SUPPLEMENTARY METHODS

Tissue collection and cell lines. Sixty-seven fresh-frozen prostate tumors were obtained from the NCI Cooperative Prostate Cancer Tissue Resource (CPCTR) (n = 37), the Department of Pathology at the University of Maryland, Baltimore, MD (UMD; n = 10), and the Department of Urology, Johns Hopkins Medical Institutions, Baltimore, MD (JHU; n = 20). Tissue collection was approved by the institutional review boards (IRB) at the participating institutions according to guidelines set by the Declaration of Helsinki and Belmont Report. Written informed consent was obtained from all donors. Institutions comprising CPCTR collected tissues and patient information as described (1). Tissues at JHU were collected under the protocol "Molecular Studies of Human Prostate Cancer" from consented patients (IRB# NA-00034840). Tissues at UMD were collected under the protocol "Resource Collection and Evaluation of Human Tissues and Cells from Donors with an Epidemiology Profile for NCI Contract #NO2RC-2010-00117" from consented patients (UMD IRB# HP-00042163 and NCI IRB # 05-C-N021). The research was also reviewed by the NIH Office of Human Subjects Research Protections (OHSRP #2657). Additional FFPE tumor specimens were collected at UMD for *in situ* hybridization (n = 22) and immunohistochemical analyses (n = 69). All tumors were resected adenocarcinomas that did not receive any therapy prior to prostatectomy. Clinicopathological characteristics of the patients, including age at prostatectomy, PSA at diagnosis, race/ethnicity, pathological stage, Gleason score, and seminal vesicle invasion were either provided (CPCTR) or extracted from medical and pathology records. Smoking information at time of surgery (current, past, never) was obtained from medical records and cancer registry entries (CPCTR and JHU). Current smokers used cigarettes at time of prostatectomy. A past smoker was defined as a patient who

did not use cigarettes at time of surgery. Thus, the past smoker category in our study is a group of men that has stopped smoking either more recently or many years ago with respect to prostatectomy and sample collection. Never smokers did not have a smoking history. For patients from UMD, smoking information was obtained from an epidemiological questionnaire that collected detailed information on the smoking habits of the subjects. Here, a current smoker was defined as a subject who reported to be a cigarette smoker at time of prostatectomy. A never smoker was defined as a subject who did not currently smoke and also smoked less than 100 cigarettes in his lifetime. Past smokers did not smoke cigarettes in the 6 months prior to prostatectomy, thus may have stopped smoking more recently or many years ago. One of the past smokers reported occasional use of cigars within the 6 months prior to surgery. The human immortalized prostate epithelial cell line, RWPE-1, and human prostate cancer cell lines (22Rv1, PC-3, LNCaP, DU145) were obtained from the American Type Culture Collection (Manassas, VA). Additional authentication of the cell lines was performed in December, 2013, using a short tandem repeat analysis with GenePrint10 (9 loci + amelogenin for sex determination).

RNA extraction from frozen bulk tissue and nicotine-treated cell lines. Total RNA was isolated from both macrodissected human tumors and mouse prostate glands after the tissues were pulverized under liquid nitrogen and dissolved in TRIzol reagent, following the manufacturer's instructions (Invitrogen, Carlsbad, CA). RNA integrity for each sample was confirmed with the Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA). To obtain macrodissected human prostate tumors, frozen tissue was trimmed to enrich for tumor tissue. H/E sections from the trimmed tumors were used to confirm that highly enriched tumor was now present in

the frozen biospecimen. For gene expression analysis of nicotine-treated prostate cancer cell lines, 22Rv1 and LNCaP cells were treated with 100 nM nicotine for 24 hours and RNA was isolated from treated and untreated cells (each n=3) using TRIzol.

