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# Vitamin C modulates TET1 function during somatic cell reprogramming

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**Vitamin C, a micronutrient known for its anti-scurvy activity in humans, promotes the generation of induced pluripotent stem cells (iPSCs)[1] through the activity of histone demethylating dioxygenases[2,3]. TET hydroxylases are also dioxygenases implicated in active DNA demethylation[4–8]. Here we report that TET1 either positively or negatively regulates somatic cell reprogramming depending on the absence or presence of vitamin C. TET1 deficiency enhances reprogramming, and its overexpression impairs reprogramming in the context of vitamin C[2,9] by modulating the obligatory mesenchymal-to-epithelial transition (MET)[10,11]. In the absence of vitamin C, TET1 promotes somatic cell reprogramming independent of MET. Consistently, TET1 regulates 5-hydroxymethylcytosine (5hmC) formation at loci critical for MET in a vitamin C–dependent fashion. Our findings suggest that vitamin C has a vital role in determining the biological outcome of TET1 function at the cellular level. Given its benefit to human health, vitamin C should be investigated further for its role in epigenetic regulation.**

We isolated mouse embryonic fibroblasts (MEFs) from wild-type (*Tet1*$^{+/+}$) or *Tet1*-null (*Tet1*$^{-/-}$; ref. 12) embryos and tested their ability to undergo reprogramming with OCT4-KLF4-SOX2 (OKS)[13]. Unexpectedly, we observed that MEFs deficient in *Tet1* underwent reprogramming more efficiently, as determined on the basis of alkaline phosphatase staining (**Supplementary Fig. 1a**), suggesting that TET1 may inhibit somatic cell reprogramming. To confirm this finding, we bred *Tet1*-null mice with OG2 mice[14] to derive OG2 MEFs from *Tet1*$^{+/+}$, *Tet1*$^{+/-}$ and *Tet1*$^{-/-}$ embryos, and we showed that, indeed, Tet1 has negatively regulates reprogramming by defined factors (**Fig. 1a** and **Supplementary Fig. 1b**). We compared the proliferation rate and expression of genes involved in pluripotency in *Tet1*$^{+/+}$ and *Tet1*$^{-/-}$ MEFs undergoing reprogramming and showed that they proliferate at a similar rate, yet expression of both *Nanog* and *Dppa3* was activated much more rapidly in *Tet1*$^{-/-}$ than in *Tet1*$^{+/+}$ MEFs (**Fig. 1b** and **Supplementary Fig. 1c**). The resulting *Tet1*$^{-/-}$ iPSCs were pluripotent and capable of generating chimeric mice after blastocyst injection (**Fig. 1c** and **Supplementary Fig. 1d**). We compared the DNA methylation status of several imprinted loci[15,16] and did not observe any consistent difference between *Tet1*$^{+/+}$ and *Tet1*$^{-/-}$ iPSCs (**Supplementary Fig. 1e**). These results suggest that TET1 has a negative role in somatic cell reprogramming. We have recently shown that pre-iPSCs, which represent a stable intermediate stage of reprogramming[1,2,17,18], can be converted into fully reprogrammed iPSCs by vitamin C through a histone H3 lysine 9 (H3K9) demethylation step[2]. We asked whether TET1 has any role in the vitamin C–induced conversion of pre-iPSCs into iPSCs. Using small interfering RNA (siRNA) for *Tet1* (**Supplementary Fig. 1f**), we showed that *Tet1* knockdown consistently promoted the conversion of two independently derived pre-iPSCs[2] into fully reprogrammed iPSCs (**Fig. 1d**,**e**), suggesting that TET1 is also part of the barrier blocking the transition of pre-iPSCs into iPSCs. Taken together, results from these two different loss-of-function approaches suggest that TET1 negatively regulates somatic cell reprogramming.

Despite the consistent results described above, we were concerned about the unexpected finding that TET1 is a negative regulator of somatic cell reprogramming, a process previously known to involve massive DNA demethylation at loci involved in pluripotency[19]. Indeed, three papers were published showing that TET1 is a positive regulator of reprogramming[20–22], thus, further compounding our concerns. Despite the high concentration of vitamin C (~76.5 μM in mouse plasma) under physiological conditions[23] and the reported positive role of vitamin C in reprogramming[1,24], we realized that the use of vitamin C might not have been adapted widely in iPSC generation and thus might be the difference between our results and those reported by others[20,21]. To resolve this discrepancy, we omitted vitamin C from the chemically defined iCD1 medium used in our

