Supplemental Information

Hedgehog signaling restrains bladder cancer progression by eliciting stromal production of urothelial differentiation factors

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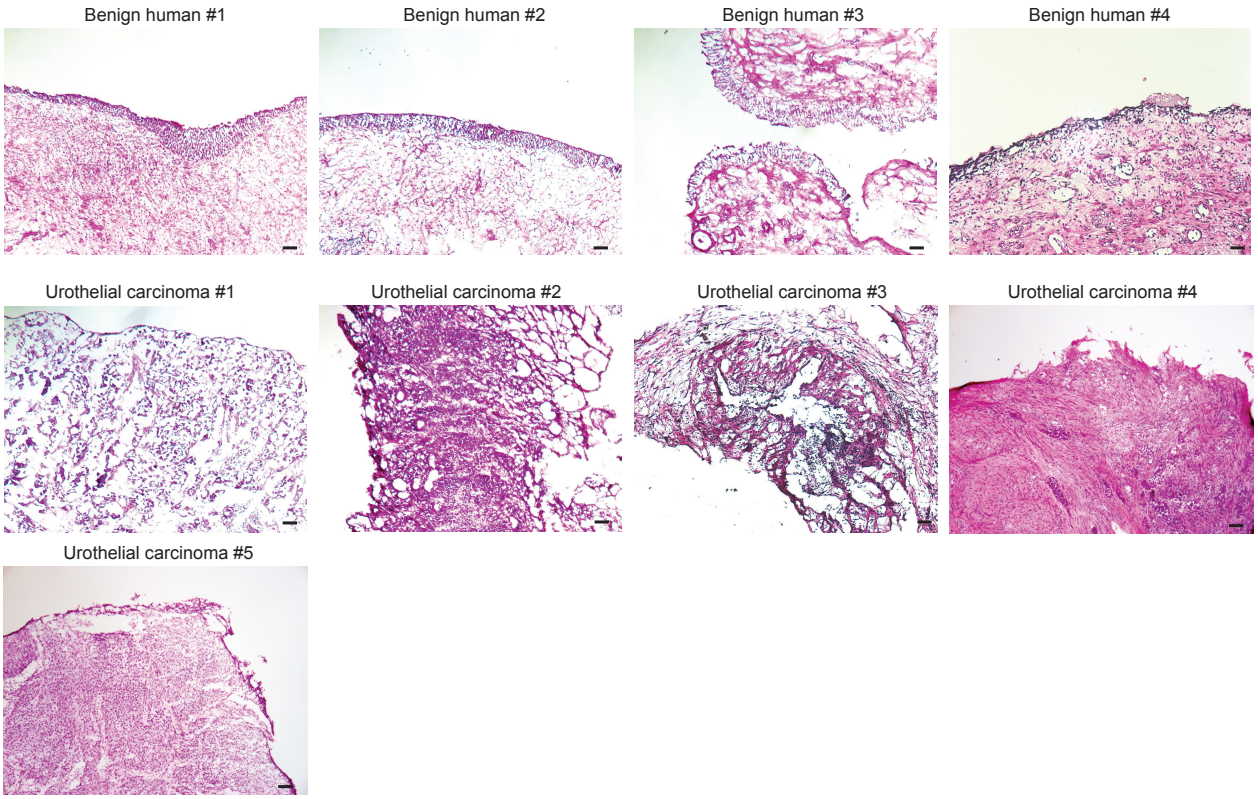
A


Benign human #1
Benign human #2
Benign human #3
Benign human #4
Urothelial carcinoma #1
Urothelial carcinoma #2
Urothelial carcinoma #3
Urothelial carcinoma #4
Urothelial carcinoma #5

