Supplemental materials and methods

Single-strand cDNA synthesis and quantitative real-time PCR

1-5 μg of total RNA was reverse-transcribed to cDNA using oligo dT primer and Superscript III reverse transcriptase (Invitrogen), according to manufacturer's instructions. cDNA samples were typically diluted 1:20 in 10 mM Tris (pH 8.0) before use in subsequent PCR reactions.

qPCR reactions were set up to quantify expression of mouse *c-Myc*, *Smchd1, Makorin3, Necdin, Lor, Igfbp5, Onecut2, Gata6, Pcdha7, Pcdhb22*, *Prkcb,* and *Jun* using specific pre-designed Taqman™ gene expression assays (Applied Biosystems, USA), with *Hmbs* as ahousekeeping gene. PCR reactions were 10 μL volume and performed in technical duplicate or triplicate. The reactions were run on the ABI 7900 HT real-time PCR machine (Applied Biosystems) with conditions as follows: 95^{o}C incubation for 15 minutes, 40 cycles of 92^{o}C for 15 seconds and 60^{o}C for 1 minute. Ct values were derived using the SDS2.2 software (Applied Biosystems). Relative gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method.

Cell lysis and protein quantification

Infected NIH3T3 cells or primary MEFs were trypsinized, washed twice with PBS and lysed with KALB lysis buffer (150 mM NaCl, 50 mM Tris-HCl, pH 7.5, 1% (v/v) Triton X-100, 1 mM EDTA) supplemented with protease inhibitors (Complete mixture tablets; Roche Applied Science) on ice for 30 mins. Insoluble material was removed by centrifugation. Total protein concentration in the whole cell extract was quantified using BCA protein assay kit (Pierce) following manufacturer's instructions.

Western Blot Analysis

Proteins were resolved by 4-12% SDS-PAGE (Invitrogen), transferred to PVDF membrane (Osmonics; GE) and blocked with 5% (w/v) skim milk powder in 0.1% (v/v) TWEEN-PBS. Membrane was incubated with anti-Smchd1 antibody (1:2000; Abcam) overnight at 4°C followed with horseradish peroxidase (HRP)-conjugated anti-Rabbit secondary antibody. Membrane was incubated with HRP-conjugated anti-Actin antibody (1:2000; Santa Cruz) for 1 hour at 22°C as a loading control. Antibody binding was visualized using ECL system (Immobilon; Millipore) according to manufacturer's instructions.

Cell death analysis (Annexin V)

Pro/pre-B cells ($B220^{+}IgM^{-}$) and immature/mature B cells ($B220^{+}IgM^{+}$) were FACS-purified from bone marrow and spleen, respectively, and cultured in Kelso DME supplemented with 10% FCS, 50 μM 2-mercaptoethanol (Sigma) and 100 μM L-asparagine (Sigma) at a concentration of 0.5-1 x 10^{6} cells/mL in 96-well plates (Falcon). Cells were harvested at 24, 48 and 72 hours, and stained with FITC-conjugated Annexin V (Annexin V apoptosis detection kit from BD Biosciences, USA) for 30 minutes on ice. Cells were then washed with FACS buffer prior to addition of 1 μg/mL PI and analysis by flow cytometry.

***In vivo* BrdU labeling**

175 mg/kg BrdU in sterile PBS (BD Biosciences, USA) was administered into mice by intraperitoneal injection. Single-cell suspensions were prepared from bone marrow (both femurs) and spleen at two hours post-injection in 5% KDS BSS-FCS. Cells were stained with monoclonal antibodies to cell surface markers B220, c-kit, IgM and IgD prior to fixation and DNase treatment using the BrdU Flow Kit (BD Biosciences), according to the manufacturer's instructions. Cells were then stained with 0.5 μg/mL DAPI (Invitrogen) for 15 minutes prior to flow cytometric analysis.

Supplementary Figure Legends

Figure S1. Validation of *Smchd1* knockdown with 2 shRNA constructs

(A) NIH 3T3 cells were infected with retroviruses that contained a non-silencing shRNA (Non-sil) or one of the five shRNA constructs targeting *Smchd1*. Expression of *Smchd1* was quantified using a Taqman™ gene expression assay and is shown relative to a non-silencing control. *Hmbs* was used to normalise samples for variation in cDNA concentration. Data represent mean and S.E.M from two independent infections. (B) NIH 3T3 cells were infected with retroviruses that contained a non-silencing shRNA (Non-sil) or shRNA 1.2 and 1.4 constructs targeting *Smchd1* and selected in puromycin (5 μg/ml; Sigma). Whole cell extracts from infected cells and S*mchd1*$^{MD1/MD1}$ MEFs were prepared and total amount of protein was quantified as described in the Supplemental materials and methods. Equal amounts of total protein from each experiment were analyzed by Western Blot with antibody against Smchd1 and Actin. * denotes a non-specific band observed in Western Blots probed with an anti-Smchd1 antibody. Experiments were performed in duplicate for each shRNA construct. (C) Tumours were harvested from athymic nude recipients from Figure 1C when tumour volume reached more than 10 mm in any dimension, and RNA extracted to quantify *Smchd1* mRNA levels as described in (A). Data represent mean and S.E.M. from two to three independent experiments (**p<0.01, ***p<0.001).

