Supplementary Information for

Toxicity modeling of Plk1-targeted therapies in genetically engineered mice and cultured primary mammalian cells

Monika Raab[1,12,a], Sven Kappel[1,a], Andrea Krämer[1], Mourad Sanhaji[1], Yves Matthess[1], Elisabeth Kurunci-Csacsko[1], Julia Calzada-Wack[3], Birgit Rathkolb[2,4], Jan Rozman[2,5], Thure Adler[2,6], Dirk H. Busch[6], Irene Esposito[3,7], Helmut Fuchs[2], Valérie Gailus-Durner[2], Martin Klingenspor [5], Eckhard Wolf [4], Nicole Sänger[1], Florian Prinz[8], Martin Hrabě de Angelis[2,9], Jost Seibler[10], Juping Yuan[1], Martin Bergmann[11], Rainald Knecht[12], Bertolt Kreft[8], Klaus Strebhardt[1*]

[a]Both authors contributed equally

To whom correspondence should be addressed E-mail: strebhardt@em.uni-frankfurt.de

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Supplementary Methods

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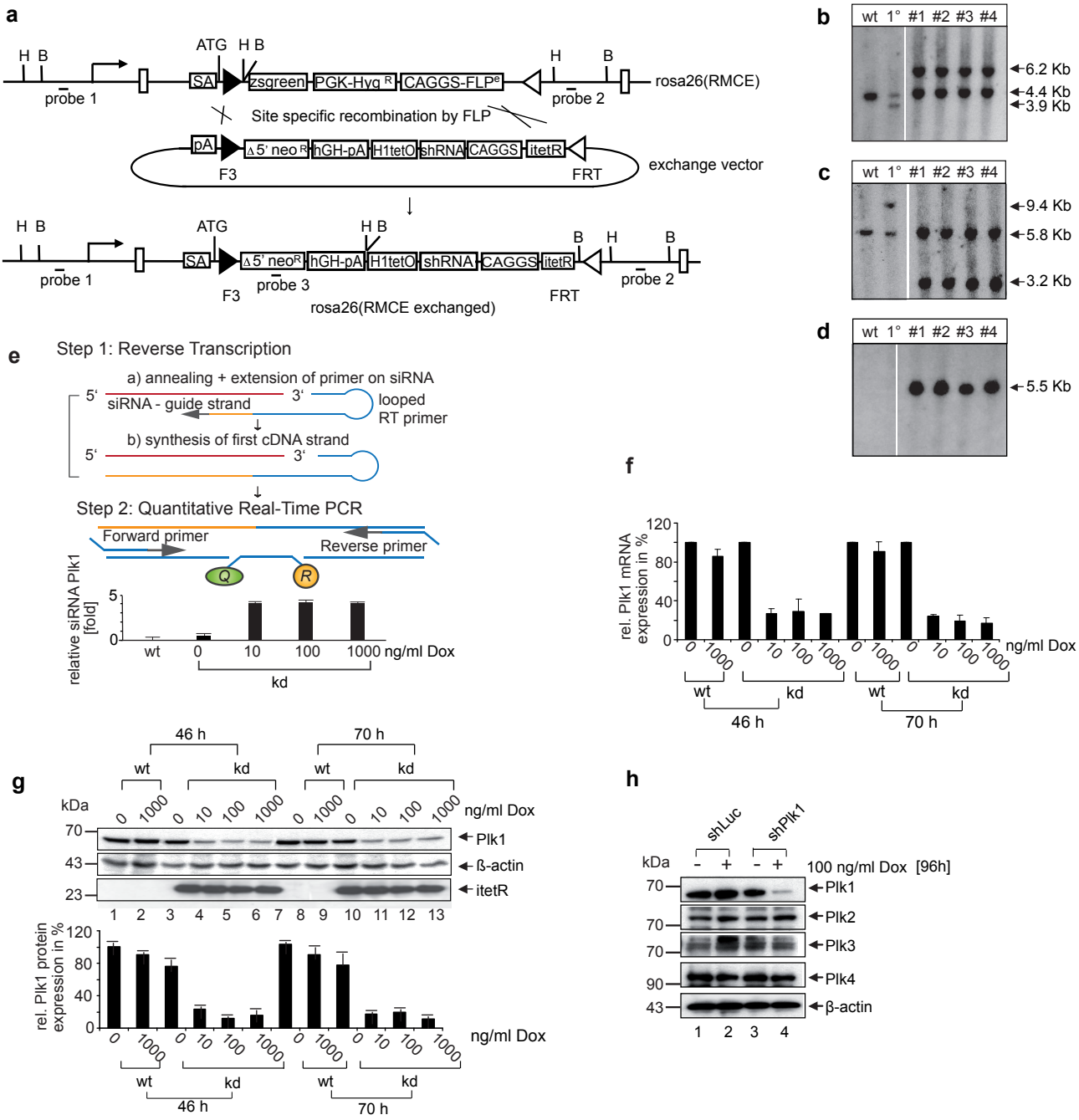
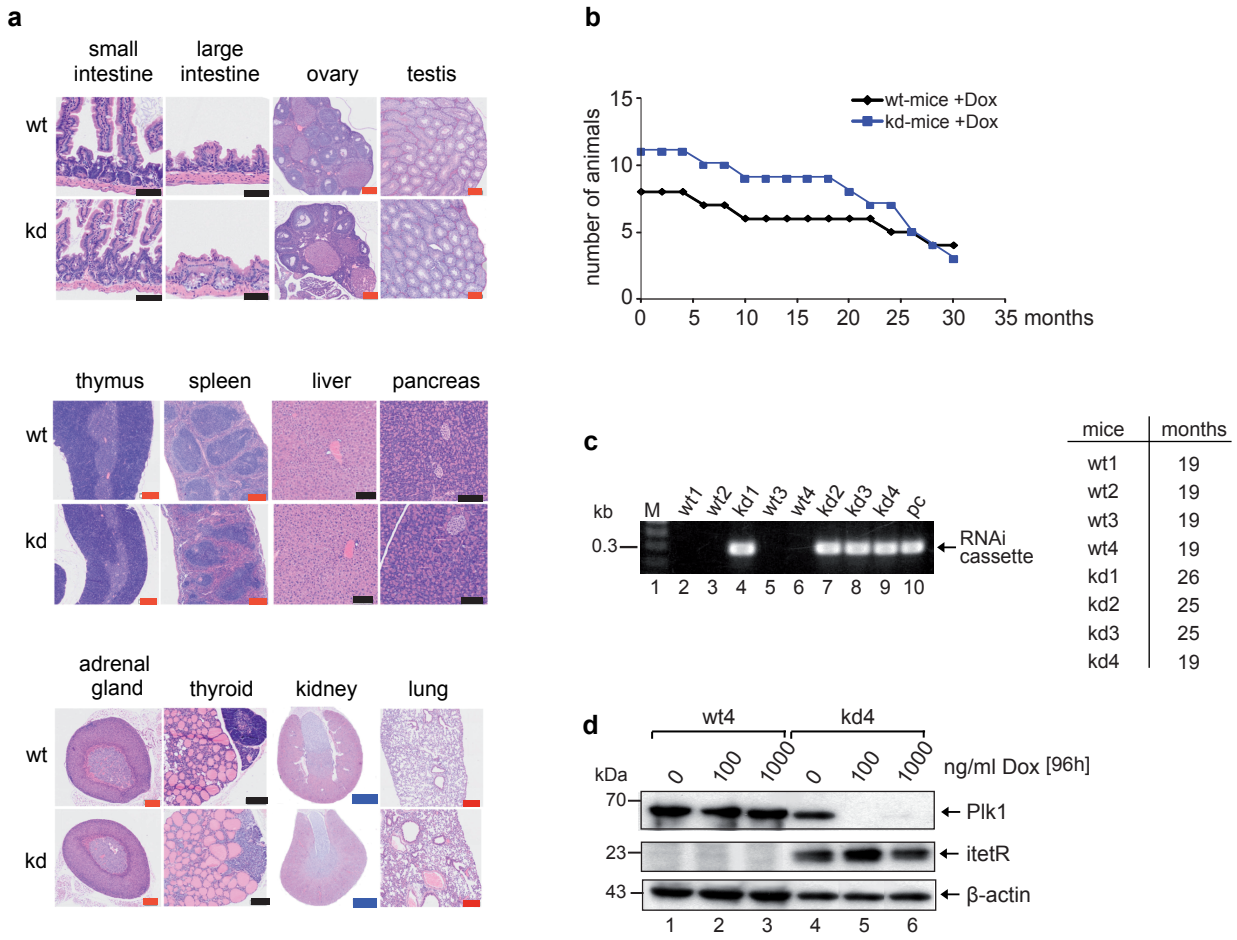
Supplementary Figure S1