Affymetrix microarrays. RNA labeling and hybridization from human samples were performed according to Affymetrix standard protocols (Santa Clara, CA) as described (2). Labeled cRNA was hybridized to Affymetrix GeneChip HG-U133A 2.0 arrays. RNA from murine prostate tumors was processed using the Ambion WT Expression kit and the Affymetrix GeneChip WT Terminal Labeling kit and was hybridized onto Affymetrix GeneChip mouse 1.0 ST arrays. In accordance with Minimum Information About a Microarray Experiment (MIAME) guidelines, we deposited the CEL files for the microarray data and additional patient information into the GEO repository (http://www.ncbi.nlm.nih.gov/geo/). The GEO submission accession number for the 47 bulk tissue tumors, which were initially analyzed, is GSE6956. GSE68138 contains the gene expression data for the additional 20 bulk tissue tumors ("JHU samples") and the laser capture microdissected tumor samples (n=10), and for prostate tumors from TRAMP mice +/- nicotine treatment (n=10), and cell lines (22Rv1 and LNCaP cells) +/- nicotine treatment (n=12).

RNA isolation from microdissected prostate tumors. Enriched tumor epithelium was obtained from 5 current and 5 never smokers (UMD prostate cancer patients) with laser capture microdissection (LCM) of frozen tissue samples. These tumors were also analyzed as bulk tissues. Approximately 5,000 to 15,000 cells per tumor were collected with LCM. Total RNA was isolated using the PicoPure protocol (Arcturus, Mountain View, CA). mRNA was amplified with two linear amplification steps by *in vitro* transcription using the MEGAscript T7 kit (Ambion, Austin, TX) followed by the labeling step using the BioArray HighYield RNA Transcript Labeling

Kit T3 from Enzo Life Sciences (Farmingdale, NY). Labeled cRNA was hybridized onto Affymetrix HG-U133A GeneChips.

Data normalization and statistical analysis of gene expression data. All chips were normalized using the robust multichip analysis (RMA) procedure (3). Because two sets of data were analyzed for human prostate tumors (expression data for tumors from JHU were obtained at a later time point), a two-way ANOVA model was applied in the Partek Genomics Suite (www.partek.com) to assess and control for the impact of a batch effect. To generate lists of differently expressed genes, the resulting data set was subjected to the significance analysis of microarray (SAM) procedure (4). We created the gene lists based on intended false discovery rates (FDRs). FDR calculation followed the method described by Storey and Tibshirani (5). Furthermore, we tested whether gene expression differences between current and past or never smokers are confounded by race/ethnicity. We built a linear regression model using expression as dependent variable and smoking status and race/ethnicity as independent variables. In this analysis, differences by smoking status were independent of race/ethnicity (F-test). We also generated gene lists for classification using the Bioconductor limma R package (http://www.bioconductor.org). Here, the batch effect for the two datasets was corrected using the ComBat package (http://www.bu.edu/jlab/wp-assets/ComBat/Abstract.html), as shown in **Supplementary Figure S4**. Differentially expressed genes were then assessed using the linear modeling features implemented in limma. *P* values < 0.05 were used to generate two gene lists for current vs. never (849 probes) and current vs. never/smokers (1331 probes). Probesets with same differential expression in both gene lists (n = 601) were then used in additional analyses (classification and pathway analysis) (see **Supplementary Table 5** for all probesets, gene names,

differential expression, and pathway analysis results). The gene symbols for the probesets were annotated by hgu133a2.db from Bioconductor. Unmapped probes were further filled in using NetAffy Query. Gene set enrichment analysis of GO terms and KEGG pathways was performed with the website tool, DAVID Bioinformatics Resources 6.7 (http://david.abcc.ncifcrf.gov/). **Supplementary Tables S7-11** describe differentially expressed genes in LCM tumor epithelium comparing current (n = 5) versus never smokers (n = 5), nicotine-treated (n = 3) vs. untreated (n = 3) 22Rv1 and LNCaP cells, and prostate tumors from nicotine-treated (n = 5) vs. untreated (n = 5) TRAMP mice, respectively.

GSEA analysis. Gene Set Enrichment Analysis (GSEA) was performed as described (6, 7). This method identifies common features between gene expression datasets based on ranking metrics. GSEA considers genome-wide gene expression differences in one dataset for enrichment analysis in another dataset and ranks all expressed genes for each dataset based on gene expression differences after which it determines whether genes in dataset #1 are either randomly distributed or found mostly at the top or bottom in dataset #2. GSEA calculates enrichment scores based on ranking metrics, measuring associations by a non-parametric, running sum statistic, and estimates significance levels for the enrichment score. We applied GSEA to find common features between the smoking-related gene signatures in prostate tumors, and published signatures archived in the molecular signature database (http://www.broad.mit.edu/gsea/msigdb). We compiled enrichment score results based on *P* values and displayed the results in color-coded heat maps. The color coding of the heatmaps is related to the enrichment score with red indicating a higher enrichment (-Log(*P* value)-based).

