Supplemental data

Supplemental Methods

Stable transgene experiments. We constructed two transgenes, one containing a promoter CGI of human *INSL6* gene, which is methylated and silenced in tissue-specific manner (1), and the second containing a promoter CGI of human *p16* gene, which is frequently methylated in cancer cells. We used pHyg-G5-eGFP vector for transfection. It carries a Hygromycin resistance gene and an eGFP reporter gene (Supplemental Figure 2A). A *NheI*/*BglII* fragment containing either the *INSL6* promoter (900bp) or *p16* promoter (882bp) was PCR amplified from normal genomic DNA and cloned into the vector in front of eGFP. To generate the construct containing the cis-element, a *NheI*/*XhoI* fragment containing the 140bp cis-element was oligo synthesized and inserted in front of the promoters. We linearized the plasmids (with or without cis-element) with *EcoRV* (Supplemental Figure 2B) and individually transfected them into a human prostate cancer cell line LNCaP and a mouse fibroblast cell line NIH3T3. The transfected cells were selected with 200 µg/ml hygromycin for 7 days. The surviving cells were then pooled and cultured in selection free medium. Cells were collected every 10 days and analyzed for DNA methylation of the integrated plasmids by bisulfite-pyrosequencing and clonal bisulfite-sequencing. The transfection and methylation analysis experiments were independently repeated three times.

Targeted knock-in experiments. A standard targeting method was used to generate germline insertions of either control-element or cis-element upstream of the mouse *p16* promoter. We used gap repair method (2) to isolate a *p16* genomic clone suitable for the construction of a targeting vector. Briefly, we obtained a BAC clone (carrying Cm^R) derived from 129 mouse strain containing *p16* from the Sanger Center. Two homologous fragments of ~500 bp each were PCR amplified from the BAC DNA and cloned into a pDTA vector which included a

diphtheria toxin A gene (DTA) as a negative selection marker. The resulting pDTA vector was linearized and introduced into a homologous recombination proficient E. coli strain (SW105). The transformed cells were plated on Lysogeny Broth containing the antibiotic ampicillin (LB/Amp). Only recombinants survived Amp selection. To insert the sequences, we employed homologous recombination as described (2). The scheme is shown in Supplemental Figure 4. First, we constructed a recombinogenic fragment consisting of the following (5' to 3'): 500bp sequence homologous to the sequence 5' to the site of insertion, LoxP, control or cis elements, Frt, PKG-Em7-Neo, Frt, LoxP, and 500bp sequence homologous to the sequence 3' to the site of insertion. This fragment was released from its vector and recombined with the *p16* genomic clone. EM7 is a synthetic promoter functional in E. coli, making Neo a dual marker. To modify one allele of *p16*, the linearized targeting construct was introduced into mouse ESCs at Mouse ES Cell Core Facility at the Baylor College of Medicine.

Southern blot, DNA sequencing and genotyping assays. For Southern blotting, 20 µg genomic DNA was digested with *BamHI*, resolved on a 0.7% agarose gel, transferred to nylon membranes and hybridized with P^{32}-dCTP-labelled 5' and 3' probes. For PCR and DNA sequencing analysis, the knock-in region was amplified from genomic DNA by using the forward primer (5'-TTTAAATCCTCCCTTCTGTCCA-3') and the reverse primer (5'-AGAGTTACCAGGGATCCACCTAAT-3'). The PCR product was directly sequenced using the same primers at Sequencing Core Facility at the Baylor College of Medicine. To facilitate genotyping, we used multiplex PCR (three primers in one reaction) to detect the presence of the endogenous and knock-in alleles (Supplemental Table 1).

Generation of targeted knock-in mouse strains. A standard targeting method was used to generate germline insertions of either control-element or cis-element upstream of the mouse *p16* promoter. Three recombinant mES clones were injected into C57BL/6J blastocysts to generate chimeric mice at the Genetically Engineered Mouse Core Facility at Baylor College of

Medicine. Chimeric mice were crossed onto the C57BL/6J background and successful germline transmission was confirmed in the offspring by both Southern blotting and DNA sequencing.

DNA methylation analysis. Quantitative bisulfite-pyrosequencing for DNA methylation analyses was performed as previously described (3). Primer sequences and PCR conditions are summarized in Supplemental Table 2. For each assay, set-up included positive controls (*SssI*-treated genomic DNA) and negative controls (whole genome amplified genomic DNA), mixing experiments to rule out bias, and repeated experiments to assess reproducibility. Annealing temperatures were optimized to overcome PCR bias as previously reported (3). For clonal bisulfite sequencing analysis, we cloned post-bisulfite PCR products into the TA vector pCR4-TOPO (Invitrogen), extracted plasmid DNA from 15-20 clones with the use of a QIAprep Spin Miniprep kit (Qiagen), and sequenced the DNA at Sequencing Core Facility at the Baylor College of Medicine.

Gene expression analysis by real-time RT-PCR. TaqMan qRT-PCR was carried out in triplicate for mouse *p16* as previously described (4). This assay was designed to have primers/probes to specifically span *p16* exon-exon junctions. Relative gene expression was calculated by the ratio of the target genes to *β-Actin* (Mm00607939_s1) expression on an ABI StepOnePlus Detection System.

5-Aza-2'-deoxycytidine (DAC) treatment of cells. Differentiated mESCs with cis-element knock-in were given either PBS or DAC (Sigma) for 72 hours. For DAC treatments, increasing concentrations from 0.25 μM to 5 μM were used.

Chromatin immunoprecipitation (ChIP) and real-time PCR. ChIP analyses were performed as described previously (5). Antibodies used were: anti-histone H3 (Abcam; catalog no. ab1791), anti-histone H3K9-acetylation (anti-H3K9Ac; Millipore; catalo no. 17-352), anti-histone H3K4-trimethylation (anti-H3K4me3; Millipore; catalog no. 17-614), anti-histone H3K9-

dimethylation (anti-H3K9me2; Abcam; catalog no. ab1220), and anti-histone H3K27-trimethylation (anti-H3K27me3; Millipore; catalog no. 17-622). Primer and probe sets corresponding to regions of interest within the cis-element, *p16* promoter and exon1 are summarized in Supplemental Table 3.

