LETTER

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Impaired hydroxylation of 5-methylcytosine in myeloid cancers with mutant *TET2*

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TET2 is a close relative of TET1, an enzyme that converts 5-methylcytosine (5mC) to 5-hydroxymethylcytosine (5hmC) in DNA[1,2]. The gene encoding TET2 resides at chromosome 4q24, in a region showing recurrent microdeletions and copy-neutral loss of heterozygosity (CN-LOH) in patients with diverse myeloid malignancies[3]. Somatic *TET2* mutations are frequently observed in myelodysplastic syndromes (MDS), myeloproliferative neoplasms (MPN), MDS/MPN overlap syndromes including chronic myelomonocytic leukaemia (CMML), acute myeloid leukaemias (AML) and secondary AML (sAML)[4–12]. We show here that *TET2* mutations associated with myeloid malignancies compromise catalytic activity. Bone marrow samples from patients with *TET2* mutations displayed uniformly low levels of 5hmC in genomic DNA compared to bone marrow samples from healthy controls. Moreover, small hairpin RNA (shRNA)-mediated depletion of *Tet2* in mouse haematopoietic precursors skewed their differentiation towards monocyte/macrophage lineages in culture. There was no significant difference in DNA methylation between bone marrow samples from patients with high 5hmC versus healthy controls, but samples from patients with low 5hmC showed hypomethylation relative to controls at the majority of differentially methylated CpG sites. Our results demonstrate that Tet2 is important for normal myelopoiesis, and suggest that disruption of TET2 enzymatic activity favours myeloid tumorigenesis. Measurement of 5hmC levels in myeloid malignancies may prove valuable as a diagnostic and prognostic tool, to tailor therapies and assess responses to anticancer drugs.

We transiently transfected HEK293T cells with Myc-tagged murine Tet2 and assessed 5mC and 5hmC levels by immunocytochemistry (Fig. 1 and Supplementary Figs 1–4). Myc–Tet2-expressing cells displayed a strong increase in 5hmC staining and a concomitant decrease in 5mC staining in the nucleus (Fig. 1b, c, quantified in Supplementary Fig. 4). In contrast, 5hmC was undetectable or barely detected in nuclei of cells expressing mutant Tet2 with H1302Y, D1304A substitutions in the signature HxD motif[1,12,17] involved in coordinating Fe^{2+}, and there was no obvious decrease in nuclear 5mC staining (Fig. 1b, c and Supplementary Fig. 4). These studies confirm that Tet2 is a catalytically active enzyme that converts 5mC to 5hmC in genomic DNA[13].

Mutations in TET2 residues H1881 and R1896, predicted to bind Fe^{2+} and 2-oxoglutarate (2OG), respectively, have been identified repeatedly in patients with myeloid malignancies[4,5,7,10]. HEK293T cells expressing Tet2 mutants H1802R and H1802Q (Fig. 1a and Supplementary Fig. 2) showed greatly diminished 5hmC staining and no loss of 5mC staining, consistent with participation of this residue in catalysis (Fig. 1b, c and Supplementary Fig. 4a, b). We analysed missense mutations identified in TET2 in our own (Supplementary Table 1) and other studies[3–6,11] (P1367S, W1291R, G1913D, E1318G and I1873T). HEK293T cells expressing Tet2 mutants P1287S, W1211R or C1834D (Supplementary Figs 2 and 3a) displayed low 5hmC staining and strong 5mC staining (Supplementary Figs 3b, c and 4c, d), indicating a role for these residues in the integrity of the catalytic or DNA binding domains. Cells expressing Tet2(R1817S/M) (Fig. 1a, Supplementary Figs 2 and 3a) were positive for 5hmC staining but changes in 5mC staining could not be assessed reliably (Fig. 1b, c, Supplementary Figs 3b, c and 4).

