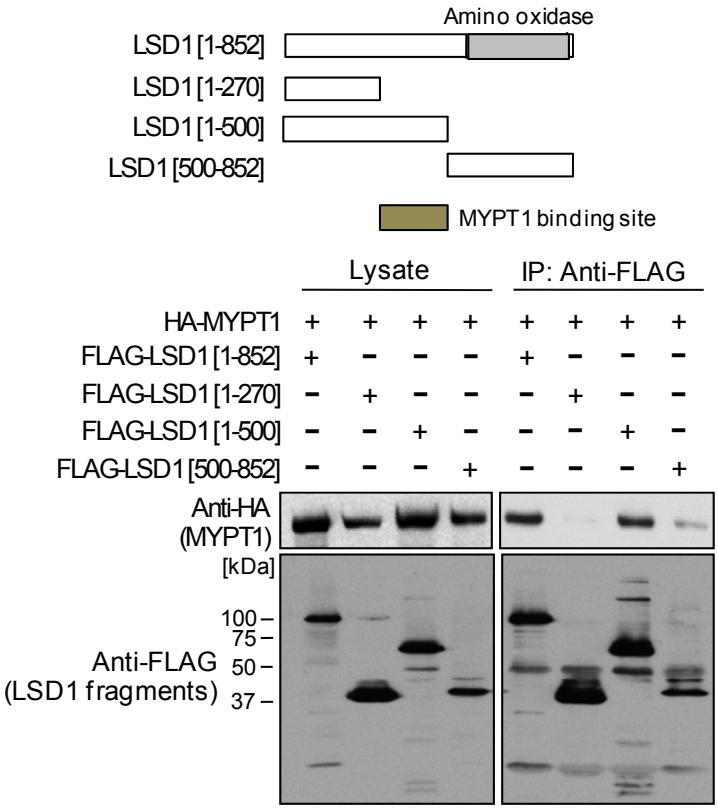
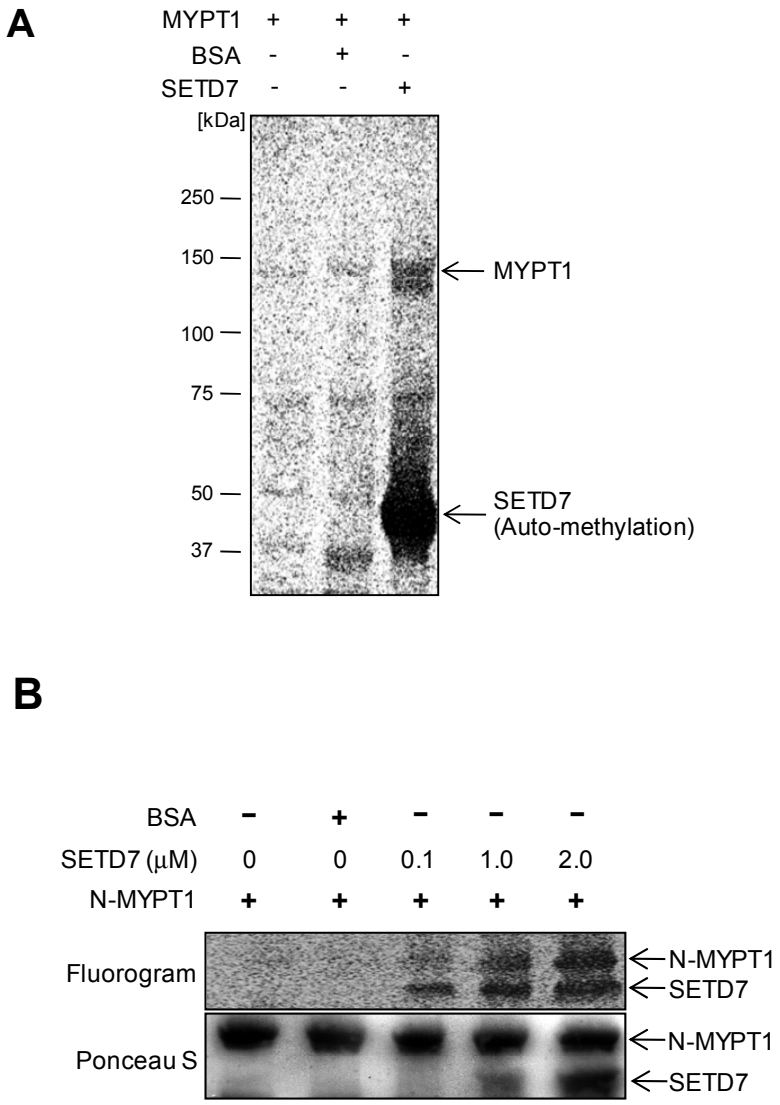
Supplementary information

