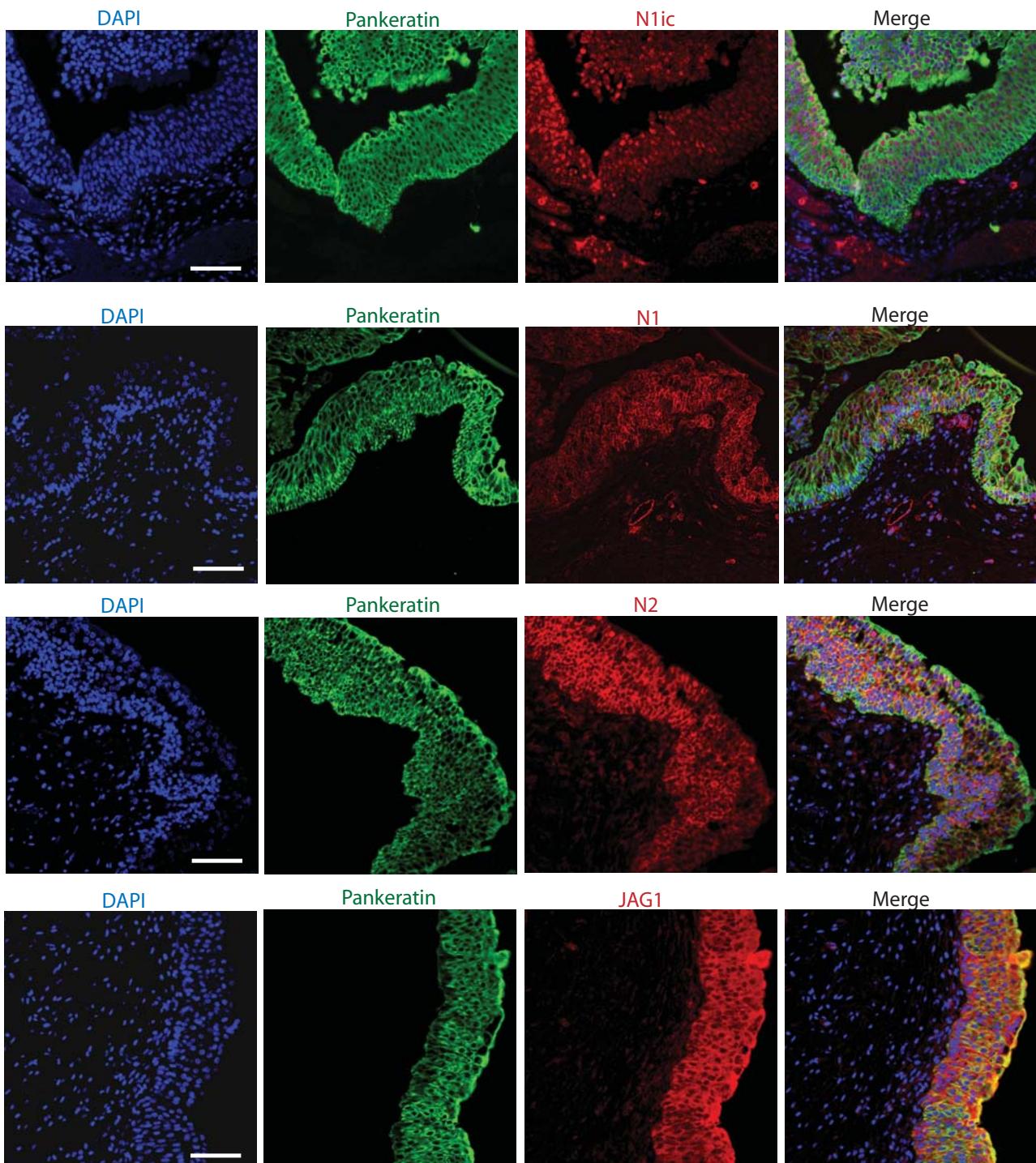


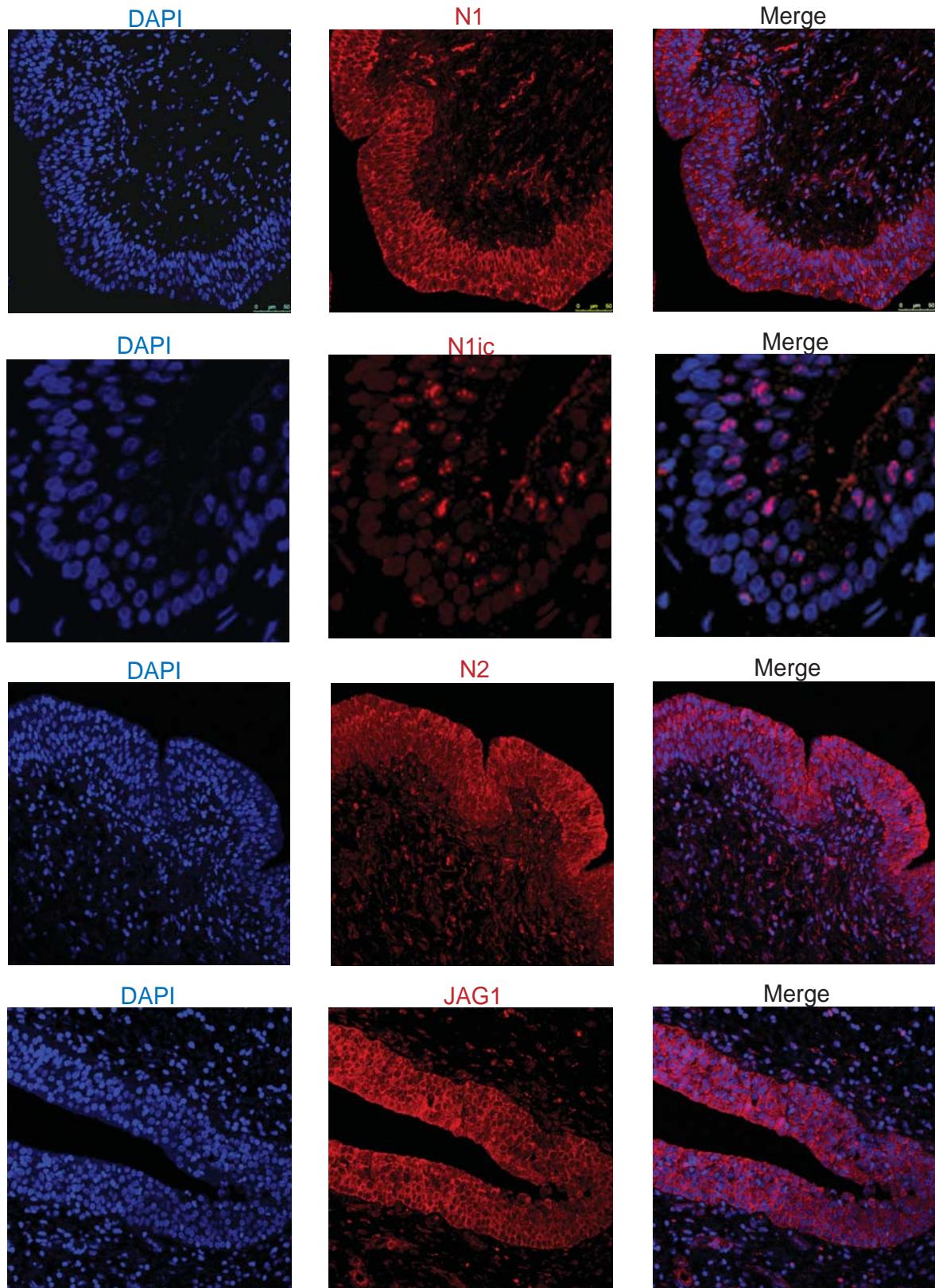
# A novel tumor suppressor role for the Notch pathway in bladder cancer

Theodoros Rampias, Paraskevi Vgenopoulou, Margaritis Avgeris, Alexander Polyzos, Konstantinos Stravodimos, Christos Valavanis, Andreas Scorilas and Apostolos Klinakis



