Supporting Information

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SI Materials and Methods

Cell Culture and Transfection. HEK293T cells were obtained from the American Type Culture Collection and maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (vol/vol) FBS, 100 U/mL penicillin G, and 100 U/mL streptomycin. Transfections of microRNA (miRNA) precursors (20 nM; Ambion) or miRCURY miR-26 power family inhibitors (20 nM; Exiqon) were performed using HiPerfect (Qiagen) according to the manufacturer's protocol. For ten eleven translocation 2 (TET2) overexpression, dissociated pancreatic $CD133^+$ cells were transfected with or without TET2 overexpression plasmid (1) using Attractene (Qiagen) according to the manufacturer's protocol.

Real-Time Quantitative RT-PCR. Total RNA was extracted using Tri-Reagent (Molecular Research Center), and cDNA was synthesized using SuperScript III reverse transcriptase (Invitrogen), as described previously (2). Real-time quantitative RT-PCR (qRT-PCR) analyses of mRNA and miRNA were performed with the Power SYBR Green PCR Master Mix (Applied Biosystems) according to the manufacturer's instructions. qRT-PCR primers are listed as follows.

Luciferase Reporter Assays. Primers sequences for 3′-UTR cloning are listed as follows.

mRNA qRT-PCR primers

Gene	Strand	Sequence (5′–3′)
Tet1 (human)	F	GAGGGAAAAGAAGCCCAAAG
	R	TCTTCCCCATGACCACATCT
Tet2 (human)	F	AGAAAAGGGAAAGGAGAGCG
	R	GAGAGGGTGTGCTGCTGAAT
Tet3 (human)	F	GAGGGAAAAGAAGCCCAAAG
	R	TCTTCCCCATGACCACATCT
TDG (human)	F	TGAAGCTCCTAATATGGCAGTTG
	R	GGTTCTGTTGTTCTGGGTTTTCT
Tet1 (mouse)	F	GAGCCTGTTCCTCGATGTGG
	R	CAAACCCACCTGAGGCTGTT
Tet2 (mouse)	F	TGTTGTTGTCAGGGTGAGAATC
	R	TCTTGCTTCTGGCAAACTTACA
Tet3 (mouse)	F	CCGGATTGAGAAGGTCATCTAC
	R	AAGATAACAATCACGGCGTTCT
TDG (mouse)	F	TAGGAAACGTGCGTGTTCAG
	R	CTCATACTGCCAAACCAGCA
β-actin (human)	F	GTTGCTATCCAGGCTGTGCT
	R	AGGGCATACCCCTCGTAGAT
β-actin (mouse)	F	TAGGCACCAGGGTGTGATG
	R	CATGGCTGGGGTGTTGAAGG

TDG, thymine DNA glycosylase.

miRNA qRT-PCR primers

Name	Sequence (5′–3′)
miRGS-T	CATGATCAGCTGGGCCAAGA
miR-26a-F	TT+CA+AGTAATCCAGGA
miR-26a-GS	CATGATCAGCTGGGCCAAGAAGCCTATCCTGG
miR-26b-F	TT+CAAGTAAT+TCAGGA
miR-26b-GS	CATGATCAGCTGGGCCAAGAACCTATCCTGA

Note: "+" means LNA (locked nucleic acid) substitution.

Dissociation of Adult Pancreas. Pancreata from 2- to 4-mo-old mice were dissected and dissociated into single-cell suspension as described (3). In short, minced tissue was placed in phosphate buffered saline (PBS)/bovine serum albumin (BSA)-containing collagenase B (2–4 mg/mL per pancreas) (Roche) and DNase I (2,000 U/mL per pancreas) (Calbiochem), and incubated at 37 °C for 20–30 min to yield a predominantly single-cell suspension. To hasten digestion, tissue was gently pipetted every 5–10 min. The single-cell suspension was filtered through 20-μm cell strainers before use.