Figure S2. Heterozygosity for *Smchd1* did not alter the latency or hematological phenotype of Eμ-*Myc*-induced lymphomas, and homozygous loss did not alter the hematological phenotype of end-stage lymphomas

(A) Kaplan-Meier survival curve of *Smchd1*$^{+/+}$/Eμ-*Myc*$^{Tg/+}$ and *Smchd1*$^{MD1/+}$/Eμ-*Myc*$^{Tg/+}$ mice on the FVB/C57 F1 background. Median survival of *Smchd1*$^{MD1/+}$/Eμ-*Myc*$^{Tg/+}$ and *Smchd1*$^{+/+}$/Eμ-*Myc*$^{Tg/+}$ mice was 106 and 113 days, respectively (n=8-10 per genotype, p=0.44, log-rank test). (B) Kaplan-Meier survival curve of *Smchd1*$^{+/+}$/Eμ-*Myc*$^{Tg/+}$ and *Smchd1*$^{gt/+}$/Eμ-*Myc*$^{Tg/+}$ mice on the C57BL/6 background. Median survival of *Smchd1*$^{gt/+}$/Eμ-*Myc*$^{Tg/+}$ and *Smchd1*$^{+/+}$/Eμ-*Myc*$^{Tg/+}$ mice was 110 and 106 days, respectively (n=11-20 per genotype, p=0.66, log-rank test). (C) The overall proportion of pro/pre-B and immature/mature B cell lymphomas arising in reconstituted Eμ-*Myc*$^{Tg/+}$ mice (n=23-32 per genotype). Single-cell suspensions of lymphoma cells were immuno-phenotyped using monoclonal antibodies to Ly5.2 and B cell surface markers (c-kit, B220, IgM and IgD) and analysed by flow cytometry. (D) Leukocytes and lymphocytes in the peripheral blood of reconstituted *Smchd1*$^{+/+}$/Eμ-*Myc*$^{Tg/+}$ and *Smchd1*$^{gt/gt}$/Eμ-*Myc*$^{Tg/+}$ mice at time of sacrifice were counted in an automated blood analyser (n=12-23 per genotype). (E) Nucleated spleen cell numbers (n=18-21 per genotype, p=0.11 compared between genotypes) and (F) Spleen weight (n=9-14 per genotype, p=0.062 compared between genotypes) of moribund reconstituted *Smchd1*$^{+/+}$/Eμ-*Myc*$^{Tg/+}$ and *Smchd1*$^{gt/gt}$/Eμ-*Myc*$^{Tg/+}$ mice. (D-F) Data represent mean and S.E.M.

Figure S3. Loss of Smchd1 does not increase expression of the Eμ-*Myc* transgene, as determined by quantitative reverse-transcriptase PCR

qPCR analysis of the expression of c-*Myc* in FACS purified Eμ-*Myc* lymphoma samples, null (n=7) or wild-type (n=5) for *Smchd1*. Expression of c-*Myc* was quantified using Taqman™ gene expression assays and is shown relative to a housekeeping gene *Hmbs*.

Hmbs was used to normalise samples for variation in cDNA concentration. 3 technical replicates for each biological sample were averaged and the mean and S.E.M are shown, along with all individual lymphoma sample points. There was no significant difference in expression between *Smchd1* null and wild-type lymphomas.

Figure S4. Loss of Smchd1 does not affect B cell development in E17.5 embryos, with or without Eμ-*Myc* transgene.
Fetal livers were harvested from E17.5 *Smchd1*$^{+/+}$/Eμ-*Myc*$^{Tg/+}$ and *Smchd1*$^{gt/gt}$/Eμ-*Myc*$^{Tg/+}$ male embryos and stained with monoclonal antibodies to c-kit, B220 and IgM for flow cytometric analysis. Fetal liver cells were counted using a haemocytometer. (A) Percentage and (B) cellularity of c-kit^{+}B220^{+} pro-B and c-kit^{-}B220^{+} pre-B cells in E17.5 foetal livers are shown (n=2-3 mice per genotype). (A-B) Data represent mean and S.E.M. from two to three independent experiments.