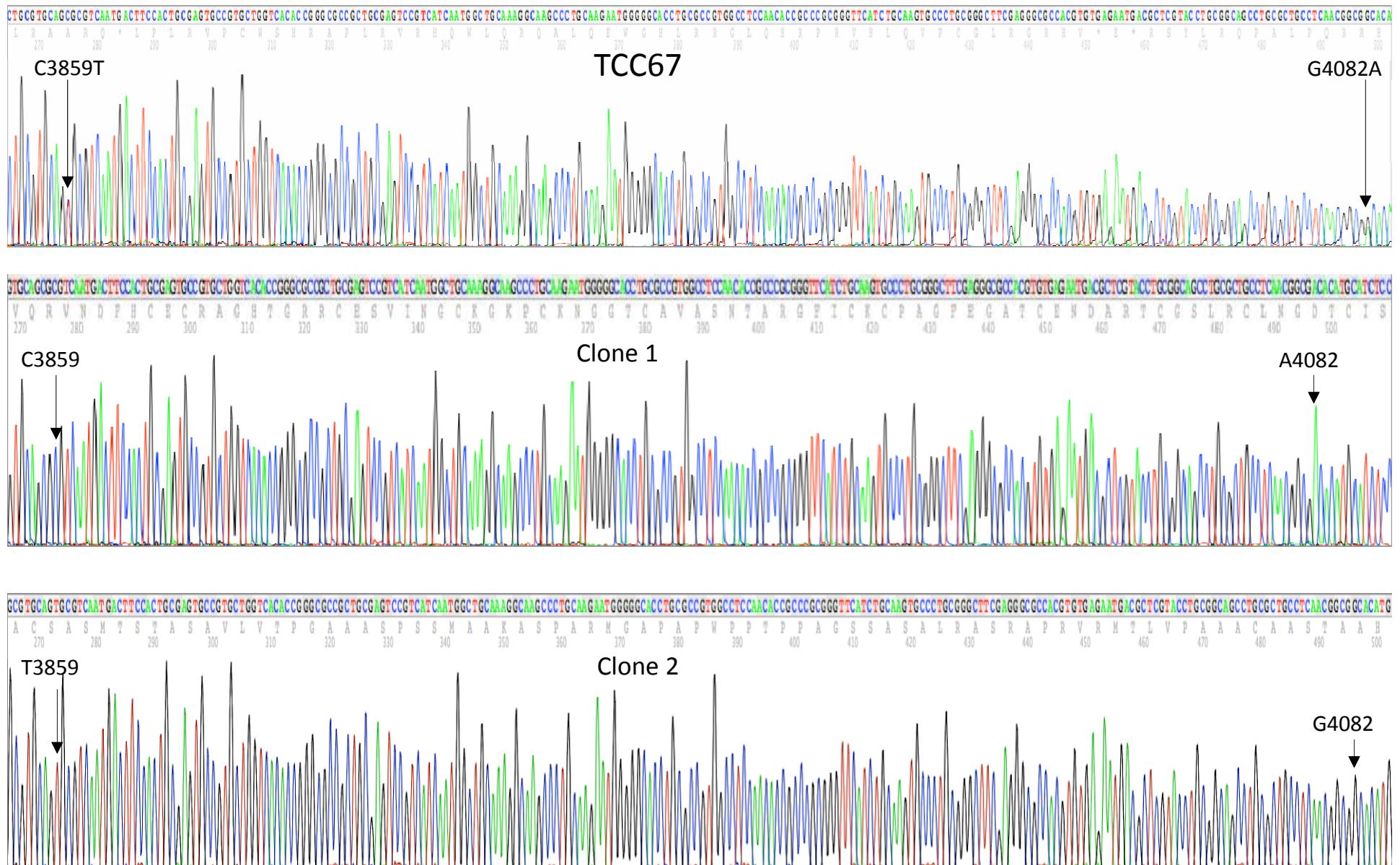
**Supplementary Figure 1. Notch signaling pathway activation in normal human bladder epithelium.**

Immunofluorescence on paraffin sections of normal human bladder stained with antibodies recognizing full-length NOTCH1 (N1), cleaved N1 (N1ic), cleaved and full-length NOTCH2 (N2), and JAG1. Sections were counterstained with a Pankeratin antibody (CK) and DAPI, and photographed with a fluorescence microscope (model BZ-9000; Keyence, Osaka, Japan) connected to a cooled CCD camera (SenSys, Photometrics). Scale bars: 50 $\mu$ m.

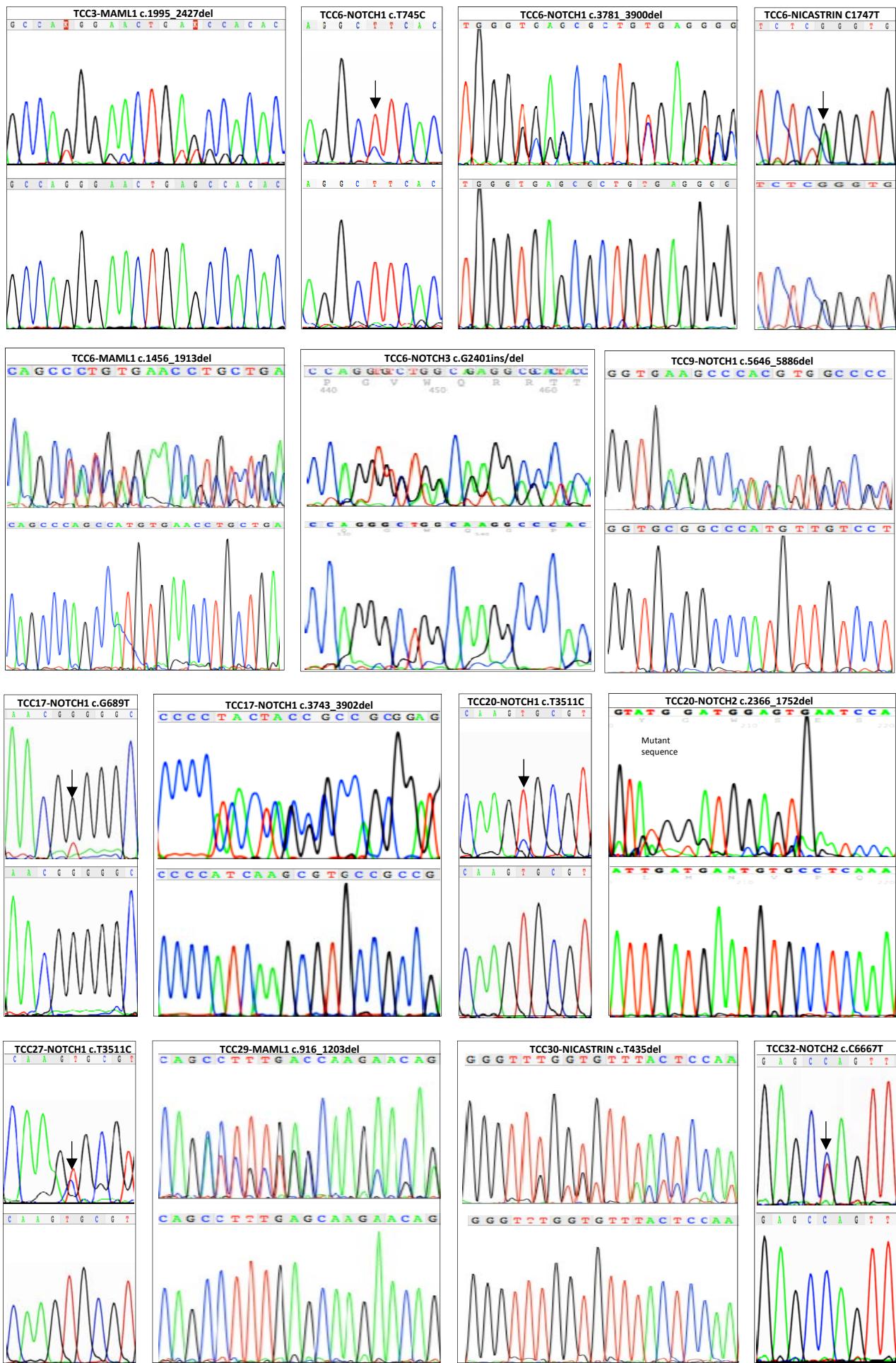


**Supplementary Figure 2. Notch signaling pathway activation in normal human ureter.**

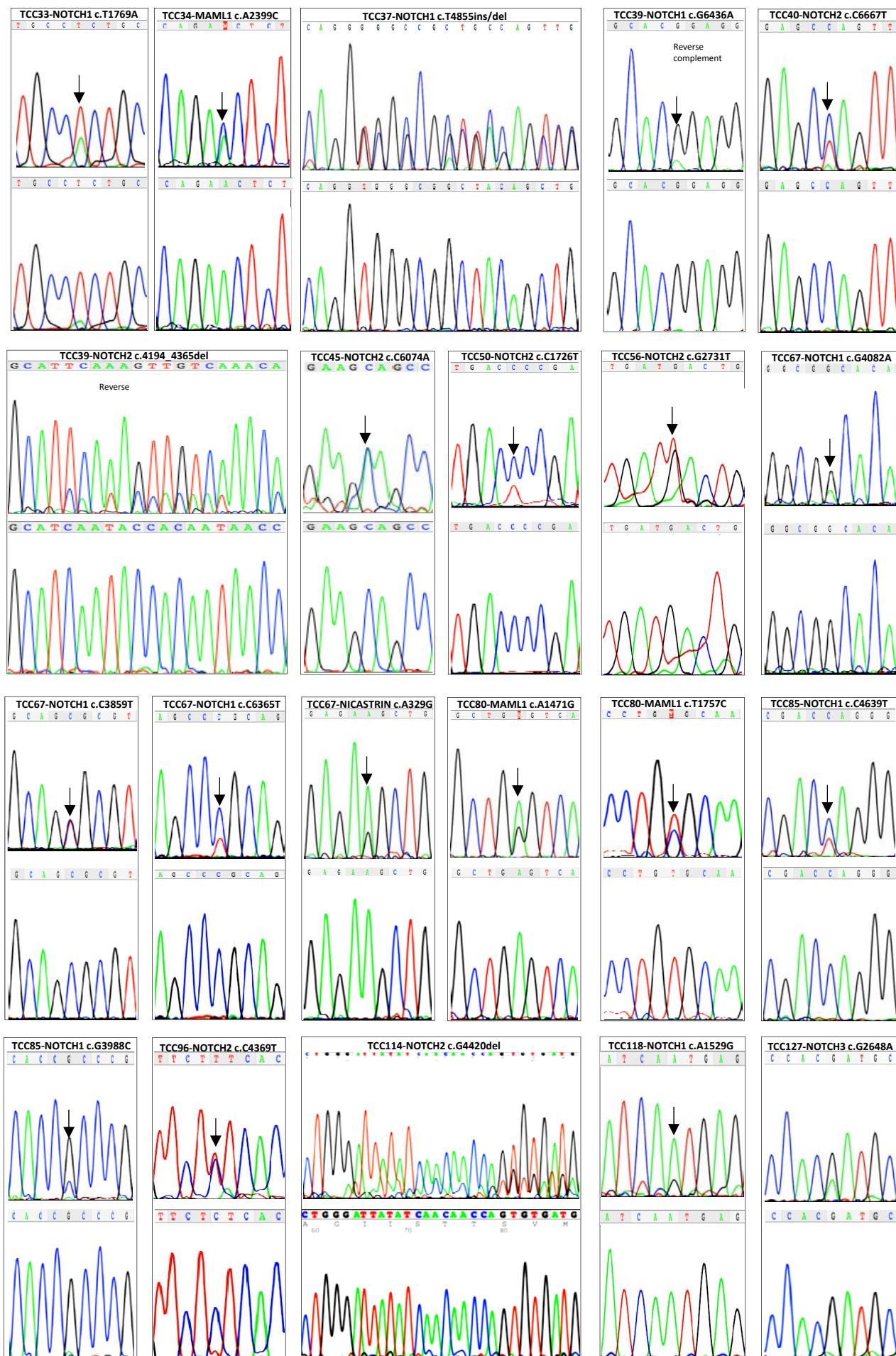
Immunofluorescence on paraffin sections of normal human ureter stained with antibodies recognizing full-length NOTCH1 (N1), cleaved NOTCH1 (N1ic), cleaved and full-length NOTCH2 (N2), and JAG1. Sections were counterstained with DAPI, and photographed with a Leica TS5 II confocal on an upright microscope (Leica DM6000 CFS). Scale bars: 50 $\mu$ m.



