Immunity drives TET1 regulation in cancer through NF-κB

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Ten-eleven translocation enzymes (TET1, TET2, and TET3), which induce DNA demethylation and gene regulation by converting 5-methylcytosine (5mC) to 5-hydroxymethylcytosine (5hmC), are often down-regulated in cancer. We uncover, in basal-like breast cancer (BLBC), genome-wide 5hmC changes related to TET1 regulation. We further demonstrate that TET1 repression is associated with high expression of immune markers and high infiltration by immune cells. We identify in BLBC tissues an anticorrelation between TET1 expression and the major immunoregulator family nuclear factor κB (NF-κB). In vitro and in mice, TET1 is down-regulated in breast cancer cells upon NF-κB activation through binding of p65 to its consensus sequence in the TET1 promoter. We lastly show that these findings extend to other cancer types, including melanoma, lung, and thyroid cancers. Together, our data suggest a novel mode of regulation for TET1 in cancer and highlight a new paradigm in which the immune system can influence cancer cell epigenetics.

INTRODUCTION

Breast cancer (BC) is a very heterogeneous disease characterized by different molecular and histopathological features, responses to therapy, and patient outcomes (1). This complexity has prompted researchers and clinicians to stratify breast tumors. Gene expression profiling has identified four main subtypes of BC (2, 3). Luminal A BCs express the estrogen receptor (ER) and the progesterone receptor (PR) and have a good prognosis. Luminal B tumors are also ER+/PR− but are associated with a worse prognosis. HER2-like tumors are characterized by amplification of the Erbb2 (HER2) gene, are high grade, and have a poor prognosis. Basal-like BCs (BLBCs) are generally negative for the three receptors (that is, ER−/PR−/HER2− or “triple negative”) and are associated with a poor outcome (4–6).

In recent years, epigenetic features have emerged as major characteristics of cancers. Epigenetic modifications interfere with gene expression, and abnormal epigenetic modification patterns are involved in cancer development and progression (7). In this regard, the discovery of DNA cytosine hydroxymethylation is of great interest (8, 9). Ten–eleven translocation enzymes (TET1, TET2, and TET3) catalyze the oxidation of 5-methylcytosine (5mC) to 5-hydroxymethylcytosine (5hmC) (10, 11), leading to DNA demethylation and gene regulation (12–15). Global loss of 5hmC, associated with TET down-regulation and/or alteration of TET functions, has been described as a hallmark of cancer. This dysregulation has been described in both hematological and solid tumors, including colon, liver, lung, skin (melanoma), prostate, and breast tumors (16–19). In mammary tumors, TET1 especially has been described as a tumor suppressor gene, and its reduced expression appears to promote cancer growth and metastasis (18, 20–22). In BC and other solid tumors, TET1 is rarely mutated, but its activity is affected by several mechanisms, such as down-regulation by MiR-29 or HMGA2 or through promoter methylation (12, 18, 20–24). Despite growing knowledge, much remains to be learned about the regulation of TET enzymes and notably about their relationship with central signaling pathways involved in cancer.

A greater attention has recently focused on the complex but essential role of immune responses in cancer. An immune response can either repress tumor development and progression (for example, through immunosurveillance and the destruction of tumor cells) or promote it (for example, through secretion of protumorigenic and proinflammatory factors) (25). The prognostic and predictive value of tumor-infiltrating lymphocytes (TILs) in many cancer types (26–28) and the recent emergence of immunotherapy in clinical oncology (29) notably illustrate the importance of the immune system in cancer.

Among the immune signaling pathways, the nuclear factor κB (NF-κB) pathway is commonly activated in cancer. NF-κB, composed of five members, p65 (RelA), RelB, c-Rel, NF-κB1, and NF-κB2, was first identified as a transcription factor crucial to the development, survival, and activation of leukocytes (including B and T lymphocytes) and macrophages (30). It is known to be involved not only in gene expression, acting either as a transcriptional activator or repressor (31–33), but also for its interactions with other key immune pathways, such as STAT3 (signal transducer and activator of transcription 3) signaling, which makes it a central regulator of immune signaling (34–36). In addition, NF-κB is a central pathway in the mammary gland, where it regulates epithelial proliferation and branching during early development, and is frequently activated in BC, particularly in the BLBC subtype (37–40). The role of NF-κB proteins in cancer is complex. Generally viewed as protumorigenic, they are involved in cell survival, invasion, angiogenesis, metastasis, and chemoresistance (41–45). However, several reviews suggest that they might also oppose cancer development (46–49). The diverse effects of the NF-κB pathway appear to be determined by the mechanisms sustaining tumor induction and by the type of immune response involved.

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Growing evidence points to a link between cancer epigenetics and immunity. For instance, changes in 5mC have been found to correlate with the degree of immune infiltration of the tumor (50). Furthermore, epigenetic drugs have been shown to enhance the antitumor immune response (51–53). In addition, epigenetic enzymes, such as TETs, can regulate immune functions within leukocytes (54–57). The cross-talk between the immune system and cancer is thus of great importance, and investigating the underlying molecular mechanisms could lead to better therapeutic strategies.

The aim of the present work was to relate TET expression levels in BC to epigenetic anomalies and genes or pathways known to affect tumor growth and progression. We demonstrate for the first time that TET1 repression and 5hmC changes are associated with activation of immune pathways and with tumor infiltration by immune cells. We further show, both in vitro and in mice, that activation of the major immune regulator NF-xB causes TET1 repression by binding to its promoter. Our results also suggest that immunity-driven repression of TET1 could occur in many cancer types. The exciting discovery that the immune system can influence the epigenetic state of cancer cells reveals a new dimension of the cross-talk between a tumor and its microenvironment.

RESULTS

TET1 regulation is associated with 5hmC changes in BLBC

To assess TET1 expression in breast tumors, we used RNA sequencing (RNA-seq) data publicly available from “The Cancer Genome Atlas” (TCGA) consortium. Given the heterogeneity of mammary tumors, we divided samples into the four main BC subtypes and compared TET1 expression in these subtypes and normal breast (Fig. 1A). As observed in previous studies (18, 22), TET1 expression was found to be decreased in luminal A (n = 376), luminal B (n = 180), and HER2-like (n = 65) tumors as compared to normal tissues (n = 100). BLBC tumors (n = 130) displayed a much wider range of TET1 expression levels (approximately four times that of the other BC subtypes), with some tumors displaying abnormally high and others displaying abnormally low expression of the TET1 gene.

