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Supporting Information for

Loss of UTX/KDM6A and the activation of FGFR3 converge to regulate differentiation gene expression programs in bladder cancer

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SI Materials and Methods

RT-qPCR analysis: RNA was isolated from cells using the RNeasy Mini Kit (Qiagen, 74104). For qPCR, cDNA was produced from that RNA using Reverse Transcriptase (Applied Biosystems, 4368814), and qPCR was performed using the Applied Biosystems StepOnePlus system with SYBR green dye. The ΔΔCt method was used for quantification where the Ct value of an experimental sample was normalized to the Ct value of GAPDH (ΔCt), and then this value was then normalized to the mean ΔCt for a control sample (ΔΔCt). Lastly, 2^(-ΔΔCt) was used as the relative expression value for that sample. The qPCR primers that were used for the uroplakin genes have been previously described (1).

Chromatin immunoprecipitation for histone post-translational modifications (fragmentation by sonication): Three days after plating, UMUC1 cells were harvested from 15 cm tissue culture plates at ~75% confluency. For each histone ChIP, ~ $1x10^7$ cells were harvested which yielded ~25 µg of chromatin DNA for immunoprecipitation after going through this protocol. For cell harvest, media was aspirated, cells were washed once in PBS, and then they were fixed directly on the plate with 1% paraformaldehyde in PBS for 5 minutes at room temperature with gentle shaking. Glycine, at a final concentration of 125 mM, was added directly to the plate, and the plate was incubated for 5 minutes at room temperature with gentle shaking. Cells were then put on ice, washed once with cold PBS, scraped off of the plate, and the fixed cells from each cell line (empty vector, wild-type UTX, and HEAA UTX) were pelleted. To get a soluble chromatin extract, cells were then washed in a series of buffers, LB1, LB2, and LB3 as specified below. Each cell line was resuspended in 1 mL LB1 buffer (50 mM HEPES pH 7.5, 140 mM NaCl, 1 mM EDTA, 10% glycerol, 0.5% NP-40, 0.25% Triton X-100, 1x protease inhibitor (Sigma, 11836170001)) and incubated while rotating at 4°C for 10 min. Samples were then centrifuged and resuspended in 1 mL LB2 buffer (10 mM Tris-HCl pH 8.0, 200 mM NaCl, 1 mM EDTA, 0.5 mM EGTA, 1x protease inhibitor), and then were incubated while rotating at 4°C for 10 min. Samples were then centrifuged and resuspended in 1 mL LB3 buffer (10 mM Tris-HCl pH 8.0, 100 mM NaCl, 1 mM EDTA, 0.5 mM EGTA, 0.1% Na deoxycholate, 0.5% N-lauroylsarcosine, 1% Triton X-100, 1x protease inhibitor). The lysates were then passed through a 27-gauge needle three times, sonicated for 14 minutes using a Covaris E220 ultra-sonicator at peak power 140 W, duty factor 5%, and cycles per burst 200, then centrifuged for 10 minutes at 20,000xG at 4°C and the supernatants were collected. 20 µL of each lysate was kept to measure DNA concentration, while the remainder was flash frozen and stored at -80°C. The 20 µL sample was added to 200 µL ChIP elution buffer (1% SDS, 50 mM Tris-HCl pH 8.0, 10 mM EDTA, 200 mM NaCl), incubated overnight at 65°C, treated with RNaseA for 1 hour at 37°C, then Proteinase K for 1 hour at 55°C, and the DNA was purified using the Qiagen PCR purification kit (28104) and lastly quantified with a Nanodrop. These quantifications were then used to dilute 25 µg of chromatin into 1 mL total of LB3, and 5% (50 µL) of each sample was set aside as an input sample. Then 75 µL of protein A Dynabeads that had been washed 3X with PBS + 0.01% Tween-20 and pre-bound with either 10 µL H3K27ac (Active Motif, 39133), 10 µL H3K4me1 (Abcam, ab8895), or 20 µL H3K27me3 (Cell Signaling Tech, 9733) antibody were added to each chromatin lysate and this mixture was incubated rotating overnight at 4°C. The beads were sequentially washed on a magnet two times each with low salt buffer (150 mM NaCl; 0.1% SDS; 1% Triton X-100; 1 mM EDTA; 50 mM Tris-HCl pH 8.0), high salt buffer (500 mM NaCl; 0.1% SDS; 1% Triton X-100; 1 mM EDTA; 50 mM Tris-HCl pH 8.0), LiCl buffer (150 mM LiCl; 0.5% Na deoxycholate; 0.1% SDS; 1% Nonidet P-40; 1 mM EDTA; 50 mM Tris-HCl pH 8.0) and once with TE buffer (1 mM EDTA; 10 mM Tris-HCl pH 8.0). After the TE wash the beads were spun down (not separated on the magnet), and 210 µL ChIP elution buffer was added to the beads and input samples. The samples were then incubated for 30 min at 65°C on