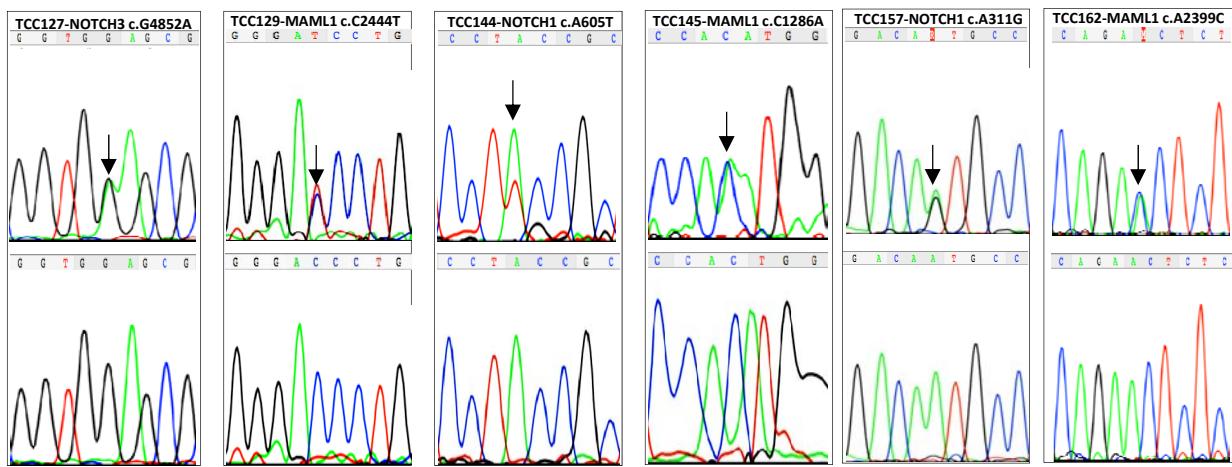
**Supplementary Figure 3. Allele-specific mutation analysis.** Chromatographs of the sequenced amplicon as well as of individual TOPO clones of the respective amplicon. Mutations c.C3859T and G4082A from patient TCC67 are found in different clones indicating that they are located in different alleles. This was the case also for mutations c.T5645C and c.C6365T of the same patient (not shown here).