Demethylation of MYPT1 by LSD1 promotes cell cycle progression in cancer cells

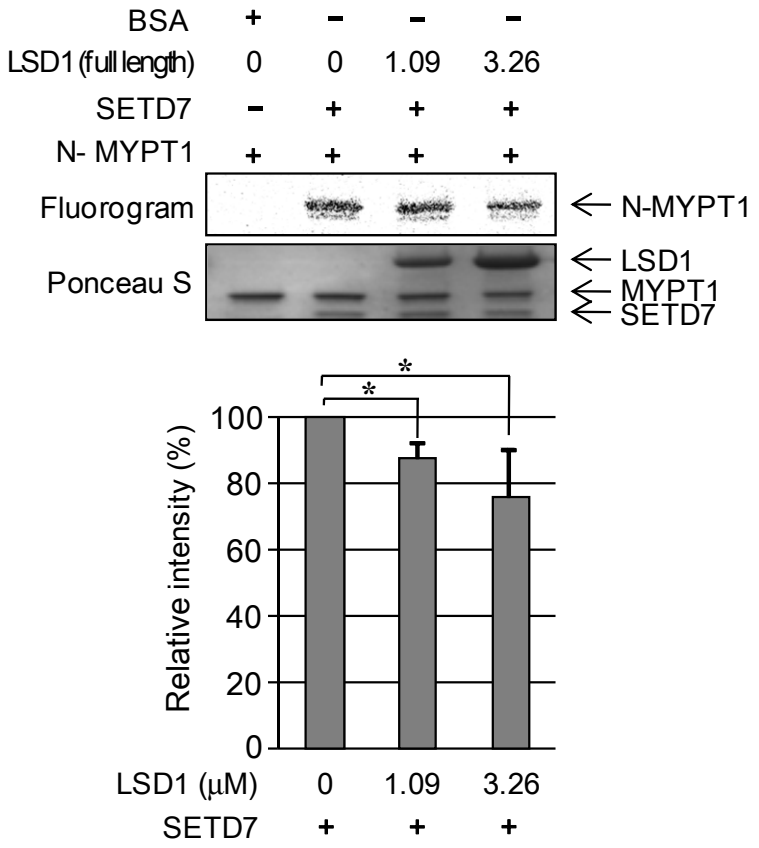
Hyun-Soo Cho, Takehiro Suzuki, Naoshi Dohmae, Shinya Hayami, Motoko Unoki, Masanori Yoshimatsu,Gouji Toyokowa, Masashi Takawa,Taiping Chen, Julia K. Kurash, Helen I. Field, Bruce A.J. Ponder,Yusuke Nakamura & Ryuji Hamamoto



Supplementary Figure S1. FLAG-tagged LSD1 deletion mutants were co-immunoprecipitated with HA tagged MYPT1. After co-transfection with HA-tagged MYPT1 and FLAG-tagged LSD1 deletion mutants in 293T cells, immunoprecipitaion was performed with FLAG antibody. The FLAG peptide was used for the elution of FLAG-tagged proteins. After SDS-PAGE, western blot was conducted using anti-HA and anti-FLAG antibodies.



Supplementary Figure S2. MYPT1 can be methylated by SETD7. A, *In vitro* methyltransferase assay of SETD7 using immunoprecipitated FLAG-tagged MYPT1 as a substrate. Purified FLAG-MYPT1 was incubated with [^{3}H]-labeled S-adenosylmethionine (SAM) as a methyl donor, in the presence of recombinant SETD7. BSA was used as a negative control. B, Dose-dependent methylation of N-MYPT1 by increasing amounts of SETD7. His-tagged N-terminal fragment (1-500) of MYPT1 (N-MYPT1) was used as a substrate and incubated with indicated concentration of SETD7. The reaction products were analyzed by SDS-PAGE followed by fluorography. Ponceau S staining was performed for the loading control.



