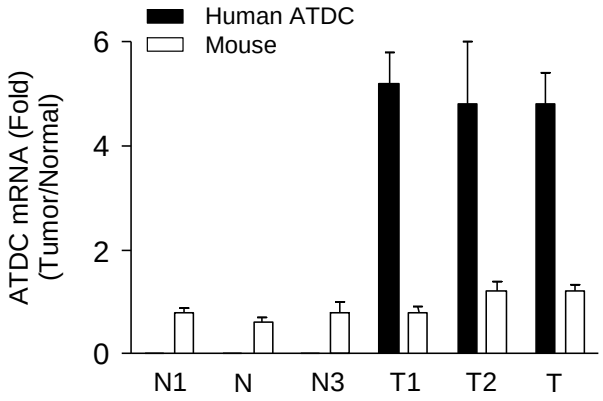
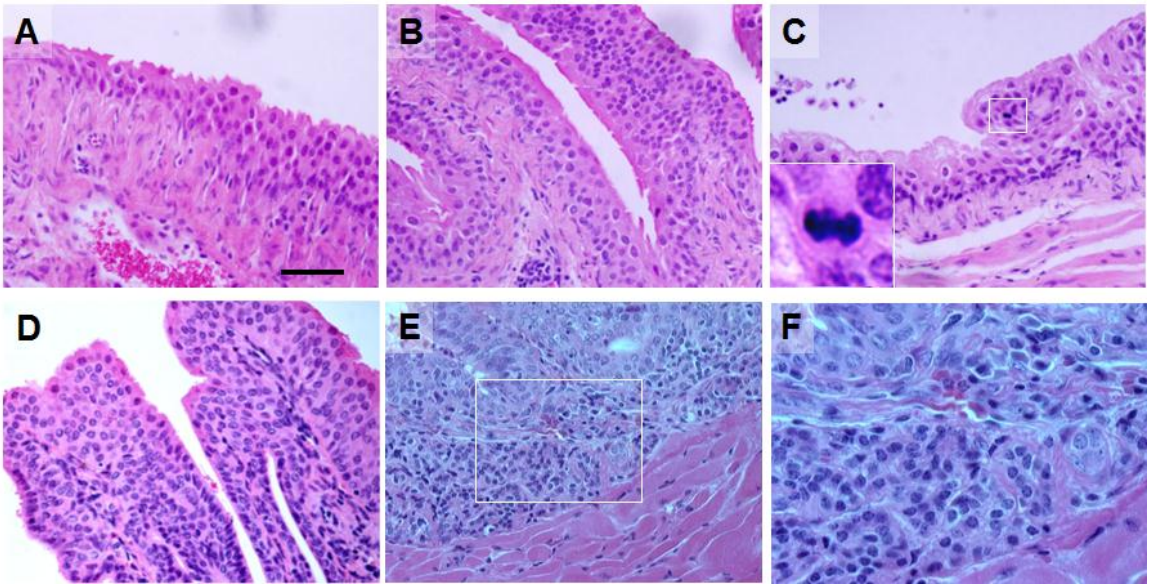
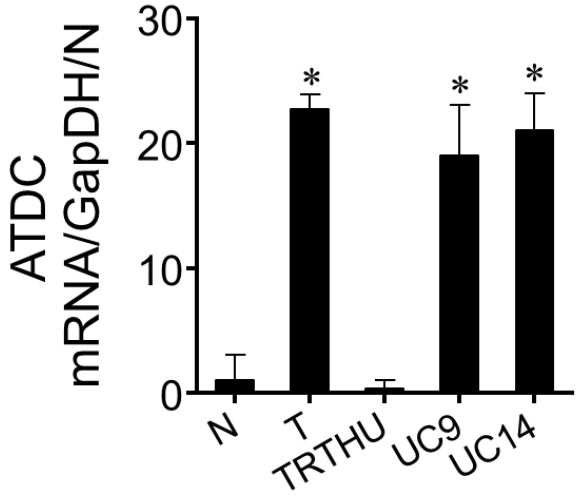
Supplemental Figures



