Supplementary Information

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Supplementary Table 1. Clinicopathological Features of patients with Hepatocellular Carcinoma

Feature	Value
Age (years)	53 ± 9.1* (22-71)
Gender	
Male	57
Female	23
Total Cholesterol	157 ± 0.66* (42-431)
AST	2176 ± 1.98* (25-11945)
ALT	862 ± 0.78* (58-3908)
Primary Diagnosis	
Liver Malignancy	5
HCV	14
Alcoholism	19
Obesity	11
NASH	9
HBV	14
Alcoholic HCV	8

*** Value = Mean ± SEM**

Supplementary Table 2. List of cellular components by gene ontology analysis in ChIP-qPCR with anti-NOTCH1 in CD133-positive vs. negative TICs. Table showing the top 10 ranked by p-value.

Rank	Term	p-value	q-value	Genes
1	Focal adhesion (GO:0005925)	0.000143	0.035426	[GRB7, ACTR2, MME, LRP1, GDI2, LMO7, SORBS3, RND3, CORO1B, ADD1, DCAF6, RPL7, ACTG1, HSP90B1, CSRP2, YWHAQ, PALLD, DLC1, GNA12, PIP5K1A, RPL27, RPL18, LIMS1]
2	Mitochondrial proton-transporting ATP synthase complex (GO:0005753)	0.000216	0.035426	[ATP5B, ATP5D, ATP5C1, ATP5J, ATP5G2]
3	Mitochondrial proton-transporting ATP synthase complex, catalytic core F(1) (GO:0000275)	0.000216	0.035426	[ATP5B, ATP5D, ATP5C1, ATP5J, ATP5G2]
4	Mitochondrial proton-transporting ATP synthase complex, coupling factor F(o) (GO:0000276)	0.000274	0.035426	[ATP5B, ATP5D, ATP5C1, ATP5J, ATP5G2]
5	Subsarcolemmal mitochondrion (GO:1990843)	0.001607	0.092517	[MRS2, MTCH2, NAXE, ECI2, ATP5C1, ATP5J, ETFA, ABCB8, PTS, MRPL41, ATP5B, NADK2, MPC1, FAM162A, ATP5D, MPV17, HARS, SLC25A20, JARID2, COX10, NKTR, POLG, CASP8AP2, FIS1, STARD5, MDH1, GK, OXSM, GOT2, MRPS18B, DARS2, TRNT1, SIRT3, PNKD, UQCRQ, NDUFS5, PCCB, OGDH, NDUFS2, SUCLG1, MXD1]
6	Interfibrillar mitochondrion (GO:1990844)	0.001607	0.092517	[MRS2, MTCH2, NAXE, ECI2, ATP5C1, ATP5J, ETFA, ABCB8, PTS, MRPL41, ATP5B, NADK2, MPC1, FAM162A, ATP5D, MPV17, HARS, SLC25A20, JARID2, COX10, NKTR, POLG, CASP8AP2, FIS1, STARD5, MDH1, GK, OXSM, GOT2, MRPS18B, DARS2, TRNT1, SIRT3, PNKD, UQCRQ, NDUFS5, PCCB, OGDH, NDUFS2, SUCLG1, MXD1]
7	Mitochondrion (GO:0005739)	0.001607	0.092517	[MRS2, MTCH2, NAXE, ECI2, ATP5C1, ATP5J, ETFA, ABCB8, PTS, MRPL41, ATP5B, NADK2, MPC1, FAM162A, ATP5D, MPV17, HARS, SLC25A20, JARID2, COX10, NKTR, POLG, CASP8AP2, FIS1, STARD5, MDH1, GK, OXSM, GOT2, MRPS18B, DARS2, TRNT1, SIRT3, PNKD, UQCRQ, NDUFS5, PCCB, OGDH, NDUFS2, SUCLG1, MXD1]
8	Nebenkern (GO:0016006)	0.001607	0.092517	[MRS2, MTCH2, NAXE, ECI2, ATP5C1, ATP5J, ETFA, ABCB8, PTS, MRPL41, ATP5B, NADK2, MPC1, FAM162A, ATP5D, MPV17, HARS, SLC25A20, JARID2, COX10, NKTR, POLG, CASP8AP2, FIS1, STARD5, MDH1, GK, OXSM, GOT2, MRPS18B, DARS2, TRNT1, SIRT3, PNKD, UQCRQ, NDUFS5, PCCB, OGDH, NDUFS2, SUCLG1, MXD1]
9	Mitochondrial derivative (GO:0016007)	0.001607	0.092517	[MRS2, MTCH2, NAXE, ECI2, ATP5C1, ATP5J, ETFA, ABCB8, PTS, MRPL41, ATP5B, NADK2, MPC1, FAM162A, ATP5D, MPV17, HARS, SLC25A20, JARID2, COX10, NKTR, POLG, CASP8AP2, FIS1, STARD5, MDH1, GK, OXSM, GOT2, MRPS18B, DARS2, TRNT1, SIRT3, PNKD, UQCRQ, NDUFS5, PCCB, OGDH, NDUFS2, SUCLG1, MXD1]
10	Cul4B-RING E3 ubiquitin ligase complex (GO:0031465)	0.001917	0.098918	[CUL5, DTL, DCAF7, DCAF6]

Supplementary Table 3. List of cellular components by gene ontology analysis in ChIP-qPCR with anti-NOTCH1 in CD133-positive TICs with/without TBC1D15 KD vs. WT. Table showing the top 10 ranked by p-value.