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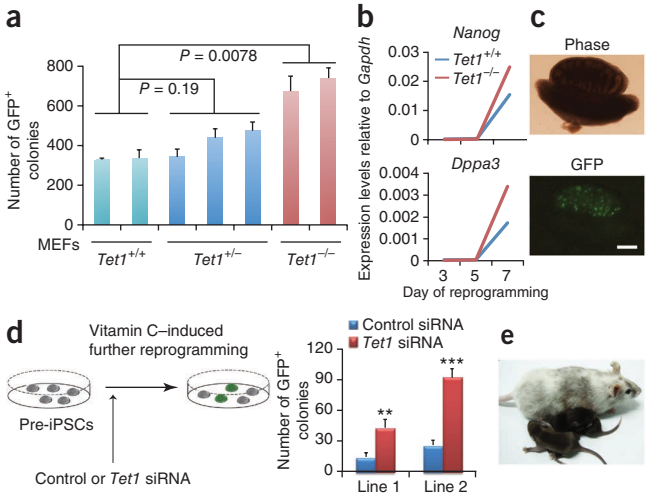


**Figure 1** *Tet1* deficiency enhances iPSC generation in the presence of vitamin C. (**a**) OG2 MEFs with the indicated genotypes were reprogrammed by OKS in iCD1 medium (with vitamin C) for 7 d, and GFP-positive colonies (expressing GFP from the *Pou5f1* (*Oct4*) promoter) were scored. *P* values were calculated by Student's two-sided *t* test. Error bars, s.d. (**b**) *Nanog* and *Dppa3* mRNA levels were measured by quantitative RT-PCR (qRT-PCR) during the reprogramming process of either $Tet1^{+/+}$ or $Tet1^{-/-}$ MEFs expressing OKS. Average values from three independent experiments are shown. (**c**) GFP expression in the genital ridge of chimeric embryos (14.5 d post-coitum (d.p.c.)) indicating germline contribution of $Tet1^{-/-}$ iPSCs. Scale bar, 100 μm. (**d**) Effect of *Tet1* ablation on the conversion of pre-iPSCs into iPSCs. Knockdown of *Tet1* enhances the reprogramming of pre-iPSCs into iPSCs. Experiments were performed on two independent pre-iPS cell lines, and GFP–positive iPSC colonies were counted; $n = 3$ technical replicates. Error bars, s.d. $^{**}P < 0.01$, $^{***}P < 0.001$ by *t* test. (**e**) A chimeric mouse was derived from the reprogrammed iPSCs with knockdown of *Tet1* in **d** and its offspring to indicate germline transmission.

experiments[9] and showed that, in the absence of vitamin C, $Tet1^{-/-}$ MEFs were less proficient than $Tet1^{+/+}$ MEFs in reprogramming, suggesting that TET1 has a positive role in the absence of vitamin C (**Fig. 2a**). We then wished to confirm the role of TET1 in reprogramming through a gain-of-function approach. We obtained an inducible expression vector for full-length *Tet1* (ref. 20) and showed that it promotes iPSC generation in the absence of vitamin C, as previously described[20], whereas it inhibits reprogramming in the presence of vitamin C (**Fig. 2b**), thus not only reconciling the discrepancies between our findings and those reported by others, but also demonstrating that vitamin C acts as a switch in the regulation of TET1 function.

We then wished to gain further insight into the relationship between vitamin C and TET1 in reprogramming. However, TET1 is a large protein and has been proven to be hard to express and measure accurately. To circumvent these issues, we employed a shorter version of TET1 containing the catalytic domain, TET1-CD (**Supplementary Fig. 2a**). Consistent with results obtained with the full-length version of TET1, we showed that the wild-type TET1-CD protein but not its mutant form that lacks dioxygenase activity (TET1-CD-mut) also robustly inhibited reprogramming when coexpressed with OKS in OG2 MEFs (**Supplementary Fig. 2b**,**c**). Because a recent study showed that TET2 positively regulates reprogramming[25], we constructed a similarly short version of TET2, TET2-CD, and its catalytically inactive mutant and showed that TET2-CD enhanced reprogramming in a catalytic activity–dependent manner (**Supplementary Fig. 2d**), despite the fact that both TET1-CD and TET2-CD catalyze similar levels of DNA hydroxylation (**Supplementary Fig. 2d**). These results illustrate the divergent specificities of TET1 and TET2 in reprogramming. Given the fact that TET1-CD recapitulates the activity of full-length TET1 well in reprogramming and that TET1-CD is readily quantifiable by protein blotting and densitometry, we decided to use TET1-CD for further analysis. We then showed that vitamin C can gradually convert TET1 from a positive regulator of reprogramming into a negative regulator (**Fig. 2c**, left). At 0 μg/ml of vitamin C, TET1-CD promoted the generation of iPSCs (**Fig. 2c**) as full-length TET1 did (**Fig. 2b**). With increasing concentrations of vitamin C from 0.01 to 20 μg/ml, reprogramming efficiencies with wild-type TET1-CD decreased below those of controls (**Fig. 2c**), whereas similar vitamin C concentrations improved reprogramming for the control cells as well as

