French, C.J., Bellomo, R. & Angus, P. (2003) Cryoprecipitate for the correction of coagulopathy associated with liver disease. *Anaesthesia and Intensive Care*, **31**, 357–361.

Liu, S., Fan, J., Wang, X., Gong, Z., Wang, S., Huang, L., Xing, T., Li, T., Peng, Z. & Sun, X. (2013) Intraoperative cryoprecipitate transfusion and its association with the incidence of biliary complications after liver transplantation: a retrospective cohort study. *PLoS ONE*, **8**, e60727.

Morrison, J.J., Ross, J.D., Dubose, J.J., Jansen, J.O., Midwinter, M.J. & Rasmussen, T.E. (2013) Association of cryoprecipitate and tranexamic acid with improved survival following wartime injury: findings from the MATTERs II Study. *JAMA Surgery*, **148**, 218–225.

Theodoulou, A., Berryman, J., Nathwani, A. & Scully, M. (2012) Comparison of cryoprecipitate with fibrinogen concentrate for acquired hypofibrinogenaemia. *Transfusion and Apheresis Science*, **46**, 159–162.

Tinegate, H., Allard, S., Grant-Casey, J., Hennem, S., Kilner, M., Rowley, M., Seeney, F. & Stanworth, S. (2012) Cryoprecipitate for transfusion: which patients receive it and why? A study of patterns of use across three regions in England. *Transfusion Medicine (Oxford, England)*, **22**, 356–361.

Warmuth, M., Mad, P. & Wild, C. (2012) Systematic review of the efficacy and safety of fibrinogen concentrate substitution in adults. *Acta Anaesthesiologica Scandinavica*, **56**, 539–548.

Wikkelsø, A., Lunde, J., Johansen, M., Stensballe, J., Wetterslev, J., Møller, A.M. & Afshari, A. (2013) Fibrinogen concentrate in bleeding patients. *Cochrane Database of Systematic Reviews*, **8**, CD008864.

Evaluation of allelic strength of human *TET2* mutations and cooperation between *Tet2* knockdown and oncogenic *Nras* mutation

The *TET2* (tet methylcytosine dioxygenase 2) gene encodes a methylcytosine dioxygenase that catalyses the hydrolysis of 5-methylcytosine (5mC) to 5-hydroxylmethylcytosine (5hmC) and promotes DNA demethylation through passive and active mechanisms (Shih *et al*, 2012). Loss-of-function mutations in *TET2* are identified in patients with myeloid and lymphoid malignancies, and are particularly frequent in patients with chronic myelomonocytic leukaemia (CMML) (36–58%) (Shih *et al*, 2012). Consistent with the patient sequencing analysis, conditional knockout of *Tet2* in mice dysregulates haematopoietic stem cell (HSC) function and promotes development of a myeloid malignancy closely resembling human CMML (Cimmino *et al*, 2011). Despite the high mutation frequency, the prognostic importance of *TET2* mutations is unclear in many cases (Shih *et al*, 2012). We postulated that this could be due to the differential allelic strengths of distinct *TET2* mutations (e.g. amorphic versus hypomorphic) and/or the influence of other concurrent genetic alterations.