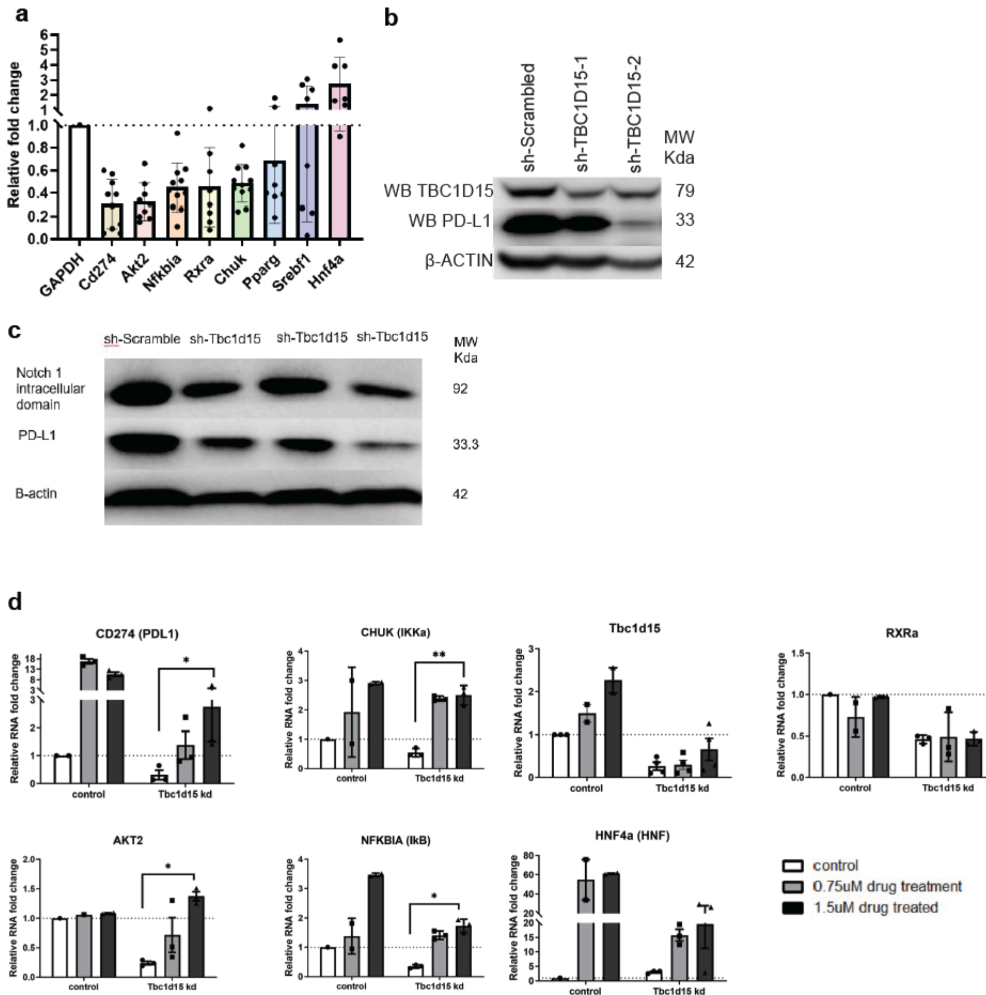
Rank	Term	p-value	q-value	overlap_genes
1	Mitochondrial matrix (GO:0005759)	6.48E-09	1.75E-07	[ALAS1, GOT2, ETFA, PDHB, ACADM, ME2, ACSF2, FARS2]
2	Intracellular organelle lumen (GO:0070013)	5.74E-06	7.75E-05	[ALAS1, GOT2, ETFA, PDHB, ACADM, ME2, ACSF2, FARS2]
3	Mitochondrial membrane (GO:0031966)	1.95E-05	1.76E-04	[SLC25A16, MRPL1, CYCS, MRPS10, ACADM, MRPL46]
4	Mitochondrial inner membrane (GO:0005743)	4.67E-05	3.15E-04	[SLC25A16, MRPL1, CYCS, MRPS10, MRPL46]
5	Organelle inner membrane (GO:0019866)	6.02E-05	3.25E-04	[SLC25A16, MRPL1, CYCS, MRPS10, MRPL46]
6	Integral component of mitochondrial membrane (GO:0032592)	3.06E-03	1.38E-02	[FIS1, MPC2]
7	Mitochondrial envelope (GO:0005740)	1.09E-02	3.74E-02	[CYCS, ACADM]
8	Peroxisome (GO:0005777)	1.11E-02	3.74E-02	[FIS1, EHHADH]
9	Integral component of peroxisomal membrane (GO:0005779)	1.61E-02	4.69E-02	[FIS1]
10	Intrinsic component of peroxisomal membrane (GO:0031231)	1.74E-02	4.69E-02	[FIS1]

Supplementary Table 4. List of enriched anti-NOTCH1/NANOG ChiP-seq-based mitochondrial relate genes in CD133-positive TICs with TBC1D15 KD vs WT.

Feature Gene	Description transcript	Start peak	End peak	Probe strand	Promoter	Relative enrichment	Fold depletion
Anti-NOTCH1 TBC1D15 KO vs WT in CD133-positive TICs							

Atp5b	ATP synthase, H+ transporting mitochondrial F1 complex, beta subunit [Source:MGI Symbol;Acc:MGI:107801]	128086681	128088681	+		2.445995	3.9267337
Fis1	fission, mitochondrial 1 [Source:MGI Symbol;Acc:MGI:1913687]	136961845	136963845	+		2.3033295	4.2162404
Slc25a16	solute carrier family 25 (mitochondrial carrier, Graves disease autoantigen), member 16 [Source:MGI Symbol;Acc:MGI:1920382]	62946026	62948026	+	Yes	2.267997	3.7568085
Fis1	fission, mitochondrial 1 [Source:MGI Symbol;Acc:MGI:1913687]	136961433	136963433	+		2.2374082	4.457248
Mrps10	mitochondrial ribosomal protein S10 [Source:MGI Symbol;Acc:MGI:1928139]	47367891	47369891	+	Yes	2.1304934	3.7568085
Slc25a16	solute carrier family 25 (mitochondrial carrier, Graves disease autoantigen), member 16 [Source:MGI Symbol;Acc:MGI:1920382]	62946075	62948075	+	Yes	2.0980718	3.9267337
Fis1	fission, mitochondrial 1 [Source:MGI Symbol;Acc:MGI:1913687]	136961077	136963077	+		1.8960276	4.7568088
Got2	glutamatic-oxaloacetic transaminase 2, mitochondrial [Source:MGI Symbol;Acc:MGI:95792]	95867045	95869045	-		1.8865674	3.9267337
Mrpl46	mitochondrial ribosomal protein L46 [Source:MGI Symbol;Acc:MGI:1914558]	78777366	78779366	-		1.8789549	4.078737
Mpc2	mitochondrial pyruvate carrier 2 [Source:MGI Symbol;Acc:MGI:1917706]	165466500	165468500	+		1.8085651	3.3417711
Me2	malic enzyme 2, NAD(+)-dependent, mitochondrial [Source:MGI Symbol;Acc:MGI:2147351]	73814392	73816392	-	Yes	1.8085651	4.7568088
Mrps10	mitochondrial ribosomal protein S10 [Source:MGI Symbol;Acc:MGI:1928139]	47368197	47370197	+	Yes	1.8085651	4.078737
Mrpl1	mitochondrial ribosomal protein L1 [Source:MGI Symbol;Acc:MGI:2137202]	96208493	96210493	+		1.7560978	4.5641637
Minos1	mitochondrial inner membrane organizing system 1 [Source:MGI Symbol;Acc:MGI:1913628]	139130113	139132113	-	Yes	1.7471647	4.341771
Fars2	phenylalanine-tRNA synthetase 2 (mitochondrial) [Source:MGI Symbol;Acc:MGI:1917205]	36458280	36460280	-		1.7345643	4.078737
Atp5b	ATP synthase, H+ transporting mitochondrial F1 complex, beta subunit [Source:MGI Symbol;Acc:MGI:107801]	128087552	128089552	+		1.7016501	4.5641637
Anti-NANOG TBC1D15 KO vs WT in CD133-positive TICs							
Atp6v1c2	ATPase, H+ transporting, lysosomal V1 subunit C2	17323665	17325665	-	Yes	3.2333727	5.286448
Atp6v1c2	ATPase, H+ transporting, lysosomal V1 subunit C2	17323703	17325703	-	Yes	3.1860666	5.239142
Atp8b4	ATPase, class I, type 8B, member 4	126490573	126492573	-		1.9345276	5.139606
Atp8b2	ATPase, class I, type 8B, member 2	89953639	89955639	-		1.8276126	5.032691
Me3	malic enzyme 3, NADP(+)-dependent, mitochondrial	89828282	89830282	+		3.2333727	5.286448
Immp2l	IMP2 inner mitochondrial membrane peptidase- like (S. cerevisiae)	41544391	41546391	+		2.985154	5.1902323
Diablo	diablo, IAP-binding mitochondrial protein	123517231	123519231	+		2.8820603	5.0871387
Mecr	mitochondrial trans-2-enoyl-CoA reductase	131859233	131861233	+		2.297098	5.5021763
Atp5h	ATP synthase, H+ transporting, mitochondrial F0 complex, subunit D	115416018	115418018	-		2.2146356	5.419714
Mtus1	mitochondrial tumor suppressor 1	41053794	41055794	-		1.9345276	5.139606
Cmpk2	cytidine monophosphate (UMP-CMP) kinase 2, mitochondrial	26472930	26474930	+		1.8820603	5.0871387
Slc25a26	solute carrier family 25 (mitochondrial carrier, phosphate carrier), member 26	94533140	94535140	+		1.8276126	5.032691

Supplementary Figures



Extended Data Figure 1
Choi *et al*.