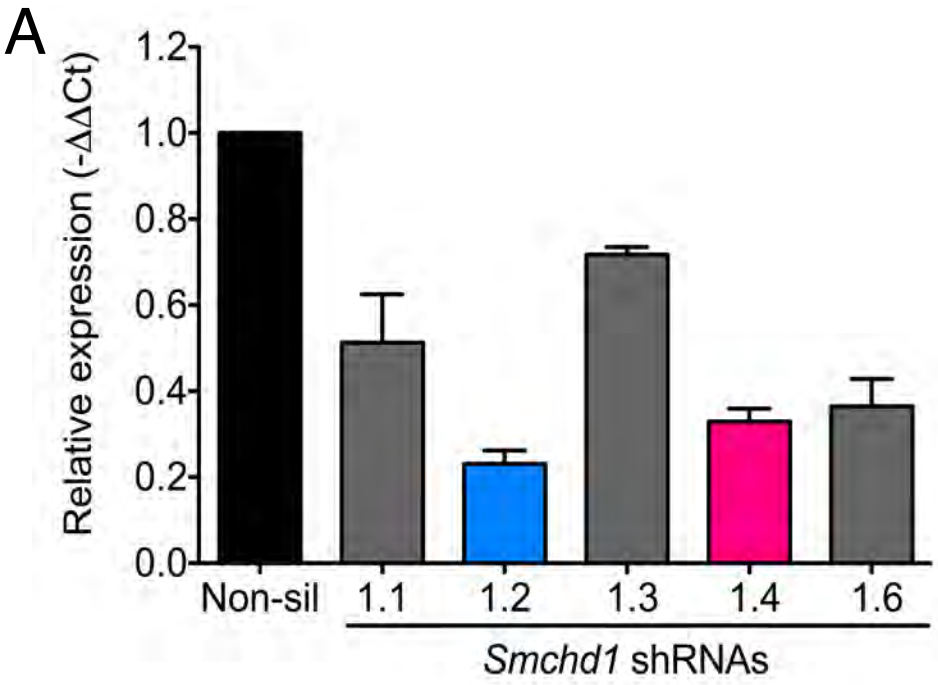
Figure S5. Genetic deletion of *Smchd1* does not have any effect on proliferation of pre-malignant B lymphoid cells *in vivo* or culture-induced apoptosis.
(A) Pre-malignant reconstituted *Smchd1*$^{+/+}$/Eμ-*Myc*$^{Tg/+}$ and *Smchd1*$^{gt/gt}$/Eμ-*Myc*$^{Tg/+}$ mice at eight weeks post-transplant were injected intra-peritoneally once with BrdU, organs harvested after two hours, cells stained with monoclonal antibodies to Ly5.2, c-kit, B220 and IgM, and analysed by flow cytometry. The percentages of BrdU^{+} pre-B (Ly5.2^{+}, c-kit^{-}, B220^{+}, IgM^{-}) and immature/mature B cells (Ly5.2^{+}, c-kit^{-}, B220^{+}, IgM^{+}) in the bone marrow and spleen are shown. (B) B220^{+}IgM^{-} pro/pre-B cells were FACS-purified from bone marrow while B220^{+}IgM^{+} immature/mature B cells were sorted from spleen of pre-malignant reconstituted *Smchd1*$^{+/+}$/Eμ-*Myc*$^{Tg/+}$ and *Smchd1*$^{gt/gt}$/Eμ-*Myc*$^{Tg/+}$ mice, and cultured in medium alone. At the indicated times, cells were stained with Annexin V and PI for flow cytometric analysis. (A-B) Data represent mean ± S.E.M of 7-9 mice per genotype from three to four independent experiments (*$p<0.05$).

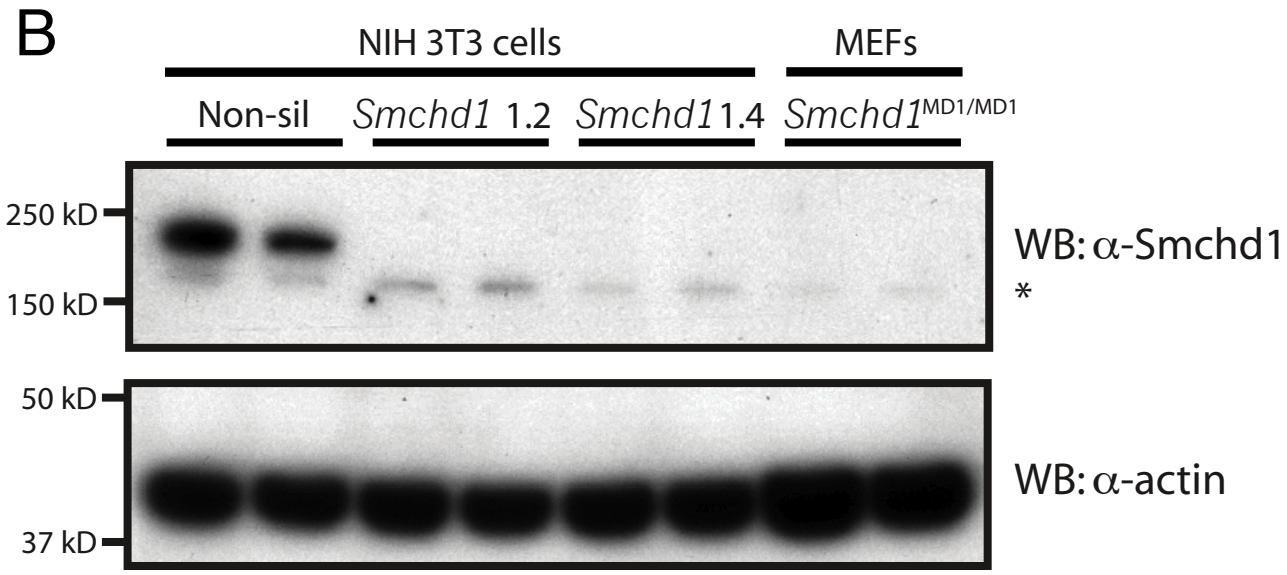
Figure S6. Increased variation in gene expression from non-malignant cells to tumours.
These plots show the $\log_2$ residual standard deviation versus average $\log_2$ expression of genes in (A) primary MEFs, transformed MEFs and tumours isolated from nude mice, and (B) E17.5 Eμ-*Myc*$^{Tg/+}$ pre-B cells, pre-malignant Eμ-*Myc*$^{Tg/+}$ pre-B cells at eight weeks post-transplant and Eμ-*Myc*$^{Tg/+}$ lymphoma cells. The number of genes listed in brackets at the top of each panel is the number of differentially expressed genes with corrected p-values less than 0.05, at the level of whole genome testing. These graphs plot log residual standard deviation versus average log expression for a fitted microarray linear model. It is used to check the mean-variance relationship of the expression data, the consistency of the variance with respect to average expression level, after fitting a linear model. These plots show the average inter-replicate variation for pooled *Smchd1* wildtype and *Smchd1* null samples. The standard deviation (SD) for each cell population is shown on the graph. The standard deviations for fibroblast tumours and lymphomas are 0.36 and 0.43, respectively, which are much larger compared with the other cell populations.