Primers for 3′-UTR cloning

Gene	Strand	Sequence (5′–3′)	Size (bp)
Tet1 (human)	F	GGGCTCGAGCCCTATAAC CATTGGGTCTGAAG	2,531
	R	ATAAGAATGCGGCCGCATCAG GAAAGAGAGTCCAACAAAG	
Tet2-s1 (human)	F	AGTCTCGAGACTTACC TGCGTTTCATCAAGTCT	279
	R	CTACTAGTAGATAACCTCT TTTGTTGCTGGTG	
Tet2-s2 (human)	F	AGTCTCGAGACTGGTTCTA TTATTGGACGAGATG	1,206
	R	CTACTAGTAGATTCACATGG AACTAACCCTGT	
Tet2-s3+4 (human)	F	TTCCTCGAGGAACTGAGAGTG AAAGCATTGTGT	383
	R	GAACTAGTTCCTGCTTGAAAC AGTCAAAAGA	
Tet2-S5+6 (human)	F	CAACTCGAGTTGGGGAGAGT TTACATAAGGAAG	433
	R	AAGGAAAAAAGCGGCCGCAAA AGGAAAACAGCTTTCAAAT AACCTGCATTC	
Tet3 (human)	F	TAGGCGATCGCCTATGCCT ACACGAAGGTCACTG	5,042
	R	GTACTAGTACTTGCAATTT CTAGGCACCCTAA	
TDG (human)	F	CCACTCGAGTGGAGTTAAGA GGAGAATCAGCTT	1,976
	R	TAACTAGTTAAAGGGGAAAGA ATACCACTCAG	

Flow Cytometry and Cell Sorting. The cell suspension was first incubated with anti-mouse CD16/32 (10 μg/m; BioLegend) for 5 min on ice to diminish nonspecific binding. Biotin-conjugated anti-mouse CD133 (clone 13A4; 5 μg/mL; eBioscience) or control biotin-conjugated rat IgG1 isotype (5 μg/mL; eBioscience) antibodies were added, and cells were incubated on ice for 20 min. After two washes with PBS/BSA, cells were incubated with streptavidin-labeled allophycocyanin (2 μg/mL; BioLegend) on ice for 15 min. Cells were washed twice in PBS/BSA and resuspended in PBS/BSA/DNase I that contained DAPI (0.2 μg/mL). Cell sorting was performed on an Aria-special order research product (SORP) (Becton Dickinson). All analyses included an initial gating of forward and side scatters to exclude cell debris. Sorting further excluded doublets by gating on forward-scatter width and side-scatter width, and live cells were selected by DAPI-negative staining. The purity of sorted population was routinely more than 95%.

In Vitro Colony Assays. Sorted CD133$^+$Sox9/EGFP$^+$ cells (Fig. 4) or CD133$^+$ cells (Fig. 5) were resuspended at 2.5×10^3 cells per 0.5 mL per well in methylcellulose-based colony culture medium as described previously (3–5). The semisolid culture mixture contained DMEM/F12 media, 1% methylcellulose (1,500 centipoise; Sinetsu Chemical), 50% (vol/vol) conditioned media from murine embryonic stem cell derived-pancreatic like cells, 5% (vol/vol) FCS, 10 mmol/L nicotinamide (Sigma), 10 ng/mL human recombinant activin-β, 0.1 nmol/L exendin-4, and 1 ng/mL vascular endothelial growth factor-A (R&D). When indicated, R-Spondin1 (RSPO1) (R&D) was used at 750 ng/mL; Matrigel, 5% (vol/vol); or laminin hydrogel (3), 100 μg/mL. Cells were plated in 24-well ultralow protein-binding plates and incubated in a humidified 5% (vol/vol) CO_2 atmosphere. To generate ring colonies for analysis, freshly sorted cells were plated in Matrigel-containing semisolid media and cultured for 3 wk. To generate endocrine/acinar colonies for analysis, total 3-wk-old primary colonies grown in Matrigel-containing semisolid media were pooled, washed twice with PBS/BSA, and dissociated into single-cell suspension by incubation with 0.25% trypsin-EDTA at 37 °C for 5 min. The single-cell suspension was then mixed in laminin hydrogel-containing semisolid media and incubated for an additional 2 wk.

Microfluidic qRT-PCR. The TaqMan probes purchased from ABI are listed as follows.

TETs and TDG Knockdown. Sorted pancreatic CD133$^+$Sox9/EGFP$^+$ cells were lentivirally transduced with appropriate short hairpin RNAs (shRNAs) as reported previously (6). shRNA sequences were cloned into the PLKO.1 vector under the control of the U6 promoter. Lentivirus was generated by cotransfecting the relevant construct and packaging plasmids into HEK293T cells. Transductions of pancreatic cells with lentiviral particles were performed using 2 mg/mL polybrene. Sixteen hours after transduction, cells were collected, washed, and plated in Matrigel with puromycin (2 μg/mL).