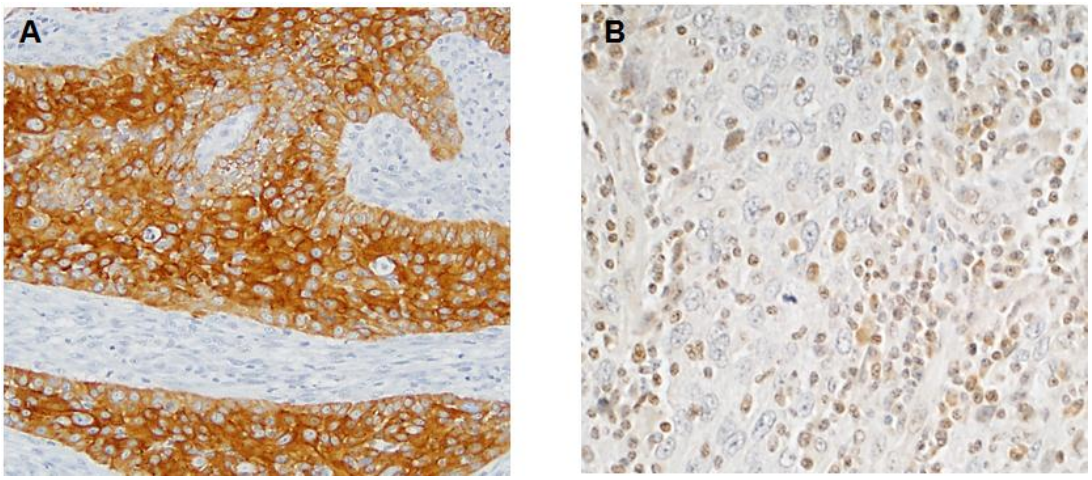
Supplemental Figure 1. ATDC mRNA expression in normal littermate (N) or CAG-ATDC transgenic bladders. Black bars indicate expression of human ATDC transgene transcript. White bars indicate expression of mouse endogenous ATDC mRNA (n=3, mean±SE).



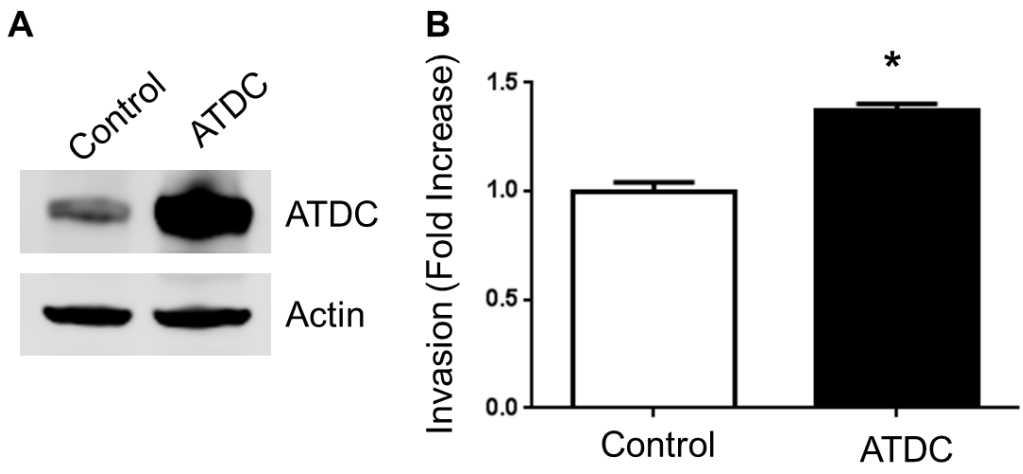
Supplemental Figure 2. Bladder urothelial lesions in CAG-ATDC transgenic line A of 8-12 months of age. (A) Bladder urothelial hyperplasia. (B) Bladder non-invasive papillary urothelial neoplasm of low malignant potential. (C) Bladder urothelial dysplasia. Inset: mitotic figures. (D) Bladder superficial papillary tumor. (E) Muscle invasive bladder cancer. Insert is area magnified in F. (F) High magnification of figure E. A-D are of the same magnification. Bar, 50 μm