Supplementary Fig. 1. The mRNA and protein levels of ChIP-seq target genes were examined after TBC1D15 silencing in Huh7 cells.

(a) *TBC1D15* regulated gene. RT-qPCR analysis of *TBC1D15*-knockdown Huh7 showed PD-L1 (*CD274*), *NFKBIA* (IκB), *RXRA* (RXRα), *CHUK* (IKKα), and *AKT2* (AKT) are significantly down-regulated, while *HNF4A* (HNF4α) is significantly up-regulated by knocking down of *TBC1D15*.
(b) Immunoblotting analysis of *TBC1D15*-knockdown Huh7 showed expression of PD-L1 protein was down-regulated by knocking down of *TBC1D15*.
(c) PD-L1 expression in Notch signaling pathway reactivated Huh7 knock down cell. Western blot showed a same pattern for Notch 1 intracellular domain and PD-L1.
(d) qRT-PCR result showed *TBC1D15* had no significant different by adding 0.75 μM or 1.5M of Notch pathway activator oxalipatin. (C-H) qRT-PCR result showed upregulation of *CD274*, *CHUK*, *AKT2*, and *NFKBIA* by activating Notch pathway induced by oxalipatin treatment; (G) however, *HNF4A* and *RXRA* was not affected by oxalipatin treatment.

Supplementary Figure 2
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Supplementary Figure 2. The CD133/CD49f TICs isolated in Lung cancer cell lines.
(**a**) Expression of TBC1D15 in various human tissues. Distribution of log10 (gene-level transcripts per kilobase million, TPM) of TBC1D15 RNA-seq expression levels in 21 human tissues were downloaded from the GTEx portal v8. ACC-Adrenocortical carcinoma, BLCA-Bladder urothelial carcinoma, BRCA-Breast invasive carcinoma, CESC-Cervical squamous cell carcinoma and endocervical adenocarcinoma, CHOL-Cholangio carcinoma, COAD-Colon adenocarcinoma, DLBC-Lymphoid Neoplasm Diffuse Large B-cell Lymphoma, ESCA-Esophageal carcinoma, GBM-Glioblastoma multiforme, HNSC-Head and Neck squamous cell carcinoma, KICH-Kidney renal Clear cell carcinoma, KIRP-Kidney renal papillary cell carcinoma, LAML-Acute Myeloid Leukemia, LGG-Brain Lower Grade Glioma, LIHC-Liver Hepatocellular carcinoma, LUAD-Lung adenocarcinoma, LUSC-Lung squamous cell carcinoma, MESO-Mesothelioma, OV-Ovarian serous Cystadenocarcinoma, PAAD-Pancreatic adenocarcinoma, PCPG-Pheochromocytoma

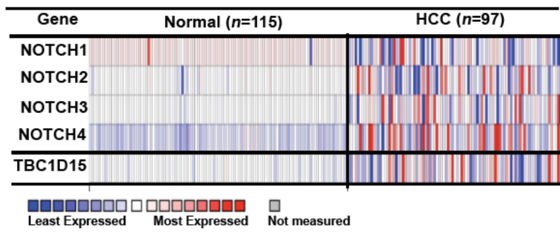
and Paraganglioma, PRAD-Prostate adenocarcinoma, READ-Rectum adenocarcinoma, SARC-Sarcoma, SKCM-Skin Cutaneous Melanoma, STAD-Stomach adenocarcinoma, TGCT-Testicular Germ Cell Tumors, THCA-Thyroid carcinoma, THYM-Thymoma, UCEC-Uterine Corpus Endometrial Carcinoma, UCS-Uterine Carcinosarcoma, UVM-Uveal melanoma

(**b**) TBC1D15 (TPM)

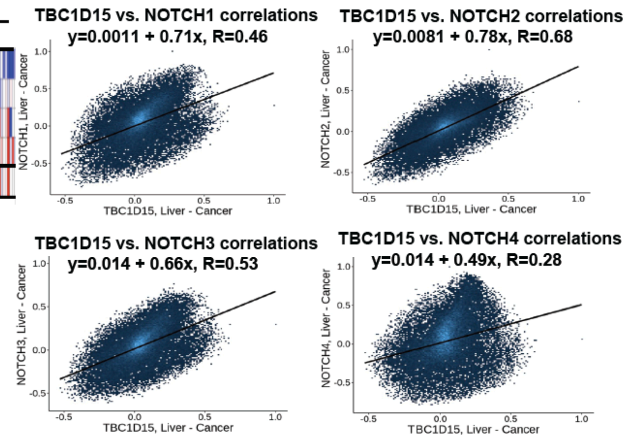
(**c**) FACS analysis of CD133+/CD49f+ TICs from isolation of CD133/CD49f TICs from H1299 and Calu3 cells using the affinity magnetic-microbead kit.

(**d**) Western blotting for TBC1D15, NUMB, and ACTIN in isolated CD133+/CD49f+ or CD133-/CD49f- TICs.

a



b



Extended Data Figure 3
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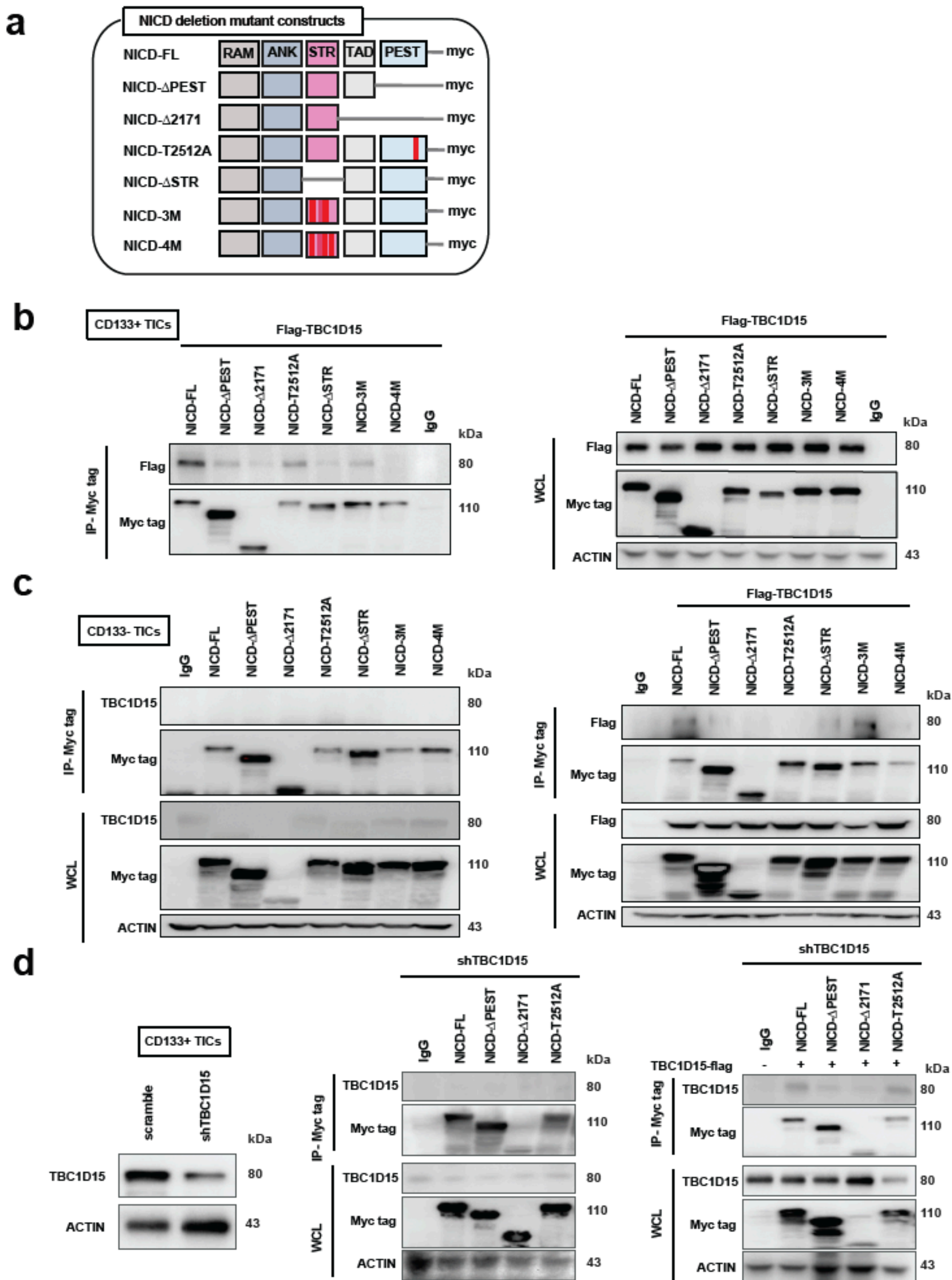
Supplementary Figure 3. Correlation between genome-wide co-expression correlations for TBC1D15 and NOTCH1-4 in liver cancer tissues by Correlation Analyzer R database