Murine gene	Assay ID from ABI
β-actin	Mm 00607939_s1
Insulin 1	Mm01259683_g1
Insulin 2	Mm 00731595_gH
Glucagon	Mm 00801712_m1
Somatostatin	Mm 00436671_m1
Pancreatic polypeptide (PPY)	Mm 00435889_m1
Amylase 2A	Mm02342487_g1
Carboxypeptidase A (CPA) 1	Mm 00465942_m1
Ngn3	Mm00436706_s1
Pdx1	Mm 00435565_m1

Dot Blot. Genomic DNA was isolated from samples with a QIAamp mini DNA kit (Qiagen) according to the manufacturer's instructions. Genomic DNA was denatured with 0.1 M NaOH and spotted on nylon membranes (Millipore). The membranes were UV cross-linked and then blocked in 5% (wt/vol) skim milk in Tris-buffered saline (TBS) containing 0.1% Tween 20 (TBST) for 1 h at room temperature. Membranes were then incubated with antibodies against 5-hydroxymethylcytosine (5hmC) (Active Motif; 1:10,000 dilution) or 5-methylcytosine (5mC) (Eurogentec; 1:1,000 dilution) overnight at 4 °C. After three washes with TBST, membranes were incubated with 1:2,000 dilution of horseradish peroxidase-conjugated anti-rabbit or anti-mouse IgG secondary antibody, respectively. The membranes were then washed with TBST and exposed to peroxidase-conjugated secondary antibodies (Amersham Bioscience). Signal of dots were quantified by ImageJ software.

Immunoblotting. Total cell lysis and Western blot analysis were performed as described previously (2). The following antibodies were used: anti-Tet1 (Millipore; 1:1,000 dilution), anti-Tet2 (Diagenode; 1:1,000 dilution), anti-TDG (Acris Antibodies; 1:1,000 dilution), and anti-HSP70 (Santa Cruz Biotechnology; 1:1,000 dilution).

Whole-Mount Immunostaining of Ring and Endocrine/Acinar Colonies. Antibody staining of DNA hydroxymethylation was carried out as previously described (3, 7). Briefly, colonies were handpicked and fixed in 4% (wt/vol) paraformaldehyde at 4 °C overnight. Colonies were then incubated with blocking buffer with 5% (vol/vol) donkey serum and 0.1% Triton X-100 at 4 °C overnight. Primary antibodies used are listed as follows and were detected with appropriate secondary antibodies conjugated to Cy3 or DyLight488 (1:2,000; Jackson ImmunoResearch). ApoTome images were captured on a LSM510 Meta Inverted 2 Photon microscope, and figures were prepared with Adobe Photoshop/Illustrator CS3.

Primary antibodies

Antigen	Species	Source	Dilution
Insulin	Guinea pig	Abcam	1:500
5hmC	Rabbit	Active Motif	1:500
Ngn3	Rabbit	Millipore	1:500
Ki67	Mouse	BD Pharmingen	1:500

Secondary antibodies

Antigen	Conjugation	Source	Dilution
Rabbit	DyLight-488	Abcam	1:2,000
Mouse/Guinea Pig	Cy3	Jackson ImmunoResearch	1:2,000

Islet Isolation from Postnatal Mice. Islet isolation was carried out as previously described with some modifications (8). Briefly, pancreata were dissected from postnatal day 4 or 7 mice, placed in a 50-mL conical tube with 1 mL of PBS containing collagenase B (2–4 mg/mL per 10 pancreata) (Roche) and DNase I (2,000 U/mL per 10 pancreata) (Calbiochem), and incubated at 37 °C for 5 min. The partially digested tissues were mixed with a cold buffer (designated as medium A; 40 mL) containing 0.1% 1 M Hepes and 0.04% Serum Replacement (Invitrogen) in HBSS, kept on ice for 2 min, and then centrifuged at $218 \times g$ for 2 min at 4 °C. Cell pellets were resuspended in 10 mL of Histopaque-1077 (Sigma), and 10 mL of medium A was slowly overlaid on top in a 50-mL conical tube, and centrifuged at $428 \times g$ for 25 min at 4 °C. The islets cell clusters enriched at the interphase were recovered and designated as the islet fraction, and the remaining clusters were designated as nonislet fraction.

Determination of Islet Number. Pancreata were dissected from postnatal day 10 mice, fixed in formalin, embedded in paraffin, sectioned (5-μm thickness), and stained with H&E. Total islet number per pancreas was determined as described previously (9).

1. Hahn MA, et al. (2013) Dynamics of 5-hydroxymethylcytosine and chromatin marks in Mammalian neurogenesis. *Cell Rep* 3(2):291–300.