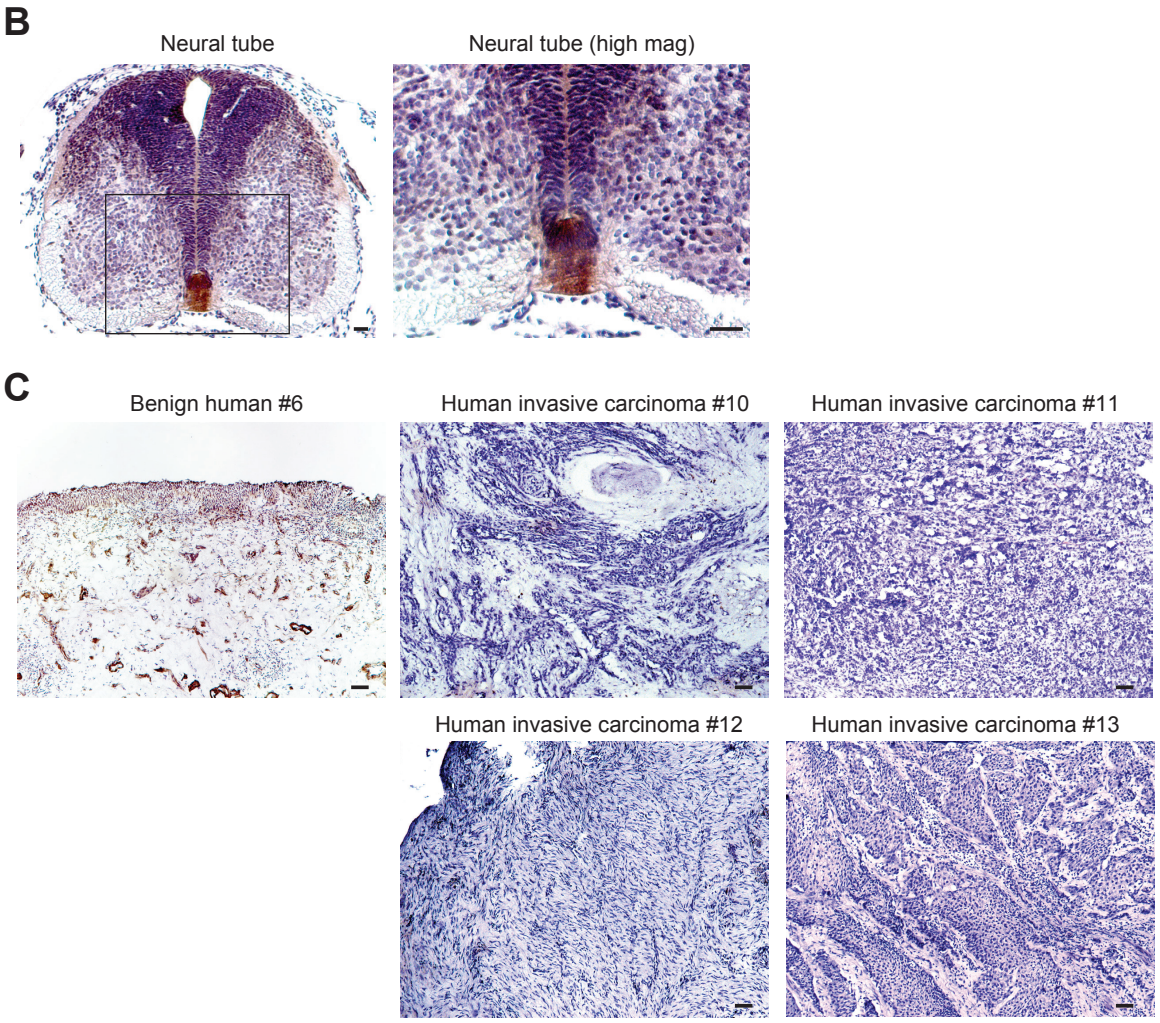

B
Neural tube
Neural tube (high mag)
C
Benign human #6
Human invasive carcinoma #10
Human invasive carcinoma #11
Human invasive carcinoma #12
Human invasive carcinoma #13
D
Relative Gene Expression (GLI1/HPRT1)
0.20
0.15
0.10
0.05
0.00
1 2 3 4 1 2 3 4 5
Benign
Invasive Carcinoma
Relative Gene Expression (PTCH1/HPRT1)
0.4
0.3
0.2
0.1
0.0
1 2 3 4 1 2 3 4 5
Benign
Invasive Carcinoma

Figure S1, related to Figure 1. Absence of Shh expression and reduced Hh pathway activity in human invasive bladder carcinoma

(A) Bladder specimens used for gene expression analyses were subjected to histological analysis. Top: Hematoxylin and Eosin (H&E) stained sections from four benign human bladder samples used for gene expression analyses. Bottom: H&E stained sections from five human invasive urothelial carcinoma samples used for gene expression analyses.

(B) The anti-Shh antibody used for immunohistochemistry was validated by its specific labeling of the floor plate in a section of an E10.5 mouse embryo.

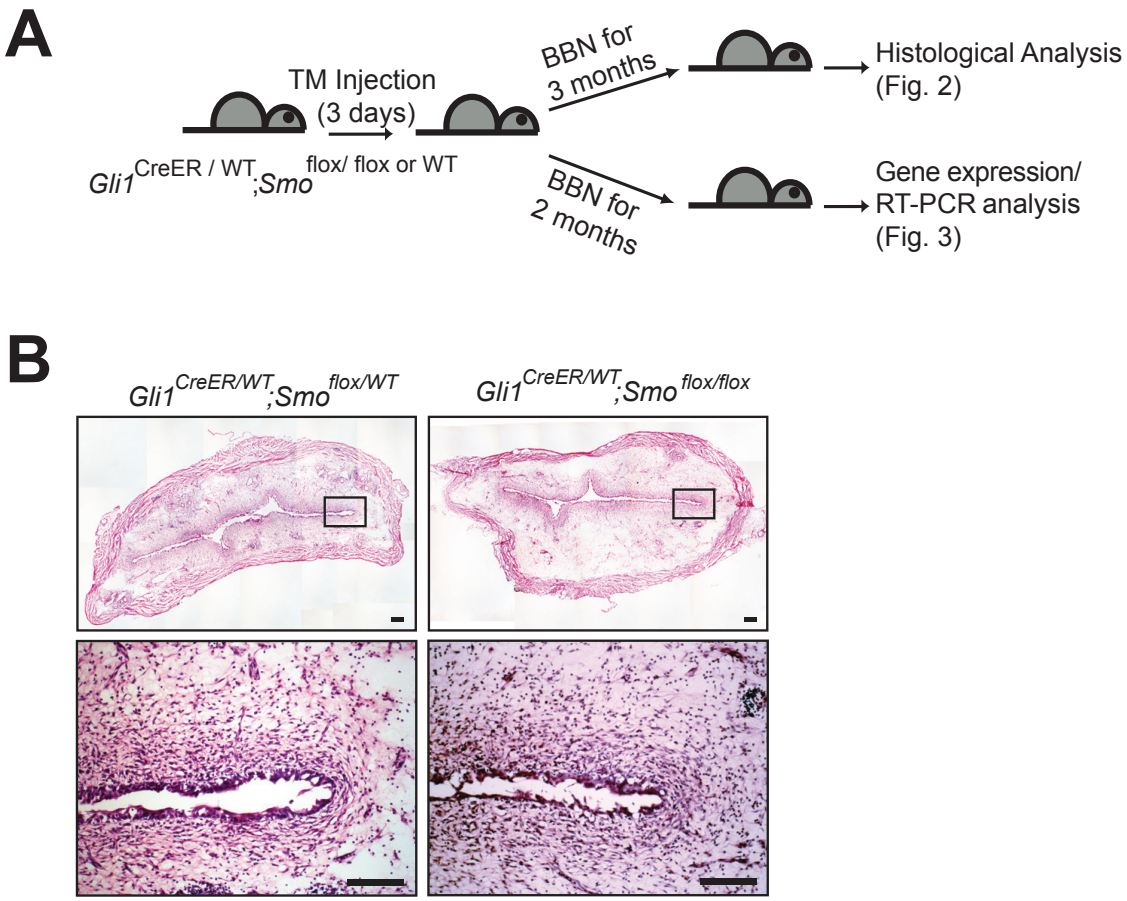
(C) One benign human bladder urothelium, and four patient-derived muscle-invasive urothelial carcinoma samples were immunostained for Shh and counterstained with Hematoxylin.