Supplementary Figure S3. MYPT1 can be demethylated by LSD1. N-MYPT1 recombinant proteins methylated by SETD7 were incubated with recombinant LSD1 in a dose dependent manner or BSA at 37°C for 4 hours. The signal intensity corresponding MYPT1 methylation was quantified by image J. Mean ± SD of three independent experiments. *P* values were calculated using Student's *t*-test (*, $P < 0.05$).

MS/MS Fragmentation of **KTGSYGALAEITASK**
Match to Query 52: 1497.758172 from(500.260000,3+)

Monoisotopic mass of neutral peptide Mr(calc): 1495.7882
Ions Score: 62 **Expect:** 2.9e-005
Matches (Bold Red): 30/164 fragment ions using 27 most intense peaks

(Mock)

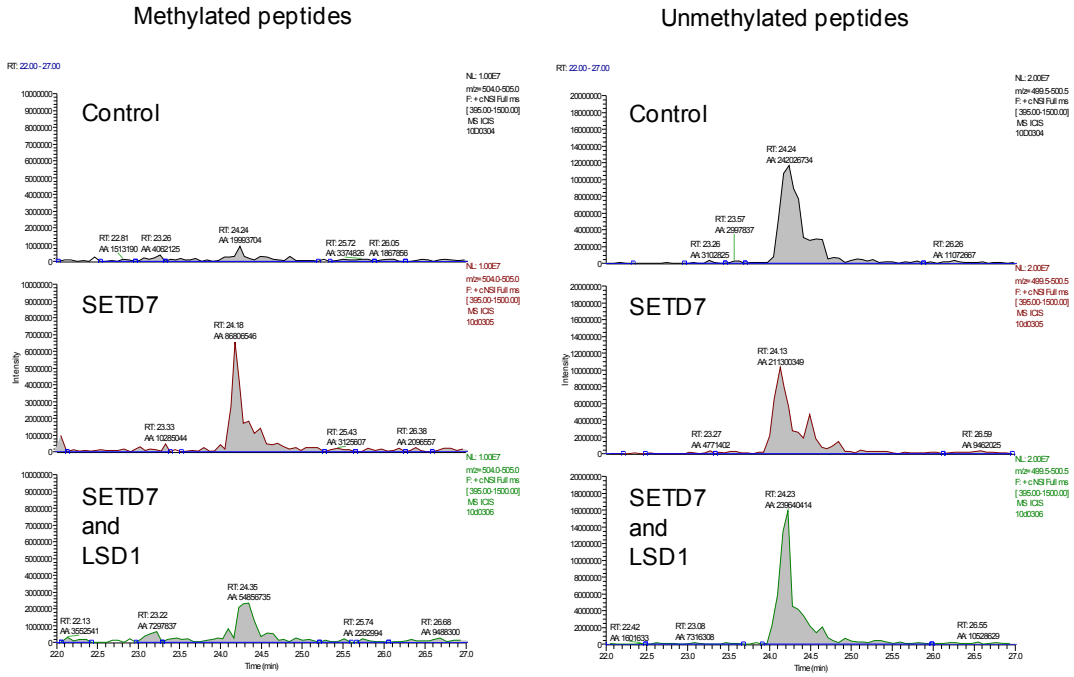
#	b	b^{++}	b*	b^{*++}	b^{0}	b^{0++}	Seq.	y	y^{++}	y*	y^{*++}	y^{0}	y^{0++}	#
1	129.1022	65.0548	112.0757	56.5415			K							15
2	230.1499	115.5786	213.1234	107.0653	212.1394	106.5733	T	1368.701	684.8539	1351.674	676.3406	1350.69	675.8486	14
3	287.1714	144.0893	270.1448	135.5761	269.1608	135.084	G	1267.653	634.3301	1250.626	625.8168	1249.642	625.3248	13
4	374.2034	187.6053	357.1769	179.0921	356.1928	178.6001	S	1210.631	605.8193	1193.605	**597.306**	1192.621	**596.814**	12
5	**537.267**	269.137	**520.24**	260.6237	**519.256**	260.1317	Y	1123.599	562.3033	1106.573	553.79	1105.589	553.298	11
6	594.2882	297.6477	577.2617	289.1345	576.2776	288.6425	G	960.536	480.7717	943.5095	472.2584	942.5255	471.7664	10
7	**665.325**	**333.166**	**648.299**	**324.653**	647.3148	**324.161**	A	903.5146	452.2609	886.488	443.7477	885.504	443.2556	9
8	**778.409**	389.7083	761.3828	381.1951	760.3988	380.703	L	**832.478**	416.7424	815.4509	408.2291	814.4669	407.7371	8
9	**849.447**	**425.227**	**832.42**	416.7136	831.4359	416.2216	A	**719.393**	**360.2**	702.3668	351.6871	701.3828	351.1951	7
10	**978.489**	489.7482	961.4625	481.2349	960.4785	480.7429	E	**648.356**	**324.682**	631.3297	316.1685	630.3457	315.6765	6
11	1091.573	**546.29**	1074.547	**537.777**	1073.563	**537.285**	I	**519.314**	260.1605	502.2871	251.6472	501.3031	251.1552	5
12	1192.621	**596.814**	1175.594	588.3008	1174.61	587.8088	T	**406.23**	203.6185	389.2031	195.1052	388.2191	194.6132	4
13	1263.658	**632.333**	1246.631	623.8193	1245.647	623.3273	A	**305.182**	153.0946	288.1554	144.5813	287.1714	144.0893	3
14	1350.69	675.8486	1333.663	667.3354	1332.679	666.8433	S	**234.145**	117.5761	217.1183	109.0628	216.1343	108.5708	2
15							K	**147.113**	74.06	130.0863	65.5468			1