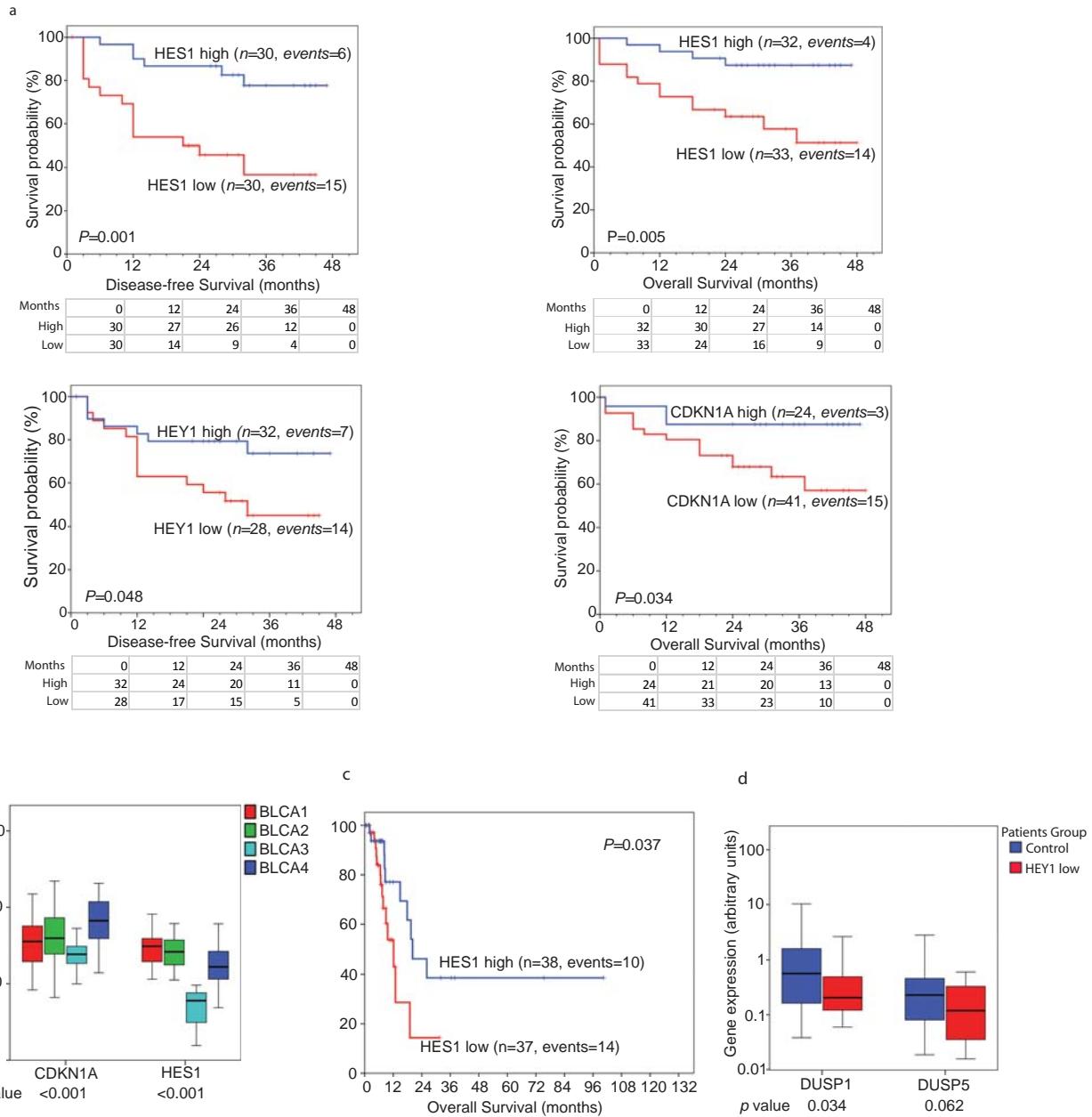
Supplementary Figure 4 continued



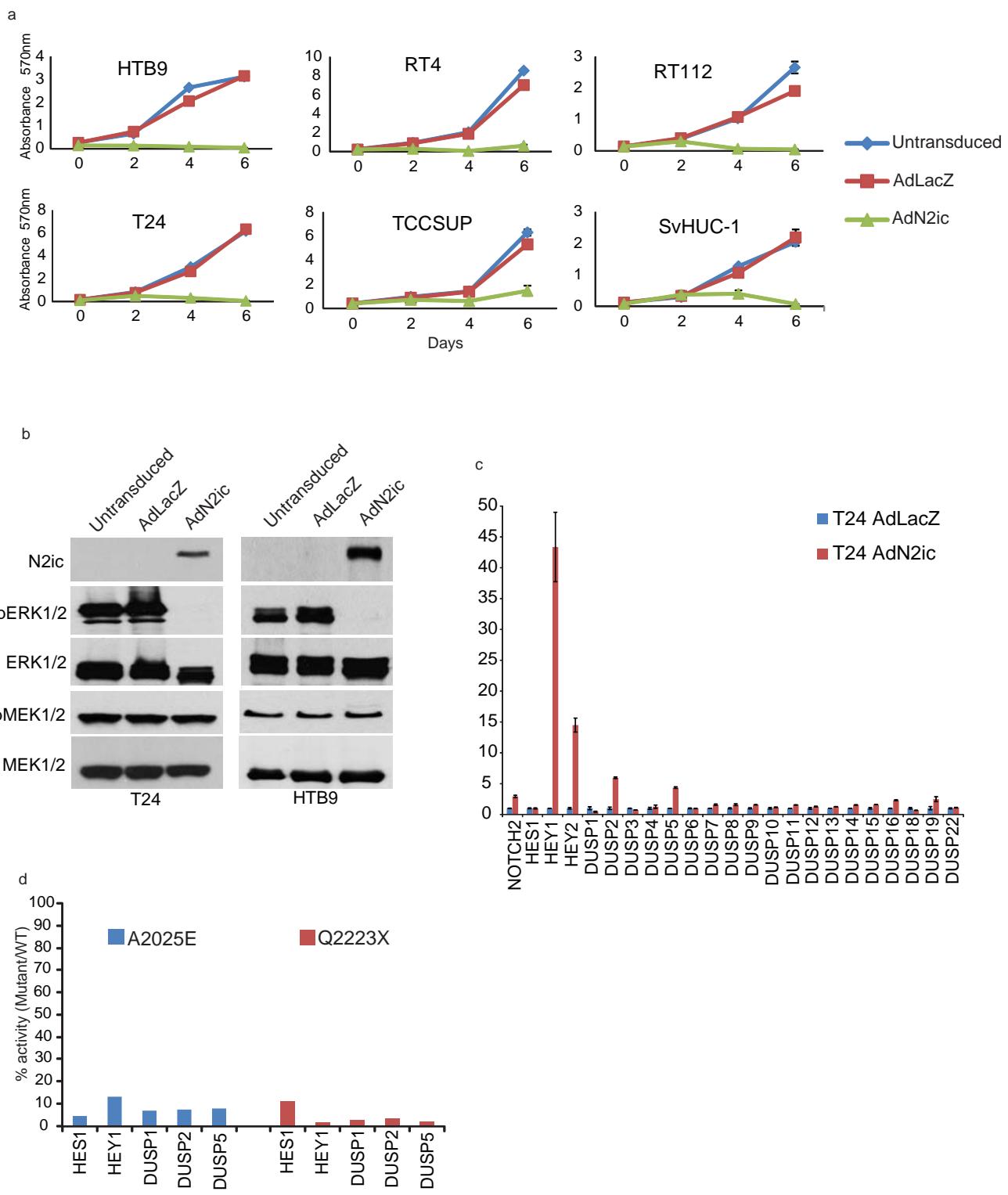
### Supplementary Figure 4 continued



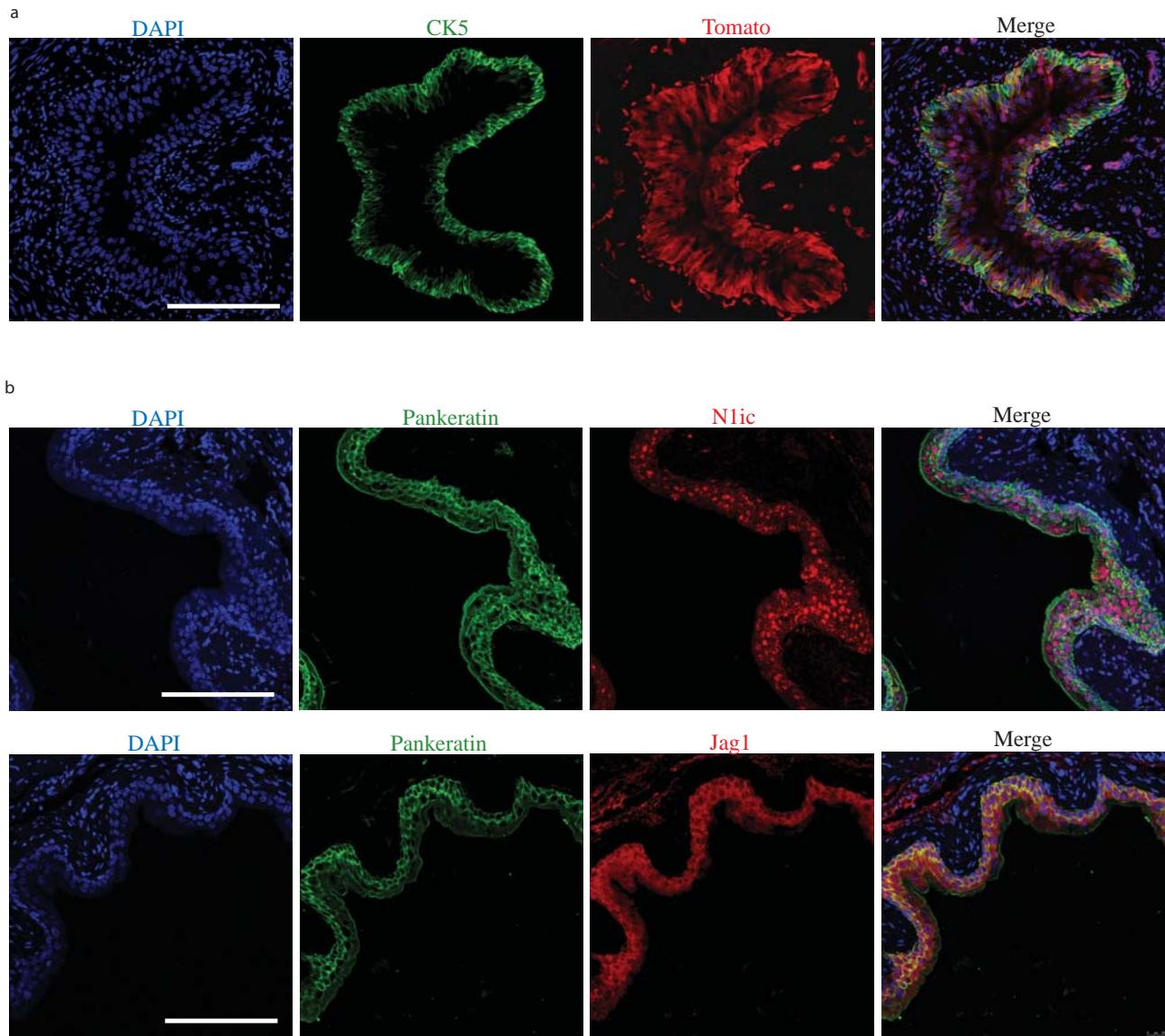
**Supplementary Figure 4. Tumor vs. normal sequence verification of somatic mutations.**  
Chromatograph pairs of tumor (top) and corresponding healthy (bottom) tissue indicating the somatic mutations identified. Arrows indicate the double peak in the tumor sample (only for single nucleotide changes).



**Supplementary Figure 5. Analysis of DUSP1, DUSP2 and DUSP5 expression levels in bladder cancer tissues.** (a) Kaplan-Meier analysis of overall and disease-free survival of bladder cancer patients in regard to expression levels of Notch target genes. According to X-tile algorithm cut-off values for HES1, HEY1 and CDKN1A were adopted at the 55th, 50th and 62nd percentile, respectively, of the patients' expression levels. "n" refers to the number of patients and "events" refers to deaths or cases of disease recurrence. *P* value calculated by log-rank test. (b) P21 and HES1 levels in the four different bladder cancer subtypes (BLCA1-4); *p* value calculated by Mann-Whitney *U* test. (c) Kaplan-Meier analysis of overall survival of the TCGA cohort of bladder cancer patients in regard to expression levels of HES1. (d) DUSP levels related to the presence of Notch pathway mutations and *NOTCH1* copy number loss. Group A: Control cohort, Group B: Bladder cancer patients with low HEY1 expression.



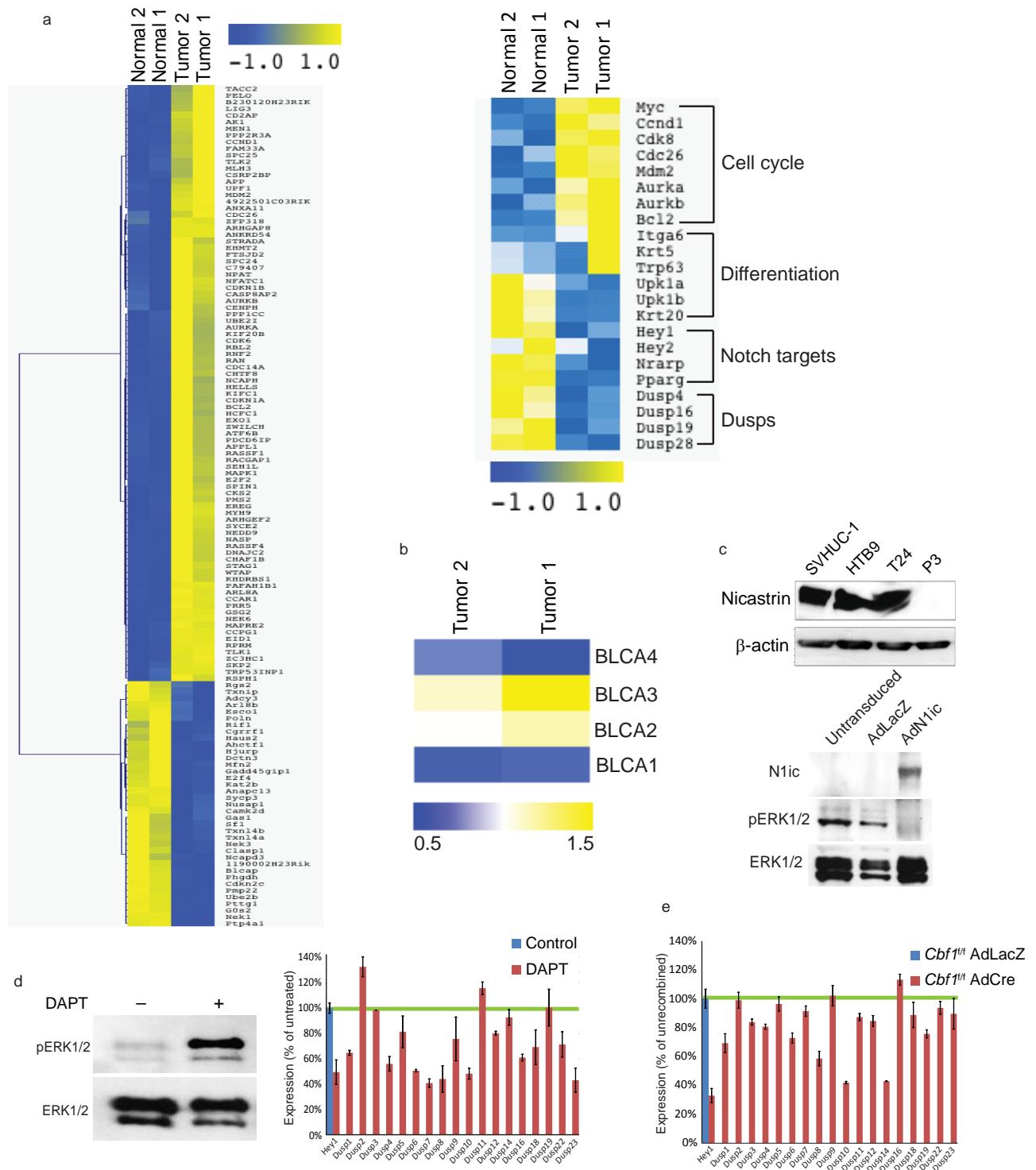
**Supplementary Figure 6. Notch2 suppresses TCC cell growth and ERK1/2 phosphorylation through DUSP overexpression.** (a) MTT assays showing growth suppression of TCC lines transduced with adenoviral vectors expressing the intracellular domain of human NOTCH2 (AdN2ic) or LacZ (AdLacZ). Measurements were taken at 0, 2, 4 and 6 days post-infection. (b) Western blot analysis on protein lysates of the indicated TCC cells. (c) Real time qPCR data from T24 cells. Lysates and RNAs were prepared 72 hours post-infection. Error bars represent the standard error of the mean (SEM). (d) Functional validation of NOTCH2 mutations A2025E and Q2223X. TCC RT4 cells were transfected with wild-type and mutant NOTCH2-expressing plasmids. RNA was harvested two days post-transfection, reverse transcribed and used in qPCR for Notch targets. Values presented correspond to normalized expression in comparison to wild-type NOTCH2 which is set as 100% for each gene.



