

Supplemental Figure Legends

Supplemental Figure S1. Validation of the shRNA knockdown efficiencies. (A) The knockdown efficiencies for shRNAs targeting *IHH*, *SMO*, *GLI2* or *GLI3* were examined in T24 cells, in which the indicated shRNAs were transduced and the expression of shRNA targets was analyzed by qPCR 2-3 days later. The knockdown efficiencies for *SHH* and *DHH* shRNAs are shown in Figure 6A-B. (B) The knockdown efficiency for *GLI1* shRNAs was similarly examined in four UC cell lines. In all cases, the gene expression of cells receiving scramble shRNA#1 was set to 1 for easy comparison. Error bars indicate S.E.M. and asterisks (*) indicate statistically significant changes when compared to cells receiving scramble shRNA#1. (C-D) Endogenous protein levels of SHH (C) and GLI1 (D) were examined in T24 cells after them being transduced with scramble shRNA#1 or shRNAs specific for *SHH* or *GLI1*. Cell lysates were harvested 3-4 days after shRNA transduction. SHH or GLI1 was first enriched by agarose beads coupled to a SHH monoclonal antibody (5E1) or by Sepharose beads conjugated with a GLI-binding oligonucleotide, and then subject to immunoblotting. Levels of α TUBULIN (TUB) in initial cell lysates were used as controls for equal loading. Representative immunoblots from two independent experiments are shown. *SMO*, *GLI2* and *GLI3* shRNAs were also validated to knockdown their specific targets at the protein levels by immunoblotting for such proteins overexpressed in HEK293 cells (data not shown).

Supplemental Figure S2. UC cell lines require HH signaling for proliferation. (A) HT1376, J82, Vmcub1 and T24 cells were transduced with four control shRNAs or two shRNAs each independently targeting *SMO* or *GLI1*. Cell proliferation was determined four days after shRNA transduction by the CellTiter Glo assay. Error bars indicate S.E.M. and asterisks (*) indicate statistically significant changes when compared to cells receiving scramble shRNA#1. (B) The indicated UC cell lines were treated with GDC-0449 (10 μ M) or vehicle for two days before determining cell proliferation by the CellTiter-Glo

assay. Error bars indicate S.D. (C) The indicated UC cell lines were transduced with scramble shRNA#1 or two shRNAs each independently targeting *SMO* or *GLII*. Cell proliferation was determined by the MTT assay four days after shRNA transduction. A representative experiment is shown here. Error bars indicate S.D.

Supplemental Figure S3. *SMO* inhibition induces apoptosis in a subset of UC cell lines. HT1376, J82, Vmcub1 and T24 cells were either untreated or transduced with a scramble shRNA or two *SMO* shRNAs for 3 days. Full-length (FL) and cleaved PARP were then examined by immunoblotting. Levels of α TUBULIN (TUB) were used as a control for equal loading. U: untreated, P: puromycin (2 μ g/ml), 1: scramble shRNA#1, 2: *SMO* shRNA#2, 3: *SMO* shRNA#3. Representative immunoblots from three independent experiments are shown.

Supplemental Figure S4. A 2507-gene signature predicts the HH-dependence of UC cells for proliferation. (A) Heat-map view of a differentially-expressed gene signature. Global expression analysis was performed using the indicated UC cell lines. The differentially-expressed gene signature is composed of 2507 genes which are regulated similarly in HH-dependent UC cells (Vmcub1 and T24), but in an opposite direction in HH-independent UC cells (HT1376 and J82). The top 147 differentially-expressed genes are shown here. (B) This 2507-gene signature is validated to predict HH-dependence in UC cell lines, by performing a meta-analysis with gene expression profiles of five UC cell lines available from the GlaxoSmithKline (GSK) dataset (1). A positive correlation (blue bar) indicates that the UC cell line is predicted to be HH-dependent. A negative correlation (red bar) indicates that the UC cell line is predicted to be HH-independent. Note that the predicted HH-dependence is consistent with what has been observed in Figure 2A. Black bar: no significant correlation. Triangle: number of overlapping genes. Asterisks (*) indicate statistically significant correlations (p-value < 0.0001 or -Log p-value > 4).

