Supplemental Experimental Procedures

Plasmids, recombinant proteins, and reagents

Human SETD3 cDNA was amplified from human brain cDNA library, and subcloned into pEGFP-N3, pCS2-FA, pCS2-3xMyc-FA, pCS2-3xFlag-FA, pFastBac-GST, pET28a, or pGEX-4T vector. GSK3β cDNAs were cloned from human cDNA library (Thermo Scientific Company), and subcloned into pCS2-3xFlag or pCS2-GST vector. All these clones were confirmed by sequencing. FBXW7α, FBXW7β, FBXW7γ, Cullin1 (Cul1), Rbx1, Skp1, Cdc20, Cdh1, Fbx4, β-TrCP, and ubiquitin-series constructs were described previously (1-3). Point mutations were generated using the QuickChange Site-Directed Mutagenesis protocol (Stratagene). His-tagged and GST-tagged full length hSETD3 were expressed in *Escherichia coli* and purified. His-tagged hSETD3 was used for antiserum production, and antisera were generated by the Proteintech Company from two New Zealand white rabbits each received 4 times booster injections in Freund's incomplete adjuvant (IFA). Production bleeds were further purified by incubating with the Affi-Gel cross-linked GST-tagged hSETD3 proteins, and the elutes were concentrated and were examined the specificity by Western blot analysis. Recombinant 6xHis-Cul1, GST-Rbx1, GST-Skp1, and FBXW7β-GST were expressed and purified from Sf9 cells. Recombinant GSK3β protein was purchased from New England Biolabs.

The following chemicals and antibodies were obtained from commercial sources: thymidine, nocodazole, hydroxyurea (HU), MG132, and Lithium Chloride (LiCl) from Sigma-Aldrich; TWS119 and CHIR-99021 from Selleck; recombinant ubiquitin and K48R mutant proteins (Boston Biochem); mouse α-GAPDH (AC002, Lot0002465), mouse α-Myc (AE010, Lot44545), and mouse α-eGFP (AE012, Lot44042) antibodies were purchased from Abclonal; and rabbit α-eGFP (50430-2-AP, 00040811), mouse α-eGFP (66002-1-1g, Lot10002792), rabbit α-Actinβ (60008-1, Lot00087452), rabbit α-Skp1 (10990-2-AP, Lot00035665), rabbit α-Cul1 (12895-1-AP, Lot00003797), α-Rbx1 (14895-1-AP, Lot00006033), rabbit α-Myc (62861-1, Lot00024343) and α-Flag (20543-1-AP, 00018570) antibodies were purchased from Proteintech; mouse anti-ubiquitin (sc-8017, LotA2312), mouse α-HA (sc-805, LotE0715), rabbit α-GST (SC-138, LotK1808) and Rabbit α-GSK3β (SC-377213，LotA3114) antibodies were purchased from Santa Cruz Biotechnology; mouse α-Flag (F1804-1MG, Lot SLBQ6349V) antibody was purchased from Sigma; rabbit α-Cyclin B1 (1495-1， LotYH071401C), α-Cyclin E1 (Ab133266， LotGR174826-1), α-phosphohistone H3 pS10 (1173-1， LotYG021101C), α-phosphoPlk1 pT210 (3646-1，LotYH092004D) antibodies were purchased from Abcam, rabbit α-FBXW7 (A301-721A, LotA301-721A-2) antibody was purchased from Bethyl; secondary HRP-conjugated α-mouse or α-rabbit antibodies were purchased from Jackson ImmunoResearch Laboratories.

Immunoprecipitation and Western blotting

293T cells were transiently transfected with various constructs expressed GFP-tagged or Flag-tagged proteins. Approximately 36 h post-transfection, cells were

lysed in buffer [1% Triton X-100, 10% glycerol, 150 mM NaCl, 20 mM Tris, pH 7.4, 2 mM EDTA, a protease inhibitor cooktail (Complete, Biotool)] on ice for 30 min. Clarified extracts were immunoprecipitated with the indicated antibodies or preimmune IgG for 4 h and then with protein G beads overnight at 4 ℃. Beads were washed 5 times with lysis buffer, and boiled in the SDS sample buffer and processed for SDS-PAGE and immunoblotting. For Western blotting, whole-cell extracts or immunoprecipitates were detected with appropriate antibodies in blocking buffer. Blots were developed using Clarity Western ECL kit (Bio-Rad), followed by exposure to X-ray film (Fuji, Japan) or scanning with a PhosphorImage (ChemiScope, 3400 Mini).