We took advantage of the wide range of TET1 expression in BLBC tumors to investigate the link between the 5hmC pattern and TET1 regulation in these tumors. We performed mapping of 5hmC in matched tumor and normal breast tissues (n = 4 matched pairs), and deposited raw data on the Gene Expression Omnibus (GEO) National Center for Biotechnology Information (NCBI) database (GSE101445). For this, we used a previously described approach (referred to here as “5hmC-seq”), combining the hMe-seal method (used to specifically select hydroxymethylated fragments) with deep sequencing (58). We then clustered breast sample pairs based on TET1 expression in the tumors. Characterization and segregation of the samples is shown in fig. S1. A comparison of TET1-low tumors with their matched normal tissues (n = 2 matched pairs) revealed 256 differentially hydroxymethylated regions (dhmRs), 58% of which were hypohydroxymethylated in the tumors. In the TET1-high tumors (n = 2 matched pairs), we identified 160 dhmRs, which were almost exclusively hyperhydroxymethylated (98%). All the identified dhmRs are displayed in a heat map in Fig. 1B, and the full list is detailed in table S1. The overlap of dhmRs between TET1-high and TET1-low groups was extremely low, with only two genes in common (DNAH14 and ABCA13). These results indicate that BLBC tumors with different TET1 expression levels display different 5hmC alteration patterns, high TET1 expression
being mostly associated with 5hmC gain and low TET1 expression being mostly associated with 5mC loss.

In each BLBC group, we next investigated potential links between 5hmC, 5mC, and gene expression. DNA methylation was profiled in the same samples as 5hmC (n = 2 matched pairs per group), while expression changes were obtained from TCGA by comparing the 25 BLBC tumors showing the lowest or highest TET1 expression with those of normal breast tissue. We focused on all the coding genes associated with identified dhmRs (Fig. 1C). In the TET1-low group, 5hmC loss was mostly associated with 5mC gain. In the TET1-high group, consistently, 5hmC gain was mostly associated with 5mC loss. Precisely, changes in 5hmC and 5mC occurred in opposite directions in 73.7 and 70.2% of the dhmRs for the TET1-high and TET1-low groups, respectively, which was significantly more than expected by chance (P = 0.003 and P < 0.0001, respectively, by one-proportion z test). In both groups, the genes displaying 5hmC changes also showed dysregulated expression, although no association could be observed between the direction of these changes (by one-proportion z test). These data suggest that there is a link between DNA hydroxymethylation, DNA methylation, and gene expression in BLBC tumors. Together, these results indicate that, in BC, regulation of TET1 expression (up or down) is associated with specific 5hmC changes, coupled with 5mC changes and altered gene expression.

In basal-like tumors, high TET1 expression is associated with low levels of immune and defense response markers

To unravel the mechanisms responsible for TET1 dysregulation in BC, we next investigated the relationship between TET1 expression and signaling pathways. As BLBC tumors can display either high or low TET1 expression, we focused on this subtype. From the TCGA RNA-seq data, we selected all the genes whose expression appeared to correlate positively (Pearson correlation coefficient r > 0.25) or negatively (r < −0.25) with TET1 expression (in what follows, these genes will respectively be called “positively correlating” and “negatively correlating” genes). We then performed a gene ontology analysis with DAVID (tables S2 and S3). In the case of the negatively correlating genes, strikingly, the pathways most overrepresented were related to immunity and defense (Fig. 2A and table S2). To illustrate this result, we computed a heat map of the top 20 genes in the immune response category (Fig. 2B). The correlation coefficient calculated for these 20 genes combined was −0.49 (P < 0.00001) (Fig. 2B). In the other BC subtypes, the correlation between TET1 expression and expression of the same 20 genes was much weaker (fig. S2), the combined r score being −0.18 (P = 5 × 10^{-4}, n = 376) for luminal A tumors, −0.18 (P = 0.016, n = 180) for luminal B tumors, and −0.16 (P = 0.18, n = 65) for HER2-like tumors.

The abovementioned overrepresented pathways included both innate and adaptive immunity and the inflammatory response (Fig. 2A, and table S2). As shown in Fig. 2C and fig. S3A, we identified genes related to the myeloid/macrophage compartment, such as TYROBP and CD14, and to the lymphoid compartment, such as CD3D, CD4, CD8A, and LST1. The expression of genes encoding key regulatory factors involved in defense pathways, such as the NF-κB family member RELA, the major histocompatibility complex class I partner B2M, and the chemokine CCL2, was also found to correlate inversely with TET1 expression (Fig. 2C, fig. S3A, and table S2).

Next, to quantify BLBC tumor infiltration by immune cells, we performed immunohistochemistry (IHC) targeting classical immune markers (n = 18). First, the percentage of CD45+ cells, commonly used to score global leukocyte infiltration, was found to correlate negatively with TET1 expression (P = 0.02; n = 13 versus n = 5 for TETI-low and TETI-high, respectively) (Fig. 3A). To score infiltration by T and B lymphocytes, we stained the CD3 and CD20 antigens, respectively, and again observed negative correlations with TET1 expression (P = 0.02 and P = 0.007, respectively) (Fig. 3A). To further investigate the infiltrating cell populations in BLBC tumors, we then used CIBERSORT, a method for characterizing the cell composition of complex tissues on the basis of their gene expression profiles. Consistently with the IHC results (Fig. 3A), we found tumors with high TET1 expression to display lower infiltration by CD4+ and CD8+ T lymphocytes (P = 5 × 10^{-5} and P = 5 × 10^{-6}, respectively). They also showed lower infiltration by M1 and M2 macrophages (P = 1 × 10^{-4} and P = 5 × 10^{-4}, respectively) (Fig. 3B and fig. S3B) and, importantly, a lower score for the NF-κB signature (P = 4 × 10^{-3}; Fig. 3C).

Given the prognostic value of immune infiltration in BC, we next assessed the potential link between TET1 expression and patient survival (fig. S3C). In public data sets, high TET1 expression is associated with worse survival in BLBC, particularly as compared to TET1-low patients with high immune infiltration. This result contrasts with previously published results for TET1 in BC (18) but agrees with the expected prognostic value of immune infiltration (59). Hence, the difference in survival might be driven by immune infiltration.

Last, we compared immune infiltration (assessed with CIBERSORT) in the four BC subtypes (fig. S4A). As described above, BLBC tumors with high TET1 expression showed significantly lower mean levels of infiltration by most immune populations than their low TET1 counterparts. In the other types, strikingly, no immune population (except B lymphocytes in luminal B) displayed any significant difference according to the level of TET1 expression. This result suggests that the link between TET1 and the immune infiltration is essentially specific to BLBC. The above findings thus indicate an association between the global immune state of BLBC and TET1 expression and, more precisely, an anticorrelation between the level of TET1 expression and the extent of infiltration by the major types of leukocytes.