Although nonsense and frameshift mutations are found spread over the entire *TET2* sequence, the majority of missense mutations occur in the two conserved regions of TET2 protein (Fig S1): a cysteine-rich region within 1134–1444 amino acids and a catalytic domain (double strand β helix, DSBH) in 1842–1921 amino acids (Fig 1A). To determine the allelic strengths of distinct *TET2* mutations, we characterized five missense mutations prevalent in the COSMIC database in the context of full length human *TET2*. Two of them (C1193W and R1261G) are located in the cysteine-rich domain and have not been examined before. The other three mutations (I1873T, H1881Q, and R1896S) are located in the DSBH domain and their equivalent mutations were previously evaluated in mouse *Tet2* (Ko *et al*, 2010). Transient expression of full-length wild-type human TET2 in HEK293T cells showed a predominant nuclear localization (~75%) and concomitant detection of 5hmC in the nucleus (Fig 1B–D). We observed that in ~25% of TET2-expressing cells, TET2 protein was distributed in both cytoplasm and nucleus and 5hmC staining was diminished (Fig 1B and C). These results suggest that intracellular localization of TET2 influences production of 5hmC. In contrast, the mutant proteins containing C1193W, R1261G, I1873T or H1881Q mutations maintained their nuclear localization but 5hmC levels were not detectable, suggesting that these mutations are amorphic. $TET2^{R1896S}$ mutant only showed partial loss of function, suggesting that this mutation is hypomorphic (Fig 1D). Importantly, our results of human $TET2^{I1873T}$ and $TET2^{R1896S}$ are not consistent with those obtained from equivalent mouse *Tet2* mutants, which did not show diminished 5hmC staining (Ko *et al*, 2010). This could be due to the differences between human TET2 and mouse Tet2, emphasizing the importance of validating discoveries from mouse genes in human genes. Nonetheless, our data indicate that leukaemia-associated *TET2* mutations lead to complete or partial loss of TET2 function, providing a rationale to further stratify leukaemia patients based on their specific *TET2* mutations in future prognostic studies.

Recent work identified a significant synergy between loss-of-*Tet2* and loss-of other epigenetic regulators (*Ezh2* (Muto *et al*, 2013) and *Asxl1* (Abdel-Wahab *et al*, 2013)) or NOTCH inactivation (loss of *Ncstn*) (Lobry *et al*, 2013) in mice. These results suggest that *TET2* mutations might indicate a poor prognosis outcome in CMML patients with concurrent *EZH2*, *ASXL1*, or *NCSTN* mutations. However, the prognostic importance of *TET2* mutations in patients with other concurrent mutations, for example, RAS signalling pathway mutations, has not been evaluated.

