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Role of Tet proteins in 5mC to 5hmC conversion, ES cell self-renewal, and ICM specification

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Abstract

DNA methylation is one of the best-characterized epigenetic modifications [1–4]. While the enzymes that catalyze DNA methylation have been characterized, enzymes responsible for the reversal process have been elusive [5]. A recent study indicates that the human Tet1 protein could catalyze the conversion of 5-methyl-C (5mC) of DNA to 5-hydroxyl-methyl-C (5hmC), raising the possibility that DNA demethylation may be a Tet1-mediated process [6]. Here we extended this study by demonstrating that all three mouse Tet proteins can also catalyze a similar reaction. Interestingly, Tet1 plays an important role in mouse ES cell maintenance through maintaining the expression of Nanog in ES cells. Importantly, Tet1 knockdown-mediated down-regulation of Nanog correlated with its promoter methylation, supporting a role for Tet1 in regulating DNA methylation status. Furthermore, knockdown of Tet1 in preimplantation embryos resulted in a bias towards trophectoderm differentiation. Thus, our studies not only uncover the enzymatic activity of the Tet proteins, but also demonstrate a role for Tet1 in ES cell maintenance and ICM cell specification.

The Tet family proteins include three members Tet1-3 (Fig. S1a). In addition to the dioxygenase motif involved in Fe(II) and alpha-ketoglutarate (α-KG) binding, they also share a conserved cysteine-rich region (Fig. S1a, indicated by D and C, respectively). Recent demonstration that the human Tet1 can convert 5mC to 5hmC in an Fe(II) and α-KG-dependent manner [6] prompted us to evaluate whether mouse Tet1 and its homologs possess a similar enzymatic activity. Results shown in Fig. S2 demonstrate that overexpression of both mouse Tet1 and Tet2 catalytic domains greatly reduced 5mC staining in both U2OS and HEK293T cells. In contrast, overexpression of mouse Tet3 catalytic domain in these cells has no apparent effect on 5mC staining. The reduced 5mC staining is not due to blocked access of the antibody by the overexpressed proteins as overexpression of a mutant Tet1 or Tet2 does not affect 5mC staining (Fig. S2a). These results suggest that the enzymatic activity of Tet1 is conserved from human to mouse and that both mouse Tet1 and Tet2 can reduce global 5mC levels when overexpressed in a manner that requires the presence of an intact Fe(II) binding site.

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Author Contributions

Y.Z. conceived the project and write the manuscript. S.I., A.C.D., O.V.T., K.H., designed and performed the experiments. L.C.S. provided the oligo substrates. The authors declare of no competing interests.

Since human Tet1 is capable of converting 5mC to 5hmC *in vitro* [6], we asked whether decreased 5mC staining in cells overexpressing Tet proteins is concomitant with generation of 5hmC. To this end, we characterized a commercial 5hmC antibody by dot blot and demonstrated that the antibody is 5hmC specific (Figs. S3a–c). Competition assays further demonstrated its specificity in immunostaining (Fig. S3d). As expected, enforced expression of wild-type Tet1 and Tet2, but not their catalytic mutants, resulted in the generation of 5hmC (Fig. 1a), indicating that Tet proteins can convert 5mC to 5hmC *in vivo* (also see Fig. S3b). Interestingly, while enforced expression of Tet3 does not cause an obvious decrease in 5mC staining (Fig. S2), it does result in the generation of 5hmC (Fig. 1a), suggesting that Tet3 is indeed enzymatically active *in vivo*.

To evaluate the enzymatic activity *in vitro*, we purified Flag-tagged Tet catalytic domains as well as their corresponding catalytic mutants from baculovirus infected SF9 cells (Fig. S4a). Incubation of the purified proteins with methylated DNA substrates followed by restriction digestion, end labeling, and TLC assays (Fig. S4b, c)[7] demonstrated that wild-type recombinant Tet proteins, but not their corresponding catalytic mutants, were able to generate a radioactive product that co-migrated with 5hmC on TLC plates (compare lanes 4, 6, and 8 with lanes 5, 7, and 9). Given that enforced expression of Tet proteins resulted in the generation of 5hmC (Fig. 1a), we conclude that all the mouse Tet proteins have the capacity to convert 5mC to 5hmC.

To understand the biological function of the Tet proteins, we examined their expression patterns in various mouse tissues and cell types by RT-qPCR. Results shown in Fig. S5 revealed that both Tet1 and Tet2, but not Tet3, are expressed in ES cells. This unique expression pattern prompted us to ask whether Tet1 or Tet2 posesses an important function in ES cells. Thus we generated two independent lentiviral knockdown shRNAs for each of the Tet proteins and verified the knockdown efficiency by RT-qPCR (Fig. S6a). Interestingly, knockdown of Tet1, but not Tet2 or Tet3, resulted in morphological abnormality as well as decreased alkaline phosphatase (AP) activity (Fig. S6b).

In addition to morphological changes, knockdown of Tet1 also resulted in a reduced ES cell growth rate (Fig. 2a), which is not due to a significant increase in apoptosis (Fig. S7), but rather due to a self-renewal defect (Fig. 2b). To gain insight into the molecular mechanism underlying the self-renewal defect, we analyzed the effects of Tet1 knockdown on the expression of key stem cell factors. We find that Tet1 knockdown reduced Nanog expression (Fig. 2c), which was confirmed by Western blotting (Fig. 2d) and immunostaining (Fig. S8a). A minor decrease in the levels of Oct4 and Sox2 was also noticed (Fig. 2c and 2d). Consistent with a role for Tet1 in ES cell maintenance, Tet1 mRNA and protein are greatly down-regulated upon LIF withdrawal or retinoic acid induced differentiation (Fig. S8b, c). Collectively, the above results suggest that Tet1 plays an important role in ES cell self-renewal and maintenance.

