q arm, including TET2 (position indicated by dashed red line). (B) Immunoblot showing TET2 protein expression in two representative parental TET2 monoallelic HEL cell clones (HEL TET2 monoallelic) and three representative TET2 CRISPR-Cas9-mutated HEL cell clones (HEL TET2 biallelic). α-tubulin was used as loading control. (C) Growth kinetics in suspension culture of HEL TET2 monoallelic (open symbols) and HEL TET2 biallelic (filled symbols) cell clones. Cells were seeded at low density and growth (relative to initial density) was determined at regular intervals. Data represents mean and SD of indicated number of clones from three independent experiments. P value calculated by one-way ANOVA. (D) CE was calculated for HEL TET2 monoallelic (open squares) and HEL TET2 biallelic (filled squares) clones after 30 days culture in soft agar. Mean and SD of indicated number of clones from seven independent experiments are shown. P value calculated by 2-tailed Student’s t-test. (E) Volcano plot demonstrating differences in CpG methylation between HEL TET2 monoallelic (n=2) and HEL TET2 biallelic (n=4) clones. Plot was constructed using fold-change (log₂FC) values and adjusted P-values and points represent individual CpG probes, colored such that significantly differentially methylated probes (P<0.05 and |Log₂FC|≥2) are in red. Orange points represent probes which reach significance (P<0.05) but are not differentially methylated (|Log₂FC|<2) and black points represent non-significant (P≥0.05) probes. (F) Unsupervised hierarchical clustering of the top 1,500 differentially methylated CpG probes across all samples resulted in distinct clustering of parental HEL TET2 monoallelic (n=2) and HEL TET2 biallelic (n=4) cell clones. Rows in the heatmap represent CpG probes and vertical columns are cell clones. Color key indicates level of methylation at CpGs.
Figure 3. Cells with biallelic TET2 mutations are sensitive to the hypomethylating agent, 5'-Aza in *in vitro* model systems.

A) Parental TET2 monoallelic HEL cell clones (HEL TET2 monoallelic; open symbols) and TET2 CRISPR-Cas9-mutated HEL cell clones (HEL TET2 biallelic; filled symbols) were cultured in soft agar supplemented with 1μM 5'-Aza (left), 20nM Ara-C (center) or 20nM daunorubicin (right) and CE (relative to respective vehicle control-treated cells) was determined after 30 days. Mean and SD of indicated number of clones from three independent experiments are shown. *P* values calculated by Student’s t-test (2-tailed).

B) Parental TET2 monoallelic HEL cell clones (HEL TET2 monoallelic; open symbols) and TET2 CRISPR-Cas9-mutated HEL cell clones (HEL TET2 biallelic; filled symbols) were treated with 5'-Aza (left), Ara-C (center) or daunorubicin (right) and cell density (relative to respective vehicle control-treated cells) was determined after 96 hrs. Data represents mean and SD of indicated number of clones from three independent experiments. *P* values calculated by two-way ANOVA.

C) Western blot showing cleaved PARP in HEL cells with monoallelic and biallelic TET2-mutation following exposure to 2μM 5'-Aza over 48 hours. GAPDH was used as a loading control.

D) Western blot (top panel) showing TET2 protein expression in a panel of 10 AML cell lines. GAPDH was used as a loading control. TET2 protein expression was quantified in each cell line and plotted against 5'-Aza IC50 (left) and IC90 (right) values. AU represents arbitrary units as measured by the Fuji LAS-300 Image Analyzer System.
Figure 4. Cells with biallelic TET2 mutations are subject to 5'-Aza-induced negative selection in an orthotopic AML mouse model.

(A) Schematic of orthotopic AML mouse model. HEL TET2 monoallelic and HEL TET2 biallelic cell clones were co-injected in a 1:1 ratio into the femurs of Rag2−/−Il2rg−/− mice. Treatment with 5-Aza (5mg/kg daily for 5 days) or VC was initiated on day 28 (post-injection) and tissues were harvested on day 35 for TET2 allele-specific qPCR analysis. (B) Tissue samples collected from mice were analyzed by custom TET2 allele-specific qPCR assay. Shown are inverse Log$_2$ [ΔCt] values which represent relative expression of the WT versus the 4bp deleted TET2 allele in individual samples, and are the means of triplicate reactions. Inverse Log$_2$ [ΔCt] of 1 indicates a 1:1 ratio between the WT and 4bp deleted TET2 alleles (and hence HEL TET2 monoallelic and HEL TET2 biallelic clones), whereas inverse Log$_2$ [ΔCt] > 1 or inverse Log$_2$ [ΔCt] < 1 indicates dominance of the WT (HEL TET2 monoallelic) or 4bp deleted (HEL TET2 biallelic) allele, respectively. Red points indicate samples which were dominated entirely by one cell clone. Horizontal dashed lines represent median inverse Log$_2$ [ΔCt] values across all samples from VC- or 5'-Aza-treated mice. The left panel shows data from all harvested tissues (BM, PB, spleen and tumors) and the right panel shows data from BM only. P values comparing inverse Log$_2$ [ΔCt] values from VC and 5'-Aza-treated mice were calculated using a Mann-Whitney test. VC, vehicle control.
Figure 5. Differential gene expression in AML cells with monoallelic and biallelic TET2 mutations.