a shaking heat block at 800 rpm. After centrifugation the eluate (supernatant) was reverse cross-linked by incubating overnight at 65°C. The eluate was then treated with RNaseA for 1 hr at 37°C then with Proteinase K for 1 hr at 55°C and DNA was recovered using the Qiagen PCR purification kit. Libraries were then prepared from this DNA using the Illumina TruSeq protocol and sequenced on a NextSeq500 sequencer.

Chromatin immunoprecipitation for UTX and MLL4 (fragmentation with micrococcal nuclease): Three days after plating, UMUC1 cells were harvested from 15cm tissue culture plates at ~75% confluency. For each UTX or MLL4 ChIP, ~$3x10^7$ cells were harvested which yielded ~75 µg of chromatin DNA for immunoprecipitation after going through this protocol. The cells were then fixed as described for the histone modification ChIPs described above. The fixed cells for each sample were resuspended in 20 mL of PBS with 0.5% NP-40 and 1x protease inhibitors, then incubated on ice for 10 minutes and pelleted. Each pellet of fixed cells was divided into two tubes, so there were ~$1.5x10^7$ cells per tube, and these cell pellets were snap frozen. After thawing the cells, each tube of cells was then resuspended in 600 µL of room temperature micrococcal nuclease (MNase) reaction buffer (50 mM HEPES pH 7.5, 2 mM $CaCl_2$, 0.2% NP-40). Before fragmenting the ChIP samples, the specific lot of MNase (Worthington, LS004798) was tested and a specific amount of MNase (~3-5uL) was determined in order to obtain fragments primarily 300-900 base pairs in length. The 600 µL of resuspended cells were then divided into three tubes of 200 µL and each tube of cells was treated with MNase for 3 minutes at 37°C. The reactions were stopped by adding 20 µL of 0.5M EDTA pH 8.0 and putting the samples on ice. The cells were centrifuged at 13,000xG for 1 minute and the supernatant was removed. The two tubes of cells for each sample were combined and these cells were resuspended in 200uL SDS lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris-HCl pH 8.1, 1x protease inhibitors). Then 800 µL of ChIP dilution buffer (0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris-HCl pH 8.1, 167 mM NaCl, 1x protease inhibitors) was added to each sample, which was then sonicated for 5 minutes using the Bioruptor sonicator. The sample was centrifuged at 20,000xG for 10 minutes at 4°C and the supernatant was transferred to a new tube. Then 20 µL was removed to measure the amount of DNA as described above, and the rest was snap frozen and stored at -80°C. After quantifying the DNA concentration for each sample, 75 µg of chromatin was diluted into 2 mL total of ChIP dilution buffer, and 5% (100 µL) of each sample was set aside as an input sample. Then 75 µL of protein A Dynabeads that had been washed 3X with PBS + 0.01% Tween-20 and pre-bound with 20 µL UTX (Cell Signaling, 33510) or MLL4 (Sigma, HPA035977) antibody were added to each chromatin lysate and this mixture was incubated rotating overnight at 4°C. The beads were then sequentially washed on a magnet two times each with low salt buffer, high salt buffer, LiCl buffer and once with TE buffer (see histone modification ChIP section for buffer compositions). After the TE wash the beads were spun down (not separated on the magnet), and resuspended in 200 µL elution buffer (1% SDS, 0.1 M $NaHCO_3$). 100 µL elution buffer was also added to each 100 µL input sample. Samples were incubated at 65°C for 30 minutes while shaking at 800 rpm. Then 8 µL of 5M NaCl was added to each sample before reverse crosslinking overnight at 65°C. The eluate was then treated with RNaseA for 1 hr at 37°C then with Proteinase K for 1 hr at 55°C and DNA was recovered using the Qiagen PCR purification kit. Libraries were prepared using the NEBNext Ultra II DNA Library Prep kit, and then sequenced on a NextSeq500 sequencer.