To quantify these findings, we developed dot blot assays to detect 5hmC in genomic DNA (Supplementary Fig. 5). In the first assay format, the blot was developed with a specific antiserum to 5hmC (Supplementary Fig. 5b, left), whose ability to recognize 5hmC depended strongly on the density of 5hmC in DNA (Supplementary Fig. 5c, top). We therefore developed a more sensitive and quantitative assay in which DNA was treated with bisulphite to convert 5hmC to cytosine 5-methylenesulphonate (CMS)[14] (Supplementary Fig. 5a), after which CMS was measured with a specific anti-CMS antiserum (Supplementary Fig. 5b, right). Unlike anti-5hmC which reacted efficiently only with DNA containing high densities of 5hmC, the anti-CMS antiserum recognized DNA with an average of only a single 5hmC per 201 base pairs (Supplementary Fig. 5c, bottom). This lack of density dependence allowed us to plot the signal obtained with twofold dilutions of a standard oligonucleotide containing a known amount of 5hmC against the amount of CMS obtained after bisulphite conversion. We assumed 100% conversion efficiency[15] and used the linear portion of the standard curve to compute the amount of CMS, and therefore 5hmC, in the DNA samples (for example, see Fig. 2a, right).

To assess 5hmC levels, we obtained uniform populations of Tet2-expressing HEK293T cells by transfection with Tet2-IRES-CD25 plasmid followed by magnetic isolation of CD25-expressing cells[1]. Wild-type and mutant Tet2 proteins were expressed at comparable levels (Fig. 1d and Supplementary Fig. 3d). Anti-5hmC/CMS dot blots of genomic DNA revealed, as expected, that 5hmC was barely detectable in DNA from cells transfected with empty vector; DNA from cells expressing wild-type Tet2 showed a substantial increase in 5hmC and a corresponding decrease in 5mC; and DNA from cells expressing the HxD mutant Tet2 protein had very low 5hmC (Fig. 1e, Supplementary Figs 3e and 6). DNA from cells expressing seven of the nine mutant Tet2 proteins tested—H1802Q/R, R1817S/M, W1211R, P1287S and C1834D—contained significantly less 5hmC than DNA from cells expressing wild-type Tet2 (Fig. 1e, Supplementary Figs 3e and 6), confirming our previous conclusion that these mutations impair enzymatic activity.

We measured 5hmC (CMS) levels in genomic DNA extracted from bone marrow or blood (with >20% immature myeloid cells) of 88

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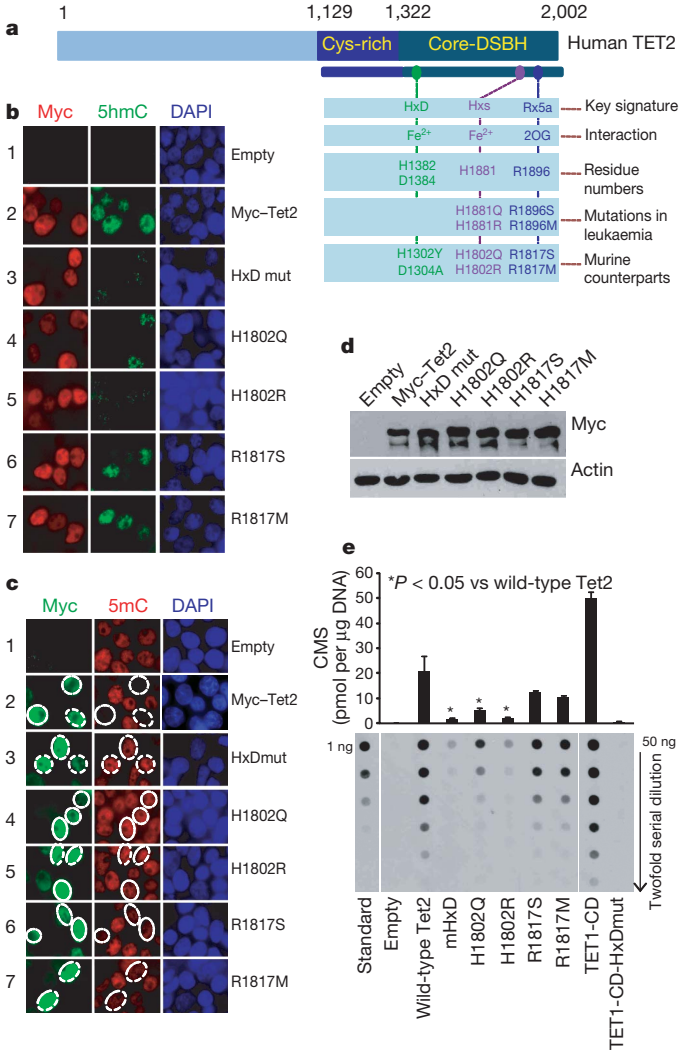

