ARTICLE

TET2 deficiency sensitizes tumor cells to statins by reducing HMGCS1 expression

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TET2 (ten-eleven-translocation) protein is a Fe(II)- and α-ketoglutarate-dependent dioxygenase that catalyzes DNA demethylation to regulate gene expression. While TET2 gene is frequently mutated in hematological cancer, its enzymatic activity is also compromised in various solid tumors. Whether TET2 deficiency creates vulnerability for cancer cells has not been studied. Here we reported that TET2 deficiency is associated with the change of lipid metabolism processes in acute myeloid leukemia (AML) patient. We demonstrate that statins, the inhibitors of β-Hydroxy β-methylglutaryl-CoA (HMG-CoA) reductase and commonly used cholesterol-lowering medicines, significantly sensitize TET2 deficient tumor cells to apoptosis. TET2 directly regulates the expression of HMG-CoA synthase (HMGCS1) by catalyzing demethylation on its promoter region, and conversely TET2 deficiency leads to significant down-regulation of HMGCS1 expression and the mevalonate pathway. Consistently, overexpression of HMGCS1 in TET2-deficient cells rescues statin-induced apoptosis. We further reveal that decrease of geranylgeranyl diphosphate (GGPP), an intermediate metabolite in the mevalonate pathway, is responsible for statin-induced apoptosis. GGPP shortage abolishes normal membrane localization and function of multiple small GTPases, leading to cell dysfunction. Collectively, our study reveals a vulnerability in TET2 deficient tumor and a potential therapeutic strategy using an already approved safe medicine.

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INTRODUCTION

Reversible DNA methylation is one of the major epigenetic modifications that can activate or repress gene expression. Dysregulated DNA methylation occurs in different types of cancer and is closely associated with tumor initiation, progression and metastasis [1, 2]. The ten-eleven translocation (TET) family of proteins catalyzes the process of DNA demethylation [3]. Among the TET family, TET2 is frequently mutated in ~15% of myeloid cancers, including 24% in acute myeloid leukemia (AML) [4, 5]. Although TET genes are rarely mutated or altered in expression in solid tumors [6], a significant loss of 5hmC has been observed in multiple cancers, such as liver, breast, lung, pancreas, prostate, brain, colorectal, gastric, melanoma and oral cancers, indicating the functional inactivation of TET proteins in solid tumor [7–10]. As TET proteins require Fe(II) as a metal cofactor and α-KG as a co-substrate, pathological accumulation of α-KG analogs, such as 2-HG produced by mutant isocitrate dehydrogenase 1/2 (IDH1/2), fumarate accumulated by mutant fumarate hydratase (FH) or succinate accumulated by mutant succinate dehydrogenase (SDH), can inhibit TET activity and lead to a hypermethylation phenotype [11–13]. Besides these oncometabolites, increased glucose level also reduces TET2 stability through suppressing AMPK-mediated phosphorylation of TET2 [14]. While how metabolites regulate TET2 activity is well-studied, whether TET2 can reciprocally affect metabolism is still not fully understood. It has been reported that TET2 reduces glycolysis by interacting with phosphofructokinase (PFK), therefore suppresses nasopharyngeal carcinoma progression (NCP) [15]. Elucidation of TET2 regulation on tumor metabolism may offer a potential strategy for treatment of cancers with TET2 dysfunction.

Cholesterol is an essential lipid for membrane functions and serves as the precursor for steroid hormones and bile acids. Cholesterol can be either taken into cells by low-density lipoprotein receptor (LDLR) or synthesized de novo from acetyl-coenzyme A (acetyl-CoA) through over 30 steps of reactions [16, 17], which is also known as the mevalonate pathway. Mevalonate is converted from HMG-CoA and further turned into isopentenyl pyrophosphate (IPP) and dimethylallyl pyrophosphate (DMAPP). DMAPP can be first elongated to the C-10 metabolite geranyl pyrophosphate (GPP) and then to the C-15 isoprenoid farnesyl pyrophosphate (FPP) [18]. Interestingly, FPP is not only the precursor of sterol isoprenoids (such as cholesterol and steroid biosynthesis), but also the precursor of non-sterol isoprenoids, (such as dolichol, heme-A, ubiquinone and geranylgeranyl pyrophosphate (GGPP)) [19]. FPP thus serves as a key node for the control of multiple biological processes. For example, FPP and

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GGPP are essential for the post-translational prenylation of many small GTP-binding proteins (GTPases), which functions as a lipid anchor and facilitates protein association with membranes [18]. The membrane localization is important for many small GTPases to regulate cell functions [20].

The statin family contains eight unique compounds that are derived from fungal fermentation (pravastatin, simvastatin, and lovastatin) or chemically synthesized (fluvastatin, atorvastatin, cerivastatin, rosuvastatin and pitavastatin) [21]. As the inhibitor of the first rate-limiting enzyme 3-hydroxy-3-methylglutaryl-CoA reductase (HMGCR) in the mevalonate pathway, statins have been used successfully in the treatment of hypercholesterolemia for the past 30 years. And this family of drugs has consistently demonstrated a positive safety and tolerability profile [22–25].

In this study, we found that TET2 mutation significantly affected the expression of lipid metabolism related genes in AML patients. We further demonstrated that TET2 deficiency attenuated cholesterol synthetic pathway in different types of cancer cells by down-regulating the expression of 3-hydroxy-3-methylglutaryl-CoA synthase 1 (HMGCS1), leading to cell sensitivity to statins treatment. Our findings imply a potential strategy for the treatment of cancer patients with TET2 functional deficiency.

RESULTS

TET2 affects expression of metabolic genes in AML

To elucidate the potential influence of TET2 dysfunction on metabolic homeostasis, we analyzed RNA seq data of AML patients from TCGA database and profiled differential gene expression associated with TET2 mutation. AML patients had a high mutation rate of TET2, which is about 9%. We also examined genes (IDH1, IDH2 and WT1) that are known to function in the same genetic pathway with TET2 in DNA demethylation [11, 26], and found they all exhibited high mutation rate (IDH1: 10%; IDH2: 10%; WT1: 7%) (Supplementary Figure 1A). We divided the 173 patients with available transcriptome data into TET2-WT and TET2-mutant groups, which included 158 and 15 patients, respectively. Metabolism-related genes with a number of 2122 were obtained from Reactome database and 739 differentially-expressed genes were filtered with |log2FC| of 0.58 and p value of 0.05 (Fig. 1A). Sixty-seven genes were intersected between these two gene sets and labeled dark blue in the volcano plot (Fig. 1B). Enrichment analysis was performed with the 67 metabolism-related differentially-expressed genes, which indicated significant enrichment of carbohydrate metabolism and lipid metabolism (including fatty acid metabolism and steroids metabolism) genes (Fig. 1C). To expand the sample size, we also included patients bearing IDH1, IDH2 and WT1 mutations, which are reported to suppress TET2 function in AML [26], into the TET2 mutation group and analyzed the alteration of metabolism-related genes. It turned out that carbohydrate metabolism and lipid metabolism were also predominantly enriched (Supplementary Fig. 1B–D).

TET2 deficiency sensitizes cells to statin-induced apoptosis in multiple cancer cell types

To address the functional significance of TET2 in regulating metabolic gene expression in AML, we knocked out *TET2* in human leukemia K562 cells (Supplementary Figure 1E) and performed RNA-seq with WT and TET2-deficient cells. In consistence with bioinformatic analysis, genes involved in carbohydrate metabolism and lipid metabolism (including fatty acid metabolism, steroids metabolism and phospholipid metabolism) exhibited the most significant changes (Fig. 1D). Inspired by these results, we asked whether the alterations in expression of metabolism enzymes make cells more dependent on specific metabolic pathways. To this end, WT or TET2 knock-out K562 cells were treated with 8 inhibitors targeting different metabolic pathways, including cholesterol synthesis (simvastatin and lovastatin, which inhibit 3-Hydroxy-3-Methylglutaryl Coenzyme A Reductase), fatty acid synthesis (TVB2460 and C75, which inhibit fatty-acid synthase), phospholipid metabolism (EB-3D, which inhibits choline kinase α; FIPI, which inhibits phospholipase D 1/2) and glucose metabolism pathways (oligomycin A, which inhibits oxidative phosphorylation; 2DG, which inhibits glycolysis) (Fig. 1E) [27]. Notably, among these inhibitors, simvastatin and lovastatin exhibited significantly higher potency to inhibit the growth of the TET2 knock-out over the WT cells, suggesting that TET2 deficiency renders cells more dependent on the *de novo* cholesterol synthesis pathway.

