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TET1 reprograms the epithelial ovarian cancer epigenome and reveals casein kinase 2α as a therapeutic target

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Abstract

Ten-eleven translocation methylcytosine dioxygenase-1, TET1, takes part in active DNA demethylation. However, our understanding of DNA demethylation in cancer biology and its clinical significance remain limited. This study showed that TET1 expression correlated with poor

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Author contributions statement

LYC and HCL initiated the project and designed the study LYC, TSH, RCW, YSR, PHS, YCW, and JLC performed the experiments LYC and RLH analyzed the data and performed the bioinformatics analysis PSY performed the MethylCap-Seq analysis TKC, YCW, MWC, IMS, and HCL supplied administrative, technical, and material support LYC, IMS, and HCL wrote the manuscript.

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survival in advanced-stage epithelial ovarian carcinoma (EOC), and with cell migration, anchorage-independent growth, cancer stemness, and tumorigenicity. In particular, TET1 was highly expressed in serous tubal intraepithelial carcinoma (STIC), a currently accepted type II EOC precursor, and inversely correlated with *TP53* mutations. Moreover, TET1 could demethylate the epigenome and activate multiple oncogenic pathways, including an immunomodulation network having casein kinase II subunit alpha (CK2α) as a hub. Patients with $TET1^{high}CK2\alpha^{high}$ EOCs had the worst outcomes, and TET1-expressing EOCs were more sensitive to a CK2 inhibitor, both *in vitro* and *in vivo*. Our findings uncover the oncogenic and poor prognostic roles of TET1 in EOC and suggest an unexplored role of epigenetic reprogramming in early ovarian carcinogenesis. Moreover, the immunomodulator CK2α represents a promising new therapeutic target, warranting clinical trials of the tolerable CK2 inhibitor, CX4945, for precision medicine against EOC.

Keywords

casein kinase-2 (CK2); DNA demethylation; epigenetics; epithelial ovarian cancer; ten-eleven translocation-1 (TET1)

Introduction

Epithelial ovarian cancer (EOC) remains the most lethal gynecological cancer, due to its frequent late diagnosis, aggressiveness, and chemoresistance. EOC is divided into two broad categories, type I and type II [1,2]. Type I tumors are genetically relatively stable, typically present at a low stage, and exhibit distinct, morphologic differences, as compared to type II tumors; they also have distinct molecular genetic profiles such as mutations of *KRAS*, *BRAF*, and *ERBB2*, but not *TP53*[3]. In contrast, type II tumors, mostly high-grade serous ovarian carcinoma (HGSOC), are highly aggressive, with a high frequency of *TP53* mutation. The heterogeneity of this disease further poses a therapeutic challenge, and the clinical application of genetic alterations has proved limited.

Aberrant DNA methylation, including global hypomethylation and gene-specific hypermethylation, is an established hallmark of cancer [4,5]. Although the mechanisms and consequences of DNA methylation have been extensively investigated, understanding of active demethylation, and its possible dysregulation in disease pathologies, remains limited. In 2009, Rao *et al.* found that the ten-eleven translocation protein 1 (TET1), an α-ketoglutarate (α-KG) and Fe^{2+}-dependent dioxygenase, oxidizes 5-methylcytosine (5mC) into 5-hydroxymethylcytosine (5hmC), a prerequisite step in initiation of demethylation [6]. In addition, TET1 can further oxidize 5hmC to 5-formylcytosine (5fC) and 5-carboxylcytosine (5caC) [7], which trigger base excision repair (BER) and activation-induced cytidine deaminase (AID) [8,9], to achieve active DNA demethylation. However, the role of TET1-mediated active demethylation in cancer remains largely unexplored. Limited studies have reported downregulated TET1 and 5hmC in cancers [10–12]. TET1 has also been reported to activate tissue inhibitor of metalloprotease (TIMP) [13], upregulate the tumor suppressors *PTEN*, *SLIT2*, *ZNF382*, and *HOXA9* [14,15] and inhibit the oncogenic WNT pathway [16], thus suppressing tumor growth.

Despite these prior reports implying TET1 as a potential tumor suppressor, TET1 has also been shown to facilitate induced pluripotency in terminally differentiated adult tissue cells, in response to the reprogramming transcription factor NANOG [17,18]. Such a role in the dedifferentiation of normal cells allows inference of similar, but aberrant, dedifferentiation roles that might facilitate the generation of cancer stem cells. The oncogenic role of TET1 remains rarely reported. The fusion protein of TET1 and MLL1 (a histone H3 Lys4 (H3K4) methyltransferase), in leukemia [19] is expressed, suggesting the possible oncogenic role of TET1. Recently, in a triple-negative breast cancer model, TET1 was found to activate multiple oncogenic pathways by DNA demethylation [20].

Here, we report that TET1 promotes malignant phenotypes and confers poor prognosis in EOC. Moreover, we found that casein kinase II subunit alpha (CK2α), a major signaling serine/threonine kinase, is a key signaling node in a TET1 regulatory network and that therapeutically targeting this enzyme may improve personalized therapy in epithelial ovarian cancer.

Materials and methods

Cell lines and cell culture studies

All cell lines were obtained from the American Type Cell Collection (ATCC, Manassas, VA, USA), and further underwent identity verification by DNA profiling of short tandem repeat (STR) sequences by MISSION BIOTECH (Taipei, Taiwan). For up-/downregulation of *TET1* expression, 3 μg pCMV6/TET1 (Origene, Rockville, MD, USA), pLKO.1/shTET1 (National RNAi Core Facility, Academia Sinica, Taipei, Taiwan), or empty plasmid were transfected using lipofectamine 2000 (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA), following manufacturer's instructions. Cells were selected and cultivated with fresh culture medium containing 400 μg/ml Geneticin®, or 2 μg/ml puromycin. The shRNA target sequences were CCTAAGGTTAAGTCGCCCTCG (shControl), CCCAGAAGATTTAGAATTGAT (shTET1-1), and CCTCCAGTCTTAATAAGGTTA (shTET1-2) obtained from the National RNAi Core Facility (Academia Sinica).

Patients and clinical samples

All human subject studies were approved by contributing sites' Institutional Review Board. For ovarian cancer tissue arrays, the acquisition of tissue samples was collected following patients' informed consent [21]. For the eight formalin-fixed, paraffin-embedded samples concurrently containing NFTE STIC, and HGSOC samples, were retrieved from the Ovarian Cancer Tissue Bank of the Johns Hopkins University School of Medicine (Baltimore, MD, USA).

Immunohistochemistry

For immunohistochemical staining, antigen retrieval was performed by steaming the sections in citrate buffer for 20 min. After incubation with the primary antibodies at room temperature for 2 h, a positive reaction in tissue sections was detected by the EnVision™ +System (DAKO, Carpinteria, CA, USA) and developed with DAB. The sources and dilution for each antibody were: TET1 (GT1462; 1:200; GeneTex, Irvine, CA, USA), 5-

hydroxymethylcytosine (39769; 1:1000; Active Motif, Carlsbad, CA, USA), p53 (760–2542; 1:1; Ventana, Basel, Switzerland), Ki-67 (790-4286; 1:1, Ventana), and LAMC1 (HPA001909; 1:200; Sigma-Aldrich, Merck KGaA, Darmstadt, Germany). The percentage and intensity of immunoreactive were determined, and a formula calculated score as (percentage of area) × (intensity level).