Figure 1 | The catalytic activity of Tet2 is compromised by mutations in predicted catalytic residues. **a**, Schematic representation of TET2. The catalytic core region contains the cysteine-rich (Cys-rich) and double-stranded beta-helix (DSBH) domains. Three signature motifs conserved among 2OG- and Fe^{2+}-dependent dioxygenases are shown[1,2]. Substitutions in the HxD signature that impair the catalytic activity of TET1 (ref. 1), leukaemia-associated mutations in the carboxy-terminal signature motifs, and corresponding substitutions introduced into murine Tet2 are indicated. **b**, Tet2 expression results in increased 5hmC by immunocytochemistry. HEK293T cells transfected with Myc-tagged wild-type and mutant Tet2 were co-stained with antibody specific for the Myc epitope (red) and antiserum against 5hmC (green). DAPI (blue) indicates nuclear staining. **c**, Tet2 expression results in loss of nuclear 5mC staining. HEK293T cells transfected with wild-type and mutant Myc-tagged Tet2 were co-stained with antibody specific for the Myc epitope (green) and antiserum against 5mC (red). **d**, Equivalent expression of wild-type and mutant Myc–Tet2. $CD25^+$ cells were isolated from HEK293T cells transfected with bicistronic Tet2-IRES-human CD25 plasmids, and Tet2 expression in whole cell lysates was detected by immunoblotting with anti-Myc. β-actin serves as a loading control. **e**, Genomic DNA purified from $CD25^+$ HEK293T cells overexpressing wild-type or mutant Tet2 was treated with bisulphite to convert 5hmC to CMS (Supplementary Fig. 5a). CMS was quantified by dot blot assay using anti-CMS and a synthetic bisulphite-treated oligonucleotide containing a known amount of CMS. As positive and negative controls, we included DNA from $CD25^+$ HEK293T cells transfected with TET1 catalytic domain (TET1-CD) or TET1-CD with mutations in the HxD motif (TET1-CD-HxDmut)[1].