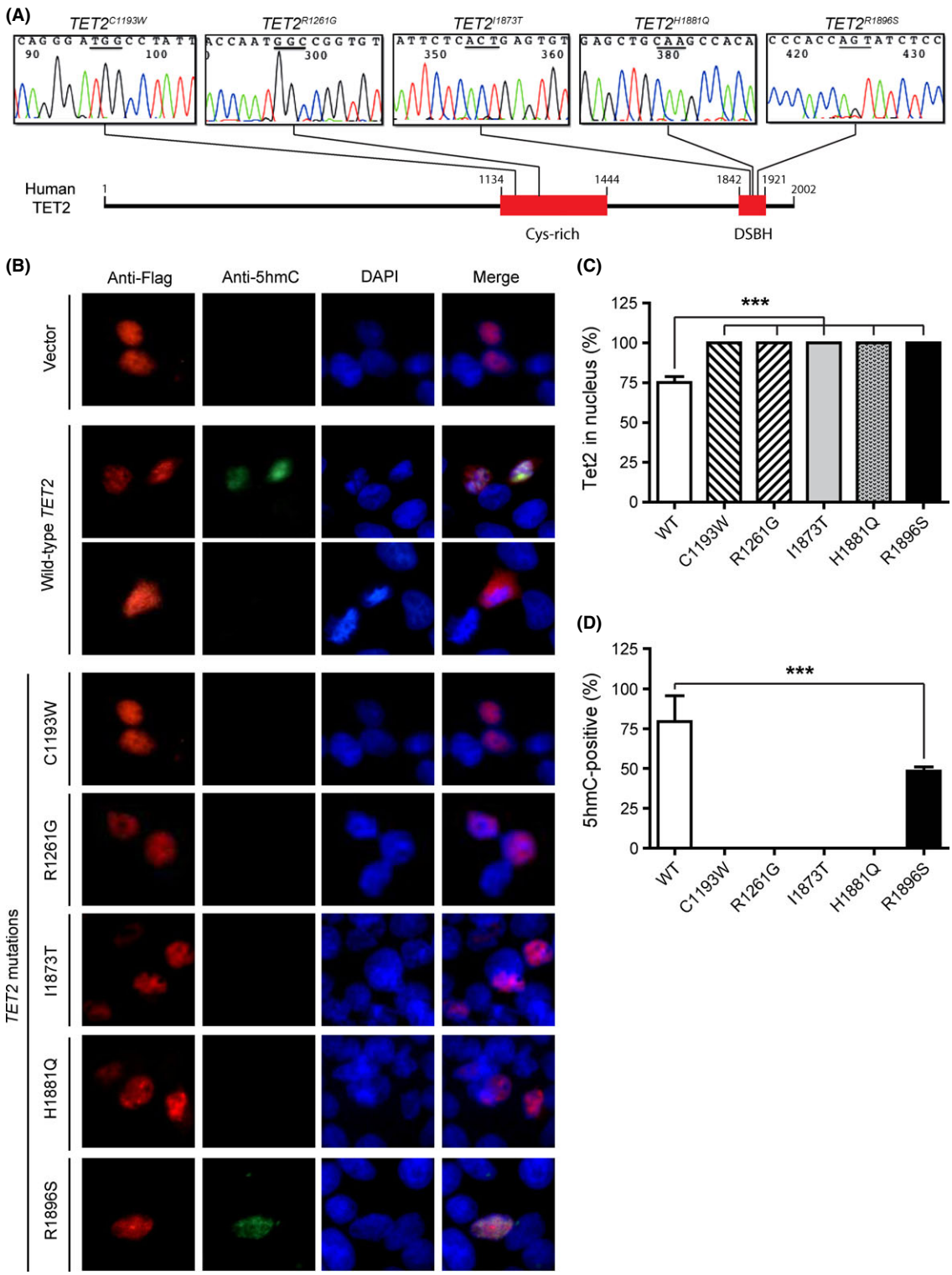

Fig 1. Missense mutations at the Cys-rich and DSBH regions of human TET2 attenuate its catalytic function. (A) Schematic illustration of TET2 protein structure and localization of constructed human *TET2* point mutations. These mutations were confirmed by sequencing. (B–D) HEK293T cells were transiently transfected with pCMV6-*TET2* or various mutant plasmids and simultaneously stained for Flag-tagged TET2 and 5hmC. (B) Representative images of transfected cells. (C, D) Quantification of nuclear TET2 (C) and 5hmC-positive cells (D). The results are presented as percentages of total TET2 expressing cells. The cytoplasm/nucleus distribution of TET2 proteins and 5hmC was quantified in at least 100 cells for each experiment under a fluorescent microscope. All experiments were performed at least three times, and values are presented as means ± standard deviation. ***$P < 0{\cdot}001$.

(A)

Patient	CMML-2	CMML-8	CMML-10	CMML-12	CMML-13
Age	73	60	70	77	74
Gender	Male	Male	Male	Male	Male
Diagnosis	sAML with antecedent of CMML	sAML with antecedent of CMML	CMML-2	CMML-1	CMML-1
Survival days after diagnosis	71	341	249	31	572
Cytogenetics	46, XY	46, XY	46, XY	46, XY	46, XY
WBC count ($\times 10^9$/l)	7·4	56·1	29·7	32·2	81·6
Monocyte count ($\times 10^9$/l)	592	23001	2673	6118	6528
Marrow blasts (%)	20	20	10	2	1
KRAS	V7E (21%)	—	—	G12D (42%)	—
NRAS	—	G12D (51%)	G12R (19%)	—	G13V (44%)
TET2	N1118fs (43%), I1762V (50%)	N7S (22%), T270fs (48%), L1721W (38%), I1762V (56%)	C1221fs (29%), M1701I (46%), L1721W (38%), I1762V (70%)	N196K (18%)	Q129K (15%), L1258fs (38%), L1340R (50%), L1721W (50%)
ASXL1	R693* (27%), L815P (50%)	L815P (50%)	L815P (50%)	R693* (27%), L815P (50%)	L815P (50%)
EZH2	Y523P (13%)	—	—	—	A103V (25%), M121I (67%), R690H (30%)
NCSTN	—	—	—	—	—

