

## Hedgehog Signaling Regulates Bladder Cancer Growth And Tumorigenicity

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## Abstract

The role of HEDGEHOG (HH) signaling in bladder cancer remains controversial. The gene encoding the HH receptor and negative regulator *PATCHED1* (*PTCH1*) resides on a region of chromosome 9q, one copy of which is frequently lost in bladder cancer. Inconsistent with *PTCH1* functioning as a classic tumor suppressor gene, loss-of-function mutations in the remaining copy of *PTCH1* are not commonly found. Here, we provide direct evidence for a critical role of HH signaling in bladder carcinogenesis. We show that transformed human urothelial cells and many urothelial carcinoma (UC) cell lines exhibit constitutive HH signaling, which is required for their growth and tumorigenic properties. Surprisingly, rather than originating from loss of *PTCH1*, the constitutive HH activity observed in UC cell lines was HH ligand-dependent. Consistent with this finding, increased levels of HH and the HH target gene product GLI1 were found in resected human primary bladder tumors. Furthermore, based on the difference in intrinsic HH dependence of UC cell lines, a gene expression signature was identified that correlated with bladder cancer progression. Our findings therefore indicate that therapeutic targeting of the HH signaling pathway may be beneficial in the clinical management of bladder cancer.

## Introduction

Cancer of the urinary bladder is one of the most common malignancies worldwide, with a lifetime risk of 1 in 42 (1). Greater than 80% of bladder cancers originate from the urothelium of the bladder and are referred to as urothelial carcinoma (UC) (2). UC is classified into two subtypes based on whether or not the cancer cells infiltrate into the muscle layer of the bladder (2). Non-muscle invasive UC (NMIUC) is a less aggressive type of cancer with good prognosis, but with a greater than 50% recurrence rate (3). Muscle-invasive UC (MIUC) frequently metastasizes, resulting in a poor five-year survival rate (4). The high recurrence rate and increased risk of metastasis of the two major UC subtypes make the effective clinical management of UC an important goal.

The deregulation of HEDGEHOG (HH) signaling has been linked to the etiology of many cancers, where it is thought to play an initiating or maintenance role (5-7). Constitutive HH pathway activity is thought to result from mutations in key regulators of the pathway, overexpression of the HH ligands or non-canonical activation of HH target genes (8, 9). The vertebrate HH ligands consist of SONIC HEDGEHOG (SHH), INDIAN HEDGEHOG (IHH) and DESERT HEDGEHOG (DHH), which engage a signaling cascade by binding to their common cellular receptor PATCHED1 (PTCH1) (10). Upon HH binding, PTCH1 releases its inhibitory effect on the transmembrane protein SMOOTHENED (SMO), ultimately leading to activation of GLI family transcription factors (GLI1 – 3) (11). Among the GLIs, GLI1 is both a transcriptional activator and HH target gene (11-13). Furthermore, GLI1 is thought to be the most reliable biomarker of HH pathway activity (13, 14). The steady-state levels of GLIs are highly regulated via proteolysis, stabilization of which is thought important for cancer progression (15). Unlike GLI1, GLI2 and GLI3 are also regulated by proteolytic cleavage to convert them from transcriptional repressor forms to activator forms in response to HH. Ultimately, the overall activation status of GLIs determines the output of HH pathway activity (11, 16).

The role HH signaling plays in human bladder cancer has been quite controversial. Loss of certain regions of chromosome 9q is one of the most common and earliest genetic alterations in UC (17-19). The *PTCH1* gene was reported to reside in a minimal deletion region of 9q by one group but not by others (20-22). Inconsistent with *PTCH1* functioning as a classic tumor suppressor gene for bladder cancer, mutations of *PTCH1* were rarely found in the remaining allele of UC samples in which one copy of *PTCH1* was deleted, nor in other bladder cancers whose *PTCH1* loss-of-heterozygosity (LOH) status were unclear (20-24). Furthermore, two groups reported on insensitivity of UC cell lines to SMO antagonists, inconsistent with HH signaling playing a major role in UC (25, 26). We recently showed that the bladder carcinogen arsenic activated HH signaling, and that GLI1 protein was highly expressed in the vast majority of human UC specimens (27). Here, we provide direct experimental evidence, *in vitro* and *in vivo*, for a critical role of HH signaling in bladder carcinogenesis.

## Materials and Methods

### *Cell lines, Reagents, and Assays*

All cells were grown and maintained as described previously (25, 28). Stable cells were selected under G418 (Invitrogen) and were used as either polyclonal or monoclonal lines as indicated. Lentiviruses expressing various shRNAs (Supplemental Table S3) were used to transduce target cells as previously described (29). Equal viral titers were determined as previously described (30). UC cells were seeded in 96-well plates, transduced with various shRNAs, and incubated for 3-4 days before determining cell proliferation and apoptosis using CellTiter-Glo and Caspase 3/7 Assays (Promega). The soft agar assay, GLI1 enrichment for immunoblotting, HH-reporter assay, and Taqman-based RT-qPCR analysis were performed as previously described (27, 28). Details for primary cilia staining, genome-wide copy number and expression profiling of UC cells, gene signature generation, meta-analysis and methylation analysis are provided in the Supplemental Materials and Methods.

### *Immunohistochemical staining*

Resected human urinary bladder tissues were fixed in formalin, paraffin embedded and sectioned at 4  $\mu\text{m}$  thickness under an approved IRB protocol at the University of Miami. Immunohistochemical (IHC) staining for GLI1 and SHH was performed by a DAKO autostainer and scored by a board certified pathologist (MJ). The scoring criteria were based on an estimate of the intensity of tumor cells stained positive for GLI1 or SHH: - (negative); + (weakly positive); ++ (moderately positive); +++ (strongly positive). The primary antibodies for IHC were rabbit polyclonal GLI1 (27) and rabbit polyclonal SHH (Santa Cruz).

### *Xenograft tumors in nude mice*

6-week-old female athymic nude mice (Charles River) were inoculated with Vmcub1 or HT1376 cells which had been transduced with control shRNAs or *SMO*- and *GLI1*-specific shRNAs. 1–5 million live cells were mixed with an equal volume of matrigel (BD Biosciences) and injected subcutaneously in the flanks of nude mice. Tumor growth was monitored for up to 40 days and tumor volume was measured by the formula:  $\text{Volume} = (S \times S \times L) / 2$ , where S and L are the short and long dimensions (29).

### *Statistics*

All experiments were independently performed at least three times unless otherwise stated. Statistical significance was determined by Student's T test. P-value  $\leq 0.05$  was considered statistically significant. Statistical analysis for representative experiments was not provided.

## **Results**

We have suggested that elevated HH activity might account for the etiology of arsenic-induced bladder cancer (27). As chronic arsenic exposure transforms human urothelial cells *in vitro* (28), we reasoned that HH activity might be required for arsenic-mediated urothelial cell transformation. Therefore, we

compared arsenic transformed urothelial cells (URO-MSC52) to their passage-matched immortalized parental cells (UROtsa), initially noting that the expression level of the HH target gene *GLI1* was higher in the arsenic transformed cells (Figure 1A). We next knocked down the expression of *SMO* or *GLI1* in these cells, using two independently targeted short-hairpin RNAs (shRNA), to examine whether URO-MSC52 cells require HH signaling to maintain their transformed phenotype. When compared to cells transduced with a control scramble shRNA (scramble shRNA#1), URO-MSC52 cells exhibited a greater than 50% reduction in their ability to grow in an anchorage-independent manner upon reduction of *SMO* or *GLI1* (Figure 1B). Moreover, although parental UROtsa cells grew poorly in soft agar, their ability to do so was dramatically improved after SHH transduction (Figure 1C). Consistent with UROtsa cells being HH responsive, we observed an induction in HH target gene expression in response to a HH pathway agonist (Figure 1D). This modest HH target gene activation is in contrast to the strong induction in anchorage-independent growth, which likely results from the different biological properties measured in these mechanistically distinct assays. Collectively, these results suggest a critical role for HH signaling in urothelial cell transformation.