Figure S7. Genes identified by microarrays as upregulated in *Smchd1* null cells are validated by quantitative reverse-transcriptase PCR.

(A) qPCR analysis of the expression of nine genes that were found upregulated in *Smchd1* null MEF-derived tumours relative to wildtype by microarrays. Expression of these genes was quantified using Taqman™ gene expression assays and is shown relative to a housekeeping gene *Hmbs*. *Hmbs* was used to normalise samples for variation in cDNA concentration. 3 technical replicates for each biological sample were averaged and the mean and S.E.M of 3 biological replicates are shown. (B) qPCR analysis of the expression of three genes found upregulated in E17.5 *Smchd1* null Eμ-*Myc*$^{Tg/+}$ pre-B cells relative to wild-type by microarrays (n=3 per genotype). The experiment performed as in (A). Data represent mean and S.E.M. (*$p<0.05$).

Figure S1


A
Relative expression (-ΔΔCt)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Non-sil
1.1
1.2
1.3
1.4
1.6
Smchd1 shRNAs


B
NIH 3T3 cells
MEFs
Non-sil
Smchd1 1.2
Smchd1 1.4
Smchd1MD1/MD1
250 kD
150 kD
WB: α-Smchd1
*
50 kD
37 kD
WB: α-actin

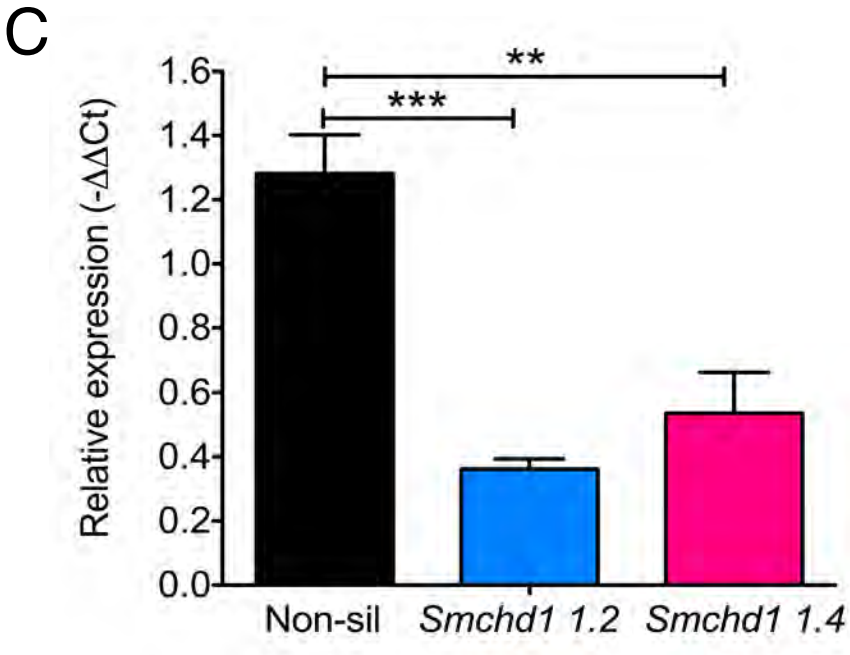

C
Relative expression (-ΔΔCt)
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
**
***
Non-sil
Smchd1 1.2
Smchd1 1.4