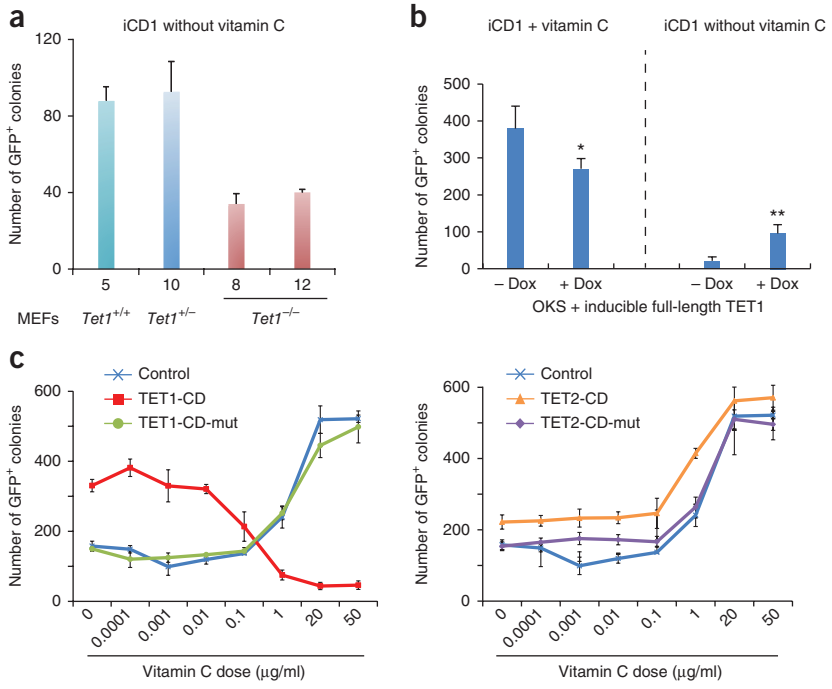


**Figure 2** *Tet1* represses reprogramming in the presence of vitamin C. (**a**) MEFs with different *Tet1* genotype were infected with virus expressing OKS and cultured in iCD1 medium without vitamin C. GFP–positive colonies (expressing GFP from the *Pou5f1* (*Oct4*) promoter) were scored; $n = 3$ technical replicates. Error bars, s.d. (**b**) MEFs expressing OKS and inducible TET1 were cultured in iCD1 medium with and without vitamin C. GFP–positive colonies were scored; $n = 3$ technical replicates. Error bars, s.d. $^{*}P < 0.05$, $^{**}P < 0.01$ by two-sided *t* test. Dox, doxycycline. (**c**) MEFs expressing OKS and the indicated genes were cultured in iCD1 medium supplemented with increasing concentrations of vitamin C. Control cells received empty vector. GFP–positive colonies were scored; $n = 3$ technical replicates. Error bars, s.d. Experiments were performed together but are plotted separately, sharing the same control data to avoid overlapping lines.