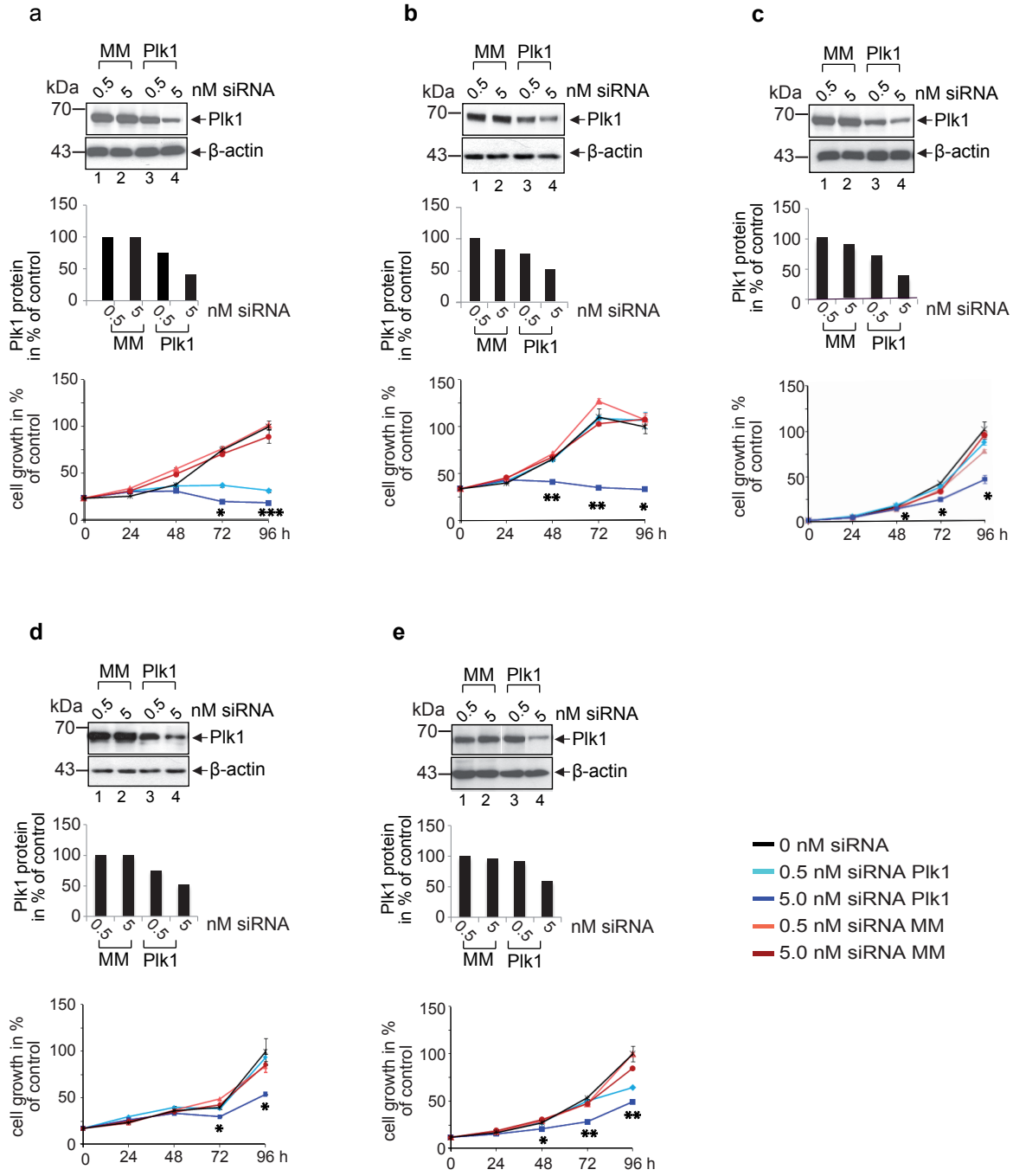
Supplementary Figure S1 | RMCE targeting system for the rosa26 locus. (a) The rosa26 targeting vector, which comprises the ZsGreen, PGK-Hyg, and cytomegalovirus-enhancer/beta-actin (CAGGS)-FLP, was inserted into the rosa26 locus via homologous recombination in ES cells. The F3/FRT sites were oriented in opposite directions. The modified locus carrying the RMCE acceptor is called rosa26(RMCE). The exchange vector carries the Plk1-shRNA1473 sequence, which is under the control of the inducible H1 promoter, and itetR is under the control of cytomegalovirus-enhancer/beta-actin (CAGGS), the F3/FRT pair, and a truncated neoR gene for positive selection. A polyA signal is included to prevent the expression of the truncated neoR gene at random integration sites. The RMCE is mediated by FLP recombination and leads to the rosa26(RMCE exchanged) allele. H: HindIII, B: BamHI. **(b-d)** Southern blot analyses of genomic ES cell DNA (wild-type, wt; 1°, rosa (RMCE), clones #1-4, transgenic ES cell) are presented. **(b)** Genomic DNA was digested with HindIII and analyzed using probe 1. The sizes of wt, rosa26(RMCE) and rosa26(RMCE exchanged) were 6.2 kb, 4.4 kb and 3.9 kb, respectively. In clones #1-4, successful RMCE was observed. **(c)** Genomic DNA was digested with BamHI and analyzed using probe 2. The sizes of wt, rosa26(RMCE) and rosa26(RMCE exchanged) were 9.4 kb, 5.8 kb and 3.2 kb, respectively. In clones #1-4, successful RMCE occurred. **(d)** Genomic DNA was digested with BamHI and analyzed using probe 3. The size of rosa26(RMCE exchanged) was 5.5 kb. In clones #1-4, no random integration was detectable, whereas, as expected, wt and rosa26(RMCE) showed no signal for the exchange vector. **(e)** A schematic representation of the TaqMan siRNA assay is shown. TaqMan-based real-time quantification of the siRNAs includes two steps: the stem–loop RT and the real-time PCR. The stem–loop RT primers bind to the 3' end of the siRNA molecules and are reverse transcribed. Then the RT product is quantified using conventional TaqMan PCR, which utilizes a siRNA-specific forward primer, reverse primer and dye-labeled TaqMan probes. The total RNA was isolated from the ES cells at Dox concentrations between 0 and 1000 ng/ml, and siRNA levels were determined by qRT-PCR. The relative levels of Plk1 siRNA to GAPDH are shown. (means ± s.d., n=3, for each concentration). **(f)** Levels of Plk1 mRNA as measured by qRT-PCR analysis in wt and transgenic ES cells treated for 46 h or 70 h with Dox (0 to 1000 ng/ml) are shown (means ± s.d., n=5, for each concentration). **(g)** The levels of protein expression in lysates of wt and transgenic ES cells treated for 46 h or 70 h with Dox (0 to 1000 ng/ml) were monitored by immunoblotting for Plk1, β-actin and itetR (means ± s.d., n=3, for each concentration). **(h)** The levels of Plk1, Plk2, Plk3, Plk4 and β-actin in lysates of Luc- and Plk1-iKD ES cells treated for 96 h with Dox (100 ng/ml) were monitored by immunoblotting (n=3).

