Supplemental material for

Myc/Mycn-mediated glycolysis enhances mouse spermatogonial stem cell self-renewal

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Supplemental information for this paper contains materials shown below.

Supplemental Figures S1-7

Supplemental Tables S1-3

Supplemental Materials and Methods

Supplemental References

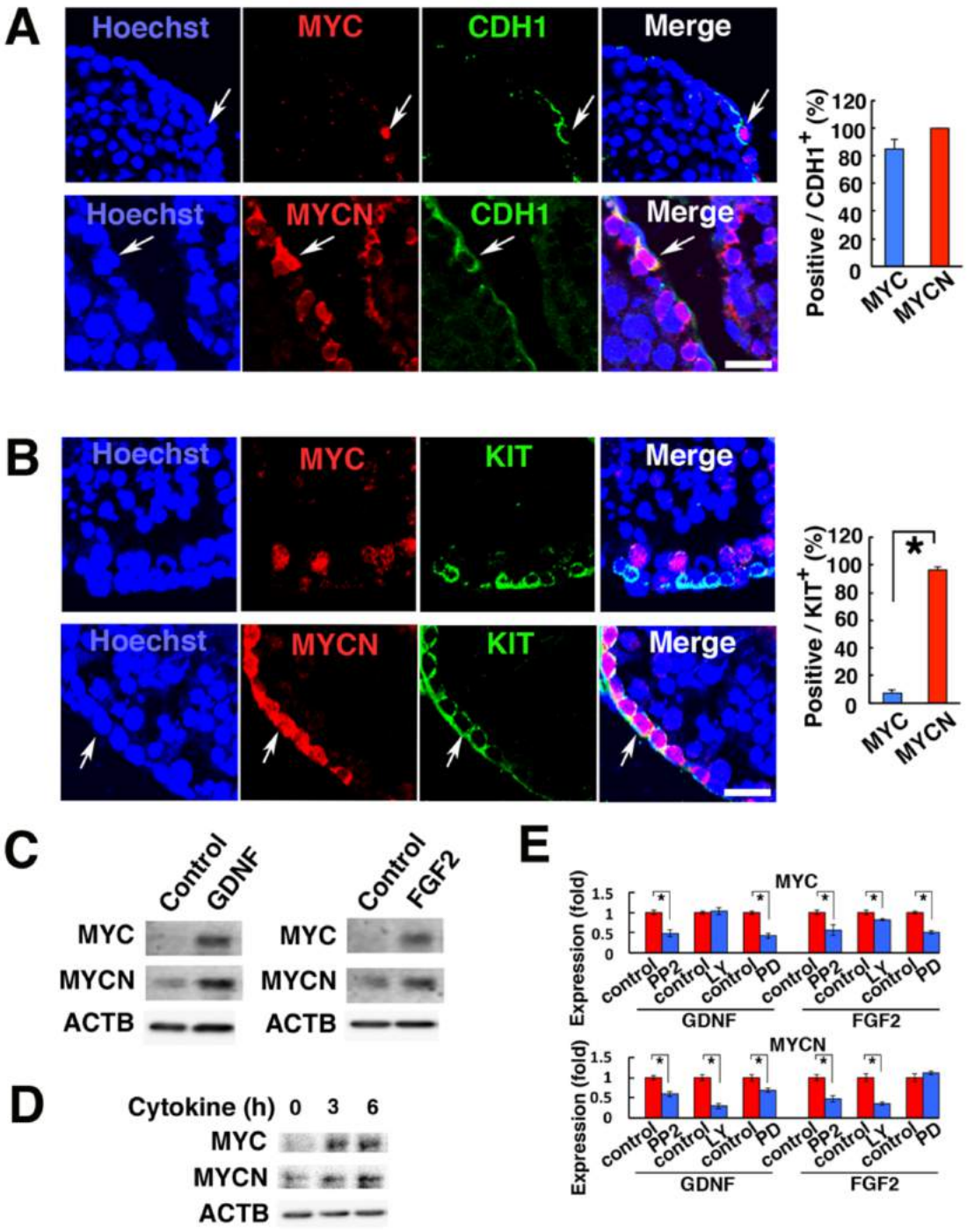

Figure S1 Expression of MYC/MYCN in vivo and in vitro. (A, B) Double immunohistochemisry of MYC/MYCN with CDH1 (A) and KIT (B) in adult testes. Arrows indicate cells expressing both antigens. Cells in 10 tubules were counted for

each antigen. MYCN was expressed more significantly than MYC in KIT^+ cells ($p < 0.0001$). (C) Western blot of MYC/MYCN after cytokine stimulation. GS cells were cultured without cytokines for 3 days. The indicated cytokines were added, and cells were collected 12 h after cytokine stimulation. (D) Time course of MYC/MYCN induction. GS cells were cultured without cytokines for 2 days. The indicated cytokines were added, and cells were collected at indicated time points after stimulation by GDNF and FGF2. (E) Quantification of Western blot in Figure 1B. Results of three independent experiments. Bar = 20 μm (A, B).