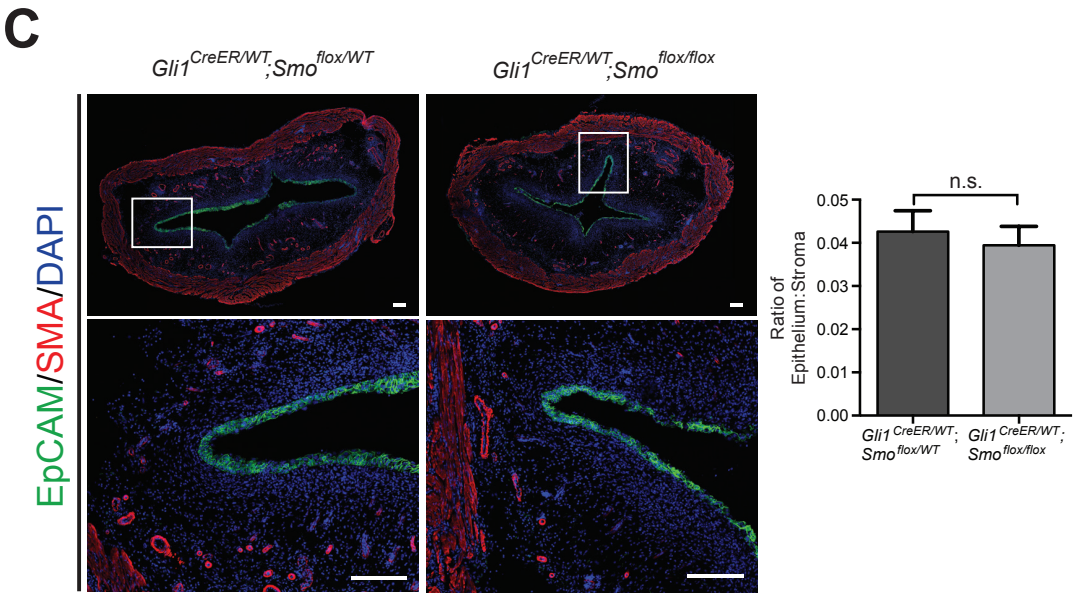
(D) Realtime quantitative PCR measurement of *GLI1* and *PTCH1* expression in four benign human urothelial samples and five invasive human urothelial carcinoma samples (same samples as examined in Fig. 1A). Data are presented as mean ± s.e.m. n = 3 technical replicates for each independent sample. Scale bars represent 50μm. Sample numbers correspond to those in Table S1.

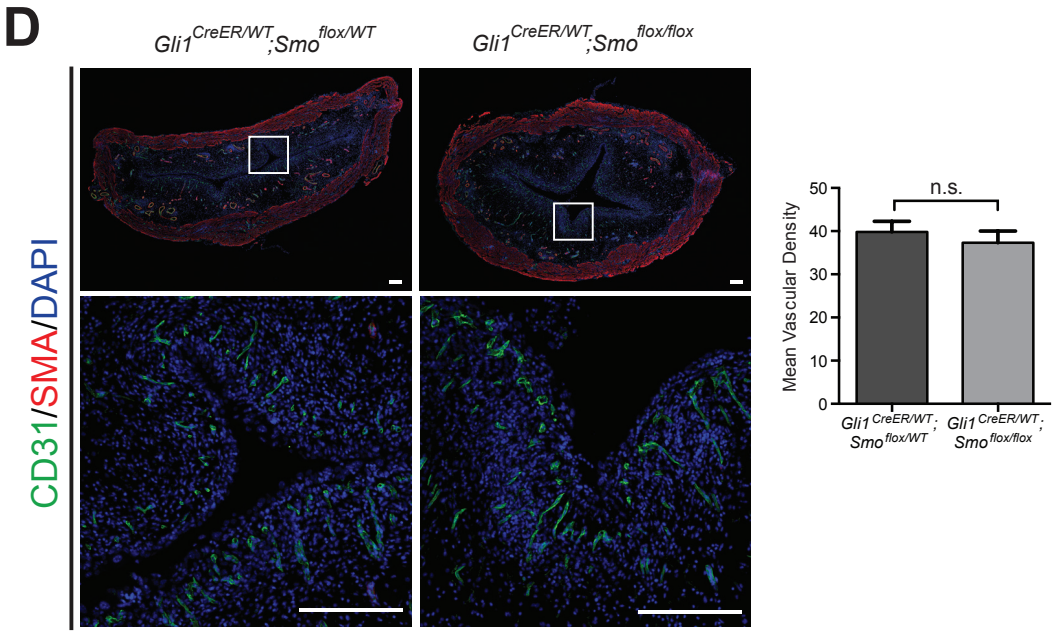
Table S1, related to Figures 1 and S1. Patient sources of benign and invasive carcinoma samples
Benign bladder tissues and invasive carcinoma samples were from radical cystectomy or transurethral resection (TUR) patients with disease and treatment histories as shown.

	#	Age	Sex	Tissue source	History of BCG	History of neo-adjuvant chemotherapy	Stage
Benign samples**	1	75	F	Cystectomy	N/A	N/A	(pT1)*
	2	76	M	Cystectomy	N/A	N/A	(pT3a)*
	3	68	M	Cystectomy	N/A	Y	(pT0)*
	4	65	M	Cystectomy	N	Y	(pT1)*
	5	N/A	N/A	Cystectomy	N/A	N/A	N/A
	6	64	M	TUR	N	N	(pTa)*
Invasive carcinoma samples	1	69	M	TUR	N	N	pT2
	2	63	M	TUR	N	N	pT2
	3	75	M	Cystectomy	N/A	N/A	pT3b
	4,12	67	M	Cystectomy	N	N	pT3a
	5,13	51	M	Cystectomy	N	N	pT2
	6	54	M	Cystectomy	N/A	N/A	pT3b
	7	70	M	TUR	N	N	pT2
	8	81	M	Cystectomy	N/A	N/A	pT1
	9	55	M	Cystectomy	N/A	N/A	pT3b
	10	75	M	Cystectomy	N/A	N/A	pT3b
	11	82	M	Cystectomy	N/A	N/A	pT3b