In vivo studies

Athymic nude mice (BALB/cByJNarl), were purchased at four weeks of age from the National Laboratory Animal Center (Taipei, Taiwan). All experiments were conducted in accord with a license from the Animal Experimentation Ethics Committee of the National Defense Medical Center.

For *TET1* tumorigenicity studies ($n = 4$), 5×10^5 cells were resuspended in 50 μl PBS and 50 μl Matrigel Matrix (BD Biosciences, Bedford, MA, USA), and injected subcutaneously into the right and left (pCMV6/TET1 versus empty vector-expressing cells, respectively) flanks of each mouse. Tumors were harvested at the end of the experiment, and all mice were euthanized.

For *in vivo* efficacy studies ($n = 20$), xenografts were initiated by subcutaneous injection of 10^6 SKOV3 pCMV6/TET1 or empty vector cells. When tumors reached a designated volume of 150-200 mm^3, mice were randomized and divided into 2 groups. 75 mg/kg of the casein kinase inhibitor, CX4945 (Abcam, Cambridge, UK), was administered by oral gavage daily for 21 d. Tumor volumes and body weights were measured twice weekly. The length and width of the tumor were measured with calipers and the tumor volume calculated as (length × $width^2$)/2.

Network analysis

A Cytoscape plugin (https://cytoscape.org/) that enables visualization and interpretation of GSEA results was used to analyze the network of 2218 genes identified as demethylated and upregulated after *TET1* overexpression. These overlapping gene sets (*P* value <0.001, FDR *q*-values <0.05, overlap coefficient <0.5) were used to organize a visualize enrichment gene sets. Nodes represent enriched gene sets, which were grouped and annotated by their similarity, according to their related gene sets.

Kaplan–Meier (KM) plotter online survival analysis

The KM Plotter (http://kmplot.com/analysis/index.php?p=service) database was used to evaluate mRNA levels of *TET1* and *CSNK2A1* (probe IDs, 228906_at and 212072_s_at, respectively) in ovarian cancer tumor datasets (2017 version), and expression levels were then correlated to patient outcomes. For PFS analysis, patient's recurrence date less than eight-month was excluded; all results were used for auto select best cutoff and exclude biased arrays.

Statistical analysis

Student's *t*-test was utilized to determine statistical significance unless otherwise specified. *P* values ≤0.05 were considered statistically significant.

Supplementary material, Supplementary materials and methods provides details of: *In vitro* functional studies, Chromatin immunoprecipitation assays, Immunofluorescence, MethylCap-Seq, and RNA Sequencing.

Results

Overexpression of TET1 and 5hmC in epithelial ovarian cancer, and their association with poor outcomes

Based on reports of TET1 overexpression in embryonic stem cells and its participation in self-renewal [17,18], we hypothesized that this mediator of DNA demethylation might contribute to EOC "stemness." We first investigated TET1 and 5hmC levels in tissue microarrays containing 89 EOC tissues, and 12 benign tumors, demonstrating strong correlation in expression levels between TET1 and 5hmC (Figure 1A, supplementary material, Figure S1A). Moreover, TET1 immuno-intensity significantly correlated with type II EOC pathogenesis, while 5hmC was significantly higher in advance-stage, serous type, and type II EOC (supplementary material, Figure S1B, Table S1). In line with this finding, serous type EOC expressed the highest TET1 among different EOC types using the CSIOVDB database [23] (supplementary material, Figure S1C). In particular, higher levels TET1 and 5hmC staining were associated with poor progression-free survival (PFS; Figure 1B, C). The median PFS of 25.0 months was not reached (PFS >follow-up time) for patients with $TET1^{low}$ tumors, nor was the median PFS reached after 25.3 months follow-up of patients with $5hmC^{low}$ tumors. To verify these observations, we examined the expression of *TET* family genes, using a GEO dataset (GSE18520) [24]. We found that patients with advanced-stage EOC expressed higher mRNA levels of *TET1*, but not *TET2* or *TET3*, as compared to normal ovarian surface epithelium brushings (Figure 1D). Further, patients with high *TET1* mRNA had poorer survival outcomes (Figure 1E), with median PFS and overall survival (OS) values shortened by 5.0 and 14.5 months, respectively (median PFS, 22.0 versus 17.0 months; median OS, 52.0 versus 37.5 months). These results support our hypothesis that TET1 may promote oncogenesis in epithelial ovarian cancer.

TET1 increases epithelial ovarian cancer malignant phenotypes *in vitro* and *in vivo*

To determine the biological effects of TET1 on ovarian tumorigenesis, we generated *TET1* gain- and loss-of-function EOC cell lines. Full-length sequences for TET1 or shTET1 were transfected into SKOV3/HeyC2 (low endogenous *TET1* expression) or CP70 (high endogenous *TET1* expression), respectively, and then we assessed correlation of *TET1* expression and 5hmC levels (supplementary material, Figure S1D–H).

To determine whether TET1 causally contributes to ovarian carcinogenesis, we assessed the effects of TET1 on malignant phenotypes. As shown in Figure 2A, B, *TET1* expression increased cell migration by 27%, and colony formation by 3.9- to 6.3-fold. Inhibition of TET1 decreased colony formation by 0.4- to 0.5-fold (Figure 2C). Analogously, knockdown of *TET1* sensitized EOCs to the routinely used chemotherapeutics cisplatin and taxol, in a dose-dependent manner (Figure 2D, supplementary material, Figure S1F). To support a role for TET1 in chemoresistance, we assessed *TET1* expression in platinum-resistant EOC cell lines and observed its overexpression (Figure 2E). Tumor xenograft experiments further

demonstrated that activation of TET1 promoted tumorigenicity *in vivo* (Figure 2F). Collectively, these results indicate that TET1 overexpression promotes epithelial ovarian cancer malignant phenotypes.

TET1 enhances stemness in epithelial ovarian cancer

TET1 can activate pluripotency-related genes, maintain self-renewal in embryonic stem cells [25], facilitate the induction efficiency of induced-pluripotent stem cells (iPSCs), and replace Yamanaka factors to generate iPSCs [7,25,26]. However, the role of TET1 in cancer stemness remains poorly understood. Since our above-described findings show that TET1 expression promotes tumor growth and correlates with poor survival in epithelial ovarian cancer, it is likely that TET1 induces cancer stem-like features. We analyzed expression patterns of Yamanaka factors and the most widely described stem or ovarian cancer stem cell (OCSC) markers. As a result, TET1 activated Yamanaka factors, as well as a panel of OCSC markers (Figure 3A–C, supplementary material, Figure S2). Moreover, spheroid formation, another phenotype of stem cells [27], was also increased with *TET1* overexpression and decreased by *TET1* knockdown (Figure 3D).

We then assessed TET1 expression in OCSCs, and their differentiated progenies, in our previously established OCSC model [28,29]. Our data showed that *TET1* expression levels were increased in OCSCs but decreased in OCSC progeny cells (Figure 3E,F). Moreover, seven out of nine patient-derived OCSCs expressed higher levels of TET1 than their parental tumor cells (Figure 3G). These results suggest that TET1 “reprograms” epithelial ovarian cancer cells to a relatively undifferentiated CSC-like state.