To determine whether Tet1 knockdown causes spontaneous differentiation, we stained the cells with the mouse ES cell surface marker SSEA-1 and found that Tet1 knockdown resulted in 10–15% increase in SSEA-1 negative cells (Fig. S9a). RT-qPCR analysis of several markers of early differentiation demonstrates that knockdown of Tet1 in ES cells resulted in selective upregulation of Cdx2, Hand1, GATA6 and GATA4 (Fig. 2e), which is further supported by positive staining of Cdx2 and Troma-1 (TE cell markers), and GATA6 (primitive endoderm marker) in some of the knockdown cells (Fig. S9b). Collectively, our data supports a role for Tet1 in ES cell maintenance.

To understand the molecular mechanism underlying Tet1's function in ES cell maintenance, we asked whether Nanog is a direct Tet1 target by performing chromatin

immunoprecipitation (ChIP) assays using a Tet1 antibody. ChIP-qPCR analysis indicates that Tet1 binds to the proximal and distal T-DMR (amplicons 3 and 4) identified by K. Shiota and colleagues [8], but it does not bind to region I (amplicon 1) (Fig. 3a). MeDIP analysis demonstrates that knockdown of Tet1 resulted in increased DNA methylation in Tet1 binding regions, but has no effect on region I (Fig. 3a), consistent with a role for Tet1 in maintaining the Nanog promoter at a hypomethylated state. Interestingly, we also observed decreased Sox2 binding in response to Tet1 knockdown (Fig. S10). The ability of Tet1 to convert 5mC to 5hmC prompted us to ask whether 5hmC exists in the Nanog promoter. Unfortunately the available 5hmC Ab failed to specifically immunoprecipitate 5hmC under our assay conditions (Fig. S3e).

Previous studies have demonstrated that the proximal T-DMR of Nanog promoter is demethylated in ES cells, and its methylation correlates with Nanog silencing in TS cells [8]. To verify the MeDIP result, we performed bisulfite sequencing of this transcriptionally relevant region. Results shown in Fig. 3b demonstrate that knockdown of Tet1 resulted in an increase, from 2.8% to 32%, in the levels of DNA methylation at this region. To determine whether increased DNA methylation in Tet1 knockdown cells is responsible for Nanog down-regulation, we performed Tet1 knockdown in normal mouse J1 ES cells and J1 ES cells lacking DNMT1, 3a, and 3b (DNMT TKO) [9]. Similar to that observed in E14 mouse ES cells (Fig. 2c, d), knockdown of Tet1 in mouse J1 ES cells also resulted in down-regulation of Nanog expression at both the RNA and protein level (Figs. 3c, S11). However, a similar result is not observed in the DNMT TKO J1 ES cells (Figs. 3c, S11), indicating that Nanog down-regulation in Tet1 knockdown cells is dependent on DNA methylation. Alternatively, failure of DNMT TKO cells to down regulate Nanog expression can be due to the inability of the DNMT TKO cells to differentiate [10]. Collectively, these results support that Nanog is a direct Tet1 target and that Tet1 regulates Nanog expression by preventing the Nanog promoter from hypermethylation.

To determine whether Tet1's function in ES cells is mediated through maintaining Nanog expression, we asked whether the phenotypes exhibited in Tet1 knockdown ES cells can be rescued by exogenous Nanog expression. To this end, we generated lentiviral constructs that express Tet1 shRNAs with or without simultaneous expression of Nanog (Fig. S12a). Transduction of ES cells with the lentiviruses expressing the above constructs followed by puromycin selection allowed for the generation of stable knockdown ES cells with or without exogenous Nanog. Interestingly, both the morphological changes and the AP activity of the two independent Tet1 knockdowns are largely rescued by expression of exogenous Nanog (Fig. 3d). In addition, the growth and self-renewal defects caused by Tet1 knockdown are also partially rescued (Figs. 3e, S12b). Consistent with the phenotypic rescue, expression of exogenous Nanog also suppressed up-regulation of differentiation genes, such as Cdx2 and GATA6, caused by Tet1 knockdown (Fig. 3f). Collectively, these results support that Nanog is a key target gene that mediates the function of Tet1 in ES cells.

The fact that knockdown of Tet1 results in increased expression of trophectoderm (TE) and primitive endoderm marker genes raises the possibility that Tet1 may play an important role in the specification of cells of the inner cell mass (ICM). To address this possibility, we examined the expression and cellular localization of Tet1 in preimplantation embryos by immunostaining. We found that Tet1 is present from the one-cell embryo to the blastocyst, and is mainly located in the nuclei. Interestingly, Tet1 is relatively enriched in the ICM comparing to TE at the blastocyst stage (Fig. 4a). Consistently, RT-qPCR analysis indicates that Tet1 mRNA level is five-fold more in ICM-derived ES cells than TE stem cells (Fig. 4b). These results suggest that Tet1 might be important for the specification of ICM cells. To address this possibility, siRNAs that target Tet1 were injected into single blastomeres at the two-cell stage and its effect on TE and ICM cell specification was evaluated. To mark

the cells derived from the injected blastomere, mRNAs that encode the red fluorescent protein fused to histone H2B (H2B-mRFP) were co-injected with Tet1 siRNAs. To identify cells of TE lineage, immunostaining of the key TE transcription factor Cdx2 was performed. An analysis of the distribution of the H2B-mRFP (red) expressing cells that are Cdx2 positive (green) demonstrated that Tet1 knockdown cells preferentially contributed to TE over ICM cell lineage (Fig. 4c, S13a). Quantification analysis indicated that the ratio of Cdx2-RFP double positive cells over total number of RFP positive cells was significantly increased in Tet1 knockdown when compared with control injections (Fig. 4d), indicating that Tet1 knockdown favors embryonic cell specification towards the TE lineage. Similarly, staining of the Oct4 positive ICM cells indicate that Tet1 knockdown prevents embryonic cell specification towards the ICM (Figs. 4e, f, S13b). Collectively, these studies support a role for Tet1 in the formation of ICM consistent with the studies performed in ES cells.