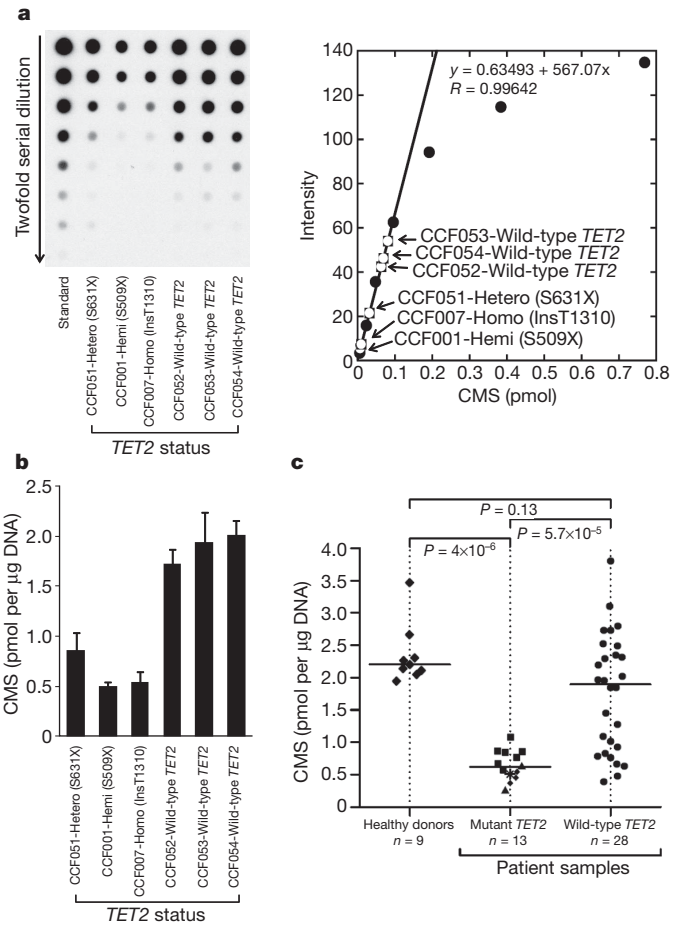

Figure 2 | TET2 mutational status correlates with 5hmC levels in patients with myeloid malignancies. **a**, Quantification of 5hmC by anti-CMS dot blot. Left, a representative dot blot of genomic DNA isolated from bone marrow aspirates of patients with MDS/MPN and *TET2* mutational status as indicated. A synthetic oligonucleotide with a known amount of CMS was used as standard. Right, the linear portion of the standard curve was used to estimate the amount of 5hmC in DNA from patient samples. **b**, Bar graph of data from panel **a**. The three patients with *TET2* mutations show lower 5hmC levels than the three patients with wild-type *TET2*. Error bars indicate s.d. ($n = 3$). **c**, Correlation of 5hmC levels with *TET2* mutational status. CMS levels in bone marrow samples from healthy donors and patients with myeloid malignancies (Supplementary Table 1) are shown as the median of triplicate measurements (Supplementary Fig. 7b). In the *TET2* mutant group, squares, triangles, diamonds and the star indicate homozygous, hemizygous, heterozygous and biallelic heterozygous mutations, respectively (for detailed definition, see Supplementary Methods). The horizontal bar indicates the median for each group. *P*-values for group comparisons were calculated by a two-sided Wilcoxon rank sum test. Patients bearing *TET2* mutations show uniformly low 5hmC expression levels.

patients with myeloid malignancies and 17 healthy controls (Supplementary Table 1). In blinded experiments, DNA was treated with bisulphite and CMS levels were measured. *TET2* mutations were strongly associated with low genomic 5hmC (Fig. 2 and Supplementary Fig. 7a). To confirm these conclusions in a statistically rigorous fashion, we tested samples for which a sufficient amount of DNA was available to make independent dilutions in triplicate, so that a median and standard deviation for 5hmC (CMS) levels in each patient could be derived (Supplementary Fig. 7b). Analysis of DNA from 9 healthy donors and 41 patients (28 with wild-type *TET2* and 13 with *TET2* mutations, Supplementary Table 1) revealed a strong, statistically significant correlation of *TET2* mutations with low 5hmC (Fig. 2c). In contrast, samples from patients with wild-type *TET2* showed a bimodal distribution, with 5hmC levels ranging from ~0.4 to ~3.8 pmol per µg DNA (Fig. 2c, Supplementary Fig. 7, also see Fig. 4).

We examined *Tet2* expression in haematopoietic cell subsets isolated from bone marrow and thymus of C57BL/6 mice (Supplementary Figs 8 and 9). *Tet2* mRNA was highly expressed in lineage-negative (Lin^-) Sca-1^+c-Kit^{hi} (Sca-1 is also known as Ly6a) multipotent

progenitors (LSK), at levels similar to those in embryonic stem cells (ESC). Expression was maintained at high levels in myeloid progenitors (common myeloid progenitors, CMPs, and granulocyte-monocyte progenitors, GMPs), was low in mature granulocytes ($Gr\text{-}1^+Mac\text{-}1^+$, also known as Ly6g and Cd11b or Itgam, respectively) and high in monocytes ($Gr\text{-}1^-Mac\text{-}1^+$) (Supplementary Fig. 9a, middle panel).