**TET1 expression is repressed by NF-κB activation**

Since both the sizes of various immune cell populations and the levels of certain immune mediators (such as cytokines) were found to correlate negatively with TET1 expression in BLBC, we hypothesized that leukocyte-driven activation of immune pathways might repress TET1 expression in BC cells. This hypothesis is backed up by the observation that, in a public data set (GSE61208), TET1 appears regulated upon immune modulation in breast tumors in mice (fig. S5A). To test this, we first treated MDA-MB-231 triple-negative BC cells with medium conditioned by myeloid U937 cells, and observed, by reverse transcription quantitative polymerase chain reaction (RT-qPCR), a significant decrease in transcript-level TET1 expression (62% decrease; P = 0.02), but no change in TET2 or TET3 expression (Fig. 3D). Western blotting confirmed decreased TET1 production (Fig. 3D). Yet since U937 cells do not represent mature infiltrating macrophages, we further tested the effect of medium conditioned by polarized M1 and M2 macrophages (fig. S5C). M1-conditioned medium, but not M2-conditioned medium, caused TET1 repression in MDA-MB-231 cells, suggesting that TET1 might be regulated in BLBC by soluble factors secreted by specific immune subpopulations.

We next sought to unravel specific mechanisms of TET1 regulation. As changes in TET1 expression appeared to correlate with changes in tumor immune status, involving both innate and adaptive pathways and inflammatory markers as well (Fig. 2A), we hypothesized that a
central regulator might affect \textit{TET1} expression. The NF-\textit{k}B family transcription factors constitute a key regulatory family affecting many immune and inflammatory functions \cite{36}. The \textit{RELA} gene, encoding the NF-\textit{k}B family member p65, was among the genes whose expression was found to correlate negatively with \textit{TET1} expression in BLBC tumors (Fig. 2C). Consistent with this, MDA-MB-231 cells treated with U937-conditioned medium displayed by Western blotting an increase in nuclear p65, indicative of activation of the canonical NF-\textit{k}B pathway (Fig. 3D). In BLBC tissues, we also detected a link between high \textit{TET1} expression and a low score for the NF-\textit{k}B signature (Fig. 3C).
In a publicly available data set (GSE52707), furthermore, p65 overexpression was found to cause reduced TET1 expression (fig. S5B).

The above data suggest that NF-κB activation contributes to TET1 repression. To test this hypothesis, different approaches were used to activate NF-κB. First, p65 was overexpressed in MDA-MB-231 cells, and reduced TET1 expression was observed (41% decrease; \( P = 0.04 \)) (Fig. 4A). This result is consistent with public data on p65 overexpression in BC cells (fig. S5B). Next, cells were treated with one of two well-established activators of the canonical NF-κB pathway (60), tumor necrosis factor (TNF) or lipopolysaccharide (LPS). This led, respectively, to a 45% \( (P = 0.01) \) or a 63% \( (P = 0.001) \) decrease in TET1 production (Fig. 4, B and C, and fig. S6). When the same experiment was performed after pretreating the cells with MG-132, a known blocker of NF-κB activation (61), TNF-dependent repression of TET1 was compromised (16% reduction; \( P = 0.15 \)) (Fig. 4D). The effect of NF-κB activation on TET1 expression was confirmed in two other triple-negative BC cell lines, Hs 578T and BT-549 (fig. S7, A and B). Of note, TNF and LPS specifically down-regulated TET1 expression, with no effect on TET2 expression and even a stimulation of TET3 expression on occasion (Fig. 4, B and C).

To confirm TET1 regulation in vivo, we used a transgenic mouse model (IKMV mice) in which aberrant NF-κB activation in the mammary epithelium leads to hyperplastic growth and ductal carcinoma in situ (62). Significantly reduced TET1 expression was detected in the carcinomas formed by both RT-qPCR (78% decrease; \( P = 0.001 \)) and Western blotting (Fig. 4E).

In contrast to BLBC, other BC subtypes did not show reduced NF-κB signaling in TET1-high tumors (fig. S4B). In luminal (MCF7 and T47D) and HER2 (SKBR3) cell lines, no TET1 repression was observed following TNF or LPS treatment (fig. S7, C to E). On the basis of control genes, the NF-κB response was also reduced as compared to triple-negative cells. This might explain, at least in part, the inability to regulate TET1 in these cell lines.

In addition, and given the occasional changes of TET2 and TET3 expression upon immune modulation in vitro, we analyzed the potential association of these genes with immunity and NF-κB signaling in BLBC tissues (fig S8). TET2 displayed a negative correlation with immune markers \( (r = −0.19, P = 0.03) \), but not to the extent of TET1. This association did not appear specifically linked to NF-κB \( (P = 0.26) \). Despite increased expression upon p65 overexpression and LPS treatment in vitro (Fig. 4, A and B), TET3 also displayed a negative correlation with immune markers \( (r = −0.48, P < 10^{-5}) \), and NF-κB signaling was reduced in TET3-high tumors \( (P = 1 × 10^{-6}) \). TET3- and TET1-high tumors thus displayed very similar patterns. In conclusion, there does not appear to be any compensation by either TET2 or TET3 in BLBC tumors.

Together, the above in vitro and in vivo results support the view that NF-κB activation negatively regulates TET1 expression. This occurs specifically in BLBC.

**TET1 is repressed through binding of NF-κB to its promoter**

We next examined whether the NF-κB family transcription factor p65 might affect expression of TET1 by binding to its promoter. In silico analyses performed with three different algorithms (JASPAR, AliBaba, and TFBIIND) predicted two putative p65-binding sites (hereafter named sites A and B) in the TET1 promoter, both located near the transcription start site (TSS) of the gene (Fig. 5A).

Luciferase assays were performed on extracts of MDA-MB-231 cells transfected with a TET1-LUC reporter. Upon NF-κB activation achieved by overexpressing p65 or by TNF treatment, the luciferase signal was found to decrease, indicating that the effect on TET1 expression was, at least in part, promoter-dependent (Fig. 5B).

To assess binding of NF-κB to the TET1 promoter in MDA-MB-231 cells, we conducted in vitro streptavidin-agarose pulldown assays. TNF treatment was found to induce binding of p65 to a TET1 promoter probe containing the putative binding sites A and B (Fig. 5C). To confirm this result in an endogenous setting, we also performed chromatin immunoprecipitation (ChIP)–qPCR with a p65-targeting antibody. In TNF-treated cells, an average 2.7-fold enrichment was obtained with
anti-p65, as compared to ChIP with a control immunoglobulin G (IgG) \((P = 0.03)\) (Fig. 5D).