Colony formation in soft agar: Both 1% and 0.7% agar were heated until fully dissolved, then equilibrated to 42°C in a water bath. 2X EMEM media (with 20% FBS) was prepared from powder (Corning, 50-011-PB) and heated to 42°C. The 1% agar was then mixed 1:1 with 2X EMEM and 1.5 mL of this mixture was added to each well of a 6-well plate, and the plate was left to dry at room temperature. UMUC1 cells were then trypsinized and washed once in 1X EMEM media. For each cell line 6 wells were plated per experiment, and enough cells to have

10,000 cells/well were put into a tube in 100 µL of 1X EMEM media. A 1:1 mixture of 0.7% agar and 2X EMEM was then added to these cells and 1.5 mL (10,000 cells) was added to each well. Once the top layer solidified, 1 mL of 1X EMEM (with FBS) was added on top of the agar, and the plate was incubated, changing the media twice a week, for 3-4 weeks until colonies were seen. The plates were then scanned, and the number and size of colonies were quantified from these images using the MetaMorph image analysis software.

Cell proliferation: Multiple 96 well plates were seeded with 500 cells per well in 200 µL. After at least three hours on the same day, cell density of a plate was measured using the CellTiter-Glo Luminescent Cell Viability Assay (Promega, G7572) - this was considered Day 0 and all future days were normalized to this measurement. Cell density of the additional plates was then measured on the specified days. For experiments involving drug treatments, only one plate was seeded with 500 cells in 100 µL, and then the drug was added on the second day in 100 µL to bring the total volume to 200 µL. Cell density was quantified after 4 days and normalized to the DMSO-treated condition.