Supplemental Figure S5. Constitutive HH signaling is required for the anchorage-independent growth of UC cells. (A) The indicated UC cells were transduced with scramble shRNA#1 or shRNAs specific for *SMO* or *GLII*, and then grown in soft agar. Colonies were quantified and the number of colonies in cells receiving scramble shRNA#1 was set to 100% for easy comparison. (B) Similar results were found when additional control shRNAs were used in HT1376 cells. Representative images for HT1376 cells grown in 35 mm dishes are also shown. (C) The expression of HH target genes was measured by qPCR in HT1376, J82, Vmcub1 and T24 cells, when they were grown in monolayer or in soft-agar. Gene expression values in monolayer HT1376 cells are set to 1 for comparison. In all cases, error bars indicate S.E.M. and asterisks (*) indicate statistically significant changes when compared to cells receiving scramble shRNA#1 or grown in monolayer.

Supplemental Figure S6. UC cells maintain HH signaling activity through ligand expression. (A) Expression of *SHH*, *IHH* and *DHH* was examined in the indicated cell lines by qPCR. The abundance of each transcript was calculated as the percent expression of *18S* rRNA. Error bars indicate S.E.M. (B and C) *SHH* expression was knocked down in the indicated UC cell lines. Subsequent changes in the expression of HH target genes *GLII* and *PTCHI*, as well as the shRNA targeted RNA, were measured by qPCR. Error bars indicate S.E.M. and asterisks (*) indicate statistically significant changes when compared to cells receiving scramble shRNA#1.

Supplemental Figure S7. SMO localizes to primary cilia in UC cell lines. (A-B)

Immunofluorescence (IF) staining was performed in T24-derived clonal cell lines stably expressing SMO-GFP or GFP. Shown are representative images from two T24 clonal isolates expressing SMO-GFP (Clone#1 and Clone#2) and one clonal isolate expressing GFP. (C) IF staining was performed in UROtsa-derived polyclonal cells expressing *SMO-GFP* and treated with either vehicle (DMSO) or SAG

(200 nM) for 24 h. In all images, the different colors depict: SMO-GFP or GFP (GFP fluorescence, green), activated-SMO (stained for phospho (p)-SMO, cyan), primary cilia (stained for acetylated (AC)- α Tubulin, red), basal body (stained for γ Tubulin, white) and chromatin (DAPI fluorescence, blue). SMO localization in primary cilia is highlighted on the merge images by white arrows. Scale bar: 10 μ m. (D) The percent SMO localization in primary cilia of UROtsa cells was quantified by counting at least 50 ciliated cells in each treatment group. Error bars indicate S.E.M. and asterisk (*) indicates a statistically significant change over DMSO treatment.

Supplemental Figure S8. *In situ* hybridization (ISH) confirms the positive staining of SHH and GLI1 IHC in human UC. Serial sections of a representative human UC resected sample were stained by ISH for *GLI1* or *SHH* using sense (A, D) or anti-sense (B, E) probes, or by IHC using GLI1 or SHH antisera (C, F).

Supplemental Figure S9. Increased expression of SHH and GLI1 in bladders associated with cystitis cystica. H&E and IHC for GLI1 and SHH were performed in serial sections of a bladder with cystitis cystica, a condition associated with chronic inflammation. U: normal urothelium area. vBn: Von Brunn's nests (indicator of cystitis cystica).

Supplemental Tables

Supplemental Table S1. List of 2507 differentially-expressed genes.

Supplemental Table S2. SHH and GLI1 levels are enhanced in primary human UC specimens.

Supplemental Table S3. pLKO-based shRNA constructs used in this study.