Supplementary Figure S2



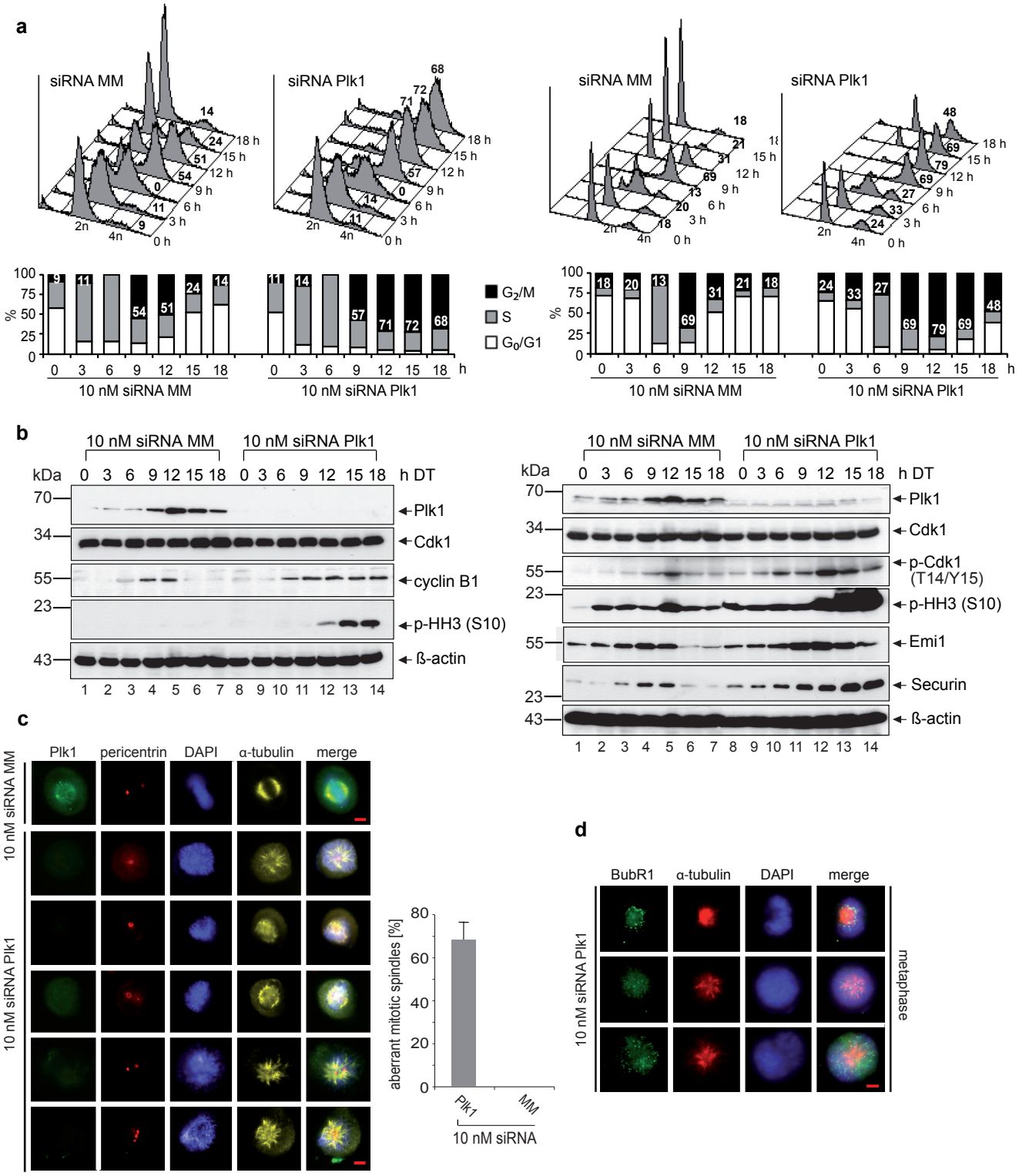
Supplementary Figure S2 | Histology and long-term analysis of iKD and wt mice. (a) Animals were sacrificed after 6 weeks of Dox treatment (drinking water containing 2 mg/ml Dox), followed by fixation, routine embedding, sectioning, and hematoxylin/eosin (HE) staining of indicated tissues. Sections representing the organs of control (wt) and iKD mice include the small intestine, large intestine, ovary, testis, thymus, spleen, liver, pancreas, adrenal gland, thyroid, kidney and lung. Bar, 100 µm (black), 200 µm (red), 1 mm (blue). **(b)** In a long-term experiment animals were treated with Dox (drinking water containing 2 mg/ml Dox) for more than 30 months and survival was monitored. **(c,d)** 4.5-5 years after the initial generation of the first Plk1-iKD-ES cells and -mice, tails clips from a small cohort of mice were used for a PCR-based genotyping. Splenocytes were isolated from wt- and iKD mice for a functional iKD test by monitoring the levels of Plk1, itetR and β-actin in lysates of wt- and Plk1-iKD cells treated for 96 h with Dox (0, 100 and 1000 ng/ml) by immunoblotting.