Upregulation of TET1 in Type II epithelial ovarian cancer precursors

A new paradigm has been proposed that many high-grade serous ovarian carcinoma (HGSOC) arises from the normal Fallopian tubal epithelium (NFTE) through a precursor stage when highly atypical tubal epithelium was harboring *TP53* mutations with increased proliferation [30]. These precursor lesions are known as serous tubal intraepithelial carcinomas (STICs) [31,32]. Analogously, we observed that TET1 expression correlated with type II pathogenesis, and rarely expressed in immortalized human Fallopian tube fimbrial epithelial cells, FE25 [33] (supplementary material, Figure S1D, Table S1). In particular, we were interested in whether TET1 upregulation may be an early event in HGSOC development. To that end, we investigated TET1 expression in paired normal Fallopian tubes, STICs, and invasive HGSOC, after laser captured micro-dissection from six patients’ tissues, following gene expression array, using GEO dataset (GSE69429) [34]. We found that STICs displayed higher TET1 expression, compared to NFTEs (Figure 4A). Moreover, analysis of RNA-seq data [35] demonstrated higher transcript expression of TET1 in HGSOCs than in NFTE (Figure 4B). Likewise, immunohistochemistry showed undetectable to very low TET1 immunoreactivity in NFTE, but TET1 upregulation in HGSOCs (Figure 4C–F). Cumulatively, these results showed that TET1 expression increases sequentially, from NFTE to STIC, to HGSOC.

To further explore the role of TET1 in early HGSOC development, we investigated the association of TET1 with other STIC markers TP53, Ki-67, and LAMC1 [36] using

immunohistochemistry in 8 STICs. We found both LAMC1 and TET1 overexpression in STICs with null and missense *TP53* mutations (Figure 4G–K). Interestingly, TP53 null STICs showed stronger TET1 expression than TP53 missense STICs (supplementary material, Figure S3). This inverse correlation between TET1 expression and *TP53* mutation, in STICs, deserves further investigation.

TET1 reprograms the epigenetic landscape, and activates oncogenic pathways, in epithelial ovarian cancer

To identify the TET1-mediated regulatory network in epithelial ovarian cancer, we performed whole-genome DNA methylation profiling and RNA-seq. Overexpression of *TET1* resulted in global DNA demethylation (supplementary material, Figure S4A). Specifically, we analyzed differentially methylation regions (DMRs) within ±5 kb of transcription start sites (TSS). Demethylated genes were categorized into three groups: group 1, genes demethylated at promoter regions (TSS ±2 kb); groups 2 and 3, genes demethylated at the upstream TSS (−5~−3 kb) and downstream TSS (3~5 kb) in SKOV3 cells, while HeyC2 cells showed general demethylated around TSS (supplementary material, Figure S4B). By combining gene expression profiles (supplementary material, Figure S4C) and DNA methylation, 2218 genes showed ≥2-fold demethylation, together with ≥1.5-fold activation, in both SKOV3 and HeyC2 cells, with 1487 (67%) of these genes previously reported as direct TET1-targets in mouse embryonic stem cells [37] (supplementary material, Figure S4D). To build a comprehensive picture of the biological/pathological role of TET1, we performed gene set enrichment analysis (GSEA) of these demethylated and upregulated genes to identify functional enrichment of gene sets. The resulting enrichment map presented the significant gene sets results by TET1 (Figure 5A). Network analysis highlighted the critical effect of TET1 on morphogenetic processes, immune response, and hormone response. Next, we explored potential epithelial ovarian cancer therapeutic targets within the TET1 signaling network. GSEA identified that the computational TET1 network possessed multiple upregulated oncogenic pathways, including RAS/RAF, ERBB2, VEGF, TGF-β, and EGFR (supplementary material, Figure S5A, Table S2). Subsequently, we validated TET1 effects on EGFR signaling in EOC cell lines, noting that TET1 upregulated AKT, but not P38 MAPK, while also increasing the ratio of phospho-AKT/AKT after EGF exposure (supplementary material, Figure S5B). Therefore, we examined the drug sensitivity targeting MAPK/ERK, PI3K/AKT, and EGFR and were disappointed to find there was no therapeutic effect either by targeting single or multiple oncogenic pathways (supplementary material, Figure S5C).

TET1 upregulates CK2α to sensitize epithelial ovarian cancer cells to a CK2 inhibitor

To further explore novel therapeutic interventions, we analyzed the effect of TET1 overexpression on 174 immunity-related genes, based on our computational predictions. These gene products were further subjected to protein-protein interaction (PPI) analysis, using the InnateDB database [38,39]. Those predictions showed that CSNK2A1 (casein kinase 2 subunit alpha, CK2α) was the largest hub (Figure 5B), containing 42 edges in the PPI network. Upregulation of CK2α, after TET1 overexpression, was experimentally confirmed in cell lines (Figure 6A), and the concordant coexpression of both genes, in human epithelial ovarian cancer tissues, was verified using the TCGA ovarian cancer

database (Figure 6B). ChIP-PCR, using anti-5hmC and anti-TET1 antibodies, demonstrated the direct binding of TET1 to the *CSNK2A1* promoter region as shown in Figure 6C, D.

To determine whether CK2α could be a potential therapeutic target in *TET1*-overexpressing EOC, a CK2 inhibitor, CX4945 [40], was tested. We found that EOC cell lines with high *TET1* expression were sensitive to this drug (Figure 6E). This *in vitro* finding was validated *in vivo*, using a mouse xenograft model to demonstrate that CX4945 inhibited EOC tumor growth, with *TET1*-overexpressing tumors more susceptible to CX4945 (Figure 6F). These results indicate that targeting CK2α may hold promise for further investigation as an anti-tumor therapy.

TET1 as a biomarker for CK2α targeting in epithelial ovarian cancer

We assessed the expression of CK2α in EOC tissues and assessed its clinical relevance. We found that CK2α expression alone was not associated with survival outcome (supplementary material, Figure S6). Therefore, we performed a combined assessment of CK2α and TET1 expression and survival using KM-plotter. Those patients with $TET1^{high}CK2\alpha^{high}$ had the worst survival outcomes (Figure 6G,H and supplementary material, Table S3), with median 5-year PFS durations of 23.6 versus 17.5 months (Hazard ratios (HR) = 1.69), and OS durations of 52.4 versus 36.6 months (HR = 1.62), compared to patients with $TET1^{low}CK2\alpha^{low}$. These data suggest that targeting CK2α in $TET1^{high}CK2\alpha^{high}$ epithelial ovarian cancer patients may provide a companion test for future therapy targeting the TET1-CK2α cancer network.

Discussion

During the preparation of this manuscript, two reports were published suggesting a tumor suppressor role of TET1 in ovarian cancer [41,42]. Li *et al.* used the TET1 catalytic domain instead of a complete *TET1* gene for experiments, which is not able to reflect the 5hmC independent effects of TET1 [43]. The author claimed that *RASSF5* reactivation was through DNA demethylation; however, there was no evidence showing the direct regulation of *RASSF5* by the TET1 domain. The only evidence was the reactivation of *RASSF5* after TET1 domain expression, which may be caused by passive demethylation or other mechanisms. It is inappropriate to link the interaction between CUL4-DDB1 E3 ubiquitin ligase and TET1 in oocytes to ovarian cancer. Logically, overexpression of TET1 in A2780 cells, which have high endogenous TET1, is not appropriate. Technically, knockdown efficiency by siRNA in the same cell line extinguished TET1 expression, which is not common. Also, the length of bisulfite sequences, the big difference in melting temperature of primers (>10 °C), a very low loading of DNA (25–100 ng) for dot bots are all beyond the general principle. In their xenograft investigation, the authors provided original tumor formation in only one mouse. Duan *et al* continued their TET1 story in ovarian cancer despite the inconsistent results between IHC of ovarian cancer tissue microarray and TCGA database. The observed effects on EMT were limited only to SKOV3 but not the other 5 cell lines. This could not exclude the possibility of clonal effects and selection bias. Moreover, the analysis of 5hmC and 5mC on target gene promoters (*SFRP2* and *DKK1* in their Figure 5C) does not appear reasonable to us. Besides, the primers for ChIP PCR experiments for