Whether DNA methylation is a reversible reaction and, if so, what the underlying mechanism is has been a hotly debated issue [5]. While there is substantial evidence suggesting genome-wide [11,12] as well as locus specific DNA demethylation [13], the molecules that mediate DNA demethylation have been elusive [5]. Interestingly, a recent study indicates that the cytidine deaminase AID is involved in DNA demethylation in mouse primordial germ cells (PGCs) [14] although significant DNA demethylation still occurs in PGCs in the absence of AID. Using live cell imaging coupled with siRNA knockdown in mouse zygotes, we have recently demonstrated that the transcriptional elongator is required for paternal genome demethylation in zygotes [15]. However, how the elongator participates in the demethylation process is not known. In search of an enzyme that can modulate DNA methylation, a recent study revealed that human Tet1 could convert 5mC of DNA to 5hmC [6]. Here we confirm and extend this study by demonstrating that all the three mouse Tet proteins have similar enzymatic activities. Whether 5hmC can be further processed to C by an enzyme-catalyzed process or by base excision repair still awaits to be determined. In this regard, we note that a 5hmC-specific DNA glycosylase activity has been previously reported [16].

In addition to demonstrating the enzymatic activity of the Tet proteins, we provide evidence suggesting that Tet1 is a novel factor of the ES cell self-renewal network. Our data supports a working model by which Tet1 and DNMTs coordinately regulate Nanog expression. In ES cells, high levels of Tet1 either block the access of DNMTs or results in the enzymatic conversion of 5mC to 5hmC, which is further processed by another enzyme (directly or indirectly through a repair-based mechanism) to C, for maintained Nanog expression. On the other hand, when Tet1 is down-regulated in ES cells, DNMTs methylate the Nanog promoter, leading to the down-regulation of Nanog expression and consequent loss of ES cell identity. Previous studies have demonstrated a role for Nanog in ES cell self-renewal [17,18]. In addition, Tet1 also appears to have a role in maintaining ES cell fate, which is consistent with its role in ICM cell specification. Future studies should reveal the precise mechanism underlying Tet1's function in these processes.

Methods

Constructs and antibodies

Mouse Tet1 (GU079948), Tet2 (GU079949) cDNAs were cloned from E14Tg2A ES cells, and mouse Tet3 (Q8BG87) was cloned from ovaries of CD1 mice. Clones were generated using MonsterScript™ 1st strand cDNA synthesis kit (Epicentre) followed by PCR amplification with FailSafe™ (Epicentre). DNA fragments were subcloned into the Invitrogen TA cloning Kit following the manufacturer's protocol and verified by DNA sequencing. Plasmids encoding Flag-tagged catalytic domains of Tet1 (aa1367-2039), Tet2 (aa916-1921), and Tet3 (aa697-1668) were generated by subcloning of the DNA fragments

into BamH1 and XbaI sites (for Tet1) or EcoRI site (for Tet2, Tet3) of an N-terminal Flag-tagged pcDNA3 vector or an N-terminal Flag-tagged pFASTBAC vector. The catalytic mutants encoded by pcDNA3-Flag-Tet1 (H1652Y, D1654A) and pcDNA3-Flag-Tet2 (H1304Y, D1306A) were generated using the QuikChange II Xl site directed mutagenesis kit (Stratagene). For mTet1 Ab production, cDNA encoding mTet1 (aa1-184) was cloned into the BamHI and XbaI sites of pProEx HTb. All the constructs generated through PCR were verified by DNA sequencing.

mTet1 His-tagged antigen (aa1-184) was expressed in *E. coli* and purified using Talon superflow metal affinity resin (Clontech). Rabbit polyclonal anti-sera against mTet1(aa1-184) was generated by Pocono Rabbit Farm and Laboratory, Inc. The specificity of the affinity purified antibodies was tested using extracts from the control and Tet1 knockdown mES cells. The rabbit 5hmC antibody was purchased from Active Motif (catalog# 39769). All the other antibodies used in this study are listed in Supplementary Table 1.

Recombinant protein expression, purification, and activity assays

For production of recombinant proteins, the catalytic domain of Tet1, Tet2, and Tet3 cDNAs were cloned into a modified pFastbac-HTb (Invitrogen) vector containing an N-terminal Flag-tag. Generation of baculovirus that expresses Flag-Tet1(CD), Flag-Tet2(CD), or Flag-Tet2(CD) was performed using the Bac-to-Bac system (Invitorogen) according to the manufacturer's instructions. The recombinant proteins were purified from infected Sf9 cells with the anti-FLAG M2 antibody agarose affinity gel (Sigma-Aldrich) and eluted with buffer containing 10 mM Tris-HCl pH8.0, 150 mM NaCl, 1 mM DTT, 15% glycerol, and 0.2 μg/μl Flag peptide.

In vitro activity assay was performed using a modified procedure previously described [20]. Briefly, 5 μg of purified proteins were incubated with 0.5 μg of double stranded oligonucleotide substrates in 50 mM HEPES, pH 8, 75 μM $Fe(NH_4)_2(SO_4)_2$, 2 mM Ascorbate, and 1mM α-ketoglutarate for 3 hours at 37°C. Oligonucleotide substrates were purified using Qiaquick Nucleotide Removal Kit (Qiagen) and then digested with *Msp*I. 5'-end of the digested DNA was treated with calf alkaline phosphatase, and labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase. Labeled fragments were ethanol-precipitated, and digested with 10 μg of DNase I and 10 μg Phosphodiesterase I in the presence of 15 mM $MgCl_2$, 2 mM $CaCl_2$ at 37°C. One μl of digestion products was spotted on a PEI-cellulose TLC plate (Merck) and separated in isobutyric acid: water: ammonium hydroxide (66:20:2) running buffer. After drying, the TLC plate was exposed to X-ray film.