2. Meng Z, et al. (2010) miR-194 is a marker of hepatic epithelial cells and suppresses metastasis of liver cancer cells in mice. *Hepatology* 52(6):2148–2157.

3. Jin L, et al. (2013) Colony-forming cells in the adult mouse pancreas are expandable in Matrigel and form endocrine/acinar colonies in laminin hydrogel. *Proc Natl Acad Sci USA* 110(10):3907–3912.
4. Ku HT, et al. (2007) Insulin-expressing colonies developed from murine embryonic stem cell-derived progenitors. *Diabetes* 56(4):921–929.
5. Winkler M, et al. (2011) A quantitative assay for insulin-expressing colony-forming progenitors. *J Vis Exp* (57):e3148.
6. Ito S, et al. (2010) Role of Tet proteins in 5mC to 5hmC conversion, ES-cell self-renewal and inner cell mass specification. *Nature* 466(7310):1129–1133.
7. Ficz G, et al. (2011) Dynamic regulation of 5-hydroxymethylcytosine in mouse ES cells and during differentiation. *Nature* 473(7347):398–402.
8. Zmuda EJ, Powell CA, Hai T (2011) A method for murine islet isolation and subcapsular kidney transplantation. *J Vis Exp* (50):2096.
9. Bock T, Pakkenberg B, Buschard K (2005) Genetic background determines the size and structure of the endocrine pancreas. *Diabetes* 54(1):133–137.

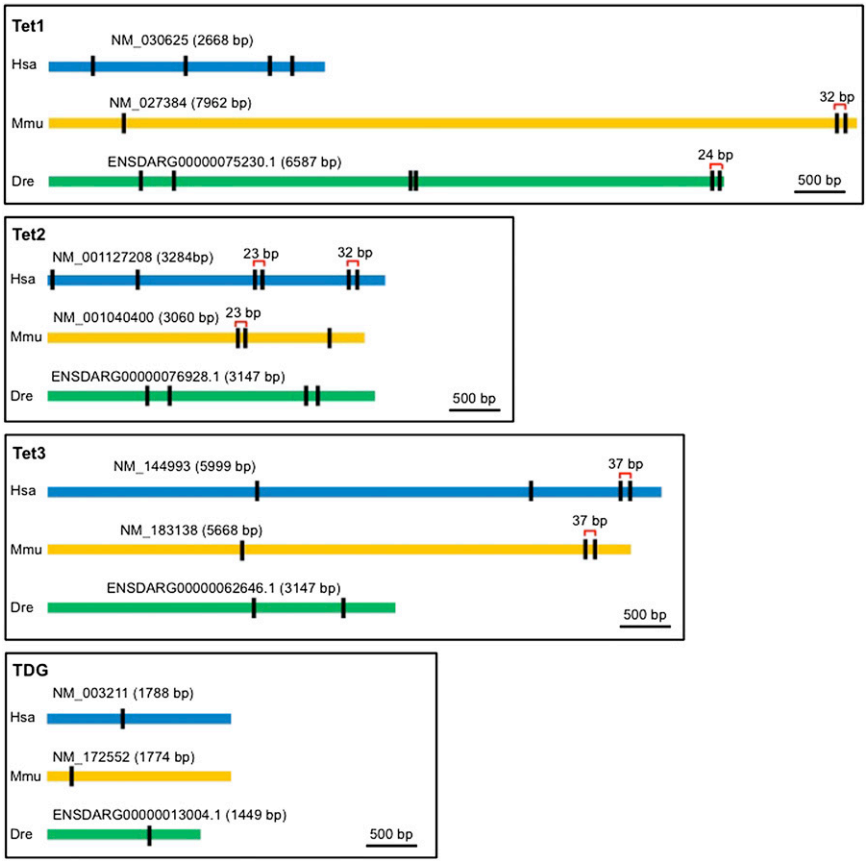

Fig. S1. Location of miR-26a target sites in the 3′-UTR of TET1/2/3 and TDG. TargetScan predicted potential miR-26 target sites in the 3′-UTR of TET1/2/3 and TDG are shown as black rectangles. Sites within cooperative distance of each other (within 40 nt, but no closer than 8 nt) are indicated (square brackets). Dre, zebrafish; Hsa, human; Mmu, mouse.

