



## TUMORIGENESIS AND NEOPLASTIC PROGRESSION

# RAMP1 Is a Direct NKX3.1 Target Gene Up-Regulated in Prostate Cancer that Promotes Tumorigenesis

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The homeodomain-containing transcription factor, NKX3.1, plays an important role in the suppression of prostate tumorigenesis. Herein, we identify the receptor activity—modifying protein 1 (*RAMP1*) as a direct NKX3.1 target gene through analysis of chromatin immunoprecipitation coupled to massively parallel sequencing and gene expression data. *RAMP1* is a coreceptor for certain G-protein—coupled receptors, such as the calcitonin gene-related peptide receptor, to the plasma membrane. We found that *RAMP1* expression is specifically elevated in human prostate cancer relative to other tumor types. Furthermore, *RAMP1* mRNA and protein levels are significantly higher in human prostate cancer compared with benign glands. We identified multiple NKX3.1 binding sites in the *RAMP1* locus in human prostate cancer cells and in the normal mouse prostate. Analyses of *Nkx3.1* knockout mice and human prostate cancer cell lines indicate that NKX3.1 represses *RAMP1* expression. Knockdown of *RAMP1* by shRNA decreased prostate cancer cell proliferation and tumorigenicity *in vitro* and *in vivo*. By using gene expression profiling and pathway analyses, we identified several cancer-related pathways that are significantly altered in *RAMP1* knockdown cells, including the mitogen-activated protein kinase signaling pathway. Further experiments confirmed a reduction in *MAP2KI* (*MEK1*) expression and phosphorylated-extracellular signal—regulated kinase 1/2 levels in *RAMP1* knockdown cells. These data provide novel insights into the role of *RAMP1* in promoting prostate tumorigenesis and support the potential of *RAMP1* as a novel biomarker and possible therapeutic target in prostate cancer. (*Am J Pathol* 2013, 183: 951–963; <http://dx.doi.org/10.1016/j.ajpath.2013.05.021>)

Receptor activity—modifying proteins (RAMPs) 1, 2, and 3 are a small family of single transmembrane spanning proteins that were first identified during an attempt to clone the human calcitonin gene-related peptide (CGRP) receptor.<sup>1</sup> Since their initial discovery, RAMPs have been shown to interact with G-protein—coupled receptors, such as calcitonin receptor-like receptor, vasoactive intestinal peptide receptor (VIPR), calcitonin receptor, calcium-sensing receptor, parathyroid hormone receptor, glucagon receptor, and corticotrophin releasing factor receptor 1, to regulate their trafficking, pharmacological characteristics, and signaling capabilities.<sup>1–5</sup> Although the cellular biological and biochemical characteristics of these G protein-coupled receptors and their ligands are well studied, the functional significance and roles of RAMPs in human diseases remain undefined.

*RAMP1* is expressed in many tissues, including the heart, uterus, bladder, brain, pancreas, skeletal muscle, and

gastrointestinal tract.<sup>1</sup> The expression of *RAMP1* in these various tissues indicates that it may have diverse physiological functions. *RAMP1* mRNA is also expressed in human adrenal tumors and meningiomas, and is up-regulated in prostate tumors.<sup>6–8</sup> However, neither the underlying mechanism for up-regulation of *RAMP1* expression in these tumors nor the functional significance of *RAMP1* in tumorigenesis is fully understood.

NKX3.1 is an androgen-regulated, prostate-specific homeodomain transcription factor that plays critical roles in prostate development and in suppressing tumorigenesis.<sup>9–13</sup>