Given the close proximity of sites A and B (Fig. 5A), we used a streptavidin-agarose pulldown assay based on DNA probes to test them separately for p65 binding, as this allows better resolution than ChIP analysis. The assay was performed with a probe carrying either the wild-type version of the consensus NF-κB–binding sequence to be tested (A or B) or a disrupted version thereof (Fig. 5E and fig. S9). Strikingly, only the probe bearing the wild-type B site showed stronger p65 binding upon TNF treatment than its mutated counterpart. The above results suggest that TET1 is repressed through binding of NF-κB to its promoter, the B site being the more potent binding site responsible for NF-κB–mediated regulation in BC cells.

**TET1 is down-regulated by NF-κB in other cancer types**

Given the broad involvement of NF-κB signaling in different cancer types, we screened the TCGA cohorts for other cancer types in which NF-κB–dependent regulation of TET1 might occur. On the basis of RNA-seq data, several cancer types displayed a global immune status shift correlating with TET1 expression, which was assessed with the 20-gene “immune signature” initially identified in BLBC (Fig. 2B). The list of TCGA cancer cohorts and their signature scores is provided in Table 1. Examples of cancers with a strong association between TET1 and immunity include thyroid carcinoma (THCA), skin cutaneous melanoma (SKCM), and lung adenocarcinoma (LUAD). As shown in Fig. 6A, the immune signature correlated negatively with TET1 expression in these three cancer types \((r = −0.39, P = 6 \times 10^{-20}; r = −0.36, P = 1 \times 10^{-15}; \text{and } r = −0.41, P = 9 \times 10^{-23})\) for THCA, SKCM, and LUAD, respectively. These cancers are all known to be infiltrated or surrounded by immunoreactive cells (63–65). Accordingly, the infiltration of most immune subpopulations, assessed with CIBERSORT, was found significantly decreased in tumors with high TET1 expression (fig. S10A) in these cancer types, as observed in BLBC. However, other cancer types failed to display any significant correlation between
TET1 and the immune signature (Table 1). The spectrum of associations between TET1 and tumor immunity is illustrated in fig. S11 for a representative panel of cancers, covering examples with strong [ovarian cancer (OV): \( r = -0.58, P = 2 	imes 10^{-22} \)], intermediate [prostate adenocarcinoma (PRAD): \( r = -0.24, P = 1 	imes 10^{-7} \)], and nonsignificant [kidney renal clear cell cancer (KIRC): \( r = -0.02, P = 0.62 \); and colon adenocarcinoma (COAD): \( r = 0.03, P = 0.68 \)] correlations. As expected, the association between TET1 and immune markers or tumor immune infiltration was strongest in OV and markedly reduced in PRAD, KIRC, and COAD.

Next, we investigated the potential link between NF-\( \kappa \)B signaling and TET1 regulation. Consistent with the results obtained for BLBC, high expression of TET1 was associated with a weak NF-\( \kappa \)B signature in THCA, SKCM, and LUAD (Fig. 6B). We tested the effect of NF-\( \kappa \)B activation by TNF in vitro in cell lines derived from THCA, SKCM, and LUAD tumors (respectively the TPC1, A375, and A579 cell lines) (Fig. 6C and fig. S10B). Decreased TET1 expression was consistently observed in all three cell lines (respectively a 69% decrease with \( P = 0.0001 \), a 47% decrease with \( P = 0.02 \), and a 38% decrease with \( P = 0.03 \)). High expression of TET1 was also associated with reduced NF-\( \kappa \)B signaling in OV (Fig. S12A), and in vivo inhibition of NF-\( \kappa \)B in ID8 mouse OV cells induced TET1 expression (fig. S12B). NF-\( \kappa \)B–dependent regulation of TET1 is thus not only restricted to BLBC but can also occur in other tumors. In contrast, the association between TET1 and NF-\( \kappa \)B signaling was either reduced or absent in PRAD, KIRC, and COAD (fig. S12, A and C to E).

Last, streptavidin-agarose pulldown assays performed with the probe bearing the wild-type NF-\( \kappa \)B binding site B confirmed that p65 can bind to the TET1 promoter upon TNF induction in TPC1, A375, and A549 cells (Fig. 6D and fig. S10C). Disruption of the B-site NF-\( \kappa \)B consensus sequence was found to decrease this binding. Only in A549 cells, however, did the A site show reduced binding upon disruption of the NF-\( \kappa \)B consensus sequence (fig. S10D). This suggests that, while p65 can also bind to the A site, the B site is favored in most cellular contexts. Together, these results suggest that the mechanism by which immunity drives TET1 down-regulation through NF-\( \kappa \)B activation and binding to the TET1 promoter is not restricted to BLBC and may instead be common to many cancer types, such as melanoma and thyroid, lung, and ovarian cancers.

**DISCUSSION**

Dysregulation of TETs and 5hmC has been described as a hallmark of cancer, with implications for progression of the disease (17, 66, 67). In BC, TET1 down-regulation has been suggested to enhance tumor progression and metastasis (18, 24). Here, our investigation of the regulation of TETs and 5hmC in cancer has led us to address the essential question of the cross-talk between cancer cells and their immune microenvironment and to uncover a mechanism through which the immune system can regulate the epigenetic state of cancer cells, and hence their gene expression pattern, via TETs.

As TET down-regulation is observed in nearly all cancer types, our starting hypothesis was that alterations in signaling pathways frequently associated with tumors could play a role in this regulation. Our gene ontology analysis, applied to the genes whose expression anticorrelates with TET1 expression in the BLBC subtype, predominantly highlighted immune pathways. Accordingly, tumors where TET1 was repressed were found to show high expression of immune genes and high infiltration by major immune populations, including B lymphocytes, CD4\(^+\) and CD8\(^+\) T lymphocytes, NK cells, and macrophages (Fig. S10). The TET1 down-regulation was associated with increased expression of immune genes in a significant fraction of TNBC cases (Fig. S10).