Monoisotopic mass of neutral peptide Mr(calc): 1509.8038
Variable modifications:
K1 : Methyl (K)
Ions Score: 61 **Expect:** 2.7e-005
Matches (Bold Red): 30/164 fragment ions using 31 most intense peaks

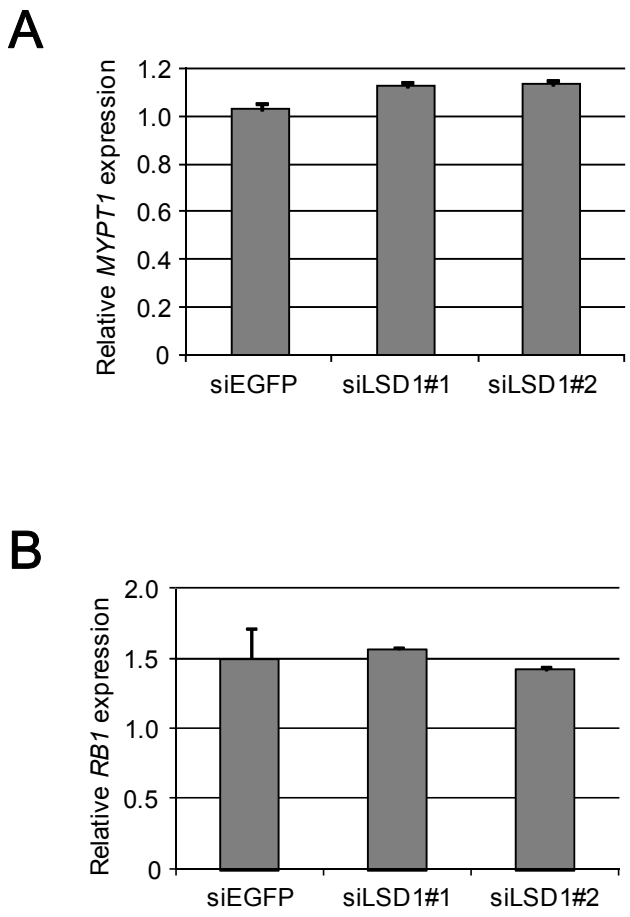
(+SETD7)