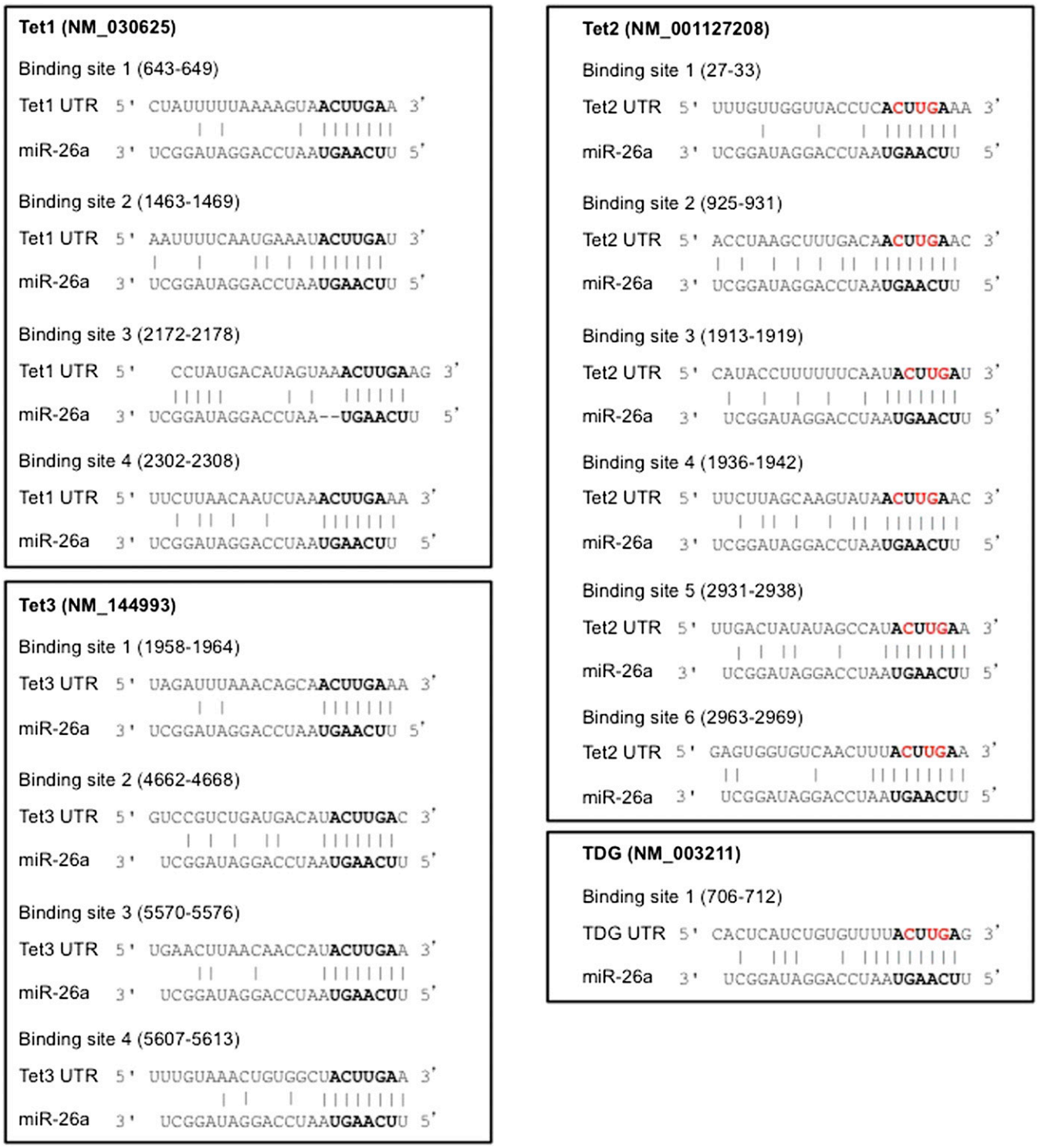

Fig. S2. Putative miR-26a target sites in the 3′-UTR of TET1/2/3 and TDG. The seed sequence of predicted miR-26a binding sites in the indicated human genes are shown by bolded sequence. Sites of point mutations made to abolish the potential pairing at the seed regions are shown in red.