Figure S2

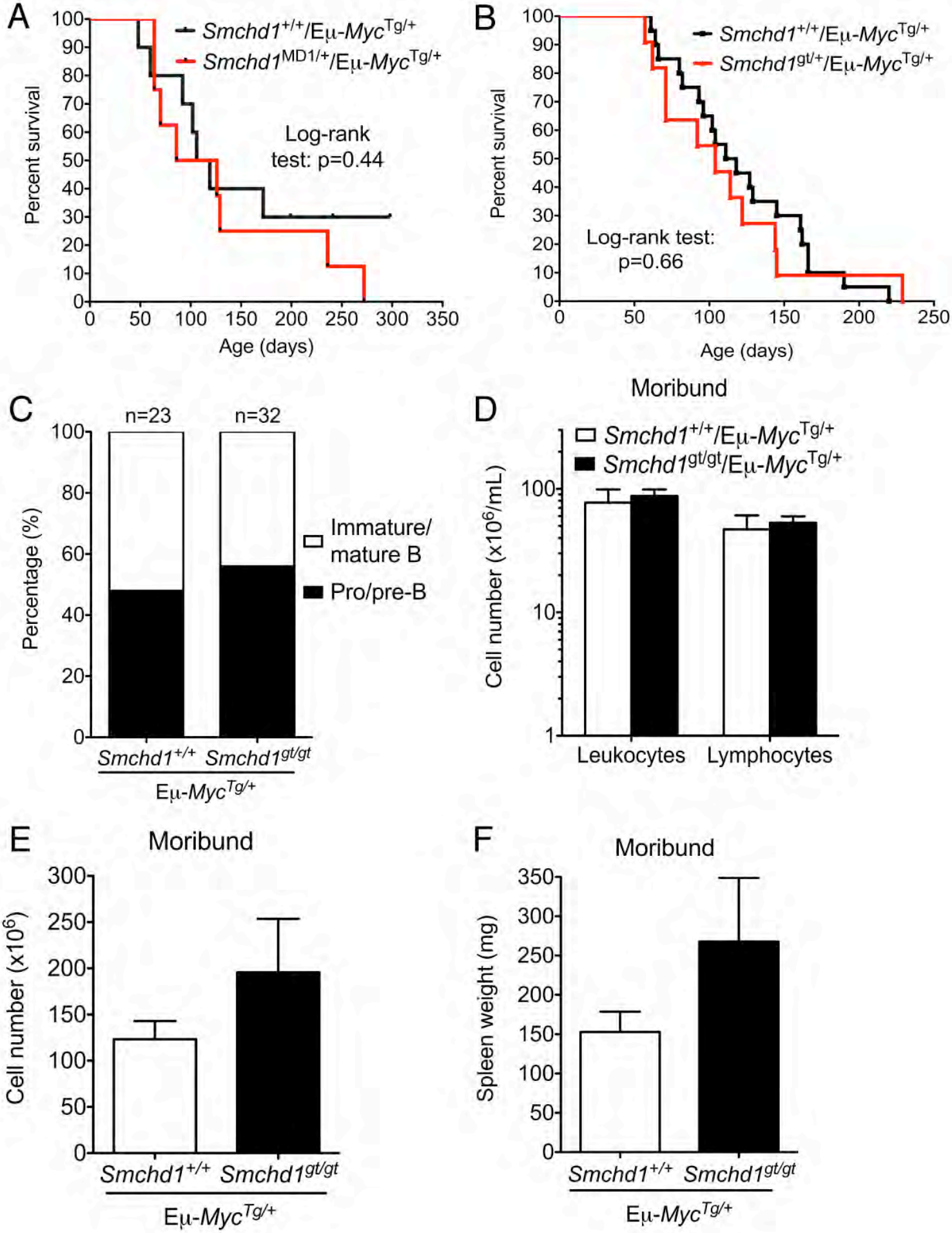

A
Percent survival
Smchd1+/+/Eμ-Myc Tg/+
Smchd1 MD1/+/Eμ-Myc Tg/+
Log-rank test: p=0.44
Age (days)
B
Percent survival
Smchd1+/+/Eμ-Myc Tg/+
Smchd1 gt/+/Eμ-Myc Tg/+
Log-rank test: p=0.66
Age (days)
C
n=23
n=32
Percentage (%)
Immature/ mature B
Pro/pre-B
Smchd1+/+ Smchd1gt/gt
Eμ-Myc Tg/+
D
Moribund
Smchd1+/+/Eμ-Myc Tg/+
Smchd1 gt/gt/Eμ-Myc Tg/+
Cell number (x10^6/mL)
Leukocytes
Lymphocytes
E
Moribund
Cell number (x10^6)
Smchd1+/+ Smchd1gt/gt
Eμ-Myc Tg/+
F
Moribund
Spleen weight (mg)
Smchd1+/+ Smchd1gt/gt
Eμ-Myc Tg/+