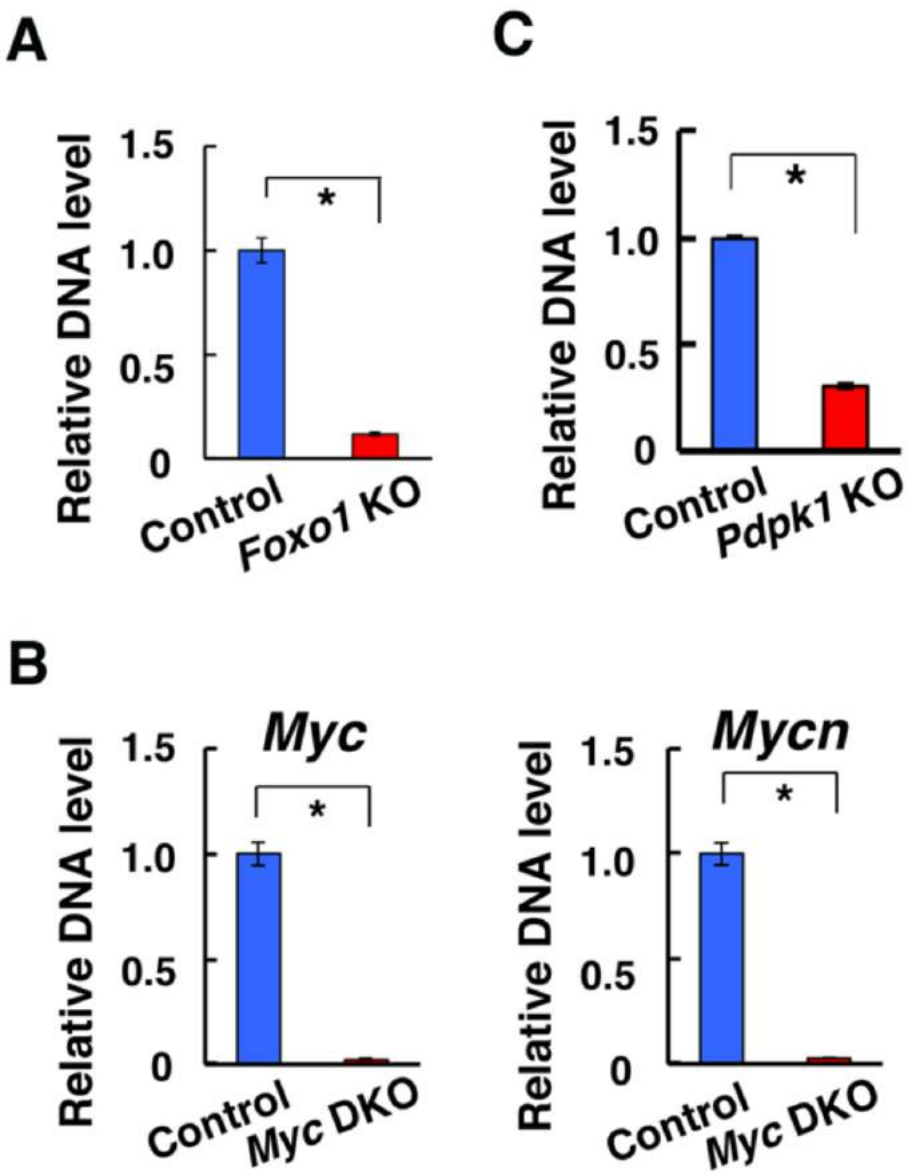

Figure S2 Estimation of deletion efficiency. Real-time PCR analysis of *Foxo1* (A; n = 3), *Myc* and *Mycn* (B; n = 4), and *Pdpk1* (C; n = 4). Cells were collected 9 (A) or 3 (B, C) days after AxCANCre treatment. The difference was statistically significant in all cases ($p = 0.0001$ for *Foxo1*; $p < 0.0001$ for *Myc*; $p < 0.0002$ for *Mycn*; $p < 0.0001$ for *Pdpk1*).