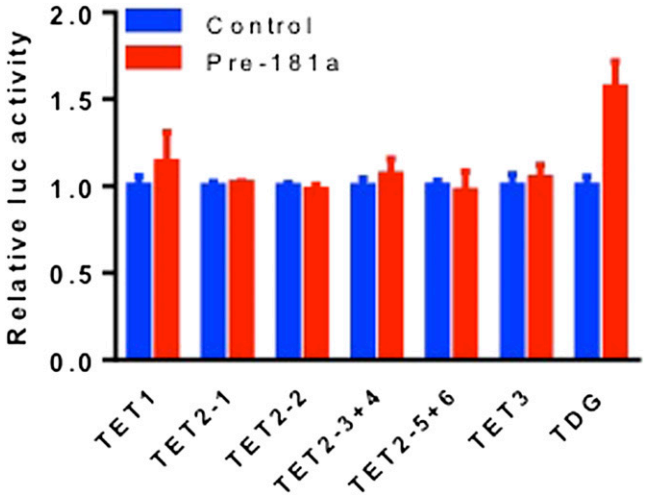

Fig. S3. TETs and TDG are not targeted by miR-181a. miR-181a does not have a putative target site within 3′-UTRs of TETs and TDG. HEK293T cells were transfected with (*i*) luciferase reporter constructs containing the 3′-UTRs of TETs or TDG and (*ii*) an miR-181a precursor (Pre-181a) or a negative control (Control). The luciferase activity of reporter construct cotransfected with the negative control was set to 1. The increased luciferase activity from the TDG construct by miR-181a may be due to an indirect effect of miR-181a on TDG expression.

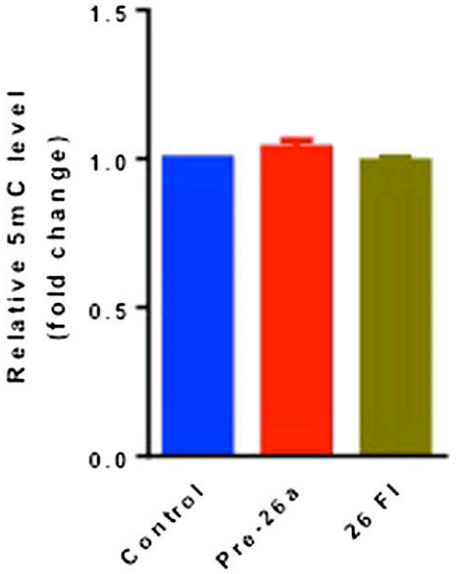

Fig. S4. miR-26a has minor roles in 5mC levels. HEK293T cells were transfected with a miR-26a precursor (Pre-26a), a miR-26 family inhibitor (26 FI), or their corresponding controls. After 48 h, the amount of 5mC in genomic DNA isolated from transfected cells was determined by dot blot analysis. Data are shown as mean ± SD.

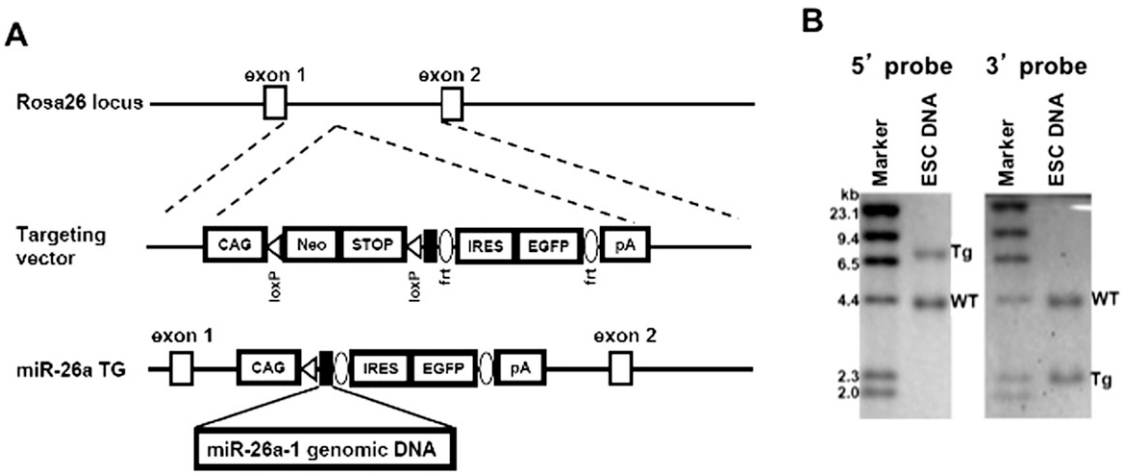

Fig. S5. Generation of miR-26a transgenic mice. (*A*) Scheme for generating miR-26a transgenic mice. (*B*) Southern blot analysis of genomic DNA from embryonic stem cells (ESCs) using 5′ probe (*Left*) and 3′ probe (*Right*), respectively.