Figure S3

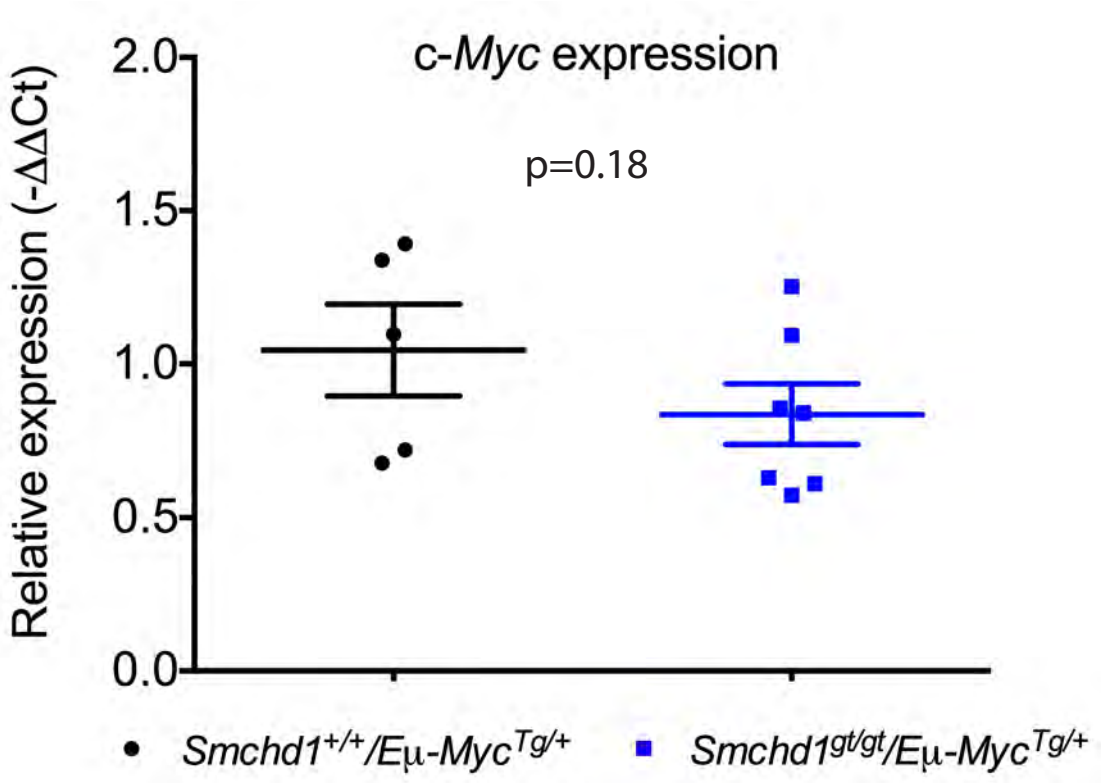

c-Myc expression
p=0.18
Relative expression (-ΔΔCt)
2.0
1.5
1.0
0.5
0.0
Smchd1+/+/Eμ-MycTg/+
Smchd1gt/gt/Eμ-MycTg/+

Figure S4

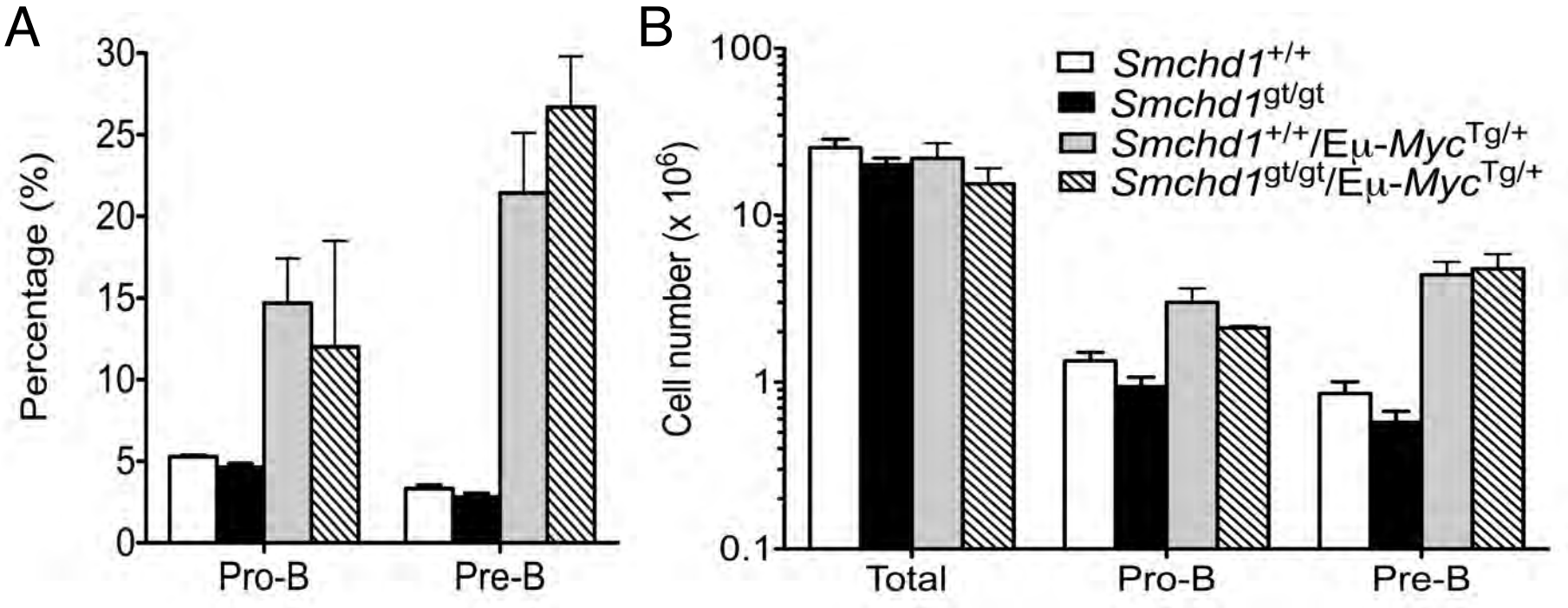

A
Percentage (%)
30
25
20
15
10
5
0
Pro-B
Pre-B
B
Cell number (x 10^6)
100
10
1
0.1
Smchd1+/+
Smchd1gt/gt
Smchd1+/+/Eμ-MycTg/+
Smchd1gt/gt/Eμ-MycTg/+
Total
Pro-B
Pre-B