Based on our results with transformed urothelial cells, we hypothesized that increased HH pathway activity might be required for the growth of bladder cancer cells. To test this hypothesis, we measured the proliferation of a large panel of well-characterized human UC cell lines and attenuated HH signaling in these cells using shRNA-based RNA interference to knockdown the expression of key HH pathway components (*SMO*, *GLI1*, *GLI2* and *GLI3*). All the shRNA constructs used here were validated at the mRNA and protein levels (Supplemental Figure S1). To further control the stringency of this shRNA-mediated approach, we used two independent shRNAs to target each HH component and four sequence-distinct control shRNAs. In general, UC cell lines showed a wide range of sensitivity to knockdown of HH signaling components (Figure 2A and Supplemental Figure S2A). For example, *GLI1* knockdown decreased the proliferation of T24 and Vmcub1 cells by 60% but had little effect on the proliferation of

HT1376 and J82 cells, although the knockdown efficiency of the targeted genes was similar among the cell lines tested (Supplemental Figure S1B). Interestingly, *GLI3* attenuation showed similar inhibitory effects on cell proliferation as other positive HH regulators did, indicating that the *GLI3* activator form likely predominated in the sensitive cells. Knocking down the expression of *SMO*, *GLI1*, *GLI2* or *GLI3* showed similar inhibitory effects on proliferation in any given UC cell line, implying that the HH signaling rather than any individual pathway component might be important for UC cell proliferation. Overexpression of *GLI1* was able to rescue the proliferation defects rendered by *SMO* shRNA in T24 cells, suggesting the shRNA-mediated *SMO* knockdown was specific (Figure 2B). The incomplete rescue observed might also implicate *GLI1*-independent, *SMO*-dependent signaling mechanisms (such as those through *GLI2* or *GLI3*) or a HH-dependent *GLI1* modification may also be important for cell proliferation (31). Similar attenuation of proliferation was also observed in a subset of UC cells treated with the *SMO* antagonist GDC-0449 (Supplemental Figure S2B). Overall, these results suggest that HH signaling is required for cell proliferation in at least a subset of UC cell lines.

We further characterized the role of HH signaling in UC cells focusing on four cell lines (HT1376, J82, Vmcub1 and T24 cells), as they represent cells in which the proliferation was least or most affected when HH signaling was inhibited. We first confirmed their relative sensitivity to HH inhibition using a second distinct proliferation assay (Supplemental Figure S2C). We next examined if HH signaling could act as a survival factor for UC cells, which had been suggested for other HH-dependent cancer cell lines (32-35). We therefore attenuated the expression of *GLI1* or *SMO* and then measured cell apoptosis. Caspase 3/7 activity was induced when *GLI1* expression was attenuated in the two cell lines whose proliferation was most dependent on HH signaling (Vmcub1 and T24 cells), but not in cells which showed the least dependence on HH signaling for proliferation (HT1376 and J82 cells) (Figure 2C). Similar results were obtained when *SMO* levels were reduced, using the cleavage of PolyADP-ribose Polymerase (PARP) as an indicator of apoptosis (Supplemental Figure S3). PARP cleavage was evident

when HT1376 and J82 cells were exposed to puromycin, suggesting that these cells have a functional apoptotic pathway (Supplemental Figure S3 and data not shown). These results indicate that HH signaling is required to maintain the viability of a subset of UC cell lines.

As the four UC cell lines showed distinct dependence on HH signaling for proliferation we reasoned that the HH signaling pathway might be differentially regulated in these cells. By comparing the expression of several HH pathway components, we observed that the HH-dependent cells (Vmcub1 and T24) expressed higher levels of *SMO*, but not other genes examined (Figure 3A and data not shown). We also compared the protein level of GLI1 in these four cell lines as a second determinant of HH activity, as stabilization of GLI1 protein is a key event for HH signaling in cancer (15). GLI1 was immunoblotted from lysates of these four UC cell lines after GLI enrichment, using Sepharose beads conjugated to a defined GLI-binding DNA oligonucleotide. High levels of GLI1 were only detected in HH-dependent cells (Figure 3B). Notably, J82 cells expressed comparable high levels of *GLI1* mRNA, but unlike the HH-dependent cells, failed to accumulate significant amount of GLI1 protein (compare Figure 3B with Figure 3A). The accumulation of *SMO* and GLI1 indicate that Vmcub1 and T24 cells harbor a higher level of HH pathway activity, correlating well with their dependence on HH signaling for proliferation.

We further compared the genome-wide expression profiles of these two groups of cell lines in order to understand the biological significance of HH-dependence. We searched for genes which were commonly expressed in HH-dependent cells or in HH-independent cells, but were differentially expressed between these two groups of cells (Supplemental Figure S4A and Table S1). Such analysis identified 2507 genes and this gene signature was able to predict the HH-dependence in UC cell lines by performing a meta-analysis. When searching for correlations between our gene signature and the published gene expression profiles of five UC cell lines in a public dataset (36), we were able to identify two HH-independent cell lines used to generate this signature, HT1376 and J82, and two HH-dependent



cell lines that were not used to generate this signature, UM-UC-3 and BFTC905 cells (Supplemental Figure S4B and compare to Figure 2A). We then used a similar approach to correlate our gene signature to publicly available gene signatures in five bladder cancer studies. This analysis identified significant positive associations with published gene signatures obtained by comparing UC to normal urothelium and by comparing metastatic UC to non-metastatic UC (Figure 3C). Such associations were lost when a scrambled gene signature, consisting of 2601 differentially expressed genes from a random grouping (T24 and HT1376 versus Vmcub1 and J82), were used to perform the meta-analysis (data not shown). These results suggest that the HH-dependent cell lines represent a more tumorigenic cell population. Moreover, this 2507-gene signature appears able to predict UC progression, and might prove of prognostic value.

We next determined the ability of these two groups of UC cell lines to grow in an anchorage-independent manner. When these cells were transduced with shRNAs targeting *SMO* or *GLII* and then embedded in soft agar, the HH-dependent T24 cells and Vmcub1 cells showed a dramatic reduction in their ability to form soft agar colonies (Supplemental Figure S5A). Surprisingly, the HH-independent HT1376 and J82 cells also showed significant reduction in colony formation. This reduction in soft-agar growth was specific to *SMO* and *GLII* shRNAs, as four control shRNAs failed to elicit the same inhibitory effect (Supplemental Figure S5B). This unexpected result indicated that the anchorage-independent growth of these latter cells require HH signaling. Indeed, HT1376 and J82 cells exhibited 50- to 100-fold increase in *GLII* expression when they were grown in soft agar relative to when these cells were grown in monolayer culture, which might explain this switch in HH-dependence (Supplemental Figure S5C).