Quantitative Real-time PCR of gene expression. About 100 ng of total RNA was reversed transcribed using the High-Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA). Quantitative Real-time qRT-PCR was subsequently performed in triplicate using TaqMan Gene Expression Assays (Applied Biosystems), which include pre-optimized probe and primer sets specific for the validated gene. We assayed 57 tumors with available RNA following the microarrays experiments. The assay ID numbers of the validated genes are as follows: Immunoglobulin heavy constant (*IGH*), Hs00920518_g1; immunoglobulin kappa constant (*IGKC*), Hs00736177_m1; immunoglobulin lambda locus (*IGL*), Hs00382306_m1; immunoglobulin heavy constant mu (*IGHM*), Hs00385871_m1; chemokine (C-C motif) ligand 5 (*CCL5*), Hs00982282_m1; chemokine (C-X-C motif) ligand 10 (*CXCL10*), Hs01124251_g1; chemokine (C-X-C motif) ligand 11 (*CXCL11*), Hs04187682_g1; indoleamine 2,3-dioxygenase 1 (*IDO1*), Hs00984148_m1; acetylcholine receptor isoform α3 (*CHRNA3*), Hs00609519_s1; acetylcholine receptor isoform α4 (*CHRNA4*), Hs00181247_s1; acetylcholine receptor isoform α5 (*CHRNA5*), Hs00181248_s1; acetylcholine receptor isoform α7 (*CHRNA7*), Hs01063373_s1; acetylcholine receptor isoform β2 (*CHRNB2*), Hs00181267_m1; and acetylcholine receptor isoform β4 (*CHRNB4*), Hs00609520_m1. Data were collected using the ABI PRISM® 7500/7900 Sequence Detection Systems. The 18s RNA was used as the internal standard reference. Because of the high abundance of 18s RNA, we diluted total RNA 1:1000 for the 18s RNA assay yielding average C_t times of 13-20. Normalized expression was calculated using the comparative C_t method as described and fold differences were derived from the $2^{-\Delta\Delta Ct}$ values for each gene (8). When expression of a gene was not detectable in a sample, a threshold value (as lowest ΔC_t value, usually 35) was imputed for analysis. Graphs were prepared using relative C_t values that

were calculated by subtracting the C_t values for the gene being measured from the 18s C_t values and plotted using GraphPad Prism 6 or the R software. To calculate fold differences in gene expression between groups, we used the mean C_t for the gene of interest and the mean C_t for 18s for each group and calculated fold change between groups exactly as described (9). In Figure 1B-D, expression was plotted as relative expression [(18s C_t - Ig C_t) +20] for all groups. We added 20 to the (-)dC_t only for display purposes, not for calculation. There is no effect on relative differences or *P* value calculations when one applies this procedure.

***In situ* hybridization for immunoglobulin kappa and lambda light chain expression in prostate tumors.** *In situ* hybridization (ISH) for kappa and lambda light chain mRNA was performed on paraffin-embedded, formalin-fixed tissues using the Kappa and Lambda ISH detection kits from DakoCytomation, Carpinteria, CA, as directed by the manufacturer (peptide nucleic acids (PNA) ISH detection kit: Y5202; fluorescein-conjugated 15-mer PNA probes: K5201), with tonsil tissue as the provided positive control. The ISH kit uses an optimized protocol to detect fluorescein-conjugated PNA probes hybridized to their target mRNA in tissue preparations. Briefly, tissue sections were deparaffinized and treated for 20-30 min with proteinase K that was diluted 1:10 in Tris-buffered saline solution. Fluorescein-conjugated PNA probes were added to the tissue sections and placed in a humidity chamber for 90 min at 55° C. Slides were washed and incubated with an alkaline phosphatase-conjugated anti-fluorescein antibody for 30 min. After a wash step, the antibody labeled PNA probes were visualized in the tissue sections with 5-bromo-4-chloro-3-indolylphosphate and nitroblue tetrazolium. Numbers of labeled B lymphocytes were counted per 250x field in 3 representative fields by a pathologist blinded to the smoking status of the patients.