DKK1 promoter appear incorrect and cannot match to *DKK1* gene sequence. Overall, we are not convinced by those reports. In our study, we have highlighted the importance of TET1 in epithelial ovarian cancer and revealed a previously uncharacterized role of TET1 in type II EOC precursors. In three EOC cell lines, we demonstrated that TET1 was a novel epigenetic modifier and its upregulation reprogrammed the epigenome into an oncogenic state including stem-like properties and tumor-promoting phenotypes. Clinical correlation showed that TET1 upregulation can be detected as early as in STICs, the precursors of many HGSOC, and patients with high TET1-expressing EOCs had poor prognosis. This poor prognostic indication of TET1 was validated in multiple public databases. We also show strong evidence that TET1 directly regulated CK2α by hydroxymethylation-dependent activation, and further revealed targeting CK2α for therapeutic intervention in EOCs as a possibility. Targeting TET1 or TET1-mediated signal pathways may provide a new direction to develop a potential therapeutic strategy for EOCs.

The heterogeneity and uncertain characteristic cell type of EOCs limited our exploration of the role of TET1 in EOCs; however, through numerous publicly available data analysis, functional studies, and molecular characterization, we provided evidence to suggest the importance of TET1 in a subset of EOC. The findings from our study should have several important biological and translational implications for studying EOC. Specifically, we found that targeted therapies, including ERK, AKT, and EGFR inhibitors, failed to inhibit TET1-mediated cancer cell growth. This may due to complicating redundancy of oncogenic pathways that compromise the therapeutic effects of target therapy, which may explain why most clinical trials, using current tyrosine-kinase inhibitors, have failed in EOC [44,45]. Among TET1-dysregulated immune genes, we establish CK2α as its prominent hub connecting to several cancer pathways. CK2α is a constitutively active serine/threonine kinase which is known to be pro-survival and anti-apoptotic [46,47], and to participate in maintaining cancer stemness [47]. In EOC specifically, CK2α works as a key signaling node in an inflammatory cytokine network [48]. Inhibition of CK2α significantly reduced cytokine release and decreased Notch signaling, which is an important pathway in EOC pathogenesis [48–50]. CK2α can be upregulated in ovarian cancer stem-like cells, in association with expression of the transcriptional activator GLI1 [51]. It has been reported that EOC cell lines were sensitive to a CK2α inhibitor, CX4945, only under 3D culture conditions [48]. Interestingly, TET1-mediated activation of CK2α can be observed only in 3D-"spheroids" and not in monolayers. As expected, a CK2α inhibitor was more potent in inhibiting the growth of ovarian cancer stem-like cells ($TET1^{high}$) in 3D culture than cells in monolayers ($TET1^{low}$). This finding is similar to a recent report studying EZH2 inhibitors [52]. The current study provides a new molecular mechanism in which TET1 promotes tumor development through collaboration with CK2α. These data support the benefit of CK2α inhibitor in epithelial ovarian cancer, in the context of TET1 overexpression, justifying clinical trials for such inhibitors.

Recently, a phase Ib trial, using CX4945 in combination with gemcitabine and cisplatin for advanced cholangiocarcinoma patients, has demonstrated no drug-drug interactions, no adverse toxicity, and a disease control rate of 64% (partial response 32% and stable disease 32%) [53]. However, CK2α alone was not prognostic for patients' survival, suggesting that the lack of a suitable biomarker might limit the application of a CK2 inhibitor. Rather, we

showed that inhibition of CK2α much more successfully suppressed EOC growth *in vitro* and *in vivo*, when both CK2α and TET1 were both highly expressed. Furthermore, in EOC patients, TET1^{high}CK2α^{high} tumors conferred the worst survival outcomes, and our preclinical and *in silico* studies strongly suggest this group might benefit most from a CK2 inhibitor. These findings strongly suggest a molecular synergism between TET1 and CK2α pathways.

Besides CK2α inhibition, targeting TET1 itself may also appear promising. Unfortunately, TET1-specific inhibitors are yet to be developed. Since TET1 is an α-ketoglutarate (α-KG)-dependent dioxygenase, inhibition of TET1 enzymatic activity would be possible via α-KG. Isocitrate dehydrogenases 1 and 2 (IDH½) are key metabolic enzymes that convert isocitrate to α-KG. While IDH½ mutations produce 2-hydroxyglutamate (2-HG), which inactivates TET1 by competitively inhibiting α-KG [54]. However, IDH½ is not mutated in epithelial ovarian cancer according to TCGA database (supplementary material, Figure S7). Recent studies suggest that 2-HG-mediated epigenetic regulation occurs in a dose-dependent manner, i.e., correlating with 2-HG synthesis rate [55,56]. A significant inhibitory effect on endogenous 2-HG, *in vivo*, would require over 100-fold excess 2-HG [54]. Even at such supraphysiologic levels, 2-HG may not even be a significant inhibitor of TET1 in IDH½ wild-type cells [54]. Alternatively, direct inhibition of IDH½ catalytic activity by oxalomalate, a competitive inhibitor of IDH½, reduced α-KG production and downregulated the activity of α-KG-dependent histone demethylases [54]. However, oxalomalate treatment alone did not inhibit epithelial ovarian cancer cell survival [57]. Thus, whether oxalomalate might be more effective in treating TET1-overexpressing EOC patients, needs further investigation. Therapeutic inhibition of TET1, in epithelial ovarian cancer, merits further exploration.

Accumulated evidence has shown that many HGSOCs originate in STIC of the Fallopian tube [58,59]. Most precisely, *TP53* mutation is the earliest molecular genetic event for HGSOC development [60], this genetic mutation may be related to epigenetic reprogramming. Experimentally, TP53 knockdown significantly correlated with TET1 upregulation, but not TET2/3, in human fibroblasts [61]. Analogously, TET1 expression in STIC inversely correlated with TP53 immunoreactivity. It appears that TET1 participates, at least in part, in high-grade ovarian carcinogenesis. Nevertheless, the mechanistic understanding of this phenomenon is far from complete.