Next, we tested whether lovastatin causes cell apoptosis. By staining with annexin V and PI, we observed significant cell death in TET2 knock-out cells under treatment with lovastatin for 48 h (Fig. 2A–B). The half maximal inhibitory concentration (IC_{50}) of lovastatin toward K562 cells was then measured. Compare to the control group, loss of TET2 resulted in a decrease of IC_{50} of lovastatin by about 3.4 fold (Fig. 2C). Consistently, TET2 deficiency also promoted the cleavage of PARP1 and caspase-3, two markers for apoptosis, in response to lovastatin treatment, indicating statins-induced cell death was mediated by caspase-dependent apoptosis (Fig. 2D). To elucidate whether the function of TET2 on lovastatin sensitivity is a common regulation among different cancer cells, we generated TET2 knock-out cells in A549 (lung adenocarcinoma) (Fig. 2E), HCT116 (colon carcinoma) (Fig. 2F) and U2OS (osteosarcoma) cell lines (Fig. 2G). Interestingly, all cell lines with TET2 deficiency were more sensitive to lovastatin treatment than their corresponding TET2 wild type cells. Overall, these results suggest a common function of TET2 in protecting cells from lovastatin-induced cell death.

Loss of TET2 reduces the flux of *de novo* cholesterol synthesis

In mammalian cells, cholesterol can be synthesized *de novo* through the mevalonate pathway started from acetyl-CoA (Fig. 3A). To elucidate the regulation of cholesterol synthesis pathway by TET2, we analyzed the metabolites involved in this pathway in WT and TET2-deficient K562 cells. We firstly measured the relative abundance of IPP, FPP and GPP, three important intermediates downstream of mevalonate, by LC-MS (Fig. 3B). Notably, loss of TET2 significantly reduced the levels of IPP, GPP and FPP. We then performed a comprehensive analysis of other metabolites by lipidomic analysis. Consistently, most intermediates in the mevalonate pathway, such as squalene, lanosterol, T-MAS, dihydro-T-MAS, zymosterol and desmosterol were significantly decreased when TET2 was deleted (Fig. 3C). To our surprise, despite of down-regulation of most intermediate metabolites in the mevalonate pathway, the final product of this pathway, cholesterol, remained unchanged in TET2 knock-out cells (Fig. 3D). It could be reasoned that K562 cells may obtain cholesterol mainly through LDLR-mediated uptake, but not the de novo synthesis pathway. This was supported by the fact that when cells were cultured in lipoprotein-depleted medium, both WT and TET2-deficient cells exhibited similar decrease of cell viability (Fig. 3F). In addition, knocking-down of *LDLR* reduced the viability of WT and TET2-deficient K562 cells to a similar extent (Fig. 3G–H). Heat map was also generated to facilitate the display of the change of metabolites in the cholesterol synthesis pathway (Fig. 3E). Collectively, these results indicate that TET2-deficiency induced statins sensitivity is not due to lack of cholesterol in cells. We speculate that alteration of some intermediates in the mevalonate pathway may be responsible for the cell death caused by statins.

TET2 regulates tumor cell sensitivity to statins by promoting HMGCS1 expression

To unravel the function of TET2 in regulating the mevalonate pathway and statin sensitivity, we analyzed the expression of sterol metabolism-related genes in WT and TET2-deficient K562 cells by RNA-seq. The results showed that loss of TET2 led to

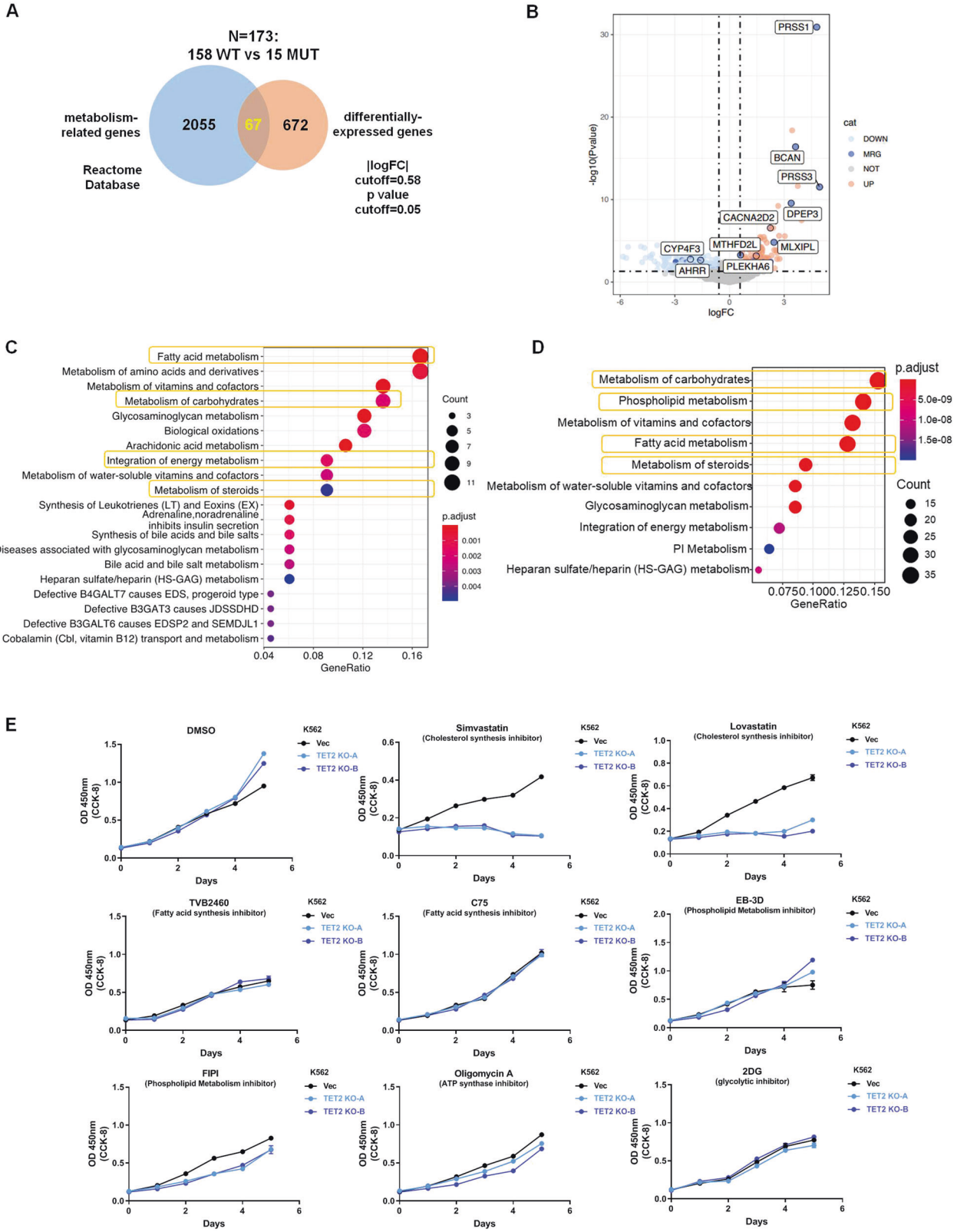
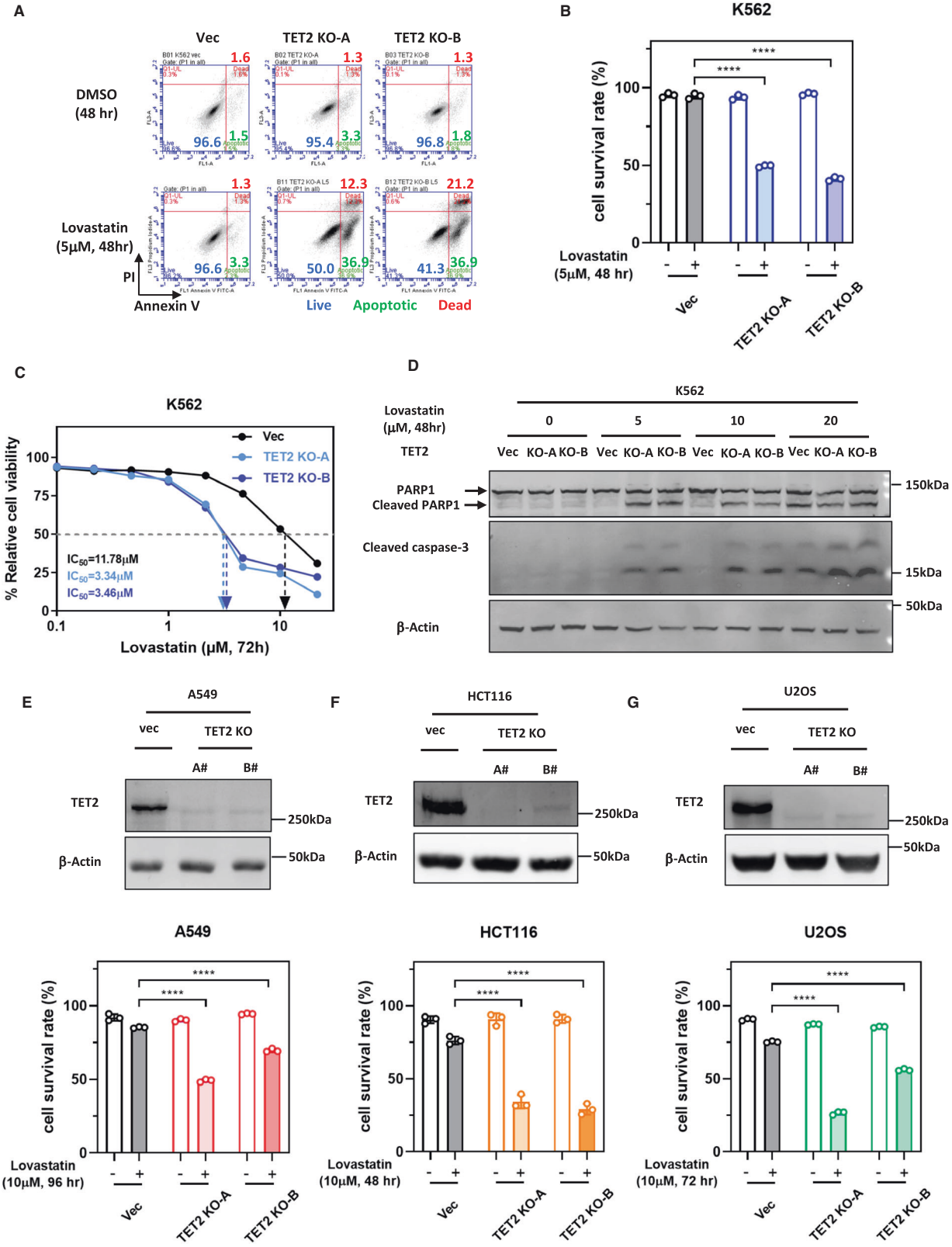