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Throughout development and adulthood, Nkx3.1 functions to maintain prostate cellular homeostasis, and in mice, targeted deletion of *Nkx3.1* in the prostate leads to developmental defects in ductal branching morphogenesis, secretory protein production, and growth.<sup>11,14</sup> In addition, conditional deletion of one or both alleles of *Nkx3.1* in the adult mouse prostate has been shown to promote the formation of premalignant lesions termed prostatic intraepithelial neoplasia.<sup>11–13</sup> In humans, loss of heterozygosity at the *NKX3.1* locus has been observed in a significant fraction of early-stage prostate cancer specimens.<sup>15,16</sup> Furthermore, loss of NKX3.1 protein has been observed in approximately 20% of human prostatic intraepithelial neoplasia lesions and 40% of prostate tumors, which correlate with prostate tumor progression.<sup>17</sup> NKX3.1 function can also be impaired by mutations in the *NKX3.1* gene that can decrease its expression or affect the stability of the homeodomain structure.<sup>18–21</sup> Although loss of Nkx3.1 does not result in invasive carcinoma, the introduction of additional mutations, such as loss of the tumor suppressor *Pten* in the *Nkx3.1* mutant mouse prostate, can lead to invasive adenocarcinoma and, in some cases, metastatic disease.<sup>22,23</sup> We and others have also shown that loss of Nkx3.1 and Myc overexpression can cooperate to promote prostate carcinogenesis.<sup>24–26</sup> Furthermore, our laboratory showed that the cooperativity of these oncogenic mutations was the result of coregulation of shared target genes between Nkx3.1 and Myc that were observed in patients with prostate cancer and in a mouse model. Thus, a full understanding of the role of NKX3.1 in tumorigenesis requires the identification of genes directly regulated by NKX3.1 that may play a role in transformation.

We have recently used chromatin immunoprecipitation coupled to massively parallel sequencing (ChIP-Seq) to identify genomic loci bound by NKX3.1 in the human and mouse genomes.<sup>26</sup> These data were integrated with gene expression profiling data from *Nkx3.1* mutant mouse prostates<sup>27,28</sup> to yield a core set of direct NKX3.1 target genes.<sup>26</sup> In the present study, we identified *RAMP1* as a novel NKX3.1 target gene and, for the first time to our knowledge, have characterized its functional role in prostate tumorigenesis.

## Materials and Methods

### Cell Lines and Constructs

Human prostate cancer cell lines, PC-3 and LNCaP, were obtained from ATCC (Manassas, VA). Cells were cultured in RPMI 1640 medium or Dulbecco's modified Eagle's medium/F-12 medium supplemented with 10% fetal bovine serum. Lentiviral-mediated gene transfer was used to generate stable knockdown of RAMP1 in PC-3 and LNCaP cells. The 293FT packaging cells were transfected with shRAMP1 (V2LHS\_196808 or VLHS\_180867) or pGIPZ vector control (RHS4346), provided by the Vanderbilt Genome Sciences Resource (Vanderbilt University Medical Center, Nashville, TN), along with  $\Delta$ 8.9 and vesicular stomatitis virus-G (provided by Dr. David Baltimore, Caltech, Pasadena, CA) to

produce lentivirus as described.<sup>29</sup> At 2 days after transfection, medium containing viral particles was collected and added to PC-3 cells for infection with 15  $\mu$ g/mL polybrene. At 48 hours after infection, medium was changed and 6  $\mu$ g/mL puromycin or 800  $\mu$ g/mL G418 was added for selection of stable clones. Transient transfection of PC-3 and DU145 was performed using polyethylenimine, with FUGW-GFP or FUGW-Nkx3.1 plasmid (a kind gift from Dr. Hong Wu, University of California, Los Angeles).

### Human Tissue Arrays

Histological slides of radical prostatectomy specimens from patients with prostatic carcinoma were reviewed to identify areas of prostatic carcinoma of various grades and areas with benign prostatic glands remote from site(s) of carcinoma. The corresponding paraffin blocks were used to generate a TMA containing 38 foci of benign prostatic tissue and 23 foci of prostatic adenocarcinoma with different Gleason scores. The TMA was produced using a manual tissue microarrayer model MTA-1 from Beecher Instruments, Inc. (Sun Prairie, WI). An additional TMA (PR483a) was obtained from US Biomax (Rockville, MD) and contained 16 foci of benign prostatic tissue and 37 foci of prostatic adenocarcinoma. RAMP1 staining in each focus on the TMAs was scored semi-quantitatively using the H-score, as previously described.<sup>30</sup> The H-score was determined as the intensity of expression (0, none; 1, weak; 2, moderate; 3, strong) multiplied by the percentage of cells (0% to 100%) stained at each intensity level to provide a score ranging from 0 to 300. Anti-RAMP1 staining was evaluated between the individual cores, and within the same core, because some cores contained both benign and malignant tissues. Two pathologists (S.T.S. and O.H.) evaluated the TMAs for RAMP1 staining, and any potential discrepancies were resolved by joint review on a double-headed microscope.