**Fig. 5.** NF-\( \kappa \)B represses TET1 gene expression by binding to its promoter. (A) Schematic view of the TET1 gene promoter. Two NF-\( \kappa \)B binding sites, named “A” and “B,” were identified. Binding site locations are indicated relative to the TET1 TSS. (B) TET1 promoter activity was assessed under NF-\( \kappa \)B activation by cotransflecting MDA-MB-231 cells with a vector encoding firefly luciferase under the control of the TET1 promoter (TET1-LUC) and a control vector encoding Renilla luciferase (R-LUC) before treating the cells with TNF (15 ng/ml, 24 hours) or overexpressing p65 (24 hours) (\( n = 3 \), data expressed as means \( \pm \) SD). Results are expressed relatively to control conditions (\( *P \leq 0.05 \)). (C) Streptavidin-agarose pulldown assays were performed to assess binding of p65 to the TET1 promoter in vitro. Pulldown of nuclear proteins extracted from MDA-MB-231 cells was achieved with biotinylated DNA probes corresponding to the TET1 promoter (TET1-LUC) and with positive/negative control probes (CTL+/CTL\(^-\)). TNF treatment (15 ng/ml, 30 min) was used to induce nuclear translocation of p65. (D) ChiP was performed with a p65-targeting antibody or a control IgG to assess p65 binding to the TET1 promoter in MDA-MB-231 cells. TNF treatment (15 ng/ml, 30 min) was used to induce nuclear translocation of p65. NS, not significant (\( *P \leq 0.05; **P \leq 0.01 \)). (E) Streptavidin-agarose pulldown assays were performed with biotinylated DNA probes corresponding to the predicted NF-\( \kappa \)B binding site A or B. To assess the binding specificity, pulldowns were done with either the wild-type site or a mutated version in which the consensus NF-\( \kappa \)B–binding sequence was disrupted (wild-type probes: A and B; mutated probes: A mut and B mut). TNF treatment (15 ng/ml, 30 min) was used to induce nuclear translocation of p65.
CD8+ T lymphocytes, and macrophages. We provide evidence that soluble factors secreted by immune cells, such as M1 macrophages, could be responsible for TET1 modulation in BLBC. One factor abundantly secreted by M1 (but not M2) macrophages is TNF (68, 69), which is sufficient to cause TET1 repression. Thus, secretion of TNF by infiltrating macrophages might be involved in TET1 repression. This is most interesting, as interactions between tumors and the immune system have emerged in recent years as important in cancer (25). Infiltration of the tumor by immune cells is observed in many types of cancer. It is, at least in part, due to secretion of recruiting factors by cancer cells themselves and has major impacts in terms of disease progression and response to treatment (36).

The immune system has a dual action in cancer (25). On the one hand, secretion of proinflammatory factors appears to enhance

### Table 1. TET1 expression correlates negatively with immune markers in many cancer types

| Cancer type                                      | TCGA cohort | Pearson | P            | Number of samples |
|-------------------------------------------------|-------------|---------|--------------|------------------|
| Ovarian serous cystadenocarcinoma                | OV          | −0.58   | 2.1 x 10⁻²⁰ | 307              |
| Glioblastoma multiforme                          | GBM         | 0.56    | 7.4 x 10⁻¹⁸ | 166              |
| Sarcoma                                          | SARC        | −0.50   | 9.6 x 10⁻¹⁸ | 263              |
| Brain lower grade glioma                         | LGG         | −0.46   | 1.0 x 10⁻¹⁸ | 530              |
| Uterine carcinosarcoma                           | UCS         | −0.44   | 5.7 x 10⁻⁴  | 57               |
| Lung adenocarcinoma                              | LUAD        | −0.41   | 9.0 x 10⁻²³ | 517              |
| Mesothelioma                                     | MESO        | −0.41   | 6.9 x 10⁻³  | 87               |
| Thyroid carcinoma                                | THCA        | −0.39   | 5.6 x 10⁻²⁰ | 509              |
| Cholangiocarcinoma                               | SARC        | −0.38   | 3.6 x 10⁻²  | 177              |
| Uterine corpus endometrial carcinoma             | UCS         | −0.36   | 9.1 x 10⁻⁷  | 177              |
| Skin cutaneous melanoma                          | SKCM        | −0.36   | 1.1 x 10⁻¹⁵ | 472              |
| Kidney renal papillary cell carcinoma            | KIRP        | −0.33   | 4.9 x 10⁻⁹  | 291              |
| Uveal melanoma                                   | UVM         | −0.32   | 4.0 x 10⁻⁴  | 80               |
| Lung squamous cell carcinoma                     | LUSC        | −0.32   | 4.3 x 10⁻¹³ | 501              |
| Pheochromocytoma and paranglioma                 | PCPG        | −0.31   | 2.2 x 10⁻⁵  | 184              |
| Testicular germ cell tumors                      | TGCT        | −0.28   | 4.0 x 10⁻⁴  | 156              |
| Thymoma                                          | THYM        | −0.28   | 2.1 x 10⁻³  | 120              |
| Adrenocortical carcinoma                         | ACC         | −0.25   | 2.9 x 10⁻²   | 79               |
| Prostate adenocarcinoma                          | PRAD        | −0.24   | 9.6 x 10⁻⁸  | 498              |
| Head and neck squamous cell carcinoma            | HNSC        | −0.18   | 3.0 x 10⁻⁵  | 522              |
| Kidney chromophobe                               | KICH        | −0.17   | 1.8 x 10⁻¹  | 66               |
| Cervical and endocervical cancers                | CESC        | −0.13   | 2.0 x 10⁻²  | 306              |
| Bladder urothelial carcinoma                     | BLCA        | −0.09   | 7.6 x 10⁻²  | 408              |
| Stomach adenocarcinoma                           | STAD        | −0.06   | 2.1 x 10⁻¹  | 415              |
| Kidney renal clear cell carcinoma                | KIRC        | −0.02   | 6.2 x 10⁻¹  | 534              |
| Liver hepatocellular carcinoma                   | LIHC        | 0.02    | 6.8 x 10⁻¹  | 373              |
| Colon adenocarcinoma                             | COAD        | 0.03    | 6.8 x 10⁻¹  | 191              |
| Esophageal carcinoma                             | ESCA        | 0.04    | 6.2 x 10⁻¹  | 185              |
| Rectum adenocarcinoma                            | READ        | 0.04    | 7.1 x 10⁻²  | 72               |
| Pancreatic adenocarcinoma                        | PAAD        | 0.19    | 9.7 x 10⁻³  | 179              |
cancer progression and favor resistance to treatment. On the other hand, antitumor immune responses, and particularly TILs, are increasingly recognized as associated with better clinical outcome in many cancers (26–28). The prognostic value of $TET1$ expression in cancer might be related to the immune status of the tumor. Also central is the recent emergence of novel and promising immunotherapeutic tools (for example, PD-L1 and PD-1 inhibitors) used to prevent cancer from escaping destruction by the immune system. These tools have raised new hopes for better cancer treatment (29).