We further evaluated the dependence of UC cells on HH signaling during tumor development in a xenograft tumor model. Vmcub1 and HT1376 were chosen because they grew well *in vivo* (data not

shown). These cells were similarly transduced with two control shRNAs or two shRNAs independently targeting either *SMO* or *GLII*, and were injected subcutaneously into athymic nude mice. Tumor volume was then monitored for up to 40 days. While palpable tumors were observed in control shRNA treated cells approximately two weeks after implantation, *SMO* or *GLII* shRNAs greatly repressed the tumor growth originating from Vmcub1 cells, but had no effect on the growth of HT1376 tumors (Figure 4). These results are consistent with Vmcub1 cells being more sensitive than HT1376 cells to HH pathway inhibition for proliferation. Overall, our *in vitro* and *in vivo* results highlight the importance of HH signaling in mediating the tumorigenic properties of a subset of UC cell lines.

Since HH signaling is required for various aspects of tumorigenicity in certain UC cells, we sought to explore the mechanism that drives this constitutive HH signaling. Deletion of the entire *PTCHI* locus and *PTCHI* mutations were reported in some primary human bladder cancers (20, 23). As *PTCHI* loss-of-function would result in increased HH pathway activity, we reasoned this could account for the constitutive HH signaling in UC cell lines. Therefore, we examined the chromosomal integrity along 9q in the four UC cell lines using a high-density single-nucleotide polymorphism (SNP) array.

Unexpectedly, the *PTCHI* locus is intact in all four UC cell lines (Figure 5A). Moreover, *PTCHI* does not appear to be epigenetically silenced or mutated (Figure 5B and data not shown). Collectively, these results argued against the contribution of *PTCHI* alterations to constitutive HH signaling in any of the UC cell lines tested. We further examined the copy number changes of all known HH pathway components, including loss of the negative regulator *SUFU* and amplification of *GLII*, but failed to find obvious genetic changes that might account for the HH pathway activity in these cells (data not shown).

Besides loss of *PTCHI* or *SUFU*, constitutive HH signaling in cancer cells commonly results from expression of the HH ligands (35, 37, 38). Indeed, over-expression of *SHH* has been observed in many UC cell lines, including the majority used in the current study (25). We confirmed the expression of all

three HH ligands in the UC cell lines (Supplemental Figure S6A). To determine the biological significance of HH ligand-production, we attenuated the expression of each of the HH ligands using shRNAs and then examined the subsequent changes in the proliferation of UC cells. Consistent with the proliferation of Vmcub1 and T24 cells being most dependent on levels of HH pathway activity, these two cell lines were similarly sensitive to knockdown of HH ligands (Figure 5C). These results suggest that production of HH ligands could account for the constitutive HH signaling that is required for the tumorigenic properties of UC cells.

We next used several loss-of-function and gain-of-function approaches to confirm the causative effect of HH ligand expression on the constitutive HH signaling we observed in UC cells. We first knocked down the highest expressed HH ligand in each of the four UC cell lines and then monitored the expression of the HH target genes *GLII* and *PTCH1* as indicators of HH activity. HH attenuation reduced the expression of *GLII*, and in one case also *PTCH1*, in T24 and Vmcub1 cells (Figure 6A-B). Similar results were obtained using GLII protein as a readout of HH activity. HH inhibition also decreased the expression of *GLII* and *PTCH1* in HT1376 and J82 cells, though to a lesser extent (Supplemental Figure S6B-C). Next, we examined whether the intrinsic HH pathway activity of the UC cells would be enhanced by exogenous HH stimuli. Indeed, a HH-driven luciferase reporter gene was readily activated when T24 and Vmcub1 cells were engineered to overexpress *SHH* or exposed to a SMO agonist (Figure 6C and data not shown). Furthermore, this increased HH activation correlated with a dramatic increase in colony size when such cells were grown in an anchorage-independent manner (Figure 6D). These results suggest that HH pathway activity is maintained in UC cell lines via the production of HH ligands.

Ligand-dependent HH signaling is thought to require primary cilium (39). We examined and identified the presence of obvious primary cilia structures in at least one UC cell line, T24, and in the immortalized UROtsa cells. We next engineered two independent clonal T24 cell lines stably expressing a SMO-GFP

fusion protein, and asked whether SMO accumulated in primary cilia and whether this localization could be regulated by modulators of HH pathway activity. In such T24-derived cell lines, SMO-GFP enriched in primary cilia in about 50% of the cells, while GFP alone failed to accumulate at the primary cilia (Supplemental Figure S7A). The cilium-localized SMO was also activated, as it could be detected by an antiserum specific for activated phospho-SMO (Supplemental Figure S7B). These results were in accordance with the constitutive active HH signaling observed in T24 cells. We further measured SMO-cilia translocation in response to small molecule modulators of SMO. Consistent with what had been previously reported (40, 41), the SMO antagonist SANT1 decreased the accumulation of SMO in primary cilia while the SMO agonist SAG increased this localization (Figure 6E). These results support the model of ligand-dependent HH pathway activation in UC cells. Similar results were observed in UROtsa cells in which SMO localized to primary cilia in response to SAG treatment (Supplemental Figure S7C-D), consistent with them also being HH responsive (See Figure 1C-D).

We extended our findings to examine the level of GLI1 and SHH in human urinary bladders and UCs by immunohistochemistry and *in situ* hybridization (Figure 7, Supplemental Figure S8 and Table S2). In general, GLI1 and SHH were either negative or weakly positive in normal bladder urothelium, although strong positivity was sometimes observed in individuals with cystitis cystica syndromes (Supplemental Figure S9). However, enhanced GLI1 and SHH levels were observed in the majority of the UC samples examined and they tended to enrich in similar areas of the tumors. Particularly, in the cases where adjacent normal urothelium was available, GLI1 and SHH staining were either absent or confined to the basal layer of normal urothelium, but more enriched in the tumor cells. These results suggest that ligand-dependent HH pathway activation likely occurs in primary human UC samples.

## Discussion

We show here that HH signaling plays an important role in mediating the tumorigenic properties of UC cells both *in vitro* and *in vivo*. These UC cell lines appeared to maintain their intrinsic HH activity through the expression of HH ligands in an autocrine-like fashion. Our results from the UC cell lines were further validated in primary human bladder cancer samples, in which SHH and GLI1 levels were frequently found elevated in tumor cells but not in normal urothelial cells, consistent with the model of ligand-dependent HH pathway activation in primary human UC.

Our model of autocrine-like signaling in UC is inconsistent with the conclusions from a recent publication in which HH signaling was proposed to regulate the regenerative proliferation of murine urothelial cells through an indirect paracrine-like mechanism (42). In this later model, SHH production from the urothelial compartment acts on stromal cells to provide a feedback mechanism that regulates the proliferation of urothelial cells. They further suggested that this epithelial-to-mesenchymal HH signaling is how the pathway functions in mediating human bladder cancer. We show here that primary human UC cells express both SHH and GLI1, consistent with an epithelial-to-epithelial HH signaling mechanism. This result is reached by us and by another group (43), using both immunohistochemistry and *in situ* hybridization as detection methods. Moreover, unlike mouse urothelial stem cells which lack *GLII* expression, *GLII* is the most commonly expressed biomarker in human bladder cancer initiating cells (44). This difference in HH signaling between human and mouse is not restricted to that found in the urinary bladder, as similar observations have been made in other tissues such as prostate and colon (35, 45). However, our results cannot rule out the contribution epithelial-to-mesenchymal HH signaling might play in human UC.