* Represents the stage of the entire surgical specimen
** Samples were obtained from benign regions of the surgical specimen


A
TM Injection
(3 days)
Gli1CreER / WT;Smo flox/ flox or WT
BBN for
3 months
Histological Analysis
(Fig. 2)
BBN for
2 months
Gene expression/
RT-PCR analysis
(Fig. 3)
B
Gli1CreER/WT;Smo flox/WT
Gli1CreER/WT;Smo flox/flox


C
Gli1CreER/WT;Smoflox/WT
Gli1CreER/WT;Smoflox/flox
EpCAM/SMA/DAPI
n.s.
Ratio of Epithelium:Stroma
0.05
0.04
0.03
0.02
0.01
0.00
Gli1CreER/WT; Smoflox/WT
Gli1CreER/WT; Smoflox/flox


D
Gli1CreER/WT;Smoflox/WT
Gli1CreER/WT;Smoflox/flox
CD31/SMA/DAPI
n.s.
Mean Vascular Density
50
40
30
20
10
0
Gli1CreER/WT; Smoflox/WT
Gli1CreER/WT; Smoflox/flox

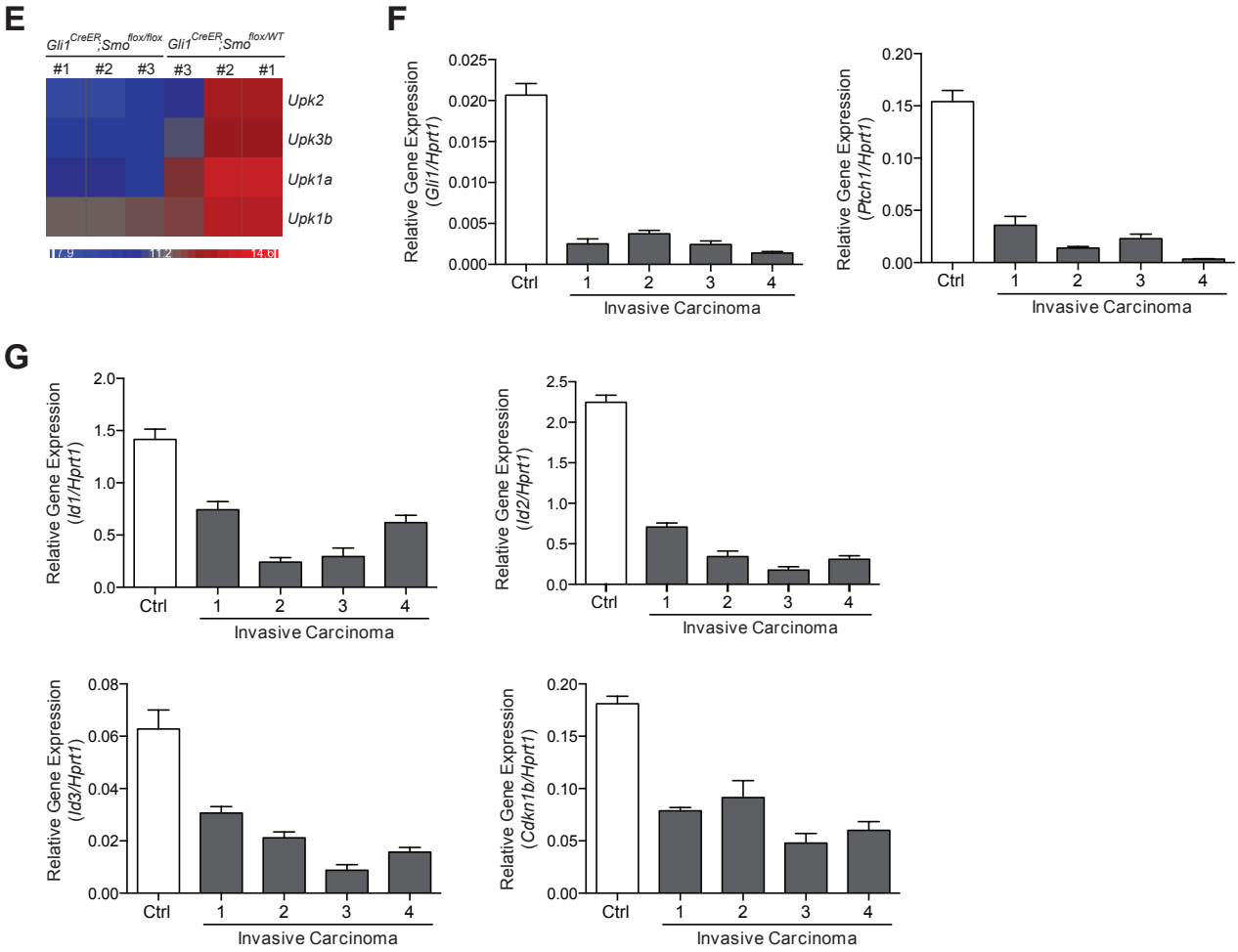

E
Gli1CreER;Smoflox/flox
Gli1CreER;Smoflox/WT
#1 #2 #3 #3 #2 #1
Upk2
Upk3b
Upk1a
Upk1b
-7.9
11.2
14.6
F
Relative Gene Expression (Gli1/Hprt1)
Relative Gene Expression (Ptch1/Hprt1)
Ctrl 1 2 3 4
Invasive Carcinoma
G
Relative Gene Expression (Id1/Hprt1)
Relative Gene Expression (Id2/Hprt1)
Relative Gene Expression (Id3/Hprt1)
Relative Gene Expression (Cdkn1b/Hprt1)
Ctrl 1 2 3 4
Invasive Carcinoma

Figure S2, related to Figure 3. Decreased cellular differentiation in Smo-ablated bladders and decreased expression of Hh and Bmp pathway target genes in urothelial invasive carcinoma

(A) Schematic diagram illustrating experimental strategies for Figs. 2 and 3. *Gli1*^{CreER}*;Smo*$^{flox/flox}$ and *Gli1*^{CreER}*;Smo*$^{flox/WT}$ mice injected with TM on three consecutive days were treated with BBN for 2 or 3 months, and the bladders harvested for microarray gene expression analysis (Fig. 3) or histological analysis by H&E staining (Fig. 2) respectively.