Histology and immunohistochemistry. For histological analyses, mouse tissues were fixed in 4% paraformaldehyde. Fixed tissues were paraffin-embedded, sectioned, and stained with haematoxylin and eosin according to standard laboratory protocols at the Cellular and Molecular Morphology Core at the Texas Medical Center Digestive Disease Center. We performed immunohistochemical staining for p16 protein on paraffin-embedded tissue sections as previously described (5). Briefly, 5-µm-thick sections were deparaffinized, rehydrated, incubated with incubated with 3% H_2O_2 to block endogenous peroxidase activity, and incubated with normal sheep serum to block nonspecific antibody binding. The sections were incubated at 4 °C overnight with a monoclonal antibody against p16 protein (1:100 dilution; Santa Cruz; catalog no. sc-1661), followed by incubation with anti-biotin secondary antibody (Vector laboratories) diluted in 1: 1000. Slides were developed using DAB kit (Vector laboratories), and imaged using a DS-Fi1 camera connected to Nikon E80i stereomicroscope. Images were processed using Nikon imaging software, NIS Elements RA3.2. p16 expression in nuclei was scored as present or absent.

References

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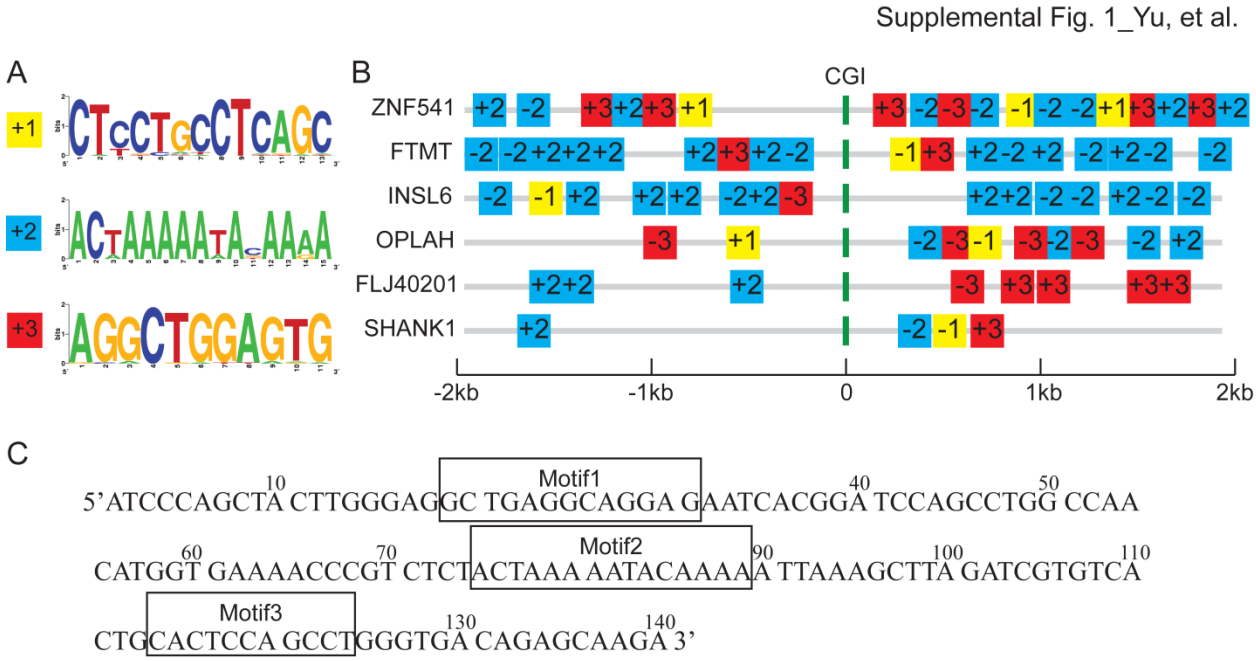
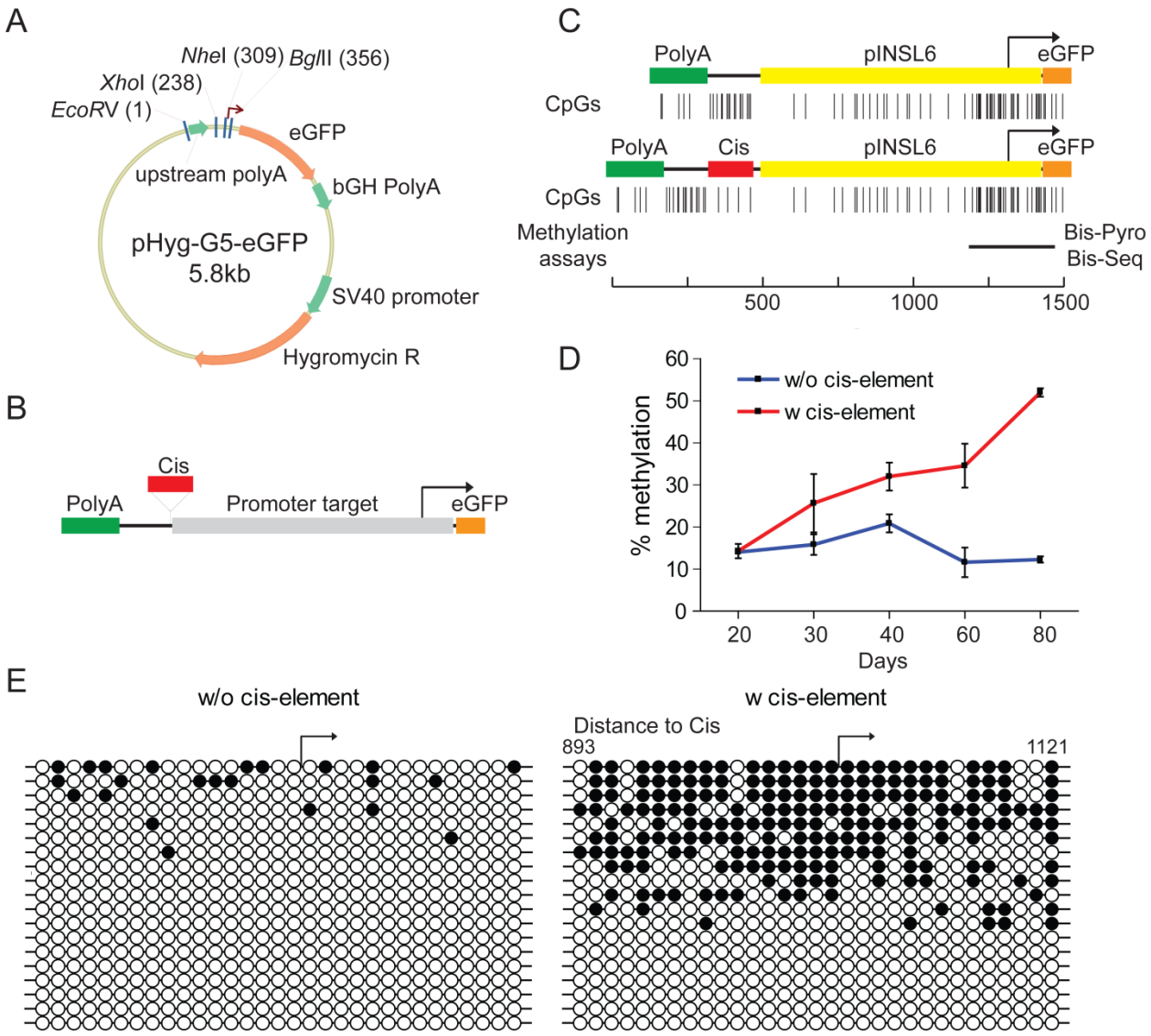
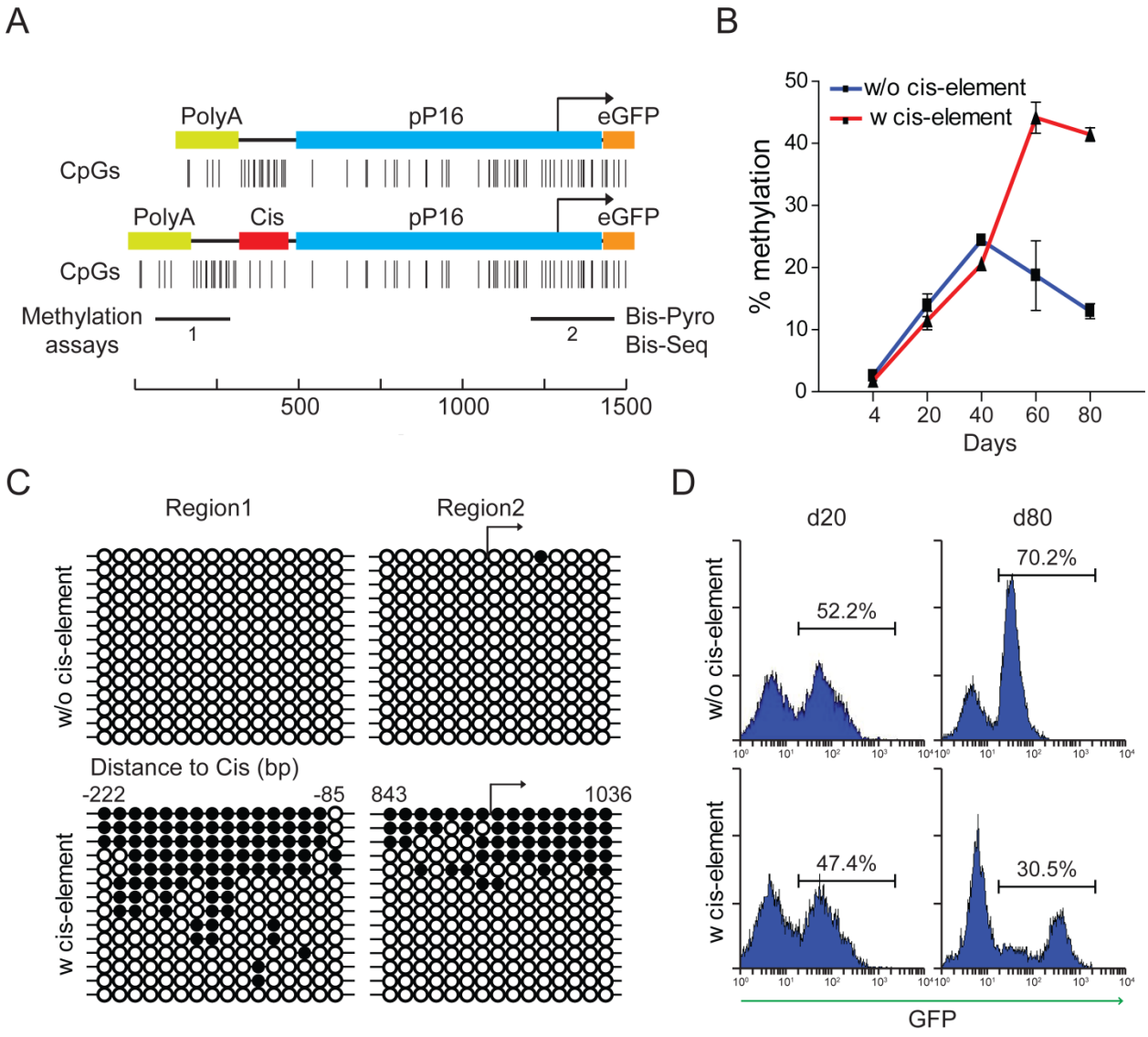
Supplemental Figures