**Supplementary Figure 7. Notch signaling pathway activation in normal mouse bladder epithelium.**

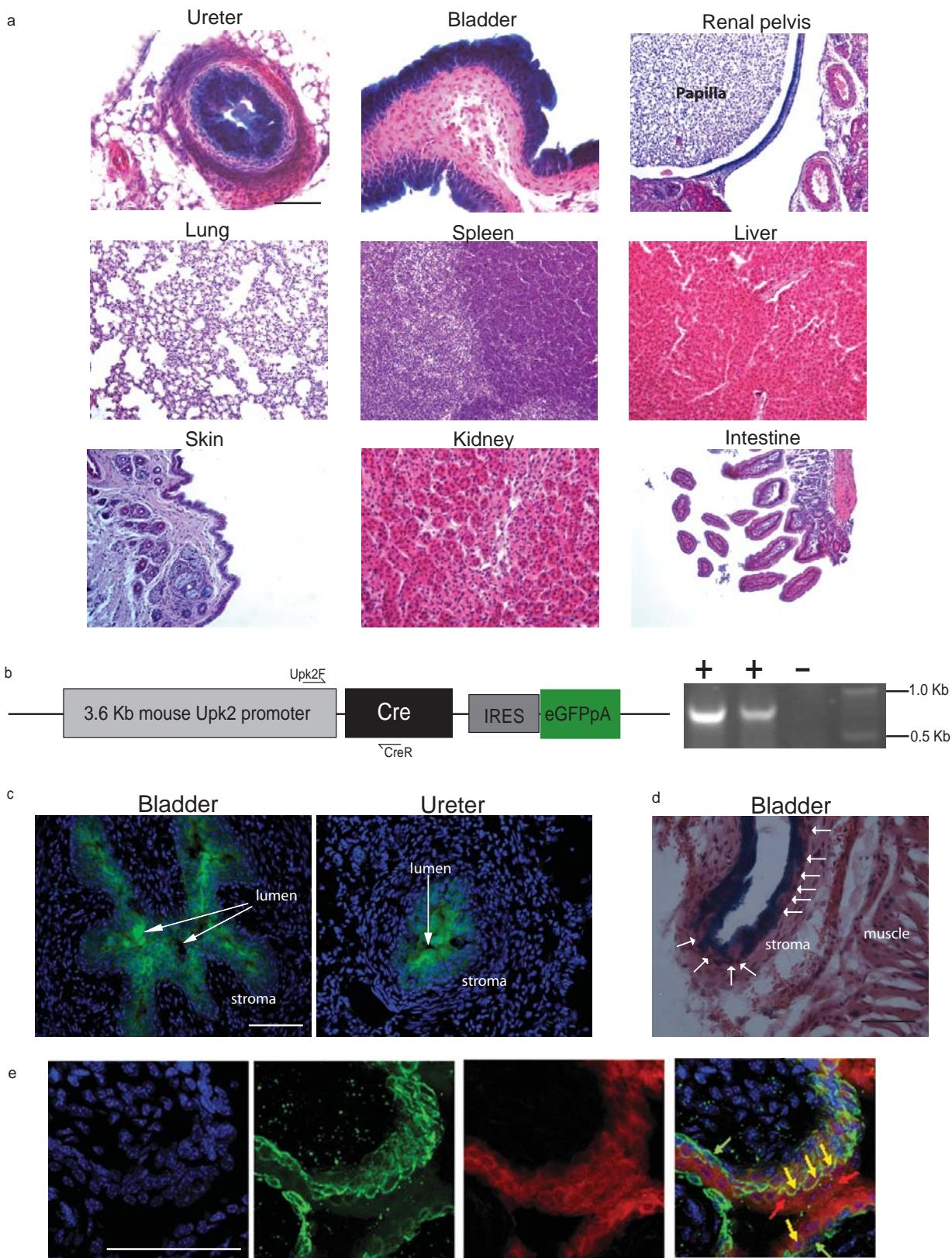
(a) Eight-week old bitransgenic *Notch1-CreERT2;R26-Tm* mice, expressing CreERT2 under the control of the *Notch1* locus<sup>36</sup> (kind donation of Dr. S. Artavanis-Tsakonas) and the fluorescent protein Tomato under the *ROSA26* locus<sup>37</sup>, respectively, were injected with Tamoxifen five consecutive times (1mg per injection) and sacrificed. Anti-Ck5 antibodies that mark the basal and intermediate layers, and DAPI were used as counterstain.

(b) Immunofluorescence on paraffin sections of normal mouse bladder stained with antibodies recognizing exclusively cleaved Notch1 (N1ic) and Jag1. Sections were counterstained with a Pankeratin antibody (CK) and DAPI. Sections were photographed with a Leica TS5 II confocal on an upright microscope (Leica DM6000 CFS). Scale bars: 50μm.



**Supplementary Figure 8. Molecular characterization of mouse urothelial tissue lacking Notch activity.**

(a) Unsupervised hierarchical clustering (Pearson correlation coefficient) of normalized values from expression arrays of normal and cancerous mouse ureters. A gene ontology cell cycle signature (left) and selected genes (right) are presented. Heatmaps and clustering were performed with the TMev software. (b) Heatmaps indicating the correlation of mouse tumors with the BLCA1-4 human bladder subtypes. Correlation for each mouse tumor was calculated based on the overlapping genes of the 2708 gene signature used for human bladder cancer subtyping<sup>16</sup>, and those exhibiting two-fold change in the tumor vs. normal comparison in mice (number of overlapping genes: 1235 for mouse tumor 1, and 1032 for mouse tumor 2). (c) Western blot with anti-Ncstn antibodies on mouse primary ureter tumor cells (P3) and control human TCC lines (top), and with pERK1/2 and total ERK1/2 antibodies on P3 cells untransduced or transduced with an adenovirus carrying the intracellular N1 (N1ic) or LacZ (bottom). (d) Western blot (left) and real time qPCR (right) from wild-type bladder tissue explants cultured *ex vivo* for three days and treated with DAPT (10uM). (e) Real-time qPCR on bladder tissue explants from conditional *Cbf1* knockout mice transduced with adenoviral vectors expressing either LacZ or Cre recombinase.



**Supplementary Figure 9. Recombination pattern of the Cre lines used.** (a) X-gal staining on tissues of tritransgenic mice which express  $\beta$ -galactosidase upon Dox administration in a Cre-dependent manner. Recombination is restricted in urothelial cells of the ureter, bladder and renal pelvis. Ubiquitous response of the teto-Cre transgene is lost presumably due to a genetic background switch from the original FVB to a mixed but primarily C57BL/6 background. (b) Schematic representation of the UpII-Cre-eGFP construct injected in CBA/C57BL/6 mice (left) and genotyping of transgenic mice (right; Upk2F: ctgaggctacagtggccca; CreR: atgttttagctggcccaaatg). (c) Native eGFP fluorescence in the ureter and bladder of UpII-Cre-eGFP mice. IRES: Internal Ribosome Entry Site; pA: polyadenylation signal. (d) X-gal staining on the bladder of bitransgenic UpII-Cre-eGFP;R26LacZ mice. Arrows indicate basal and/or intermediate cells in which recombination did not occur. (e) Immunofluorescence against Ck5 on frozen sections from Up2-Cre;R26-Tm mice<sup>37</sup> showing Ck5-positive cells in which Cre is active and recombination has occurred (yellow arrows) or Ck5-positive cells with no recombination (green arrows) and Ck5-negative cells showing Tomato fluorescence (red arrows). Scale bars: 50 $\mu$ m.