### Oncomine Database Analysis

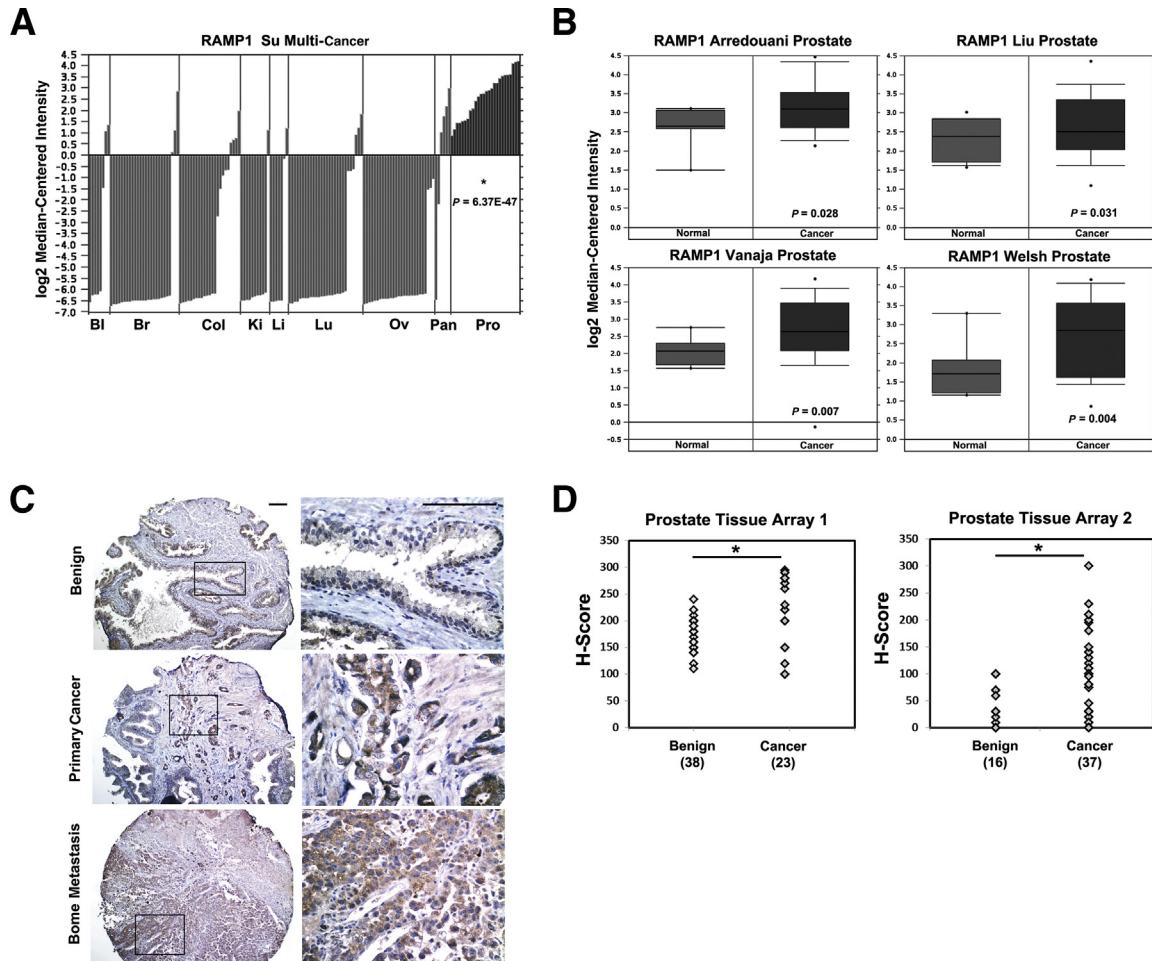
We used the Oncomine (Compendia Bioscience, Ann Arbor, MI) Cancer Microarray database<sup>31</sup> to query the profile of RAMP1 expression in multicancer data sets and in prostate cancer data sets.