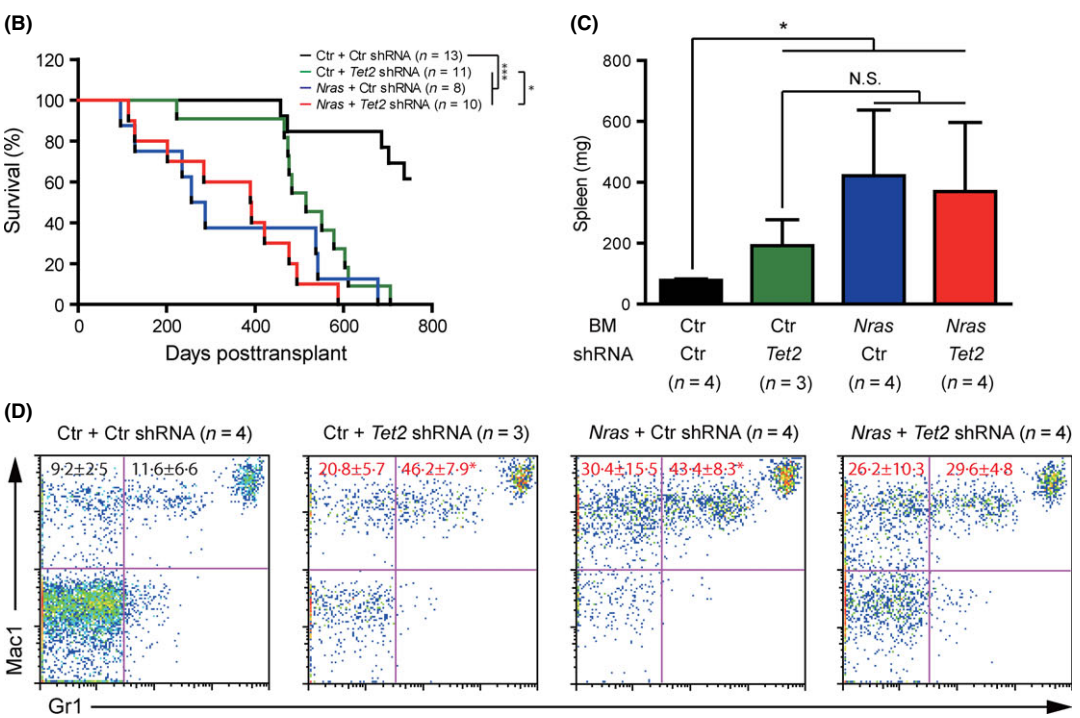

Fig 2. *Tet2* knockdown does not promote $Nras^{G12D/+}$-induced CMML. (A) Summary of *KRAS*, *NRAS*, *TET2*, *ASXL1*, *EZH2* and *NCSTN* mutation status in five human CMML patients. The percentages of mutant alleles are shown in parentheses. (B–D) Bone marrow cells from control or $Nras^{G12D/+}$ mice were infected with retrovirus encoding control or *Tet2* shRNA and transplanted into lethally irradiated recipient mice. (B) Kaplan-Meier survival curves of different groups of recipient mice were plotted against days post-transplant. *P* values were determined by the Log-rank test. (C) Splenomegaly in different groups of recipient mice. (D) Quantification of donor-derived myeloid cells in peripheral blood of moribund mice. The percentages of monocytes ($Mac1^+$ $Gr1^-$) and neutrophils ($Mac1^+$ $Gr1^+$) are indicated in their corresponding quadrants. The results are presented as mean ± standard deviation. Red font indicates significant changes compared with recipients transplanted with control cells infected with control (Ctr) shRNA. Asterisks indicate significant changes compared with recipients transplanted with $Nras^{G12D/+}$ cells infected with *Tet2* shRNA. *$P < 0{\cdot}05$; ***$P < 0{\cdot}001$. BM, bone marrow; N.S., not significant; CMML, chronic myelomonocytic leukaemia; WBC, white blood cell.