We previously showed that the bladder carcinogen arsenic activates HH signaling (27). This finding was substantiated using a cohort of bladder cancer patient samples, correlating increased arsenic exposure with higher HH activity in primary bladder cancer samples. Here, we further ascertained the functional

significance of increased HH activity by arsenic in urothelial cell transformation. HH induction was both sufficient and necessary to allow urothelial cells to grow in an anchorage-independent manner. Notably, *SMO* depletion could partially reverse the growth of these arsenic-transformed cells, which was contradictory to our previous conclusion that arsenic activated HH signaling downstream of *SMO* (27). We speculate that a second HH-activating event, such as HH ligand expression, might have occurred to sustain a high-level of HH activity during UROtsa transformation by arsenic. Alternatively, it is possible that HH activation might arise from mechanistically distinct ways in response to the different arsenic species used in these studies (27, 28).

Here, we showed that a subset of UC cell lines harbor an active HH signaling pathway driven by ligand production. The cell lines used to obtain these findings, where known, were derived from MIUC (46, 47). However, we had previously shown a highly significant association between arsenic exposure in bladder cancer patients and increased HH activity in NMIUC, which we proposed was through loss of *GLI3* repressor (27). Consistent with a role of *GLI3* in NMIUC, certain SNPs of *GLI3* could serve as prognostic markers for poor survival of NMIUC patients (48). Moreover, loss of the *PTCHI* locus was also found primarily in NMIUC (20). Therefore, while HH pathway activity may be commonly required in human bladder cancer, such activation might result from multiple routes in different UC subtypes: (I) NMIUC acquires HH signaling activity primarily by genetic alterations of HH pathway components; (II) MIUC often elaborates HH signaling activity by HH ligand production. In this manner, HH signaling might play an initiating role in NMIUC but a maintenance role in MIUC, consistent with a suggestion that these two subtypes of UC might have distinct etiologies (49, 50). Regardless of the mechanism of activation, our results suggest that the therapeutic targeting of the HH signaling pathway will be beneficial for bladder cancer patients.

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## Figure Legends

**Figure 1. HH signaling is required for urothelial cell transformation.** (A) Relative expression of *GLII* (left) or *SMO* (right) was examined by qPCR in arsenic-transformed urothelial cells (URO-MSC52) and passage matched control cells (UROtsa) after transduction of scramble shRNA#1 or shRNAs specific for *GLII* or *SMO*. (B) Knockdown of *GLII* (left) or *SMO* (right) attenuates the anchorage-independent growth of URO-MSC52 cells. (C) UROtsa cells stably overexpressing SHH exhibit enhanced anchorage-independent growth. (D) UROtsa cells were treated with SAG (200 nM) or DMSO for 48 h before examining the expression of HH target genes by qPCR. Error bars: standard error of the mean (S.E.M.). #: statistically significant changes when comparing UROtsa to URO-MSC52 cells. \*: statistically significant changes when comparing within each cell line.

**Figure 2. HH signaling mediates the proliferation and survival of human UC cell lines.** (A) UC cell lines were transduced with indicated shRNAs. Cell proliferation was determined four days later and normalized to cells receiving scramble shRNA#1. (B) T24 cells overexpressing GFP or GLI1 were transduced with scramble shRNA#1 or *SMO* shRNA#3. Cell proliferation was determined three days after shRNA transduction. (C) UC cell lines were transduced with indicated shRNAs and caspase 3/7 activity was measured four days later. Error bars: S.E.M. \*: statistically significant changes comparing to control cells.

**Figure 3. Levels of HH pathway activity correlate with UC progression.** (A) The expression of *SMO* and *GLI1* was examined by qPCR in HH-independent cell lines (HT1376 and J82) and HH-dependent cell lines (Vmcub1 and T24), normalizing to the expression values of UROtsa cells (set to 1). Error bars: S.E.M. (B) An immunoblot for GLI1 from the indicated cell lines was performed after enriching for GLI proteins using the GLI-binding oligonucleotide beads (first four lanes) or the non-specific oligonucleotide (Ctrl, last lane). Equal loading was verified by immunoblotting for  $\alpha$ TUBULIN (TUB) in the original cell lysates. (C) A gene signature, consisting of 2507 genes differentially expressed between HH-dependent and HH-independent UC cell lines, correlated with UC progression. A meta-analysis was performed to compare this gene signature with gene signatures from available bladder cancer studies. Blue bar: positive correlation. Red bar: negative correlation. Triangle: number of overlapping genes. CIS: carcinoma *in situ*.

**Figure 4. HH signaling is required for UC tumor growth *in vivo*.** Vmcub1 (A and B) and HT1376 (C) cells were transduced with control shRNAs or two shRNAs targeting either *GLI1* (A) or *SMO* (B and C). Equal amount of viable cells were then injected subcutaneously in the flanks of athymic nude mice (N =

7-10 mice per group). Tumor volumes were measured for a total of 40 days. Error bars: S.E.M. \*: statistically significant changes comparing to control-shRNA transduced cells.

**Figure 5. HH ligand-production is required for UC cell proliferation.** (A) The *PTCH1* locus (highlighted in red box) is retained in UC cell lines. Virtual karyograms of chromosome 9 were generated based on a high-density SNP array analysis. The red or blue triangles denote loss or gain of chromosomal regions respectively. (B) Methylation analysis for a region of the *PTCH1* promoter. Numbers denote CpG islands within this promoter region. An arrow denotes the position of the GLI binding sites. (C) The indicated UC cell lines were transduced with either control shRNAs or shRNAs targeting *SHH*, *IHH* or *DHH*. Cell proliferation was determined four days after shRNA transduction and normalized to the cells receiving scramble shRNA#1. Error bars: S.E.M. \*: statistically significant changes comparing to control-shRNA transduced cells.

**Figure 6. The constitutive HH signaling in UC cell lines is ligand-dependent.** *SHH* or *DHH* levels were knocked down in T24 (A) or Vmcub1 (B) cells respectively. Changes in the expression of the HH target genes, as well as the shRNA targeted transcripts, were measured by qPCR (top). The immunoblots for GLI1 were performed after GLI enrichment (bottom). Equal loading was verified by immunoblotting for  $\alpha$ TUBULIN (TUB) in the original cell lysates. (C) A GLI-driven luciferase plasmid and a constitutive Renilla plasmid were cotransfected with or without *SHH* overexpression in T24 or Vmcub1 cells. Luciferase activity was measured 48 h after transfection and normalized to Renilla activity. (D) Vmcub1 cells stably overexpressing either GFP or SHH were grown in soft agar. Shown is the quantification for the number of colonies larger than 500  $\mu$ m in diameter (per 35mm dish). (E) Quantification of SMO localization to primary cilia in a T24 clone isolate stably expressing SMO-GFP. Cells were treated with DMSO, SANT1 (100 nM) or SAG (100 nM) before performing the