(**a**) Co-expression analysis of NOTCHs and TBC1D15. Left panel, Genes that co-expressed with NOTCHs and TBC1D15 revealed by the Oncomine database. Range of the of mRNA expression values is represented by the color palette. Red color represents the higher expression and blue represents the lower expression of the mRNA.

(**b**) Scatter plot showing the relationship between genome-wide co-expression correlations for TBC1D15 and NOTCH1-4 in liver cancer tissues by Correlation Analyzer R database. Displayed R value determined by Pearson correlation.



Supplementary Figure 4. The NICD C-terminal PEST domain interacts with TBC1D15 in CD133-positive TICs.

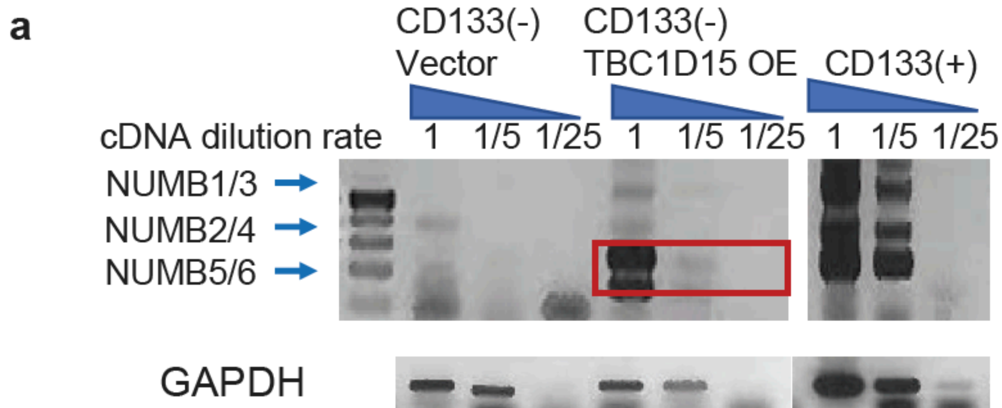
(**a**) Schematic illustration of NICD domain deletion mutant constructs**.** The red bars represent alanine-substitution sites for NOTCH STR phosphorylation sites.

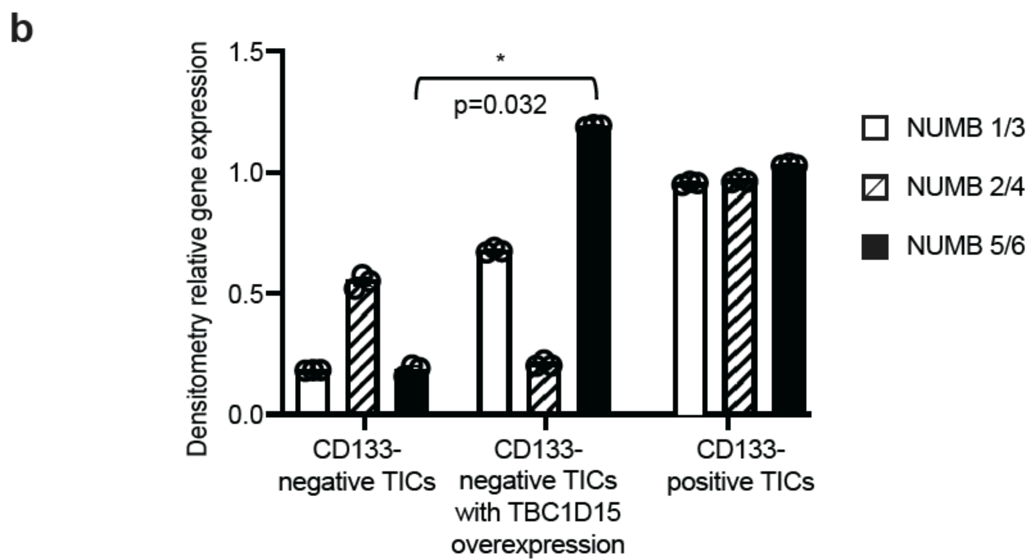
(**b**) Co-IP-Western blot analysis for identified interaction domains of NICD and TBC1D15 in CD133-positive TICs with or without TBC1D15-overexpressing.

(**c**) Co-IP-Western blot analysis for identified interaction domains of NICD and TBC1D15 in CD133-negative TICs with or without TBC1D15-overexpressing.

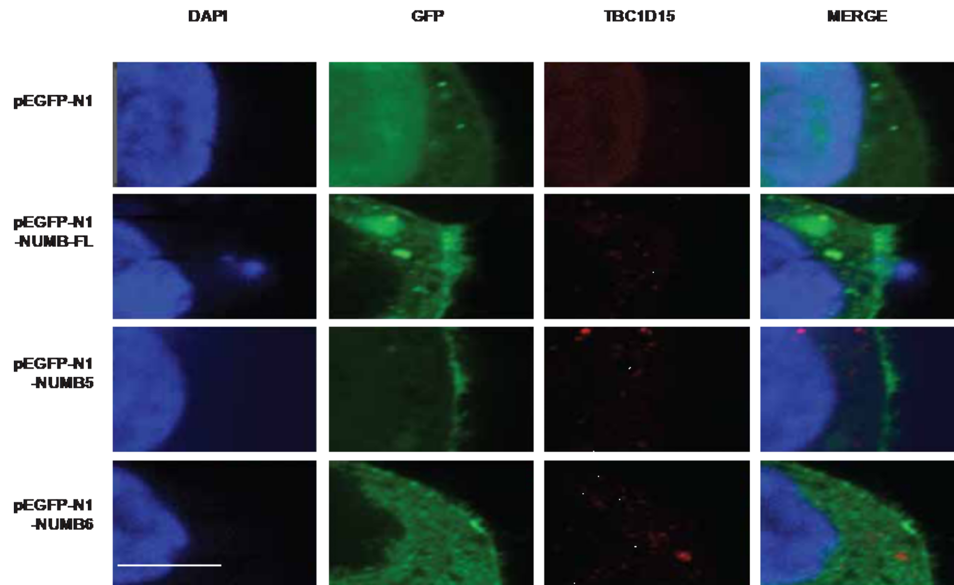
(**d**) **Left panel**, Western blot analysis for knockdown of TBC1D15 expression in shTBC1D15 lentiviral transducing-CD133-positive TICs. **Middle panel**, Co-IP-Western blot analysis for

confirmed interaction PEST domains of NICD and TBC1D15 in shTBC1D15 lentiviral transducing-CD133-positive TICs. **Right panel**, Co-IP-Western blot analysis for confirmed interaction PEST domains of NICD and TBC1D15 in shTBC1D15 lentiviral transducing-CD133-positive TICs with TBC1D15-overexpressing.