**Supplementary Table 1. Clinical characteristics of the TCC patients**

Patient	Grade (WHO 1973)	Grade (WHO 2004)	Tumor Stage	Age (years)	Gender
TCC1	3	High	T2	67	F
TCC2	3	High	T2	76	M
TCC3	2	Low	Ta	84	F
TCC5	3	High	T3	66	M
TCC6	3	High	T3	79	M
TCC7	2	Low	Ta	68	M
TCC8	1	Low	Ta	62	M
TCC9	3	High	T3	54	M
TCC14	3	High	T2	45	M
TCC15	2	Low	Ta	82	M
TCC16	3	High	T3	74	M
TCC17	3	High	T1	88	M
TCC20	3	High	T4	Unknown	M
TCC23	3	High	T3	88	M
TCC24	3	High	T1	78	M
TCC25	3	High	T3	71	M
TCC27	2	Low	Ta	63	M
TCC29	1	Low	Ta	76	M
TCC30	3	High	T4	72	F
TCC31	3	High	T1	71	M
TCC32	2	High	T1	76	M
TCC33	2	Low	Ta	73	M
TCC34	2	Low	Ta	85	M
TCC35	3	High	T2	Unknown	M
TCC36	1	Low	Ta	58	M
TCC37	3	High	T1	78	M
TCC39	2	Low	T1	62	M
TCC40	2	Low	Ta	58	M
TCC41	3	High	T2	85	M
TCC43	3	High	T2	64	M
TCC45	3	High	T3	69	M
TCC48	3	High	T3	81	M
TCC49	1	Low	T1	52	F
TCC50	3	High	T4	79	M
TCC51	3	High	T4	62	M
TCC53	3	High	T3	80	M
TCC56	1	Low	Ta	50	M
TCC60	2	Low	Ta	78	F
TCC67	3	High	T4	74	M
TCC73	3	High	T3	72	M
TCC80	3	High	T2	74	M
TCC85	2	Low	Ta	85	M
TCC90	2	Low	T1	73	M
TCC91	3	High	T2	75	M
TCC92	3	High	T1	60	M
TCC96	2	Low	T2	75	M
TCC98	3	High	T2	65	M
TCC99	3	High	T3	72	F
TCC114	2	Low	Ta	48	M
TCC115	2	Low	Ta	Unknown	M
TCC116	2	Low	Ta	87	M
TCC117	2	Low	Ta	54	F
TCC118	2	Low	Ta	61	M
TCC119	2	Low	T1	Unknown	M
TCC126	1	Low	Ta	78	M
TCC127	3	High	T2	85	M
TCC128	3	High	T1	Unknown	F
TCC129	2	High	T1	Unknown	M
TCC130	3	High	Ta	50	M
TCC131	2	Low	T1	62	M
TCC132	2	Low	Ta	70	M
TCC141	3	High	T2	75	M
TCC142	3	High	T1	76	M
TCC144	3	High	T2	72	F
TCC145	3	High	T4	75	M
TCC146	3	High	T2	88	M
TCC148	2	Low	Ta	Unknown	M
TCC157	2	Low	Ta	79	M
TCC159	3	High	T1	74	M
TCC161	2	Low	Ta	63	F
TCC162	3	High	Ta	56	M
TCC164	3	High	T2	51	F

**Supplementary Table 2. Distribution of mutations with regard to the tumor grade (WHO 2004) and stage**

Variable	<i>NOTCH 1</i>	<i>NOTCH 2</i>	<i>NOTCH 3</i>	<i>MAML1</i>	<i>NCSTN</i>	Notch pathway	
	No. of patients (%)						
<b>Grade</b>							
Low	29 (40.3)	6 (20.7)	5 (17.2)	0	3 (10.3)	0	13 (44.8)
High	43 (59.7)	8 (18.6)	4 (9.3)	2 (4.6)	6 (14.0)	3 (7.0)	18 (41.9)
<b>Stage</b>							
Superficial (Ta, T1)	40 (55.6)	8 (20.0)	5 (12.5)	0	5 (12.5)	0	17 (42.5)
Muscle-invasive (T2-T4)	32 (44.4)	6 (18.8)	4 (12.5)	2 (6.2)	4 (12.5)	3 (9.4)	14 (43.8)

**Supplementary Table 3. Description of Notch pathway alterations and PolyPHEN-2 predictions**

Patient	Gene	Nucleotide	Protein	PolyPHEN-2 <sup>a</sup>	N1 Copy
TCC3	<i>MAML1</i>	c.2235_2667del	p.F746LfsX5		Loss
TCC6	<i>NOTCH1</i>	c.T745C	p.F249L	D/D	
	<i>NOTCH1</i>	c.3781_3900del	p.V1261_H1300del		Neutral
	<i>NOTCH3</i>	c.G2401ins/del	p.G800LfsX1		
	<i>MAML1</i>	c.1456_1813del	p.P485L_S604del		
	<i>NCSTN</i>	c.C1747T	p.R583X		
TCC9	<i>NOTCH1</i>	c.5646_5886del	p.G1882_G1962del		Neutral
TCC17	<i>NOTCH1</i>	c.G689T	p.G230V	D/D	
	<i>NOTCH1</i>	c.3743_3902del	p.V1249AfsX142		Neutral
TCC20	<i>NOTCH1</i>	c.T3511C	p.C1171R	D/D	
	<i>NOTCH2</i>	c.2366_2752del	p.P881_F1009del		Loss
TCC27	<i>NOTCH1</i>	c.T3511C	p.C1171R	D/D	Loss
TCC29	<i>MAML1</i>	c.916_1203del	p.A306_DfsX60		Loss
TCC30	<i>NCSTN</i>	c.T435del	p.F145LfsX57		Loss
TCC32	<i>NOTCH2</i>	c.C6667T	p.Q2223X		Loss
TCC33	<i>NOTCH1</i>	c.T1769A	p.L590H	B/B	Gain
TCC34	<i>MAML1</i>	c.A2399C	p.N800T	D/D	Loss
TCC37	<i>NOTCH1</i>	c.T4855ins/del	p.Y1619TfsX70		Neutral
TCC39	<i>NOTCH1</i>	c.G6436A	p.R2179Q	D/B	
	<i>NOTCH2</i>	c.4194_4365del	p.Q1497HfsX47		Loss
TCC40	<i>NOTCH2</i>	c.C6667T	p.Q2223X		Loss
TCC45	<i>NOTCH2</i>	c.C6074A	p.A2025E	D/D	Loss
TCC50	<i>NOTCH2</i>	c.C1726T	p.P576S	D/D	Neutral
TCC56	<i>NOTCH2</i>	c.G2731T	p.D914Y	D/D	Neutral
TCC67	<i>NOTCH1</i>	c.G4082A	p.G1361D	D/D	
	<i>NOTCH1</i>	c.C3859T	p.R1287C	D/D	Neutral
	<i>NOTCH1</i>	c.C6365T	p.P2122L	D/D	
	<i>NOTCH1</i>	c.T5645C	p.F1882S	D/D	
	<i>NCSTN</i>	c.A329G	p.K110R	B/B	
TCC80	<i>MAML1</i>	c.A1471G	p.S491G	B/B	
	<i>MAML1</i>	c.T1757C	p.V586A	B/B	Loss
TCC85	<i>NOTCH1</i>	c.C4639T	p.Q1548X		
	<i>NOTCH1</i>	c.G3988C	p.A1330P	B/B	Loss
TCC96	<i>NOTCH2</i>	c.C4369T	p.L1457F	D/D	Gain
TCC114	<i>NOTCH2</i>	c.G4420del	p.D1474IfsX79		Loss
TCC118	<i>NOTCH1</i>	c.A1529G	p.N510S	B/B	Neutral
TCC127	<i>NOTCH3</i>	c.G2648A	p.R883Q	D/D	
	<i>NOTCH3</i>	c.G4852A	p.E1618K	B/D	Gain
TCC129	<i>MAML1</i>	c.C2444T	p.T815I	D/D	Neutral
TCC144	<i>NOTCH1</i>	c.A605T	p.Y202F	B/B	Loss
TCC145	<i>MAML1</i>	c.C1286A	p.T429K	D/B	Neutral
TCC146	<i>NOTCH1</i>	c.G226A	p.V76M	B/B	Loss
TCC157	<i>NOTCH1</i>	c.A311G	p.N104S	B/B	Loss
TCC162	<i>MAML1</i>	c.A2399C	p.N800T	D/D	Loss
TCC164	<i>MAML1</i>	c.C2444T	p.T815I	D/D	Neutral

<sup>a</sup>PolyPHEN-2 is an algorithm that utilizes homology (Humdiv) and structural (Humvar) parameters for the prediction of possible impact exclusively of amino acid replacements on protein structure and function.