Taken together, we propose a model for the role of TET1 in the pathogenesis of epithelial ovarian cancer (supplementary material, Figure S8). TET1 pathway is initially activated by the various carcinogenic events such as oxygen radicals and other DNA damage agents in the Fallopian tube epithelium. TET1 collaborates with other molecular alterations including *TP53* mutations in reprogramming the epigenome, promoting CSC-like state, upregulating multiple RTK signaling pathways, and a group of immune response genes, all of which may accelerate the tumor progression. Future studies should be undertaken to elucidate the mechanisms of how TET1 and CK2 together promote disease aggressiveness and how we can harness both pathways to develop a new therapy. Nevertheless, our study demonstrates the oncogenic role of TET1 in epithelial ovarian cancer and illuminates the participation of TET1 in cancer biology besides those known epigenetic modifiers including HDAC and

EZH2. TET1 could be a valuable biomarker for targeting CK2α and is itself a potential a drug target.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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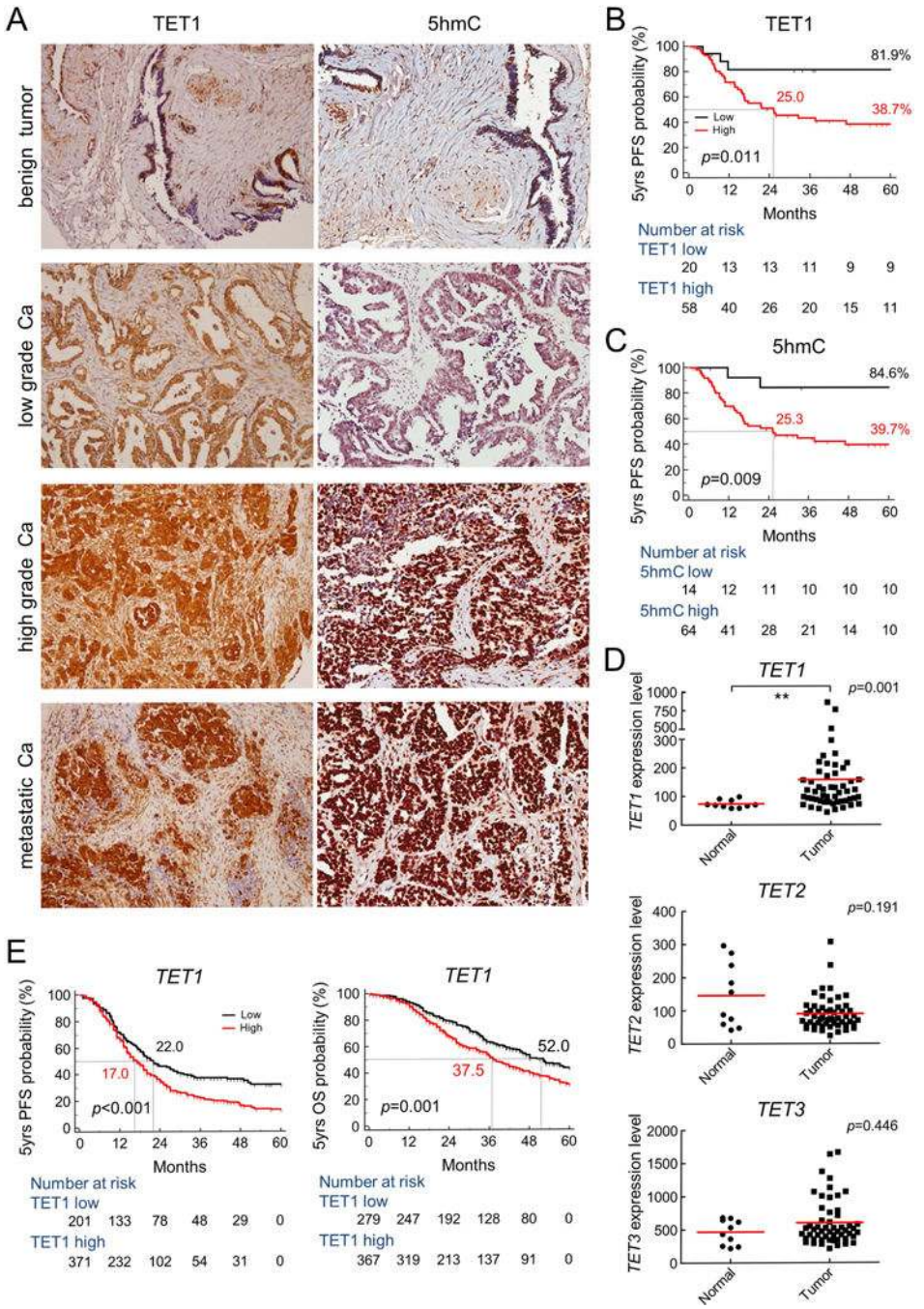

Figure 1. TET1 and 5hmC are upregulated in human epithelial ovarian cancer tissues and correlate with poor patient survival.

(A) IHC detection of TET1 and 5hmC in ovarian tissue microarray. Original magnification, ×200. (B, C) Correlation of five-year PFS with TET1 (B) and 5hmC (C) expression levels in 78 EOC patient tumors, according to Kaplan–Meier analysis. (D) Relative *TET1*, *TET2*, *TET3* mRNA expression in normal tissue and tumor specimens. Normal = 10, Tumor = 53; *p*-values, by Mann–Whitney test. (E) Kaplan–Meier analysis of 5-year PFS (left panel, n = 470) and 5-year OS (right panel, n = 571), based on tumor *TET1* expression, in EOC patients, using the KM plotter database [62]. Log-rank tests are shown.