Supplementary Figure S3



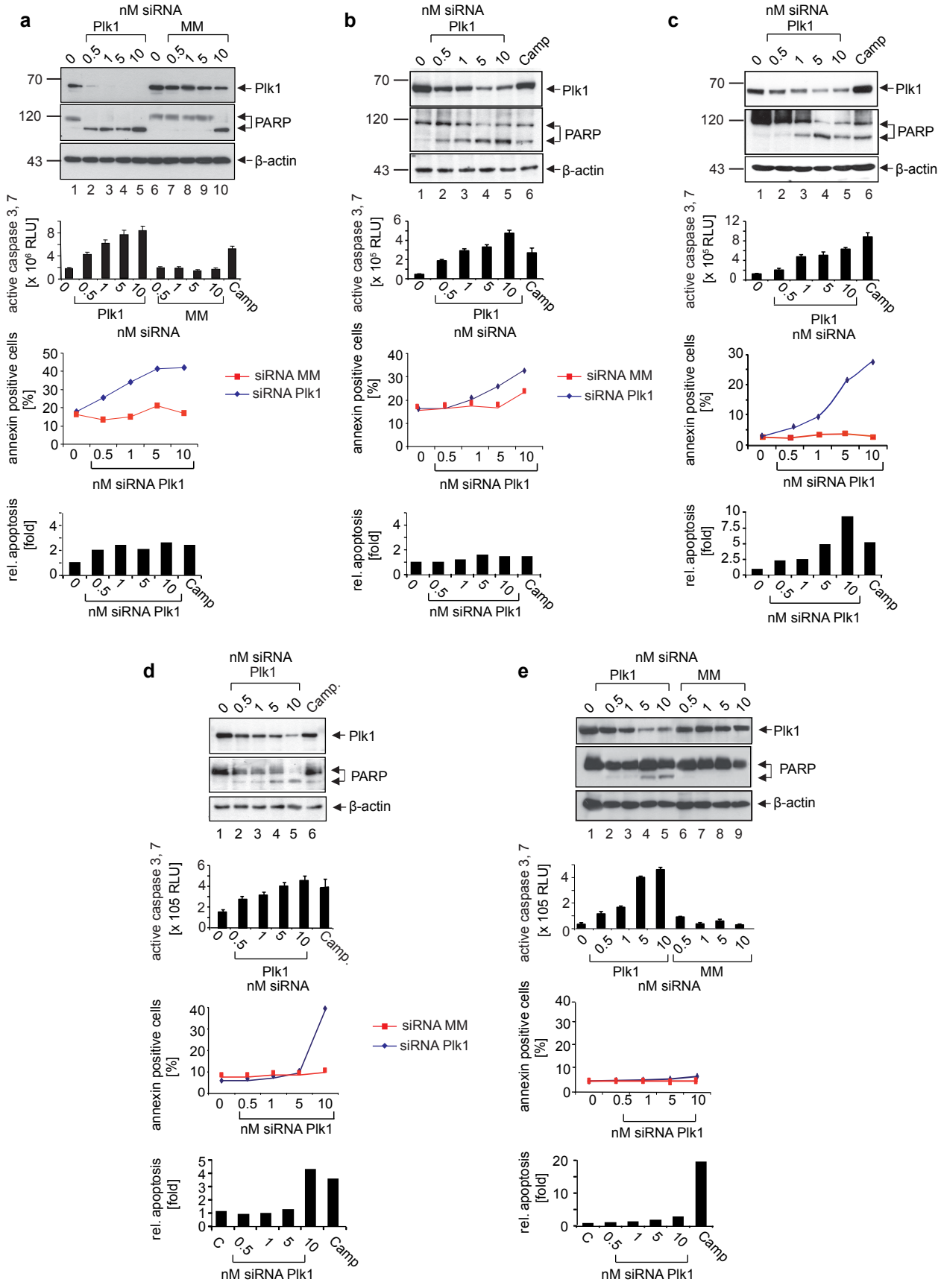
Supplementary Figure S3 | The effect of Plk1 silencing on the proliferation of cancer cells. Western blot analysis of Plk1 protein in cancer cell lines (a) HeLa, **(b)** SW480, **(c)** MDA-MB-231, **(d)** SK-OV-3 and **(e)** 293T cells was conducted 24 h after transfection with siRNA Plk1 and siRNA MM at the indicated concentrations. Cell lysates were immunoblotted for Plk1, and β-actin was the loading control. Plk1 protein expression in the cancer cells was standardized to the expression of β-actin levels and relative to the levels of Plk1 in cells treated with 0.5 nM siRNA MM (=100%). The proliferation of the cancer cell lines at the indicated siRNA concentrations is shown. Cells treated with the transfection agent alone served as the control (100%) (means ± s.d., n=3, for each time point). * p<0.05, ** p<0.01, *** p<0.001, student's t-test, unpaired and two-tailed.

Supplementary Figure S4



Supplementary Figure S4 | Influence of Plk1 silencing on cell cycle distribution of cancer cell lines. (a) HeLa and SW480 cells were synchronized at the G1/S transition by double thymidine arrest followed by transfection with Plk1-specific siRNA (siRNA Plk1) or a Plk1 mismatch control (siRNA MM). After release from the thymidine arrest, the cell cycle status was monitored every 3 h by FACS (upper panels). Representative examples are shown. Quantification of the cell cycle analysis is shown. **(b)** Western blot analysis of the Plk1 protein was conducted after cancer cell lines (HeLa, SW480) were transfected with 5 nM of siRNA Plk and siRNA MM. Cell lysates were immunoblotted for Plk1, Cdk1, cyclin B1, histone H3 phosphorylated at Ser10 (pHH3(S10)), pCdk1(T14/Y15), Emi1, Securin and β-actin. **(c)** HeLa cells were labeled using Plk1, pericentrin and α-tubulin antibodies and DAPI for the analysis by immunofluorescence microscopy. Representative examples of siRNA MM- and siRNA Plk1-transfected HeLa cells are shown. The percentage of cells with aberrant mitotic spindles was determined by counting 250-300 cells. (means ± s.d., n=6, for each sample of cells). Bar, 5 µm. **(d)** Plk1-depleted HeLa cells were labeled using BubR1 and α-tubulin antibodies and DAPI for the analysis by immunofluorescence microscopy. Representative examples of Plk1-transfected cells are depicted. Bar, 5 µm.