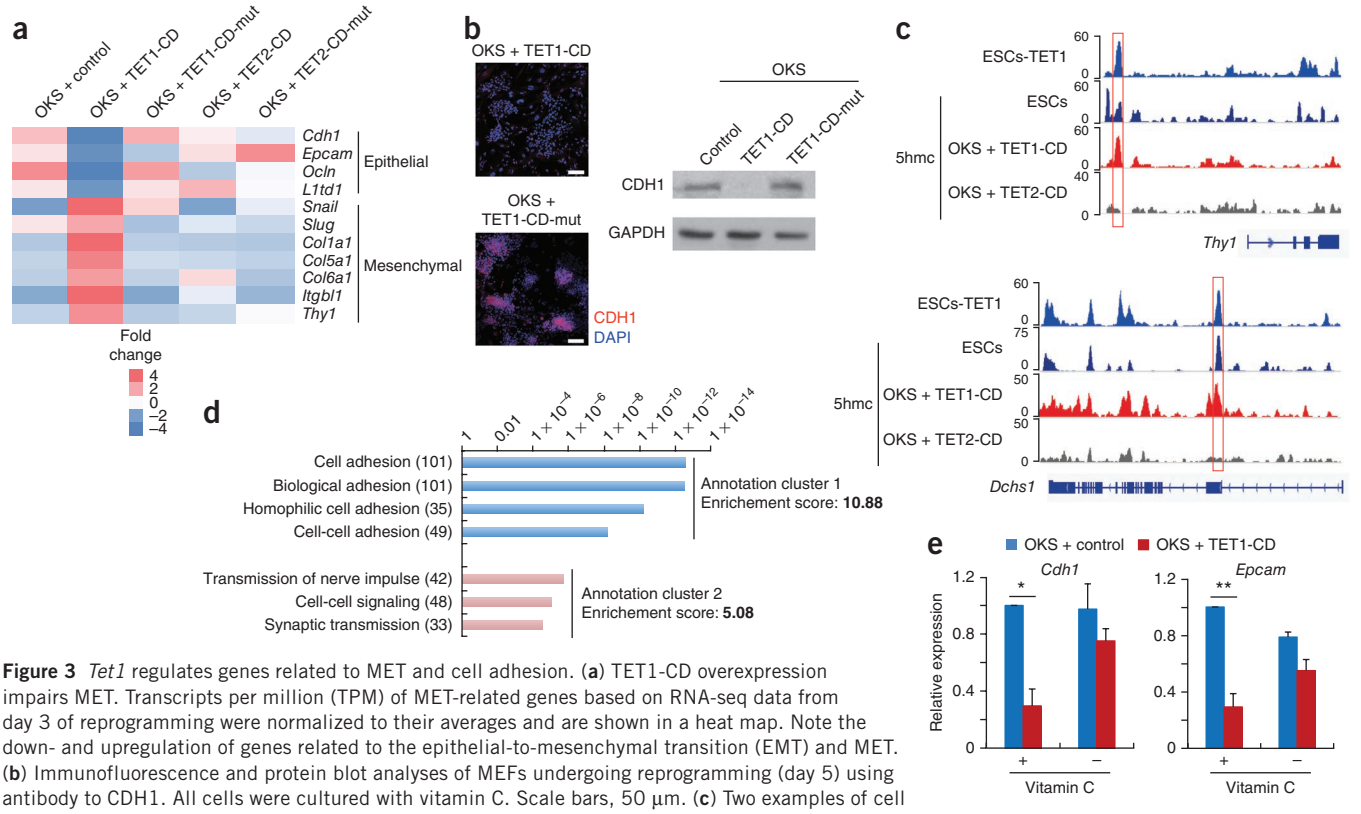


**Figure 3** *Tet1* regulates genes related to MET and cell adhesion. (**a**) TET1-CD overexpression impairs MET. Transcripts per million (TPM) of MET-related genes based on RNA-seq data from day 3 of reprogramming were normalized to their averages and are shown in a heat map. Note the down- and upregulation of genes related to the epithelial-to-mesenchymal transition (EMT) and MET. (**b**) Immunofluorescence and protein blot analyses of MEFs undergoing reprogramming (day 5) using antibody to CDH1. All cells were cultured with vitamin C. Scale bars, 50 μm. (**c**) Two examples of cell adhesion–related genes occupied by TET1-CD–specific 5hmC peaks (red boxes) in cells undergoing reprogramming. ESCs-TET1, TET1 chromatin immunoprecipitation and sequencing in ESCs. (**d**) Top 2 clusters of GO categories overrepresented in 1,775 genes with TET1-CD–mediated 5hmC peaks in cells undergoing reprogramming. (**e**) Expression of epithelial markers in MEFs undergoing reprogramming treated with or without vitamin C was examined by qRT-PCR at day 3 of reprogramming; $n = 2$ independent experiments. Error bars, s.d. $^{*}P < 0.05$, $^{**}P < 0.01$ by two-sided $t$ test.

those expressing mutant TET1-CD (**Fig. 2c**). Interestingly, vitamin C at a concentration of 1 μg/ml (~3.1 μM), which is even lower than the plasma concentration under physiological conditions[23], turned TET1 into a strong inhibitor of reprogramming (**Fig. 2c**). In contrast, TET2-CD showed a constitutively positive effect on the generation of iPSCs at all concentrations of vitamin C (**Fig. 2c**, right), suggesting that the switch in TET1 function mediated by vitamin C is specific to this protein. These results demonstrate that TET1, both endogenous and exogenous, as a full-length protein or just its catalytic domain, regulates somatic reprogramming in a vitamin C–dependent fashion.

We were intrigued by the potential mechanism through which TET1 inhibits reprogramming in the presence of vitamin C. We first performed RNA sequencing (RNA-seq) to profile the genes affected by TET1-CD during reprogramming and found that it regulates the expression of genes controlling the cell cycle, cell adhesion and epithelial differentiation (**Supplementary Fig. 3a** and **Supplementary Table 1**). Although TET1-CD apparently regulates the expression of genes related to cell cycle pathways and MEFs infected with retroviruses expressing OKS and TET1-CD proliferated much more slowly than those infected with viruses expressing OKS and TET1-CD-mut or DsRed (**Supplementary Fig. 3b**,**c**), we did not observe any differences in proliferation between *Tet1*$^{+/+}$ and *Tet1*$^{-/-}$ MEFs during reprogramming, suggesting that endogenous TET1 may not regulate the cell cycle (**Supplementary Fig. 1b**). We next decided to focus on other genes. Given the fact that the reprogramming of MEFs by defined factors starts with MET[10], the mesenchymal-to-epithelial transition with characteristic changes in cell shape and adhesion molecules, we hypothesized that TET1 inhibits reprogramming by regulating MET. Indeed, RNA-seq results suggested that TET1-CD impeded the MET process induced by the OKS reprogramming factors (**Fig. 3a**). TET1-CD–expressing cells at day 5 after infection still lacked E-cadherin expression (CDH1), in contrast to the robust CDH1 expression seen in cells expressing mutant TET1-CD, as evidenced by immunostaining and protein blot assays (**Fig. 3b**), confirming that MET was blocked by TET1-CD.