(B-D) Bladders from *Gli1*^{CreER}*;Smo*$^{flox/flox}$ and *Gli1*^{CreER}*;Smo*$^{flox/WT}$ mice injected with TM on three consecutive days and treated with BBN for 2 months were analyzed. Lower panels show magnified views of the regions highlighted by boxes in the upper panels. Scale bars represent 200 μm.

(B) H&E staining of bladders from *Gli1*^{CreER}*;Smo*$^{flox/flox}$ and *Gli1*^{CreER}*;Smo*$^{flox/WT}$ mice.

(C) Epithelial:stromal ratios in bladders from *Gli1*^{CreER}*;Smo*$^{flox/flox}$ and *Gli1*^{CreER}*;Smo*$^{flox/WT}$ mice (no statistically significant difference). Left: Epithelial cells are marked by expression of EpCAM, bladder wall muscle is marked by the expression of smooth muscle actin (SMA), and stromal cells are located between the epithelial muscle layers. Right: The y-axis represents the ratio of the total number of epithelial cells to the total number of stromal cells in each tissue section. n=6 tissue sections from two mice per genotype, n.s, not significant.

(D) Vascular density in bladders from *Gli1*^{CreER}*; Smo*$^{flox/flox}$ and *Gli1*^{CreER}*; Smo*$^{flox/WT}$ mice (no statistically significant difference). Left: Blood vessels are marked by the expression of CD31. Right: Mean vascular density (MVD) was quantified as the number of CD31+ vessels per high powered field of view, averaged over 5 fields of view per tissue section. n=6 tissue sections from two mice per genotype. (C, D) Data are presented as mean ± s.e.m., and significance was calculated by an unpaired Student's t-test, n.s, not significant.

(E) Heat map of microarray data showing expression of Uroplakins, markers of urothelial differentiation. Color bar indicates RMA-normalized intensity values, with blue indicating low and red indicating high levels of gene expression.

(F and G) Expression of Hh and Bmp pathway target genes in bladders from control (Ctrl) wild-type mice with no BBN exposure, or bladders with invasive carcinoma from four mice exposed to BBN for 6 months was analyzed.

(F) Expression of Hh pathway target genes *Gli1* (all samples compared to control: $p<0.0001$) and *Ptch1* (Ctrl vs. 1: $p<0.001$; Ctrl vs. 2,3,4: $p<0.0001$) in invasive carcinoma samples as compared to the control.

(G) Expression of Bmp pathway target genes I*d1* (all samples compared to control: $p<0.001$), *Id2* (all samples compared to control: $p<0.0001$), *Id3* (Ctrl vs. 1: $p<0.01$; Ctrl vs. 2,3,4: $p<0.001$) and *Cdkn1b* (Ctrl vs. 2: $p<0.001$; Ctrl vs. 1,3,4: $p<0.0001$) in invasive carcinoma samples as compared to the control. (F, G) Data are presented as mean ± s.e.m. and significance was calculated by an unpaired Student's t-test. n = 3 technical replicates, and the entire experiment was repeated three times.

Table S2, related to Figure 3. Gene ontology analysis of genes down-regulated in Gli1CreER; Smoflox/flox mouse bladders following 2 months of BBN treatment

Biological Process Term (Level 3)	Count	Percentage	p-value
GO:0006811: Ion transport	26	8.099688474	5.08E-04
GO:0050793: Regulation of developmental process	21	6.542056075	1.80E-03
GO:0030154: Cell differentiation	42	13.08411215	3.61E-03
GO:0007267: Cell-cell signaling	13	4.049844237	4.25E-03
GO:0006897: Endocytosis	10	3.115264798	5.08E-03
GO:0010324: Membrane invagination	10	3.115264798	5.08E-03
GO:0021537: Telencephalon development	6	1.869158879	7.73E-03
GO:0032879: Regulation of localization	15	4.672897196	1.03E-02
GO:0007010: Cytoskeleton organization	13	4.049844237	1.04E-02
GO:0048731: System development	49	15.26479751	1.06E-02
GO:0044262: Cellular carbohydrate metabolic process	13	4.049844237	1.07E-02
GO:0006810: Transport	55	17.13395639	1.11E-02
GO:0005975: Carbohydrate metabolic process	16	4.984423676	1.12E-02
GO:0005996: Monosaccharide metabolic process	9	2.803738318	1.71E-02
GO:0021549: Cerebellum development	4	1.246105919	2.16E-02
GO:0044057: Regulation of system process	9	2.803738318	2.24E-02
GO:0048513: Organ development	40	12.46105919	2.45E-02
GO:0009653: Anatomical structure morphogenesis	29	9.034267913	2.53E-02
GO:0051049: Regulation of transport	11	3.426791277	2.54E-02
GO:0051239: Regulation of multicellular organismal process	22	6.853582555	2.57E-02
GO:0007411: Axon guidance	6	1.869158879	2.64E-02
GO:0048522: Positive regulation of cellular process	33	10.28037383	2.72E-02
GO:0030902: Hindbrain development	5	1.557632399	2.76E-02
GO:0016192: Vesicle-mediated transport	15	4.672897196	2.97E-02
GO:0022037: Metencephalon development	4	1.246105919	3.27E-02
GO:0048518: Positive regulation of biological process	36	11.21495327	3.28E-02
GO:0045595: Regulation of cell differentiation	13	4.049844237	3.38E-02
GO:0031175: Neuron projection development	9	2.803738318	3.41E-02
GO:0048634: Regulation of muscle development	4	1.246105919	3.69E-02
GO:0051093: Negative regulation of developmental process	9	2.803738318	4.08E-02