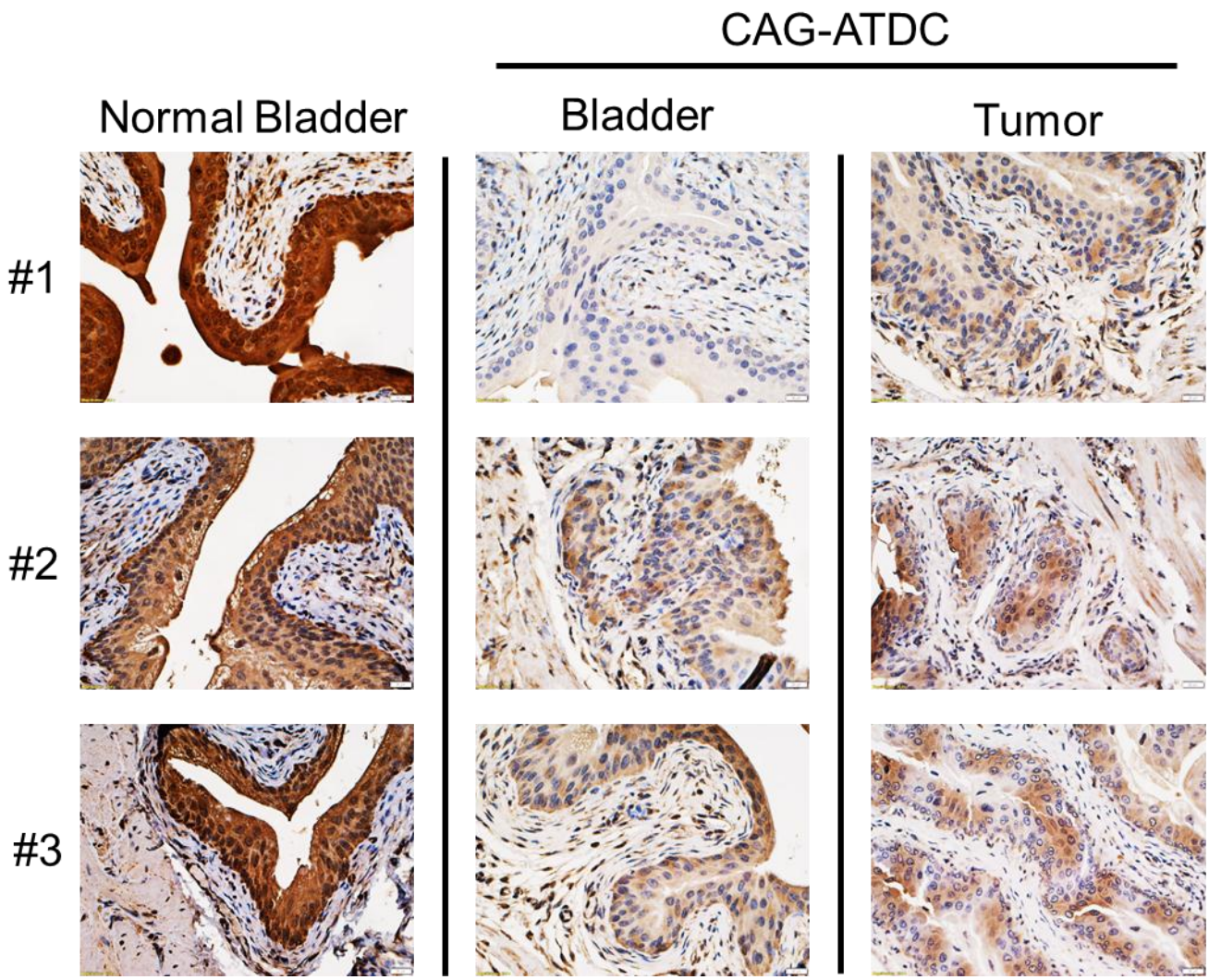
Supplemental Figure 3. ATDC mRNA levels in CAG-ATDC tumors (T) are similar to human bladder cancers (UC9 and UC14) and ~20 fold higher than normal mouse (N) or human urothelial cell (TRTHU) expression. Mean±SE, n = 3 for each column, * $p < 0.05$ compared to N.



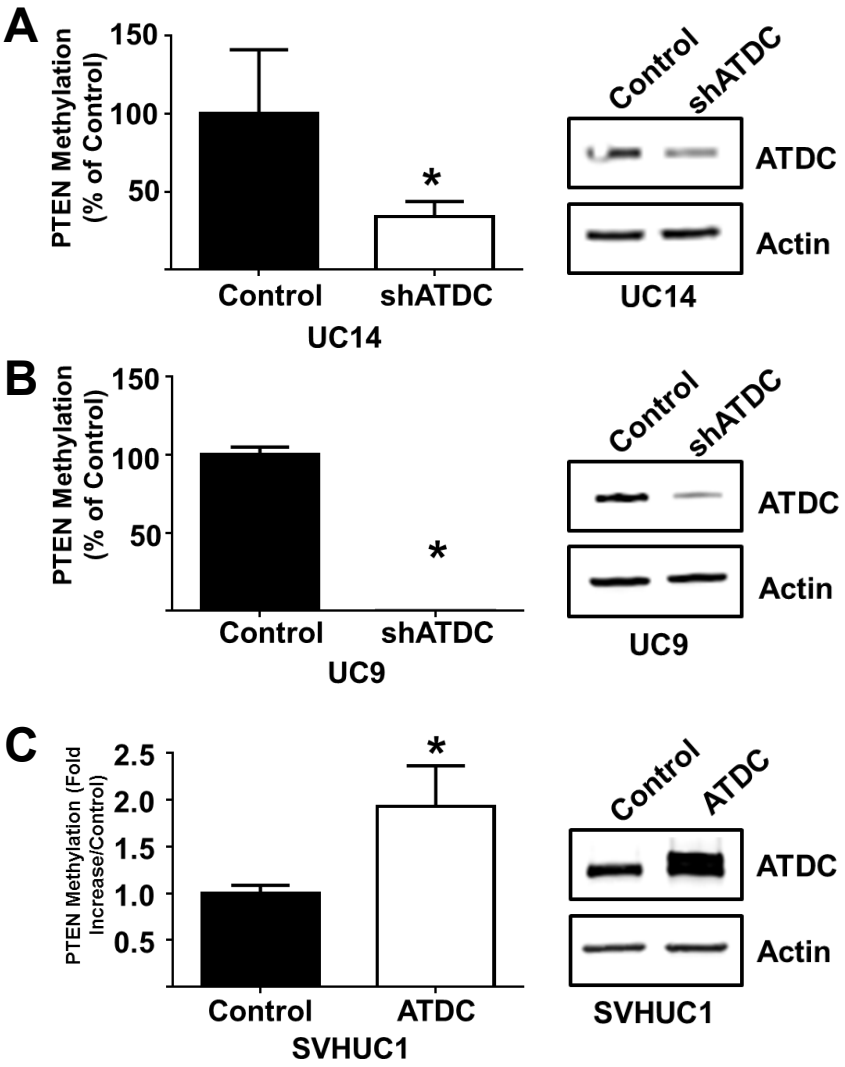
Supplemental Figure 4. ATDC immunostaining in two representative human invasive urothelial carcinomas demonstrates predominantly cytoplasmic staining (A) in most samples with rare tumors showing nuclear localization (B).