Fig. 1 TET2 aberrations affect lipid and carbohydrate metabolism pathways. A 739 genes are differentially expressed between AML patient groups with or without TET2 mutation, among which 67 genes are involved in metabolism processes. Data is from TCGA database. **B** Volcano plot indicates altered gene expression in the TET2 mutation group compared to the wildtype group. Upregulated and downregulated genes are marked with orange and light blue, respectively. Metabolism-related genes are marked with dark blue. **C**, **D** Genes in metabolic pathways are altered by TET2 deficiency. Enrichment analysis of 67 metabolism-related genes, which are differentially expressed between AML patients with or without TET2 mutation (**C**), and between K562 cells with or without TET2 expression (**D**), showed that pathways associated with lipid metabolism were significantly enriched based on REACTOME database. **E** Effects of chemical inhibitors (cholesterol synthesis inhibitors, fatty acid synthesis inhibitors, phospholipid metabolism inhibitors, ATP synthase inhibitor and glycolytic inhibitor) on cell growth in K562 with or without TET2 expression. Simvastatin: 10 μM; lovastatin: 5 μM; TVB2460: 10 μM; C75: 5 μM; EB-3D: 10 μM; FIPI: 10 μM; oligomycin A: 2 μM; 2DG: 5 mM.



down-regulation of a bunch of genes including HMGCS1 and SQLE, two essential enzymes involved in the mevalonate pathway (Fig. 4A). HMGCS1 catalyzes the synthesis of HMG-CoA from acetyl-CoA, which serves as a substrate for HMGCR, the specific target of statins; SQLE catalyzes the conversion of squalene to squalene-2,3-epoxide (Fig. 3A). We examined the effects of TET2 knock-out on the expression of HMGCS1 and SQLE in A549, HCT116, and U2OS cells, and found that HMGCS1 was commonly down-regulated in all TET2-deficient cells, whereas SQLE expression was reduced in K562 and U2OS cells, but not in A549 and

Fig. 2 TET2 deficiency sensitizes cells to statin-induced apoptosis in multiple cancer cell types. A, **B** TET2 is required to protect cells from lovastatin-induced apoptosis. WT or TET2-deficient K562 cells were treated with lovastatin for 48 h, and viable cell (both PI and annexin V negative) was determined by flow cytometry. **C** TET2 deficiency decreases the IC_{50} of lovastatin toward K562 cells. Cell viability was determined by flow cytometry. **D** TET2 deficiency promotes lovastatin induced cleavage of PARP1 and caspase-3. WT or TET2-deficient K562 cells were treated with 0, 5, 10 or 20 μM lovastatin for 48 h. The protein levels of cleaved PARP1 and caspase-3 were determined by Western blot. **E**–**G** TET2 deficiency sensitizes multiple cancer cell lines to lovastatin-induced apoptosis. A549 cells (**E**), HCT116 cells (**F**), and U2OS cells (**G**) were treated with 10 μM lovastatin for 96 h, 48 h and 72 h, respectively. Cell viability was determined by flow cytometry and TET2 knockout efficiency was determined by Western blot. Asterisks denote statistical significance with one-way ANOVA. ****$p < 0.0001$ for the indicated comparison.

HCT116 cells, upon TET2 knock-out (Fig. 4B). We thus focused on HMGCS1. ChIP-qPCR analysis demonstrated that TET2 directly bound to the promoter region of HMGCS1 (Fig. 4C). Consistent with the TET2 binding data, 5hmC in HMGCS1 promoter was abolished in the TET2 knockout cells (Fig. 4D). As expect, loss of TET2 led to a decrease of global genomic 5hmC level in K562 cells (Supplementary Figure 2). Then we investigated the function of HMGCS1 expression in statin-induced apoptosis. We ectopically expressed HMGCS1 in TET2-deficient K562 cells (Fig. 4E) and HMGCS1 overexpression significantly reduced the lovastatin-induced cell death (Fig. 4F). Likewise, HMGCS1 overexpression also suppressed the cleavage of PARP1 and caspase-3 upon lovastatin treatment (Fig. 4G). Together, our data demonstrate that TET2 deficiency sensitizes tumor cells to statins at least in part due to the reduction of HMGCS1 expression.