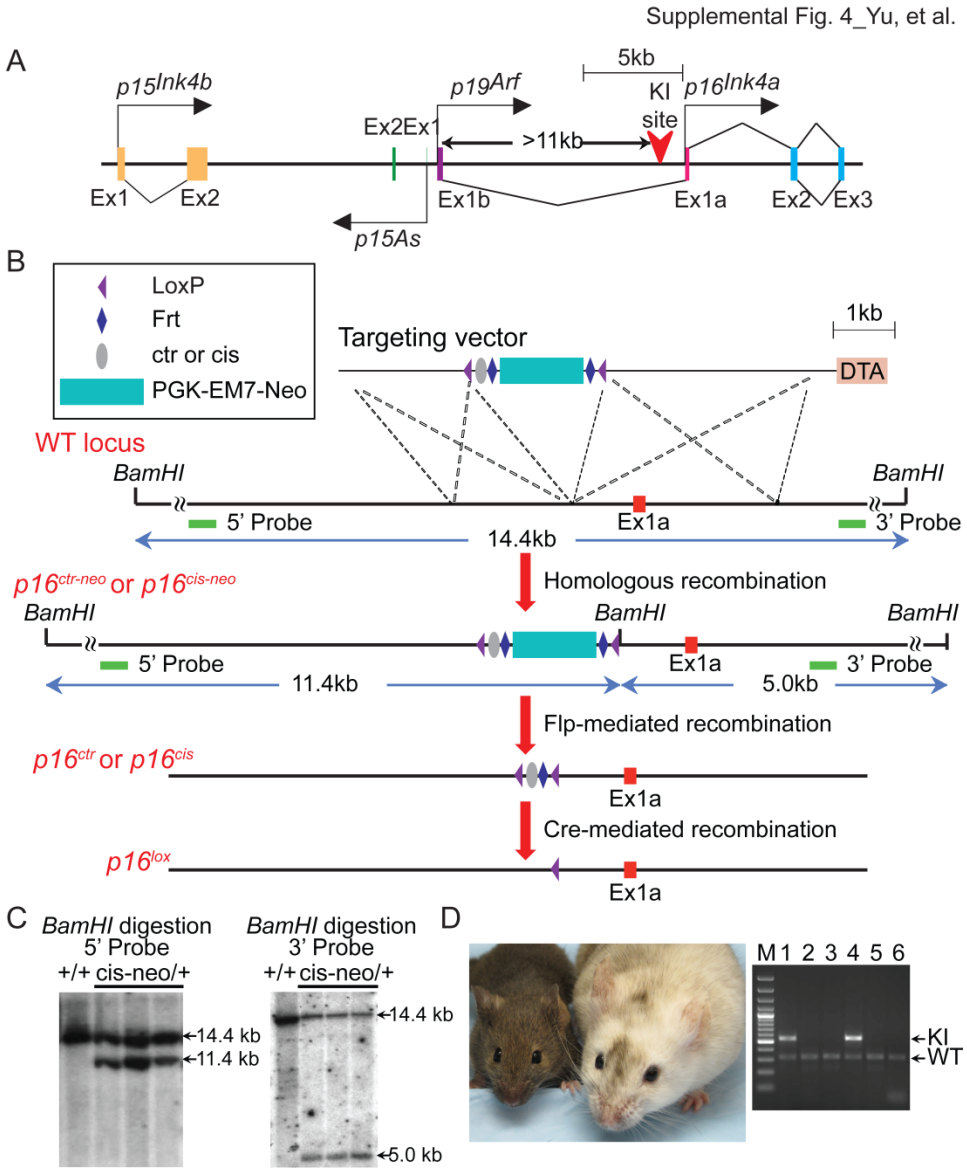
Supplementary Figure 1. DNA sequence motifs significantly enriched in genes with methylated promoter CGIs. (A) Sequence logos represent the consensus sequence for each of three candidate motifs. The +/- signs indicate presence in forward and reverse DNA strands, respectively. **(B)** Occurrences and positions of the motifs flanking the center of the indicated methylated promoter CGIs (green bar). **(C)** Insertion construction of a 140bp DNA sequence element which comprises the top three identified motifs.