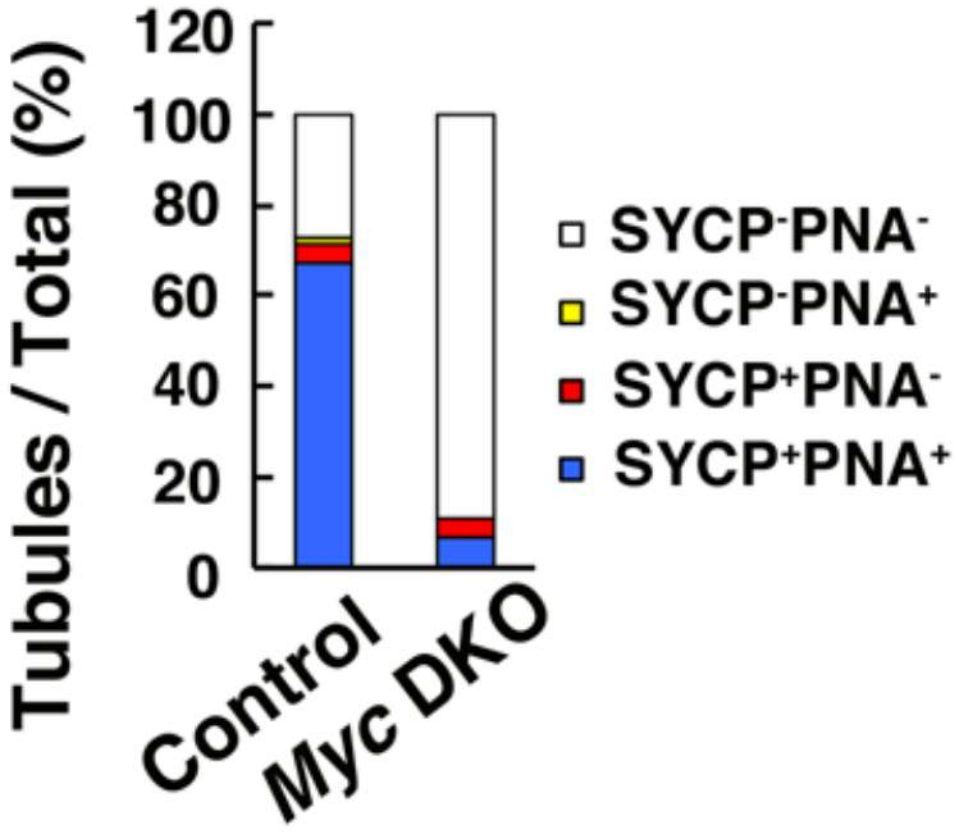

Figure S3 Quantification of seminiferous tubules in primary recipient testis that contain meiotic (SYCP) or haploid (PNA) marker-expressing cells. At least 153 tubules were counted in six testes.

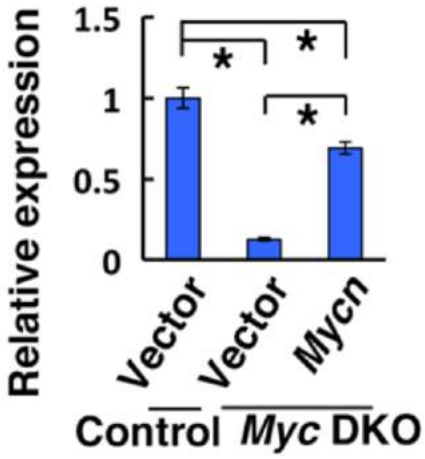

Figure S4 Real-time PCR analysis of *Mycn* expression in *Myc* DKO cells after transfection (n = 3-4). Cells were transfected with indicated vectors and passaged on the next day after transfection. Cell were recovered 7 days after passage. The differences were statistically significant in all cases ($p = 0.001$ for vector in control and *Myc* DKO cells or vector and *Mycn* in *Myc* DKO cells; $p = 0.004$ for vector in control cells and *Mycn* in *Myc* DKO cells).