#	b	b^{++}	b*	b^{*++}	b^{0}	b^{0++}	Seq.	y	y^{++}	y*	y^{*++}	y^{0}	y^{0++}	#
1	**143.118**	72.0626	126.0913	63.5493			K							15
2	244.1656	122.5864	227.139	114.0732	226.155	113.5811	T	1368.701	684.8539	1351.674	676.3406	1350.69	675.8486	14
3	301.187	151.0972	284.1605	**142.584**	283.1765	142.0919	G	1267.653	634.3301	1250.626	625.8168	1249.642	625.3248	13
4	**388.219**	194.6132	371.1925	186.0999	370.2085	185.6079	S	1210.631	605.8193	1193.605	597.3061	1192.621	596.8141	12
5	551.2824	276.1448	534.2558	267.6316	533.2718	267.1396	Y	1123.599	562.3033	1106.573	**553.79**	1105.589	**553.298**	11
6	608.3039	**304.656**	591.2773	296.1423	590.2933	295.6503	G	960.536	480.7717	943.5095	472.2584	942.5255	471.7664	10
7	**679.341**	**340.174**	662.3144	331.6608	661.3304	331.1688	A	903.5146	452.2609	886.488	443.7477	885.504	443.2556	9
8	**792.425**	**396.716**	775.3985	**388.203**	774.4145	**387.711**	L	**832.478**	416.7424	815.4509	408.2291	814.4669	407.7371	8
9	**863.462**	**432.235**	846.4356	423.7214	845.4516	423.2294	A	**719.393**	**360.2**	702.3668	351.6871	701.3828	351.1951	7
10	**992.505**	**496.756**	975.4782	**488.243**	974.4942	**487.751**	E	**648.356**	324.6818	631.3297	316.1685	630.3457	315.6765	6
11	1105.589	**553.298**	1088.562	544.7848	1087.578	544.2928	I	**519.314**	260.1605	502.2871	251.6472	501.3031	251.1552	5
12	1206.637	**603.822**	1189.61	595.3086	1188.626	594.8166	T	**406.23**	203.6185	389.2031	195.1052	**388.219**	194.6132	4
13	1277.674	**639.34**	1260.647	630.8272	1259.663	630.3352	A	**305.182**	153.0946	288.1554	144.5813	287.1714	144.0893	3
14	1364.706	682.8565	1347.679	674.3432	1346.695	673.8512	S	**234.145**	117.5761	217.1183	109.0628	216.1343	108.5708	2
15							K	147.1128	74.06	130.0863	65.5468			1

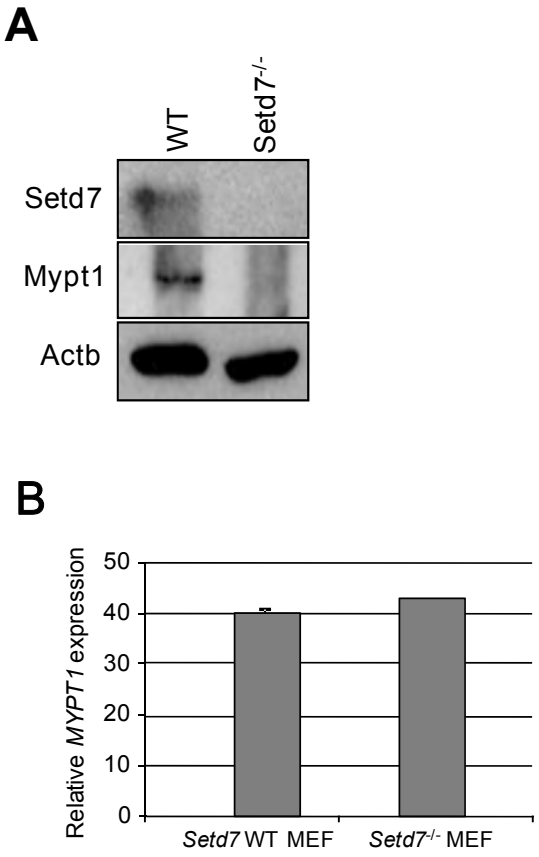
Supplementary Figure S4. MS/MS spectra of MYPT1 peptide (KTGSYGALAEITASK). Typical MS/MS spectra of unmethylated (upper panel) and methylated (bottom panel) MYPT1 peptides. His-N-MYPT1 (1-500) was incubated with SETD7 and digested with bovine trypsin. An aliquot of digest was analyzed by nano LC-MS/MS using LCQ Deca XP plus. The peptides were separated using nano ESI spray column packed with a reversed-phase material. The mass spectrometer was operated in the positive-ion mode and the spectra were acquired in a data-dependent MS/MS mode. The MS/MS spectra were searched against in-house database using local MASCOT server (version: 2.2.1).