Figure S1

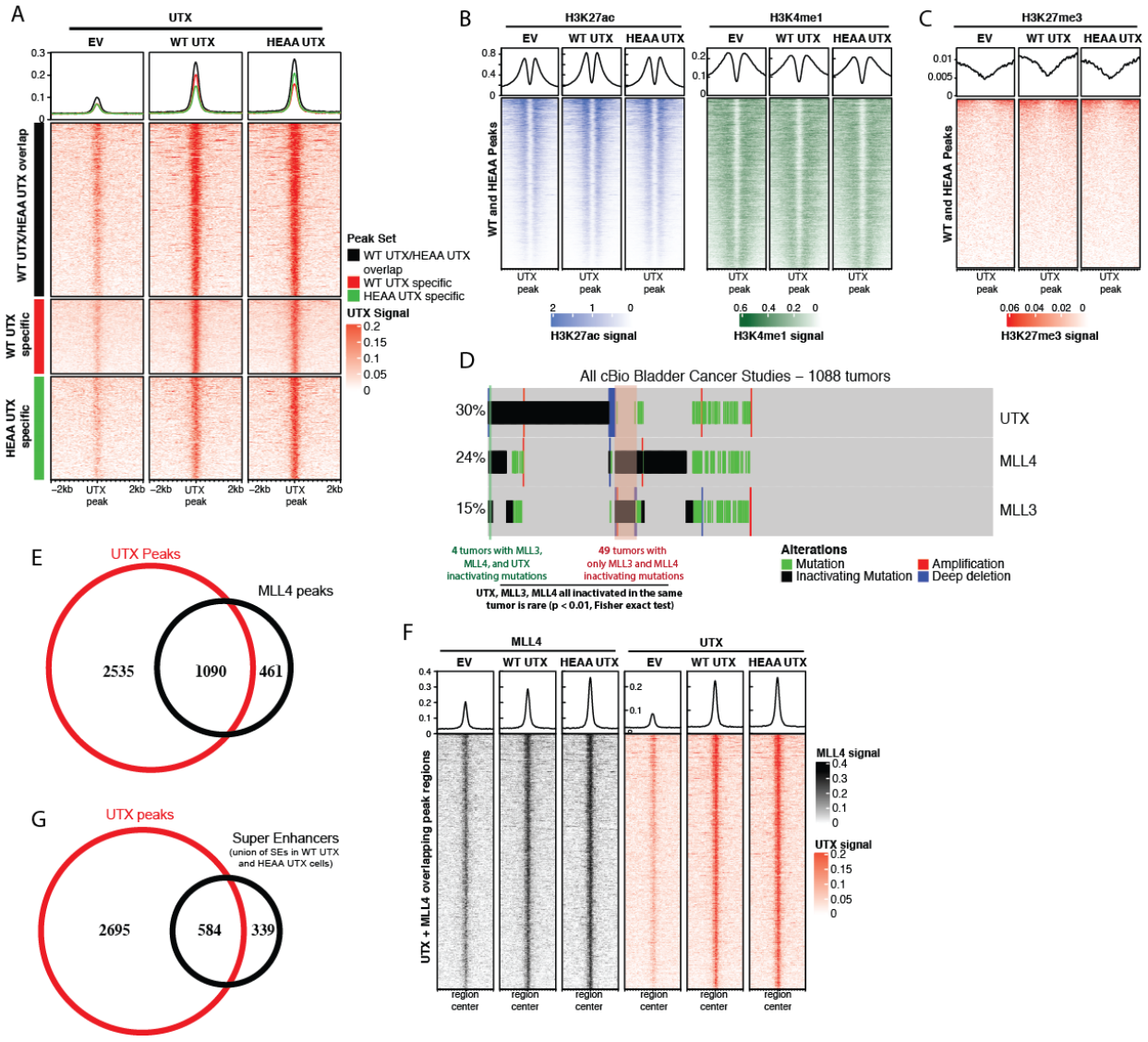


Fig. S1. UTX acts as a tumor suppressor in bladder cancer cells through a catalytic-independent mechanism and localizes to enhancers of luminal genes

A) Quantification of ChIP-seq read signal for UTX 2kb upstream and 2kb downstream from the center of UTX peaks in either empty vector (EV) control, wild type (WT) or HEAA mutant UTX expressing UMUC1 cells. Peaks are grouped based on whether they were called in the wild type UTX cells only, HEAA UTX cells only, or in both.

B) Quantification of ChIP-seq signal for histone H3K27ac and H3K4me1 2kb upstream and 2kb downstream from the center of UTX peaks in either empty vector (EV) control, wild type (WT) or HEAA mutant UTX-expressing UMUC1 cells.

C) Quantification of ChIP-seq signal for histone H3K27me3 2kb upstream and 2kb downstream from the center of UTX peaks in either empty vector (EV) control, wild type (WT) or HEAA mutant UTX-expressing UMUC1 cells.