Figure S5

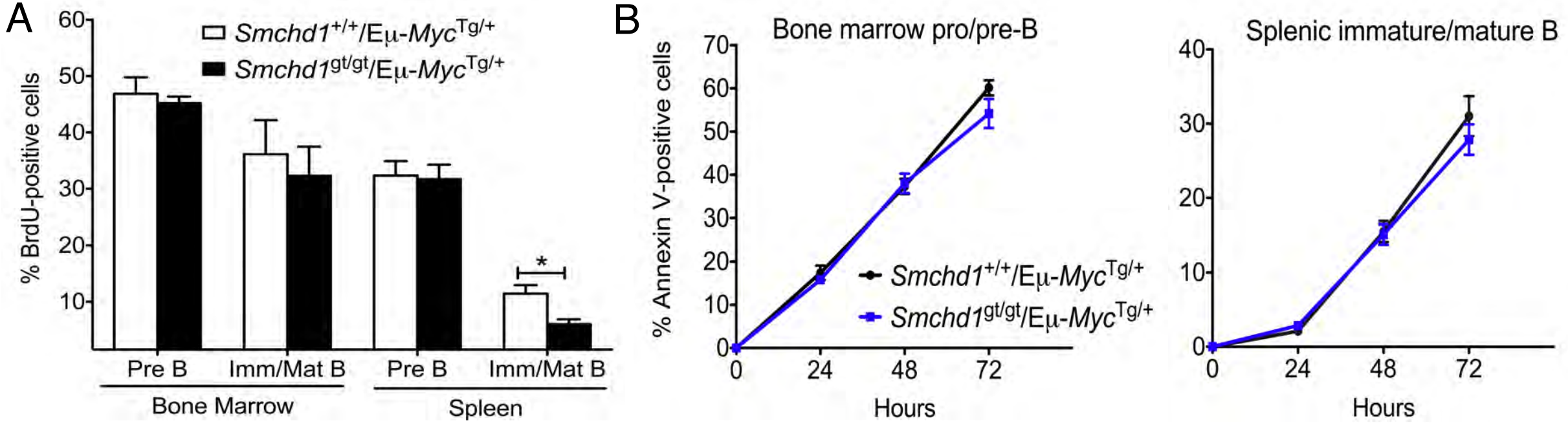

A
% BrdU-positive cells
60
50
40
30
20
10
Smchd1+/+/Eμ-MycTg/+
Smchd1gt/gt/Eμ-MycTg/+
*
Pre B
Imm/Mat B
Pre B
Imm/Mat B
Bone Marrow
Spleen
B
% Annexin V-positive cells
Bone marrow pro/pre-B
70
60
50
40
30
20
10
0
Smchd1+/+/Eμ-MycTg/+
Smchd1gt/gt/Eμ-MycTg/+
0
24
48
72
Hours
Splenic immature/mature B
40
30
20
10
0
0
24
48
72
Hours