Supplementary Figure S5



Supplementary Figure S5 | Assessment of apoptosis in Plk1-depleted cancer cells. A small panel of cancer cell lines **(a)** HeLa, (b) MDA-MB-231, **(c)** SW480, **(d)** SK-OV-3 and **(e)** 293T cells was treated with siRNA Plk1 or a siRNA control (siRNA MM) at doses of 0.5-10 nM. Cell lysates were immunoblotted for Plk1, PARP and β-actin. Caspase-3 activity was determined in the cell lysates from transfected cells using the Caspase-Glo® 3/7 Assay (means ± s.d., n=3, for each concentration). Propidium iodide was used in conjunction with annexin V staining for discriminating among the viable, apoptotic and necrotic cells using dual parameter FACS analysis. Annexin V-based measurements of cells treated with siRNA Plk1 or a siRNA control (siRNA MM) were correlated with the relative levels of apoptosis.

Supplementary Table S1

siRNA	Target site (NM_011121.2)	kd efficiency in NIH 3T3 [%]	kd efficiency in RENCA [%]
1	1484-1504	10	n.d.
2	1903-1923	20	n.d.
3	1061-1081	11	n.d.
4	658-678	0	n.d.
5	2048-2066	10	n.d.
6	2076-2094	7	n.d.
7	1785-1803	30	7
8	1896-1914	0	n.d.
9	711-729	5	n.d.
10	835-853	29	24
11	608-626	29	n.d.
12	1502-1522	25	n.d.
13	401-419	25	n.d.
14	381-401	40	n.d.
15	970-990	22	n.d.
16	**1473-1493**	**70**	**70**
17	1740-1760	40	65
18	1866-1886	25	n.d.

Supplementary Table S1 | Selection of suitable shRNA sequences for Plk1 depletion. Levels of Plk1 mRNA measured using qRT-PCR analysis 72 h after the transfection of NIH3T3 and RENCA cells (n=3). The Plk1 mRNA depletion is shown as a percentage of the Plk1 mRNA levels in cells transfected with siRNA MM.

Supplementary Table S2

	control Dox-treated		Plk1-iKD Dox-treated		2. control untreated females		2-way-ANOVA (p)		
	treated		treated		untreated females		genotype	sex	interaction
parameter	male (n=6-8)	female (n=6-8)	male (n=6-8)	female (n=6-8)	control (n=4)	Plk1 (n=4)	ad libitum food reduced	ad libitum food reduced	* treatment effect
body weight (g)	29.3 ± 0.7	25.1 ± 0.7	33.0 ± 1.6	25.9 ± 0.7	25.8 ± 0.5	26.7 ± 0.9	<0.05	<0.001	n.s. -
rectal body temperature (°C)	36.0 ± 0.1	36.5 ± 0.2	35.5 ± 0.1	36.1 ± 0.1	37.0 ± 0.3	36.8 ± 0.2	<0.001	<0.001	n.s. *

Supplementary Table S2 | Selected metabolic parameters from the in vivo screen of Dox-treated wt and Plk1-iKD mice. Body weight and rectal body temperature were determined in male and female mice (wt, iKD, + Dox) (means ± s.e.m., n=6-8, for each parameter). For statistical analysis, two-way ANOVA was applied.

Supplementary Table S3

Large intestine	Area	Nucleus	Ratio nucleus/area
Control mouse	2797	277	0.099
Plk1-iKD mouse	2354	156	0.066

Supplementary Table S3 | Proliferation index of cells within the mucosal folds of large intestine. For the determination of the proliferative index Ki-67-positive cells/tissue area were quantified. The numbers in the table represent the positive cells counted in 12-15 folds.

Supplementary Table S4

Follicle Ovary	Area	Nucleus	Ratio nucleus/area
Control mouse	2991	342	0.1143
Plk1-iKD mouse	1012	105	0.1037

Supplementary Table S4 l Proliferation index of cells within ovarian follicles. For the determination of the proliferative index Ki-67-positive cells/tissue area were quantified.

Supplementary Table S5

Parameter	wt male n=10	Plk1-iKD male n=10	wt female n=10	Plk1-iKD female n=9	Two-way-ANOVA genotype	sex	interaction
WBC [10^3/µl]	3,87 ± 1,99	3,81 ± 1,60	3,27 ± 1,62	4,05 ± 0,81	n.s.	n.s.	n.s.
RBC [10^6/µl]	11,06 ± 0,39	11,16 ± 0,28	10,97 ± 0,81	10,85 ± 0,36	n.s.	n.s.	n.s.
MCV [fl]	45,1 ± 0,74	45,6 ± 0,84	45,8 ± 0,63	45,7 ± 0,71	n.s.	n.s.	n.s.
RDW [%]	13,9 ± 0,30	14,2 ± 0,33	13,9 ± 0,22	14,2 ± 0,17	0,015	n.s.	n.s.
HGB [g/dl]	15,6 ± 0,41	16,0 ± 0,53	16,0 ± 1,1	15,9 ± 0,55	n.s.	n.s.	n.s.
PLT [10^3/µl]	987 ± 95,5	1024 ± 185,9	876 ± 151,0	797 ± 114,8	n.s.	<0,001	n.s.
MPV [fl]	5,19 ± 0,32	5,29 ± 0,18	5,28 ± 0,16	5,43 ± 0,17	n.s.	n.s.	n.s.
CD3+ T cells [%]	18,7 ± 2,53	18,2 ± 4,25	21,9 ± 5,12	21,0 ± 3,29	n.s.	0,023	n.s.
CD19+ B cells [%]	52,3 ± 7,48	61,7 ± 8,20	47,0 ± 5,89	53,5 ± 4,58	<0,001	0,003	n.s.
GR1CD11b+ Gran. [%]	10,3 ± 2,64	6,9 ± 2,72	12,8 ± 8,84	7,7 ± 3,54	0,015	n.s.	n.s.
NK cells [%]	5,8 ± 0,85	4,4 ± 1,02	6,4 ± 1,37	6,3 ± 1,02	0,031	<0,001	n.s.