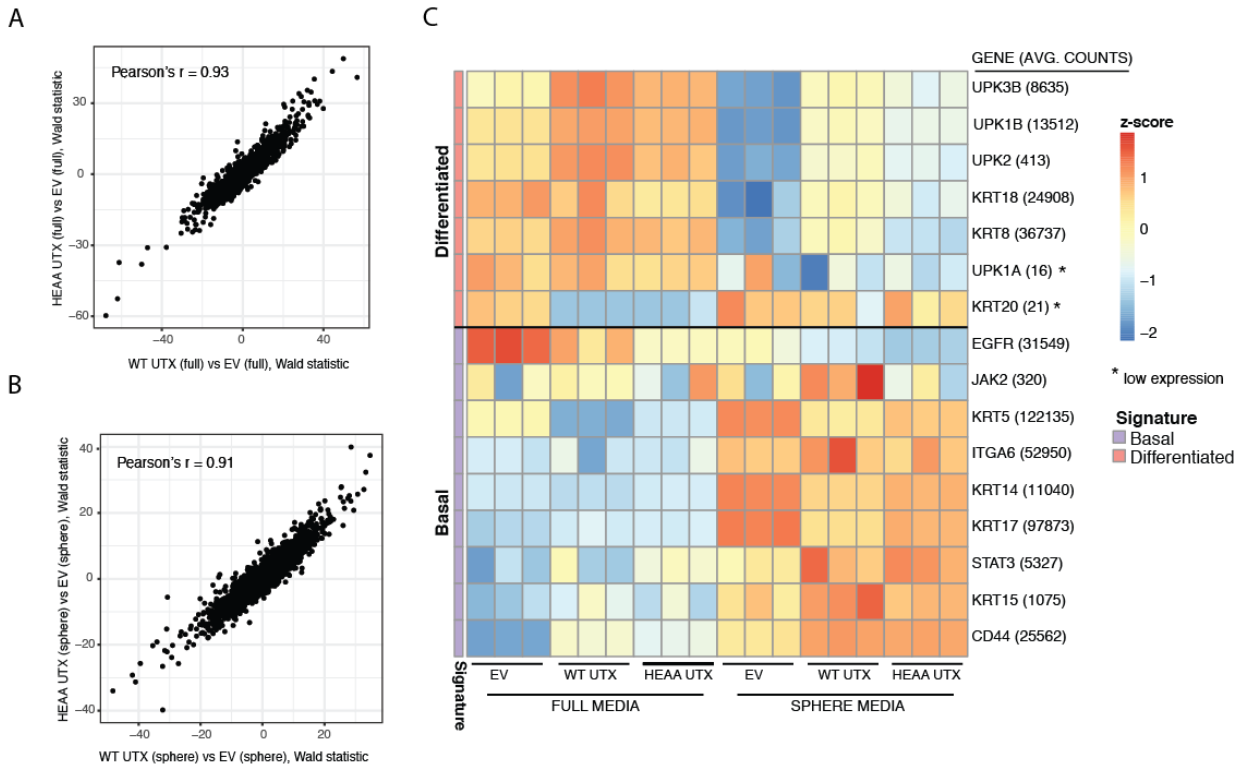
D) Mutational spectrum of 1088 bladder cancers available on the cBioPortal.

E) Venn diagram showing overlap between UTX ChIP-seq peaks (either in wild type (WT) or HEAA UTX cells) and MLL4 peaks from either empty vector (EV) control, wild type (WT) or HEAA mutant UTX-expressing UMUC1 cells.

F) Quantification of ChIP-seq signal for MLL4 and UTX 2kb upstream and 2kb downstream from the center of UTX and MLL4 overlapping peak regions identified in E.

G) Venn diagram showing overlap between UTX ChIP-seq peaks (either in wild type (WT) or HEAA UTX cells) and 'super enhancers' (as defined by the ROSE algorithm) from either wild type (WT) or HEAA mutant UTX-expressing UMUC1 cells.

Figure S2



E

Clone Label in Figure 2D	Clone Label in Figure S2D	UTX KO Clone #	UTX/UTY KO Clone #
Control - 1	7-4	7	4
Control - 2	7-16	7	16
UTX/UTY KO - 1	9-5	9	5
UTX/UTY KO - 2	9-19	9	19
UTX/UTY KO - 3	17-16	17	16
UTX/UTY KO - 4	17-22	17	22

Fig. S2. UTX hinders transition to more stem-like cellular state by regulating bladder differentiation genes

A) Wild type (WT) and HEAA mutant UTX gene expression changes in full media (both versus empty vector (EV) cells) are plotted.

B) Wild type (WT) and HEAA mutant UTX gene expression changes in tumorsphere media (both versus empty vector (EV) cells) are plotted.

C) Heatmap representing z-scores for the expression of genes from the tumor differentiation gene signature to distinguish basal and luminal tumors (2) in UMUC1-derived cell lines in both regular and tumorsphere media conditions. Genes that were not attributed reads in the RNA-seq experiment were not included.

D) Western blot analysis of sequential clonal isolations of RT4 cells infected with Cas9 and gRNAs for UTX (left) and UTY (right). UTX knockout clones that were used for subsequent UTY knockout (left) or UTX and UTY double knockout clones used for qPCR experiments (right) are indicated with an arrow.

E) Nomenclature for the labels of UTX/UTY knockout clones was changed to a simpler version for Figure 2D, and the conversions of the labels are shown here.