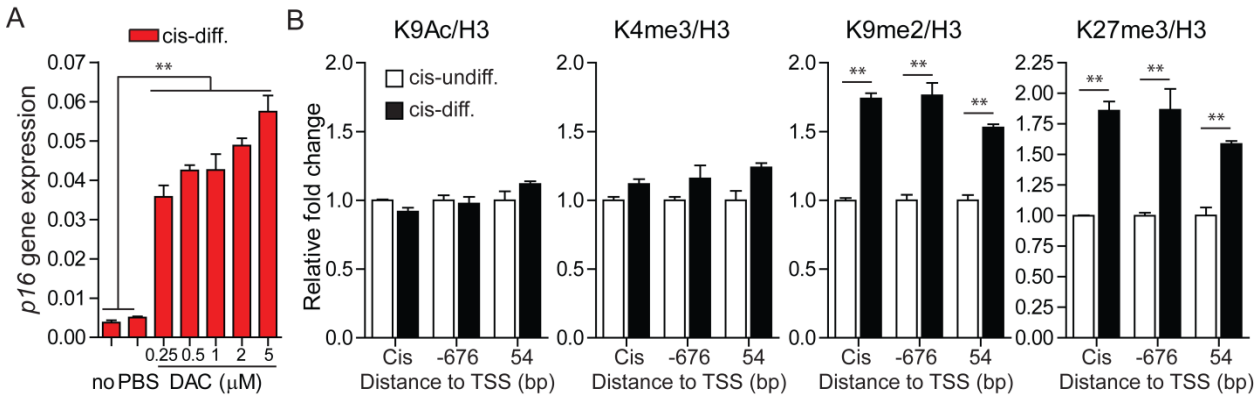
Supplementary Figure 2. Cis-element induces CGI methylation on the *INSL6* transgene in a human prostate cancer cell line LNCaP. (A) Map of pHyg-G5-eGFP vector. **(B)** Map of linearized plasmid with cis-element inserted in front of targeted promoter. **(C)** CpG maps of the two constructs without (w/o) or with (w) cis-element. (Vertical lines indicate CpG sites.) The locations of methylation assays for both bisulfite-pyrosequencing (Bis-Pyro) and clonal bisulfite-sequencing (Bis-Seq) are indicated below. **(D)** Quantitative methylation analysis by bisulfite-pyrosequencing at various time-points after transfection. Results are shown from 3 independent experiments. Significantly increased methylation was found in constructs including the cis-element. **(E)** Clonal bisulfite sequencing confirms that the cis-element induces methylation at 60 days after transfection. Each row represents an individual cloned allele. Circles represent the location of CpG sites; open circles represent unmethylated cytosines, and filled circles indicate methylation. The 31 CpGs assayed (from 893bp to 1121bp relative to cis-element) were specific to the constructs; the first 22 CpG sites from left to right are located within the *INSL6* promoter CGI, and the last 9 are within the vector sequence.



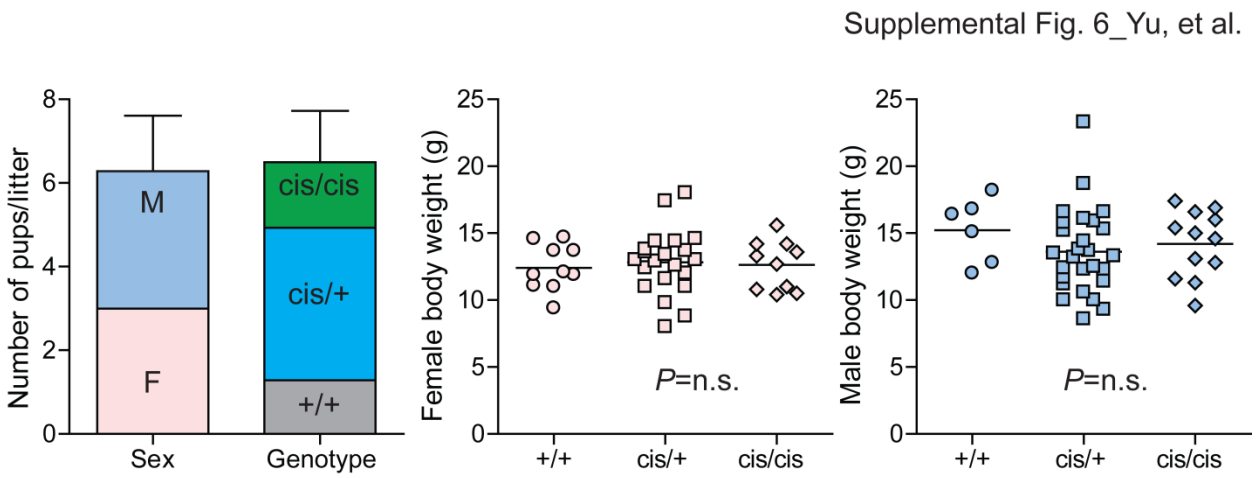
Supplementary Figure 3. Cis-element induces CGI methylation on the *p16* transgenes in a mouse fibroblast cell line NIH3T3. (A) CpGs assayed for DNA methylation. **(B)** DNA methylation kinetics of transgenes (with or without cis-element) was determined by quantitative bisulfite-pyrosequencing at region 2. **(C)** Bisulfite-sequencing confirms that cis-element induces methylation at 80 days after selection. The induced de novo methylation extends both 5' (region 1, from -222bp to -85bp relative to cis-element) and 3' (region 2, from 843bp to 1036bp relative to cis-element) from the cis-element. **(D)** FACS analyses shows cis-element reduces the *p16* transgene expression at day 80 after selection.