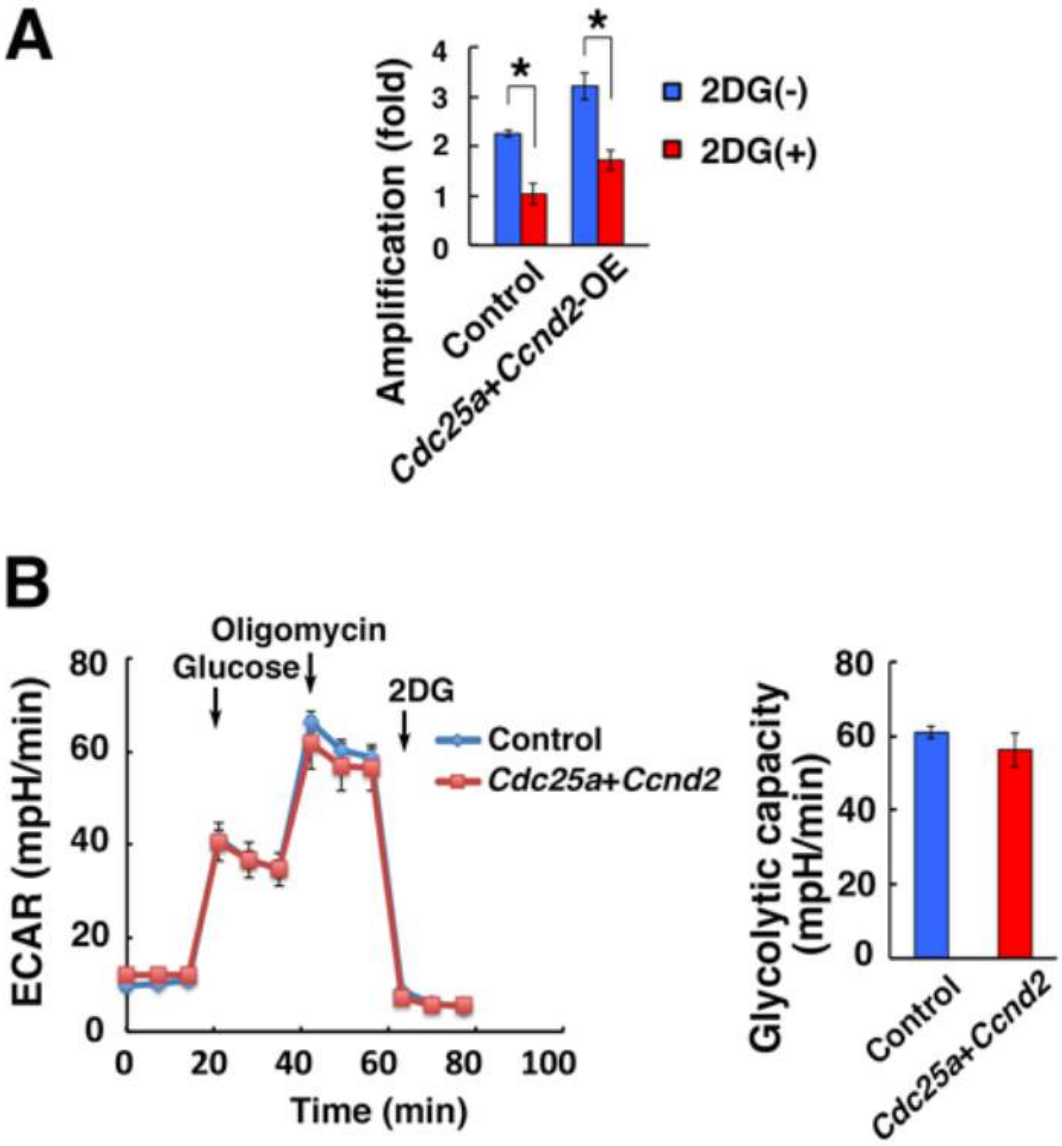

Figure S5 Characterization of *Myc* DKO GS cells transfected with *Cdc25a* and *Ccnd2*. (A) Suppression of proliferation by 2DG (5 mM; n = 3). Cells were passaged on the next day after transfection and cultured for 6 days. The differences were statistically significant in both cases (p = 0.007 for control; p = 0.01 for *Cdc25a* and *Ccnd2*). (B) ECAR (n =14). Cells were analyzed 3 days after transfection.

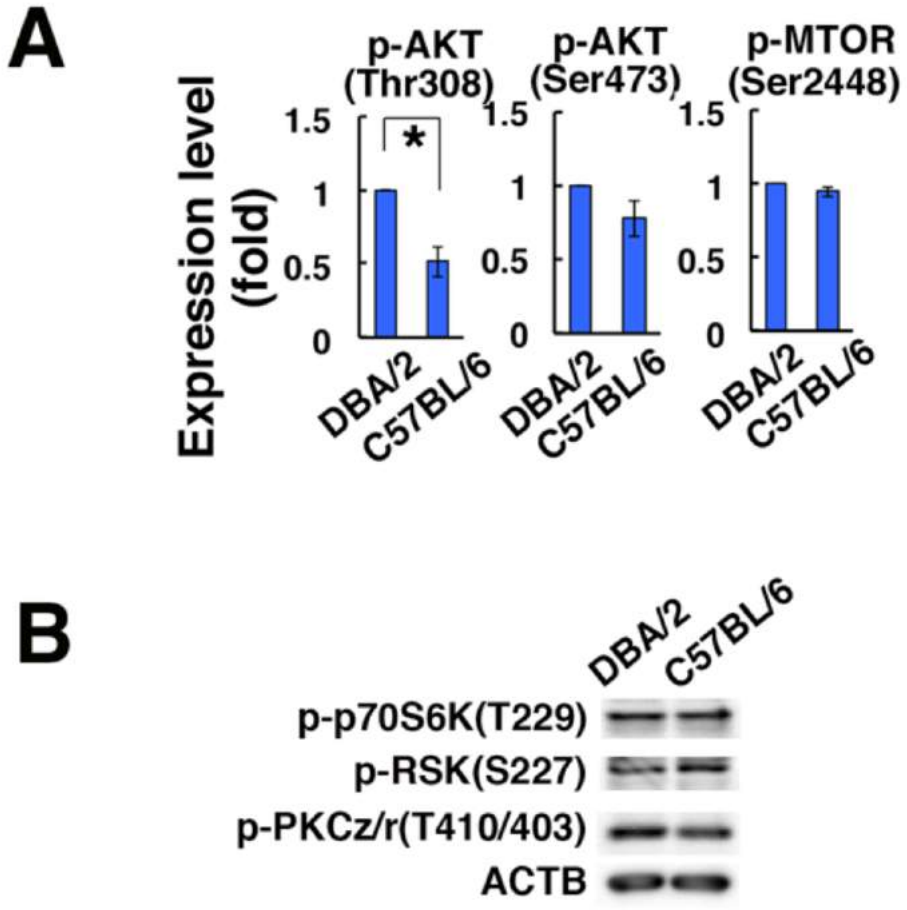

Figure S6 Activation of AKT in DBA/2 germ cells. (A) Quantification of AKT and MTOR phosphorylation in Figure 6B. Results of three independent experiments. Only p-AKT (Thr308) showed statistical difference ($p = 0.003$). (B) Western blot of 8-day-old DBA/2 and B6 germ cells after overnight culture with GDNF and FGF2. No significant changes were found between the two cell types using the indicated antibodies.