Real-time quantitative PCR analyses

Cell samples were extracted using TRIzol (Invitrogen) and further purified via chloroform extraction methods. After drying, an aliquot of 1 μg RNA was subjected to reverse transcription using the cDNA synthesis kit (with genomic DNA removal, Tiangen Company) according to the manufacturer's protocol. RT-qPCR was performed using primer sets targeting FBXW7β gene (Forward primer: 5'-TTGTCAGAGACTGCCA-3'; Reverse primer: 5'-GTTGGTGTTGC-TGAACATGG-3') or GAPDH gene (Forward primer: 5'-GACCCCTTCATTG-ACCTCAAC-3'; Reverse primer: 5'-CTTCTCCATGGTGGTGAAGA-3'). The relative mRNA levels of FBXW7β are normalized to GAPDH, and error bars indicate mean±SD of three individual repeats.

Polyubiquitination Assays

In order to distinguish the type of ubiquitin linkage of the poly-Ub chains, 293T cells were transiently co-transfected with SETD3-GFP, 3xFlag-FBXW7β and equal amounts of HA-Ub (WT, K48O, K63O, K0) plasmids. Ubiquitin mutants represent that all but one lysine residues were simultaneously mutated to arginines (K-O) or all seven lysine residues were individually mutated to arginine (K-R). After transfection over 48 h, cells were harvested and subjected to the same procedure described above. One quarter of the supernatants were subjected to immunoblotting to confirm the expression of each protein, while the rest were incubated with GFP antibody and protein G beads for 6 hr. The recovered beads were then washed 5 times and finally boiled in SDS sample loading buffer, followed by SDS-PAGE and immunoblotted by using anti-HA.

RTCA assay

Cells were plated onto the E-16 plates at a density of 1,500/well with fresh medium to a final volume of 200 μl. Cells were incubated for 30 min at 37 ℃ and 5% CO_2 in the RTCA cradle. The impedance signals were recorded every 30 min until the end of the experiment (up to 21 h). Background measurements were taken from the wells by adding 50 μl of the same medium to the plate. RTCA software was used to calibrate the proliferation.

Supplemental References

1. Wang, R., Wang, Y., Liu, N., Ren, C., Jiang, C., Zhang, K., Yu, S., Chen, Y., Tang, H., Deng, Q., Fu, C., Wang, Y., Li, R., Liu, M., Pan, W., and Wang, P. (2013) FBXW7 regulates endothelial functions by targeting KLF2 for ubiquitination and degradation. *Cell Res.* **23**, 803-819
2. Seki, A., Coppinger, J. A., Du, H., Jang, C. Y., Yates, J. R., 3rd, and Fang, G. (2008) Plk1- and beta-TrCP-dependent degradation of Bora controls mitotic progression. *J. Cell Biol.* **181**, 65-78
3. Zhong, B., Zhang, Y., Tan, B., Liu, T. T., Wang, Y. Y., and Shu, H. B. (2010) The E3 ubiquitin ligase RNF5 targets virus-induced signaling adaptor for ubiquitination and degradation. *Journal of immunology* **184**, 6249-6255

Supplementary Figure Legends

FIGURE S1. **Characterize and validate the SETD3 antibody.** *A*, Hela cells were infected with shRNA control (shControl) or shRNA targeting SETD3 (shSETD3), and cell lysates were loaded onto SDS-PAGE gel and subjected to Western blot by probing with the purified SETD3 antibody. *B*, SETD3 antibody pre-incubated with bovine serum albumin (BSA) or recombinant His-SETD3. SETD3 levels from cells bearing with shRNA or shSETD3 were examined by probing with pre-treated SETD3 antibody. GAPDH levels served as a loading control. *C*, Hela cells transfected with shRNA or with shSETD3 immunostained for SETD3 (green) and DNA (blue). *Scale bar*: 20 μm. *D*, The apercentage of apoptotic cells in Hela cells transfected with shRNA or with shSETD3 were detected by staining with FITC-Annexin V Apoptosis Detection Kit (BD, USA) and analyzed by flow cytometry (Beckman CytoFLEX) . Viable cells did not bind annexin V or PI (lower left quadrant), early apoptotic cells bound to Annexin V but excluded PI (lower right quadrant), and late apoptotic cells were both annexin V and PI-positive (upper right quadrant).