Supplementary Figure 5. Semi-qPCR analysis of NUMB isoforms expression in TICs and non-TICs.
(**a**) Semi-qPCR analysis of *NUMB* isoforms expression in indicated cells.
(**b**) Graph data from agarose images of semi-qPCR analysis were used for this bar graph. The densitometry quantification was done using the NIH image J program.



Supplementary Figure 6. TBC1D15 and NUMB isoforms are colocalized in CD133-negative TICs.

CD133-negative TICs was transfected with GFP-NUMB full-length, -NUMB5, and -NUMB6 and analyzed by immunofluorescence microscopy. Merge images show TBC1D15 (endogenous, red) and GFP-NUMBs (green). Scale bars: 7.4 μm.

Supplementary Methods

STAR Methods

- **Key Resources Table**

Reagent or Resource	Source	Identifier
Antibodies		
Anti-Flag	Sigma-Aldrich	Cat# F1804
Anti-NUMB (C29G11)	Cell Signaling	Cat# 2756S
Anti-NUMB (P20)	Santa Cruz	Cat# SC-15590
Anti-TBC1D15	Abcam	Cat# ab121396
Anti-Myc-tag (9B11)	Invitrogen	Cat#PA1-981
Anti-β-Actin (AC-15)	Santa Cruz	Cat# SC-69879
Anti-CDK8 (P455)	Cell Signaling	Cat# 4106S
Anti-CDK19	Abcam	Cat# ab168633
Anti-Phospho-NUMB	Cell Signaling	Cat# 9878S
Anti-NUMB	Thermo Fisher scientific	Cat# BS-3311R
Anti-cleaved NOTCH1 (Val1744)	Cell Signaling	Cat# 4147S
Anti-NOTCH1 (C-10)	Santa Cruz	Cat# SC-373891
Anti-c-Jun	Cell Signaling	Cat# 9165S
Anti-c-Myc (A-14)	Santa Cruz	Cat# SC-789
Anti-CD133 epitope	Thermo Fisher scientific	Cat# 12-1338-42
Anti-GFP (9F9.F9)	Abcam	Cat# ab1218
Anti-Fbxw7	Abcam	Cat# ab109617

Primers	
NUMB Fwd	5'-AGGAATGCACATCTGTGAAG-3'
NUMB Rev	5'-CTCAGAGGGAGTACGTCTAT-3'
ACTIN Fwd	5'-ACCCACACTG TGCCCATCTACGA-3'
ACTIN Rev	5'-CAGCGGAACCGCTCATTGCCAAT-3'

Chemicals and Reagents		
Normal Goat serum	Thermo Fisher Scienfic	
FBS	GeminiBio	
1X NEAA	Gibco-BRL	
Glutamax-1	Gibco-BRL	Cat# 35050-061
Antibiotic-Antimycotic	Thermo Fisher Scienfic	
EMEM	Gibco-BRL	

DMEM	Sigma-Aldrich	
Triple	Gibco-BRL	Cat# 12563-029
DAPI	Invitrogen	
Triton X-100	Invitrogen	
10X PBS	Invitrogen	
Para-formaldehyde	Sigma-Aldrich	
Protease inhibitor	Invitrogen	
6X Laemmli sample buffer	Bioland	Cat# SAB03-02
CD133 isolation kit	Miltenyi Biotec	Cat# AC133
Protein A/G bead	Santa Cruz	Cat# SC-2003
40% Acrylamide/Bis solution (29:1)	Bio-Rad	Cat# 1610146
Glutathione-Sepharose 4B bead	GE Healthcare	
DTT	Sigma-Aldrich	
Lysozyme	Sigma-Aldrich	
IPTG	Sigma-Aldrich	Cat# 420291
HEPES		
Anti-Flag M2 Affinity gel	Thermo Fisher Scienfic	Cat# A2220
Flag peptide	Sigma-Aldrich	
MACS LS columns	Miltenyi Biotec	Cat# 130-042-401
RPMI 1640 Medium	Gibco-BRL	Cat# 11875
TRIzol	Thermo Fisher Scientific	Cat# 15596026
TEMED	VWR Life science	Cat# 110-18-9
BSA (Bovine Serum Albumin)	VWR Life science	Cat# 9048-46-8
Agarose	Fisher Scientific	Cat# 9012-36-6
Methanol	VWR Life science	Cat# 67-56-1
Donkey anti-Mouse IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor 488	Thermo Fisher Scientific	Cat# A-21202
Goat anti-Rabbit IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor 568	Thermo Fisher Scientific	Cat# A-11036
Rabbit anti-Goat IgG (H+L) Cross-Adsorbed Secondary Antibody, Alexa Fluor 488	Thermo Fisher Scientific	Cat# A-11078
CompactPrep Plasmid Midi Kit	QIAGEN	Cat# 12843
SuperSep™ Phos-tag™ 7.5%	FUJIFILM Wako Pure Chemical Corporation	Cat# 192-18001
PRKCZ (PKC ZETA)	Thermo Fisher Scientific	Cat# P2268
CDK8/CYCLIN C	Thermo Fisher Scientific	Cat# PV4402
MiniElute PCR purification kit	QIAGEN	Cat# 28004
DNeasy Blood and Tissue kit	QIAGEN	Cat# 69504

Cas9-D10A Nickase Protein	Sigma	Cat# CAS9D10APR
Bio T	Bioland Scientific LLC	B01-01

Recombinant DNA		
p3X-Flag-TBC1D15-full length		
p3X-Flag-TBC1D15-N terminal		
pcDNA3-N1ICD-myc		
pcDNA3-∆PEST-myc		
pcDNA3-∆2171-myc		
pcDNA3-∆STR-myc		
pcDNA3-3M (S2122A/T2133A/S2137A)-myc		
pcDNA3-4M (S2122A/T2133A/S2137A/S2142 A)-myc		
pcDNA3-PEST-S2490A-myc	This paper	N/A
pcDNA3-PEST-S2493A-myc	This paper	N/A
pcDNA3-PEST-S2500A-myc	This paper	N/A
pcDNA3-PEST-S2514A-myc	This paper	N/A
pcDNA3-PEST-S2514E-myc	This paper	N/A
pcDNA3-PEST-T2512E-myc	This paper	N/A
pcDNA3-PEST-T2512A-myc		
pGEX-GST-NUMB-full length	Kaibuchi lab	Mol Biol Cell 2011, 22(17):3103-19
pGEX-GST-PTB	Kaibuchi lab	Mol Biol Cell 2011, 22(17):3103-19
pGEX-GST-PRR/1	Kaibuchi lab	Mol Biol Cell 2011, 22(17):3103-19
pGEX-GST-PRR/2	Kaibuchi lab	Mol Biol Cell 2011, 22(17):3103-19
pGEX-GST-PRR/3	Kaibuchi lab	Mol Biol Cell 2011, 22(17):3103-19
NUMB4-eGFP-M107K	Addgene	Cat #37805
pEGFP-N1-NUMB5	Karaczyn lab	Neural Dev 2010, 5:31
pEGFP-N1-NUMB6	Karaczyn lab	Neural Dev 2010, 5:31
pEGFP-N1	Karaczyn lab	Neural Dev 2010, 5:31
pGreen-puro-TBC1D15	This paper	N/A
pBabe-puro-CDK8-Flag	Addgene	Cat #19758
pBabe-puro-CDK8-KD-Flag	Addgene	Cat #19759
p3X-Flag-FBW7a		

Software		

Adobe Photoshop 2020	Adobe	
Adobe illustrator	Adobe	
ImageJ	NIH	https://imagej.nih.gov/ij/
Prism8	Graphpad	https://www.graphpad.com/scientific-software/prism/
Enrichr	Ma'ayan Lab	https://maayanlab.cloud/Enrichr/
UCSC Genome Brower	UCSC Genomics Institute	http://www.genome.ucsc.edu

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- **RESOURCE AVAILABILITY**

- **Material Availability**

  The unique materials and reagents created in this study are available upon request from the lead representative along with a completed Material Transfer Agreement.