MVA or GGPP replenishment blocks the lovastatin-induced cell death in TET2 knock-out cells

Previous results indicate that decrease of some intermediate metabolites in the mevalonate pathway may result in elevated sensitivity to statins. In addition to synthesis of cholesterol, the mevalonate pathway also produces several non-sterol products, including heme, ubiquinone, dolichol, FPP, and GGPP [17]. Ubiquinone acts as an important antioxidant, while FPP and GGPP are essential for the prenylation of small GTPases [18, 28]. We asked if change of these intermediates is involved in statins-induced cell death. We first replenished K562 cells with mevalonate, the product of HMGCR, and observed an effective suppression of lovastatin-induced cell death in TET2-deficient cells (Fig. 5A–B). Moreover, mevalonate replenishment significantly suppressed the cleavage of PARP1 and caspase-3 induced by lovastatin treatment in TET2-deficient cells. These observations suggest that inhibition of HMGCR enzyme activity by lovastatin, rather than other off-target effects, contributes to cell apoptosis (Fig. 5C). Furthermore, our data also supports that TET2 deficiency sensitizes cells to statin due to the downregulation of mevalonate pathway.

As a key node in the mevalonate pathway, FPP initiates the branches of the pathway that generate cholesterol (by producing squalene) and non-sterol isoprenoids (such as GGPP and CoQ_{10} and dolichol). To clarify which branch is important for statin sensitivity of TET2-deficient cells, we replenished cells with squalene, idebenone (a hydrophilic analog of CoQ_{10}) or GGPP. Interestingly, supplementation of GGPP, but not squalene or idebenone, efficiently rescued TET2-deficient cells from lovastatin-induced apoptosis (Fig. 5D–E). Similar effect of GGPP was observed in A549 and U2OS cells (Supplementary Fig. 3A). Consistently, addition of GGPP, but not squalene or idebenone, inhibited cleavage of PARP1 and caspase-3 (Fig. 5F). To validate the findings above, mevalonate and GGPP levels was measured by LC-MS (Supplementary Fig. 3B). Although there's no change in GGPP level in TET2-knockout cells under normal conditions, lovastatin treatment resulted in significant reduction of GGPP level, accompanying with reduced viability of TET2-deficient cells. Taken together, we propose that disruption of the GGPP branch in TET2 knock-out cells leads to increased cell sensitivity to statins.

TET2 deficiency reduced membrane localization of small GTPases upon lovastatin treatment

Geranylgeranyl modification of small GTPases, including Rho family of proteins (e.g. RhoA/B/C, RhoG), the Ras-related proteins (e.g. Rap1A, Rac1 and Rab), and Cdc42, is essential for them to be anchored on the plasma membrane to promote cell signaling, survival, and proliferation [20]. Therefore, we measured cellular localization of RhoA, Rac1 and Cdc42 in response to lovastatin treatment by subcellular fractionation. Notably, lovastatin treatment preferentially diminished membrane localization of RhoA, Rac1 and Cdc42 while increased cytosolic distribution of these proteins in the TET2 knockout cells (Fig. 6A). Due to the poor quality of RhoA, Rac1 and Cdc42 antibodies for immunofluorescence staining, we examined the localization of another small GTPase, RhoG protein, by immunofluorescence analysis. Consistently, TET2 deficiency disrupted the accumulation of RhoG on cell membrane (Fig. 6B). In addition, HMGCS1 overexpression or GGPP replenishment rescued small GTPase membrane localization in TET2 knockout cells after lovastatin treatment (Fig. 6C–D). Overall, our results indicate that TET2 deficiency sensitizes cancer cells to statins by decreasing the expression of HMGCS1 and GGPP levels, leading to reduced membrane localization of proteins, such as small GTPases, which may contribute to cell death (Fig. 7).

DISCUSSION

The interplay of metabolism and epigenetics has been gradually acknowledged as a key mechanism in physiological regulation and cancer development [29, 30]. One of the most common characteristics of tumor cells is uncontrolled proliferation, leading to their demands for substances and energy supporting rapid growth and thus tumor cells undergo metabolic reprogramming to survive under a variety of stress, including hypoxia and starvation. Notably, some metabolic alterations are known to occur at epigenetic level, and metabolites produced in several metabolic pathways, including glycolysis and oxidative phosphorylation, can in turn serve as cofactors, substrates, or inhibitors to modulate epigenetic modifications. Hence, metabolic reprogramming and epigenetic alterations are highly intertwined, such as in the process of tumor development [31].

Epigenetic changes have been reported to regulate metabolic reprogramming in cancer. For example, hypomethylation of promoter regions is responsible for the upregulation of hexokinase 2 (HK2) in glioblastoma and liver cancer [32], whereas hypermethylation of promoter regions leads to the silence of fructose-1, 6-bisphosphatase (FBP1) in gastric, liver and colon cancers [33]. However, whether the enzymes involved in control of DNA methylation/demethylation play a role in cancer metabolic reprogramming remains unclear. In this study, we found that deficiency of TET2, which is frequently observed in myeloid leukemia (12–32%) [34–37], exhibits significant influence on the lipid metabolism and carbohydrate metabolism pathway in AML patients. Intrigued by this observation, we demonstrate that TET2 deficiency markedly increases cell sensitivity to statins (such as lovastatin and simvastatin). The statin family of drugs specific inhibits HMGCR, which can effectively lower serum cholesterol