Supplementary Table S5 | Hematological examination of Dox-treated wt and Plk1-iKD mice. Basic hematological parameters were measured by automated peripheral blood cell counts. Leukocyte subsets were analyzed by FACS analysis, and the data represent the percentage of CD45+ cells (all leukocytes). WBC, total white blood cell count; RBC, red blood cell count; MCV, mean corpuscular volume; RDW, red cell distribution width; HGB, hemoglobin; PLT, platelet count; and MPV, mean platelet volume (means ± s.d., n=10, for each parameter). For statistical analysis, two-way ANOVA was applied.

Supplemental Methods

Inducible shRNA expression vector (exchange vector). The RMCE system has been described previously[20, 21]. For the shRNA expression vector, the following oligos representing the shRNA sequence 1473 against the murine Plk1 mRNA were cloned into pH1tetOflex by Bbs1/Xho1: (oPlk_1473 s: TCCCGCAGCAGGAAACCTCTCAAAGTTCAAGAGAC TTTGAGAGGTTTCCTGCTGCTTTTTC) and (oPlk_1473 as: TCGAGAAAAAGC AGCAGGAAACCTCTCAAAGTCTCTTGAACTTTGAGAGGTTTCCTGCTGC). The resulting vector was called pPlk_1473. pPlk-INV exchange vector: The vector contained the F3 site and the FRT site in a similar configuration[21]. pPlk-INV was generated by inserting the H1-tet-shPlk1 fragment from pPlk_1473 into the MCS of pINV-2 containing the following elements in 5' to 3' direction: synthetic polyA signal, F3-site, neomycin-resistance gene lacking the start ATG, hgH polyadenylation signal, MCS, loxP, CAGGS promoter[56], the codon-optimized tet-repressor gene, synthetic polyA signal, loxP, and FRT-site.

Southern Blot. To determine the integration of vector DNA carrying the Plk1-shRNA 1473 sequences, total DNA was isolated from ES cells and electrophoresed[31, 57]. To depurinize and denature DNA, gels containing DNA bands were incubated first for 15 minutes in 0.25 M HCl on a shaker to induce double-strand breaks, then for 30 minutes under denaturing conditions in a solution of 1.5 M NaCl and 0.5 M NaOH, and then for two 15-minute periods in a neutralizing solution containing 1.5 M NaCl, 0.5 M Tris–HCl (pH 7.2), and 1 mM EDTA (pH 8.0). DNA bands were then transferred to nylon membranes that were dried at room temperature, and DNA was fixed to the membranes by a 5-minute exposure to UV radiation on a UV transilluminator.

Plasmid DNA was detected with 3 different α-^{32}P–labeled probes**.** Blots were prehybridized for 20 minutes at 68 °C in QuickHyb buffer (Stratagene, Amsterdam, The Netherlands) and hybridized in fresh QuickHyb buffer containing probes (10^6 cpm/mL) for 1 hour at 68 °C. Membranes were washed twice in 2× SSC (1× SSC = 0.15 M NaCl plus 15 mM trisodium citrate dihydrate) for 15 minutes at 36 °C and exposed to MP Hyperfilms (Amersham Pharmacia Biotech, Freiburg, Germany). Bands were quantified by use of a Kodak gel documentation system (model 1D 3.5).

Generation of ES mice by tetraploid embryo complementation. Mice heterozygous for the targeted allele for the shRNA and the control were generated via tetraploid embryo complementation[20].

Mice. All animal studies were approved according to the German Animal Welfare Act. Mice were kept in the animal facility at Taconic Artemis GmbH in microisolator cages (Tecniplast Sealsave, Hohenpeißenberg). B6D2F1 mice used for the generation of tetraploid blastocysts were obtained from Harlan, NL. The drinking water which contained 2 mg/ml Dox (Sigma, München) and 10% sucrose was prepared every other day (kept in the dark). Athymic mice for tumor modeling were kept at the ZFE (Goethe University, Frankfurt). The use of animals complied with the regulations of the ZFE (Goethe University, Frankfurt) and the German Mouse Clinic (München). Details of the animal experiments that may influence results were included in the manuscript based on the ARRIVE reporting guidelines for the documentation of animal trails[58].

Analysis of mouse genotypes. To test the genotype of wt or iKD mice, genomic DNA was prepared from tail clips 0.5-0.8 mm in length with Viagen Direct PCR-Tail reagent (Peqlab Biotechnologie, Erlangen) according to the manufacturer's protocol. For the standard PCR, 10 ng genomic DNA was amplified using the sense primer 5'-ATCGCGGGCCCAGTGTCACTAGGC-3' and the antisense primer 5'-CTAGTACGCGCCTGCAGGCTAGCC-3'. The original plasmid containing the cDNA of the kd cassette served as the positive control.