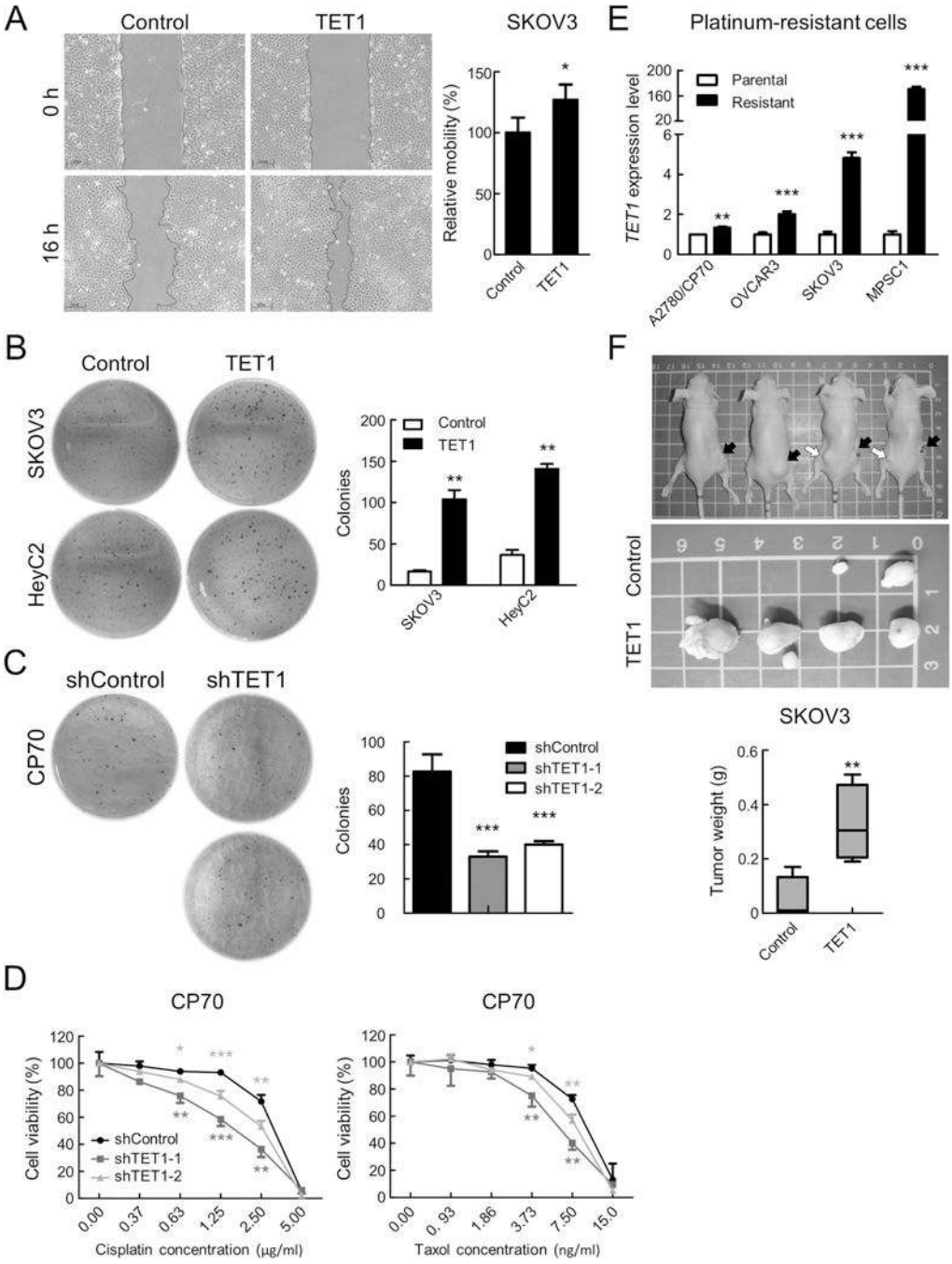

Figure 2. TET1 promotes malignant phenotypes in epithelial ovarian cancer cells.

(A) Migration rates of control and TET1-overexpressing SKOV3 EOCs, as measured by wound-healing assays. (B,C) Anchorage-independent growth was assessed by soft-agar assay in TET1 overexpressing (B) and TET1 knockdown (C) cells. (D) Effects of *TET1* knockdown on chemosensitivity of CP70 EOCs to cisplatin (left) and taxol (right), as determined by MTS cytotoxicity assays. (E) Increased *TET1* expression levels in the platinum-resistant EOCs. (F) Effects of TET1 on *in vivo* tumor growth were determined by a xenograft model. 5×10^5 SKOV3 EOCs stably transfected with *TET1* (black arrow) or

empty vector (white arrow) were injected subcutaneously into nude mice ($n = 4$). The weight of tumors of *TET1*-overexpressing was significantly increased compared to the vector controls (box plots). Data are represented as means ± SD. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

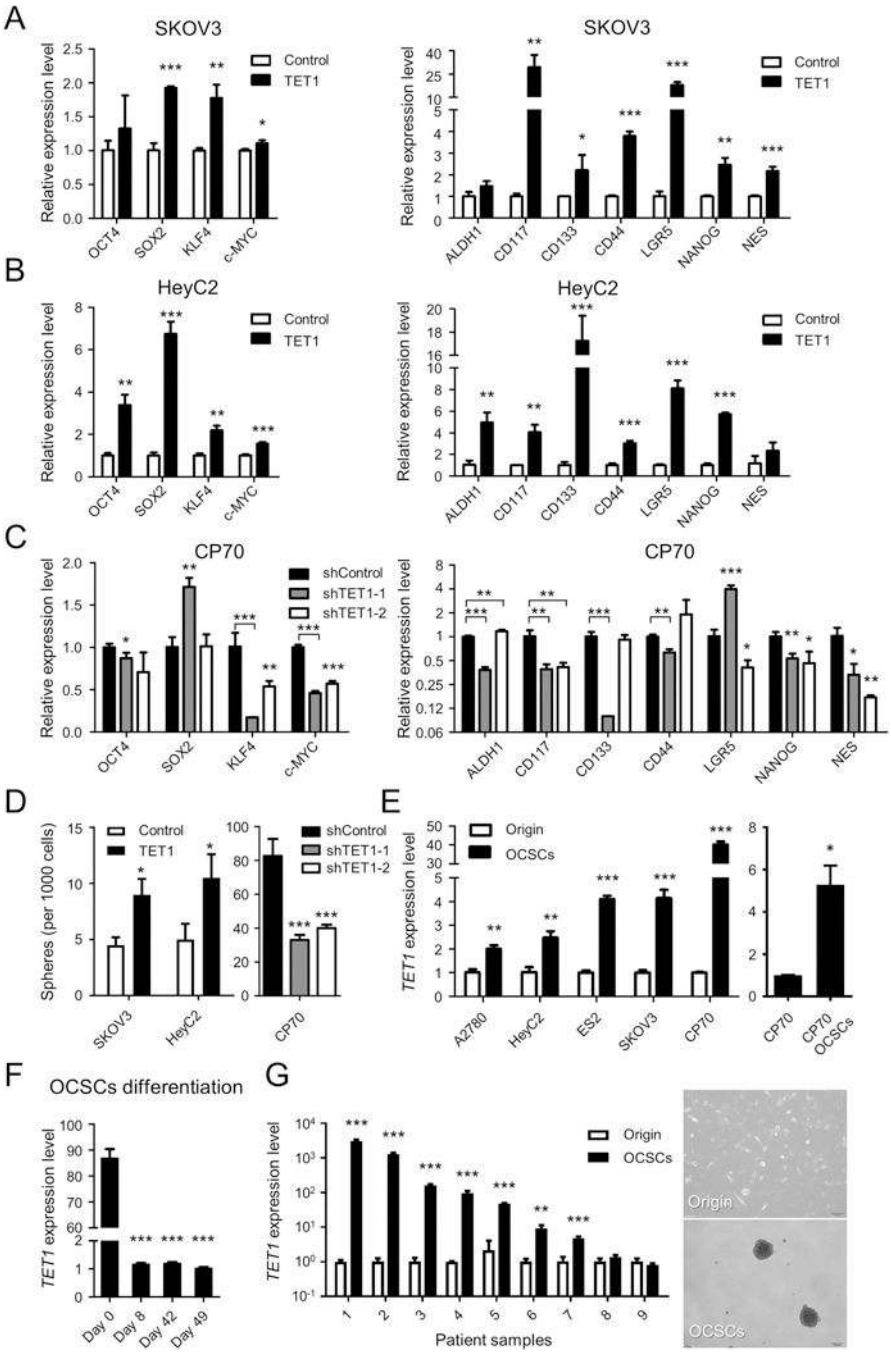

Figure 3. TET1 promotes epithelial ovarian cancer stem identity.

(A-C) Expression of Yamanaka factors and stem/cancer stem markers were assessed in *TET1*-overexpressing SKOV3 (A) and HeyC2 (B), as well as in TET1-knocked-down CP70 (C) EOCs. (D) TET1 enhances spheroid-forming abilities of ovarian cancer cells. (E) *TET1* expression levels were increased in ovarian cancer stem cells (OCSCs) using two enrichment methods. Relative *TET1* mRNA expression in parental cancer cells and OCSCs were examined by RT-qPCR. (F) Expression of *TET1* is markedly decreased in OCSC-differentiated progeny cells. (G) EOC patient-derived stem cells express high levels of

TET1. Representative pictures of stem-like spheres generated from patient 1 are shown. Original magnification, ×400.