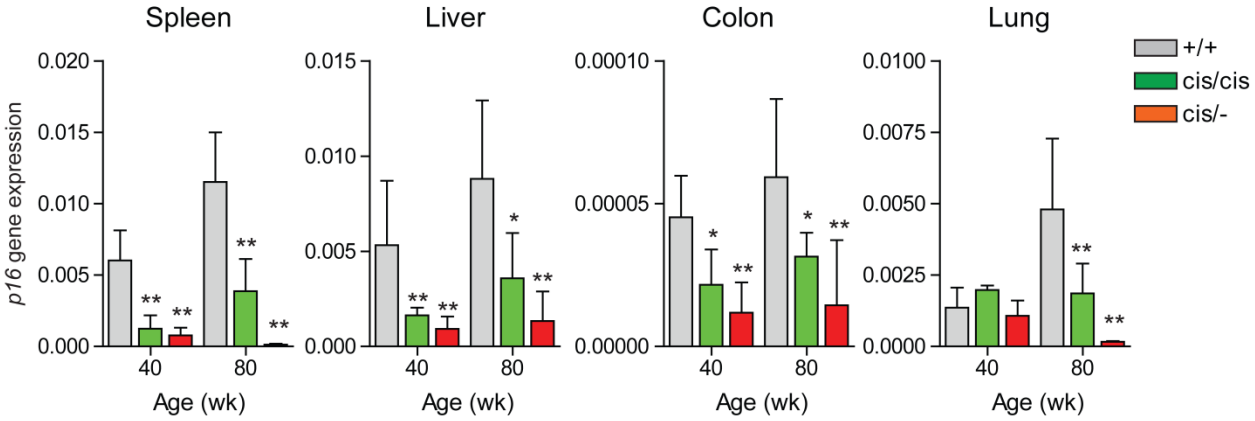
Supplementary Figure 4. Targeted knock-in of control or cis-elements upstream of mouse *p16* promoter. (A) Genomic structure of mouse *p16* locus. The exons 1α ($p16^{Ink4a}$) and 1β ($p19^{Arf}$) are separated by >11kb. Red arrow indicates the knock-in site**. (B)** Targeting strategy to insert either cis- or control (ctr) elements 1kb upstream of *p16* promoter. Schematic diagram showing structures of targeting vector, wild-type (WT) *p16* allele, targeted $p16^{cis\text{-}neo}$ or $p16^{ctr\text{-}neo}$ allele, $p16^{cis}$ or $p16^{ctr}$ allele after Flp mediated recombination and $p16^{lox}$ allele after Cre mediated recombination. The crossed broken lines indicate the areas of homology. Digestion with *BamHI* distinguishes the targeted allele from the WT allele. The neomycin resistant cassette is flanked by Frt sequences and the inserted cis- or ctr-elements are flanked by LoxP sequences. **(C)** Southern blot analysis. The expected sizes of wild type (+) (14.4 kb) and *p16* gene-targeted (cis-neo) bands (11.4 kb for 5' probe and 5.0 kb for 3' probe) are shown. **(D)** Germline transmission of targeted $p16^{cis\text{-}neo}$ allele. Left panel: the male chimera and his agouti offspring. Right panel: PCR genotyping of the wild-type (WT) and *p16* gene-targeted (KI) alleles. M, 100bp DNA marker; 1 and 4 are $p16^{cis\text{-}neo}$ positive offspring; and 2, 3, 5 and 6 are the germline-negative offspring.



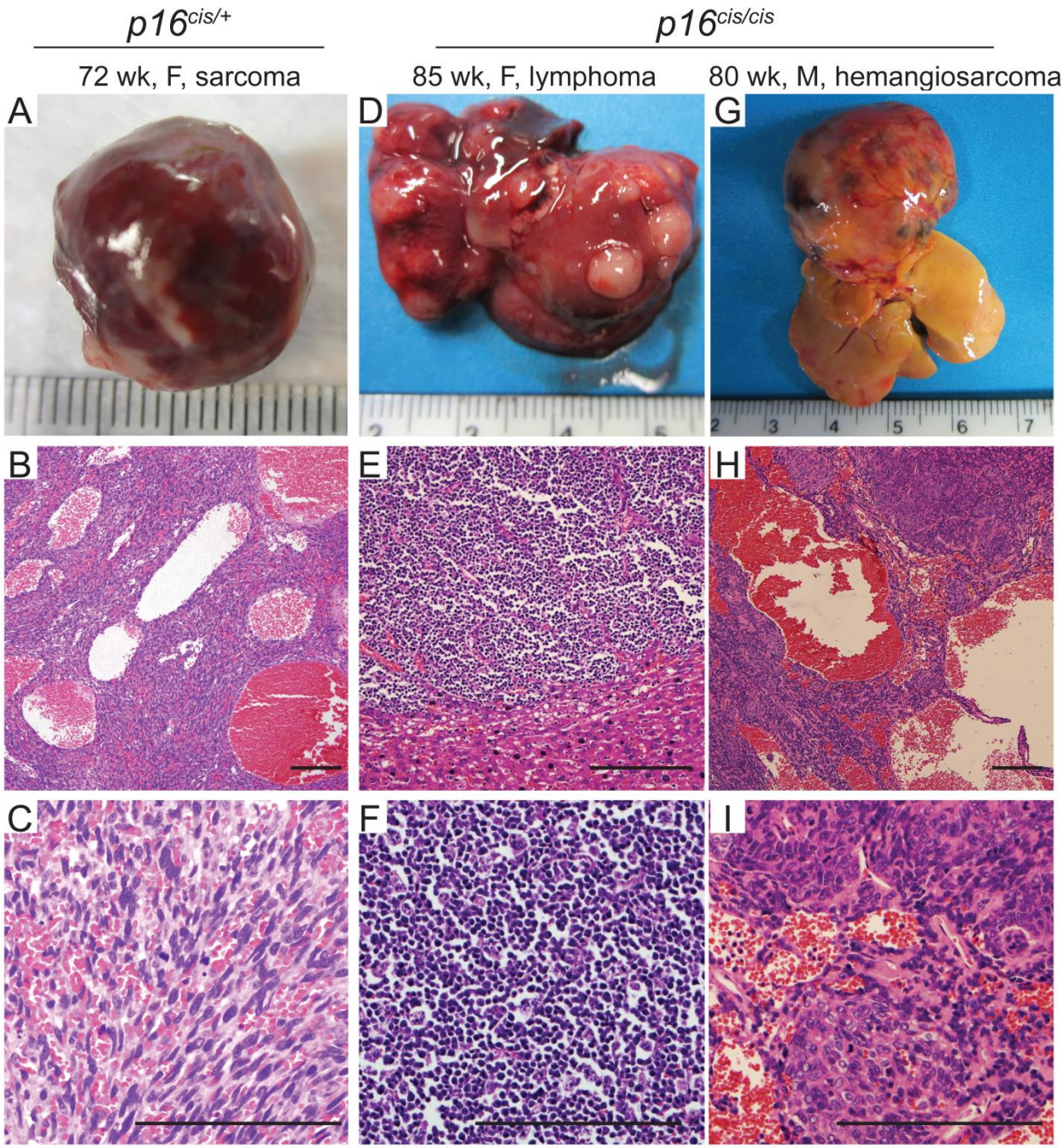
Supplementary Figure 5. (A) The effect of DAC treatment on the expression of *p16*. Differentiated *p16*$^{+/cis\text{-}neo}$ mESCs were cultured in either PBS or increasing amounts of DAC (from 0.25 to 5 μM) for 72 hours. Compared with untreated (no) or PBS treated samples, treatment of DAC restored *p16* expression in a dose-dependent manner. **(B)** Repressive chromatin modifications are associated with cis-mediated promoter methylation and *p16* silencing. ChIP-qPCRs for the enrichments of H9K9Ac, H3K4me3, K9Me2, and K27me3 were performed in *p16*$^{+/cis\text{-}neo}$ mESCs before and after differentiation. Three regions spanning the *p16* promoter were analyzed (indicated on the x axis). The y axis represents the relative fold change of the ratio of the H3-acetylation or methylation immunoprecipitation to a core histone H3 immunoprecipitation. Values are given as means and SDs. ***P* < 0.01 by Student's *t* test.