Immunohistochemistry. Five micron slides from paraffin-embedded formalin-fixed tumor specimens were deparaffinized and microwaved in citrate buffer for antigen retrieval. Endogenous peroxidase was blocked using the DakoCytomation Envision System-HRP blocking buffer according to the manufacturer's protocol (DakoCytomation, Carpinteria, CA). NF-κB phosphorylation was evaluated using a 1:200 diluted rabbit polyclonal antibody from abcam, Cambridge, MA (#ab28856). The IHC analysis for nuclear p-NF-κB has been described (10) and immunohistochemical staining was scored by a pathologist blinded to the smoking status of the patients. Accumulation of nuclear NF-κB phosphorylation was categorized as present or absent in the tumor epithelium.

Proliferation, motility, and invasion assays of nicotine-treated cells. Cell proliferation was determined using the WST-1 reagent (Roche Diagnostics, Mannheim, Germany) in 96-well plate assays according to a Roche standard protocol or by cell counting using the Bio-Rad TC10 automatic counter. For the latter method, 22Rv1 and PC-3 cells were cultured in 100 mm dishes for 4 days +/- 100 nM nicotine. At day 4, cells were 70-80% confluent. BrdU incorporation during DNA synthesis was determined with a BrdU colorimetric ELISA (Roche Diagnostics). Cells were plated in 96-well plates and serum starved for 24 hours. After starvation, cells were treated with 100 nM or 1 μM nicotine for 24 hours using the culture conditions described for the ELISA and BrdU incorporation was determined per manufacturer's protocol. Invasion and motility assays were performed as previously described (11). For the motility assay, 22Rv1 or PC-3 cells ($1x10^5$) were cultured in RPMI 1640/1% FBS medium and plated into the BD Biosciences HTS multiwell insert system ($1x10^5$cells each) and cells were treated with 100 nM, 500 nM, and 1 μM nicotine. After 24 hours, the number of cells that migrated to the outer side

of the insert were determined by counting cell numbers in 5 fields per well at 200X magnification. To determine cell invasion, cells were plated in BD Biocoat Matrigel Invasion Chamber (# 354480, BD Biosciences, San Jose, CA) and were treated with either various amounts of nicotine, 10 ng/ml HGF, or both. To inhibit nicotine-induced phenotypes, 10 µM mecamylamine (mecamylamine hydrochloride, M9020, Sigma-Aldrich, St.Louis, MO), which blocks responses to nicotine, was added to the culture medium. Medium (0.5 ml) was added to the well underneath the chamber. Cells were incubated at 37°C for 48 hours. After that, the inner side of the insert was wiped with a wet swab to remove the cells while the outer side of the insert was gently rinsed with PBS and stained with 2.3% (w/v) crystal violet (HT9031, Sigma-Aldrich) for 5 min, rinsed again and then allowed to dry. The inserts were then viewed under the microscope and the number of cells per field in 5 random fields was counted at 200x magnification. The total cell count over 5 fields per insert was used to calculate the relative number of invading cells. Results are reported as the average relative number of invading cells over three inserts per condition.

Integrin cell surface expression and extracellular matrix (ECM) protein binding assays. Integrin cell surface expression profiles were examined using the α/β integrin cell adhesion array combo assay, colorimetric (Millipore, ECM532). This Chemicon product uses mouse monoclonal antibodies generated against human alpha and beta integrin subunits that are immobilized onto a goat anti-mouse antibody-coated microtiter plate. The plate is then used to capture cells expressing these integrins on their cell surface, per manufacturer's protocol. Alpha Integrin (PN: 90599) and Beta Integrin (PN: 90600) 96-well plates with 12 strips were re-hydrated with 200 µl of PBS per well for 10 min at room temperature. Plates were tapped on tissue paper to remove