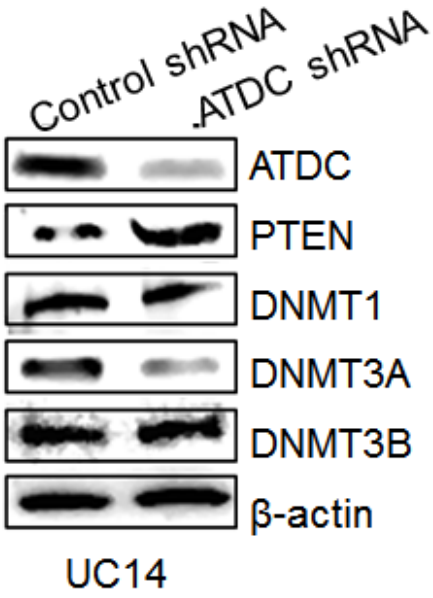
Supplemental Figure 5. ATDC overexpression drives invasion in UC10 bladder cancer cell line. (A) Transduction of UC10 with control or ATDC expression lentiviral vectors results in increased ATDC expression as measured by western blot. (B) Increased ATDC expression resulted increased invasion as measured by boyden chamber assay. Invasion is plotted as fold increased compared to control. ($n = 5$, * mean±SE, $p < 0.0001$).



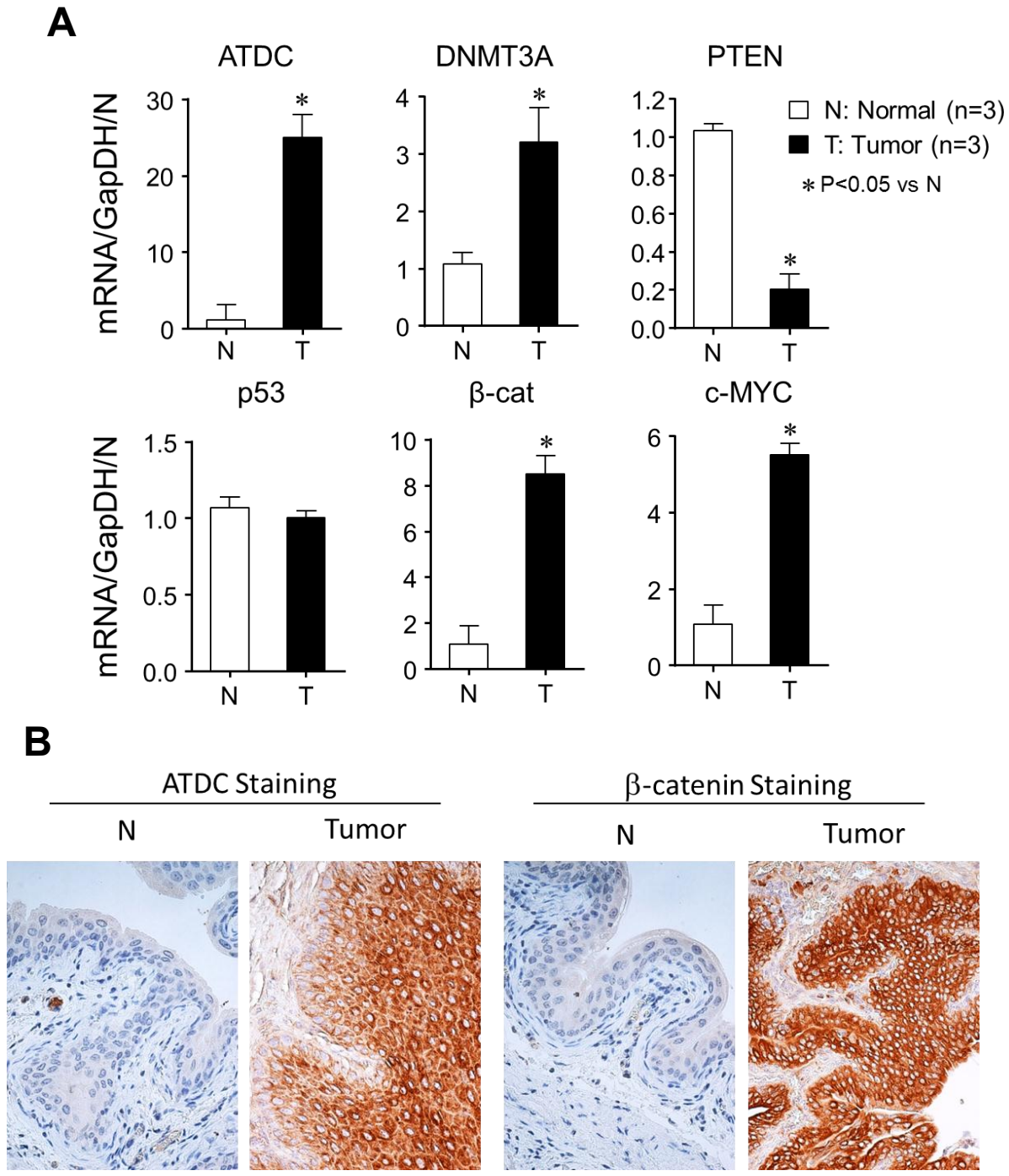
Supplemental Figure 6. PTEN immunostaining in three representative images of normal mouse bladder, CAG-ATDC mouse bladder or CAG-ATDC mouse tumor specimens demonstrating decreased PTEN expression in ATDC transgene expressing tissues