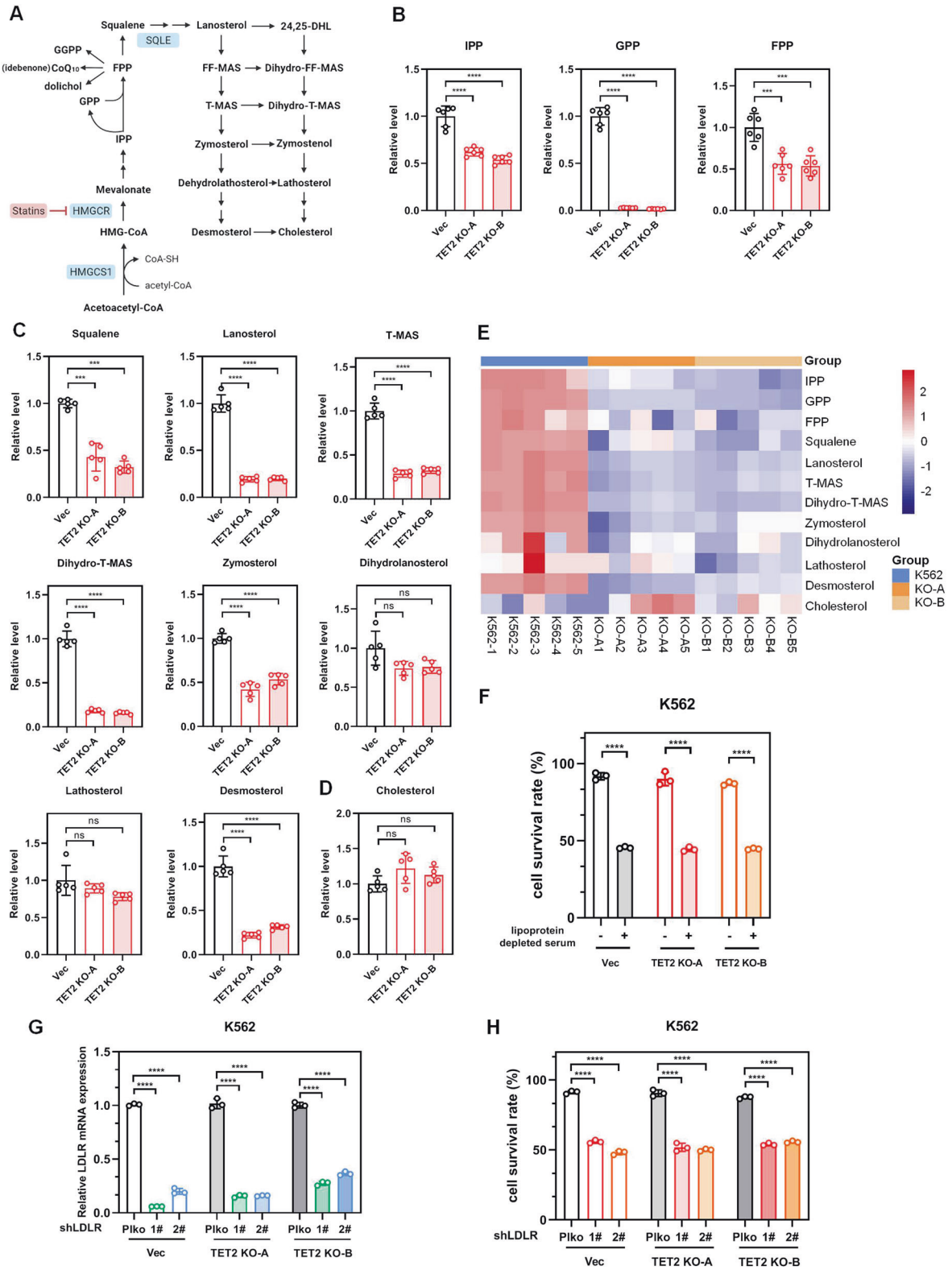
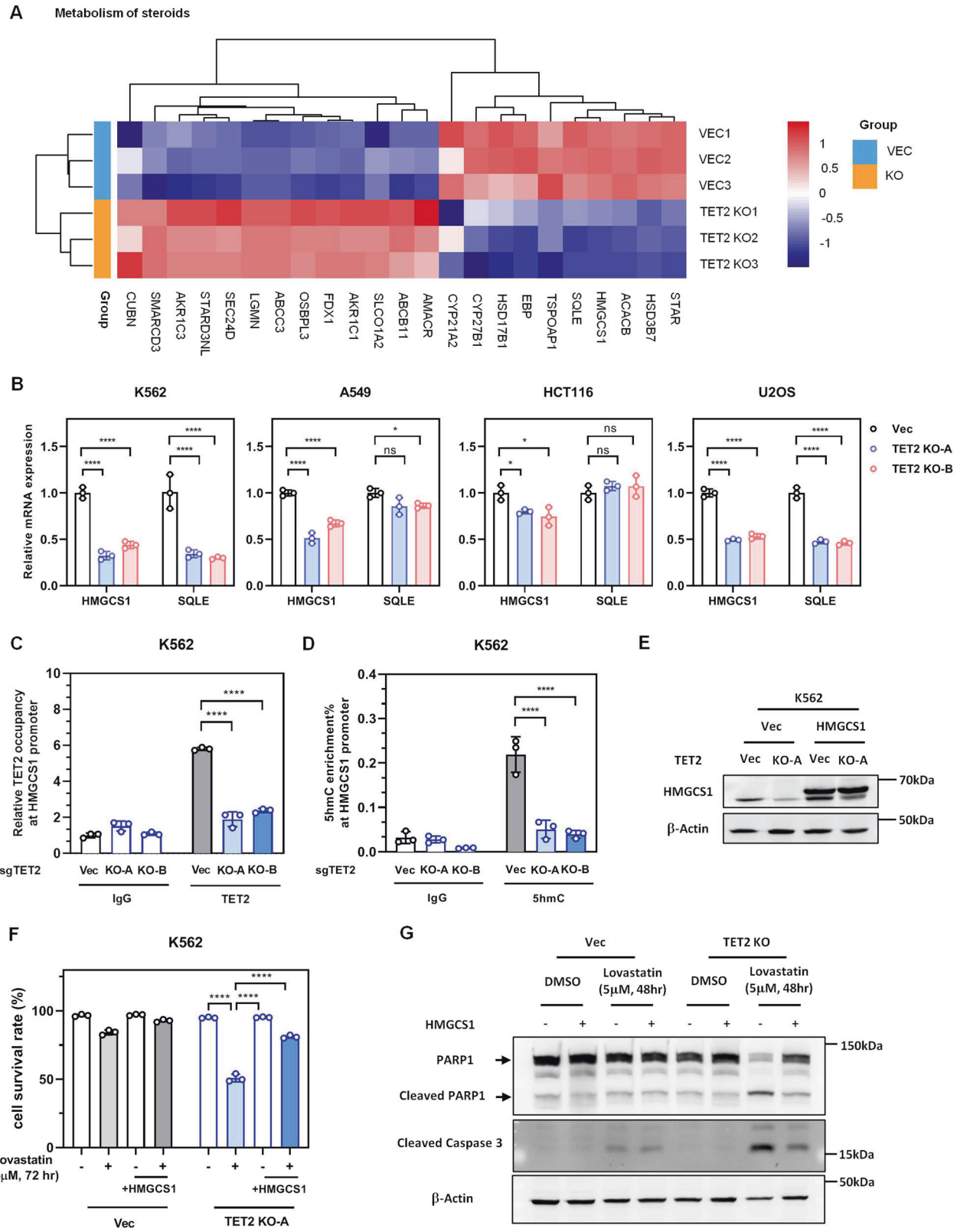


Fig. 3 Loss of TET2 attenuates the mevalonate pathway. A Cholesterol synthesis pathway in mammalian cells. Statins, the inhibitors of HMGCR, is shown in red. **B**, **C**) Alteration of metabolites in the mevalonate pathway derived from TET2 deficiency in K562 cells presented by histogram. **D** Cholesterol level remains unchanged in TET2-deficient cells. The relative concentration of metabolites was determined by LC-MS (IPP, GPP, FPP) or by the Lipidall Technologies company. **E** Heatmap of metabolites involved in the cholesterol synthesis pathway. **F** Uptake of exogenous cholesterol is essential for K562 cells to survive. WT or TET2-deficient K562 cells were cultured for 7 days in medium supplemented with fetal bovine serum or lipoprotein-depleted serum, and viable cell (both PI and annexin V negative) was determined by flow cytometry. **G**, **H** WT or TET2-deficient K562 cells were infected with shRNAs targeting LDLR and grown for five days, viable cell (both PI and annexin V negative) was determined by flow cytometry (**H**). Knocking-down efficiency of LDLR was measured by RT-PCR (**G**). Asterisks denote statistical significance with one-way ANOVA. ***$p < 0.001$; ****$p < 0.0001$ for the indicated comparison.



levels and are widely prescribed for hypercholesterolemia treatment in the past thirty years [38]. Given the superb safety record of statins, future study is warranted to test the beneficial effect of statins on cancer patients with TET2 mutation or compromised activity.

As for the mechanism of statins-induced cell death, we show TET2 deficiency reduces HMGCS1 expression, leading to decrease of most intermediates in the cholesterol synthesis pathway except the final product cholesterol, suggesting that lovastatin promotes cell death through mechanisms independent of cholesterol. It has

Fig. 4 TET2 regulates the cholesterol synthesis pathway by promoting HMGCS1 expression. A Heatmap of differentially expressed genes in steroid metabolism pathway in WT and TET2 knockout K562 cells revealed by RNA sequencing. **B** Real-time qPCR confirms that HMGCS1 gene expression is significantly decreased by TET2 deficiency in K562, A549, HCT116 and U2OS cells. Asterisks denote statistical significance with one-way ANOVA. *$p < 0.05$; ****$p < 0.0001$ for the indicated comparison. **C** TET2 binds to HMGCS1 promoters in K562 cells. The occupancy of endogenous TET2 on the promoters of HMGCS1 was determined by ChIP-qPCR. Asterisks denote statistical significance with one-way ANOVA. ****$p < 0.0001$ for the indicated comparison. **D** TET2 deficiency reduces 5hmC at the promoter region of HMGCS1 in K562 cells. 5hmC levels were determined by hMeDIP-qPCR. Asterisks denote statistical significance with one-way ANOVA. ****$p < 0.0001$ for the indicated comparison. **E**–**G** Ectopically expressed HMGCS1 rescues TET2-defecient cells from lovastatin-induced apoptosis. The protein levels of HMGCS1 (**E**), cleaved PARP and caspase-3 (**G**) was determined by Western blot. Cell viability was determined by flow cytometry (**F**). Asterisks denote statistical significance with one-way ANOVA. ****$p < 0.0001$ for the indicated comparison.

been reported that multiple cancer cells rely on uptake to meet their need for cholesterol [39]. We also demonstrated that K562 cells relied on exogenous cholesterol for growth, which is TET2-independent. Furthermore, we reveal that the reduced GGPP is responsible for lovastatin-induced cell death in the TET2 knockout cells. FPP is a known branch-point in the biosynthesis of cholesterol, dolichol, ubiquinone and prenylated proteins. Although the FPP level was reduced to ~50% in TET2-knockout cells under normal condition (Fig. 2), the level of GGPP remained steady, suggesting a possible favor of tumor cells to reduce the flux of other branches to meet their need for GGPP. In line with this, lovastatin treatment exaggerated the crisis of GGPP in TET2-deficient cells, leading to increased cell apoptosis (Fig. 7).