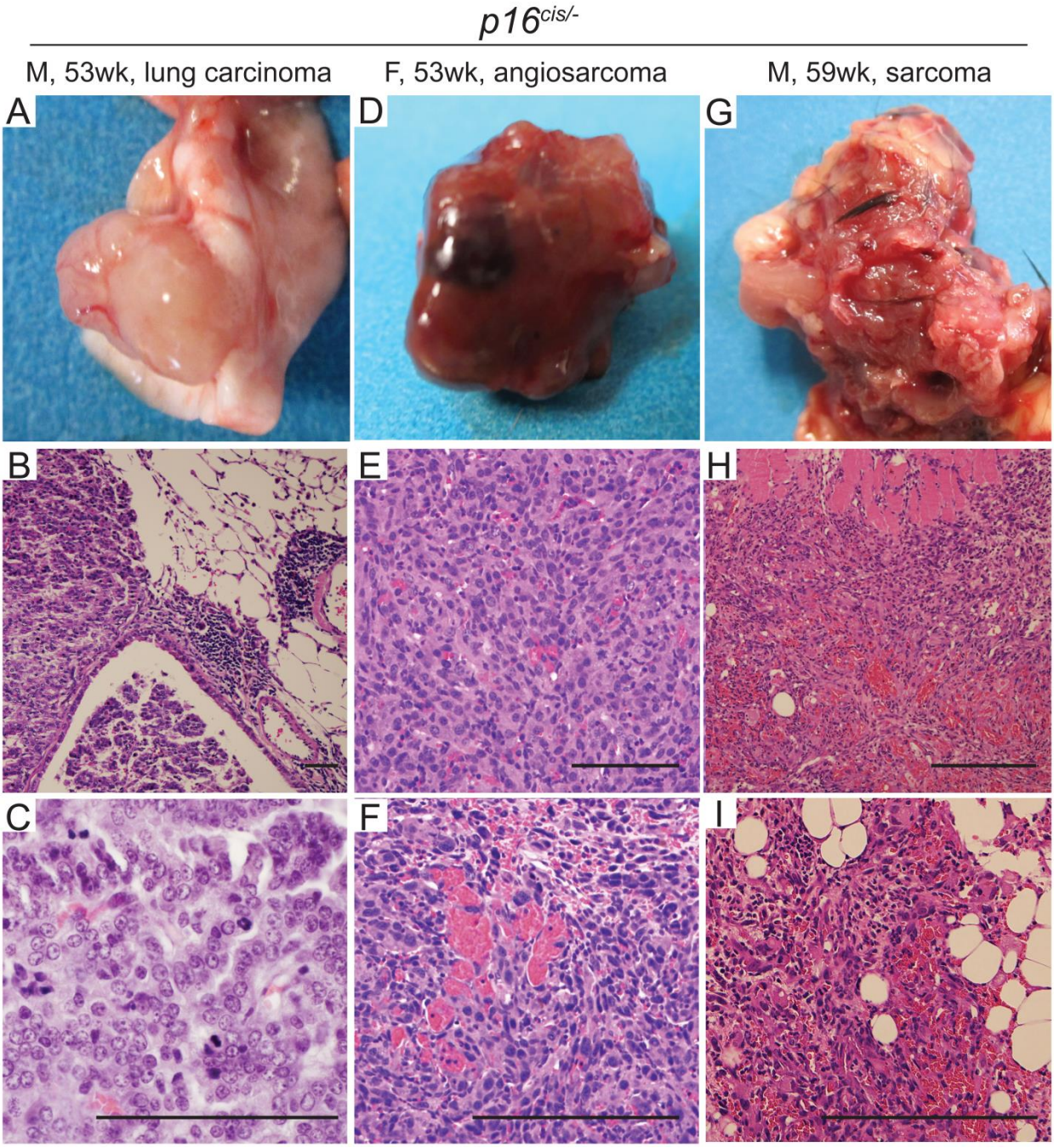
Supplementary Figure 6. Litter size, ratios of sex and genotype and body weight at weaning of pups from *p16*^{cis} heterozygous intercrosses. F, female; and M, male. Results are means ± SD.