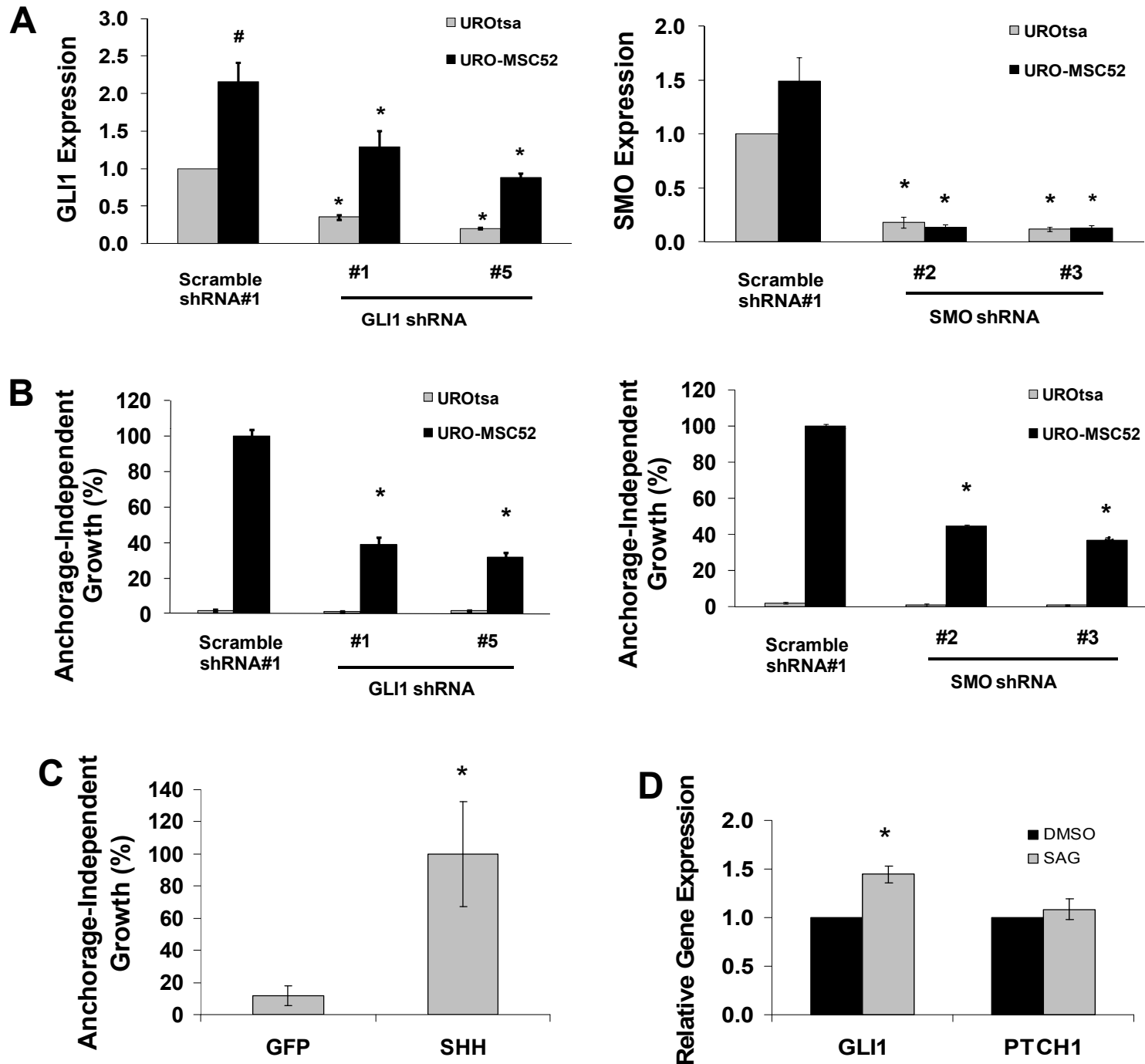
immunostaining and quantification. At least 150 ciliated cells were counted in each treatment group.

Error bars: S.E.M.. \*: statistically significant changes comparing to the control cells.

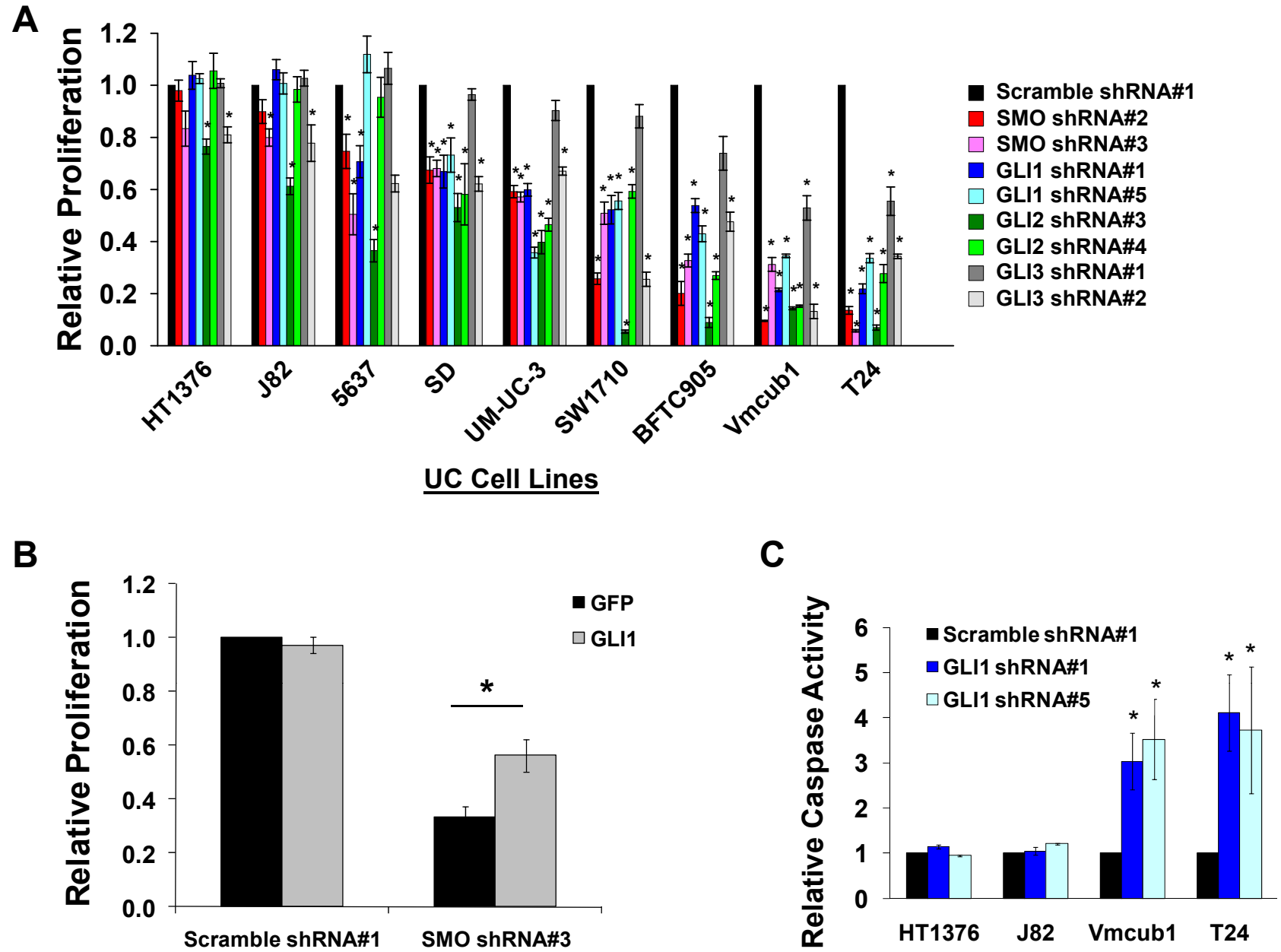
**Figure 7. GLI1 and SHH are enriched in primary human UC samples.** H&E or

immunohistochemical staining for GLI1 and SHH was performed in malignant and normal human bladder samples. Shown are representative cases of: a high grade T1 tumor (UC#1, A-C), a high grade T2 tumor (UC#2, D-F), the matched adjacent normal urothelium to UC#2 (G-I), as well as one case of normal bladder (J-L). (A, D, G, J) H&E; (B, E, H, K) GLI1 staining; (C, F, I, L) SHH staining. The staining results are summarized in Supplemental Table S2.