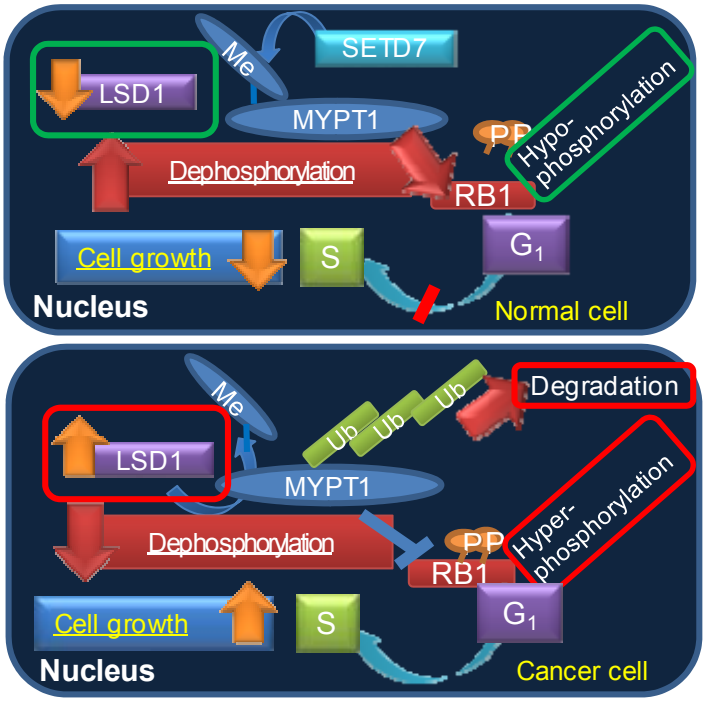
Supplementary Figure S5. Lys 442 of MYPT1 can be an essential target of methylation/demethylation dynamics regulated by SETD7 and LSD1. Typical mass chromatograms of methylated and unmethylated MYPT1 peptides. MYPT1 samples were digested with bovine trypsin, and an aliquot of digest was analyzed by nano LC-MS/MS using LCQ Deca XP plus.



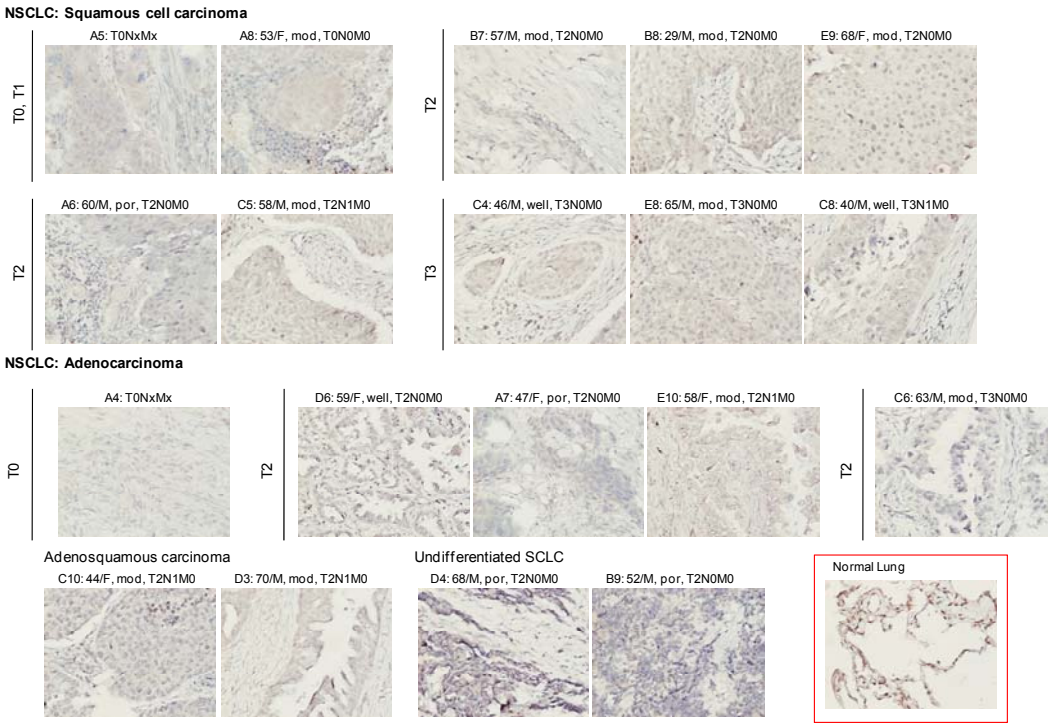
Supplementary Figure S6. Quantitative real-time PCR analysis. mRNA levels of *MYPT1* (A) and *RB1* (B) after treatment with two independent LSD1 siRNAs. siEGFP was used for a negative control.