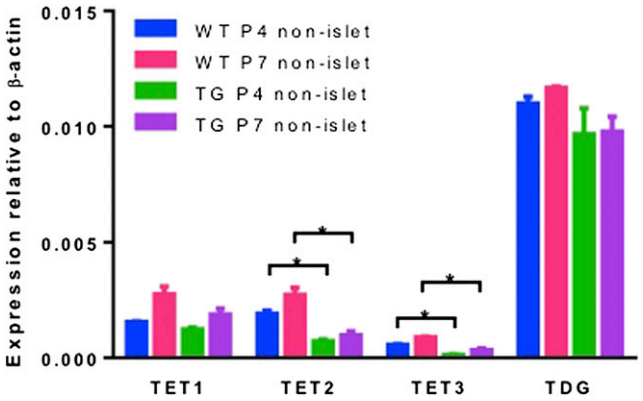

Fig. S6. Expression of TETs/TDG in nonislet cells. Primary islets and nonislet cells were isolated from miR-26a transgenic (TG) or wild-type (WT) mice on postnatal day 4 (P4) or P7. Expression of TETs/TDG in nonislet cells was determined by qRT-PCR. Data are shown as mean ± SD.

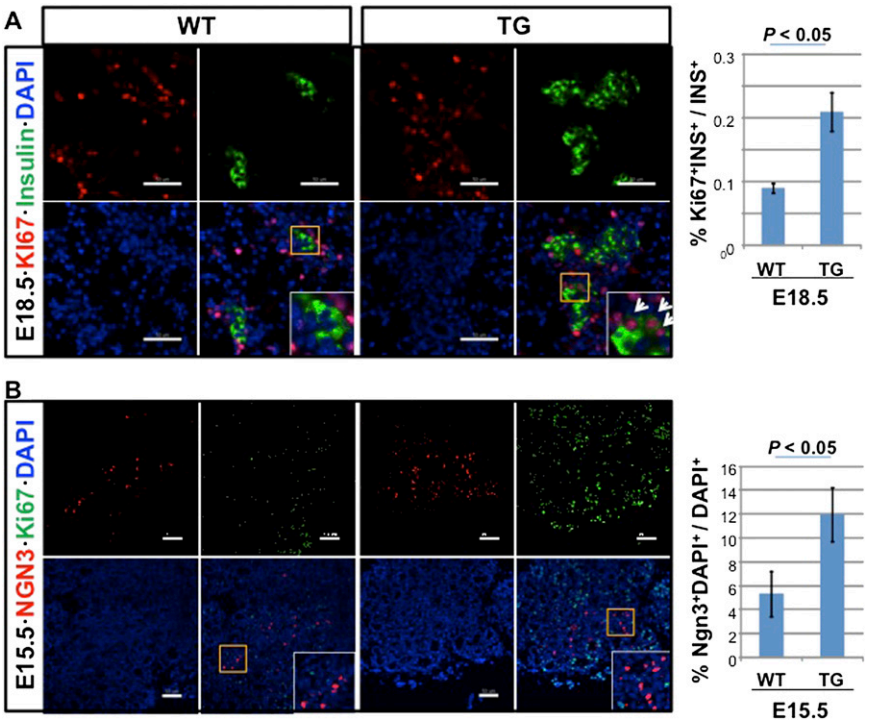

Fig. S7. miR-26a enhances proliferation and differentiation of the developing endocrine pancreas. Confocal images of double immunostaining for insulin and Ki67 (*A*) or Ngn3 and Ki67 (*B*) in E18.5 (*A*) or E15.5 (*B*) pancreas. (Scale bars, 50 μm.) (*A*) Insulin-expressing cells are actively proliferating (arrows). (*B*) Note the higher percentage of $Ngn3^+$ cells among total cells in miR-26a transgenic (TG) compared with wild-type (WT) pancreas.

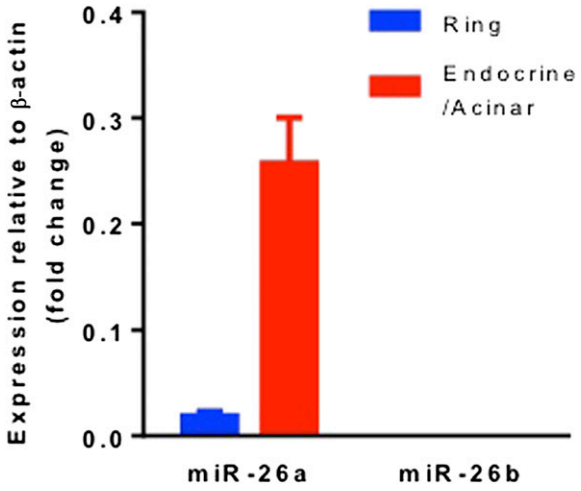

Fig. S8. miR-26a is the major miR-26 family member being expressed by in vitro differentiated cells. Pancreatic $CD133^+Sox9/EGFP^+$ cells were FACS sorted from adult Sox9/EGFP transgenic mice and plated in colony assays. miRNA expression in the resulting ring and endocrine/acinar colonies was determined by qRT-PCR. Data are shown as mean ± SD.