**Figure 1**

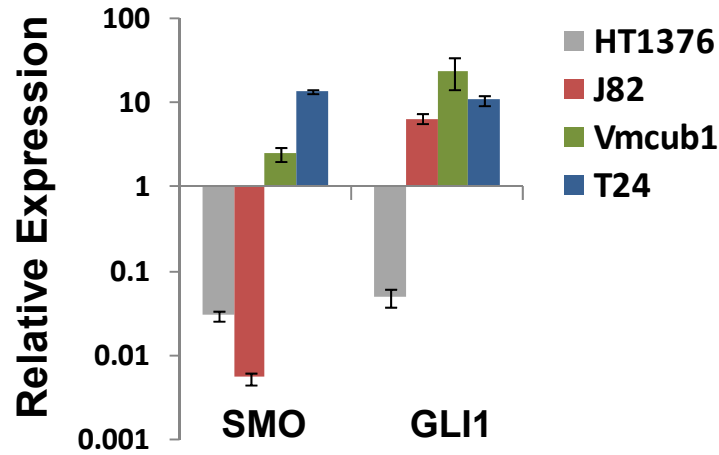


**Figure 2**

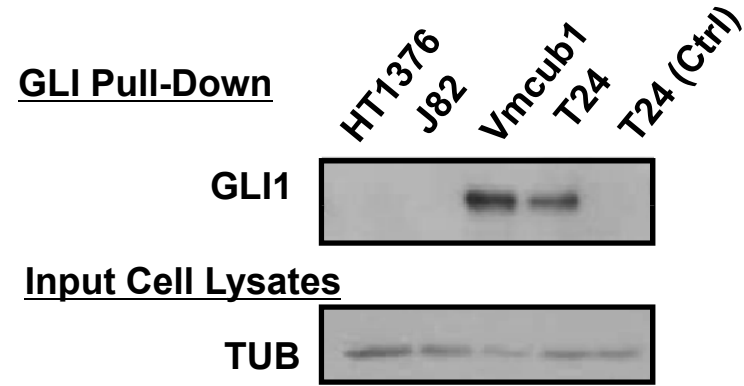


**Figure 3**

**A**



**B**



**C**

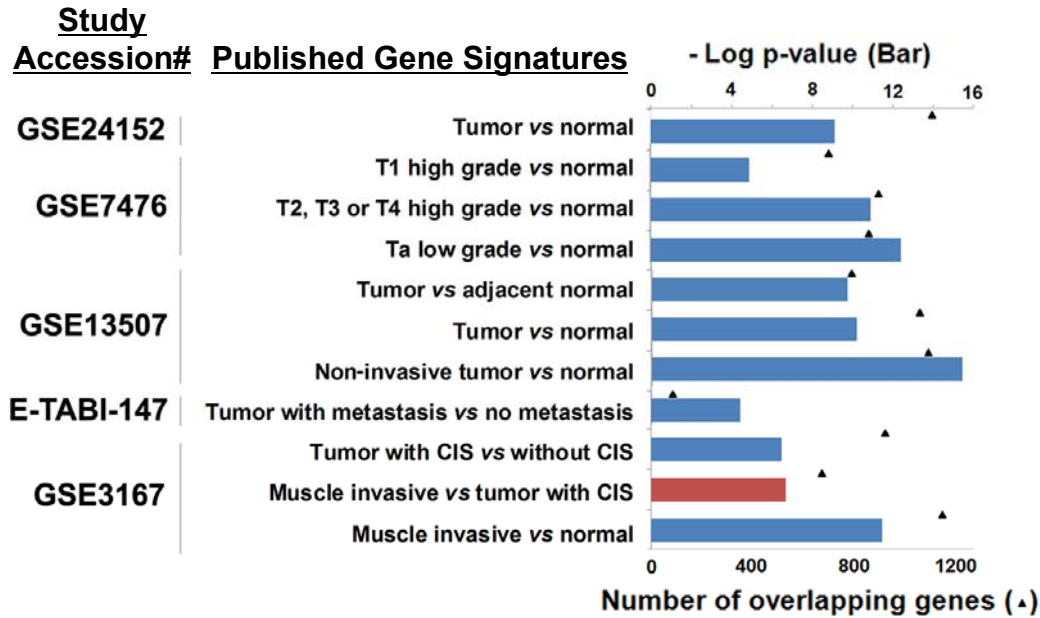
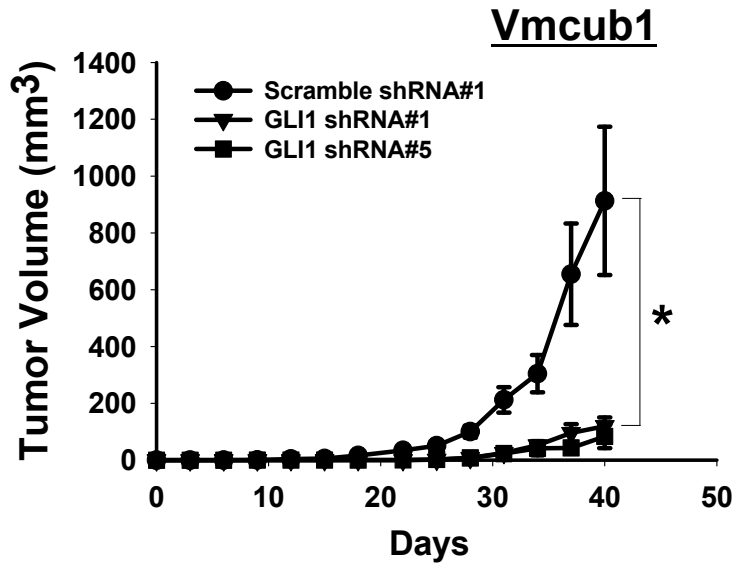
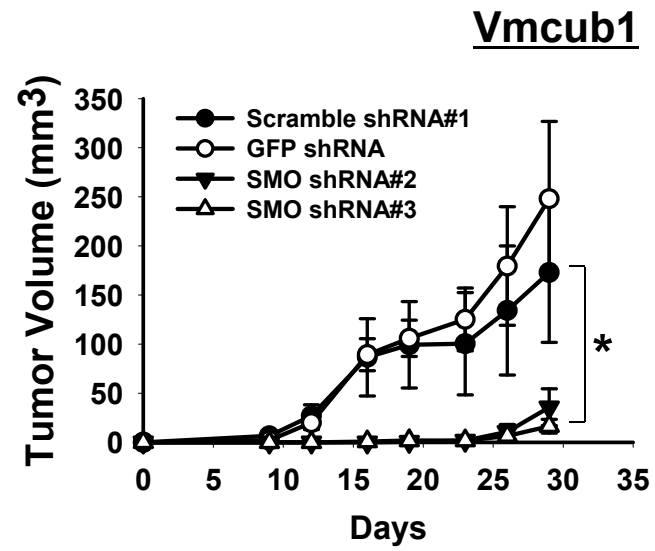