To test the role of Tet2 in myelopoiesis, we transduced bone marrow stem/progenitor cells with *Tet2* shRNA (Supplementary Fig. 10a), effectively downregulating *Tet2* mRNA and protein relative to control cells transduced with empty vector or scrambled shRNA (Fig. 3a, b) (refer to Supplementary Fig. 10b for choice of *Tet2* shRNA). Tet2 depletion promoted expansion of $Mac\text{-}1^+$ $F4/80^+$ (also known as Emr1) and $Mac\text{-}1^+$ $CD115^+$ (also known as Csf1r or M-CSFR, macrophage colony stimulating factor receptor) monocyte/macrophage cells in the presence of G-CSF (granulocyte colony-stimulating factor) or GM-CSF (granulocyte-macrophage colony-stimulating factor), cytokines that support granulocyte and granulocyte/ monocyte development respectively, but not in the presence of M-CSF (macrophage colony-stimulating factor), which promotes growth of monocytic progenitors (Fig. 3c and Supplementary Fig. 10d). Simultaneous treatment with GM-CSF and M-CSF, or GM-CSF and G-CSF, also led to increased numbers of monocyte/macrophage cells (Fig. 3c). These results indicate that Tet2 has an important role in normal myelopoiesis. However, Tet2 does not markedly influence short-term proliferation of myeloid-lineage cells: when shRNA-transduced Lin^- cells were cultured in the presence of GM-CSF and pulse-labelled with bromodeoxyuridine (BrdU), Tet2 depletion promoted monocyte/macrophage expansion but $CD115^+$ ($M\text{-}CSFR^+$) cells from the two cultures showed no difference in acute BrdU incorporation (Supplementary Fig. 11).

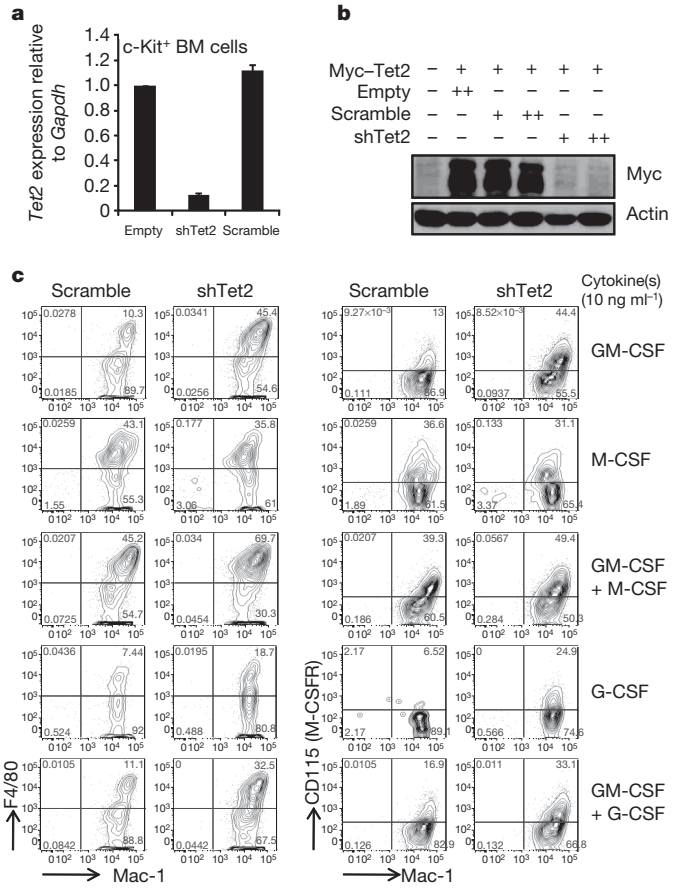

Figure 3 | Tet2 regulates myeloid differentiation. **a**, **b**, *Tet2* shRNA represses *Tet2* mRNA and protein expression. **a**, $c\text{-}Kit^+$ stem/progenitor cells from bone marrow of C57BL/6 mice were transduced with retroviruses (Supplementary Fig. 10). After selection with puromycin for 3 days, *Tet2* mRNA expression was assessed by quantitative RT–PCR (PCR with reverse transcription). Error bars show the range of duplicates. **b**, HEK293T cells were cotransfected with expression plasmids encoding Myc-tagged Tet2 and retroviral shRNAs. Tet2 protein expression was quantified 48 h later by anti-Myc immunoblotting of whole-cell extracts. **c**, Effect of Tet2 depletion on myeloid differentiation. Lin^- cells purified from bone marrow of C57BL/6 mice were transduced with control (scramble) or shTet2 retroviruses, then grown in the presence of 50 ng ml^{-1} stem cell factor (SCF), puromycin (2 μg ml^{-1}) and cytokines (10 ng ml^{-1}) as indicated (also see Supplementary Fig. 10). After 4 days, flow cytometric analysis of Mac-1 versus F4/80 (left panel) or CD115 (right panel) was performed. All cells were GFP^+ on the day of analysis.