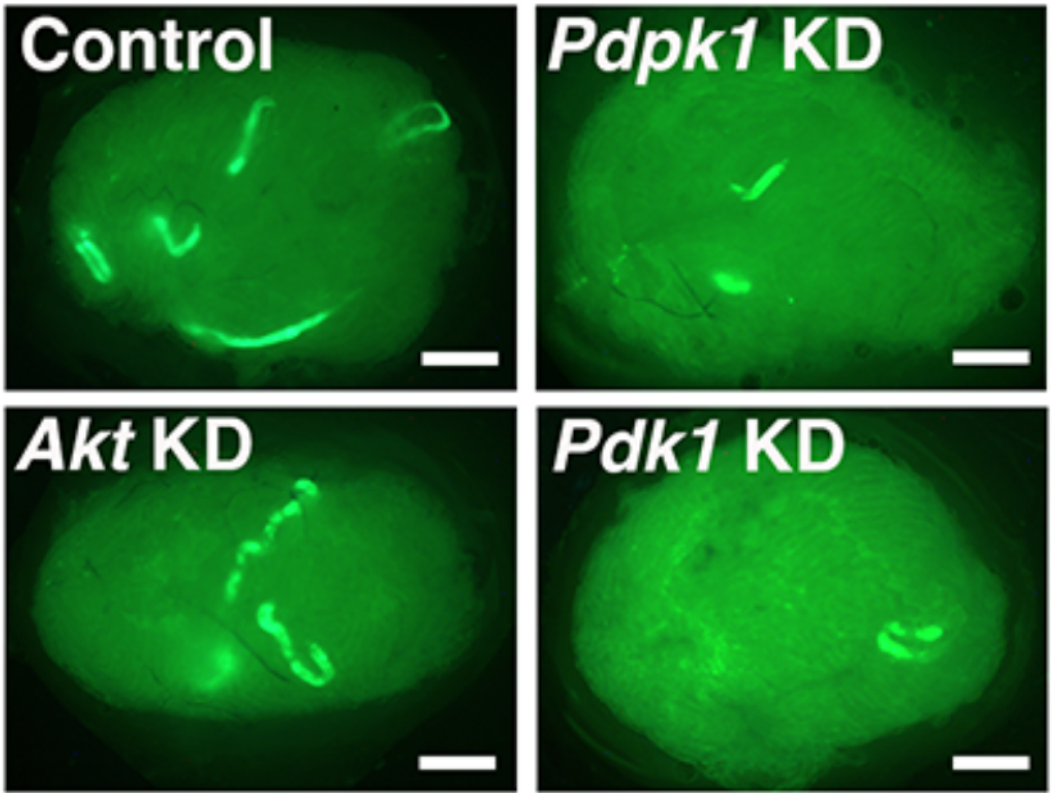

Figure S7 Macroscopic appearance of recipient testis that received transplantation of testis cells that had been transfected with lentivirus vectors expressing shRNA against *Pdpk1*, *Akt*, or *Pdk1*. Bar = 1 mm.

Supplemental Table S1 KD vectors used in this study

	Vector
Pdpk1	TRCN0000022734, TRCN0000022736, TRCN0000022737
Akt1	TRCN0000022934, TRCN0000022935, TRCN0000022936, TRCN0000022937
Akt2	TRCN0000055258, TRCN0000055261, TRCN0000055262, TRCN0000022670, TRCN0000022671
Akt3	TRCN0000054729, TRCN0000054725, TRCN0000054726, TRCN0000054727
Pdk1	TRCN0000078808, TRCN0000078809, TRCN0000078810, TRCN0000078811, TRCN0000078812

Supplemental Table S2 PCR primers used in this study

Genotyping	
Myc	TAAGAAGTTGCTATTTTGGC and TTTTCTTTCCGATTGCTGAC Mutant = 530 bp, Heterozygote = 530 bp and 400 bp, Wild type = 400 bp
Mycn	Common : GTCGCGCTAGTAAGAGCTGAGATC Forward : CACAGCTCTGGAAGGTGGGAGAAAGTTGAGCGTCTCC Mutant = 260 bp, Heterozygote = 217 bp and 260 bp, Wild type = 217 bp
Pdpk1	PDPK1-39**:** GCCTCTTTAGGCTTTTGGAGTCGGC PDPK1-40**:** GGAGAGGAGGAATGTGGACAAACAGC Mutant = 326 bp, Heterozygote = 326 bp and 264 bp, Wild type = 264 bp
R26YFP	oIMR8052: AAGACCGCGAAGAGTTTGTC Rosa26R_316: GGAGCGGGAGAAATGGATATG