Given the high mutation rate of *TET2* in CMML patients, we set out to find and characterize additional gene mutations concurrent with *TET2* mutations. We performed whole exome sequence analysis of five CMML patients with a normal karyotype and at different stages of CMML development, including two collected from patients transformed to acute myeloid leukaemia (AML) with antecedent of CMML, one with Type II CMML, and two with Type I CMML (Fig 2A). All of them contained *TET2* mutations, including 4 frameshift and 11 missense mutations. Among the missense mutations, L1721W and I1762V were reported before (Nibourel *et al*, 2010; Kohlmann *et al*, 2011), L1340R was found in the COSMIC database (http://cancer.sanger.ac.uk/cancergenome/projects/cosmic/), while N7S, Q129K, and N196K have not been described but are absent from the SNP database (http://www.snp-nexus.org/). Detection of more than one *TET2* mutation in individual patients suggests the presence of multiple leukaemic clones. The mutation frequency of *TET2* in our small cohort is much higher than previously reported (Shih *et al*, 2012). This could be due to the small sample size and the selection of myeloproliferative variant of CMML (indicated by high white blood cell and monocyte counts) and transformed AML in our study.

Consistent with the mouse studies, our sequencing results revealed that all patients carried *ASXL1* mutations and two patients carried *EZH2* mutations. However, *NCSTN* mutations were not detected in any of the patients (Fig 2A). In addition, four patients carried canonical oncogenic mutations in *NRAS* or *KRAS*, and one patient carried the $KRAS^{V7E}$ mutation, which has not been reported in human cancers. However, V7 codon was recently suggested as a key residual in regulating oncogenic Kras activity (Maurer *et al*, 2012). Our finding of concurrent *TET2* mutations with oncogenic *RAS* mutations in CMML patients is consistent with our data-mining result of the COSMIC database (Table SI) and other reports (Table SII).

To determine whether loss-of-*Tet2* co-operates with oncogenic *Ras* to promote CMML development, we knocked down *Tet2* expression in $Nras^{G12D/+}$ bone marrow cells (Fig 2B–D). Compared with recipients transplanted with control cells expressing a scrambled shRNA, recipients transplanted with control cells expressing *Tet2* shRNA (Ko *et al*, 2010) developed CMML-like phenotypes after a prolonged latency, consistent with previous reports of *Tet2* knockout mice (Cimmino *et al*, 2011). To our surprise, knockdown of *Tet2* did not accelerate $Nras^{G12D/+}$ induced CMML (Fig 2B) or further promote CMML phenotypes (Fig 2C and D). All CMML mice displayed comparably enlarged spleen and significantly higher percentage of monocytes ($Mac1^+$ $Gr1^-$) and neutrophils ($Mac1^+$ $Gr1^+$) in peripheral blood compared to controls.

It is likely that *Tet2* knockdown does not result in long-term abrogation of Tet2 expression. Alternatively, oncogenic *Ras* signalling might alter the subcellular localization of Tet2 protein to promote *Tet2* loss-of-function during CMML development as shown in *BCR-ABL1*-driven chronic myeloid leukaemia (Mancini *et al*, 2012). Thus, further downregulation of *Tet2* expression in $Nras^{G12D/+}$ bone marrow cells does not significantly accelerate CMML progression. It is also possible that the order of mutational acquisition is important. However, current technologies do not allow us to assess this possibility under physiological conditions.

In summary, our results provide a rationale to further stratify leukaemia patients based on their specific *TET2* mutations and presence of specific additional genetic mutations in future prognostic studies.