PBS. Cells were cultured serum-free +/- nicotine for 24 hours before $1x10^5$ cells were plated per well and incubated for 1 hour at 37° C. Medium and unbound cells were removed from the wells and adherent cells were lysed and quantified colorimetrically with Cell Stain Solution at 570 nm. ECM adhesion assays were performed on CytoMatrix™ screening kits (Millipore, ECM205). 96-well plates pre-coated individually with fibronectin, vitronectin, laminin, collagen I or collagen IV were re-hydrated with 200 µl of PBS per well for 15 min at room temperature, then the PBS was removed. Cells were cultured serum-free +/- nicotine for 24 hours before $1x10^5$ cells were plated per well and incubated for 1 hour at 37º C. Bound cells were stained with 0.2% crystal violet in 10% ethanol for 10 minutes and gently washed with PBS. 100 µl of Solubilization Buffer (a 1:1 mixture of 0.1M NaH_2PO_4, pH 4.5 and 50% ethanol) was added to each well and incubated at room temperature until the cell-bound stain was completely solubilized. To determine relative cell attachment, the optical density was measured at 570 nm.

Western blot analysis of nicotine-treated cells. Cells were grown to 70% confluency and serum-starved overnight. Nicotine (nicotine hydrogen tartrate salt, Sigma, St. Louis, MO) was added at the zero time point and cell culture was continued (e.g., 5 min, 15 min, 30 min, 1 hour, 2 hours, 8 hours and 24 hours). At various time points, culture dishes were placed on ice and cells were rinsed with ice cold PBS and then scraped off into PBS using a cell scraper. This cell suspension was centrifuged at 1000 rpm for 5 min at 4°C and cell pellets were dissolved in 0.5% Nonidet P-40 protein lysis buffer containing a protease inhibitor cocktail. Western blot analysis was performed according to standard procedures and 50 µg of total protein were loaded per lane. The following antibodies were used to detect the membrane-bound proteins: rabbit monoclonal anti-pAkt Ser473 from Cell Signaling, Beverly, MA, 1:500 (#4060); rabbit polyclonal

anti-pAkt Thr308 from Cell Signaling, 1:250 (#9275); rabbit polyclonal anti-Akt from Cell Signaling, 1:2000 (#9272); rabbit polyclonal anti-pGSK3β Ser9 from Cell Signaling, 1:500 (#9336); rabbit polyclonal anti-pHDM2 Ser166 from Cell Signaling, 1:1000 (#3521); rabbit polyclonal anti-pBad Ser136 from Cell Signaling, 1:500 (#9295).

Measurement of IL-8 in human plasma samples. Heparinized plasma was collected from consented prostate cancer patients and population-based controls (men without the clinical disease) under an IRB approved protocol # 05-C-N021 and stored at -80°C. Cases were recruited at two hospitals in Baltimore, Maryland, while controls were identified through Maryland Department of Motor Vehicles records. Plasma IL-8 concentrations were determined at a Frederick National Laboratory for Cancer Research core facility using the human IL-8 electrochemiluminescence immunoassay (ECLIA) from Meso Scale Discovery (Gaithersburg, MD) under standardized conditions. Ultrasensitive 10-plex ECLIA plates were custom-designed and were analyzed on the Meso Scale Discovery 6000 instrument, following manufacturer's assay and analysis protocols. We analyzed plasma samples from 26 current [age: 59 (mean) ± 8 (standard deviation)], 34 past (age: 64 ± 8) and 37 never smokers (age: 62 ± 10) among the cases (no prior therapy) and from 29 current (age: 68 ± 7), 29 past (age: 68 ± 10) and 29 never smokers (age: 65 ± 9) among population-based controls. In this population, plasma IL-8 concentrations and age did not show an association (r^2 = -0.02, *P* = 0.85; Pearson correlation test).

Glutamine consumption in nicotine-treated prostate cancer cells. To determine glutamine consumption of nicotine-treated cells, 22Rv1 and LNCaP cells were plated in T150 flasks and allowed to attach for 24 hours. After an additional 24 hour serum starvation period, cells were