	Rosa26R_883: AAAGTCGCTCTGAGTTGTTAT Mutant = 320 bp, Heterozygote = 320 bp and 600 bp, Wild type = 600 bp	
qPCR	Forward	Reverse
Nanos2	CCATATGCAACTTCTGCAAGC	TGAGTGTATGAGCCTGGTCG
Nanos3	CTTCTGTCTACTGCTACACCACC	TTGGAACCTGCATAGACACC
Ngn3	AGCAGAGAGGCTCAGCTATCC	AACTGAGCACTTCGTGGTCC
Zbtb16	TCGCTCACATACAGGTGACC	CACACTCAAGAGCCACAAGC
Pou5f1	GAAGTTGGAGAAGGTGGAACC	CTTAAGGCTGAGCTGCAAGG
Bcl6b	CCCGGGCTCAAGAGACTTC	TTCCTGGCGGTGGATTAGC
Etv5	AACTTGGTGCTTCATGCTCC	ACTTAGCACCAAGAGCCTGC
Ccnd1	GTTCATTTCCAACCCACCC	CTCAGATGTCCACATCTCGC
Ccnd2	TTCATTGAGCACATCCTTCG	TTCATCATCCTGCTGAAGCC
Ccnd3	CTATACGGACCAGGCTGTGG	ATGGATGGAGGATACATCGC
Cdkn1b	AGGAGAGCCAGGATGTCAGC	GAATCTTCTGCAGCAGGTCG
Mycn	CCAGAGCGGAGGTCTTGG	CCGGAGAGGATACCTTGAGC
Hprt	GCTGGTGAAAAGGACCTCT	CACAGGACTAGAACACCTGC
Deletion	Forward	Reverse
Foxo1	GTGAAGAGCGTGCCCTACTT	TCCTTCATTCTGCACTCGAA
Hprt	GCTGGTGAAAAGGACCTCT	CACAGGACTAGAACACCTGC
Myc-exon1	CTCGCTGTAGTAATTCCAGC	ACTCCAGAGCTGCCTTCTTA
Myc-exon2	AAAGACAGCACCAGCCTGAG	AAGACCACTGAGGGGTCAAT

Mycn-exon1	GTGACAGTCATCTGTCTGGAC	TGCAGTGTGTGCGCGCTTAC
Mycn-exon3	AACAACAAGGCGGTAACCAC	GATGGGAACACAGCGCTTGA
Pdpk1-exon2	ACCACAGCCTCGCAAGAAAC	AACAAGCAACAGCAGCAGAG
Pdpk1-intron3-4	AGGCAATCTGGGTAGTCGCA	GTTGTCCCAAAACCACCCAC

Supplemental Table S3 Antibodies used in this study

Flow cytometry		
Antigen	**Name**	**Company**
KIT	APC-conjugated rat anti-mouse c-kit	eBioscience (clone ACK2)
EPCAM	Rat anti-mouse EpCAM	BioLegend (clone G8.8)
ITGA6	APC-conjugated rat anti-mouse CD49f (α6-integrin)	BD Biosciences (clone GoH3)
ITGB1	Biotin-conjugated hamster anti-rat CD29 (β1-integrin)	BD Biosciences (clone Ha2/5)
GFRA1	Biotin-conjugated goat anti-rat Gfrα1	R&D systems (BAF560)
FUT4	Biotin-conjugated anti-mouse/human SSEA-1	eBioscience (clone MC-480)
CD9	APC-conjugated rat anti-mouse CD9	eBioscience (clone KMC8)
Secondary reagents		
APC-conjugated Streptavidin		eBioscicence (17-4317)
APC-conjugated goat anti-rat IgG+IgM		BD Bioscience (551019)
Western blotting		

Antigen	**Name**	**Company**
MYC	Rabbit anti-c-Myc	Cell Signaling (5605)
MYC	Rabbit anti-c-Myc	Cell Signaling (9402)
MYCN	Rabbit anti-n-Myc	Cell Signaling (9405)
CCND1	Mouse anti-human Cyclin D1	Cell Signaling (2926)
CCND2	Rabbit anti-Cyclin D2	Cell Signaling (2924)
CCND3	Mouse anti-human Cyclin D3	Cell Signaling (2936)
CCNE	Rabbit anti-rat Cyclin E	Abcam (ab7959)
CDC25A	Mouse anti-mouse Cdc25A	Santa Cruz Biotechnology (sc-7389)
CDC25B	Rabbit anti-mouse Cdc25B	Santa Cruz Biotechnology (sc-326)
CDC25C	Rabbit anti-human Cdc25C	Santa Cruz Biotechnology (sc-327)
CDKN2A	Mouse anti-mouse p16	Santa Cruz Biotechnology (sc-1661)
CDKN2D	Rat anti-mouse p19 ARF	Santa Cruz Biotechnology (sc-32748)
CDKN1A	Mouse anti-human p21	BD Pharmingen (556431, clone SXM30)
CDKN1B	Rabbit anti-mouse p27 Kip1	Cell Signaling (2552)
CDKN1C	Rabbit anti-human p57 Kip2	Abcam (ab4058)
p-AKT(Thr308)	Rabbit anti-mouse p-AKT(Thr308)	Cell Signaling (9275)