GGPP is essential for post-translational prenylation of many small GTP-binding proteins [18]. Taking RhoA, Rac1 and Cdc42 as examples, we show that shortage of GGPP caused by TET2 deficiency disrupts membrane localization of small GTPases that undergo prenylation. Small GTPases are known to regulate multiple fundamental processes in mammalian cells, including morphogenesis, polarity, movement, and cell division [40]. It can be speculated that increased statins sensitivity of TET2-deficient may result from the dysfunction of numerous small GTPases. Further studies are needed to elucidate whether one or more small GTPases are critical in this process and the underlying mechanisms.

Revealing the mechanisms of metabolic reprogramming in cancers with specific genetic alterations is important for developing new targeted therapies. In this study, we uncover a previously undescribed TET2-HMGCS1 regulatory axis that connecting epigenetic modification to statin sensitivity. This regulatory axis may serve as a potential therapeutic strategy for patients having TET2 mutation or reduced TET2 enzyme activity.

MATERIALS AND METHODS

Cell culture, transfection and infection

K562, A549, U2OS and HCT116 cells were obtained from the American Type Culture Collection (ATCC). K562, A549 and U2OS cells were grown in RPMI-1640 medium (Invitrogen) while HCT116 cells were grown in Dulbecco's modified Eagle's medium (GIBCO), supplemented with 10% fetal bovine serum (Gibco) and 50 μg/ml of penicillin/streptomycin. As for depletion of exogenous cholesterol uptake, medium was supplemented with 10% lipid depleted fetal bovine serum (vivacell, c3840-0050). Plasmid transfections were performed with polyethylenimine (PEI) or lipofectamine 2000 reagent (Invitrogen). For the establishment of stable cell lines with gene knockout, lentiviruses carrying TET2 sgRNA were produced by co-transfecting target and packaging plasmids into HEK293T cells. K562, A549, U2OS and HCT116 cells were then infected with the lentivirus-containing medium together with 8 μg/mL of polybrene. Stable cell lines were obtained after 5-day selection with 10 μg/mL of puromycin. To generate K562 cells with LDLR knockdown, shRNA oligo targeting LDLR were custom-synthesized, annealed, and inserted into the pLKO.1 plasmid, according to standard cloning protocol for producing lentivirus-containing medium. K562 cells were then infected together with 8 μg/mL of polybrene. To generate K562 cells stably expressing Flag-HMGCS1, lentiviruses carrying pCDH-EF1-vector and pCDH-EF1-HMGCS1 were produced and infected as described above. Primers involved were listed below:

sgTET2-A TGGAGAAAGACGTAACTTCG
sgTET2-B GGGAGATGTGAACTCTGGGA
shLDLR-1 ACAGAGGATGAGGTCCACATT
shLDLR-2 CCAGCGAAGATGCGAAGATAT
pCDH-Flag-HMGCS1-R
ATTCTAGAGCTAGCGAATTCATGGATTACAAGGATGACGACGATAAGATGCC TGGATCACTTCCT
pCDH-Flag-HMGCS1-F
TCCTTCGCGGCCGCGGATCCTTAATGTTCCCCATTACT

RNA sequencing

Samples were harvested using TRIzol reagent (Invitrogen) according to manufacturer's instruction. RNA extraction, library preparation and transcriptome sequencing were performed by Shanghai Majorbio.

Proliferation assay

Cells were seeded in 96-well plates (3000 cells/well) and treated with different drugs for 5-day proliferation assay. CCK-8 solutions were added to each well, then cultured for 1 h and microplate readers were used to measure the absorbance at 450 nm for each well every day.

Flow cytometry

Apoptosis flow cytometry assay was carried out using standard immunology protocols with annexin V FITC/PI kit (BD). Cells were treated with different agents in 6-well plates and collected according to their condition. Cell pellets were resuspended in 500 μL of cold fixing buffer and 100 μL of suspension was extracted for dual staining with FITC-conjugated annexin V and PI. After 15-minute incubation protected from light at 25 °C, samples were subjected to flow cytometry for fluorescence detection and Annexin V/PI double-negative staining referred to viable cells.

Liquid Chromatography/Mass Spectrometry (LC/MS)

Pre-cooled 80% methanol (300 μL for 60-mm plate) was added to each sample and reacted at −20 °C for 30 min. Mixture of cells and methanol was collected, rotated at 4 °C for 30 min and then centrifuged at 14,000 rpm for 15 min. Supernatant was transferred to LC-MS vials for detection.

Western blotting

Western blotting was performed on cell lysates with SDS–polyacrylamide gel electrophoresis. The separated proteins were transferred onto a nitrocellulose membrane and blocked with 5% fat-free milk (BD Biosciences) for 30 min. After incubation with primary antibody overnight at 4 °C and secondary antibody for 1 h at room temperature (RT), expression level was detected by Typhoon FLA 9500 (GE Healthcare) image scanning and quantified by Image Quant TL software (GE Healthcare). The antibodies used were listed below: TET2 (CST, 18950 S), PARP (CST, 9532 S), Cleaved Caspase-3 (CST, 9664 S), RhoA (CST, 2117 S), Rac1 (Jingjie PTM BioLab, PTM-5329), Cdc42 (CST, 2466 T), β-actin (genscript, A00702).

Quantitative RT-PCR

RNA was extracted using TRIzol reagent following the manufacturer's instruction. cDNA was synthesized from total RNA with Oligo-dT primers using SuperScript III First-Strand Synthesis SuperMix (Invitrogen). Real-time PCR was performed, β-actin utilized as a housekeeping control, using SYBR Green (TaKaRa) and gene-specific primers in a 7900HT Sequence Detection System (Applied Biosystems). The primers involved were listed below:

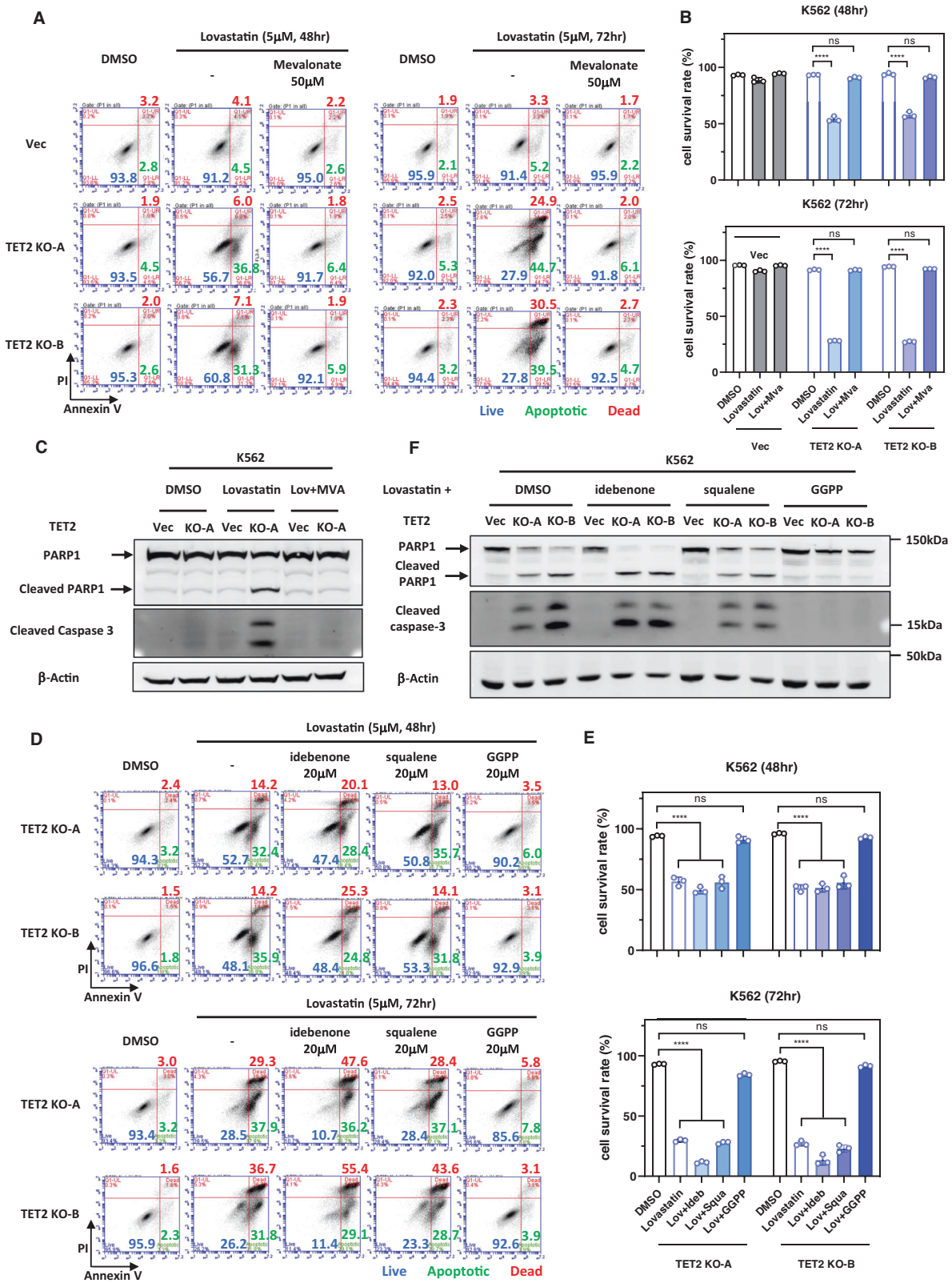
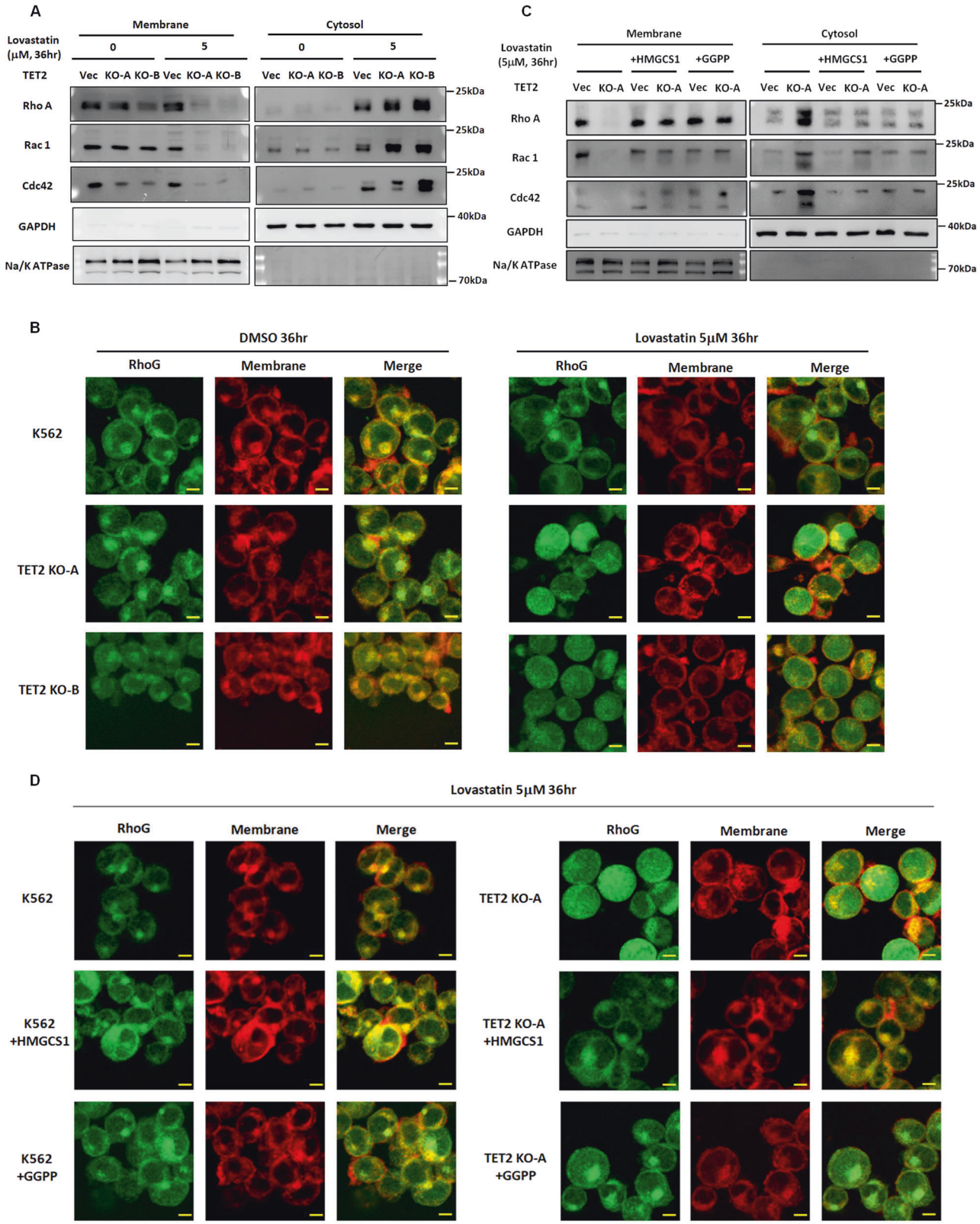


Fig. 5 Addition of MVA or GGPP rescues the lovastatin-mediated apoptosis in TET2 KO cells. A–C Supplement of mevalonate protects cells from lovastatin-induced apoptosis. WT or TET2-defecient K562 cells were treated with lovastatin (5 μM) plus mevalonate (10 μM) for 48 h or 72 h. Cell viability was determined by flow cytometry (**A**, **B**). Protein levels of cleaved PARP and caspase-3 were determined by Western blot (**C**). Asterisks denote statistical significance with one-way ANOVA. ****$p < 0.0001$ for the indicated comparison. **D–F** Supplement of GGPP rescues the lovastatin-induced apoptosis. The indicated K562 cells were treated with lovastatin (5 μM) plus idebenone (20 μM), squalene (20 μM) or GGPP (20 μM), respectively. Cell viability was determined by flow cytometry (**D**, **E**) and protein levels of cleaved PARP and caspase-3 were determined by Western blot (**F**). Asterisks denote statistical significance with one-way ANOVA. ****$p < 0.0001$ for the indicated comparison.



ACTIN-F GCACAGAGCCTCGCCTT
ACTIN-R GTTGTCGACGACGAGCG
HMGCS1-F AAGTCACACAAGATGCTACACCG
HMGCS1-R TCAGCGAAGACATCTGGTGCCA
SQLE-F CGTGCTGGTGTTCCTCTCG
SQLE-R TTGGTTCCTTTTCTGCGCCTC
LDLR-F CTACAGCTACCCCTCGAGAC
LDLR-R GGGACTCCAGGCAGATGTTC

Immunofluorescence Staining

K562 suspension cells were seeded in 24-well plates and centrifuged at 1000 rpm for 5 min to adhere to cell sheets. After fixation with 4%

Fig. 6 Combination of TET2 loss and Lovastatin treatment reduce the membrane localization of small GTPases. A TET2 deficiency suppresses RhoA, Rac1 and Cdc42 membrane localization under lovastatin treatment. WT or TET2-defecient K562 cells were treated with or without lovastatin (5 μM) for 36 h. Cells were subjected to subcellular fractionation. Protein levels of RhoA, Rac1 and Cdc42 in the membrane or cytoplasmic fraction were determined by Western blot. **B** TET2 deficiency decreases RhoG membrane localization under lovastatin treatment. Immunofluorescence staining was performed by using RhoG antibody and Dil for detection of RhoG protein and plasma membrane, respectively. Scale Bars: 10 μm. **C** HMGCS1 overexpression or GGPP replenishment rescues RhoA, Rac1 and Cdc42 membrane localization upon lovastatin treatment in TET2 deficient cells. Protein levels of RhoA, Rac1 and Cdc42 in the membrane or cytoplasmic fraction were determined by Western blot. **D** HMGCS1 overexpression or GGPP replenishment rescues RhoG membrane localization in TET2 deficiency cells under lovastatin treatment. Immunofluorescence staining was performed by using RhoG antibody and Dil for detection of RhoG protein and plasma membrane, respectively. Scale Bars: 10 μm.