FIGURE S2**. SETD3 interacts with FBXW7β and SETD3 is degraded through a FBXW7β-dependent K48-linked polyubiquitinated manner.** *A*, 293T cells were treated with cycloheximide, and SETD3 protein stability was examined by Western blot probing with an α-SETD3 antibody. *B* and *C*, Relative FBXW7β mRNA levels in samples described in Figure 2E (panel *B*) or Figure 2F (panel *C*) were examined by RT-qPCR analysis. Three different siRNAs targeting FBXW7β were used in *B.* Erorr bars indicate mean±SD of three individual samples. *$p < 0.05$, **$p < 0.01$. *D*, FBXW7β targeted SETD3 for K48-linked polyubiqiutination. SETD3-GFP and different forms of ubiquitin, including WT, K48 only (K48O), K63 only (K63O) or the mutant bearing all lysine residues of ubiquitin were mutated to arginine (K0), were cotransfected with or without Flag-FBXW7β in 293T cells, and cells were harvested after 48 h transfection. Cell lysates were subjected to α-GFP IP, the immunoprecipitates were analyzed by Western blot with an α-HA (upper panel) or an α-GFP (lower panel) antibody. The whole cell lysates were analyzed by Western blot

with α-GFP or α-Flag antibodies as indicated.

FIGURE S3**. The CPD motif is required for SETD3 degradation.** ***A,*** Coomassie Brilliant Blue staining showed the purified proteins from Sf9 insect cells. The asterisks represent the indicated proteins that were used in Figure 3F. *B*, Alignment of conserved CPD sequences between different FBXW7 substrates. 'X' indicates any residue. *C*, Alignment of the conserved CPD1 sequence of SETD3 between different species. *D*, Cells expressing exogenous wild-type SETD3, but not the CPD1 mutant, cotransfected with GSK3β or FBXW7β plasmid, displayed a decreased level of SETD3 protein by Western blot with an α-GFP antibody.

FIGURE S4. **SETD3 promotes cellular proliferation and tumorigenesis in liver cancer cells.** *A*, Relative SETD3 protein levels in various cell lines as indicated were examined by Western blot with GAPDH as a loading control. *B*, Relative SETD3 protein levels in various tissues samples as indicated were examined by Western blot with β-actin as a loading control. *C-F*, L02 or BEL7402 cells stably overexpressed SETD3 (O/E) or had a knockdown of SETD3 by shRNA. Cellular proliferation of the indicated cell lines was assessed by MTT assays in panels *B* or by RTCA assay in panel *E*. Colony formation assays using the indicated cell lines were performed. Three representative images (3 cm x 3 cm) from three independent plates were shown in panels *D* and *F* (left), and quantifications of colony numbers from the representative images were shown (right). *Scale bar*: 1 cm. *G.* Summary of numbers and rates of tumor formation in nude mice shown in Figure 5G and 5H.

FIGURE S5**. SETD3 protein levels correlate with liver tumor.** *A*, Relative mRNA expression of *Setd3, Fbw7,* and *GSK3β* in 30 normal liver tissue (Normal), and 146 liver hepatocellular carcinoma tumors (Cancer). Data were obtained from The Cancr Genome Atlas (TCGA) database. *B*, Representative IHC staining images of FBXW7 in different clinical pathological staging (grade II or grade III) obtained from the same samples shown in Fig 6E.

FIGURE S6. **Knockdown of SETD3 does not affect global methylation levels of various histone marks.** Indicated methyl status of histone H3 or H4 were examined by Western blotting using different antibodies specifically recognizing various histone methylation sites in the control or knockdown of SETD3 cell lines. Three shRNAs targeting different regions of SETD3 were used.