(A) Unsupervised hierarchical clustering of the top 1,500 differentially expressed transcripts in parental TET2 monoallelic HEL cell clones (HEL TET2 monoallelic; n=3) and TET2 CRISPR-Cas9-mutated HEL cell clones (HEL TET2 biallelic; n=3). Horizontal rows of the heatmap represent individual transcripts and each vertical column is a cell clone. Color indicates relative expression with down-regulated and up-regulated transcripts indicated in blue and red, respectively. (B) Volcano plot demonstrating significant differential gene expression (P-value < 0.05 and |Log_{2} FC| ≥ 0.3) in TET2 CRISPR-Cas9-mutated HEL cell clones (HEL TET2 biallelic; n=3) relative to parental HEL clones with monoallelic TET2 mutation (HEL TET2 monoallelic; n=3). Plot was constructed using fold-change (Log_{2} FC) values and adjusted P-values and points represent individual gene transcripts. Shown are 326 significantly down-regulated transcripts (blue) and 369 significantly up-regulated transcripts (red). Non-significant (NS) transcripts (P-value ≥ 0.05) are represented by grey points. Genes with particularly significant differential expression are labelled. (C) Heatmap showing differential expression of components of the spliceosomal snRNP complex (GO:009752) in parental TET2 monoallelic HEL cell clones (HEL TET2 monoallelic; n=3) and TET2 CRISPR-Cas9-mutated HEL cell clones (HEL TET2 biallelic; n=3). Horizontal rows represent genes and each vertical column is a cell clone. Color indicates relative expression, as in (A). (D) Transcript expression (expressed as counts per million (CPM) reads) of LSM8 (top) and ABCB1 (bottom) in parental TET2 monoallelic HEL cell clones (HEL TET2 monoallelic; open symbols) and TET2 CRISPR-Cas9-mutated HEL cell clones (HEL TET2 biallelic; filled symbols). Data represents the mean and SD of indicated number of clones. P values are from differential expression analysis. Western blots to the right of the charts show corresponding protein expression in the individual cell clones included in RNA sequencing analysis. α-tubulin was used as a loading control.
Figure 6. ABCB1 affects sensitivity to 5′-Aza in HEL AML cells.
(A) Mean synergy scores for verapamil and 5′-Aza combination and for (B) tariquidar and 5′-Aza in combination, stratified by TET2 mutant allele dosage. Synergy scores for verapamil/5′-Aza and tariquidar/5′-Aza were significantly higher for TET2 monoallelic mutant HEL cell clones compared to TET2 biallelic HEL cell clones (P = 0.0003 and P < 0.0001, respectively, paired t-test). Data are derived from six independent experimental replicates using two independent cell clones for each TET2 genotype. Data represents the mean and SD of indicated number of clones. (C) HEL AML cells clones were exposed to escalating dose of 5′-Aza to generate significantly resistant sub-clones (P = 0.0008, unpaired t-test). Data shows IC50 values for 5′-Aza-resistant sub-clones derived from parental cells with either TET2 monoallelic mutation (open symbols) or TET2 biallelic mutation (closed symbols). Data represents the mean and SD of indicated number of clones. (D) Western blots show ABCB1 protein levels in three representative 5′-Aza-resistant derivatives derived from cells with either TET2 monoallelic mutation (left panel) or TET2 biallelic mutation (right panel). (E) ABCB1 protein levels were quantified in parental HEL cells and nine independent 5′-Aza-resistant derivatives with either TET2 monoallelic mutation (open symbols) or TET2 biallelic mutation (closed symbols). ABCB1 protein levels were quantified and normalized to GAPDH with the expression in each parental cell given a nominal value of 1. ABCB1 protein levels of 5′-Aza-resistant derivatives were significantly higher than their respective parental cells (P=0.0069, paired t-test). Solid horizontal line represents the median fold change in ABCB1 protein expression in 5′-Aza-resistant sub-clones relative to their respective parental cells (represented by the dashed horizontal line).
Figure 7. Somatic mutations affecting the TET2 locus in AML patients with cytogenetic abnormality of chromosome 4 and response to treatment in AML patients with TET2 mutation. 

(A) Illustrated are regions of copy number gain (green), gain with concomitant LOH (blue) and loss (orange) affecting chromosome 4 (discerned using high-density SNP array) in 18 AML patients with cytogenetically detectable abnormalities of chromosome 4. Base substitution mutations (indicated by black triangles) were determined by TET2 exon sequencing. The vertical dashed red line indicates the location of the TET2 gene. The mutation status of the 7 patients with loss of function TET2 mutations are indicated to the right. Patient ID numbers are shown in parentheses for these patients. 

(B) Swimmer plots showing patients with TET2 mutated AML treated with either 5’-Aza (left) or low-dose Ara-C plus fludarabine (FLUGA) (right). The AML index case (UPN01) is included in the 5’-Aza swimmer plot for reference. Patients with biallelic TET2 mutation (UPN01, UPN31, UPN33, UPN47, UPN68, UPN73 and UPN78) are represented by purple bars. All other patients had monoallelic TET2 mutation discerned by whole exome sequencing and are represented by pale yellow bars. European LeukemiaNet (ELN) favorable, intermediate and adverse risk groups are represented by green, blue and red squares, respectively. ECOG, Eastern Cooperative Oncology Group performance score.