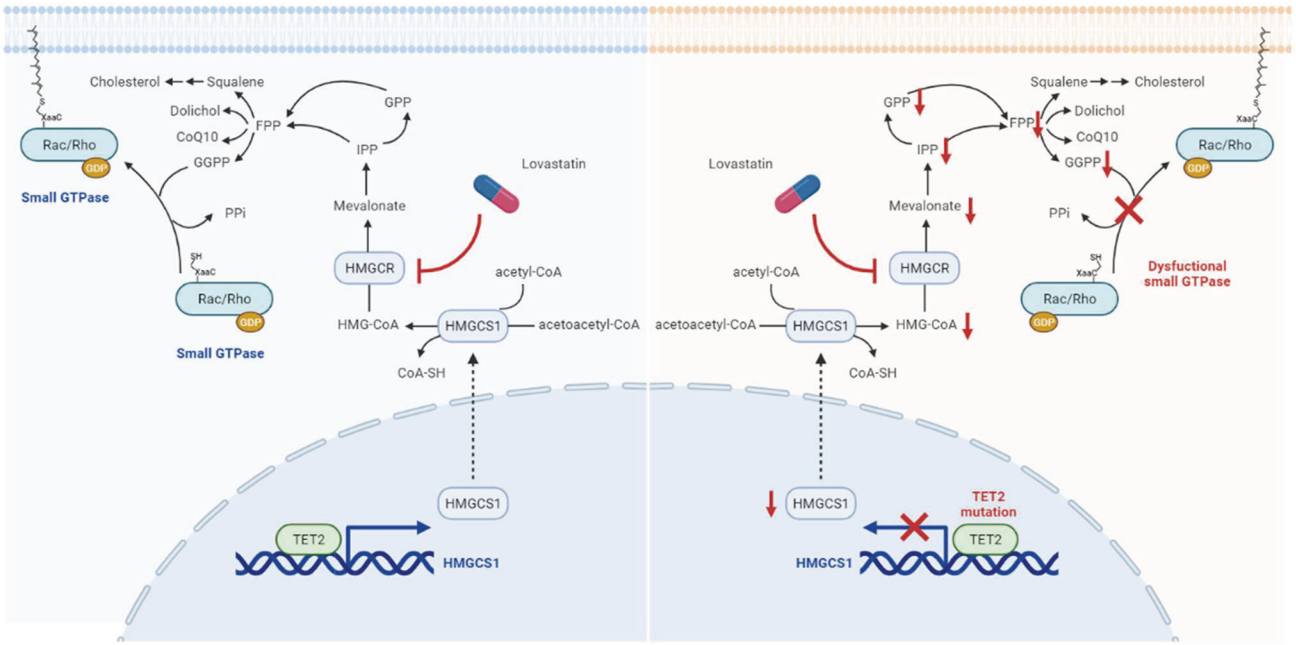


Fig. 7 Model of TET2 sensitizes tumor cells to statins by reducing HMGCS1 expression. A proposed model of TET2-HMGCS1-GGPP axis that regulates the membrane localization of small GTPases and tumor cell sensitivity to statins. Model was created with BioRender.com.

paraformaldehyde, permeabilization with 0.3% Triton X-100 and blockage with 5% bovine serum albumin, cells were incubated with primary antibody against Rho G (Santa Cruz, sc-80015) overnight at 4 °C and Alex Fluor 488 (Green) conjugated secondary antibodies (Invitrogen) for 1 h at RT. Cell membrane was stained with Dil (Beyotime). Fluorescence microscope images were captured using Leica fluorescence confocal scanning microscope.

Chromatin Immunoprecipitation (ChIP)-qPCR Assay

Cells were cross-linked with 1% paraformaldehyde for 10 min at RT, and then quenched by adding 0.125 M glycine. Chromatin were sheared into 0.2–1 kb fragment with sonication (Bioruptor) at 4 °C for 25 min and immunoprecipitated at 4 °C for 2.5 h with the antibody against TET2 (YouKe). Antibody-chromatin complexes were pulled-down using protein A beads (GE) with rotation for 1 h at 4 °C. Subsequently, beads combined with specific binding DNA was washed with high salt buffer for 3 times (50 mM HEPE NaOH pH 7.5, 500 mM NaCl, 1 mM EDTA, 0.1% Na-Deoxycholate, 1% TritonX-100), low salt buffer for 2 times (10 mM Tris-HCl pH 8.0, 250 mM LiCl, 1 mM EDTA, 0.5% NP40, 0.5% Na-Deoxycholate), and finally TE buffer once. After treated with cross-link reversal and proteinase K (TaKaRa), DNA fragments were extracted with PCR Purification Kit (QIAGEN) and further analyzed by quantitative PCR with the primers listed below:

HMGCS1-1F ACAAACAGGCACAACCTCAC
HMGCS1-1R CTTCGGGACCAGCTTTAGGT
HMGCS1-2F TGGGATTTCAGAACACCAAGGA
HMGCS1-2R TGATGGAAAGAAGGGAAGTGGG
HMGCS1-3F CTATATGTGGCAGCAGGACA
HMGCS1-3R CTAACGACAGAGGAGTTGGAGG
HMGCS1-4F GAATACCTAGACCTCAACGCCC
HMGCS1-4R CCTTGACTAGGCCCGAAGG
HMGCS1-5F CACCGCCTCTGTCTGGC
HMGCS1-5R CTGTGGGAGCCACCGTC
HMGCS1-6F CAATCGCGGCCGGTAGAGTT
HMGCS1-6R TATCTCGCAGCTCCGTCATTG
HMGCS1-7F GAGATACGGCGAGAGCCAAC
HMGCS1-7R TCAGAAATCTTTCAGGTGAAGCC
HMGCS1-8F ACGTTTTCTAGCAAAGTGTGCG
HMGCS1-8R CCCCACGGATGCCTAATCC
HMGCS1-9F GATTAGGCATCCGTGGGGAGG
HMGCS1-9R GCAGTCCGTATCCGATGGATTT

hMeDIP-qPCR assay

Genomic DNA was extracted from cells using a phenol–chloroform method as previously described. genomic DNA was first denatured and immunoprecipitated with 5hmC antibody (Active Motif, 39769) and pulled-down using protein A beads (GE). Beads were washed for three times. After proteinase K treatment for 4 h at 65 °C, DNA was purified with PCR Purification Kit (QIAGEN) and then analyzed by quantitative PCR with the primers same as the ChIP-qPCR assay.

Statistics

All data were analyzed and presented using Prism 8.0 software (GraphPad Software) with at least three biological or technical replicates. Continuous variables are reported as mean ± standard error (SE) and statistical testing significance was calculated using one-way ANOVA. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$.

The sample sizes were chosen based on previous experience in the laboratory and pre-specified effect sizes considered to be biologically

significant. No samples were excluded from any analyses and all replicates were authentic biological replicates. No blind analysis was performed in this study.

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AUTHOR CONTRIBUTIONS

S-JS, YX, K-LG and H-XY conceived the general framework of this study. Y-JA contributed to the bioinformatics analysis. S-JS, Y-JA and H-XY designed the experiments. S-JS, Y-JA, K-LD, J-YZ, CZ and Y-PS performed experiments. S-JS, Y-JA, YX, K-LG and H-XY prepared the manuscript.

COMPETING INTERESTS

KLG is a co-founder of Vivace Therapeutics. YX is a co-founder of Cullgen Inc. Other authors declare no competing financial interests.

ADDITIONAL INFORMATION

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