Figure S3

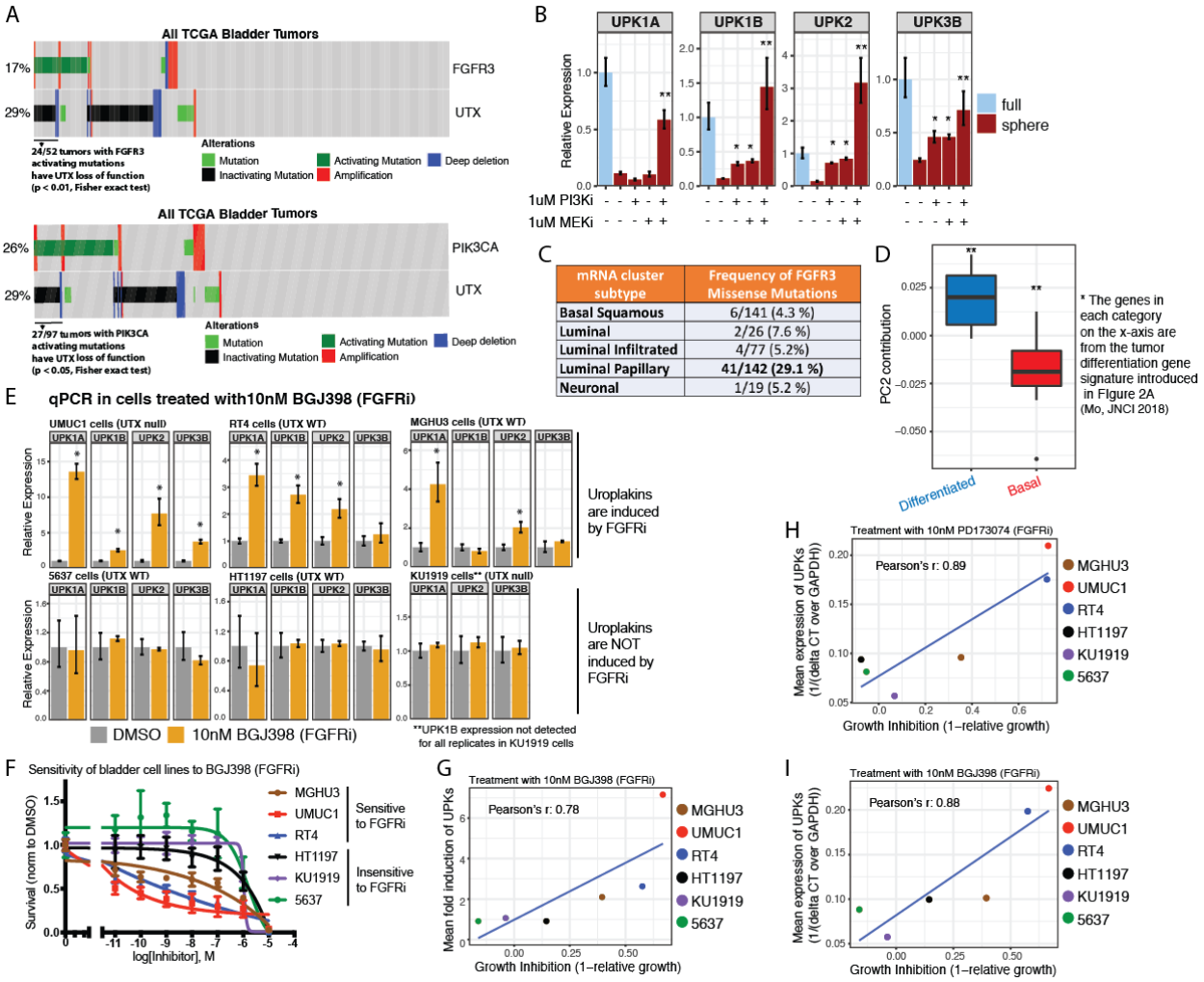


Fig. S3. Loss of function UTX mutations co-occur with activating mutations of upstream kinases that regulate differentiation pathways

A) Mutational spectrum of TCGA invasive bladder tumors comparing UTX to FGFR3 (top) and PIK3CA (bottom).