**Fig. 6. $TET1$ and NF-κB in other cancer types.** (A) Heat map illustrating expression (RSEM z score) of the “20–immune response gene” signature of Fig. 2B in several cancer types (from left to right: THCA SKCM, and LUAD). Data were taken from the TCGA cohort and ordered by $TET1$ expression in each cancer type. (B) High expression of $TET1$ is associated with a weak NF-κB signature in THCA ($n = 509$), SKCM ($n = 472$), and LUAD ($n = 510$) tumors. Gene expression data (RSEM z scores) were obtained from TCGA. (C) NF-κB was activated by treating TPC1 thyroid cancer cells, A375 melanoma cells, and A549 lung cancer cells with TNF for 4 hours. $TET$ expression was measured by RT-qPCR ($n = 3$; data expressed as means ± SD, relative to control) (*$P \leq 0.05$, **$P \leq 0.01$, and ***$P \leq 0.001$). (D) Streptavidin-agarose pulldown assays were performed as described above to assess in vitro the binding of NF-κB family member p65 to the $TET1$ promoter in TPC1, A375, and A549 cells [TNF (15 ng/ml), 30 min].
We have specifically linked TET1 repression to NF-κB. This major immunoregulatory transcription factor is known to be activated in many cancer types (36). It is mostly viewed as protumorigenic, particularly when its activation is associated with an inflammatory context, but NF-κB signaling can also be a marker of an immune response targeting malignant cells (35, 70). Here, we have found the NF-κB family member p65 to be involved in TET1 repression. Generally speaking, the p65-p50 heterodimer is recognized as promoting the expression of many cytokines and chemokines, while repressive activity is more often attributed to the p50-p50 homodimer. Yet there are also reports of a repressive effect of p65-p50, exerted through interaction with co-repressors such as histone deacetylases (HDACs) or DNA methyltransferases (33, 71). Our study thus highlights the dual function (both activating and repressing) of this transcription factor in gene regulation while revealing a new facet of its role in cancer signaling: an involvement in epigenetic regulation.

We have extended to other cancers our findings concerning BLBC. We have notably demonstrated in THCA, SKCM, LUAD, and OV an anticorrelation between TET1 expression and the 20-gene immune signature described for BLBC. We have also shown in cell lines derived from these tumor types that NF-κB represses TET1 by binding to its promoter. We have thus uncovered what appears as a commonly occurring novel link between TET1 regulation and the immune system in cancer.

The finding that epigenetics and immunity are interwoven in cancer has been increasingly highlighted. Altered DNA methylation, particularly, has been linked to the presence of infiltrating immune cells (50). The present study highlights a new dimension of the epigenetics-immunity connection: immunity-driven repression of TET1 in cancer cells. Thus far, TET enzymes have been implicated only in the regulation of immune cells themselves. In regulatory T cells, for instance, TETs promote FOX3 expression and Treg-associated immune homeostasis (72). In myeloid cells, TET2 controls inflammation by repressing the proinflammatory cytokine IL6 (54). TET2 has also been found to promote activation of cytokine genes in CD4+ T cells (55). Furthermore, TET1 has been reported as an epigenetic regulator involved in T helper cell differentiation (57). Here, for the first time, we show that the link between immune pathways and TETs extends beyond the immune system itself. Specifically, we provide evidence that NF-κB–mediated regulation of TET1 occurs in both BC and other cancer cells.

In conclusion, our data reveal a novel function of NF-κB, a factor known to orchestrate immune and inflammatory responses and oncogenesis (63–65, 70). Although identified in BLBC, NF-κB–mediated repression of TET1 appears to be common to many cancer types. Given the link between TET1 repression and immunity and the importance of immune infiltration in predicting clinical outcome, it is worth rethinking how TET1 expression relates to cancer. This is of great importance, as epigenetic drugs have been shown to modulate the antitumor immune response, and dissecting the epigenetic mechanisms underlying the cross-talk between the immune system and cancer could help optimize therapeutic strategies.

**MATERIALS AND METHODS**

**Human cancer data sets**

TCGA gene expression data sets (RNA-seq expression RPKM and RSEM) were downloaded from the “firehose” website (https://gdac.broadinstitute.org/). For correlation analysis, a log2 transformation was applied to the expression values, and then the Pearson scores for the correlation of all genes with TET1 expression were computed. Functional enrichment analysis was performed with DAVID (version 6.7) (73). For all TCGA analyses, the top 10% of each cohort was considered “TET1-high,” the rest being considered “TET1-low.” The same cutoff was applied for TET2 and TET3 in TCGA analyses. For the other cohorts, given the smaller number of samples available, all percentiles were computed, and the best-performing threshold (in terms of the P value for the corresponding observation, regardless of the direction of the change) between the two groups was selected as the cutoff.

Immune infiltration in TCGA cohorts was quantified on the basis of expression data with the CIBERSORT algorithm (https://cibersort.stanford.edu/). Briefly, this algorithm can accurately estimate levels of many different leukocyte subtypes in bulk tumor samples profiled by array or RNA-seq on the basis of a signature of 547 distinct genes distinguishing leukocyte subpopulations (74). The signature was built from public expression data sets of leukocyte subpopulations and was optimized to include the most relevant differentially expressed genes. Then, a deconvolution method was applied to separately quantify each population. The NF-κB signature score was based on the mean expression (RNA-seq RPKM) of eight target genes, as previously reported (75).

**Cell culture and treatments**

MDA-MB-231 and A549 cells were purchased from Caliper Life Sciences. Hs 578T, BT-549, MCF-7, T47D, SKBR3, 786-O, HT29, PC3, THP-1, and U937 cells were obtained from the American Type Culture Collection. A375 and TPC1 cells were provided, respectively, by J.-C. Marine (Katholieke Universiteit Leuven, Belgium) and C. Maenhaut [Université libre de Bruxelles (ULB), Belgium]. All cells were grown at 37°C under 5% CO2. MDA-MB-231, A549, Hs578T, A375, BT-549, MCF-7, T47D, SKBR3, and TPC1 cells were cultured in Dulbecco’s modified Eagle’s medium (Gibco) supplemented with 10% fetal bovine serum (FBS) (Gibco), 1% L-glutamine (Gibco), and 1% penicillin and streptomycin (Gibco). PC3 cells were cultured in Ham’s F-12K (Kaighn’s) Medium (Gibco) supplemented with 10% FBS (Gibco) and 1% penicillin and streptomycin (Gibco). U937, HT29, THP-1, and 786-O cells were cultured in RPMI 1640 (Invitrogen) supplemented with 10% FBS.