Mouse ES cell culture, Tet knockdown, RNA isolation and qPCR

Mouse feeder free E14Tg2A ES cells were maintained on gelatin-coated dishes in Glasgow Minimum Essential medium (GMEM; GIBCO), supplemented with 15% heat-inactivated fetal bovine serum, 55 μM β-mercaptoethanol (GIBCO), 2 mM L-glutamine, 0.1 mM MEM nonessential amino acid, 5,000 units/ml penicillin/streptomycin and 1,000 units/ml of ESGRO (Chemicon) under feeder-free conditions. J1 ES cells and Dnmt1−/−Dnmt3a−/−Dnmt3b−/− (DNMT TKO) ES cells were maintained on gelatin-coated dishes with mitotically inactivated mouse embryonic fibroblasts. Alkaline phosphatase staining was performed with Alkaline Phosphatase Detection Kit (Chemicon). For growth curve analysis, control and knockdown ESCs were plated at 1×10^3 cells/cm_2 and counted for 6 consecutive days. For self-renewal analysis cells were plated on 96 well plates at single cell density and the number of colonies on each plate was counted 6 days after plating.

To knockdown Tet proteins, lentiviral transduction was performed in mouse ES cells as described previously [19]. Short-hairpin RNA (shRNA) sequences (Supplementary Table 2) were cloned into pTY vetctor under the U6 promoter. To rescue Tet1 knockdown with Nanog, cDNA of Nanog was placed downstream of puromycin-resistant gene and foot-and-mouth disease virus 2A segment (Fig. S12), which enables multicistronic expression of transgenes in ES cells using a single promoter [21].

Total RNA from mouse tissues was isolated using Trizol reagent (Invitrogen) and total RNA from cultured cells was isolated using RNeasy Mini Kit (Qiagen), and cDNA was generated with Improm-IITM Reverse Transcription System (Promega). Real-time quantitative PCR reactions were performed on an ABI PRISM 7700 Sequence Detection System (Applied Biosystems) using SYBR Green reagent (Invitrogen). cDNA levels of target genes were analyzed using comparative Ct methods, where Ct is the cycle threshold number and normalized to GAPDH. RT-qPCR primers are listed in Supplementary table 3.

Immunostaining, confocal images, FACS analysis, and apoptosis assay

Mouse ES cells, U2OS cells, or HEK293T cells were plated onto glass coverslips and U2OS cells, or HEK293T cells were transfected with constructs encoding the wild-type or catalytic mutants and cultured for 2 days. Cells were fixed in 4% paraformaldehyde for 15 min. The cells were then washed with cold PBS and permeabilized for 15 min with cold PBS containing 0.4% Triton X-100. Permeabilized cells were then washed and incubated for 1 hour with blocking buffer (10% donkey serum, 3% bovine serum albumin in PBS containing 0.1% Triton X-100) before incubation with primary antibodies overnight in a humidified chamber at 4°C. For 5mC or 5hmC staining, permeabilized cells were denatured with 2N HCl for 15 min, neutralized with 100 mM Tris-HCl (pH 8.5) for 10 min before blocking. The primary antibodies used are listed in Supplementary table 1. After three consecutive 5-min washes with PBS, cells were incubated with secondary antibodies and 4,6-diamidino-2-phenylindole dihydrochloride (DAPI) for 30 min. Cells were washed again three times with PBS and then mounted in fluorescent mounting medium (Dako). Images were acquired using Zeiss immunofluorescence microscope Axiovert 200 and Axiovision software.

For preimplantation embryo staining, embryos at various developmental stages were incubated in blocking solution containing anti-Tet1, anti-Cdx2, or anti-Oct4 antibody for 1hr at room temperature. After washing with PBS, cells were incubated in FITC or Rhodamine-conjugated secondary antibody for 1hr at room temperature. Embryos were counterstained with DAPI. Fluorescent images were captured using a confocal microscope with a spinning disk (CSU-10, Yokogawa) and an EM-CCD camera (ImagEM, Hamamatsu). All images were acquired as 2μm-Z-axis intervals and reconstituted using Axiovision (Zeiss).

For apoptosis analysis, cells were stained with active caspase and Annexin V-FITC apoptosis detection kit with propidium iodide (EBiosciences) following manufacturer's instruction. Fluorescence intensity was determined by flow-cytometry on a Becton Dickinson FACscan. Data acquisition and analysis were performed with the Summit 4.0 software.

Knockdown of Tet1 in 2 cell embryos

Five to six week-old female BDF1 (C57BL6 X DBA2) hybrid mice were superovulated by injecting 7.5 I.U of PMSG (Harbor, UCLA) and 7.5 I.U of hCG. (Sigma Aldrich). For knockdown at the 2-cell stage, siRNAs (KD1 and KD2, 2μM each) that target Tet1 or siControl were co-injected into one of the blastomeres with histone H2B fused to monomeric red fluorescent protein (mRFP, 50ng/μl). The injected embryos were cultured in the KSOM media (EmbryoMax, Millipore) to blastocyst stage.

ChIP assays and bisulfite sequencing

Cells were fixed with a final concentration of 1% formaldehyde. After incubation at room temperature for 10 minutes, the reaction was stopped by the addition of 125 mM glycine. Chromatin immunoprecipitation (ChIP) assays were performed using a protocol associated with the ChIP assay kit (Upstate Biotechnology). After extensive washing, ChIPed DNA was eluted from the beads, and analyzed on an ABI 7300 Real Time PCR System (Applied Biosystems) using SYBR Green reagent (Invitrogen). Primer sequences and antibodies are listed in Supplementary Tables 4 and 1, respectively.

Bisulfite sequencing was performed as described previously with minor modifications [22]. Five microgram of sodium bisulfite-treated DNA samples was subjected to PCR amplification using the first set of primers; PCR products were used as templates for a subsequent PCR reaction utilizing nested primers. The PCR products of the second reaction were then subcloned using the Invitrogen TA cloning Kit following the manufacturer's instruction. PCRs and subcloning were performed in duplicate for each sample. The clones were sequenced using the M13 reverse primer. Primers for bisulfite sequencing are listed in Supplementary Table 5.