To gain further insight into vitamin C–dependent TET1 function, we mapped 5hmC sites by immunoprecipitation with antibody to 5hmC followed by quantitative PCR (hMeDIP-qPCR) in *Tet1*$^{+/+}$ and *Tet1*$^{-/-}$ embryonic stem cells (ESCs). We showed that sites occupied by both 5hmC and TET1 (for example, P2 and P5) according to previously published data sets[7,8] (**Supplementary Table 2**) were indeed enriched for 5hmc in a TET1-dependent manner compared to other sites (**Supplementary Fig. 4a**), indicating that this method can be used to analyze TET1 targets. Given the opposite effects exerted by TET1 and TET2 on reprogramming in the presence of vitamin C (**Fig. 1a** and **Supplementary Fig. 2d**), we performed hMeDIP sequencing to map 5hmC sites catalyzed specifically by TET1-CD and TET2-CD during reprogramming and showed that TET1-CD has a similar, although not identical pattern as that of endogenous TET1 in ESCs (**Fig. 3c**, **Supplementary Fig. 4b**,**c** and **Supplementary Table 3**). Of the 1,775 genes with TET1-CD–specific 5hmC sites mapped by hMeDIP sequencing, there were 94 genes that were upregulated and 113 genes that were downregulated (including *Cdh1*) according to RNA-seq data (**Supplementary Fig. 4d**,**e** and **Supplementary Table 4**). Several representative TET1-CD– or TET2-CD–specific 5hmC sites were also confirmed by hMeDIP-qPCR (**Supplementary Fig. 4f**). Interestingly, unbiased gene ontology (GO) analysis of these 1,775 TET1-CD–specific genes showed that the top ranked clusters were related to