- **Data and Code Availability**

  The ChIP-seq datasets generated during this study are available at Enrichr.

- **EXPERIMENTAL MODEL AND SUBJECT DETAILS**

Mouse studies

All experiments on mice were approved by the USC Institutional Animal Care and Use Committee. All mice were raised in a specific pathogen-free environment at the University of Southern California Keck School of Medicine. All surgeries were performed under deep anesthesia, and all efforts were made to minimize suffering. Tbc1d15 Fl/Fl: Ns5aTg transgenic mice will be crossbred with LysM-Cre mice to obtain single-positive offspring or double-positive transgenic mice. LysM-Cre:Tbc1d15 Fl/Fl transgenic mice will be further crossbred with Ns5a Tg mice to obtain triple-positive offspring or double-positive transgenic mice.

Mice were anesthetized with isoflurane and skin was sterilized with iodophor three times before surgery. For the intrahepatic inoculation of TBC1D15 KO (sgTBC1D15 232, 260,295) cells, the surgery was conducted on left lobes along the left rib edge. $2x10^5$ Hepa1-6 cells were suspended in 50 µl PBS and injected into left lobes of the liver with a syringe at 30° angle. The injection site was gently pressed with cotton balls to reduce bleeding and leakage of cell suspensions afterwards. Then, the peritoneum and skin were closed with 4–0 sutures.

Cells

Human hepatoma cell line (Huh7) and transformed liver cell line (Hep3B) were obtained from ATCC (Manassas, VA). Cells were cultured in growth media [Dulbecco’s modified Eagle’s medium (DMEM, Sigma-Aldrich) with 10% fetal bovine serum (FBS, GeminiBio), 0.1% non-essential amino acid (Gibco-BRL), 1% Glutamax-1 (Gibco-BRL) and 1% penicillin/streptomycin (P/S, Gibco-BRL)]. Cells were cultured

with Eagle's Minimum Essential Medium (Gibco-BRL) containing 1% Glutamax-1, 1% P/S, and 10% FBS. Isolation of CD133-posivie/negative cells were performed on Huh7, Hep3B and H1299 cell lines using magnetic-microbeads conjugated with CD133 (AC133, Cell isolation Kit, Miltenyi Biotec). Magnetic separation was performed twice. CD133-positive cells were cultured in growth media as described above. The quality of isolating was controlled by flow cytometry with an antibody against a different CD133 epitope (Santacruz Biotechnology).

Cell transfection

Transfection of cells was performed using BioT (Bioland Scientific LLC.) at a transfection reagent:DNA ratio of 3:1 for 48 hours. For HEK293A cells, BioT was used for 72 hours according to manufacturer's instructions.

Immuno-precipitation, and Immunoblot

Cells were co-transfected with Flag-tag TBC1D15 full-length constructs together with Myc-tag NICD full-length or deletion mutants (ΔPEST, Δ2171, T2512A, ΔSTR, 3M, and 4M; from Dr. Del Sal), Myc-tag FBW7 full-length (Addgene). And cells were transfected with Flag-tag TBC1D15 full-length, Myc-tag NICD full-length or deletion mutants, Myc-tag FBW7 full-length, Myc-tag PEST mutants (S2490A, S2493A, S2500A, T2512A, S2514A, T2512E, S2514E) constructs. Bio T was used as the transfection reagent (Bioland Scientific LLC). After 48 hours transfection, cells were harvested with Triple solution (Invitrogen) and lysed in lysis buffer (50mM Tris-Cl, pH 7.5, 150mM NaCl, 1% NP-40, 0.1% SDS) supplemented with protease inhibitors (Invitrogen), incubated on ice for 30 min, and cleared by centrifugation at 13,000 rpm at 4°C for 10 min. For immuno-precipitation, protein lysate was incubated 2 hours in 4°C with primary antibodies [1 mg of antibody as indicated, or isotype control antibodies; anti-Flag tag (Sigma-Aldrich) or anti-Myc tag (Invitrogen)] on a rotation device. Protein A/G beads (Santacruz Biotechnologies) were pre-cleared for 1 hour in 4°C and added protein lysate with primary antibodies for 1 hour in 4°C on a rotation device. After 1 hour, protein A/G beads were pulled down and three times washed with 1X PBS (Invitrogen) and boiled in 6 X Laemmli sample buffer (Bioland) for 5 min. And the precipitants subjected to SDS-PAGE and immunoblot, and protein analysis by nitrocellulose membrane (Invitrogen). Immunoprecipitation was performed at least twice per cell line.

Immunofluorescence Staining

Cells were transfected with EGFP-N1, EGFP-NUMB4, EGFP-NUMB5, and EGFP-NUMB6 constructs by Bio T. After 48 hours transfection, cells were fixed by 4% para-formaldehyde (Sigma-Aldirch) for 15 min at room temperature. Cells were blocked with 10% normal goat serum (Invitrogen) with 0.25% Triton X-100 (Invitrogen) in PBS for 1 hour on shaker, and then incubated with primary antibodies [anti-TBC1D15 (1:1,000) and anti-NOTCH1 (Cell signaling, 1:500)] overnight at 4°C followed by the appropriate secondary fluorescently labeled antibodies [anti-rabbit (1:1,000), anti-mouse (1:1,000), and anti-goat (1:1,000); Molecular Probe] for 1 hour at room temperature on shaker. Nuclei were counterstained with DAPI (Sigma-Aldrich). Images were acquired using a confocal microscope (Leica).

Production of Lentivirus

Lentivirus constructs [sh-Scramble and sh-TBC1D15] and assemble particles were generated by co-transfecting sub-confluent HEK-293T cells with lentivirus construct along with the psPAX2 packaging vector and an envelope vector (pMD2.G). Lentivirus was collected from the culture media at 72 hours post-transfection and purified by ultracentrifugation (Beckman, SW 28 rotor) with 25,000 rpm for 2 hours at 4°C. Lentivirus pellets were resuspended in de-ionized water and stored at -80°C until use. Cells were transduced with lentivirus in presence of polybrene (5 mg/ml). Lentivirus transduced-cells were selected in culture media containing puromycin (Gibco-BRL, 1 mg/ml).

Quantitative real-time PCR

Total RNA was isolated using the ISOLATE II RNA Mini Kit (Bioline) as per manufacturer's instructions and reverse transcribed to cDNA using oligo(dT) nucleotides and SuperScript III Reverse Transcriptase (Thermo Fisher Scientific). Quantitative real-time PCR was performed using Advanced Taq (Thermo Fisher Scientific) on Bio-Rad PCR system. Primers were designed using the Integrated DNA Technologies online tool and listed in the Key Resources Table.