Dot-blot assay

Different amounts of standard DNA (949bp, Zimo Research-Cat#D5405) where C are either as C, 5mC, or 5hmC, or genomic DNA were denatured with 0.1 N NaOH and spotted on nitrocellulose membranes (BioRad-Cat#162-0112). The membrane was baked at 80°C and then blocked in 5% skim milk in TBS containing 0.1% Tween 20 (TBST) for 1 h at room temperature. The membranes were then incubated with 1:10000 dilution of 5hmC (Active Motif) or 1:1000 dilution of 5mC (Eurogentec) overnight at 4°C. After 3 times of washes with TBST, membranes were incubated with 1:2000 dilution of HRP-conjugated anti-rabbit or anti-mouse IgG secondary antibody, respectively. The membranes were then washed with TBST and treated with ECL.

HmeDIP

10 ng of control methylated or hydroxymethylated DNA were diluted in 480 μl 1x TE buffer. DNA was heat-denature for 10 min at 95 °C, and quickly cooled on ice for 5 min. Then 100 μl of 5X IP buffer (50 mM Sodium phosphate, pH7.0; 700 mM NaCl; 0.25% Triton X-100) was added to the denatured DNA. 5 μl of monoclonal antibody for 5-methylcytosine (Eurogentec), HmeC (active Motif) or rabbit IgG (Santa Cruz) were added, and the DNA/antibody mixture was incubated overnight at 4 °C on a rotating platform. Antibody/DNA complexes were captured for 2 hrs with protein G magnetic Dynabeads (Invitrogen) pre-blocked in 0.5% BSA in 1xPBS. Beads were washed three times with 1X IP buffer and resuspended in 250 μl digestion buffer (50 mM Tris-HCl, pH8.0; 10 mM EDTA; 0.5% SDS). To elute the DNA, 3.5 μl proteinase K was added and incubated overnight on a rotating platform at 55 °C. After a brief spin, the DNA was extracted with phenol-chloroform followed by ethanol precipitation and qPCR with primers H/me-1- F (AGGTGGAGGAAGGTGATGTC) and H/me-1- R (ATAAACCGAACCGCTACACC).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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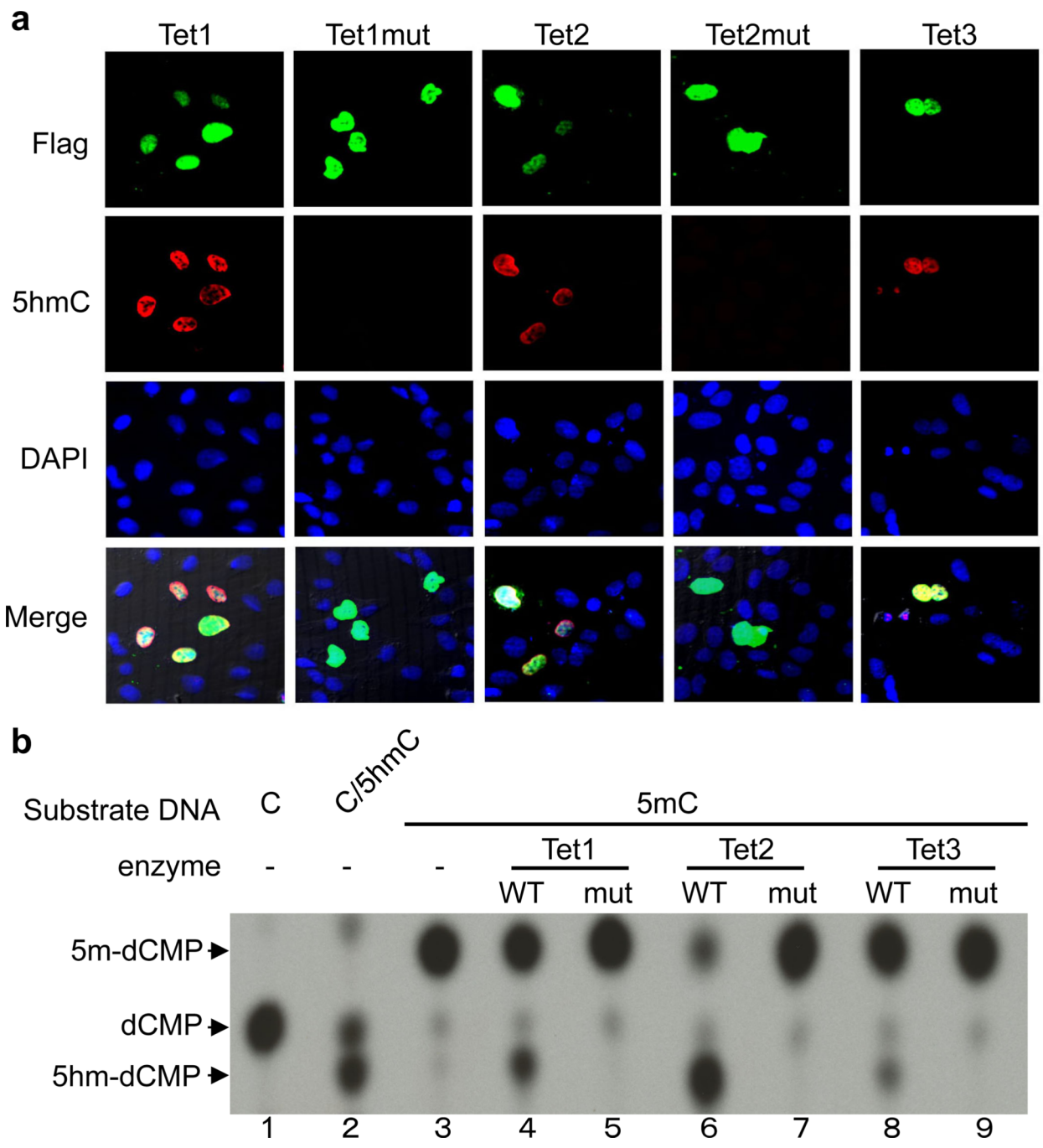