We asked whether 5hmC levels in tumour samples correlated with DNA methylation status. A histogram of normalized values from 88 patients and 17 healthy individuals showed the expected bimodal distribution (see Supplementary Methods): healthy controls and most patient samples with wild-type *TET2* had high 5hmC, whereas the majority of patient samples with mutant *TET2* had low 5hmC (Fig. 4b). The DNA methylation status of 62 samples was interrogated at 27,578 CpG sites. As expected[16], the resulting histograms were strikingly bimodal, with sites within and outside CpG islands showing low and predominantly high methylation, respectively (Fig. 4c). Comparison of 28 control samples with 24 high 5hmC tumour samples (22 wild-type *TET2*, 2 mutant *TET2*) showed no significant difference in DNA methylation; in contrast, comparison of the control samples with 29 low 5hmC tumour samples (7 wild-type *TET2* , 22 mutant *TET2*) yielded 2,512 differentially methylated sites, of which the majority (2,510 sites) were hypomethylated compared to controls (Fig. 4d and Supplementary Table 2). Thus TET2 loss-of-function is predominantly associated with decreased methylation at CpG sites.

To summarize, our studies demonstrate a strong correlation between myeloid malignancies and loss of TET2 catalytic activity. The leukaemia-associated missense mutations associated with diminished 5hmC levels provide clues to the structure of the TET2 catalytic domain. The W1211R, P1287S and C1834D mutations affect positions that are highly conserved within the catalytic domain of the TET subfamily of dioxygenases[2]: W1211 is located at the beginning of the strand just amino-terminal to the core of the double-stranded beta-helix (DSBH), and is predicted to constitute part of the 'mouth' of the active site pocket of the enzyme; P1287 is predicted to stabilize the conformation of the junction between the N-terminal helix and the first core strand of the DSBH; and G1913/C1834 is predicted to be the N-terminal capping residue of a helix that lines the 'mouth' of the DSBH and potentially interacts with substrate DNA[2]. The E1238G mutation had no detectable effect on 5hmC production in our overexpression assays; however, the patient with this mutation also showed CN-LOH spanning 4q24, a feature that likely contributes to the significant reduction in 5hmC levels observed in the bone marrow.

Low 5hmC levels were observed in a subset of patients with apparently wild-type *TET2*, whose clinical phenotypes resembled those of patients with mutant *TET2*. In several of these patients, *TET2* mRNA expression was not significantly different from controls; mutations in other TET proteins have not been described (Supplementary Text). Some patients in the wild-type *TET2*/low 5hmC category may harbour mutations in regulatory or partner proteins for TET2, or in *cis*-regulatory regions controlling *TET2* mRNA expression. Alternatively, the primary event in some of these patients may be CpG hypomethylation, resulting in decreased 5hmC secondary to depletion of the substrate, 5mC.

There is little consensus on whether *TET2* mutations correlate with clinical outcome. One study reported an association with decreased survival in AML[4], whereas others report little prognostic value in MPN diseases[7,10,12]. Assays for 5hmC may increase our options for the molecular classification of myeloid malignancies, making it possible to ask whether patients with high or low levels of genomic 5hmC show differences in disease progression or therapeutic response. Notably, histone deacetylase and DNA methyltransferase inhibitors show clinical efficacy