treated with 100 nM nicotine for 24 and 48 hours. One ml of media was collected and the cells were washed with PBS and trypsinized. A cell count was done with the Bio-Rad TC-10 Automatic Cell counter and cell pellet was generated. Sample preparation for Liquid Chromatography/Mass Spectrometry (LC/MS): Cell pellets and media were stored at -80°C until analysis. For extraction of metabolites, either equal numbers of cells or 100 µl of media were mixed/sonicated in a 1:4 ice cold water:methanol mixture containing isotopic labeled standard compounds (e.g., L-glutamic acid-d_5). This was followed by sequential addition of ice cold chloroform and water in 3:1 ratio and separation of the organic (methanol and chloroform) and aqueous solvents (water:methanol:chloroform:water; ratio 1:4:3:1). The aqueous extract was de-proteinized using a 3 KDa molecular filter (Amicon Ultracel-3K Membrane, Millipore Corporation, Billerica, MA) and the filtrate containing metabolites was dried under vacuum (Genevac EZ-2plus, Gardiner, NY). Prior to mass spectrometry, the dried extract was resuspended in identical volume of injection solvent composed of water:methanol (50:50) and subjected to LC/MS as follows: 10 µL of suspended samples was injected and analyzed using a 6495 triple quadrupole mass spectrometer (Agilent Technologies, Santa Clara, CA) coupled to a HPLC system (Agilent Technologies, Santa Clara, CA) via Multiple reaction monitoring (MRM) for steady-state analyses of samples. Samples were delivered to the MS via normal phase chromatography using either a 4.6 mm i.d ×10 cm Amide XBridge HILIC column (Waters). Gradients were run starting from 85% buffer B (HPLC grade acetonitrile or 0.1% formic acid in acetonitrile) to 35% B from 0–3.5 minutes; 35% B to 2% B from 3.5–11.5 minutes; 2% B was held from 11.5–16.5 minutes; 2% B to 85% B from 16.5–17.5 minutes; 85% B was held for 7 minutes to re-equilibrate the column. Peak area for each metabolite was

integrated using MassHunter Workstation Software Quantitative Analysis Version B.06.00 software (Agilent Technologies, Santa Clara, CA).

Nicotine treatment of prostate cancer-prone TRAMP mice and evaluation of lung metastasis. Male TRAMP mice were bred at the Assisted Reproduction Laboratory, Frederick National Laboratory for Cancer Research, Frederick, Maryland, using *in vitro* fertilization (B6xFVB F1). These mice usually start to develop metastatic lesions at 24 weeks of age and after 28 weeks of age, two-thirds of the mice will develop lung metastasis. Beginning at 8 to 9 weeks of age, TRAMP mice received either tap water or a solution of either 100 or 250 μg/ml of nicotine [nicotine tartrate salt (Sigma-Aldrich, St. Louis, MO)] in tap water, which is similar to a previous described protocol (12). The three groups consisted of 20-25 animals each. At the selected concentration, nicotine is not toxic and generates nicotine plasma concentrations comparable to those of active smokers and causes some weight loss (**Supplementary Figure S1**). All mice were euthanized after 80 days or when they became moribund because of prostate cancer. Retro-orbital sinus/plexus blood samples were obtained to determine plasma nicotine concentration at day 7 and were taken from 6 mice per treatment group. Nicotine was measured at the Laboratory of Proteomics and Analytical Technologies, Frederick National Laboratory for Cancer Research, using LC/MS with nicotine-d_3 as internal standard. To assess the effects of nicotine on prostate cancer development and metastasis, the prostate glands (as whole urogenital tracts with seminal vesicles) and lungs were collected and were formalin-fixed for histological examination by a boarded veterinary pathologist. The size of the cancerous prostate in TRAMP mice was estimated from whole urogenital tract weights. A histological

evaluation of the prostate and the lungs was performed for 20 TRAMP mice on tap water, 22 TRAMP mice treated with 100 μg/ml of nicotine, and for 23 TRAMP mice with 250 μg/ml of nicotine. We also collected a frozen tissue specimens for gene expression analysis of the primary prostate tumors. Total RNA was isolated from 10 cancerous glands, from TRAMP mice on tap water or treated with 250 μg/ml of nicotine for 80 days, n = 5 each group, after the prostate tissue was pulverized under liquid nitrogen, dissolved in TRIZOL reagent, and total RNA was isolated according to the manufacturer's instructions. All described animal procedures were reviewed and approved by the NCI-Frederick Institutional Biosafety Committee (IBC registration #06-060 and 11-041). NCI-Frederick is accredited by AAALAC International and follows the Public Health Service Policy for the Care and Use of Laboratory Animals.

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