Figure 1. The Tet family proteins convert 5mC of DNA to 5hmC

a. Expression of wild-type Tet proteins, but not Fe(II)-binding mutants of Tet1 or Tet2, in U2OS cells results in the generation of 5hmC. Forty-eight hours post-transfection, the cells were co-stained with Flag and 5hmC antibodies. Nuclei are counterstained by DAPI.

b. Recombinant catalytic domains of Tet proteins, but not their catalytic mutants, convert 5mC in DNA oligonucleotides to hmC *in vitro*. Double stranded DNA oligonucleotides containing a fully methylated MspI site were incubated with wild-type and catalytic mutant forms of Flag-Tet1(aa1367-2039), Flag-Tet2(916-1921),

or Flag-Tet3(aa697-1668) proteins (1:10 enzyme to substrate ratio) in the presence of Fe(II) and α-KG. Recovered oligonucleotides were digested with MspI, end labeled with T4 DNA kinase, digested with DNaseI and phosphodiesterase, and analyzed by TLC. Unmethylated or 5hmC oligonucleotides containing the same sequences were used as a control for marking the migration of dCMP and hmC on TLC plates.

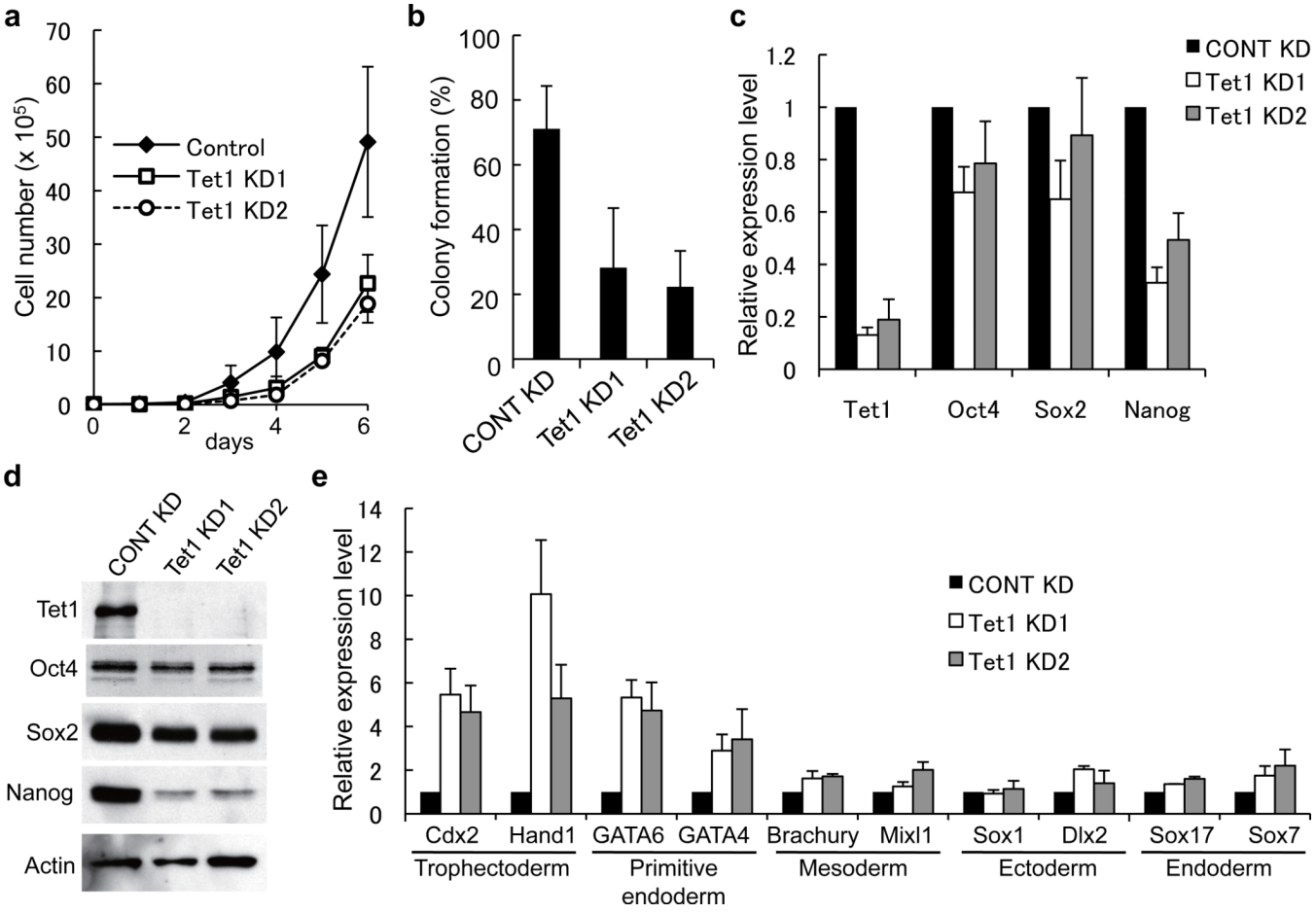

Figure 2. Knockdown of Tet1, but not Tet2 or Tet3, impairs ES cell self-renewal and maintenance

a. Tet1 knockdown impairs ES cell proliferation. Growth curves were determined for control and Tet1 knockdown (KD1 and KD2) cells by counting the cell numbers everyday. The average cell numbers with s.d. from three independent experiments are shown.

b. Tet1 knockdown impairs ES cell self-renewal. A single control or knockdown cell was plated in each well and its ability to form colonies was evaluated at day 6 after plating. There is no obvious difference between the colony size, but the colony number is greatly reduced in Tet1 knockdown cells when compared to that from control cells. Error bars represent s.d. of three independent experiments.

c. RT-qPCR analysis of expression levels of Tet1 and selected stem cell factors in control and knockdown cells. The expression levels in control cells are set as 1. Error bars represent s.d. of three independent experiments.

d. Western blot analysis of Tet1 and selected stem cell factors in control and knockdown cells. Actin is used as a loading control.

e. RT-qPCR analysis of the expression of various cell lineage marker genes in control and Tet1 knockdown ES cells. The expression level in control knockdown cells is set as 1. Error bars represent s.d. of three independent experiments.