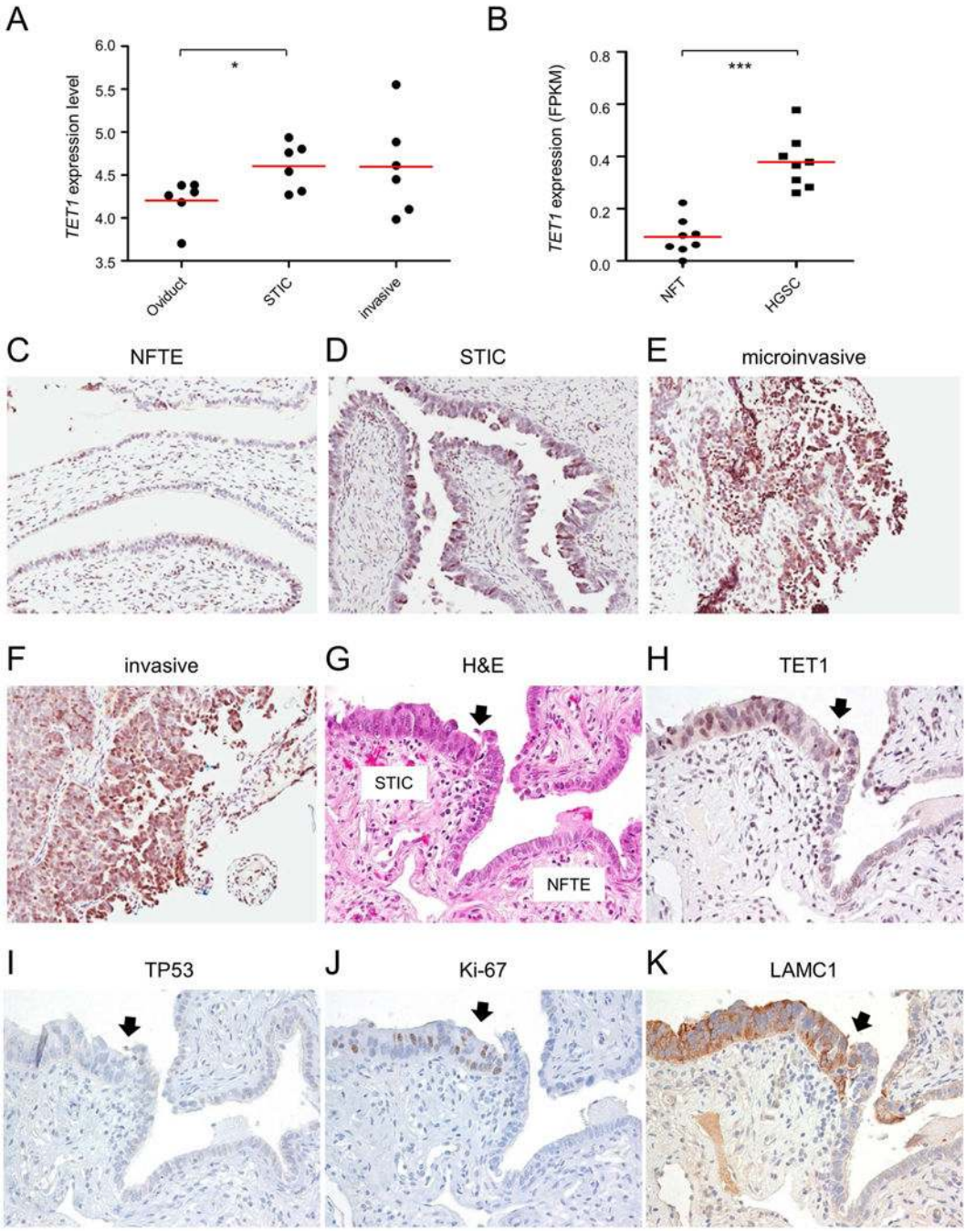

Figure 4. TET1 Upregulation is an early event in Type II epithelial ovarian cancer development.

(A) Increased *TET1* expression in serous tubal intraepithelial carcinoma (STIC). Relative *TET1* mRNA expression in six paired normal oviducts, STICs, and invasive serous cancers. $P < 0.05$ by Mann–Whitney test. (B) Increased *TET1* expression in HGSOC. *TET1* transcript abundances were determined by genome mapping of RNA-seq data obtained from human NFTEs ($n = 8$) and HGSOCs ($n = 8$). (C-F) Representative histologies of TET1 IHC staining in individual cases of normal Fallopian tube (C), STIC (D), microinvasive serous ovarian carcinoma (E), and invasive serous carcinoma (F). Photomicrograph original

magnification 200x. (G-K) TET1 colocalizes with LAMC1 in STICs. Immunoreactivity of H&E (G), TET1 (H), TP53 (I), Ki-67 (J), and LAMC1 (K) in a representative fimbrial STIC location. Arrows denote junctions between STICs and NFTEs. Original magnification, ×400. See also supplementary material, Figure S3.

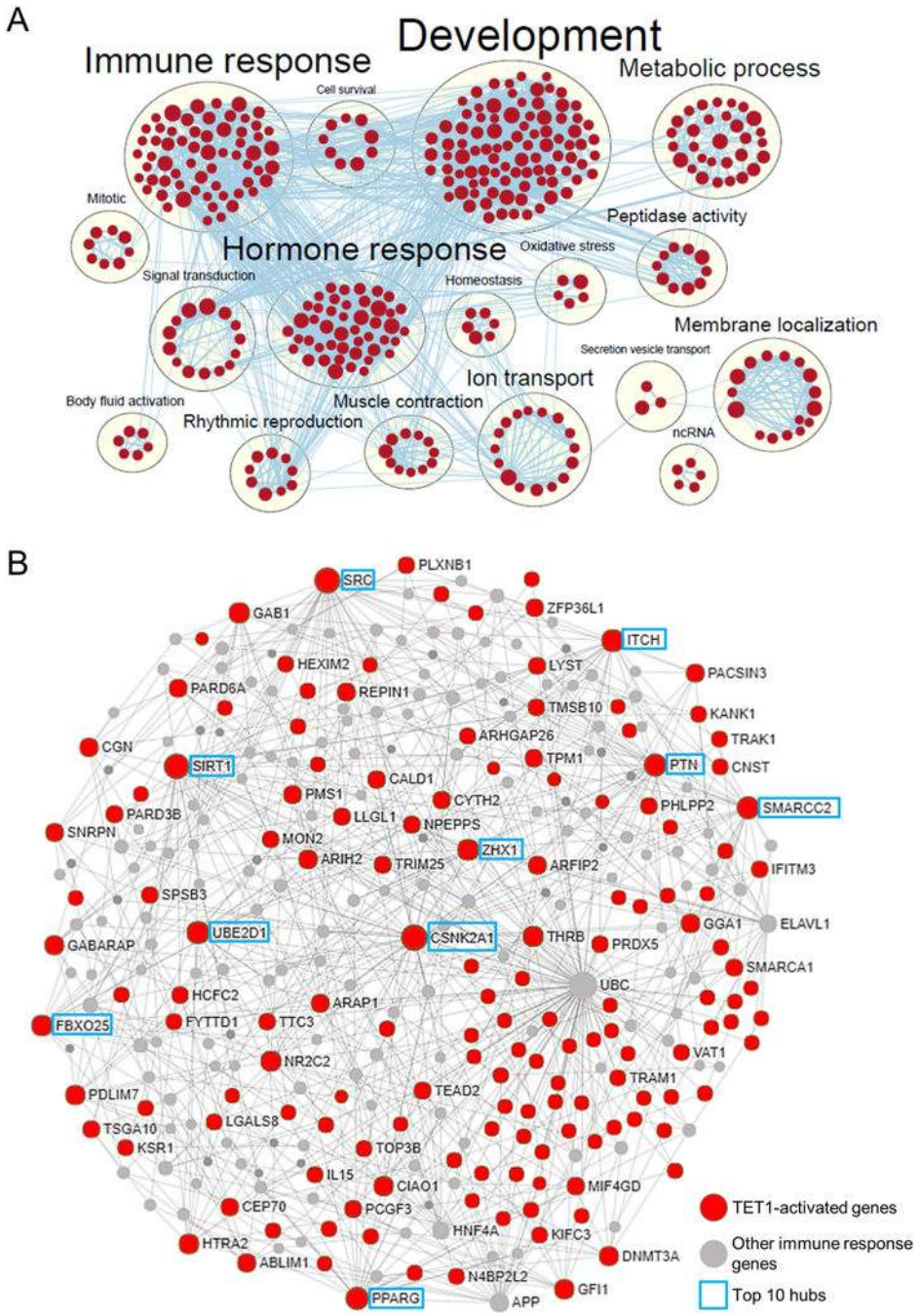

Figure 5. Functional map of global changes in gene expression and dna demethylation, in response to TET1 overexpression.