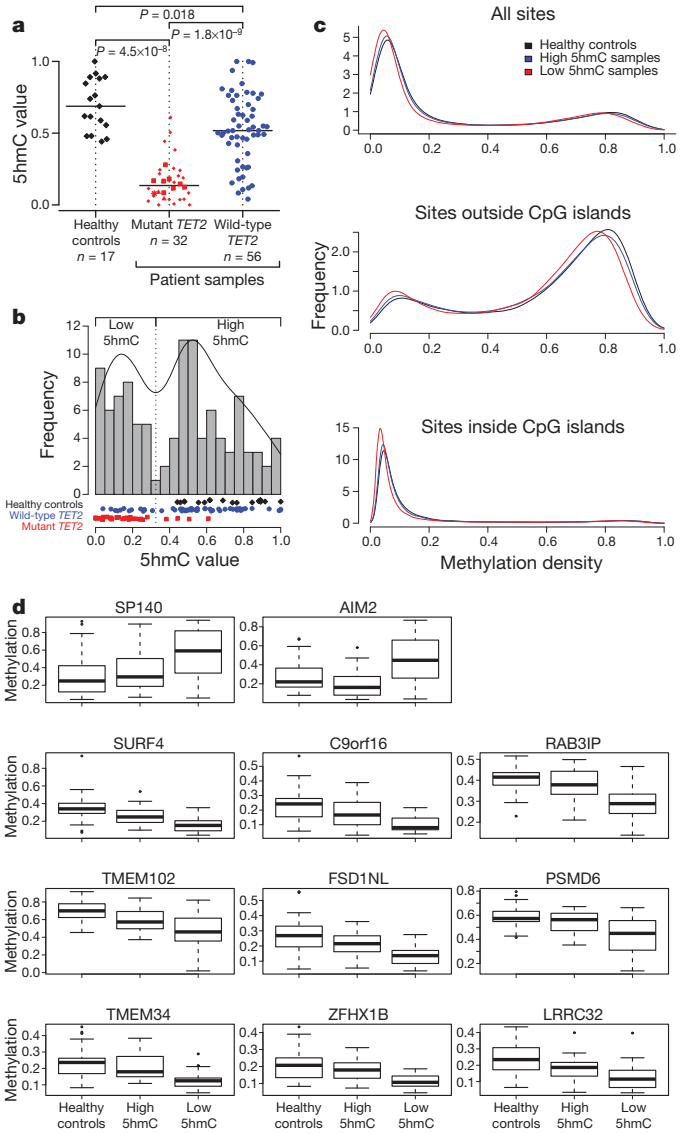

Figure 4 | Relation of 5hmC levels to DNA methylation status. **a**, Normalized 5hmC (CMS) levels in DNA from three different groups: healthy controls (black diamonds), patients with mutant *TET2* (red symbols) and patients with wild-type *TET2* (blue circles). Among *TET2* mutants, we distinguish homozygous (squares), hemizygous (triangles), heterozygous (small diamonds) and biallelic heterozygous (star) mutations (for definitions see Supplementary Methods). The horizontal bar indicates the median for each group. The number of samples in each group is indicated. **b**, Histogram of normalized 5hmC (CMS) levels in DNA from healthy donors (black diamonds), patients with mutant *TET2* (red rectangles) and patients with wild-type *TET2* (blue circles). The frequency was calculated based on a Gaussian kernel estimator. The local minimum between both modes was used as a threshold (vertical dotted line) between low and high 5hmC values. **c**, Density of methylation values for healthy controls (black), high 5hmC samples (blue) and low 5hmC samples (red) of all sites (top panel), sites outside CpG islands (middle panel) and sites inside CpG islands (lower panel). **d**, Box plot for group-specific methylation for the only two hypermethylated sites (*SP140*, *AIM2*; top panel) and the top nine hypomethylated sites (lower panels) between healthy controls and low 5hmC samples (total number of differentially methylated sites was 2,512).

in patients with CMML and AML[17]; and genomic 5hmC levels could potentially be a useful prognostic indicator or predictor of patient responses or refractoriness to 'epigenetic' therapy with demethylating agents.