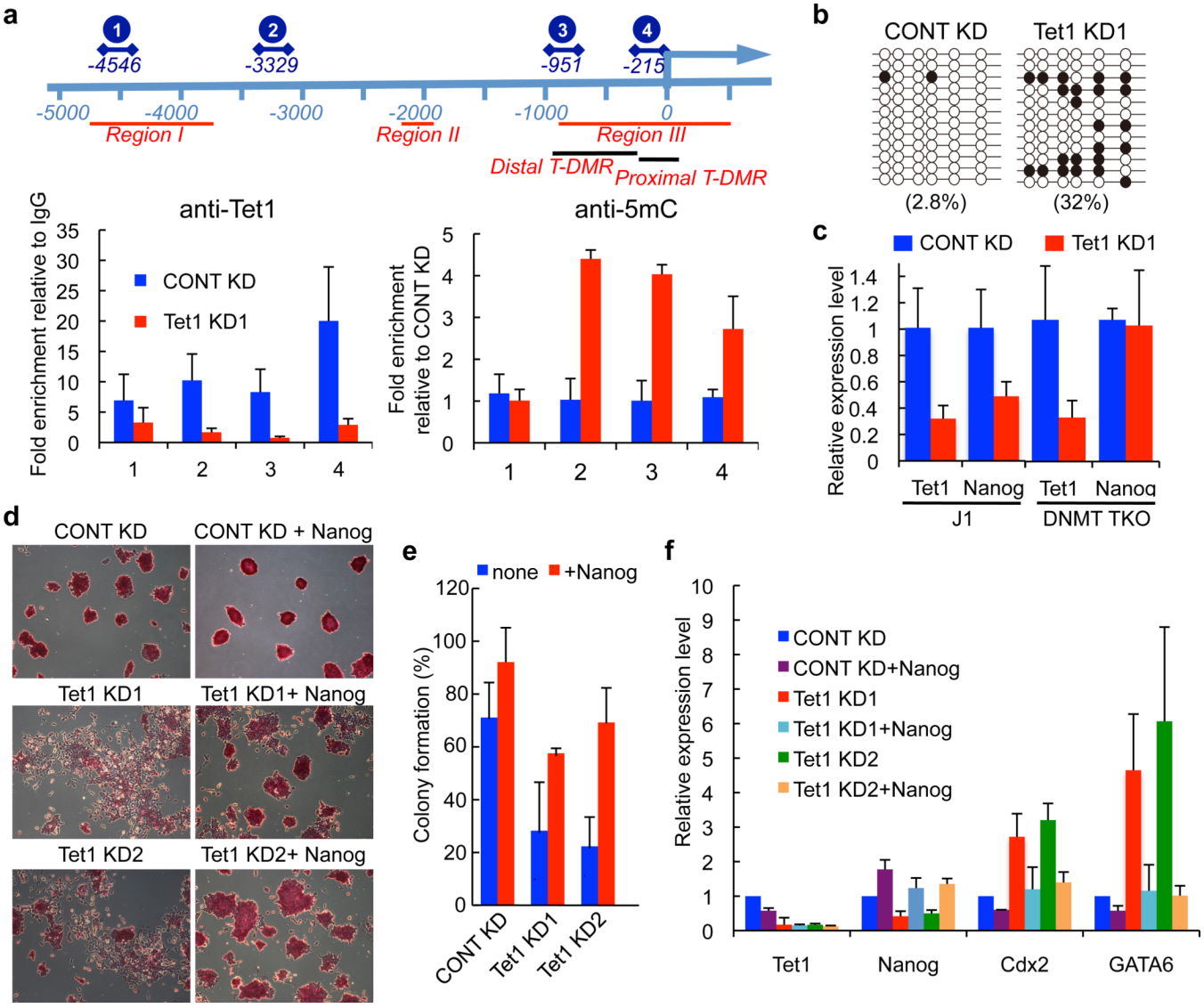

Figure 3. Nanog is a direct Tet1 target and Tet1 knockdown phenotypes can be partially rescued by expression of exogenous Nanog

a. ChIP analysis demonstrates that Nanog is a direct target of Tet1. Top panel is a diagram of the Nanog gene with the four amplicons indicated. The proximal and distal T-DMR as well as regions I and II are defined as that in a previous publication [8]. The numbers in the diagram refer to the gene coordinates. Bottom left panel is Tet1 enrichment in control and Tet1 knockdown cells relative to IgG controls. Bottom right panel is 5mC enrichment in Tet1 knockdown relative to control knockdown. Results presented are the average of three independent experiments with s.d.

b. Bisulfite sequencing results indicate that Tet1 knockdown in ES cells results in an increase in DNA methylation at the proximal T-DMR (Fig. 3a) of Nanog promoter. Open circles indicate unmethylated CpG dinucleotides, closed circles indicate methylated CpGs.

c. RT-qPCR analysis demonstrates that knockdown of Tet1 in wild-type J1 ES cells, but not in the DNMT TKO J1 ES cells, results in Nanog down-regulation. Error bars represent s.d. of two independent experiments.

d. AP staining of mouse ES cells after puromycin selection of cells infected with lentiviruses expressing control and Tet1 knockdown shRNAs with or without co-expression of Nanog.

e. The self-renewal defects associated with Tet1 knockdown can be partially rescued by expression of exogenous Nanog. Self-renewal assay was performed as that described in Fig. 2b.

f. RT-qPCR analysis demonstrates that expression of exogenous Nanog suppressed up-regulation of differentiation genes (Cdx2, GATA6) caused by Tet1 knockdown. Data shown is the average of three independent experiments with error bars (s.e.m).