Supplementary Figure S7. Protein stability of MYPT1 regulated by SETD7. A, Western blot analysis of lysates from WT/*Setd7*$^{-/-}$ MEF cells using antibodies against SETD7 and MYPT1. Expression of Actb was used as an internal control. B, mRNA levels of *MYPT1* in Setd7 WT and Setd7 knockout MEF cells analyzed by quantitative real-time PCR.



Supplementary Figure S8. Schematic model for the dynamic regulation of RB1 phosphorylation through methylation and demethylation of MYPT1.



Supplementary Figure S9. Tissue microarray images of lung tumors stained by standard immunohistochemistry for protein expression of MYPT1. Clinical information for each section is represented above histological pictures. Counterstaining was done with hematoxylin and eosin.

Supplementary Table S1. siRNA sequences

siRNA name	Sequence
siEGFP	Sense: 5' GCAGCACGACUUCUUCAAG 3' Antisense: 5' CUUGAAGAAGUCGUGCUGC 3'
siLSD1#1	Sense: 5' CUAUGUAGCUGAUCUUGGA 3' Antisense: 5' UCCAAGAUCAGCUACAUAG 3'
siLSD1#2	Sense: 5' GUGAUACUGUGCUUGUCCA 3' Antisense: 5' UGGACAAGCACAGUAUCAC 3'
siMYPT1#1	Sense: 5' CAUCUAAAGAGGGUCAGAA 3' Antisense: 5' UUCUGACCCUCUUUAGAUG 3'
siMYPT1#2	Sense: 5' GAGAUACACCUUUAGAUAU 3' Antisense: 5' AUAUCUAAAGGUGUAUCUC 3'

Supplementary Table S2. Primer sequences for quantitative RT-PCR

Gene name	Primer sequence
GAPDH (housekeeping gene) - f	5' GCAAATTCCATGGCACCGTC 3'
GAPDH (housekeeping gene) - r	5' TCGCCCCACTTGATTTTGG 3'
SDH (housekeeping gene) - f	5' TGGGAACAAGAGGGCATCTG 3'
SDH (housekeeping gene) - r	5' CCACCACTGCATCAAATTCATG 3'
MYPT1 – f1	5' AGGAGGCAATGGAAGAGCTA 3'
MYPT1 – r1	5' CCTGGCATCTCTAAGCATGA3'
RB1 – f1	5' ACCCAGAAGCCATTGAAATC 3'
RB1 – r1	5' TCTGGGTGCTCAGACAGAAG 3'

Supplementary Methods

Cell lines

293T and SBC5 lines were purchased from the American Type Culture Collection (ATCC). *Setd7*$^{-/-}$ MEF cell line was kindly provided by Dr. Julia K. Kurash (Novartis Institutes for BioMedical Research, USA).

Cell culture

All cell lines were grown in monolayers in appropriate media: Eagle's minimal essential medium (E-MEM) for SBC5 small cell lung cancer cells supplemented with 10% fetal bovine serum and 1% antibiotic/antimycotic solution (Sigma); Dulbecco's modified Eagle's medium (D-MEM) for 293T cells supplemented with 10% fetal bovine serum and 1% antibiotic/antimycotic solution (Sigma); MEF-medium (High glucose DMEM) for wild and *Setd7*$^{-/-}$ MEF cells supplemented with 10% fetal bovine serum, 1x penicillin-streptomycin, 2 mM L-glutamine, 0.1 mM beta-mercaptoethanol, 1x non-essential amino acids and1 mM Na-pyruvat); All cells were maintained at 37°C in humid air with 5% CO_2 condition. Cells were transfected with FuGENE6^{TM} (Roche Applied Science, Basel, Switzerland) according to manufacturers' protocols.

Immunoprecipitation

Transfected 293T cells were washed with PBS and lysed in CelLytic^{TM} M Cell Lysis Reagent (Sigma) containing complete protease inhibitor cocktail (Roche). In a typical immunoprecipitation reaction, whole-cell extract was incubated with an optimum concentration of the following antibodies: anti-FLAG (M2; Sigma), anti-HA (Y-11; Santa Cruz, Santa Cruz, CA), anti-MYPT1 (H-130; Santa Cruz), anti-LSD1 (Sigma),

anti-SETD7 (2967C2a-1; Cosmo Bio), anti-Ubiquitin (FL-76; Santa Cruz), anti-pRb (Ser 807/811; Santa Cruz), anti-CDK4 (B-10; Santa Cruz) or anti-Actin (I-19; Santa Cruz), and 30 μl of Protein A/G Plus-Agarose beads (Santa Cruz) for 1 hour at 4°C. After the beads were washed 3 times with 1 ml of TBS buffer (pH 7.6), the proteins bound to the beads were eluted by boiling in Lane Marker Reducing Sample Buffer (Thermo Scientific).