DNA methylation is highly aberrant in cancer[18–20]. Because TET operates on 5mC, we were surprised to find that TET2 loss-of-function in myeloid tumours was associated with widespread hypomethylation rather than the expected hypermethylation at differentially-methylated CpG sites. Tumour samples with low 5hmC may have expanded cells with localized hypomethylation at these sites, or TET2 may control DNA methylation indirectly, for instance by regulating the expression or recruitment of one or more DNA methyltransferases, perhaps via 5hmC-binding proteins. Alternatively, if TET2 and 5hmC are required for cells to exit the stem cell state, loss of TET2 function in myeloid neoplasms may reactivate a stem-like state characterized by generalized hypomethylation and consequent genomic instability[21,22]. Indeed, hypomorphic *DNMT1* mutations associated with genome-wide DNA hypomethylation skew haematopoietic differentiation towards myeloerythroid lineages[23], and promote the development of aggressive T-cell lymphomas due to activation and insertion of endogenous retroviruses[24,25]. Further studies of the role of TET2 in haematopoietic differentiation should uncover the relation between TET2 loss-of-function, DNA methylation changes and myeloid neoplasia.

METHODS SUMMARY

Patient samples. Genomic DNA was extracted from bone marrow/ peripheral blood samples from healthy donors and patients with MDS, MDS/MPN, primary and secondary AMLs. Clinical features and other detailed information pertaining to the patient samples are summarized in Supplementary Table 1.

Quantitative analysis of 5hmC and CMS levels using dot blot. For CMS detection, genomic DNA was treated with sodium bisulphite using the EpiTect Bisulfite kit (Qiagen). DNA samples were denatured and twofold serial dilutions were spotted on a nitrocellulose membrane in an assembled Bio-Dot apparatus (Bio-Rad). The blotted membrane was washed, air-dried, vacuum-baked, blocked and incubated with anti-5hmC or anti-CMS antibody (1:1,000) and horseradish peroxidase-conjugated anti-rabbit IgG secondary antibody. To ensure equal spotting of total DNA on the membrane, the same blot was stained with 0.02% methylene blue in 0.3 M sodium acetate (pH 5.2). To compare results obtained in different experiments, we used the normalization procedure described in Supplementary Methods (see Fig. 4a, b, which incorporate data from Fig. 2 and Supplementary Fig. 6).

Methylation analysis. The DNA methylation status of bisulphite-treated genomic DNA was probed at 27,578 CpG dinucleotides using the Illumina Infinium 27k array (Illumina)[26]. Methylation status was calculated from the ratio of methylation-specific and demethylation-specific fluorophores (β-value) using BeadStudio Methylation Module (Illumina). We removed sites on the Y and X chromosomes from the analysis because of inconsistent methylation status with respect to gender (a known problem based on communication with Illumina). Calculations are based on β values, which correspond to the methylation status of a site ranging from 0 to 1, returned by Illumina's BeadStudio software. We tested sites for differential methylation using an empirical Bayes approach employing a modified *t*-test (LIMMA). The false discovery rate (FDR) is controlled at a level of 5% by the Benjamini–Hochberg correction.

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

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Author Contributions M.K. analysed the biochemical effects of patient-associated TET2 mutations and performed the *in vitro* differentiation studies; Y.H. generated and characterized the anti-CMS antiserum, developed the quantitative dot-blot assay and quantified 5hmC in DNA samples from patients and healthy controls. A.M.J., R.G. and J.P.M. provided patient and control DNA for 5hmC quantification, performed DNA methylation arrays and analysed TET2 mutational status in patients. U.J.P. and X.S.L. carried out the statistical analysis of 5hmC levels and methylation data; M.T., H.S.B. and K.P.K. provided critical reagents; J.A. and E.D.L. contributed to molecular cloning and mouse maintenance respectively; and L.A. and S.A. provided essential intellectual input. A.R. set overall goals, coordinated collaborations and wrote the manuscript.

Author Information Data have been deposited at GEO under accession number GSE25706 (methylation status of each CpG site (beta value) can be found in Supplementary Table 3). Reprints and permissions information is available at www.nature.com/reprints. The authors declare no competing financial interests. Readers are welcome to comment on the online version of this article at www.nature.com/nature. Correspondence and requests for materials should be addressed to A.R. (arao@idi.harvard.edu and arao@liai.org) or J.P.M. (maciejj@ccf.org).