### Cell Cycle Analysis

Cell cycle analysis was performed as previously described.<sup>32</sup> Cells were harvested, washed thoroughly with PBS, fixed with cold 70% ethanol, and stained with propidium iodide (0.05 mg/mL) in a solution containing 0.1% Triton X-100 and 0.005 mg/mL RNase A. DNA content was analyzed by flow cytometry.

### ChIP-qPCR

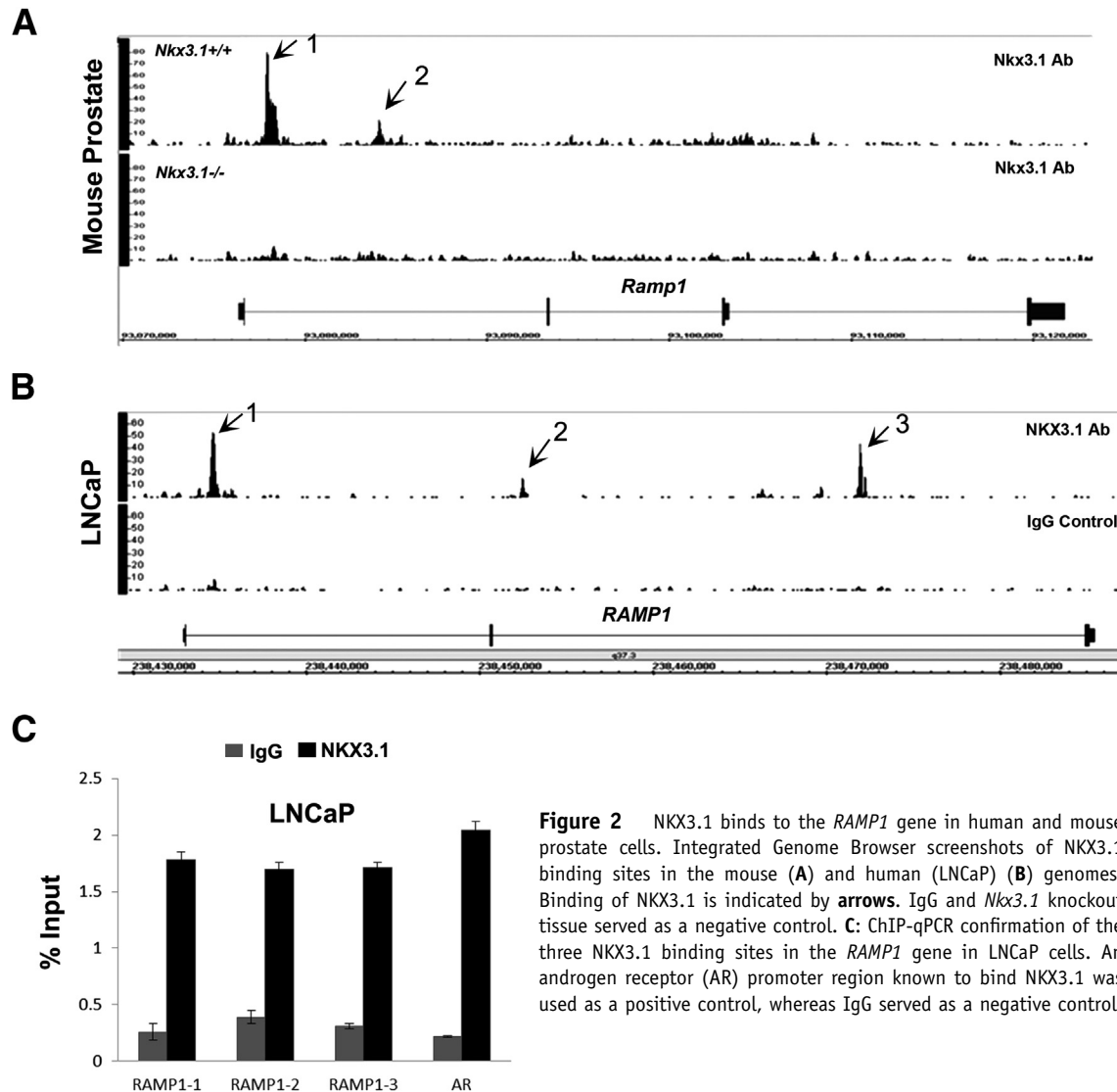
ChIP was performed using the ChIP Assay kit (Millipore, Billerica, MA), as described by the manufacturer, with the