B) qPCR analysis of select genes from UMUC1 empty vector-expressing cells in either regular full media or tumorsphere media after treatment for 72 hours with 1uM of GDC0941 (PI3K inhibitor), 1uM of Selumetinib (MEK inhibitor), or both. Mean expression was calculated from a representative experiment of three replicates and is relative to the DMSO-treated cells in the full media condition. * represents $p < 0.05$ by t-test compared to the DMSO-treated tumorsphere condition, while ** represents $p < 0.05$ by t-test compared to both the GDC0941 and Selumatinib single treatments.

C) Frequencies of FGFR3 mutations in the five mRNA subtypes from TCGA.

D) PC2 contribution from the PCA plot in Figure 3C of 'basal' and 'differentiated' genes from the tumor differentiation gene signature to distinguish basal and luminal tumors (2). ** represents $p < 0.05$ by one sample t-test compared to zero.

E) qPCR analysis of a panel of bladder cancer cell lines treated with either DMSO or 10nM of BGJ398 (FGFR inhibitor) for 48 hours. Treatments began 24 hours after the cells were plated. Mean expression is relative to the DMSO-treated cells from a representative experiment of three replicates, and * represents $p < 0.05$ by t-test compared to DMSO-treated cells.

F) Cell viability of a panel of bladder cancer cell lines after treatment with increasing doses of the BGJ398. Cells were plated, treated the following day, and cell density was quantified after 96hrs by CellTiter-Glo. These measurements were then normalized to the DMSO condition. At least two replicate experiments (each with three technical replicates) were performed for each cell line at the indicated doses.

G) Growth inhibition by the FGFRi after 96hrs of treatment is plotted against the mean induction of gene expression of UPK1A, UPK1B, UPK2 and UPK3B for the panel of bladder cancer cell lines. Note that UPK3A was not expressed in majority of cell lines so was excluded and UPK1B was excluded for the KU1919 calculation due to being on the edge of detection.

H and I) Growth inhibition by the FGFRi after 96hrs of treatment with either PD173074 (H) or BGJ398 (I) is plotted against the mean expression of UPK1A, UPK1B, UPK2, and UPK3B for the panel of bladder cancer cell lines.