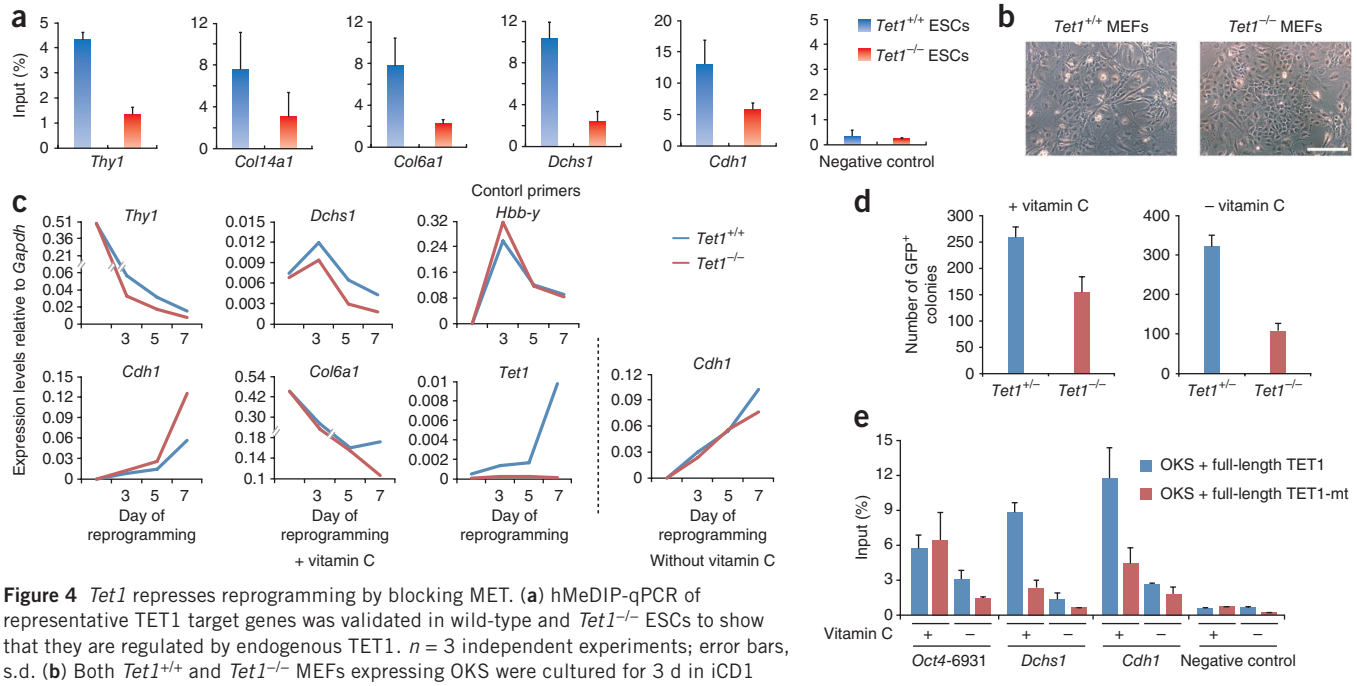


**Figure 4** *Tet1* represses reprogramming by blocking MET. (**a**) hMeDIP-qPCR of representative TET1 target genes was validated in wild-type and *Tet1*$^{-/-}$ ESCs to show that they are regulated by endogenous TET1. $n = 3$ independent experiments; error bars, s.d. (**b**) Both *Tet1*$^{+/+}$ and *Tet1*$^{-/-}$ MEFs expressing OKS were cultured for 3 d in iCD1 medium, and images were taken showing epithelial-like cells as an indication of MET. Scale bar, 250 µm. (**c**) qRT-PCR results from *Tet1*$^{+/+}$ and *Tet1*$^{-/-}$ MEFs expressing OKS. *Thy1*, *Col6a1*, *Dchs1* and *Cdh1* are representative MET-related genes that are regulated by TET1. Averages from three independent experiments are shown. (**d**) MECs derived from *Tet1*$^{+/-}$ and *Tet1*$^{-/-}$ mice were infected with viruses expressing OKS and allowed to reprogram with or without vitamin C. GFP–positive colonies (expressing GFP from the *Pou5f1* (*Oct4*) promoter) were scored; $n = 3$ technical replicates. Error bars, s.d. (**e**) MEFs expressing OKS and full-length TET1 or its catalytic mutant form were cultured in iCD1 medium with or without vitamin C for 72 h. Enrichment of 5hmC at the indicated sites was examined by hMeDIP-qPCR. $n = 2$ independent experiments. Error bars, s.d.

cell adhesion (**Fig. 3d**), consistent with RNA-seq data showing a similar top ranking for cell adhesion genes. We then confirmed that genes involved in the MET process and cell adhesion were enriched for 5hmC sites as a result of TET1-CD expression (**Supplementary Fig. 4g**,**h**). Given the effect of vitamin C on the functional outcome of TET1, we next examined whether TET1 also regulates MET-related genes in a vitamin C–dependent fashion. Indeed, TET1-CD significantly repressed the expression of *Cdh1* and *Epcam* only in the presence of vitamin C, suggesting that MET might be a crucial target step for the switch in TET1 activity mediated by vitamin C (**Fig. 3e**).

To test the idea that endogenous TET1 regulates MET during reprogramming, we first confirmed that 5hmC levels and TET1 binding around MET-related genes were markedly reduced in *Tet1*$^{-/-}$ ESCs compared to in wild-type ESCs (**Fig. 4a** and **Supplementary Fig. 4i**), indicating that endogenous TET1 regulates 5hmC levels around these select targets in MET. We then analyzed the MET process during the reprogramming of *Tet1*$^{+/+}$ and *Tet1*$^{-/-}$ MEFs, showing that TET1 deficiency led to obvious enhancement of the epithelial morphological transition during reprogramming in the presence of vitamin C (**Fig. 4b**). Analysis of gene expression confirmed the rapid induction of *Cdh1* and downregulation of the mesenchymal markers *Thy1*, *Dchs1* (ref. 26) and *Col6a1* during the reprogramming of *Tet1*$^{-/-}$ MEFs only in the presence of vitamin C (**Fig. 4c**), suggesting that endogenous TET1 also blocks the obligatory MET process during reprogramming in a vitamin C–dependent fashion. In contrast, TET1 deficiency did not affect *Tet2* expression and the early induction of control target gene *Hbb-y* (**Fig. 4c** and **Supplementary Fig. 5a**), suggesting that endogenous TET1 and vitamin C function specifically to preserve the mesenchymal identity of MEFs independent of TET2 activity. Consistent with the observation that *Tet1* is not required for the activation of loci involved in pluripotency (**Fig. 1b**), we observed even more rapid demethylation of the pluripotency locus *Pou5f1* (*Oct4*) in *Tet1*$^{-/-}$ than in *Tet1*$^{+/+}$ cells (**Supplementary Fig. 5b**).

We then asked whether TET1 and vitamin C have any role beyond the regulation of MET. We have previously shown that mammary epithelial cells (MECs) undergo reprogramming without MET[10]. Therefore, we isolated MECs from *Tet1*$^{+/-}$ and *Tet1*$^{-/-}$ mice and showed that, unlike MEFs, MECs behave similarly with or without vitamin C, suggesting that vitamin C can not switch the function of TET1 in this context (**Fig. 4d**). It is of interest that TET1 deficiency impaired the reprogramming of MECs independent of vitamin C (**Fig. 4d**), suggesting that TET1 may enhance reprogramming once the cells complete MET. Pre-iPSCs are stable reprogramming intermediates and have been shown to express less *Cdh1* than ESCs or iPSCs, with these expression levels indicative of incomplete MET[2]. Indeed, we showed that *Tet1* siRNA further enhanced the expression of *Cdh1* in two independent pre-iPSC lines and affected the expression of other cell adhesion genes (**Supplementary Fig. 6a–c** and **Supplementary Table 5**), demonstrating that endogenous TET1 represses further reprogramming of pre-iPSCs by also suppressing the epithelial activation part of MET.