**Supplementary Table 4. List of reported SNPs identified in human TCC patients**

<b>Gene</b>	<b>SNP</b>	<b>Type</b>	<b>rs</b>	<b>Genomic</b>	<b>Transcript</b>	<b>Protein</b>	<b>rs MAF<sup>a</sup></b>
<i>NOTCH1</i>	N104N	synonymous	4489420	NC_000009.11:g.139418260A>G; NG_007458.1:g.26979T>C	NM_017617.3:c.312T>C	NP_060087.3:p.Asn104Asn	2.93E-01
	N104S	nonsynonymous	199654211	NC_000009.11:g.139418261T>C; NG_007458.1:g.26978A>G	NM_017617.3:c.311A>G	NP_060087.3:p.Asn104Ser	NA
	P284P	synonymous	2229975	NC_000009.11:g.139413908C>T; NG_007458.1:g.31331G>A; NG_007458.1:g.31331G>C	NM_017617.3:c.852G>A; NM_017617.3:c.852G>C	NP_060087.3:p.Pro284Pro	1.01E-01
	N755N	synonymous	2229971	NC_000009.11:g.139407932A>G; NG_007458.1:g.37307T>C	NM_017617.3:c.2265T>C	NP_060087.3:p.Asn755Asn	4.44E-01
	S1098S	synonymous	61751546	NC_000009.11:g.139402715G>A; NG_007458.1:g.42524C>T;	NM_017617.3:c.3294C>T	NP_060087.3:p.Ser1098Ser	3.21E-03
	V1260V	synonymous	201354526	NC_000009.11:g.139401289C>G; NG_007458.1:g.43950G>C;	NM_017617.3:c.3780G>C	NP_060087.3:p.Val1260Val	4.59E-03
	R1279H	nonsynonymous	61751543	NC_000009.11:g.139401233C>T; NG_007458.1:g.44006G>A;	NM_017617.3:c.3836G>A	NP_060087.3:p.Arg1279His	1.01E-02
	P1377S	nonsynonymous	61751542	NC_000009.11:g.139400219G>A; NG_007458.1:g.45020C>T;	NM_017617.3:c.4129C>T	NP_060087.3:p.Pro1377Ser	8.72E-03
	D1698D	synonymous	10521	NC_000009.11:g.139397707G>A; NG_007458.1:g.47532C>T	NM_017617.3:c.5094C>T	NP_060087.3:p.Asp1698Asp	4.17E-01
	T1996T	synonymous	186453356	NC_000009.11:g.139393658C>T; NG_007458.1:g.51581G>A	NM_017617.3:c.5988G>A	NP_060087.3:p.Thr1996Thr	1.84E-03
<i>NOTCH2</i>	G1893G	synonymous	2229973	NC_000009.11:g.139395259G>A; NG_007458.1:g.49980C>T	NM_017617.3:c.5679C>T	NP_060087.3:p.Gly1893Gly	8.26E-03
	D2185D	synonymous	rs2229974	NC_000009.11:g.139391636G>A; NG_007458.1:g.53603C>T	NM_017617.3:c.6555C>T	NP_060087.3:p.Asp2185Asp	3.05E-01
	V2229Met	nonsynonymous	202096917	NC_000009.11:g.139391506C>T; NG_007458.1:g.53733G>A	NM_017617.3:c.6685G>A	NP_060087.3:p.Val2229Met	NA
	P394P	synonymous	312262794	NC_000001.10:g.120510784G>A; NG_008163.1:g.106493C>T	NM_024408.3:c.1180C>T	NP_001186930.1:p.Pro394Ser; NP_077719.2:p.Pro394Ser	NA
	C1078C	synonymous	7543643	NC_000001.10:g.120480583G>A; NG_008163.1:g.136694C>T	NM_001200001.1:c.3234C>T; NM_024408.3:c.3234C>T	NP_001186930.1:p.Cys107Cys; NP_077719.2:p.Cys107Cys	6.06E-02
	D1327G	nonsynonymous	61752484	NC_000001.10:g.120469147T>C; NG_008163.1:g.148130A>G	NM_024408.3:c.3980A>G	NP_077719.2:p.Asp1327Gly	2.75E-03
	L1413H	nonsynonymous	41313282	NC_000001.10:g.120468201A>T; NG_008163.1:g.149076T>A	NM_024408.3:c.4238T>A	NP_077719.2:p.Leu1413His	3.67E-03
	p.Gly801delins GlyLeufs	frameshift	35308509	NC_000019.9:g.15295724_15295725insT; NG_009819.1:g.21068_21069insA	NM_000435.2:c.2402_2403insA	NP_000426.2:p.Gly801delins GlyLeufs	NA
	V1183M	nonsynonymous	10408676	NC_000019.9:g.15290007C>T; NG_009819.1:g.26786G>A	NM_000435.2:c.3547G>A	NP_000426.2:p.Val1183Met	6.66E-02
	A2146A	synonymous	1044008	NC_000019.9:g.15272001C>T; NG_009819.1:g.44792G>A	NM_000435.2:c.6438G>A	NP_000426.2:p.Ala2146=	2.30E-02
<i>MAML1</i>	S529S	synonymous	3797776	NC_000005.9:g.179193598C>T; NT_023133.13:g.24004871C>T	NM_014757.4:c.1580C>T	NP_055572.1:p.Ser529=	NA
	S583N	nonsynonymous	41285557	NC_000005.9:g.179195867G>A; NT_023133.13:g.24007140G>A	NM_014757.4:c.1748G>A	NP_055572.1:p.Ser583Asn	1.61E-02
	P824L	nonsynonymous	61753466	NC_000005.9:g.179201298C>T	NM_014757.4:c.2471C>T	NP_055572.1:p.Pro824Leu	1.40E-03
	S1007N	nonsynonymous	6895902	NC_000005.9:g.179201847G>A	NM_014757.4:c.3020G>A	NP_055572.1:p.Ser1007Asn	2.53E-01
<i>NCSTN</i>	E79E	synonymous	34445546	NC_000001.10:g.160318835G>A; NG_027935.1:g.10773G>A	NM_015331.2:c.237G>A	NP_056146.1:u.G1u79G1u	7.81E-03
	L212L	synonymous	12239747	NC_000001.10:g.160321065A>G; NG_027935.1:g.13003A>G	NM_015331.2:c.636A>G	NP_056146.1:p.Leu212Leu	8.95E-02

<sup>a</sup> Frequency in the population

**Supplementary Table 5. SNPs found in individual TCC patients**

<i>NOTCH1</i>	N104N, N104K, N104S	P284P	N755N	S1098S	V1260V	R1279H	P1377S	D1698D	G1893G	T1996T	V2229M	D2185D
	TCC148	TCC118	TCC9	TCC115	TCC117	TCC128	TCC16	TCC40	TCC16	TCC7	TCC16	TCC40
	TCC162	TCC36	TCC30			TCC149 (Homo)		TCC9				TCC39 (Homo)
	TCC157 (N104S)	TCC45	TCC40					TCC30				TCC9 (Homo)
	TCC3	TCC16	TCC20					TCC127				TCC17 (Homo)
	TCC5	TCC127	TCC115					TCC126				TCC67 (Homo)
	TCC118 (Homo) <sup>a</sup>	TCC85						TCC161 (Homo)				TCC126
	TCC30	TCC7						TCC7				TCC129
	TCC119	TCC24						TCC162				TCC85 (Homo)
	TCC96 (Homo)	TCC162						TCC80				TCC33 (Homo)
	TCC41							TCC3				TCC115 (Homo)
	TCC126							TCC1				TCC127 (Homo)
	TCC24											TCC131 (Homo)
	TCC127 (N104K)											TCC53
	TCC30											TCC162
												TCC141
												TCC49
												TCC8
<i>NOTCH2</i>	<b>P394P</b>	<b>C1078C</b>	<b>D1327G</b>	<b>L1413H</b>								
	TCC16	TCC27	TCC45	TCC39								
	TCC20	TCC22	TCC17	TCC45								
		TCC20	TCC128									
<i>NOTCH3</i>	<b>p.Gly801delinsGlyLeufs</b>	<b>V1183M</b>	<b>A2146A</b>									
	TCC30	TCC33	TCC114									
		TCC127	TCC56									
			TCC129									
<i>MAMLI</i>	<b>S529S</b>	<b>S583N</b>	<b>P824L</b>	<b>S1007N</b>								
	TCC56	TCC131	TCC114	TCC114								
	TCC129	TCC67	TCC9	TCC127								
	TCC114			TCC56								
	TCC37			TCC129								
	TCC142			TCC9								
				TCC32								
				TCC36								
				TCC96								
				TCC161								
<i>NCSTN</i>	<b>E79E</b>	<b>L212L</b>										
	TCC127	TCC115										

<sup>a</sup> Homozygous

**Supplementary Table 6. Synonymous mutations found in human TCC patients**

<b><i>NOTCH1</i></b>	<b>Protein</b>	<b>Transcript</b>	<b>Sample</b>
	H75H	NM_017617.3:c.225C>T	TCC146
	I258I	NM_017617.3:c.774C>T	TCC53
	G519G	NM_017617.3:c.1557C>T	TCC17
	D932D	NM_017617.3:c.2799C>T	TCC9
	K1228K	NM_017617.3:c.3714G>T	TCC67
	T2132T	NM_017617.3:c.6399G>A	TCC67
	Q2395Q	NM_017617.3:c.7188G>A	TCC16
<b><i>NOTCH2</i></b>			
	G1337G	NM_024408.3:c.4311C>T	TCC27
<b><i>NOTCH3</i></b>			
	T993T	NM_000435.2:c.3055G>A	TCC36
	P2051P	NM_000435.2:c.6229T>A	TCC16
<b><i>MAML1</i></b>			
	S474S	NM_014757.4:c.1421C>A	TCC67