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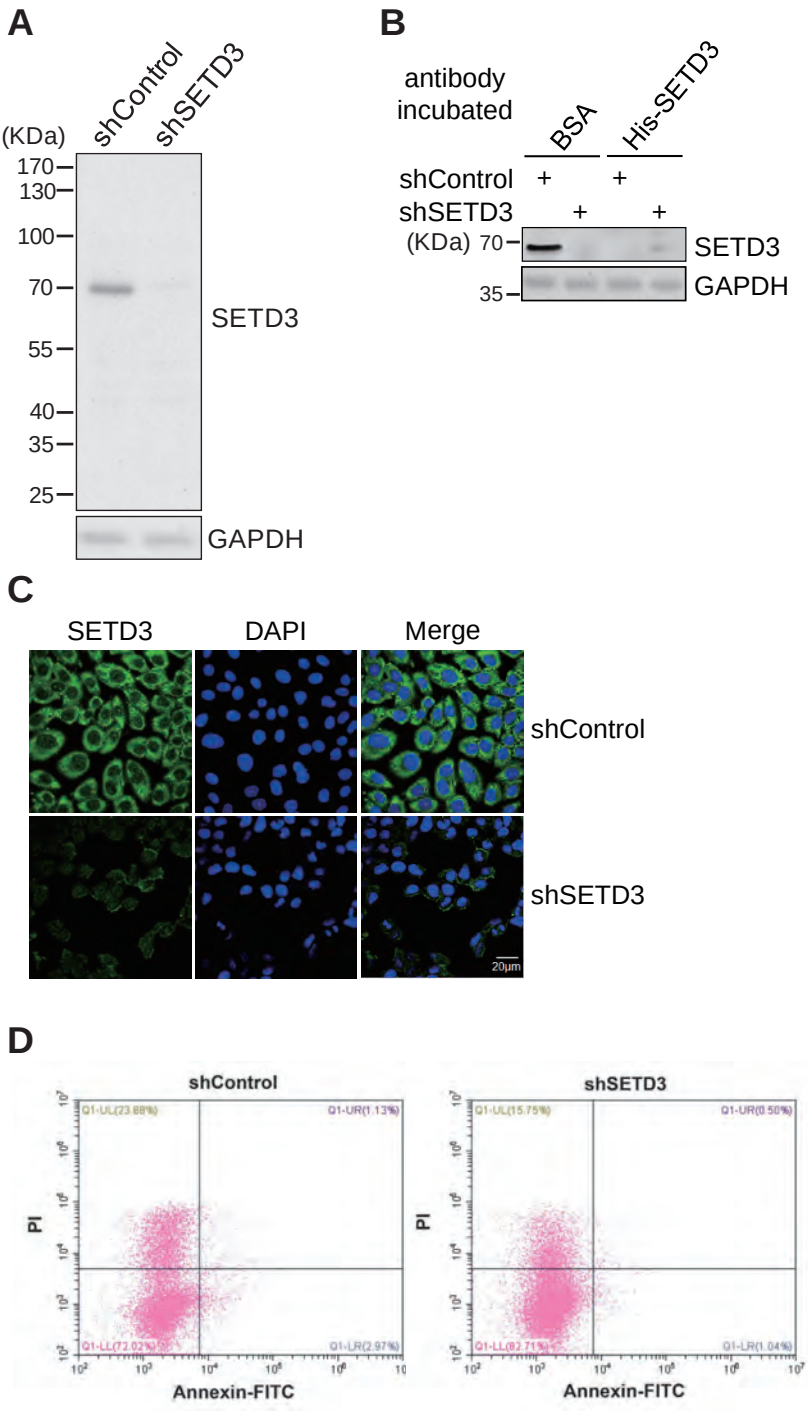