Figure S6

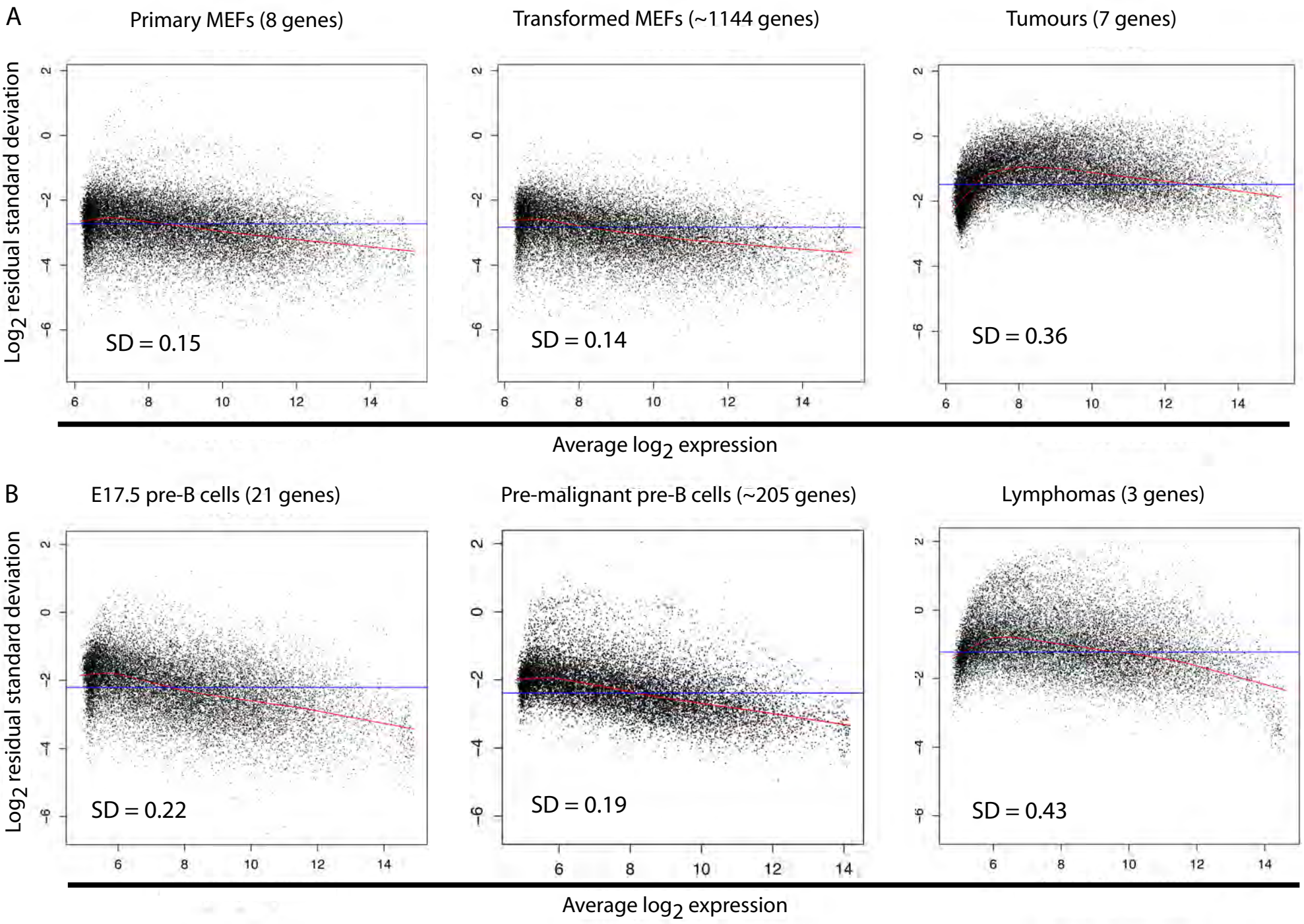
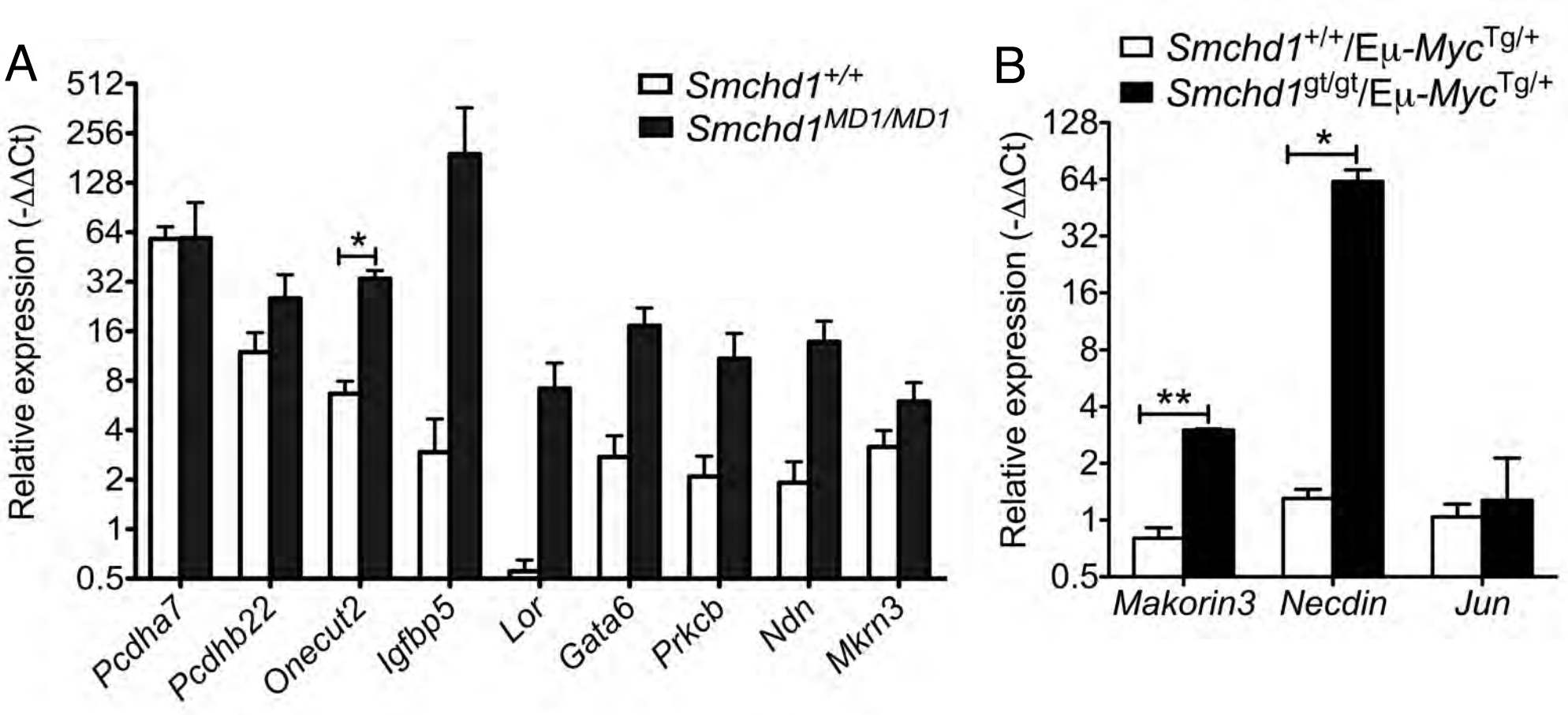

A
Primary MEFs (8 genes)
Transformed MEFs (~1144 genes)
Tumours (7 genes)
Log2 residual standard deviation
SD = 0.15
SD = 0.14
SD = 0.36
Average log2 expression
B
E17.5 pre-B cells (21 genes)
Pre-malignant pre-B cells (~205 genes)
Lymphomas (3 genes)
Log2 residual standard deviation
SD = 0.22
SD = 0.19
SD = 0.43
Average log2 expression

Figure S7


A
Relative expression (-ΔΔCt)
512
256
128
64
32
16
8
4
2
1
0.5
Smchd1+/+
Smchd1MD1/MD1
*
Pcdha7
Pcdhb22
Onecut2
Igfbp5
Lor
Gata6
Prkcb
Ndn
Mkrn3
B
Smchd1+/+/Eμ-MycTg/+
Smchd1gt/gt/Eμ-MycTg/+
Relative expression (-ΔΔCt)
128
64
32
16
8
4
2
1
0.5
**
*
Makorin3
Necdin
Jun