Purification of Recombinant Proteins

To purified GST-tag NUMB constructs (NUMB full-length, NUMB-PTB, NUMB-PRR1, NUMB-PRR2, and NUMB-PRR3), BL21 E. coli (EMD Millipore) harboring the pGEX vector were cultured at 37°C until reaching A600=0.6, then induced with 0.5 mM IPTG (Sigma-Aldrich) and grown for an additional 3 hours at 30°C. Bacterial pellets were resuspended in ice-cold bacterial lysis buffer [0.5% Triton X-100, 1 mM DTT (Sigma-Aldrich), 0.2 mg/ml lysozyme (Sigma-Aldrich) in PBS] for 1 hour, then sonicated three times in 30-s pulses with 1 min on ice between pulses. Following a centrifugation (12,000 rpm, 5 min) the supernatant was recovered and purified on Glutathione-Sepharose 4B beads (GE Healthcare). To purify flag-tag TBC1D15, HEK-293A cells grown to 70-80% confluence was transfected with flag-tag TBC1D15. After 72 hours post-transfection, cells were washed once with cold PBS, and lysed in 1ml cold lysis buffer (10 mM HEPES pH 7.5, 10 mM KCl, 1.5 mM MgCl2, 0.5% NP-40) for 1 hour on ice, followed by centrifugation at 13,000 rpm for 10 min and recovery of the supernatant. Immunoprecipitations were performed by incubating 1 ml supernatant with 40 µl of a 40% slurry of M2 anti-Flag agarose (Invitrogen) for 2 hours at 4°C with rotating. Beads were washed once in buffer W (20 mM Tris pH 7.3, 300 mM NaCl, 0.5% triton X-100, 2% glycerol), once in PBS containing 0.2% Triton X-100, and once in PBS. Flag-tag TBC1D15 was eluted by incubation in Flag peptide (1 mg/ml, Sigma-Aldrich) overnight at 4°C. The samples were stored at -80°C in PBS containing 5% glycerol and 1 mM DTT.

***In vitro* Protein Interaction assay**

Purified GST-NUMB constructs (NUMB full-length, NUMB-PTB, NUMB-PRR1, NUMB-PRR2, and NUMB-PRR3) were immobilized onto Glutathione-Sepharose 4B beads. The beads were incubated with purified flag-tag TBC1D15 protein for 1 hour

at 4°C. Then the beads were washed three times, eluted by boiling in 6 X Laemmli sample buffer for SDS-PAGE, and subjected to immunoblot analysis with anti-Flag or anti-TBC1D15 antibodies.

Immunohistochemistry

In immunohistochemical (IHC) analysis, tissue slides were deparaffinized, rehydrated through an alcohol series followed by antigen retrieval with sodium citrate buffer. Tumor sections were blocked with 5% normal goat serum and 0.1% Triton X-100 in PBS for 60 min at room temperature and then incubated with appropriate antibodies at 4°C overnight. IHC staining was performed with horseradish peroxidase (HRP) conjugates using DAB detection.

- **QUANTIFICATION AND STATISTICAL ANALYSIS**

  All quantitative experiments have been repeated using at least three independent biological repeats. Unpaired Student's t test was used for two-group comparisons. All grouped data are presented as mean ± SD or mean ± SEM. Statistical comparisons between two groups were accessed by two-tailed student's t-test. GraphPad Prism Software (GraphPad Software, Inc.) was used to examine statistical significance.

- **DATA AND SOFRWARE AVAILABILITY**

  The list of software for data analysis and processing can be found in the Key Resources Table.

Cell transfection

Transfection of cells was performed using BioT (Bioland Scientific LLC.) at a transfection reagent: DNA ratio of 3:1 for 48 hours. According to the manufacturer's instructions, for HEK293A cells, BioT was used for 72 hours.

Quantitative Real-time PCR

Total RNA was isolated using the ISOLATE II RNA Mini Kit (Bioline) as per the manufacturer's instructions and reverse transcribed to cDNA using oligo(dT) nucleotides and SuperScript III Reverse Transcriptase (Thermo Fisher Scientific). Quantitative real-time PCR was performed using Advanced Taq (Thermo Fisher Scientific) on a Bio-Rad PCR system. Primers were designed using the Integrated DNA Technologies online tool and listed in below.
NUMB Fwd 5'-AGGAATGCACATCTGTGAAG-3', Rev 5'-CTCAGAGGGAGTACGTCTAT-3'; *ACTIN* Fwd 5'-ACCCACACTG TGCCCATCTACGA-3', Rev 5'-CAGCGGAACCGCTCATTGCCAAT-3'; *SLC25A16* Fwd 5'-AATCATTGTCTTTCAGTACCATGCG-3', Rev 5'-ATGGCAACCTCTTGAACCCC-3'; *FIS1* Fwd 5'-CTTAAAGTACGTCCGCGGGT-3', Rev 5'-CCGCGTCTCCTTCAGGATTT-3'; *ATP5B* Fwd 5'-CTGGTGTTGGTGAGAGGACC-3', Rev 5'-TGGGCAAACGTAGTAGCAGG-3'; *MRPS10* Fwd 5'-AATGGTGGCTTGCTTCTCAGT-3', Rev 5'-GGAGCAGTTAGGGCAGACTC-3'.

Purification of Recombinant Proteins

To purified GST-tag NUMB constructs (NUMB full-length, NUMB-PTB, NUMB-PRR1, NUMB-PRR2, and NUMB-PRR3), BL21 E. coli (EMD Millipore) harboring the pGEX vector were cultured at 37°C until reaching A600=0.6, then induced with 0.5 mM IPTG (Sigma-Aldrich) and grown for an additional 3 hours at 30°C. Bacterial pellets were resuspended in ice-cold bacterial lysis buffer [0.5% Triton X-100, 1 mM DTT (Sigma-Aldrich), 0.2 mg/ml lysozyme (Sigma-Aldrich) in PBS] for 1 hour, then sonicated three times in 30-s pulses with 1 min on ice between pulses. Following

centrifugation (12,000 rpm, 5 min), the supernatant was recovered and purified on Glutathione-Sepharose 4B beads (GE Healthcare). To purify flag-tag TBC1D15, HEK-293A cells grown to 70-80% confluence were transfected with flag-tag TBC1D15. After 72 hours post-transfection, cells were washed once with cold PBS and lysed in 1ml cold lysis buffer (10 mM HEPES pH 7.5, 10 mM KCl, 1.5 mM MgCl2, 0.5% NP-40) for 1 hour on ice, followed by centrifugation at 13,000 rpm for 10 min and recovery of the supernatant. Immunoprecipitations were performed by incubating 1 ml supernatant with 40 μl of a 40% slurry of M2 anti-Flag agarose (Invitrogen) for 2 hours at 4°C with rotating. Beads were washed once in buffer W (20 mM Tris pH 7.3, 300 mM NaCl, 0.5% Triton X-100, 2% glycerol), once in PBS containing 0.2% Triton X-100, and once in PBS. Flag-tag TBC1D15 was eluted by incubating Flag peptide (1 mg/ml, Sigma-Aldrich) overnight at 4°C. The samples were stored at -80°C in PBS containing 5% glycerol and 1 mM DTT.

In vitro Protein Interaction assay
Purified GST-NUMB constructs (NUMB full-length, NUMB-PTB, NUMB-PRR1, NUMB-PRR2, and NUMB-PRR3) were immobilized onto Glutathione-Sepharose 4B beads. The beads were incubated with purified flag-tag TBC1D15 protein for 1 hour at 4°C. Then the beads were washed three times, eluted by boiling in 6 X Laemmli sample buffer for PAGE, and subjected to immunoblot analysis with anti-Flag or anti-TBC1D15 antibodies.