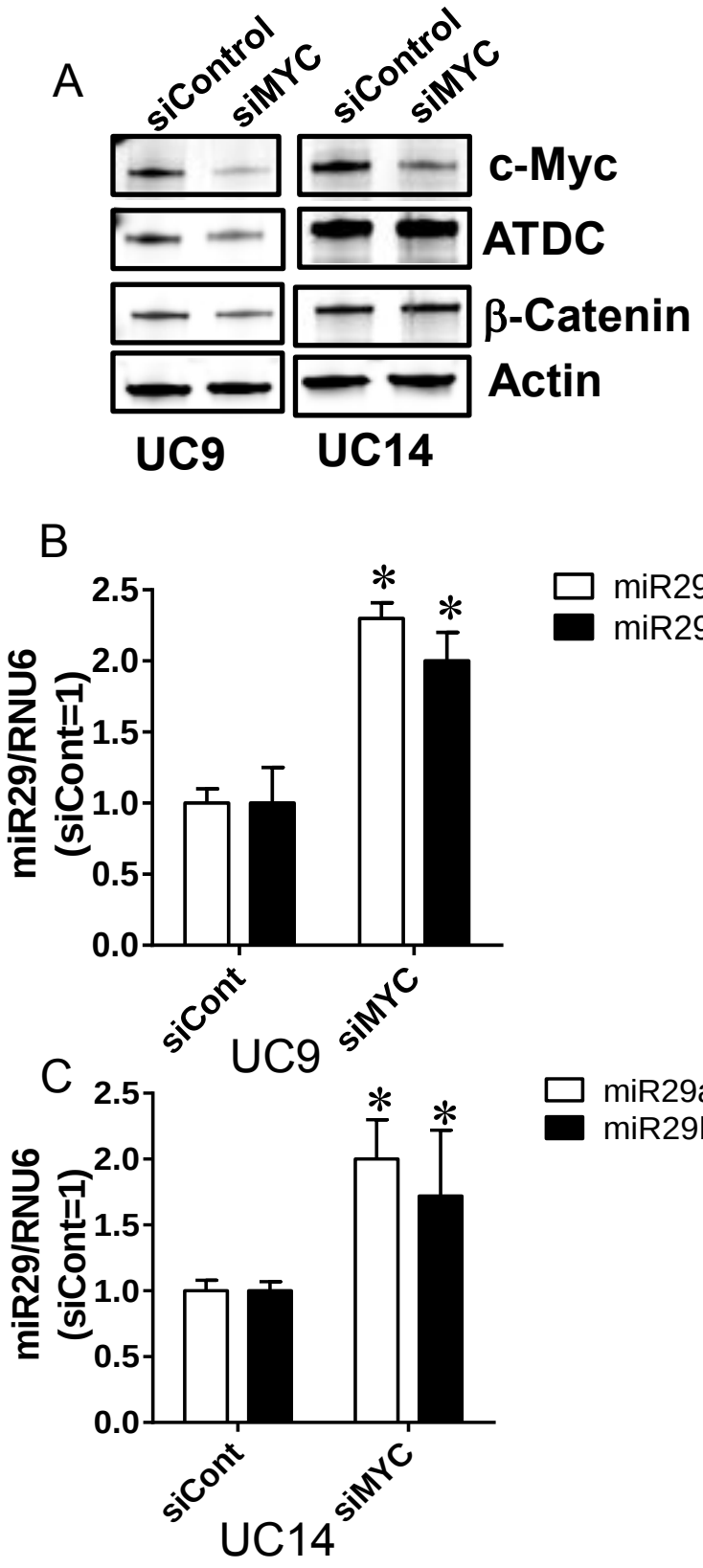
Supplemental Figure 7. PTEN Quantitative methylation specific PCR. (A) Knockdown of ATDC in UC14 bladder cancer cell lines by targeting shRNA as compared to scrambled control reduced PTEN promoter methylation in the UC14 cell line (n = 3 samples per column, * indicates p = 0.05) (B) Knockdown of ATDC by targeting shRNA as compared to non-coding control reduced PTEN promoter methylation in UC9 (n = 3 samples per column, * indicates $p < 0.001$). (C) Overexpression of ATDC but not control vector in SVHUC1 cells resulted in increased PTEN promoter methylation (n = 3 samples per column, p = 0.02). All error bars represent standard deviation.