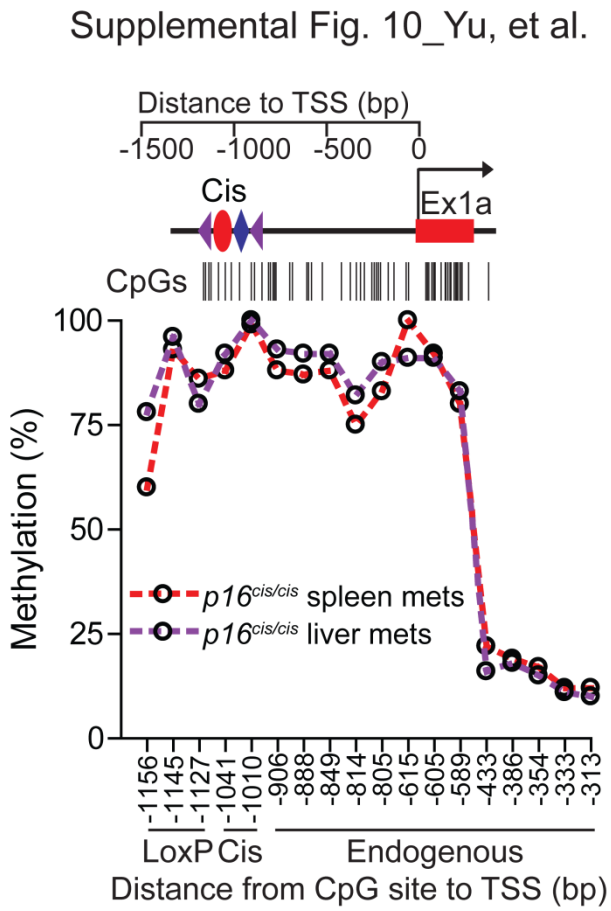
Supplementary Figure 7. Real-time *p16* mRNA expression analysis in aging tissues. qRT-PCR of *p16* expression shows that, in multiple tissues, the induction of *p16* expression during aging was significantly repressed in the $p16^{cis/cis}$ and $p16^{cis/-}$ mice (n=3-5). * *P* <0.05 and ***P* < 0.01by Student's *t* test; error bars are ± SD.



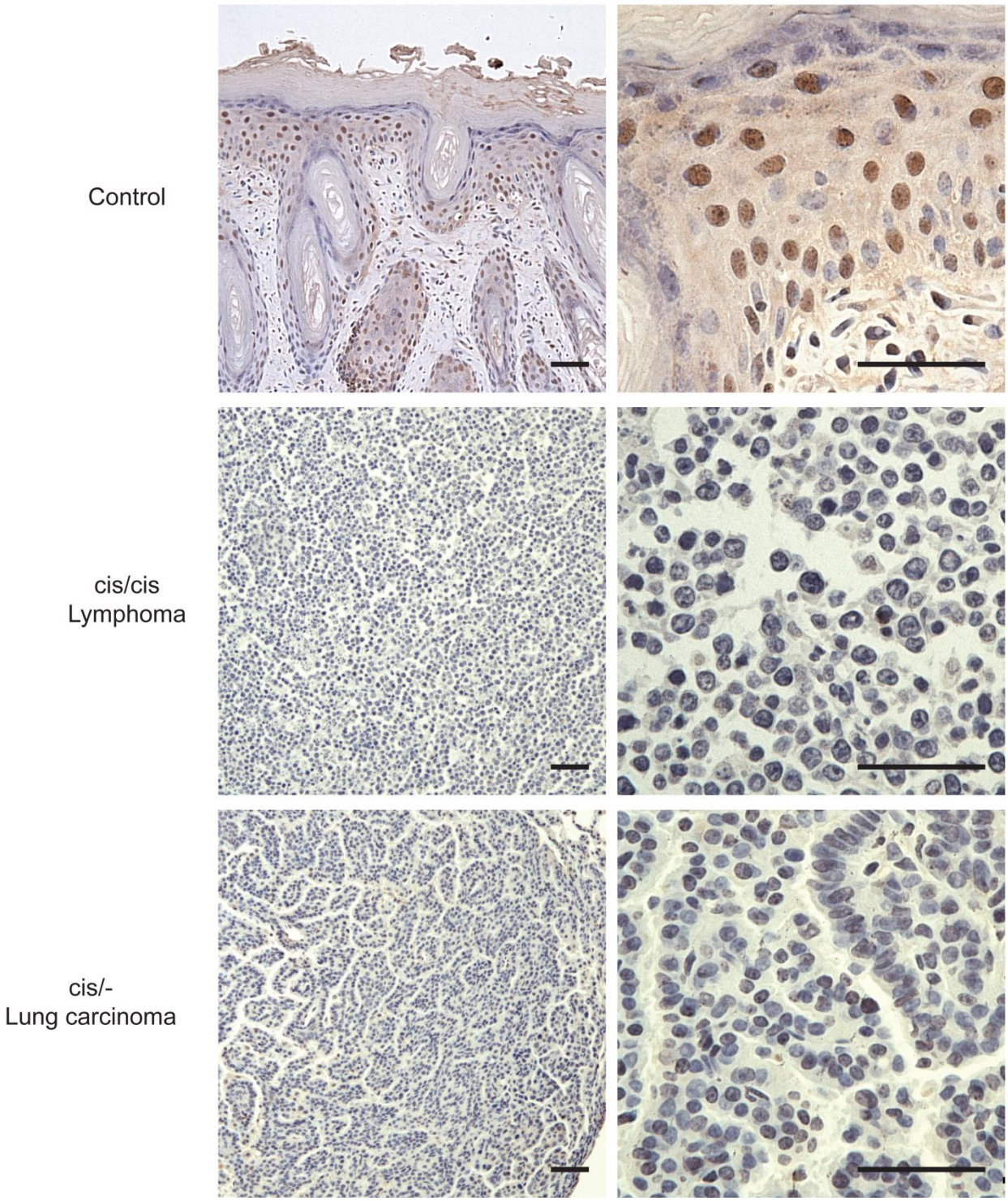
Supplementary Figure 8. Gross appearance and histology of tumors collected from one *p16*$^{cis/+}$ (A-C) and two *p16*$^{cis/cis}$ (D-I) mice. The age and sex of each animal are indicated. **(A-C)**: A sarcoma arising from the neck region. **(D-F)**: A hepatic lymphoma metastasis. **(G-I)**: A liver hemangiosarcoma. Scale bars equal 200 µm.



Supplementary Figure 9. Gross appearance and histology of tumors collected from *p16*$^{cis/-}$ mice. The age and sex of each animal are indicated. **(A-C)**: a lung papillary carcinoma. **(D-F)**: an angiosarcoma arising from the rear leg. **(G-I)**: a sarcoma arising from the tailbone. Scale bars equal 200 µm.



Supplementary Figure 10. DNA methylation profiling in metastatic *p16*$^{cis/cis}$ tumor cells. Tumors were collected from metastatic lymphoma in the spleen and liver respectively. Top panel shows a schematic of the *p16* promoter of cis targeted allele, including CpG map and distance to TSS. Bottom panel shows a plot of methylation level versus genomic location of CpG sites analyzed.