To produce U937-conditioned medium, U937 cells (seeded at 1 × 106 cells/ml) were grown for 48 hours, washed with phosphate-buffered saline (PBS), and incubated for another 24 hours in a serum-free medium. The supernatant was collected and centrifuged at 4°C. Cancer cells were split at low density (40% confluence) into 1×106 cells/ml for 48 hours, washed with phosphate-buffered saline, and incubated at 4°C. Cancer cells were split at low density (40% confluence) and

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maintained in culture for 24 hours before treatment with U937-, M1 macrophage–, or M2 macrophage–conditioned medium, human recombinant TNF (15 ng/ml) (PHC3015, Gibco), or LPS (5 μg/ml) (L2630, Sigma-Aldrich). Cells were collected at different times according to the analysis (30 min, 4 hours, or 24 hours). To inhibit the NF-κB pathway, cells were pretreated with 20 μM MG-132 (M7449, Sigma-Aldrich) for 3 hours before TNF treatment. In some assays, cells transfected with p65 complementary DNA (cDNA) were used. Transfection was performed with Lipofectamine 2000 reagent according to the manufacturer’s instructions, and cells were collected after 24 hours.

**Mouse experiments**

RNA and nuclear proteins were extracted from mammary glands of the transgenic IMVM mice, a doxycycline-inducible transgenic mouse model in which active IKK2 is expressed in the mammary epithelium. The mice were provided by F. Yull of the Vanderbilt-Ingram Cancer Center, Nashville and are described in the study of Barham et al. (62). Three wild-type and three IMVM mice treated with doxycycline for 3 days were used for analyses.

C57BL/6 mice injected intraperitoneally with ID8 mouse ovarian cancer cells were treated thrice weekly intraperitoneally for 10 days with vehicle (1% dimethyl sulfoxide in PBS) or the NF-κB inhibitor thymoquinone (TQ; 40 mg/kg). Expression of TET1 was measured in 20 μg of whole-cell protein extracts from harvested peritoneal tumors by Western blot. Three control mice and three TQ-treated mice were used for analyses. The experimental protocols were reviewed and approved by the Institutional Animal Care and Use Committee at Vanderbilt University.

**Reverse transcription quantitative polymerase chain reaction**

Total RNA was extracted with the High Pure RNA Isolation Kit (Roche) or the RNeasy Mini Kit (Qiagen). RNA was quantified with an ND-100 NanoDrop Spectrophotometer. One microgram of total RNA was reverse-transcribed with the First Strand cDNA Synthesis Kit (Roche) according to the manufacturer’s recommendations. Real-time PCR was performed with the LightCycler 480 Probes Master Kit (Roche) and the Universal ProbeLibrary System (Roche) or Brilliant SYBR Green QPCR Master Mix (Roche). Gene expression was normalized to human glyceraldehyde phosphate dehydrogenase and hypoxanthine phosphoribosyltransferase 1 (HPRT1) or to mouse actin and 18S. Primer sequences are indicated in table S4.

**Western blotting and streptavidin-agarose pulldowns assays**

Nuclear and cytosolic extracts were prepared according to methods described previously (77). Fifty micrograms of extract was electrophoresed through an 8% SDS–polycrylamide gel, transferred onto a polyvinylidene difluoride membrane (PerkinElmer) at 110 V for 80 min, and subjected to Western blot analysis. Antibodies against TET1 (1:500; 89-872, EMD Millipore), p65 (1:500; ab7970, Abcam), HDAC1 (1:1000; C15410053, Diagenode), actin (1:2000; A5316, Sigma), or Flag (1:1000; F3165, Sigma-Aldrich) were used and diluted in 5% (w/v) nonfat dry milk in PBS containing 0.1% Tween 20. Secondary antibodies were GE Healthcare NA934V (1:5000) for anti-rabbit antibodies and NAX931 (1:3000) for anti-mouse antibodies. Actin and HDAC1 were used as loading controls. Western blots were visualized with the ECL Plus system (Amersham Biosciences).

Streptavidin-agarose pulldowns were performed to evaluate protein binding to DNA. The protocol was adapted from Deng et al. (78). Briefly, 500 μg of nuclear proteins and 5 μM biotinylated DNA probe were incubated overnight at 4°C under rotation in buffer A [10 mM Hepes (pH 7.9), 10 mM KCl, 0.1 mM EDTA, 1 mM dithiothreitol, 0.4% NP-40, and antiproteases (1183615001, Roche)]. The extracts were then incubated for 1 hour at room temperature with 50 μl of streptavidin beads (20357, Thermo Fisher Scientific). After four washes with 400 μl of buffer A, the beads were heated for 5 min at 95°C in 20 μl of Laemmlli buffer and analyzed by Western blotting.

**Immunohistochemical staining**

Quantification of immune cells by pathologists was performed as previously described (79). Staining of formalin-fixed paraffin-embedded tissue sections (4 μm thick) was performed with a BenchMark XT IHC/ISH automated slide stainer (Ventana Medical Systems Inc.). The antibodies used for immunohistochemical staining were anti-CD45 (Dako Denmark A/S), anti-CD3 (Dako Denmark A/S), and anti-CD20 (Dako Denmark A/S). They were revealed with the ultraView Universal DAB Detection Kit (Ventana Medical Systems Inc.). All staining reagents used were manufactured by Roche (F. Hoffmann–La Roche Ltd.). Images were analyzed with VisionorPathD software (Visiopharm) to quantify the CD45−, CD3+, and CD20+ areas within the invasive tumor area defined for each digital image. The total positively stained area was scored as a percentage of the defined region, and the mean percentage of the scores obtained by two or three different pathologists was calculated for each sample. TET1 expression data for the IHC samples were taken from the Affymetrix data set GSE20711. The BLBC samples from this cohort were then separated into two groups (TET1-low and TET1-high), the cutoff being chosen to optimize the significance for CD45+ quantification between the two groups. The same split was then used for CD3+ and CD20+ quantification.

**Survival analyses**

Kaplan-Meier survival curves and log-rank tests were used to assess the prognostic value of TET1 expression in BLBC. Several sets from the GEO data repository were combined (regardless of treatment), and the entire collection of probe sets of the Affymetrix Human Genome U133 Plus 2.0 Array was reannotated, as previously described (80). Groups were distinguished on the basis of TET1 expression alone (left panel), or in combination with the TIL score (middle panel), and relapse-free survival was analyzed. For overall survival analysis, data were obtained from GSE16446. In this cohort, all patients were specifically treated with anthracycline (epirubicin) neoadjuvant therapy. TET1 expression was obtained for each sample from the microarray data of the corresponding cohort. Then, all percentiles were computed, and the best-performing threshold between the two groups (TET1-high and TET1-low) was selected as the cutoff.