Stem-loop RT-PCR. Reverse transcriptase reactions including purified total RNA, 50 nM stem-loop RT primer (Applied Biosystems), 1x RT buffer, 0.25 nM of each dNTP, 3.33 U/µl MultiScribe reverse transcriptase and 0.25 U/µl RNase inhibitor (both Applied Biosystems) were used. The 7.5 µl reactions were incubated in an Applied Biosystems Step One for 30 min at 16°C, 30 min at 42°C, 5 min at 85°C and then held at 4°C. All reverse transcriptase reactions, including no-template controls and RT minus controls, were run in triplicate.

Western blot. At the indicated time points, cells were lysed using RIPA buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% NP-40, 0.5% Na-desoxycholate, 0.1% SDS, 1 mM Na_3VO_4, 1 mM PMSF, 1 mM DTT, 1 mM NaF and protease inhibitor cocktail Complete and phosStop, (both Roche, Mannheim))[59]. Total protein was separated with 10%, 12% or 15% SDS-PAGE

and then transferred to Immobilon-P membranes (Millipore, Bedford, MA, USA). The membranes were exposed to corresponding antibodies for 1 h in 5% skim milk, washed with PBST and incubated for 30 minutes with the secondary HRP-conjugated antibodies. Finally, protein bands were visualized using the Super Signal® West Pico Chemiluminescent Substrate (Thermo Scientific, Rockford, IL, USA).

Pathology screen. Mice were sacrificed with CO_2 and analyzed macroscopically and weighed. The heart weight was determined. The tibia, thymus, spleen, kidney and left lobe of the liver were measured. Blood samples were taken and centrifuged, and the serum was stored at –20°C. The tails were preserved at -70°C for further genetic analysis. Following a complete dissection, an x-ray of the complete bone structure was taken when indicated (Hewlett Packard, Cabinet X-Ray System Faxitron Series, Böblingen). All organs were fixed in 4% buffered formalin and embedded in paraffin for the histological examination. Sections (2 µm) from the skin, heart, muscle, lung, brain, cerebellum, thymus, spleen, cervical lymph nodes, thyroid, parathyroid, adrenal gland, stomach, intestine, liver, pancreas, kidney, reproductive organs, and urinary bladder were cut and stained with hematoxylin and eosin (HE). The testicular specimen was fixed by immersion in 4% paraformaldehyde or Bouin´s fixative and embedded in paraffin using standard techniques. Sections (5 µm) were stained with hematoxylin/eosin and scored for spermatogenesis[27].

Determination of proliferation indices. Definiens Architect Software (Definiens Understanding Images, München) was used to determine the proliferative index, which was defined as the number of Ki-67-positive cells/tissue area. To quantify the proliferative index, the staining intensity of the sections was analyzed. Only the organs with similar intensity were analyzed using the same threshold for positive nuclei. The proliferative activity of spermatogonia was defined as the percentage of Ki-67-positive cells in seminiferous tubules of stages VII/VIII[26].

Immunohistochemistry. After deparaffinization and rehydration of the sections, immunohistochemistry was performed as follows: sections of thymus, spleen, small and large intestine, ovary and testis were microwaved for 15 min at 1000 watts in sodium citrate buffer (pH 6.0), treated with 3% H_2O_2 for 30 min, blocked with BSA (5%) for 30 min, and incubated with the monoclonal anti-Ki-67 antibody (1:50) overnight at 4°C. Sections were then exposed to the biotinylated secondary antibody (rabbit anti rat IgG, 1:200, DAKO, Hamburg) for 45

min, followed by incubation with avidin-biotin-peroxidase (ABC) complex (Vector/Biozol, Eching) for 45 min. The immunoreactivity was visualized using AEC for 25-30 min. Sections were counterstained with hematoxylin. Control immunostaining reactions were performed by substituting the buffer with the respective primary antibodies, and the results were negative.

Collection and analysis of blood samples. Blood samples were collected from ad libitum fed isoflurane-anesthetized mice by puncturing the retro-orbital sinus with non-heparinized capillaries (1.0 mm in diameter, Neolab, München) and transferred to heparinized tubes (KABE, Nümbrecht). Additionally, a small amount of blood (about 50 µl) was collected in an EDTA-coated tube (KABE, Nümbrecht) for the hematological analysis. Each tube was immediately inverted five times to achieve a homogeneous distribution of the anticoagulant. The EDTA-containing blood was mixed thoroughly and used to measure basic hematological parameters using a blood analyzer, which was carefully validated for the analysis of mouse blood (ABC-Blutbild-Analyzer, Scil Animal Care Company, GmbH, Viernheim) within 2 h after blood collection.

Heparinized samples were stored at room temperature for 1-2 h before centrifugation (10 min, 9503 x g; 8°C, Biofuge Fresco, Heraeus, Hanau) and aliquots were distributed between different blood based screens.

Plasma values of 21 biochemical parameters were determined using an AU400 Autoanalyzer (Olympus, Hamburg) and adapted reagents from Olympus (Hamburg)/ Beckman Coulter (Krefeld). Free fatty acids (NEFA) were measured using a kit from Wako Chemicals GmbH (Neuss), and creatinine was measured using an enzymatic test produced by Biomed (Oberschleißheim).

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