Supplemental Figure 8. Knockdown of ATDC results in reduction in DNMT3A, but not DNMT1 or DNMT3B in UC14 cell line as measured by immunoblot.



Supplemental Figure 9. ATDC overexpression up-regulates DNMT3A, β-catenin and c-MYC mRNA expression and suppresses expression of PTEN. P53 mRNA levels are unaffected. (A) qRT-PCR analysis of mRNA levels in normal littermate control bladders (N) vs CAG-ATDC tumor (T). n = 3 for N and T, * indicates p value < 0.05 compared to N, mean±SE. (B) ATDC and β-catenin IHC in normal littermate control bladders and CAG-ATDC tg bladder tumors.



Supplemental Figure 10. Knockdown of MYC increases miR-29A and B1 in bladder cancer cell lines. (A) Western blot demonstrating effective Myc knockdown in UC9 and UC14 bladder cancer cells. (B and C) Transfection of MYC-targeting but not control siRNA resulted in increased miR-29A and B1 levels as measured by TaqMan qRT-PCR (mean±SE, n=3, * $p < 0.05$ vs siCont). .

Supplemental Table 1. Summary of bladder urothelial lesions in CAG-ATDC mouse line A.

	Percentage of total	Number of cases
Flat Urothelial Lesions		
Hyperplasia	14%	3/21
Dysplasia	24%	5/21
Carcinoma in situ	10%	2/21
Papillary Urothelial Lesions		
Urothelial papilloma	10%	2/21
PUNLMP	10%	2/21
Non-invasive urothelial carcinoma	14%	3/21
Invasive Urothelial Carcinoma		
Invasive urothelial carcinoma	19%	4/21

PUNLMP: non-invasive papillary urothelial neoplasm of low malignant potential

Supplemental Table 2. ATDC is significantly over-expressed in human bladder cancer.

	ATDC Staining					
	Absent	Low	Moderate	High	Total	Moderate and High (% of total)
Normal	21	6	1	0	28	4
Non-invasive tumor	6	12	4	1	23	22
Carcinoma in situ	4	0	2	2	8	50
Invasive Tumor	78	41	73	62	252	53

Supplemental Table 3. Genes up-regulated in all three CAG-ATDC invasive bladder tumors.

ADAMTS2	APOC1	CEACAM1	CYP1B1	FBN1	IL33	MCF2L	PENK	SOCS3	TOP2A
ADAMTSL2	APOD	CILP	CYP26B1	FLT1	KCNK2	MCM4	PER1	SPON1	TPSAB1
ADCY7	ASPN	CNTN1	CYR61	GFPT2	LOX	MFAP5	PIK3AP1	SPON2	VCAN
AHSP	C4B	COL1A1	DUSP1	HELLS	LOXL1	NID1	PLA1A	TFPI2	VWF
ALAS2	CD163L1	COL1A2	ELN	HIGD1B	LOXL2	PADI1	PLVAP	THBS1	WISP2
ANGPT4	CD38	COL5A2	EXOC3L2	IGF1	LY6E	PADI4	RND3	TMEM100	
ANKRD1	CD93	COL8A1	FAM101B	IGFBP2	MAFF	PAPPA	SCARB1	TMEM173	
ANXA3	CDT1	CTGF	FBLN2	IGJ	MASP1	PCSK5	SNAI1	TNC	

Supplemental Table 4. Genes up-regulated in CAG-ATDC bladder cancers show significant overlap with the top 5-10% of overexpressed genes in bladder cancer in the Oncomine database.

Associated Concepts:								
Concept Type	Concept Name	Overlap	P-Value	Q-Value	Odds Ratio	Dataset	Expression	Top %
Oncomine Gene Expression Signatures	Bladder Urothelial Carcinoma Type: Infiltrating Bladder Urothelial Carcinoma - Top 10% Over-expressed (Stransky Bladder)	31	1.03E-18	4.39E-15	13.5	Stransky Bladder	Over-expressed	10
Oncomine Gene Expression Signatures	Bladder Urothelial Carcinoma Type: Infiltrating Bladder Urothelial Carcinoma - Top 5% Over-expressed (Sanchez-Carbayo Bladder 2)	26	1.88E-16	3.62E-13	11.4	Sanchez-Carbayo Bladder 2	Over-expressed	5
Oncomine Gene Expression Signatures	Bladder Urothelial Carcinoma Type: Infiltrating Bladder Urothelial Carcinoma - Top 10% Over-expressed (Modlich Bladder 2)	33	2.89E-15	3.88E-12	8	Modlich Bladder 2	Over-expressed	10
Oncomine Gene Expression Signatures	Bladder Urothelial Carcinoma Type: Infiltrating Bladder Urothelial Carcinoma - Top 10% Over-expressed (Lee Bladder)	32	2.56E-13	1.95E-10	6.6	Lee Bladder	Over-expressed	10

Supplemental Table 5. Molecular concepts showing enrichment with expression signature of genes over-expressed in CAG-ATDC bladder cancers through Oncomine analysis.