Acknowledgements

We are grateful to Coral Wille for sharing reagents and protocols with us and Dr. Anjana Rao for providing retroviral constructs encoding Tet2 shRNA and scrambled shRNA. We would like to thank the University of Wisconsin Carbone Comprehensive Cancer Center (UWCCC) for use of its Shared Services to complete this research. This work was supported by R01 grants CA152108 and HL113066, a Shaw Scientist Award from the Greater Milwaukee Foundation, a Scholar Award from the Leukemia and Lymphoma Society, and an Investigator Initiated Grant from UWCCC to J.Z. This work was also supported in part by NIH/NCI P30 CA014520–UW Comprehensive Cancer Center Support.

Author contributions

Yuan-I Chang and Alisa Damnernsawad for experimental design and execution as well as writing manuscript; Laura K. Allen, Guangyao Kong, Jinyong Wang, and Yangang Liu for experimental execution; Jingfang Zhang, Hsu-Yuan Fu, and Chii-Shen Yang for exome sequencing analysis; David Yang, Erik A. Ranheim, and Ken H. Young for obtaining CMML patient samples; Junjie Guo and Hongjun Song for providing human TET2 construct; Jing Zhang for experimental design and writing manuscript.

Conflicts of interest

We declare no competing financial interests.

Yuan-I Chang[1,*]
Alisa Damnernsawad[1,*]
Laura K. Allen[2]
David Yang[3]
Erik A. Ranheim[3]
Ken H. Young[4]
Jingfang Zhang[1]
Guangyao Kong[1]
Jinyong Wang[1]
Yangang Liu[1]
Hsu-Yuan Fu[5]
Chii-Shen Yang[5,6]

Junjie Guo[7]
Hongjun Song[7]
Jing Zhang[1]
[1]McArdle Laboratory for Cancer Research, University of Wisconsin-Madison, Madison, WI, USA, [2]Faculty of Health Sciences, the University of Western Ontario, London, ON, Canada, [3]Department of Pathology & Laboratory Medicine, University of Wisconsin School of Medicine and Public Health, University of Wisconsin Carbone Cancer Center, Madison, WI, [4]Department of Hematopathology, The University of Texas MD Anderson Cancer Center, Houston, TX, USA, [5]Department of Biochemical Science and Technology, College of Life Science, National Taiwan University, [6]Institute of Biotechnology, College of Bio-Resources and Agriculture, National Taiwan University, Taipei, Taiwan and [7]Departments of Neurology and Neuroscience, Institute for Cell Engineering, Johns Hopkins University School of Medicine, Baltimore, MD, USA.
E-mail: zhang@oncology.wisc.edu

*These authors contributed equally to this work.

Keywords: oncogenic RAS, TET2, 5hmc, chronic myelomonocytic leukaemia, whole exome sequencing

First published online 3 April 2014
doi: 10.1111/bjh.12871

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig SI. *TET2* missense mutations are predominantly localized in two conserved regions.

Table SI. Summary of COSMIC samples (v66) with both *RAS* and *TET2* mutations.

Table SII. Summary of reported patients with both *TET2* and *RAS* mutations.

Data SI. Methods.

References

Abdel-Wahab, O., Gao, J., Adli, M., Dey, A., Trimarchi, T., Chung, Y.R., Kuscu, C., Hricik, T., Ndiaye-Lobry, D., Lafave, L.M., Koche, R., Shih, A.H., Guryanova, O.A., Kim, E., Li, S., Pandey, S., Shin, J.Y., Telis, L., Liu, J., Bhatt, P.K., Monette, S., Zhao, X., Mason, C.E., Park, C.Y., Bernstein, B.E., Aifantis, I. & Levine, R.L. (2013) Deletion of Asxl1 results in myelodysplasia and severe developmental defects *in vivo*. *Journal of Experimental Medicine*, **210**, 2641–2659.

Cimmino, L., Abdel-Wahab, O., Levine, R.L. & Aifantis, I. (2011) TET family proteins and their role in stem cell differentiation and transformation. *Cell Stem Cell*, **9**, 193–204.