p-AKT(Ser473)	Rabbit anti-mouse p-AKT(Ser473)	Cell Signaling (9271)
AKT	Rabbit anti-mouse AKT	Cell Signaling (9272)
P-MTOR(Ser2448)	Rabbit anti-mouse p-MTOR (Ser2448)	Cell Signaling (5536)
MTOR	Rabbit anti-mouse mTOR	Cell Signaling (2983)
MAP2K1	Mouse anti-MEK1	Cell Signaling (2352)
p-MAP2K1/2 (Ser217/221)	Rabbit anti-human p-MEK1/2 (Ser217/221)	Cell Signaling (9121)
RPS6KB1 (T229)	Rabbit anti-human P70 S6Kinase alpha (T229)	Abcam (ab195982)
p-RSK (Ser 227)	Rabbit anti-human p-RSK (Ser 227)	Santa Cruz Biotechnology (sc-12445)
p-PKCζ/λ (Thr410/403)	Rabbit anti-human p-PKCζ/λ	Cell Singnaling (9378)
p-PDHA1 (Ser293)	Rabbit anti-human p-PDHA1 (Ser293)	Abcam (ab92696)
PDPK1	Rabbit anti-human PDPK1	Cell Singnaling (3062)
Foxo1	Rabbit anti-human Foxo1A	Abcam (ab39670)
ACTB	Mouse anti- β-actin	Sigma (clone AC-15)
Secondary reagents		
HRP (horseradish peroxidase)-conjugated horse anti-mouse IgG		Cell Signaling (7076)
HRP-conjugated horse anti-rabbit IgG		Cell Signaling (7074)
HRP-conjugated goat anti-rat IgG+IgM		Jackson Immunoresearh

		(112-035-044)
Immunofluorescence		
Antigen	**Name**	**Company**
MYC	Rabbit anti-human c-Myc	Santa Cruz (sc-764)
MYCN	Rabbit anti-human n-Myc	abcam (ab24193)
GFRA1	Goat anti-rat Gfrα1	R&D systems (AF560)
CDH1	Alexa Fluor 647-conjugated rat anti-CD324 (CDH1)	eBioscience (clone DECMA-1)
CDH1	Rat anti-CD324 (CDH1)	eBioscience (clone DECMA-1)
KIT	Rat anti-mouse CD117 (c-kit)	eBioscience (clone 2B8)
SYCP3	Rabbit anti-human SYCP3	Abcam (ab15093)
Secondary reagents		
Alexa Fluor 647-conjugated goat anti-rabbit IgG		Molecular Probes (A21245)
Alexa Fluor 647-conjugated goat anti-rat IgG		Molecular Probes (A21247)
Alexa Fluor 647-conjugated donkey anti-rabbit IgG		Molecular Probes (A31573)
Alexa Fluor 568-conjugated goat anti-rabbit IgG		Molecular Probes (A11011)
Alexa Fluor 488-conjugated goat anti-rat IgG		Molecular Probes (A11006)
Alexa Fluor 488-conjugated goat anti-rabbit IgG		Molecular Probes (A11008)
Alexa Fluor 488-conjugated donkey anti-goat IgG		Molecular Probes (A11055)
Rhodamine peanut agglutinin		Vector Laboratories (RL-1072)

Supplemental Materials and Methods

Cell culture

Wild-type (WT) and green GS cells were cultured using GDNF and FGF2 (Peprotech, London, UK), as described previously (Kanatsu-Shinohara et al., 2011). PP2 (10 μM), LY294002 (33 μM; both from Selleck Chemicals, Houston, TX), and PD0325901 (5 μM; Stemgent, San Diego, CA) were added to the culture at the time of plating. We also used PS48 (5 μM) and 2DG (both from Wako, Kyoto, Japan) for culture. In some experiments, cells were cultured on C166 (ATCC, Tokyo, Japan). For virus transfection, cDNAs encoding mouse *Myc*, *Mycn* (gift from Dr. S. Yamanaka, Kyoto University, Kyoto, Japan), *MycV394D* (Dr. M. Eilers, Biozentrum Universität Würzburg, Würzburg , Germany), omomyc (Dr. L. Soucek, Vall d'Hebron Institute of Oncology, Barcelona, Spain), *Cdc25a*, constitutive active *Akt1* (Dr. T. Kimura, Osaka University, Osaka, Japan), constitutive active *Pdpk1* (Dr. K. Aoyanagi, Kryorin University), and *Pdk1* (transOMIC technologies; Huntsville, AL) were cloned into CSII-EF-IRES2-Venus vector. *Ccnd2*–expressing lentivirus was previously described (Lee et al., 2009). Virus particles were produced by transient transfection of 293T packaging cells, as previously described (Kanatsu-Shinohara et al., 2008). Cells were maintained on mitomycin C-treated MEFs. Titer of the virus was determined by infecting 293T cells, and moi was adjusted to 10.0. However, moi for B6 testis cell transfection by constitutive active *Pdpk1* or *Akt1* was adjusted to 4.0, and *Foxo1* KO GS cell rescue experiment by *Mycn* transfection was carried out at moi 8 and 24.

For shRNA-mediated gene KD, KD vectors were purchased from Open

Biosystems (Huntsville, AL). A mixture of lentiviral particles was used to transfect testis cells. pLKO1-Scramble shRNA (Addgene) was used as a control. The lentivirus titer was determined by a Lenti-X-p24 rapid titer kit (Clontech, Mountain View, CA). Moi of the virus was adjusted to 4.0. All KD vectors are listed in Supplemental Table S1.