Supplemental Materials and Methods

Assays

UC cells were seeded in 96-well plates allowed to attach overnight, followed by shRNA transduction or GDC-0449 (Selleck) treatment. Cell proliferation was determined by the CellTiter-Glo Luminescent Cell Viability Assay (Promega) or MTT assay as previously described (2). Endogenous SHH was enriched by agarose beads coupled to the SHH monoclonal antibody (5E1, Hybridoma Bank) and immunoblotted using a rabbit polyclonal SHH (Santa Cruz). For measuring PARP cleavage, shRNA-transduced or puromycin-treated UC cells were directly lysed in 2x Laemmli sample buffer and immunoblotted using a mouse monoclonal PARP (Enzo). Immunoblot for α TUBULIN (mouse monoclonal, Calbiochem) was used as a control for equal loading. *In situ* hybridization for *SHH* and *GLII* on paraffin-embedded formalin-fixed tissue sections was performed as previously described (3).

Immunofluorescence staining

T24 or UROtsa cells stably expressing GFP or SMO-GFP were plated on cover slips and grown to confluence to maximize primary cilia formation. These cells were treated with SANT1 (Calbiochem), SAG (Alexis) or vehicle under low serum condition (0.5%) for 24 hr, followed by cell fixation and staining as described previously (4). The primary antibodies for IF staining were mouse monoclonal acetylated- α Tubulin (Invitrogen), rabbit polyclonal γ Tubulin (Sigma) and rabbit polyclonal SMO PS1 (gift from Dr. Jin Jiang, UT Southwestern Medical Center) (5). Samples were examined and imaged with a Leica SP5 inverted confocal microscope or Zeiss Axiovert 200M automated inverted microscope.

SNP array analysis

A high-density SNP array (Affymetrix SNP array 6.0) was used as a surrogate for examining the genome-wide chromosome rearrangement events in the UC cell lines. Genomic DNA was isolated from UC cell lines and purified using the DNeasy Blood and Tissue kit (Qiagen). DNA quality assessment and array hybridization were conducted at the Human Genomics Core Facility (University of Miami).

Array data were clustered along with 21 normal diploid samples (International HapMap Project) using the birdseed-v2 algorithm to discover genomic rearrangements in UC cell lines. The virtual karyograms of chromosome 9 were generated using the Genotyping Console 3.0.1 Browser.

PTCH1 promoter methylation analysis

Genomic DNA was extracted from HT1376, J82, Vmcub1, T24 and UROtsa cells using the DNeasy Blood & Tissue Kit (Qiagen). Bisulfite conversion of DNA was performed using the Cells-to-CpG Bisulfite Conversion Kit (Applied Biosystems) following vendor's instructions. Conversion efficiency was monitored using the Cells-to-CpG Bisulfite Conversion and Quantitation Control Kit (Applied Biosystems) and only those samples with a conversion rate above 99.5% were subsequently used for sequencing analysis. A region of *PTCH1* promoter, which contains the GLI binding sites, was PCR-amplified and sequenced as previously described (6). These sequencing results were used to determine the methylation status of CpG islands in all five cell lines. Primers: (Forward 5' – 3'): GAAGATATTGTTGAAAAGAAAAGGAA; (Reverse 5' – 3'): CTATCAAATAACTTAAATTTCTA.

Genome-wide expression analysis and generation of the differentially expressed gene signature

The genome-wide expression profiles were determined using the Human-HT12 V4 Expression BeadChip (Illumina) in two groups of UC cell lines whose proliferation were either HH-dependent (Vmcub1 and T24) or HH-independent (J82 and HT1376). Total RNA was isolated from these four cell lines in triplicate using the Qiagen miRNeasy kit (Qiagen). Quality assessment, array hybridization and raw data normalization were conducted at the Oncogenomics Core Facility at the University of Miami. The expression array raw and normalized data have been deposited at the NCBI Gene Expression Omnibus (GEO) database (GEO accession number: GSE28255). The commonly up- or down-regulated genes in each group of cell lines were obtained and further compared using the Agilent GeneSpring software (Agilent Technologies). Significant differences between the two cell line groups were

determined by Student's T-test. The p-values were further adjusted for multiple comparisons using the Benjamini-Hochberg FDR multiple testing correction. 3316 differentially-expressed array probes (corresponding to 2507 genes) were obtained by using p-value < 0.05 and fold change > 1.2 as the cutoff.