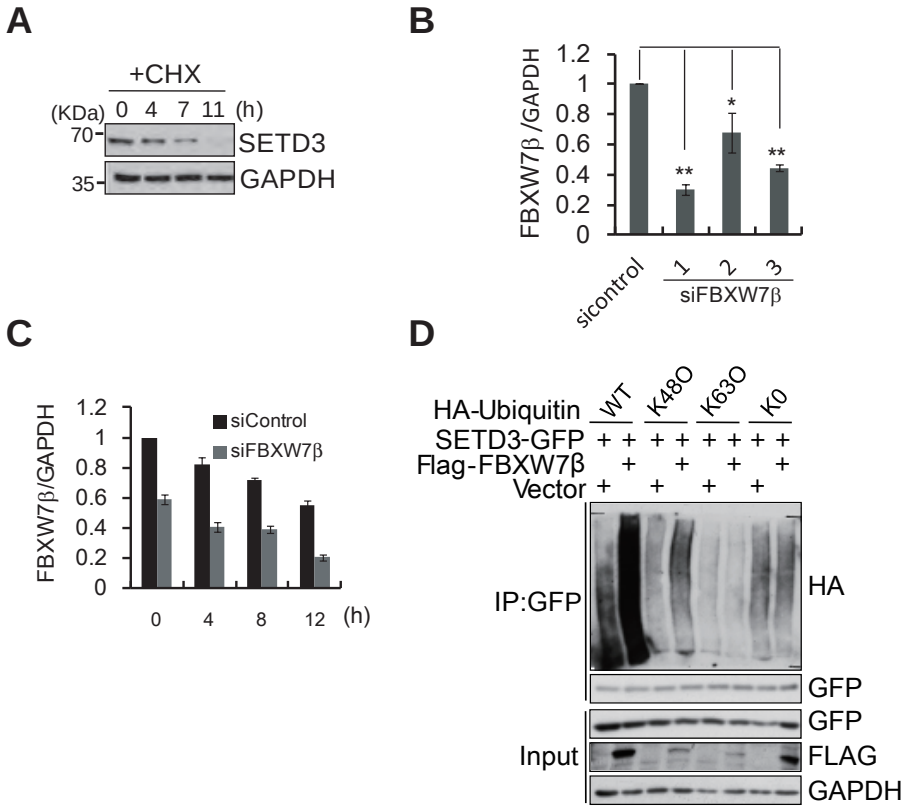
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0.4
0.2
0
**
*
**
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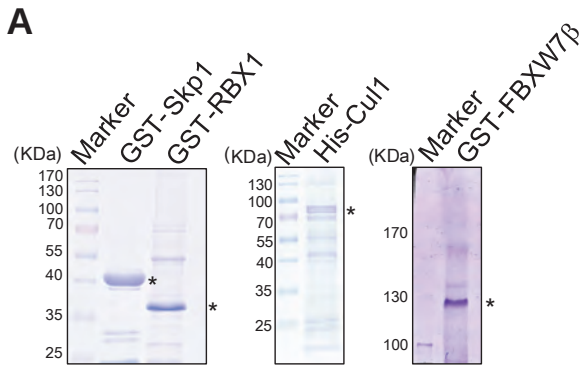
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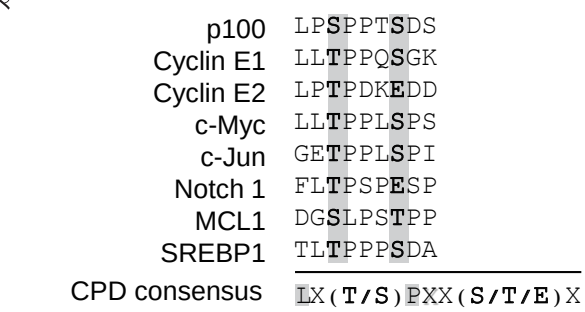
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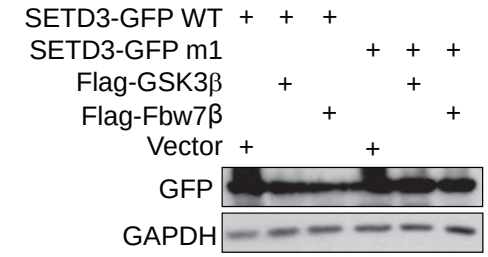
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SETD3-GFP m1 + + +
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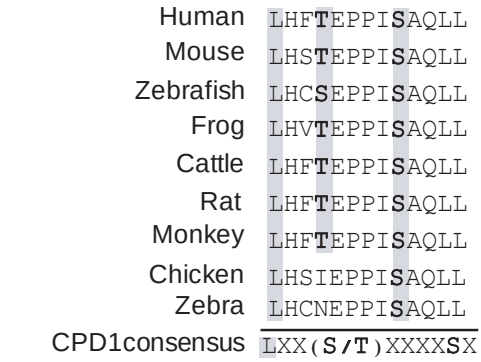
GFP
GAPDH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CPD1consensus LXX(S/T)XXXXSX