Figure S4

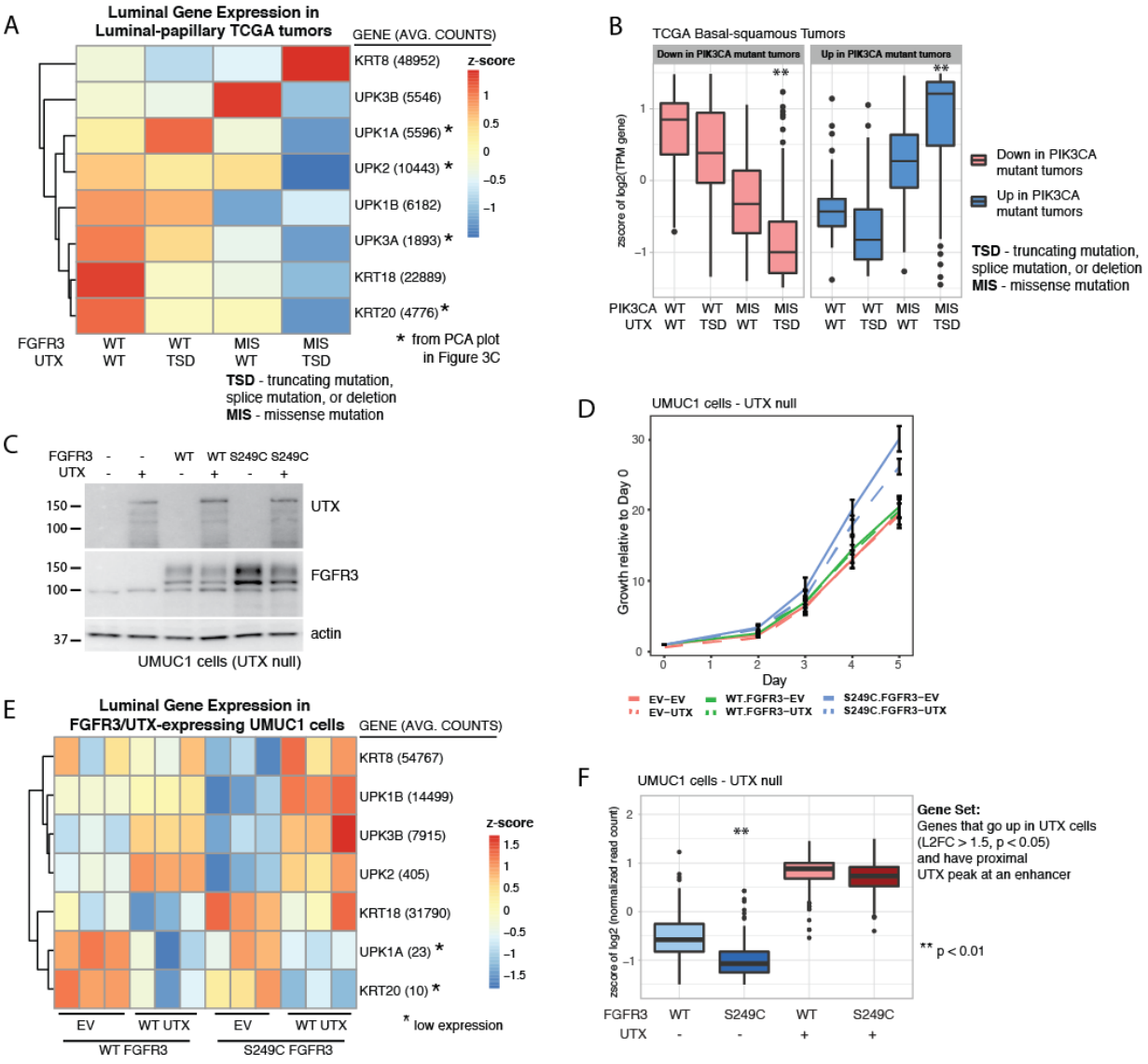


Fig. S4. UTX counteracts FGFR3 activation

A) Heatmap representing z-scores for the expression of genes from the 'differentiated' group within the tumor differentiation gene signature to distinguish basal and luminal tumors (2) in TCGA tumors from the Luminal-papillary mRNA subgroup across UTX and FGFR3 genotypes. Genes that were not attributed reads in the RNA-seq experiment were not included.

B) Plot showing the distribution of z-scores for gene expression as measured by RNA-seq across PIK3CA and UTX genotypes for genes that go down (left) or up (right) in basal-squamous (TCGA mRNA subtype) tumors with an PIK3CA missense mutation (MIS) compared to tumors with wild type (WT) PIK3CA (log2 fold change > 2 and p < 0.05). TSD for UTX genotype indicates either a truncating, splice, or deletion mutation. Only tumors with wild type MLL3 and MLL4 (KMT2C and KMT2D) were included in this analysis. ** represents p < 0.05 by

Wilcoxon Rank Sum test compared to the FGFR3 missense/UTX wild-type tumors for that group of genes.

C) Western blot analysis of UMUC1 cells expressing wild type or S249C FGFR3 with or without UTX (pCDH-blast backbone).
D) Cell growth measurement using CellTiter-Glo over a time course of five days for UMUC1 cells expressing FGFR3 and/or UTX.

E) Heatmap representing z-scores for the expression of genes from the 'differentiated' group within the tumor differentiation gene signature to distinguish basal and luminal tumors (2) in UMUC1 cells expressing wild type or S249C FGFR3 with or without UTX. Genes that were not attributed reads in the RNA-seq experiment were not included.

F) Plot showing the distribution of z-scores for gene expression as measured by RNA-seq from UMUC1 cells expressing FGFR3 and/or UTX for genes that go up in UTX-expressing cells versus either S249C or wild type FGFR3-expressing cells that do not express UTX and have a UTX peak at a nearby enhancer. ** represents $p < 0.05$ by Wilcoxon Rank Sum test compared to the WT FGFR3/UTX null sample.

Table S1 – Characteristics of bladder cancer cell lines used in this study

Cell Line	UTX Status	Luminal/Basal (3)	Prior UTX Phenotype
UMUC1	NULL	luminal	
RT4	WT	luminal	
MGHU3	WT	N.D.	Yes (4)
5637	WT	basal	
KU1919	NULL	basal	Yes (5)
HT1197	WT	basal	

SI References

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