Meta-analysis by NextBio tool sets

The web-based software package NextBio Professional (<http://www.nextbio.com/b/nextbio.nb>) was used to perform meta-analyses of our gene signature with gene signatures from publicly available bladder cancer datasets (described below). Statistical significance was determined by Fisher's exact test. The directional correlation was determined as previously described (7). We arbitrarily set p-value less than 0.0001 (or - Log p-value > 4) as the cutoff for significant correlations.

Descriptions of the public bladder cancer datasets

(1) **GlaxoSmithKline (GSK) Transcript Profiling of Cancer Cell Lines** (1). This study contains gene expression profiles from 315 human cancer cell lines, including those from five UC cell lines (HT1376, J82, 5637, UM-UC-3 and BFTC905). The platform was Affymetrix GeneChip Human Genome U133 Plus 2.0 Array. The available gene expression profiles include:

- Every cancer cell line *versus* mean gene expression of all cancer cell lines

(2) **Comparative Gene Expression Profiling Analysis of Urothelial Carcinoma of Renal Pelvis and Bladder**. GEO accession number: GSE24152. Samples included in the study: urothelial carcinoma of the bladder (n = 10) and benign bladder mucosa (n = 7). The platform was Affymetrix GeneChip Human Genome U133 Plus 2.0 Array. The following gene signature has a significant correlation with the 2507-gene signature:

- Bladder urothelial cell carcinoma samples *versus* normal bladder samples

(3) Analysis of clinical bladder cancer classification according to microarray expression profiles

(8). GEO accession number: GSE7476. Samples included in the study: normal bladder tissues, low grade superficial tumor samples (Ta low grade), high grade superficial tumors (T1 high grade) and high grade muscle invasive tumors (T2, T3 or T4). The platform was Affymetrix GeneChip Human Genome U133 Plus 2.0 Array. The following gene signatures have significant correlations with the 2507-gene signature:

- T1 high grade bladder tumor *versus* normal bladder
- T2, T3 or T4 high grade bladder tumor *versus* normal bladder
- Ta low grade bladder tumor *versus* normal bladder

(4) Predictive Value of Prognosis-Related Gene Expression Study in Primary Bladder Cancer (9).

GEO accession number: GSE13507. Samples included in the study: 165 primary bladder cancer samples, 23 recurrent non-muscle invasive tumor tissues, 58 normal looking bladder mucosae surrounding cancer and 10 normal bladder mucosae. The platform was Illumina human-6 v2.0 expression beadchip. The following gene signatures have significant correlations with the 2507-gene signature:

- Recurrent non-muscle invasive tumor *versus* normal bladder mucosa
- Primary bladder cancer *versus* normal bladder mucosa
- Primary bladder cancer *versus* bladder mucosa surrounding cancer

(5) Transcriptome analysis of 57 bladder carcinomas (10). EMBL-EBI ArrayExpress Archive

number: E-TABM-147. Samples included in the study: 57 invasive and non-invasive UC specimens of various stages, and normal bladder specimens. The platform was Affymetrix GeneChip Human Genome U95 (hgu95a and hgu95av2). The following gene signature has a significant correlation with the 2507-gene signature:

- Human urothelial bladder carcinoma with metastasis *versus* no metastasis

(6) **Classification of carcinoma *in situ* lesions in human bladder cancer** (11). GEO accession number:

GSE3167. Samples included in the study: 60 specimens from UC patients of various stages, including invasive cancers with or without adjacent carcinoma *in situ* (CIS); CIS without adjacent invasive cancer; and normal bladder specimens. The platform was Afymetrix GeneChip Human Genome U133 Array Set HG-U133A. The following gene signatures have significant correlations with the 2507-gene signature:

- Transitional cell carcinoma with surrounding CIS lesion *versus* without CIS
- Muscle invasive carcinoma *versus* transitional cell carcinoma with surrounding CIS lesion
- Muscle invasive carcinoma *versus* normal bladder from healthy donors

Supplemental References

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