**Supplementary Table 7. Copy Number Variations in the NOTCH1 locus**

Patient	$\Delta C_T$ value Average <sup>a</sup>	$\Delta\Delta C_T$ <sup>b</sup>	$2^{-\Delta\Delta C_T}$	NOTCH1 calculated copy number	NOTCH1 Copy Number Variation
TCC1	-0.60	-0.39	1.31	2.62	GAIN
TCC2	-0.08	0.08	0.95	1.89	NEUTRAL
TCC3	0.76	0.97	0.51	1.02	LOSS
TCC5	-0.08	0.13	0.91	1.82	NEUTRAL
TCC6	-0.51	-0.31	1.24	2.48	NEUTRAL
TCC7	0.18	0.38	0.77	1.54	NEUTRAL
TCC8	-0.75	-0.53	1.44	2.89	GAIN
TCC9	-0.11	0.05	0.97	1.93	NEUTRAL
TCC14	0.08	0.29	0.82	1.64	NEUTRAL
TCC15	0.74	0.95	0.52	1.03	LOSS
TCC16	1.25	1.32	0.40	0.80	LOSS
TCC17	0.14	0.21	0.86	1.72	NEUTRAL
TCC20	0.50	0.70	0.62	1.23	LOSS
TCC24	1.36	1.57	0.34	0.67	LOSS
TCC25	0.68	0.89	0.54	1.08	LOSS
TCC27	0.30	0.50	0.71	1.42	LOSS
TCC29	1.39	1.60	0.33	0.66	LOSS
TCC30	2.13	2.34	0.20	0.40	LOSS
TCC31	-0.21	0.00	1.00	2.00	NEUTRAL
TCC32	0.44	0.65	0.64	1.27	LOSS
TCC33	-0.57	-0.36	1.29	2.58	GAIN
TCC34	0.46	0.66	0.63	1.27	LOSS
TCC35	-0.87	-0.66	1.58	3.16	GAIN
TCC36	1.03	1.10	0.47	0.93	LOSS
TCC37	-0.15	0.01	0.99	1.98	NEUTRAL
TCC39	2.20	2.35	0.20	0.39	LOSS
TCC40	0.95	1.16	0.45	0.89	LOSS
TCC41	-0.12	0.09	0.94	1.88	NEUTRAL
TCC43	-0.57	-0.36	1.28	2.56	GAIN
TCC45	0.79	1.00	0.50	1.00	LOSS
TCC48	0.51	0.72	0.61	1.21	LOSS
TCC49	-0.28	-0.12	1.09	2.17	NEUTRAL
TCC50	0.12	0.27	0.83	1.65	NEUTRAL
TCC51	-0.18	-0.10	1.07	2.14	NEUTRAL
TCC53	-0.55	-0.47	1.39	2.78	GAIN
TCC60	-0.41	-0.19	1.14	2.29	NEUTRAL
TCC67	0.11	0.18	0.88	1.76	NEUTRAL
TCC73	-0.21	0.00	1.00	2.00	NEUTRAL
TCC80	0.34	0.49	0.71	1.42	LOSS
TCC85	0.24	0.45	0.73	1.47	LOSS
TCC90	1.59	1.80	0.29	0.57	LOSS
TCC91	3.00	3.19	0.11	0.22	LOSS
TCC92	0.40	0.47	0.72	1.44	LOSS
TCC96	-0.90	-0.86	1.81	3.62	GAIN
TCC99	0.65	0.86	0.55	1.10	LOSS
TCC114	1.30	1.54	0.35	0.69	LOSS
TCC119	0.60	0.82	0.56	1.13	LOSS
TCC127	-0.79	-0.63	1.54	3.08	GAIN
TCC130	0.09	0.30	0.81	1.62	NEUTRAL
TCC131	-0.09	0.12	0.92	1.85	NEUTRAL
TCC132	-0.11	0.11	0.93	1.85	NEUTRAL
TCC141	-0.38	-0.16	1.12	2.24	NEUTRAL
TCC142	-0.20	0.00	1.00	2.00	NEUTRAL
TCC144	1.28	1.48	0.36	0.72	LOSS
TCC145	0.21	0.41	0.75	1.51	NEUTRAL
TCC146	0.88	0.95	0.52	1.04	LOSS
TCC148	1.33	1.55	0.34	0.69	LOSS
TCC157	1.31	1.52	0.35	0.70	LOSS
TCC159	-0.25	-0.04	1.03	2.06	NEUTRAL
TCC161	1.35	1.42	0.37	0.75	LOSS
TCC162	0.92	1.13	0.46	0.91	LOSS
TCC164	-0.21	-0.05	1.04	2.07	NEUTRAL

<sup>a</sup> Average (Avg.)  $\Delta C_T$  value of two replicates per sample

<sup>b</sup>  $\Delta\Delta C_T = \text{Avg. } \Delta C_T^{\text{sample}} - \text{Avg. } \Delta C_T^{\text{calibrator}}$

**Supplementary Table 8. Mutations in *FGFR3*, *HRAS*, *KRAS* and *PIK3CA* genes**

PATIENT	<i>FGFR3</i>	<i>HRAS</i>	<i>KRAS</i>	<i>PIK3CA</i>
TCC1		G13V		P539R
TCC2	S249C			
TCC3	S249C/Y375C			
TCC5				
TCC6				
TCC7	Y375C			
TCC8	R248C			
TCC9				
TCC14				
TCC15		G12A		
TCC16				
TCC17				
TCC20				
TCC23	S249C			
TCC24	S249C		E545K	
TCC25		Q61H		
TCC27				
TCC29	R248C			
TCC30	G382R	G12S		
TCC31	R248C		E545K	
TCC32				
TCC33	S249C			
TCC34	S249C			
TCC35		Q61H		
TCC36	R248C/G382R			
TCC37				
TCC39				
TCC40				
TCC41				
TCC43	S249C			
TCC45				
TCC48		Q61R		
TCC49	Y375C			
TCC50				
TCC51				
TCC53			E542K	
TCC56	Y375C		E545Q	
TCC60			E542K	
TCC67	S373C			
TCC73				
TCC80	S249C			
TCC85	R248C			
TCC90	R248C			
TCC91	S249C			
TCC92	R248C			
TCC96	S249C			
TCC98				
TCC99				
TCC114				
TCC115			H1047Y	
TCC116		Q61R		
TCC117	S249C			
TCC118	Y375C			
TCC119		G13V		
TCC126	S249C		E545K	
TCC127				
TCC128				
TCC129				
TCC130		Q61L	P539R	
TCC131	R248C		E545K	
TCC132		Q61E		
TCC141	S249C			
TCC142				
TCC145				
TCC146			E545K	
TCC148			H1047R	
TCC149				
TCC157		Q61R	H1047L	
TCC159				
TCC161	Y375C			
TCC162				
TCC164	R248C			

**Supplementary Table 9. Mutation profile and grade of the TCC cell lines used**

Cell line	Notch pathway	<i>FGFR3</i>	<i>RAS</i>	<i>PIK3CA</i>	<i>p53</i>	<i>RB</i>	Grade
T24			HRAS <sup>G12V</sup>		Null		III
RT112					Het		I
RT4	N3 <sup>P844S</sup> ; N4 <sup>C733S</sup>	Overexpression					I
HTB9					Null	Null	II
TCCSUP				E545K	Null	Null	IV
SVHUC-1 <sup>a</sup>							

<sup>a</sup> SVHUC-1 originates from primary urothelial cells immortalized with the SV40 large T antigen

Only known mutations are shown.

**Supplementary Table 10. Oligonucleotides used for cDNA amplification.**

<i>NOTCH1</i>	<i>NOTCH2</i>	<i>NOTCH3</i>	<i>MAML1</i>	<i>NCSTN</i>
A1F ggcgtgtcgcccagc	agcgttagcgccaggggctga	aggctggccggacgcg	agccccggccatggtgc	aggccgctaacaaggagc
A1R ccgacctcggtggcagg	tctgccacagggtgcctcc	caggaagacaggcacagtgc	tcaggctcagaggaaatgact	tgtatttataaggcttagcat
A2F aggatgtcaacgagtgtggc	accagtgcgcgtgcctcagg	ctgtgcacccctcaccatgcgg	agccttcaacttgaatggaggc	agcatcaacccagaatcgtc
A2R aggggcagggtcgagatgg	acaaggattgttgcattggcat	gccctgcgttgcacgcacgtcc	tctgtgcgcgtgtggacatct	gaccttaggtcctgcctgagg
A3F tcaacgcgcgtgcacgc	catctggatgtgcacgc	tgccacgcggatgttatctg	agcaggccacccggcaccag	atgggttcgtattaaagcca
A3R agcagaggtaggcgttgtcg	cagttgtcaatgttcctc	ccctaccacacaggAAC	cagaggaccctgtgaactgtc	tcagtatgacacagctcctgg
A4F aggctacacggccaccact	atcgatccccgaatggcta	tgtaacgtggagatcaatgag	acaggagaagcaacagttc	
A4R caacgtcgtaatcacacgtg	ttgtccacttcacagttgtg	caggtggtcagataccatg	tgggagccatgggcctgttg	
A5F gtggctacgtgtgcacctgc	gtgaacgaatgcctgagcaa	tccaggacgtcgtgtgaact	actgcagcctcagttccac	
A5R atcgctggcagtagctgc	cactccatccatacaggAAC	gcaagcttcgcgttcacag	agtcffffcagggcatgggct	
A6F ctgcttcaacgggtggcacc	atggactgtgaggaggacat	agcctgtcaaaacgggggtc		
A6R ggcaggagcactgttaggtg	tgcgtgtgcgcattgtatg	gtctgacagcgaggacctgaga		
A7F ggtgaactgctctgaggag	atgtccatctggatgggctgg	gcctacacaatggcacctgcg		
A7R aggatgtggcacaagagc	actggcacagcgtactcg	tcggcgcagtacttctcg		
A8F tgctacaaccaggggacctg	agagcagctgtggacaagtga	tgcaagctcgccgcctgcct		
A8R gtgcgccacgttgaagacc	tcaaacaggcactcgaccgt	cgccactagcgtggcagcag		
A9F agcagctgcacacagctcc	atggcgtctgtgtggcct	cctgagaatgtactgttc		
A9R aacttctggtctccagg	agctgctgctgcattcg	cagccaggtgcaaaagcgtc		
A10F gaagaacgcctcagacggtg	attgacaaccgcgtgtgt	catcaagcttagcatcatctcc		
A10R ggtccctccagcatgcctcc	ttcatcactcaaattgtggct	cgactgtgcgccttggggc		
A11F tgtcttccagatctgtatcc	aggacaccatcgctggct	acgtagcccaggagagactg		
A11R cacgtctgacaggtagcc	acagatgacaggtagagagc	gggctggcccagtgtca		
A12F tggacagctccggcatgctc	atgaatacataatgtgacccaa	agtcccaggacatggcagga		
A12R gctgcaggcttgcgtgtgc	aggatgggtgcctcagctgg	tcagggccaacacttgcctt		
A13F tctggccacagggtgcctcc	agttgtctatcccaccacc	agcccagccactgccactggg		
A13R tggcatccacagagcgcaca	gagaatggtctgagttacc	tacttggtacatacctgggt		
A14F	cagattccagaaaatggccc			
A14R	tcacgcataaacctgtcatgt			