Concept #	Concept ID	Concept Name	Concept Type	Concept Size	Overlap	P-Value	Odds Ratio	Dataset	Expression
	NA	Over-expressed in CAG-ATDC bladder tumors	PRIMARY CONCEPT	77	NA	NA	NA	NA	NA
1	10147	Bladder Cluster ID n9869 (Modlich 2)	Oncomine Clusters	878	32	6.04E-19	11.4	Modlich Bladder 2	
2	122139909	Bladder Urothelial Carcinoma Infiltrating vs. Superficial (Stransky Bladder)	Oncomine Gene Expression Signatures	806	31	1.03E-18	13.5	Stransky Bladder	Top 10% over-expressed
3	122139850	Bladder Urothelial Carcinoma Infiltrating vs. Superficial (Sanchez-Carbayo Bladder 2)	Oncomine Gene Expression Signatures	631	26	1.88E-16	11.4	Sanchez-Carbayo Bladder 2	Top 5% over-expressed
4	122139789	Bladder Urothelial Carcinoma Infiltrating vs. Superficial (Modlich Bladder 2)	Oncomine Gene Expression Signatures	1262	33	2.89E-15	8	Modlich Bladder 2	Top 10% over-expressed
5	122202785	Bladder Urothelial Carcinoma Infiltrating vs. Superficial (Lee Bladder)	Oncomine Gene Expression Signatures	1884	32	2.56E-13	6.6	Lee Bladder	Top 10% over-expressed
6	122147305	Bladder Cluster ID n3488 (Sanchez-Carbayo 2)	Oncomine Clusters	45	9	2.51E-12	50.5	Sanchez-Carbayo Bladder 2	
7	27633	Bladder Urothelial Carcinoma N+ vs. N- (Stransky Bladder)	Oncomine Gene Expression Signatures	806	24	1.38E-11	7.9	Stransky Bladder	Top 10% over-expressed

The top 77 genes over-expressed in CAG-ATDC bladder tumors were uploaded into the Oncomine database to generate a concept for automated analysis for enrichment by disproportionate overlap. The over-expressed in CAG-ATDC bladder tumor concept (PRIMARY CONCEPT) and visualized concepts in Figure 3 showing significant interaction are listed. For each concept, the concept number in Figure 3, the Oncomine ID, name, type, size (number of genes in the concept), number of genes overlapping with the PRIMARY CONCEPT , P-value of the overlap by Fisher's exact test and Odds ratio, Oncomine dataset, expression and percentage cutoff in the expression signature are given.

Supplemental Table 6. ATDC and DNMT3A IHC Expression Correlation.

	DNMT3A (# cores)			
ATDC (IHC Staining Intensity)	**1**	**2**	**3**	**Total**
1	4	7	6	17
2	6	13	7	26
3	5	29	14	48
4	1	14	14	29
Total	16	63	41	120

Jonckheere-Terpstra Test p-value = 0.0361

Supplemental Materials and Methods

Construction of CAG-ATDC Expression Vector

Full length human ATDC cDNA with unique restriction *Hind III* and *Bgl II* sites was generated by PCR with the primers 5'-AAGCTTCACCCTGCGATGGAAG-3', 5'-AGATCTTTGGCCTCTGAGCACAGGAATGAT-3' using an ATDC-pBluescript vector as a template (provided by Dr. J.P. Murnane, University of California, San Francisco), and subcloned into p3XFLAG-CMV-10 vector (Sigma, Saint Louis, Missouri). A 1.8 kb FLAG-ATDC fragment with unique restriction *EcoR I* and *Bgl II* sites was created by PCR with primers 5'-GGCACTGGGGAGGGGTCACA, 5'-GCGTGTACGGTGGGAGGTCTA-3' using the ATDC- p3XFLAG-CMV-10 vector as a template. The FLAG-ATDC fragment was amplified and inserted at *EcoR I* and *Bgl II* restriction sites downstream of the CMV enhancer and the chicken β-actin promoter in the pCAGGS expression vector (purchased from BCCM/LMBP, Ghent University, Gent-Zwijnaarde, Belgium). The pCAGGS vector contains a bipartite promoter, consisting of the 384-bp cytomegalovirus (CMV) immediate early (IE) enhancer fused to the 284-bp chicken β-actin promoter, followed by the 917-bp chicken β-actin intron 1, and a rabbit β-globin poly A (Figure 1A) (1). The orientation and fidelity of the FLAG-ATDC transgene construct was confirmed by restriction digestion and DNA sequencing. FLAG-ATDC specific primers: sense primer 5'-CAA AGACCATGACGGTGATTATAAAGATCA-3' located in flag (P1) and an antisense primer 5'-GGAGTTCTTGTCGTCCCCGGACTCGAC-3' in human ATDC fragment (P2) were used to confirm presence or absence of construct in transgenic animals.