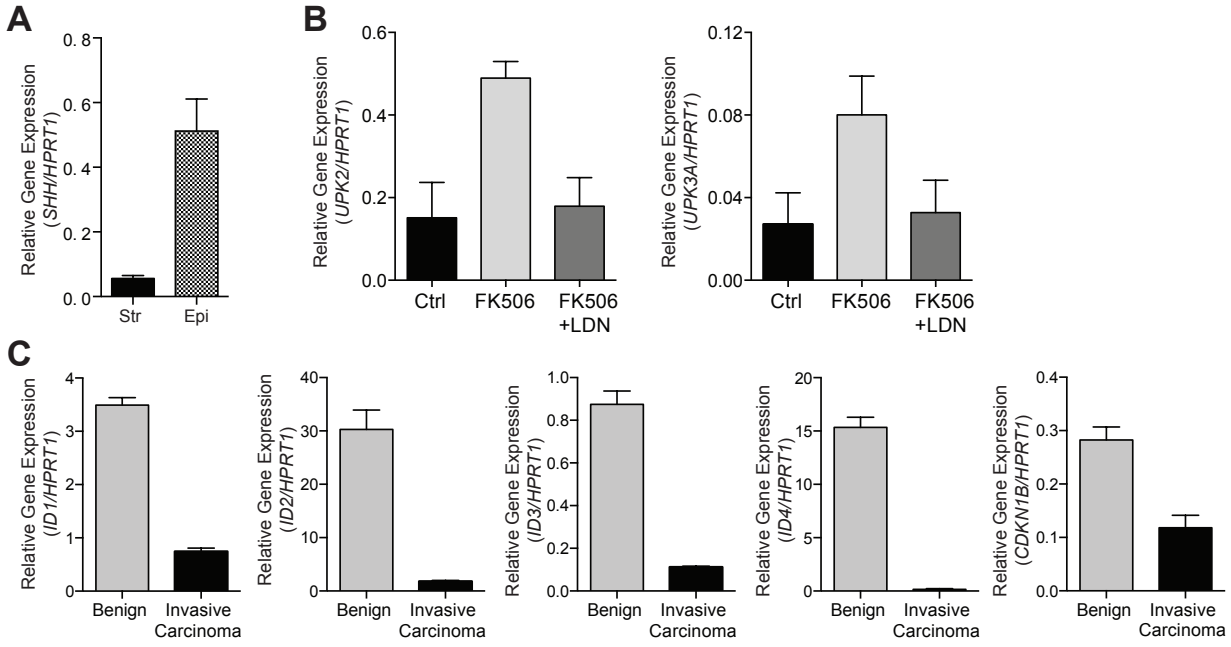
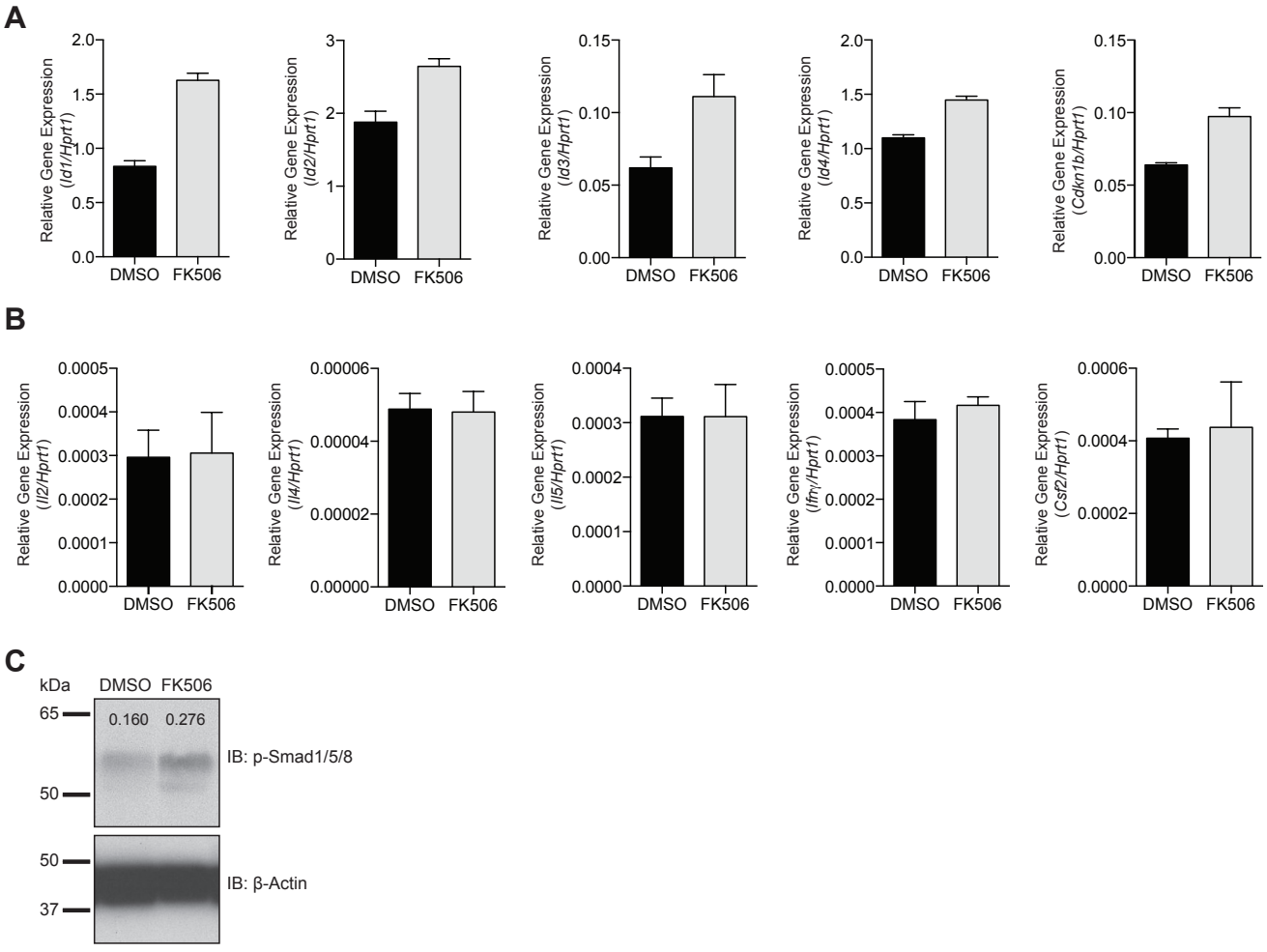