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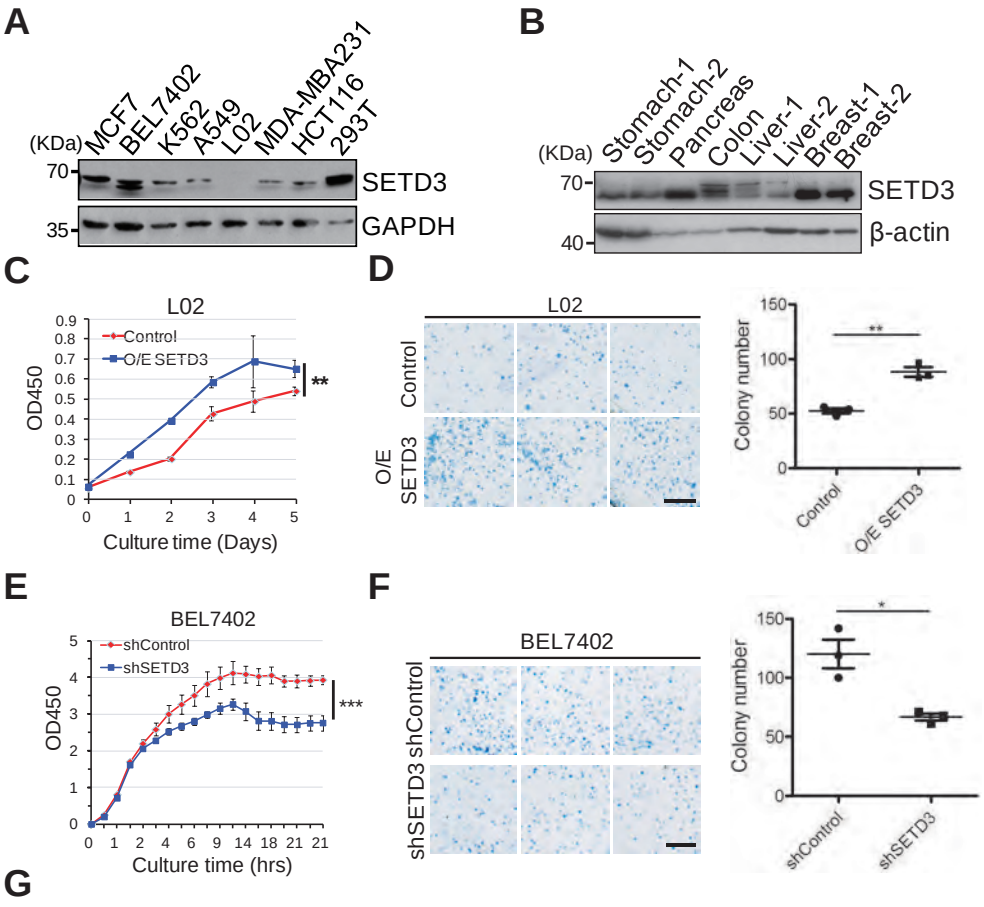
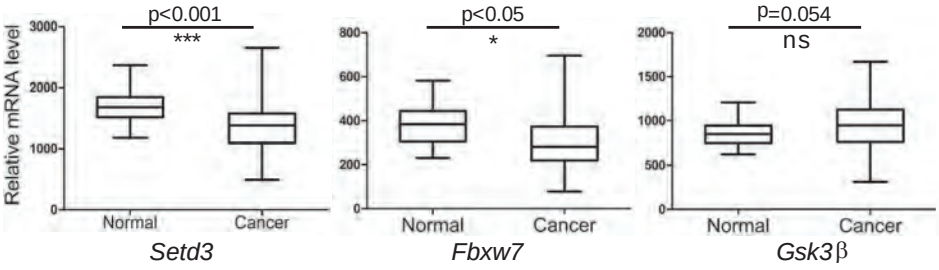


G

Samples	Ejection No.	Tumor No.	Efficiency
Control	12	12	100%
shSETD3	12	9	75%
SETD3	12	12	100%
SETD3+ Fbw7β	12	5	41.6%
SETD3+ Fbw7β$^{\triangle F\ box}$	14	14	100%

A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p<0.05
*
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800
600
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500
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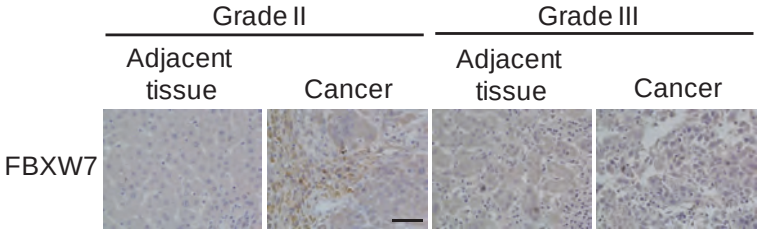
Cancer
Normal
Cancer
Normal
Cancer
Setd3
Fbxw7
Gsk3β

B


Grade II
Grade III
Adjacent tissue
Cancer
Adjacent tissue
Cancer
FBXW7

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