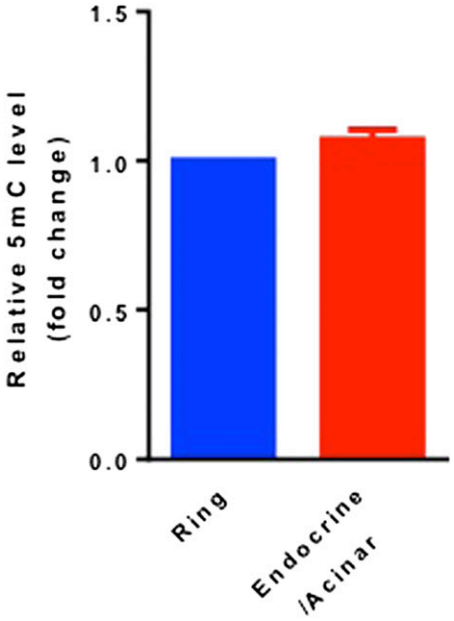

Fig. S9. 5mC levels are not altered in in vitro differentiated pancreatic cells. Amount of 5mC in genomic DNA isolated from ring or endocrine/acinar colonies was determined by dot blot analysis. Data are shown as mean ± SD.

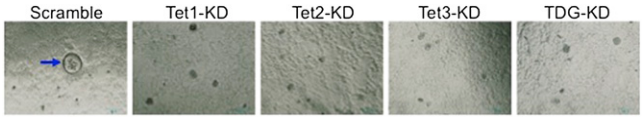

Fig. S10. Knockdown of TETs and TDG reduces the formation of ring colonies in vitro. CD133^{+}Sox9/EGFP^{+} cells were FACS sorted from adult (2- to 4-mo-old) pancreata of Sox9/EGFP transgenic mice and transduced with lentiviral vectors expressing shRNA against Tet1, Tet2, Tet3, or TDG. After 24 h of incubation, cells were plated in Matrigel-containing colony assay for 3 wk. The arrow indicates a resulting ring colony.

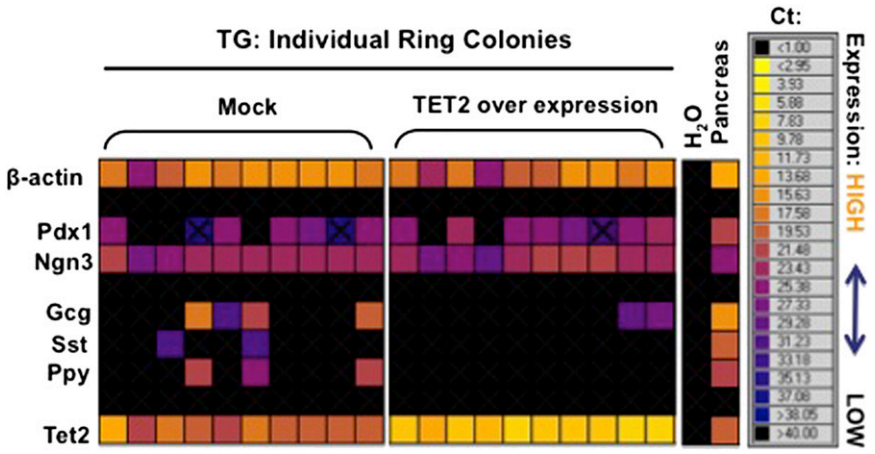

Fig. S11. TET2 overexpression inhibits endocrine cell differentiation in vitro. Microfluidic qRT-PCR analysis of individually handpicked ring colonies originated from adult miR-26a TG mice. FACS-sorted pancreatic CD133^{+} cells were transfected with or without TET2-overexpressing plasmids. After 48 h, cells were plated in Matrigel-containing colony assay and cultured for 3 wk for ring colony formation. Each column indicates a single colony.