Figure 4

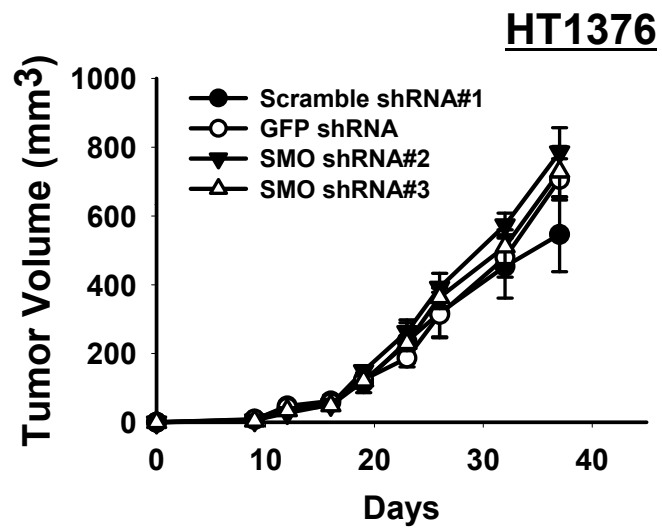
A



B

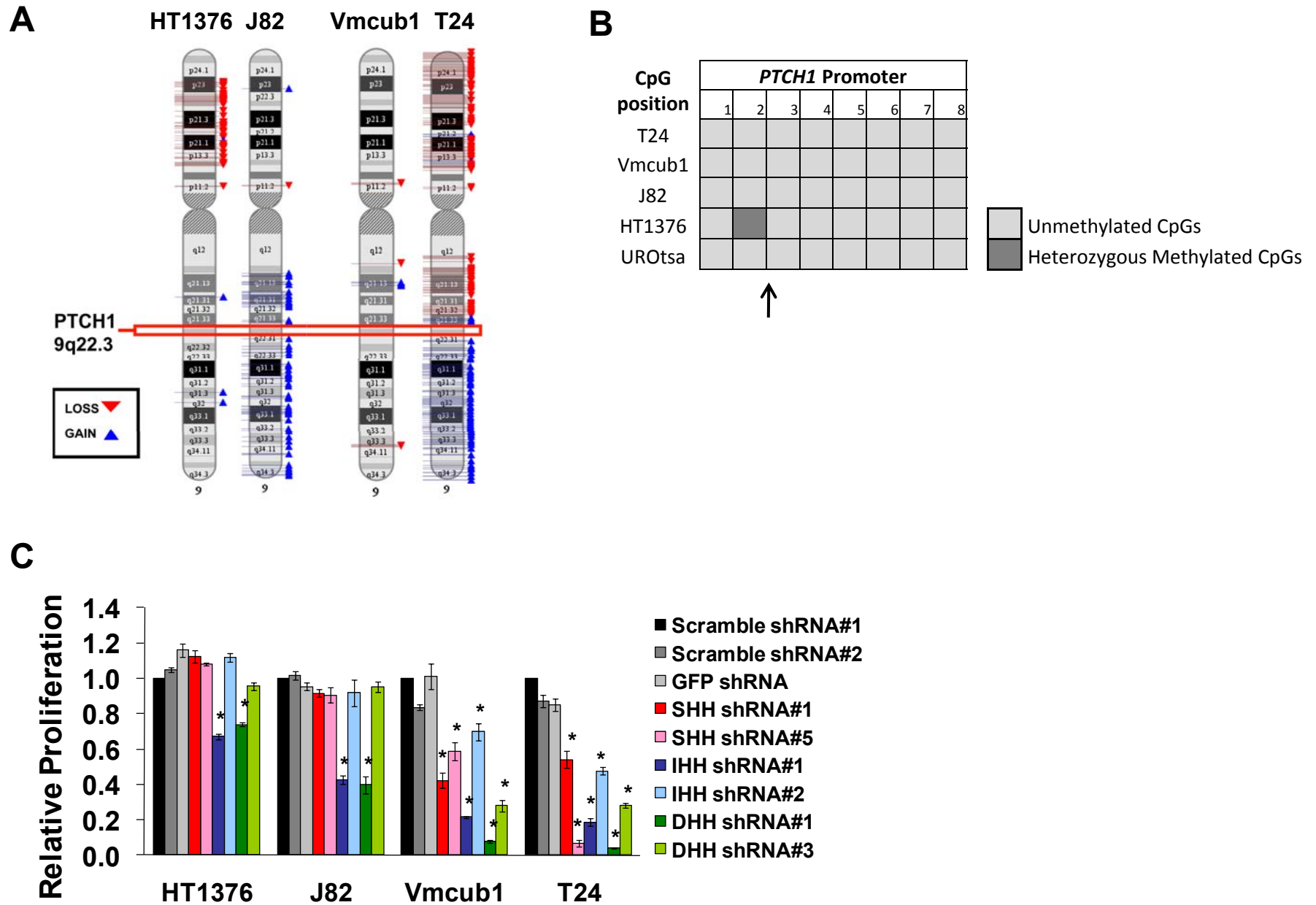


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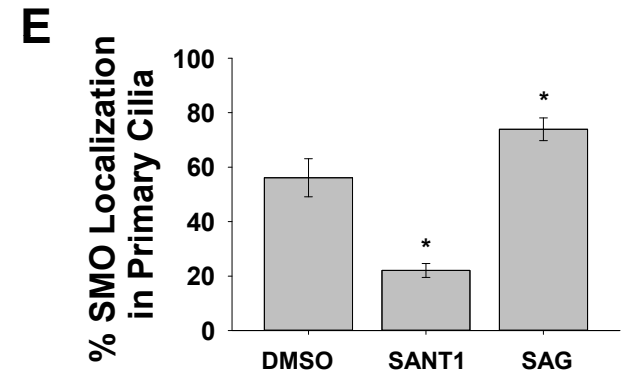
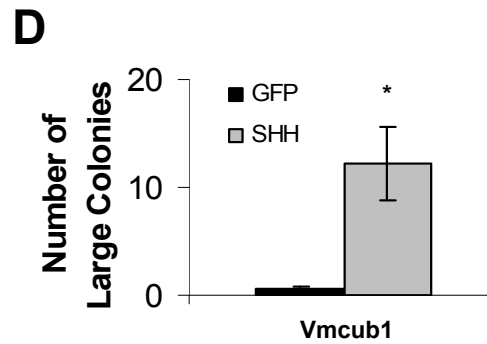
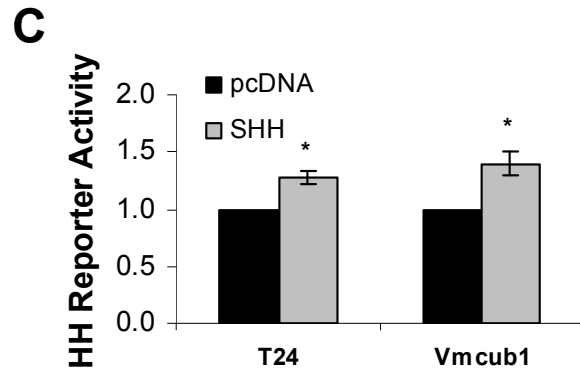
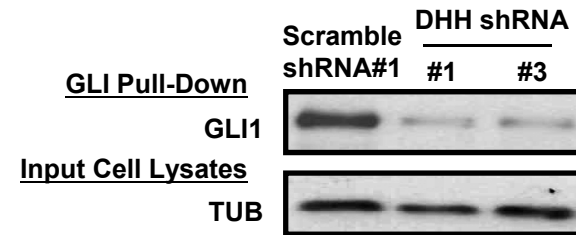
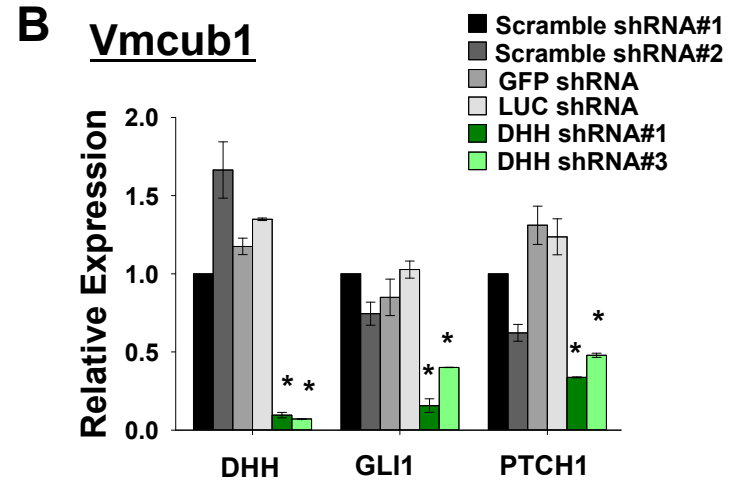