Metabolic analysis

Assays were performed in accordance with manufacturer's instructions (Seahorse Bioscience, North Billeric, MA). Cells (3-4.5 × 10^5 cells) were cultured in XF96 well plate, and OCR and ECAR were measured using the Seahorse XF96 analyzer, as previously described (Takubo et al., 2013). In brief, respiration was measured under basal conditions, in the presence of mitochondrial inhibitor oligomycin (2 μM), mitochondrial uncoupling compound FCCP (4 μM), and respiratory chain inhibitor antimycin A and rotenone (1 μM each). Glycolytic function was measured with glucose (10 mM), oligomycin (2 μM), and 2DG (100 mM). In experiments to test the effect of PS48, dimethylsulfoxide was used as a control.

Analysis of gene expression

Total RNA was isolated using Trizol reagent (Invitrogen). First-strand cDNA was synthesized using a Verso cDNA Synthesis Kit (Thermo Fisher Scientific, Waltham, MA) for RT-PCR. For quantifying mRNA expression using real-time PCR, StepOnePlus™ Real-Time PCR system and *Power* SYBR Green PCR Master Mix were

used (Applied Biosystems, Warrington, UK). All transcript levels were normalized to those of *Hprt*. The PCR conditions were 95°C for 10 min, followed by 40 cycles at 95°C for 15 s, and 60°C for 1 min. The experiments were performed on two independent samples. Each PCR was run in triplicate. The primers used for PCR are listed in Supplemental Table S2.

Flow cytometry

Cultured cells were dissociated by Cell Dissociation Buffer (Invitrogen). The antibodies used were shown in Supplemental Table S3. Stained cells were analyzed using the FACSCalibur system (BD Biosciences), as described previously (Kanatsu-Shinohara et al., 2011).

Transplantation

Donor cells were microinjected into the seminiferous tubules of W mice via an efferent duct (Japan SLC, Shizuoka, Japan), as described previously (Ogawa et al., 1997). We also used busulfan (44 mg/kg)-treated B6 × DBA F1 mice for transplantation of B6-GS cells. Serial transplantation was carried out, as described previously (Lee et al., 2009). In brief, primary recipient testis was dissociated into single cells by two-step enzymatic digestion procedure using collagenase and trypsin (Ogawa et al., 1997). A portion of dissociated cells from one testis was transplanted into two testes of the secondary recipient mouse. The Institutional Animal Care and Use Committee of Kyoto University approved all of the animal experimentation protocols.

Analysis of the recipient testes

SSC colonization levels were also evaluated by counting the number of germ cell colonies based on fluorescence under UV light. Colonies were defined as germ cell clusters longer than 0.1 mm occupying the entire circumference of the tubule. For quantification of colonization by seminiferous tubule counts, recipient testes were processed for paraffin sectioning after fixation, and stained with hematoxylin and eosin. The number of tubule cross sections showing (and not showing) spermatogenesis, defined as the presence of multiple layers of germ cells in the entire circumference of the seminiferous tubule, was counted for one section from each testis. For immunohistochemistry, testis samples were fixed in 4% paraformaldehyde for 2 h and treated with 0.1% Triton-X in phosphate-buffered saline (PBS). After immersing them in blocking buffer (0.1%Tween 20, 3% bovine serum albumin and 10% goat or donkey serum in PBS) for > 1 h, samples were incubated with primary antibodies at 4°C overnight. Secondary antibodies were incubated for 1 h at room temperature. Samples were counterstained with Hoechst 33342 (Sigma). The images were collected using a confocal microscope (Fluoview FV1000D; Olympus, Tokyo, Japan). The antibodies used are listed in Supplemental Table S3.

Western blot analysis

We used SDS-PAGE to separate cell lysates (20 μg), which were transferred to Hybond-P membranes (Amersham Biosciences, Buckinghamshire, UK). For collection of fresh germ cells from pup testes, testis cells were cultured overnight on

gelatin-coated plates and germ cells were collected by gentle pipetting. The antibodies used for Western blotting is shown in Supplemental Table S3.

Supplemental References

Kanatsu-Shinohara M, Muneto T, Lee J, Takenaka M, Chuma S, Nakatsuji N, Horiuchi T, Shinohara T. 2008. Long-term culture of male germline stem cells from hamster testes. *Biol Reprod* 78: 611-617.

Kanatsu-Shinohara M, Inoue K, Ogonuki N, Morimoto H, Ogura A, Shinohara T. 2011. Serum- and feeder-free culture of mouse germline stem cells. *Biol Reprod* 84: 97-105.

Lee J, Kanatsu-Shinohara M, Morimoto H, Kazuki Y, Takashima S, Oshimura M, Toyokuni S, Shiohara T. 2009. Genetic reconstruction of mouse spermatogonial stem cell self-renewal in vitro by Ras-cyclin D2 activation. *Cell Stem Cell* 5: 76-86.

Ogawa T, Aréchaga JM, Avarbock MR, Brinster RL. 1997. Transplantation of testis germinal cells into mouse seminiferous tubules. *Int J Dev Biol* 41: 111-122.

Takubo K, Nagamatsu G, Kobayashi CI, Nakamura-Ishizu A, Kobayashi H, Ikeda E, Goda N, Rahimi Y, Johnson RS, Soga T, et al. 2013. Regulation of glycolysis by Pdk functions as a metabolic checkpoint for cell cycle quiescence in hematopoietic stem

cells. *Cell Stem Cell* 12: 49-61.