Ko, M., Huang, Y., Jankowska, A.M., Pape, U.J., Tahiliani, M., Bandukwala, H.S., An, J., Lamperti, E.D., Koh, K.P., Ganetzky, R., Liu, X.S., Aravind, L., Agarwal, S., Maciejewski, J.P. & Rao, A. (2010) Impaired hydroxylation of 5-methylcytosine in myeloid cancers with mutant TET2. *Nature*, **468**, 839–843.

Kohlmann, A., Klein, H.U., Weissmann, S., Bresolin, S., Chaplin, T., Cuppens, H., Haschke-Becher, E., Garicochea, B., Grossmann, V., Hanczaruk, B., Hebestreit, K., Gabriel, C., Iacobucci, I., Jansen, J.H., te Kronnie, G., van de Locht, L., Martinelli, G., McGowan, K., Schweiger, M.R., Timmermann, B., Vandenberghe, P., Young, B.D., Dugas, M. & Haferlach, T. (2011) The Interlaboratory RObustness of Next-generation sequencing (IRON) study: a deep sequencing investigation of TET2, CBL and KRAS mutations by an international consortium involving 10 laboratories. *Leukemia*, **25**, 1840–1848.

Lobry, C., Ntziachristos, P., Ndiaye-Lobry, D., Oh, P., Cimmino, L., Zhu, N., Araldi, E., Hu, W., Freund, J., Abdel-Wahab, O., Ibrahim, S., Skokos, D., Armstrong, S.A., Levine, R.L., Park, C.Y. & Aifantis, I. (2013) Notch pathway activation targets AML-initiating cell homeostasis and differentiation. *Journal of Experimental Medicine*, **210**, 301–319.

Mancini, M., Veljkovic, N., Leo, E., Aluigi, M., Borsi, E., Galloni, C., Iacobucci, I., Barbieri, E. & Santucci, M.A. (2012) Cytoplasmatic compartmentalization by Bcr-Abl promotes TET2 loss-of-function in chronic myeloid leukemia. *Journal of Cellular Biochemistry*, **113**, 2765–2774.

Maurer, T., Garrenton, L.S., Oh, A., Pitts, K., Anderson, D.J., Skelton, N.J., Fauber, B.P., Pan, B., Malek, S., Stokoe, D., Ludlam, M.J., Bowman, K.K., Wu, J., Giannetti, A.M., Starovasnik, M.A., Mellman, I., Jackson, P.K., Rudolph, J., Wang, W. & Fang, G. (2012) Small-molecule ligands bind to a distinct pocket in Ras and inhibit SOS-mediated nucleotide exchange activity. *Proceedings of the National Academy of Sciences of the United States of America*, **109**, 5299–5304.

Muto, T., Sashida, G., Oshima, M., Wendt, G.R., Mochizuki-Kashio, M., Nagata, Y., Sanada, M., Miyagi, S., Saraya, A., Kamio, A., Nagae, G., Nakaseko, C., Yokote, K., Shimoda, K., Koseki, H., Suzuki, Y., Sugano, S., Aburatani, H., Ogawa, S. & Iwama, A. (2013) Concurrent loss of Ezh2 and Tet2 cooperates in the pathogenesis of myelodysplastic disorders. *Journal of Experimental Medicine*, **210**, 2627–2639.

Nibourel, O., Kosmider, O., Cheok, M., Boissel, N., Renneville, A., Philippe, N., Dombret, H., Dreyfus, F., Quesnel, B., Geffroy, S., Quentin, S., Roche-Lestienne, C., Cayuela, J.M., Roumier, C., Fenaux, P., Vainchenker, W., Bernard, O.A., Soulier, J., Fontenay, M. & Preudhomme, C. (2010) Incidence and prognostic value of TET2 alterations in *de novo* acute myeloid leukemia achieving complete remission. *Blood*, **116**, 1132–1135.

Shih, A.H., Abdel-Wahab, O., Patel, J.P. & Levine, R.L. (2012) The role of mutations in epigenetic regulators in myeloid malignancies. *Nature Reviews Cancer*, **12**, 599–612.