(A) Enrichment map analysis, following *TET1* overexpression, reveals several distinct gene clusters. Enrichment results from Gene Set Enrichment Analysis (GSEA) were mapped as a network after TET1 overexpression. Nodes represent TET1-activated gene sets (upregulated and demethylated); edges represent mutual overlap. Node size is proportional to the total number of genes in each set, and edge thickness represents the number of overlapping genes between the sets. (B) TET1-mediated activation of an immune response gene network reveals *CSNK2A1* (CK2α) as the largest hub. Red nodes represent TET1-activated genes;

grey nodes represent other immune response genes. Node size is proportional to the number of edges. Light blue boxes represent top 10 hubs.

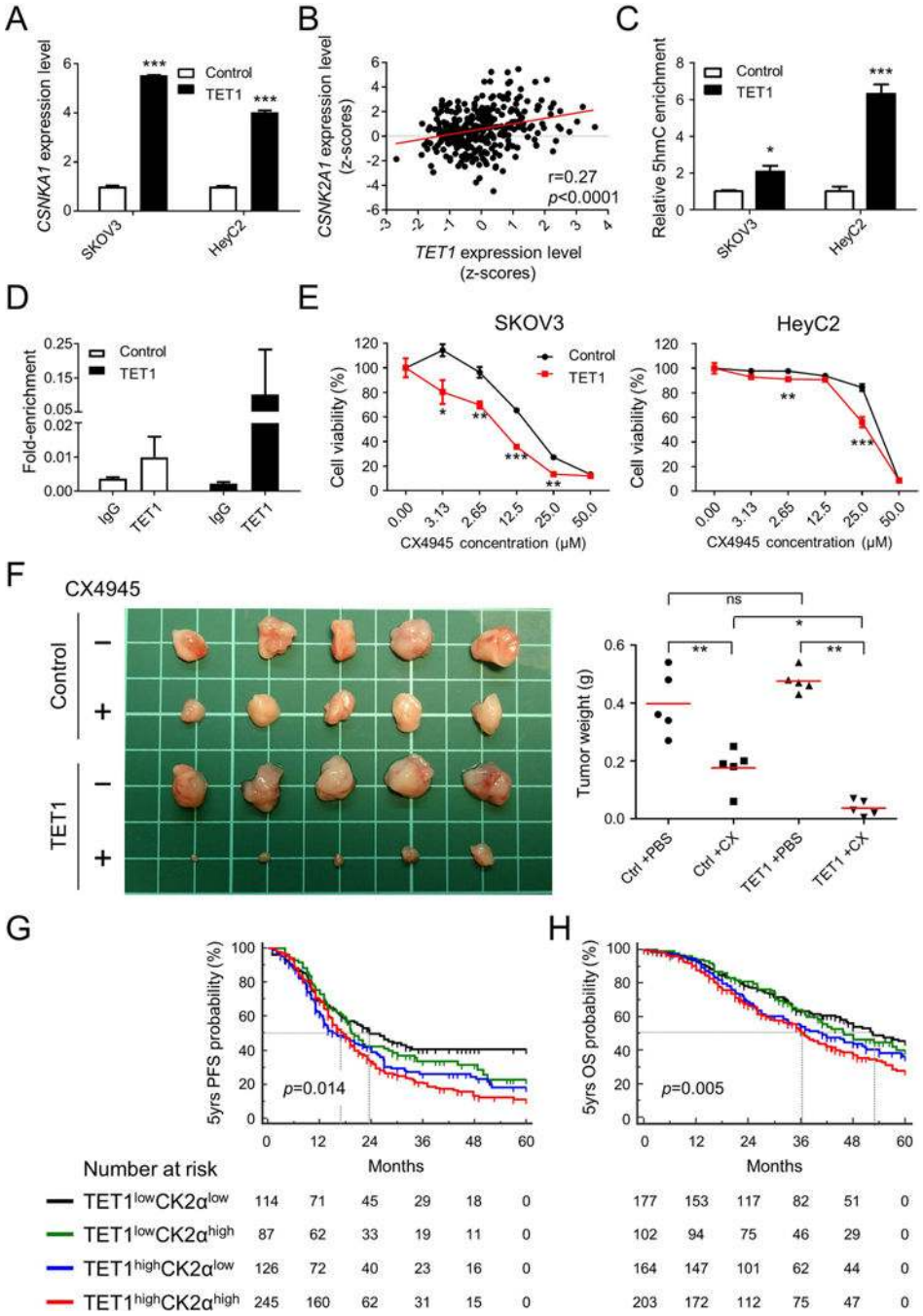

Figure 6. TET1 activation of CK2α by DNA demethylation and enhanced chemosensitivity to a CK2 inhibitor.

(A) RT-qPCR assessed *CSNK2A1* expression levels after TET1 overexpression in EOC cell lines. (B) Concordant coexpression of *TET1* and *CSNK2A1* in EOC. Correlation between *TET1* and *CSNK2A1* mRNA expression levels in EOC patient tumors. (C) Relative 5hmC levels, within the *CSNK2A1* promoter, were measured by β-glycosyl-5-hydroxymethylcytosine (5gmC) ChIP-qPCR. Considerable enrichment of 5hmC levels was detected in both of two *TET1*-overexpressing EOC cell lines. (D) TET1 ChIP-PCR was conducted on TET1-overexpressing SKOV3 EOCs. Experiments were conducted with IgG

or TET1-specific antibody and show enrichment of *CSNK2A1* fragments in TET1-ChIP over IgG controls. (E) The effect of *TET1* overexpression on chemosensitivity to a CK2 inhibitor (CX4945) was determined by MTS cytotoxicity assay. (F) Antitumor efficacy of CX-4945 to TET1-overexpressing SKOV3 EOCs was determined by a xenograft model. Vehicle (PBS) or CX-4945 (75 mg/kg) was administered by oral gavage daily. Tumor weights are expressed as means ± SEM. (G, H) Kaplan–Meier analysis of probabilities of five-year progression-free survival (PFS; G) and overall survival (OS; H) of epithelial ovarian cancer patients, according to their tumors' TET1 and CK2α expression. Patients with $TET1^{high}CK2\alpha^{high}$ coexpression (red lines) had significantly worse outcomes than those patients with $TET1^{low}CK2\alpha^{low}$ (black lines), $TET1^{low}CK2\alpha^{high}$ (green lines), and $TET1^{high}CK2\alpha^{low}$ (blue lines). *p*-values were calculated from log-rank tests for four groups. The hazard ratio (HR) was calculated from the univariate Cox regression model described in supplementary material, Table S3.