Three recent reports demonstrated that vitamin C increases 5hmC levels by three- to sevenfold in cellular DNA[27–29]. Consistently, we showed that vitamin C increased 5hmC levels in both MEFs and ESCs (**Supplementary Fig. 6d**). We then determined whether TET1 and vitamin C differentially regulate 5hmC levels at loci involved in pluripotency and MET. By overexpressing full-length TET1 or its mutant form during reprogramming, we examined 5hmC levels around the *Pou5f1* (*Oct4*), *Dchs1* and *Cdh1* loci and showed that TET1 only enhances 5hmC levels for the MET-related genes *Dchs1* and *Cdh1* in the presence of vitamin C (**Fig. 4e**). In contrast, 5hmC levels at loci involved in pluripotency such as *Pou5f1* (*Oct4*) can be increased either

with TET1 or vitamin C but not with both, suggesting that TET1 and vitamin C do not function in synergy (**Fig. 4e**). These results suggest that TET1 and vitamin C cooperate to regulate MET-related loci but not ones involved in pluripotency.

In sum, we have presented evidence here that TET1 regulates somatic cell reprogramming in a vitamin C–dependent manner. The finding that TET1 behaves as a barrier for somatic cell reprogramming in the presence of vitamin C is counterintuitive, as reprogramming is characterized by the demethylation of 5-methylcytosine (5mC) to cytosine at the promoters of core pluripotency genes such as *Pou5f1* (*Oct4*), *Nanog* and *Sox2*. On the other hand, the fact that TET1 and vitamin C work to impede MET, a requisite step in reprogramming, raises the possibility that TET1 may negatively regulate reprogramming by maintaining the roadblocks established in MEFs during differentiation. Because TET1 is dispensable for the self-renewal of ESCs and for pre- and postnatal development[30], it is not required for the maintenance of pluripotency, despite its high expression in ESCs. TET1 functions both positively and negatively regulates gene expression in ESCs[7,8,31], rather than simply activating gene expression through demethylation, suggesting that the mechanism by which TET1 functions is complex and that other members of the TET family—TET2 and TET3 (refs. 32,33)—should be investigated in greater detail. Current data indicate that TET2 has a positive role in reprogramming[25], and its genetic deficiency significantly impairs reprogramming (X.H., L.Z., S.-Q.M., Zheng Li, J.C. *et al.*, unpublished data), suggesting that members of the TET family may have distinct and complementary roles. Indeed, while our study was under review, Costa *et al.* reported that TET1 physically associates with NANOG and facilitates the reprogramming of neural stem cells to pluripotency[21], and Gao *et al.* reported that TET1 promotes reprogramming and is able to replace OCT4 (ref. 20). Similarly, we also observed a positive role for TET1, but only in the absence of vitamin C (with very inefficient reprogramming). This modulatory role of vitamin C on TET1 function warrants further investigation at the biochemical level. Because vitamin C is a cofactor for α-ketoglutarate–dependent dioxygenases[34], including the TET enzymes and several histone demethylases such as JHDM1B (KDM2b), KDM3 and KDM4 (refs. 2,3), it would be interesting to determine whether similar modulatory insight could be gained for these enzymes. Finally, given the vital role of vitamin C in human health, our results may inspire future work on this micronutrient in epigenetic regulation of both physiological and pathological processes.

## METHODS

Methods and any associated references are available in the online version of the paper.

**Accession codes.** hMeDIP sequencing data (OKS with TET1-CD or TET2-CD) and RNA-seq data in this study can be accessed from the Gene Expression Omnibus (GEO) under accessions GSE44935 and GSE48206, respectively.

*Note: Any Supplementary Information and Source Data files are available in the online version of the paper.*


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### AUTHOR CONTRIBUTIONS

J.C., L.G. and L.Z. initiated the study, designed and performed experiments and analyzed data. Haoyu Wu performed the experiments and analyzed data. J.Y. and H.L. performed qPCR and chromatin immunoprecipitation experiments and prepared some cell samples. J.C. and X.W. performed bioinformatics analysis. X.H. performed reprogramming assays and chromatin immunoprecipitation. T.G. generated the antibody to TET1. Z.Z. bred the *Tet1*-null mice and derived primary cells. Jing Liu isolated primary cells and performed reprogramming assays. Jiadong Liu performed bisulfite genomic sequencing. Hongling Wu performed proliferation and apoptosis assays. S.-Q.M. generated the *Tet1*-null mice. K.M. performed blastocyst injections. Y.L. produced recombinant growth factor and assisted in antibody production. K.L. performed karyotype analysis. J.Q. performed protein blotting. H.Y. assisted in the construction of sequencing libraries. G.P. assisted in next-generation sequencing. G.-L.X. and D.P. conceived and supervised the entire study. D.P. and J.C. wrote the manuscript.

### COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.




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## ONLINE METHODS

**Cell culture and generation of iPSCs.** OG2 transgenic MEFs isolated from embryonic day (E) 13.5 mouse embryos were cultured in high-glucose DMEM (Hyclone) supplemented with 10% FBS (PAA), GlutaMax and non-essential amino acids (NEAA) for viral infection as described previously[9]. After 2 d of infection, iCD1 medium[9] was changed every day for the generation of iPSCs. The derivation of *Tet1*$^{-/-}$ mice was described in another study[12]. The FUW-TetOn-*Tet1* vector (provided by S. Gao) was used to produce lentiviruses carrying full-length TET1 (TET1 FL)[20]. After 6 h of infection, cells were cultured in mES medium (DMEM supplemented with 15% FBS (Gibco), GlutaMax, NEAA, penicillin-streptomycin, sodium pyruvate and leukemia inhibitory factor (LIF)) for recovery and were then cultured in indicated medium supplemented with 1 μg/ml doxycycline to initiate reprogramming. Vitamin C used in this study was purchased from Sigma as 2-phospho-L-ascorbic acid in the trisodium salt form (49752).

**Mouse strains.** OG2 mice had a CBA/CaJ × C57BL/6J background. CD-1 mice were used as embryo donors and the pseudopregnant recipients for blastocyst injection. Protocols were approved by the relevant institutional animal care and use committee (IACUC).

**Generation of pre-iPSCs and siRNA transfection.** MEFs infected with viruses expressing the four Yamanka factors were cultured in mES medium. GFP-negative pre-iPSC colonies emerged approximately 7 d after infection and were picked to establish cell lines in mES medium[2]. Oligonucleotide siRNA was synthesized (RiboBio) and used at a final concentration of 50 nM. DharmaFECT 4 (ThermoFisher) was used for suspension transfection into pre-iPSCs. Vitamin C was used to convert pre-iPSCs into iPSCs at a concentration of 50 μg/ml (ref. 2).

**Immunofluorescence staining.** Cells were fixed in 4% paraformaldehyde for 30 min, washed twice with PBS and permeabilized with 0.2% Triton X-100. For immunofluorescence staining for 5hmC, cells were first denatured with 2N HCl for 30 min and then neutralized with 100 mM Tris-HCl (pH 8.0). Antibodies used in this study included antibodies to hmC (Active Motif, 39769; 1:500 dilution), Flag (Sigma, F1804; 1:200 dilution) and Cdh1 (Abcam, ab11512; 1:200 dilution). Antibodies used in this study are also listed in **Supplementary Table 6**.

**DNA immunoprecipitation and qPCR.** Genomic DNA was extracted with the Genomic DNA Purification kit (Promega, A1125). Sonicated DNA was denatured at 95 °C for 10 min and then kept on ice for 10 min. Antibodies for 5mC (Eurogentec, BI-MECY-0100) and 5hmC (Active Motif) were preincubated with Dynabeads (Invitrogen) in PBS (pH 7.4) supplemented with 0.02% Tween-20. DNA and Dynabeads were then added to 500 μl of IP buffer (pH 7.0, 10 mM sodium phosphate, 140 mM NaCl, 0.05% Triton X-100) and incubated overnight. DNA was purified with 10% Chelex and applied for qPCR on a CFX96 real-time system (Bio-Rad). Sequences for the primers used in this study are listed in **Supplementary Table 7**.

**Chromatin immunoprecipitation (ChIP).** ChIP assays were performed as described previously[2] but with minor revisions. Cultured cells were cross-linked with 1.42% formaldehyde and incubated at 25 °C for 15 min. Glycine (final concentration of 125 mM) was used to terminate cross-linking. Washed cells were scraped and treated as described previously[2].

**Dot blots.** Genomic DNA was digested overnight with EcoRI and BamHI (NEB). Digested DNA was incubated at 95 °C for 10 min and then incubated on ice for 10 min. Denatured DNA was diluted in a series of gradients with double-distilled water and was fixed on a nylon membrane using UV cross-linking. The membrane was blocked with blocking buffer (10% skim milk, 1% BSA and 0.1% Tween-20 in PBS) for 2 h at room temperature and was then incubated with antibody for 2 h at room temperature.

**RNA-seq.** Total RNA was extracted with a TRIzol-based protocol. Libraries were prepared according to the instructions for the Illumina TruSeq RNA Sample Prep kit. Sequencing was performed on a MiSeq instrument. Data were analyzed with RSEM software.

**hMeDIP sequencing.** Genomic DNA was sonicated to fragments of 100–500 bp for library construction. Illumina adaptors were ligated before hMeDIP following the instruction manual for the NEBNext DNA Library Prep Reagent Set for Illumina, and adaptor-ligated DNA was denatured and DIP using antibody to hmC. After hMeDIP, DNA was purified and amplified. Sequencing was performed on a HiSeq instrument. Sequencing reads were mapped to the mouse genome mm9, and peak calling was performed with MACS software. Functional annotation analysis was performed with DAVID as described[35,36].


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