Supplementary Figure 11. Immunohistochemical analysis of p16 expression in tumors collected from *p16*$^{cis/cis}$ and *p16*$^{cis/-}$ mice. Immunohistochemistry confirms the lack of p16 expression in the transformed tumor cells. For each staining, there is a magnified view on the right panel. Scale bars equal 50 μm.

Supplementary Table 1. Primers and PCR conditions for genotyping assays

Genotype	Primer set	Primer sequence	PCR condition (Tm_cycle)	PCR product
p16-Ctr-KI	common	TTTTTAAATCCTCCCTTCTGTCC	55°C_35	
	wt	GGGCTGTTGTTTGTTTAAATGAGT		156 bp_wt
	KI	AGCCTGGGTGACAGAATGAG		300 bp_KI
p16-Cis-KI	common	TTTAAATCCTCCCTTCTGTCCA	55°C_35	
	wt	GGGCTGTTGTTTGTTTAAATGAGT		156 bp_wt
	KI	AGAGTTACCAGGGATCCACCTAAT		326 bp_KI
P16-null	common	GACTCCATGCTGCTCCAGAT	60°C_35	
	wt	GGCAAATAGCGCCACCTAT		189 bp_wt
	KO	GCCGCTGGACCTAATAACTTC		243 bp_KO

Supplementary Table 2: Primer sequences, PCR conditions and assays for quantitative DNA methylation analysis by bisulfite-pyrosequencing (Btn, biotin)

		PCR		Pyrosequencing	
Assays	Regions	PCR Primers	Condition (Tm_cycle)	Sequencing Primers	Sequence Analyzed
Ctr	1	AGAAAGGGTTATTGTTTTTTTGGTGAG	55°C_45	TTTTAAATTTTTTTTTTTGTTTAGT	TTC/TGAGATAATTTC/TGTATAATGTATGTTATAC/TGAAGTTATAT
		[Btn]CTAACCTCCAACTCCTAACCTCAAATAATC		AGAAAATGTTTTGGAGTAG	GAC/TGC/TGGTGGTTTATATTTGTAATTTTAGTATTTTGGGAAGTC/TGAGGC/TGG
	2	AGGTTAGTTTGGTTAAAATGGTGATAT	55°C_45	GGTTGAGGTAGGAGAA	TC/TGTTTGAATTC/TGGGAGGTAGAGGTTGTAGTAAGTC/TGAGATC/TGC/TGTTATT
		[Btn]CTCCTCTTCAAACCTAAAATTCCTATACTT		GGAGGTAGAGGTTGTA	GTAAGTC/TGAGATC/TGC/TGTTATTATTTTTTAGTTTGGGTGAT
Cis	3	AGAAAGGGTTATTGTTTTTTTGGT	55°C_45	TTTAAATTTTTTTTTTTGTTTAGTA	TTC/TGAGATAATTTC/TGTATAATGTATGTTATAC/TGAAGTTATAT
		[Btn]TTCATCCCAACTACTTAAAAAACT		TTTTTGTATTTTTAGTAGAG	AC/TGGGTTTTTATTATGTTGGTTAGGTTGATATC/TGTGATTT
p16 promoter	4	AGTATAGGAATTTTATTAGTTAGGTATAT	55°C_45	TTAGGTATATAATATAATTT	C/TGTATAATGTATGTTATAC/TGAAGTTATTAGGTGGATT
		[Btn]AACAACCCTAACTCAAACAAC		ATTTTTGGTAATTTTGTTTA	AAGC/TGTGTTTTTTTTTTTGTTTTATAAGATTGTAAAGAC/TGTTTTTAAC/TGAATAA
				AAATGTTTTTGTAAATTTTT	C/TGTAAAGATTC/TGGATTTTATATTGGGC/TGTGGTATTTTTTA
	5	AGGTGGATTTTTGGTAATTTTGTT	55°C_45	TTTTTGTTTTATAAGATTGTAA	AGAC/TGTTTTTAAC/TGAATAATTTAAATC/TGGTGTAAC/TGTTTATGC/TGTAGTAT
		[Btn]AACCCTAACTCAAACAACTCATTTT		TATTTTATAAGTAGATTGTT	TTTC/TGATGATTTTATTTC/TGTTATTTTTTTATAGTTGTGTATAG
	6	GTTAGGGTTGTTGGGATTTTAGTT	60°C_45	GTTTTAGTGTAGTGATGAAAATTA	TTTTTTTTC/TGTTTTTTTAATATTTGGGTGTTG
		[Btn]CCCCATACCTAATCACCCTTTAAC		GGAGGAAGGAGAGATT	TC/TGAGAAGGATTAGTTTATTTTTTTAGAAGATAC/TGTGTG
				GAGAAGGATTAGTTTATTTTTTTA	GAAGATAC/TGTGTGTATTTTTTTGTTGTGC/TGGGTTTAGAAGGAGTTTAGC/TGT
	7	TGTTAAAGGGTGATTAGGTATGGG	55°C_45	GGGGGAGGGGTGTTA	GC/TGTGGGTAGTAGGC/TGGGGGTTGTTC/TGATTTTTTAGC/TGTTGTTTTAA
		[Btn]TAAACACCCCTAAAAACACTACTATT			
	8	TGTTAAAGGGTGATTAGGTATGGG	60/57/54/51°C_45	GTTATATTTAGGGTAAATAG	C/TGTTATTTATGGC/TGGGTTGTGGAGTTAGGTTAGGAGTA
		[Btn]TCCCTCCTTCCTTCCTCTAAAAAT			
	9	CCCCCCTAACCAATCTATCTACA	55°C_45	GGAGGGATTTATTGGTTAT	AC/TGATTGGGC/TGATTGGGC/TGGGTATTGAATTTTC/TGC/TGAGGAAAGC/TGAATT
		[Btn]CCCCCCTAACCAATCTATCTACA			

Supplementary Table 3. Primer sequences for real-time ChIP assays

Region analyzed	Forward primer	Reverse primer	Probe
cis-element	TGGAAGAAAGGGCCATTGC	CTGGACAGAAGGGAGGATTTAAAA	6FAM-TTTCTGGTGAGGACTGTC
p16 promoter (-676bp to TSS)	GATTGCCCTCCGATGACTTC	GTATCAGTGCTAGGATTCTGTACACAACT	6FAM-CCCCGTCACTTTTTTA
p16 exon1 (54bp to TSS)	ACTGAATCTCCGCGAGGAAA	TGTCTGCAGCGGACTCCAT	6FAM-AACTCGAGGAGAGCCAT