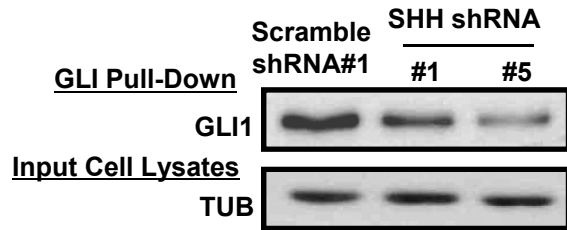
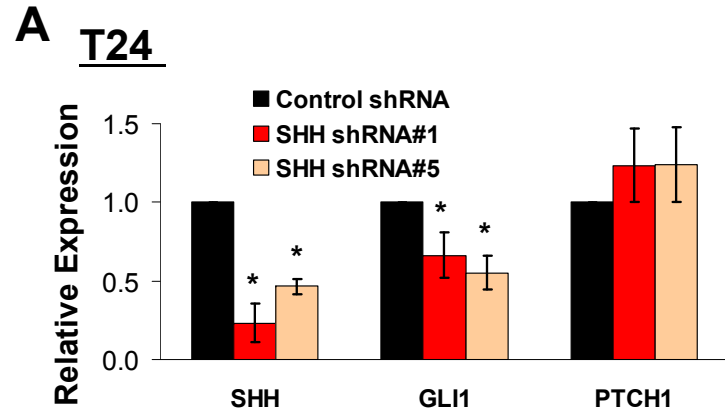




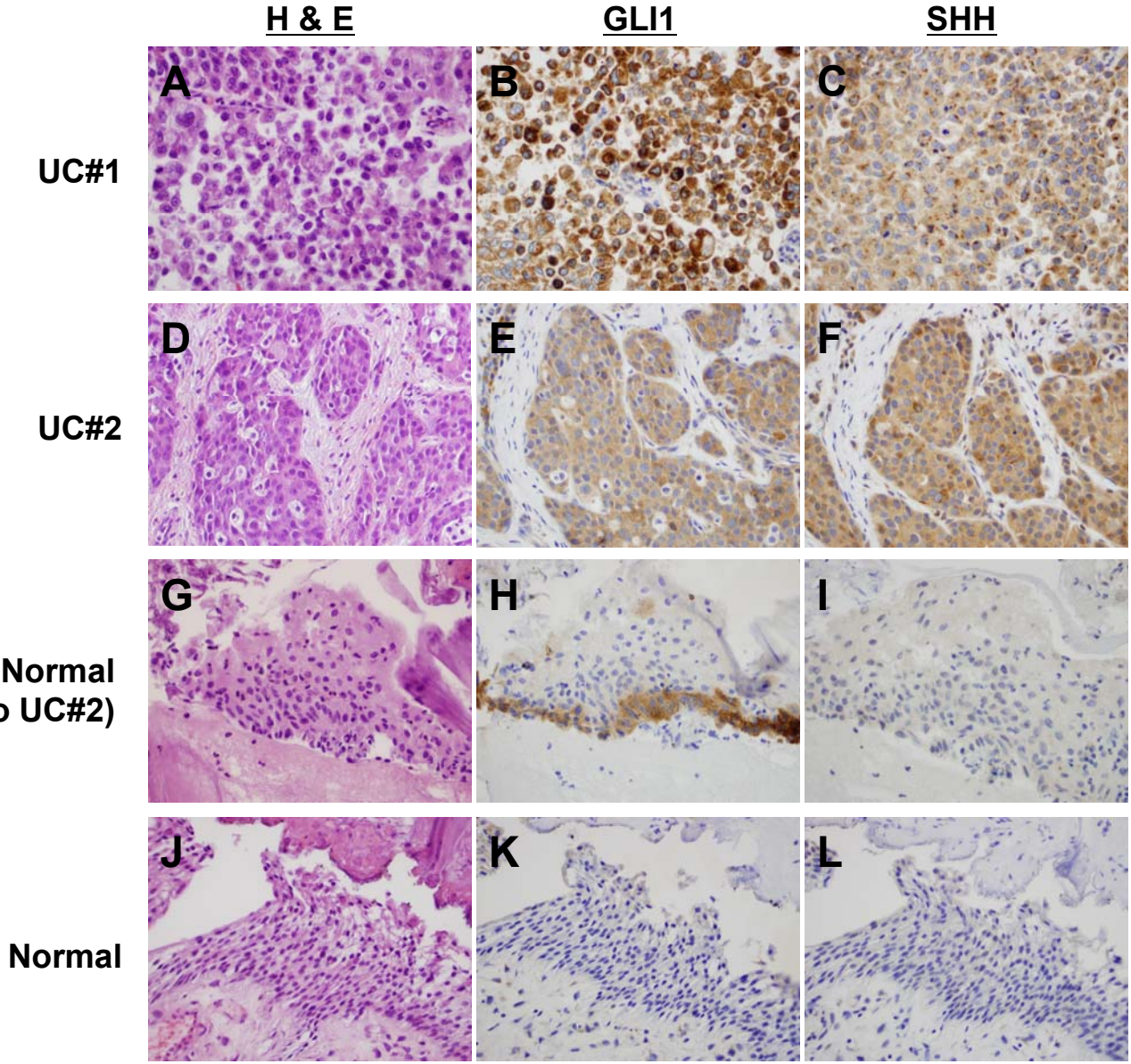
**Figure 5**



**Figure 6**



**Figure 7**



# Cancer Research

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## Hedgehog Signaling Regulates Bladder Cancer Growth And Tumorigenicity

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