Figure S3, related to Figure 4. Expression of Uroplakins is induced by FK506 and BMP target gene expression is decreased in human urothelial invasive carcinoma
(A) Expression of *SHH* in primary human bladder stromal (Str) or urothelial (Epi) cells.
(B) Expression of *UPK2* in urothelial cells treated with FK506 as compared to the DMSO control (3.2 fold increase, $p<0.01$), or as compared to urothelial cells pre-incubated with LDN-193189 (LDN) and FK506 (2.7 fold increase, $p<0.01$). Expression of *UPK3A* in urothelial cells treated with FK506 as compared to the DMSO control (3 fold increase, $p<0.05$), or as compared to urothelial cells pre-incubated with LDN and FK506 (1.8 fold increase, $p<0.05$).
(C) Expression of *ID1* (4.7 fold decrease, $p<0.0001$), *ID2* (16.6 fold decrease, $p<0.005$), *ID3* (7.8 fold decrease, $p<0.0005$), *ID4* (101 fold decrease, $p<0.0001$) and *CDKN1B* (2.4 fold decrease, $p<0.01$) in human invasive urothelial carcinoma as compared to benign bladder tissue. (A-C) Data are presented as mean ± s.e.m. and significance was calculated by an unpaired Student's t-test. n = 3 technical replicates, and the entire experiment was repeated three times.


A
Relative Gene Expression (Id1/Hprt1)
2.0
1.5
1.0
0.5
0.0
DMSO FK506
Relative Gene Expression (Id2/Hprt1)
3
2
1
0
DMSO FK506
Relative Gene Expression (Id3/Hprt1)
0.15
0.10
0.05
0.00
DMSO FK506
Relative Gene Expression (Id4/Hprt1)
2.0
1.5
1.0
0.5
0.0
DMSO FK506
Relative Gene Expression (Cdkn1b/Hprt1)
0.15
0.10
0.05
0.00
DMSO FK506
B
Relative Gene Expression (Il2/Hprt1)
0.0005
0.0004
0.0003
0.0002
0.0001
0.0000
DMSO FK506
Relative Gene Expression (Il4/Hprt1)
0.00006
0.00004
0.00002
0.00000
DMSO FK506
Relative Gene Expression (Il5/Hprt1)
0.0004
0.0003
0.0002
0.0001
0.0000
DMSO FK506
Relative Gene Expression (Ifnγ/Hprt1)
0.0005
0.0004
0.0003
0.0002
0.0001
0.0000
DMSO FK506
Relative Gene Expression (Csf2/Hprt1)
0.0006
0.0004
0.0002
0.0000
DMSO FK506
C
kDa
DMSO FK506
65
0.160 0.276
IB: p-Smad1/5/8
50
50
IB: β-Actin
37