Mitochondria fraction
According to the manufacturer's instruction, the mitochondria, nucleus, and cytoplasm fraction was performed using the Mitochondria Isolation Kit (Thermo Fisher Scientific). Briefly, the cells were lysed with I ml of ice-cold lysis buffer, and then cells were homogenized with a 26G syringe 30 times on the ice. The homogenates lysates were centrifuged for 10 min at 1500x g to obtain a nucleus fraction. The supernatant obtained was centrifuged for 10 min at 10,000xg, the supernatant contained the cytoplasm fraction, and the pellet contained the crude mitochondrial fraction.

Cell-based ubiquitylation assay
Cultured cells were lysed with a lysis buffer (120 mM NaCl, 50 mM HEPES (pH7.2), 1 mM EDTA (pH 8.0), 1% NP-40, 0.5% sodium deoxycholic acid (Sigma-Aldrich), 10 mM N-ethylmalemide (Sigma-Aldrich), and protease inhibitor cocktail for 30 min on ice. The lysates were centrifuged at 20,000 × g for 10 min at 4°C. Then 1% SDS was added to the lysates, then heated at 95°C for 10 min and diluted 5-fold with the lysis buffer. Myc-tagged NICD-FL, NICD-T2512E, and NICD-T2512A mutants were immunoprecipitated with anti-Myc-tag at 4°C overnight, respectively. Immuno-precipitates were analyzed by immunoblotting to detect ubiquitylated.

Immunohistochemistry
In immunohistochemical (IHC) analysis, tissue slides were deparaffinized, rehydrated through an alcohol series followed by antigen retrieval with sodium citrate buffer. Tumor sections were blocked with 5% normal goat serum and 0.1% Triton X-100 in PBS for 60 min at room temperature and then incubated with appropriate antibodies at 4°C overnight. IHC staining was performed with horseradish peroxidase (HRP) conjugates using DAB detection.

Intrahepatic Injection
Prepare mice for surgery in a chamber supplying 5% (v/v) inhaled Isoflurane in 1 L/min of oxygen. For intrahepatic xenografting, shave the ventral thorax and abdomen of the animals from the axillae down to the inguinal region and cleanse the skin with 70% ethanol. Using a 27 G 1/2 needle on a 1 ml syringe, administer 350 μl of sterile standard saline solution subcutaneously in the dorsum of the anesthetized animal's neck to compensate for intraoperative fluid losses. For analgesia, administer 350 μl of sterile normal saline containing buprenorphine (0.1 mg/kg) subcutaneously on the animal's flank, using a 27 G 1/2-in needle on a 1 ml syringe. Place the mouse supine on a pre-heated pad with the nose and mouth positioned inside the mouthpiece to deliver maintenance inhalational isoflurane anesthesia (2% (v/v) in 1 L/min O2).

Extend the limbs and secure them with tape to the operating surface to optimize the ventral abdomen and thorax exposure. Perform the procedure under a magnifying lamp to optimize visualization. Sterilize the shaved skin on the ventral abdomen and thorax with Betadine surgical scrub followed by 70% ethanol and lastly with povidone-iodine solution. Using sterile sharp scissors, make a transverse bilateral subcostal skin incision and divide the muscle layers to enter the peritoneal cavity to allow adequate exposure of the entire liver. Place a stitch in the skin above the xiphoid process and secure it to the mouthpiece with tape to better expose the liver and surrounding structures. Load the tumor cell suspension (prepared in step 1.13) into an insulin syringe using a 29 G 1/2 needle. With the liver stabilized using a cotton-tip applicator, insert the insulin syringe needle into the liver and advance the tip a few millimeters beyond the puncture site along a subcapsular plane. Gently discharge the contents of the syringe and then remove the needle from the liver. Place Surgical over the puncture site and apply gentle pressure with a cotton-tipped applicator to prevent leakage of the tumor cell suspension and achieve complete hemostasis. Close the incision with sutures and provide postoperative care. Remove the mouse from the inhalational anesthesia mouthpiece. Place the mouse in a cage under a heat lamp for approximately 20 min until recovered from anesthesia and mobilizing fully. Repeat the buprenorphine dose every 8-12 hours during the first 2-3 postoperative days.

Immunofluorescence Staining

Cells were transfected with EGFP-N1, EGFP-NUMB-FL, EGFP-NUMB5, and EGFP-NUMB6 constructs by Bio T. After 48 hours of transfection; cells were fixed by 4% paraformaldehyde (Sigma-Aldrich) for 15 min at room temperature. Cells were blocked with 10% normal goat serum (Invitrogen) with 0.25% Triton X-100 (Invitrogen) in PBS for 1 hour on shaker, and then incubated with primary antibodies [anti-TOM20 (1:1,000), anti-TBC1D15 (1:1,000) and anti-NOTCH1 (Cell signaling, 1:500)] overnight at 4°C followed by the appropriate secondary fluorescently labeled antibodies [anti-rabbit (1:1,000), anti-mouse (1:1,000), and anti-goat (1:1,000); Molecular Probe] for 1 hour at room temperature on shaker. Nuclei were counterstained with DAPI (Sigma-Aldrich). Images were acquired using a confocal microscope (Leica).

Immunoprecipitation and Immunoblot

Cells were co-transfected with Flag-tag TBC1D15 full-length constructs together with Myc-tag NICD full-length or deletion mutants (ΔPEST, Δ2171, T2512A, ΔSTR, 3M, and 4M), Flag-tag FBW7 full-length. And cells were transfected with Flag-tag TBC1D15 full-length, Myc-tag NICD full-length or deletion mutants, Myc-tag FBW7 full-length, Myc-tag PEST mutants (S2490A, S2493A, S2500A, T2512A, S2514A, T2512E, S2514E) constructs. Bio T was used as the transfection reagent (Bioland Scientific LLC). After 48 hours of transfection, cells were harvested with Triple solution (Invitrogen) and lysed in lysis buffer (50mM Tris-Cl, pH 7.5, 150mM NaCl, 1% NP-40, 0.1% SDS) supplemented with protease inhibitors (Invitrogen), incubated on ice for 30 min, and cleared by centrifugation at 13,000 rpm at 4°C for 10 min. For immunoprecipitation, protein lysate was incubated 2 hours in 4°C with primary antibodies [1 mg of the antibody as indicated, or isotype control antibodies; anti-Flag tag (Sigma-Aldrich) or anti-Myc tag (Invitrogen)] on a rotation device. Protein A/G beads (Santacruz Biotechnologies) were pre-cleared for 1 hour at 4°C and added protein lysate with primary antibodies for 1 hour at 4°C on a rotation device. After 1 hour, protein A/G beads were pulled down and three times washed with 1X PBS (Invitrogen) and boiled in 6 X Laemmli sample buffer (Bioland) for 5 min. And the precipitants were subjected to SDS-PAGE, immunoblot, and protein analysis by blotting onto nylone membrane. Immunoprecipitation was performed at least twice per cell line.

Mitochondria assessment

Mitochondria were identified by size and staining using the TOM20 outer membrane of the mitochondria-specific antibody and then analyzed using Graphpad Prism (version 8.0) and ImageJ.

Production of Lentivirus

Lentivirus constructs [sh-Scramble and sh-TBC1D15] and assemble particles were generated by co-transfecting sub-confluent HEK-293T cells with a lentivirus construct along with the psPAX2 packaging vector and an envelope vector (pMD2.G). Lentivirus was collected from the culture media at 72 hours post-transfection and purified by ultracentrifugation (Beckman, SW 28 rotor) with 25,000 rpm for 2 hours at 4°C. Lentivirus pellets were resuspended in de-ionized water and stored at -80°C until use. Cells were transduced with lentivirus in the presence of polybrene (5 mg/ml). Lentivirus transduced cells were selected in culture media containing puromycin (Gibco-BRL, 1 mg/ml).