Quantitative RT-PCR

Total RNA isolation, generation of cDNA and RT-PCR was performed utilizing TaqMan Fast Universal PCR Master Mix and TaqMan Gene Expression assay probes for human *ATDC* (Hs00232590_m1), human ribosomal protein S6 (RPS6) (Hs02339423_g1), mouse *Atdc* (Mm01175102_m1), beta-catenin (Hs00355049_m1), *CMYC* (Hs00153408_m1) and mouse *Rprs6* (Mm02342456_g1) (Applied Biosystems, Carlsbad, CA) as previously described (2). All reactions were performed in triplicate. *ATDC* mRNA expression was normalized to endogenous ribosomal protein S6.

Methylation Specific PCR

Equal amounts of genomic DNA from the normal bladder from control littermates, *CAG-ATDC* mouse invasive bladder cancer samples, or from bladder cancer cell lines were modified by treatment with sodium bisulfite using the EZ DNA Methylation^{TM} Kit (Zymo Research Corporation, Orange, CA). PCR was carried out using the Promega PCR Master Mix (Promega, Madison, WI), and approximately 50 ng bisulfite-modified DNA. PCR products were visualized using standard methods. MSP experiments were performed in duplicate.

The sequences of the methylated and unmethylated primer pairs were: 5'-GGT TTC GGAGGT CGT CGG C-3'(sense) and 5'-CAA CCG AAT TAC TACTAC GAC G-3' (antisense), yielding a 155-bp product for the methylated reaction, and 5'-TGG GTTTTG GAG GTT GTT GGT-3' (sense) and 5'-ACT TAA CTCTAA ACC ACA ACC A-3' (antisense), yielding a 173-bp product for the unmethylated reaction. PCR conditions were initial denaturing at 95°C for 2 min, followed by 35 cycles of denaturing at 94°C for 30s, annealing respectively at 58°C and 56°C for 30s and polymerization at 72°C for 50s, with final extension for 7 min at 72°C.

Quantitative Methylation Specific PCR

Total genomic DNA was purified from cell lines using AllPrep DNA/RNA Mini Kit (Qiagen, Valencia, CA). 30 ng of DNA from each cell line was restriction digestioned according to protocol for EpiTect II DNA Methylation Enzyme Kit (Qiagen, Valencia, CA) overnight. PTEN promoter sequence was quantitated using SYBR Green ROX (Qiagen, Valencia, CA) PCR reagents and the Applied Biosystems ViiA 7 thermocycler and primers specific for the PTEN CpG island (EPHS101755-1A, Qiagen, Valencia, CA). The data was analyzed and compared to control DNA per EpiTect II Methylation Kit protocol (Qiagen, Valencia, CA).

Affymetrix Gene Expression Profiling Analysis

Total RNA from mouse normal or bladder cancer samples was extracted using the Trizol reagent (Invitrogen, Carlsbad, CA), followed by application of the sample to a RNeasy spin column (Qiagen, Inc., Valencia, CA). According to the protocol of RNA amplification and biotin labeling for microarray analysis, biotinylated aRNA (amplified RNA) was prepared from 250 ng total RNA using the MessageAmpTM Premier RNA Amplification Kit (Applied Biosystems/Ambion, Austin, TX). Following labeling, 16 μg of aRNA was hybridized for 16 hr at 45^{o}C on an Affymetrix GeneChip Mouse 430 2.0 Array. GeneChips were washed and stained in the Affymetrix Fluidics Station 450, then scanned using the Affymetrix 3000 7G GeneChip Scanner with Autoloader. Data was analyzed using the Limma and AffyBioconductor packages implemented in the R-statistical language. Robust Multi-Array (RMA) was used to normalize the data and fit log2 transformed expression values (3).

Supplemental References

1. Niwa H, Yamamura K, Miyazaki J. Efficient selection for high-expression transfectants with a novel eukaryotic vector. Gene 1991;108(2):193-9.
2. Wang L, Heidt DG, Lee CJ, Yang H, Logsdon CD, Zhang L, et al. Oncogenic function of ATDC in pancreatic cancer through Wnt pathway activation and beta-catenin stabilization. Cancer Cell 2009;15(3):207-19.

3. Irizarry RA, Hobbs B, Collin F, Beazer-Barclay YD, Antonellis KJ, Scherf U, et al. Exploration, normalization, and summaries of high density oligonucleotide array probe level data. Biostatistics 2003;4(2):249-64.