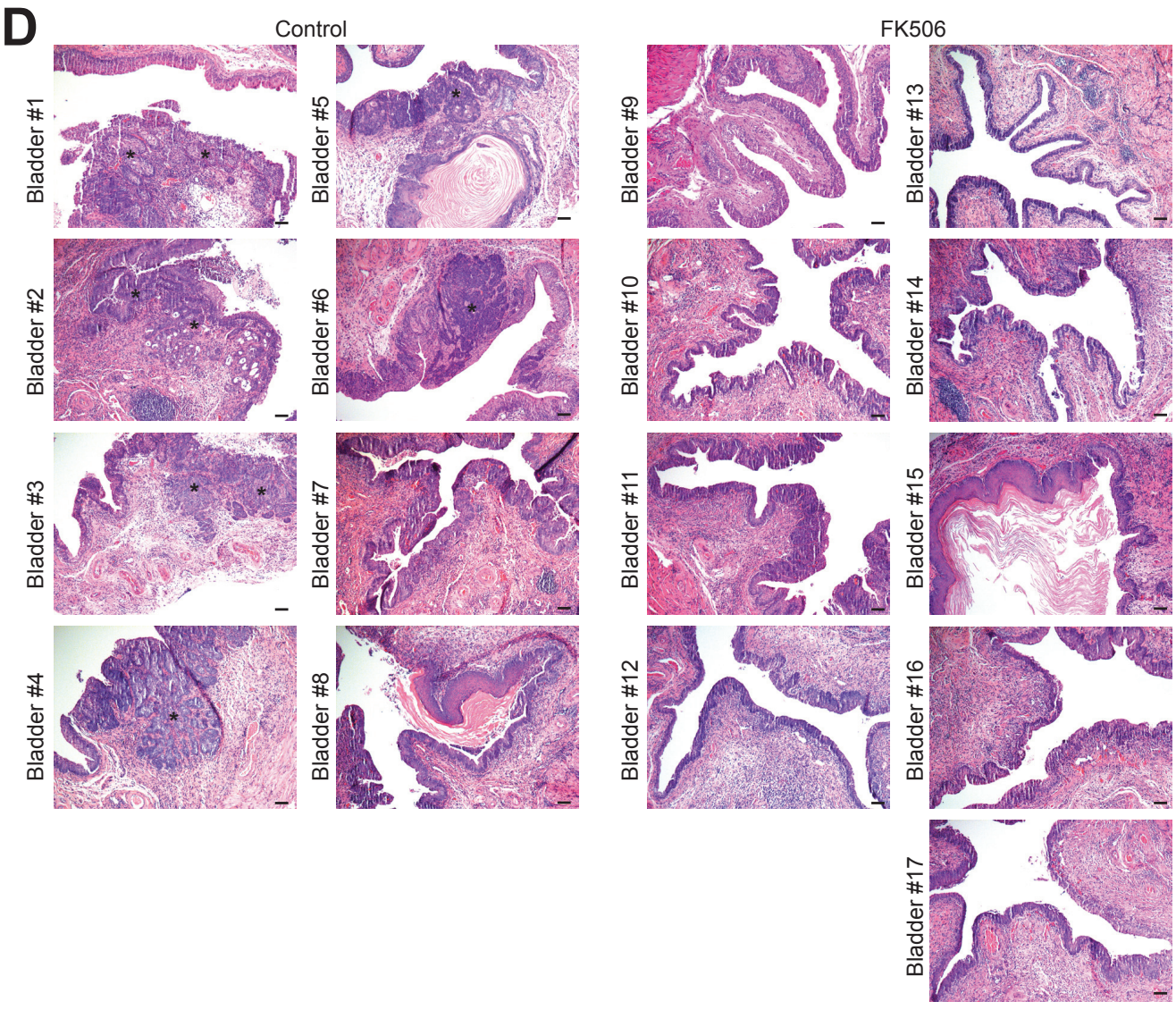

D
Control
FK506
Bladder #1
Bladder #2
Bladder #3
Bladder #4
Bladder #5
Bladder #6
Bladder #7
Bladder #8
Bladder #9
Bladder #10
Bladder #11
Bladder #12
Bladder #13
Bladder #14
Bladder #15
Bladder #16
Bladder #17

Figure S4, related to Figure 5. Pharmacological activation of BMP signaling *in vivo* impedes tumor progression to invasive urothelial carcinoma

(A and B) Gene expression in bladders from FK506-treated or control (DMSO-treated) mice.
(A) Expression of BMP target genes *Id1* (2 fold increase, $p<0.001$), *Id2* (1.4 fold increase, $p<0.05$), *Id3* (1.8 fold increase, $p<0.05$), *Id4* (1.3 fold increase, $p<0.01$) and *Cdkn1b* (1.5 fold increase, $p<0.01$) in bladders from FK506-treated mice as compared to control mice.
(B) Expression of genes in the calcineurin/NFAT pathway in bladders from FK506-treated and control mice (no significant difference). (A, B) Data are presented as mean ± s.e.m. and significance was calculated by an unpaired Student's t-test. n = 3 technical replicates, and the entire experiment was repeated three times.
(C) Total protein extracted from bladders from FK506-treated or control (DMSO-treated) mice was subjected to Western blot for phospho-Smad1/5/8 levels in FK506-treated bladders (right lane) as compared to control bladders (left lane). Numbers indicate the intensity of each Phospho-Smad1/5/8 band normalized to the intensity of the β-Actin band from the same sample.
(D) Bladder sections from mice exposed to BBN for 5 months, including 1 month of treatment with FK506 or a vehicle control, stained with Hematoxylin and Eosin (H&E). Asterisks indicate regions of invasive carcinoma in bladder samples #1-6. Scale bars represent 50μm.

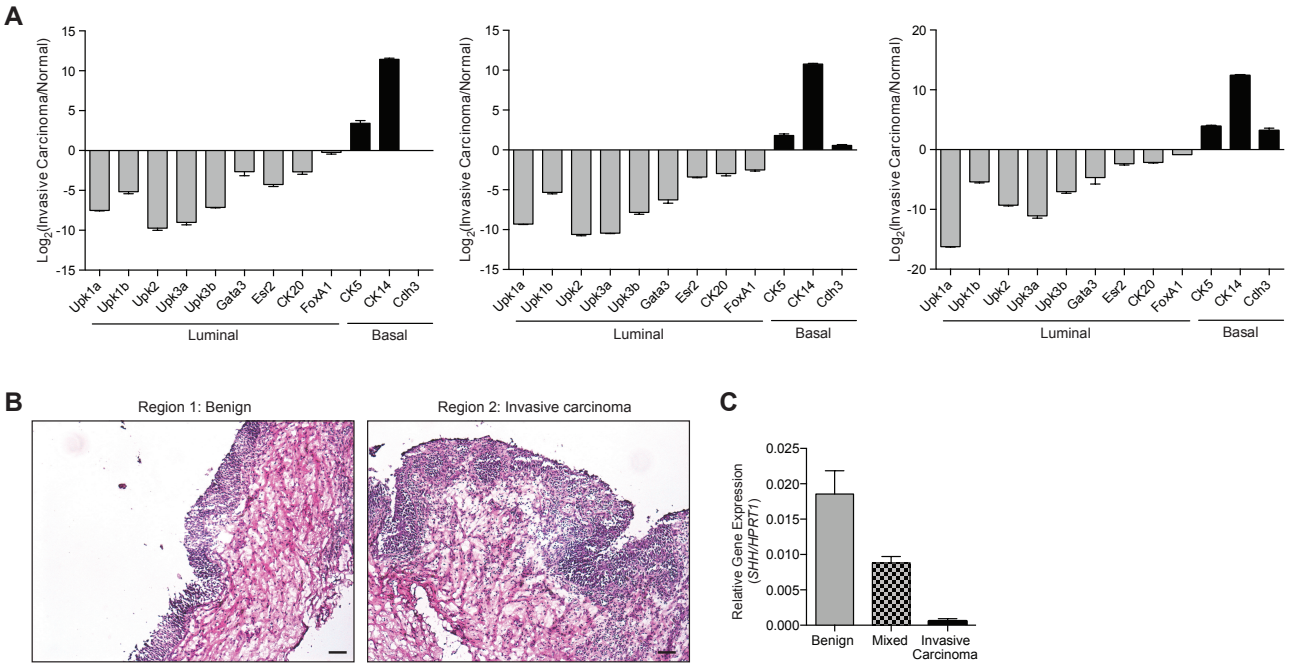


Figure S5, related to Figure 6. Basal character of BBN-induced murine urothelial carcinoma and *Shh* expression in mixed human sample containing benign and invasive carcinoma tissue

(A) Expression analysis of luminal and basal markers in three independent BBN-induced urothelial invasive carcinomas as compared to normal bladders. Data are presented as mean ± s.e.m. n = 3 technical replicates, and the entire experiment was repeated three times.

(B) Adjacent regions are shown from a tissue sample that contains both benign (left) and invasive carcinoma regions (right). Scale bars represent 50μm.

(C) *SHH* expression in this tissue sample ("mixed") as compared to samples exclusively containing benign and invasive carcinoma samples. Data are presented as mean ± s.e.m.