**Chromatin immunoprecipitation**

MDA-MB-231 cells were treated with TNF (30 min), and then chromatin was extracted with the ChIP-IT High Sensitivity Kit (Active Motif). Briefly, the cells were cross-linked for 10 min with Complete Cell Fixation Solution (1:10 growth medium volume). The reaction was stopped with 1/20 volume of Stop Solution. Extracts were washed twice with cold PBS. Sonication was performed with Bioruptor Plus. The following settings were used to get chromatin fragments 200 to 500 base pairs (bp) long: 35 min of sonication, strength set at high, with 30-s on/off intervals. Sheared chromatin (30 μg) was incubated overnight at 4°C with 4 μg of rabbit polyclonal antibody against p65 (SC-372, Santa Cruz Biotechnology) or control IgG (SC-2027, Santa
Cruz Biotechnology). Antibody-bound protein/DNA complexes were then immunoprecipitated with Protein G agarose beads. Finally, the eluted chromatin was subjected to reverse cross-linking, digestion with proteinase K, and DNA purification, according to the instructions of the IT High Sensitivity Kit. Enrichment in the p65 immunoprecipitate was measured by qPCR. Positive control sequences 1 and 2 (NF-κB response genes PTGES2 and IL-10) and negative control sequences 1 and 2 (intergenic regions of chromosomes 14 and 10) were chosen on the basis of public p65 ChIP-seq data (GSM1055811).

Luciferase assay
Luciferase assays were performed with the Dual-Glo Luciferase Assay System (E2920, Promega). Briefly, cells at 70 to 80% confluency were cotransfected with the TET1-LUC and Renilla-LUC vectors at ratio 1:5. Twenty-four hours after transfection, the cells were lysed, and the firefly signal was measured using a luminometer (Promega Turner Designs Luminometer TD-20/20). TET1-LUC reporter activity was then normalized to Renilla-LUC activity.

5hmC-seq and bioinformatics analyses
*TET1* expression was measured by RT-qPCR in four pairs of matched tumor (BLBC) and normal breast tissues. Tumors (and their corresponding normal tissues) were divided into *TET1*-high (*n* = 2 pairs) and *TET1*-low (*n* = 2 pairs) on the basis of *TET1* expression.

The genome-wide distribution of 5hmC was determined by hydroxymethylated DNA fragment affinity purification (hMe-seal), as previously described (58). DNA was fragmented using a Bioruptor sonicator (Diagenode) to obtain fragments averaging 300 bp in size, and enrichment in hydroxymethylated fragments was performed from 500 ng of DNA with the hydroxymethyl collector (Active Motif) according to the manufacturer’s protocol. Library preparation was done with the TruSeq ChiP Sample Prep Kit (Illumina) according to the manufacturer’s instructions.

The Bowtie2 software was used to map sequencing reads to the human genome [NCBI Build 37/University of California, Santa Cruz (UCSC) hg19]. Raw data are available in the GEO database (GSE101445). After removing duplicate reads (that is, reads mapping to the same location) with Picard Tools software, read density was computed by counting the reads in nonoverlapping 2-kb windows tiling the whole genome, thanks to the featureCounts software. Reads mapping to multiple locations in the reference genome or overlapping two windows were fractionated among the associated windows. Windows presenting a significantly different 5hmC level between tumor samples and adjacent normal tissues were identified with edgeR software (log2 fold change > 3 and false discovery rate < 0.05). For each patient, a tumor sample and an adjacent normal tissue were available, and paired analysis was applied.

For annotation purposes, enhancers were obtained from the EnhancerAtlas prediction database (www.enhanceratlas.org/; downloaded in June 2016), gene bodies were defined as regions from TSS to transcription termination site of RefSeq genes (downloaded from UCSC on 25 November 2015), and promoters were defined as ranging from −2 kb to TSS. CGI positions were obtained from UCSC (downloaded on 2 March 2017), and shores were defined as regions surrounding CGIs by up to 2 kb. All windows were annotated by comparing the window center genomic position with the positions of the aforementioned features with bedtools annotate. For visualization, sequencing tracks were uploaded as WIG files onto the UCSC genome browser. Read densities shown in the different figures were normalized to the total number of reads and expressed as log2 CPM.

**Infinium Human Methylation 450K**
Genomic DNA (300 to 800 ng) was converted with sodium bisulfite using the Zymo EZ DNA Methylation Kit (Zymo Research). Methylation assays were performed with 4 ml of converted DNA at 50 ng/ml, according to the manufacturer’s protocol. Infinium Human Methylation 450K raw data were submitted to the GEO database (GSE101445).

Raw Infinium data were filtered by removing low-quality data using a detection *P* value threshold of 0.05. Cross-reactive and single-nucleotide polymorphism–containing probes were filtered out using the extended annotation provided by Price et al. (81). *B* values were computed with the formula: *b* = *M*/(*U + M*), where *M* and *U* are the raw “methylated” and “unmethylated” signals, respectively. The *b* values were corrected for type I and type II bias by peak-based correction. Finally, the delta-*b* value was computed as the mean of the absolute difference between the tumor *b* value and the *b* value of the adjacent normal tissue.

**Statistical analyses**
Unless otherwise indicated, all experiments included technical replicates and were repeated at least three independent times. Data and graphs are presented as averages ± SDs. Data were compared by means of two-tailed *t* tests. When more than two groups were compared, one-way ANOVA analyses were performed. The statistical significance criterion was *P* ≤ 0.05. *P* ≤ 0.05, **P* ≤ 0.01, and ***P* ≤ 0.001.

**SUPPLEMENTARY MATERIALS**
Supplementary material for this article is available at http://advances.sciencemag.org/cgi/content/full/4/6/eaap7309/DC1

fig. S1. Characterization of BC samples used in genome-wide analyses.

fig. S2. *TET1* and immune markers in non-BLBC BC subtypes.

fig. S3. *TET1* expression and immunity in BLBC.

fig. S4. Non-BLBC subtypes do not show a strong correlation between *TET1* expression and immunity.

fig. S5. Immune pathways modulate *TET1* expression in BC.

fig. S6. Activation of NF-κB in BC cells.

fig. S7. NF-κB and *TET1* in additional BC cell lines.

fig. S8. Immunity and *TET2* and *TET3* in BLBC.

fig. S9. *TET1* promoter and streptavidin–agarose pulldown probes

fig. S10. *TET1* and immunity in thyroid, melanoma, and lung cancers.

fig. S11. *TET1* and immunity in additional cancer types.

fig. S12. *TET1* expression and NF-κB in additional cancer types.

table S1. List of dhmRs in BLBC (Excel file).

table S2. Genes negatively correlating with *TET1* in BLBC (TCGA)—Top 5 of gene ontology categories.

table S3. Genes positively correlating with *TET1* in BLBC (TCGA)—Top 5 of gene ontology categories.

table S4. List of primers.

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Immunity drives \textit{TET1} regulation in cancer through NF-\kappa B

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