Mass spectrometry

A protein band of SDS–polyacrylamide gel electrophoresis was excised and reduced with dithiothreitol and propionamidated by acrylamide. After washing the gel, the band was digested with bovine trypsin (TPCK treated, Worthington Biochemical Corp., Lakewood NJ) at 37°C overnight. An aliquot of digest was analyzed by nano LC–MS/MS using LCQ Deca XP plus (Thermo Fisher Scientific, San Jose, CA). The peptides were separated using nano ESI spray column (100 μm i.d.x 50 mm L) packed with a reversed-phase material (Inertsil ODS-3, 3 μm, GL Science, Japan) at a flow rate 200 nl/min. The mass spectrometer was operated in the positive-ion mode and the spectra were acquired in a data-dependent MS/MS mode. The MS/MS spectra were searched against the in-house database using local MASCOT server (version: 2.2.1, Matrix Sciences, UK). A peak area of selected mass chromatogram was calculated using Qual Browser V1.3 (Thermo Fisher Scientific, San Jose, CA) or iCarta (KYA Technologies, Japan).

Transfection with small interfering RNAs

The small interfering RNA (siRNA) oligonucleotide duplexes were purchased from SIGMA Genosys for targeting the human *LSD1* and *MYPT1* transcripts. siEGFP was used as a control siRNA. The siRNA sequences are described in Supplementary Table S1. siRNA duplexes (100 nM final concentration) were transfected to SBC5 and 293T with lipofectamine 2000 (Invitrogen) for 72 hours.

***In vitro* binding assay**

For the *in vitro* binding assay, GST-N-MYPT1 (residues 1-500) and His-LSD1 were purified by Glutathione Sepharose 4B (Amersham, GE Healthcare) and TALON Metal affinity Resin (Clontech), respectively, and mixed them in TBS buffer (pH 7.6). After binding with TALON beads for 1 hour, samples were washed three times with TBS buffer, and boiled in sample buffer for subjecting to SDS-PAGE

***In vivo* labeling experiments**

Cells were starved for 1 hour in methionine-free medium, including cycloheximide (100 μg/ml) and chloramphenicol (40 μg/ml). They were then labeled with L-[methyl-^{3}H] methionine (10 μCi/ml, Perkin Elmer) for 5 hours. FLAG-MYPT1 was immunoprecipitated with FLAG-M2 agarose (Sigma) and methylated MYPT1 was visualized by fluorography.

Immunostaining

Cultured cells were fixed by 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) at room temperature for 30 minutes and permeabilized with 0.5% Triton X-100 in PBS (Sigma). Fixed cells were blocked with 5% BSA or 5% skim milk in PBS for 30

minutes and incubated with primary antibodies overnight at 4°C. Then they were incubated with Alexa Fluor conjugated second antibodies (Molecular Probes, invitrogen) and observed using a Leica confocal microscopy.

Quantitative real-time PCR

For quantitative RT-PCR reactions, specific primers for all human *GAPDH* (housekeeping gene), *SDH* (housekeeping gene) and *MYPT1* and *RB1* were designed (Primer sequences in Supplementary Table S2). LightCycler® 480 SYBR Green I Master (Roche), 50 nM each of the forward and reverse primers and 2 µl of reversely-transcribed cDNA were applied. mRNA levels were normalized to *GAPDH* and *SDH* expression.

Luciferase assay

We analyzed the transcriptional activity of E2F by the Cignal™ E2F Reporter Assay Kit (SuperArray Bioscience Corporation). Cells were transfected with an E2F-responsive luciferase construct, which encodes the firefly *luciferase* reporter gene under the control of a minimal (m) CMV promoter and tandem repeats of the E2F transcriptional response element [TRE], negative control or positive control. After 24 hours of transfection, dual luciferase assay was performed using Dual-Luciferase Reporter Assay System (Promega), and promoter activity values are expressed as arbitrary units using a Renilla reporter for internal normalization. Experiments were done in triplicate, and Student's *t*-test was used for statistical analysis.