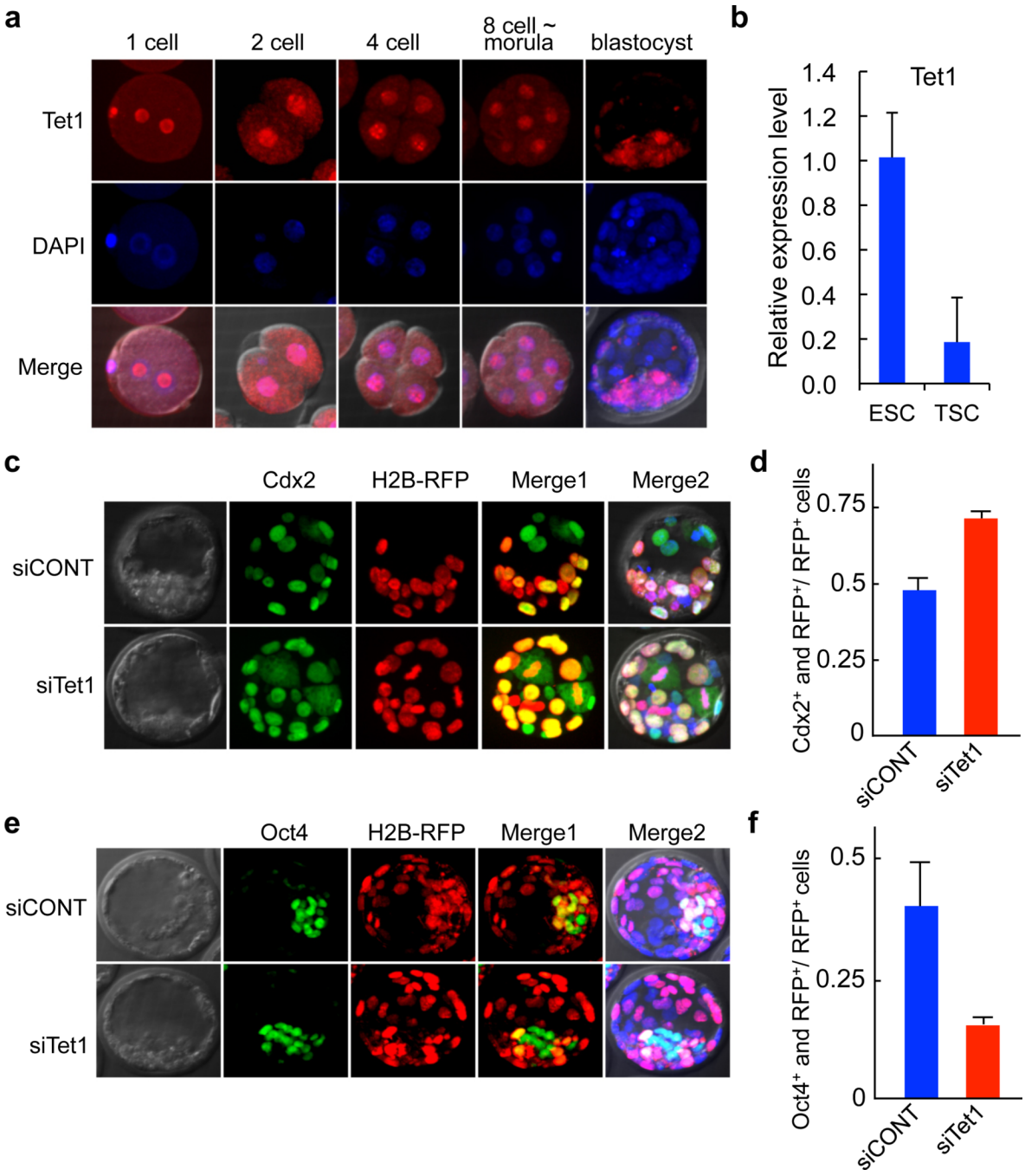

Figure 4. Tet1 is required for ICM cell specification in blastocysts

a. Tet1 protein is present in the nuclei of preimplantation mouse embryos. Mouse embryos at different developmental stages (one-cell to blastocyst) were stained with Tet1 antibody. Note that Tet1 is relatively enriched in the ICM when compared to that in the trophectodermal cells at blastocyst stage.

b. RT-qPCR demonstrates that ICM derived ES cells expressed higher levels of Tet1 when compared with TE stem cells. Error bars represent s.d. of three independent experiments.

c. Knockdown of Tet1 at the two-cell stage promotes trophectoderm cell specification. Representative Z-stack image of a control and a knockdown blastocyst are shown. Trophectoderm lineage cells are Cdx2 positive (green). Control knockdown and Tet1 knockdown cells are marked in red. Cells with yellow color in merge are the cells of TE lineage that were derived from the injected blastomere.

d. Ratio of Cdx2+RFP double positive cells over RFP positive cells from control knockdown and Tet1 knockdown. Eight control-injected embryos and 9 Tet1 siRNA injected embryos were used for the quantification analysis ($p<0.001$). Error bars represent s.e.m.

e. Knockdown of Tet1 prevents cells from contributing to ICM. Representative Z-stack images with Oct4 staining (green) in control and Tet1 knockdown cells (red). Cells with yellow color in merge are the cells derived from the injected blastomere that contributed to the ICM.

f. Ratio of Oct4+RFP double positive cells over RFP positive cells from control knockdown and Tet1 knockdown. Seven control-injected embryos and 7 Tet1 siRNA injected embryos were used for the quantification analysis ($p=0.004$). Error bars represent s.e.m.