**Figure 1** RAMP1 is up-regulated in human prostate cancer. **A:** RAMP1 mRNA expression in various human cancers in a multicancer data set from the Oncomine cancer microarray database shows significantly higher RAMP1 levels in prostate relative to other tumors. BL, bladder; Br, breast; CL, colon; Ki, kidney; Li, liver; Lu, lung; Ov, ovarian; Pan, pancreatic; Pro, prostate. **B:** RAMP1 mRNA expression in normal prostate versus prostate cancer from four different prostate cancer microarray data sets from the Oncomine database. **C:** Examples of immunostaining for anti-RAMP1 in benign prostate, primary prostate cancer, and bone metastasis from a human prostate TMA. Original magnifications: ×10 (left panel); ×40 (right panel). Scale bar = 0.1 mm. **D:** H-score analysis of anti-RAMP1 staining of samples from two independent human prostate TMAs. The expression levels of RAMP1 in benign and malignant prostatic tissue were compared using the unpaired Student's *t*-test. \**P* < 0.05.

following modifications. LNCaP cells were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum, 100 U/mL penicillin and streptomycin, and 1 nmol/L dihydrotestosterone (Sigma, St. Louis, MO) for 48 hours. Cells were fixed in 1% formaldehyde at room temperature for 10 minutes to cross-link protein-DNA complexes. Next, cells were thoroughly washed with ice-cold PBS, pelleted, and resuspended in SDS lysis buffer (1% SDS, 10 mmol/L EDTA, and 50 mmol/L Tris at pH 8.1). Chromatin was sheared to a size of approximately 300 to 500 bp and diluted 1:10 with ChIP dilution buffer. An aliquot of the diluted sample (1%) was saved as input. Samples were precleared and precipitated overnight at 4°C with anti-NKX3.1 (Santa Cruz Biotechnology, Santa Cruz, CA) or normal goat IgG (Santa Cruz Biotechnology) antibodies. Antibody complexes were collected with Protein A Agarose/Salmon Sperm DNA (Millipore, Billerica, MA) for 2 hours and washed

extensively, per manufacturer's instructions. Samples were reverse cross-linked at 65°C overnight with 0.3 mol/L NaCl and 30 μg of RNase A (Qiagen, Germantown, MD). Input and bound DNAs were purified with a PCR Purification kit (Qiagen) and analyzed by quantitative PCR (qPCR; model 7300; Applied Biosystems, Grand Island, NY) using SYBR Green (Applied Biosystems). Primers were designed to flank the three NKX3.1 binding sites in the *RAMP1* gene in LNCaP cells. The following primers were used for qPCR: RAMP1 (site 1), 5'-ACGATCATATAAAGACCTTC-CTTGT-3' (forward) and 5'-AGGCCACTCAAATAAC-GTTAAAATT-3' (reverse); RAMP1 (site 2), 5'-ATTTCA-GGGCCTCCTTTTCTAAG-3' (forward) and 5'-AATGA-CTCAGCTGTGGCAGAAG-3' (reverse); and RAMP1 (site 3), 5'-CACTCACCCGTAGGAGTTCCA-3' (forward) and 5'-ATGAAAAGCACTTCAGCACACTGT-3' (reverse). Immuno-precipitated DNA was normalized to 1% input.



**Figure 2** NKX3.1 binds to the *RAMP1* gene in human and mouse prostate cells. Integrated Genome Browser screenshots of NKX3.1 binding sites in the mouse (A) and human (LNCaP) (B) genomes. Binding of NKX3.1 is indicated by arrows. IgG and *Nkx3.1* knockout tissue served as a negative control. C: ChIP-qPCR confirmation of the three NKX3.1 binding sites in the *RAMP1* gene in LNCaP cells. An androgen receptor (AR) promoter region known to bind NKX3.1 was used as a positive control, whereas IgG served as a negative control.

### Real-Time RT-qPCR Analyses

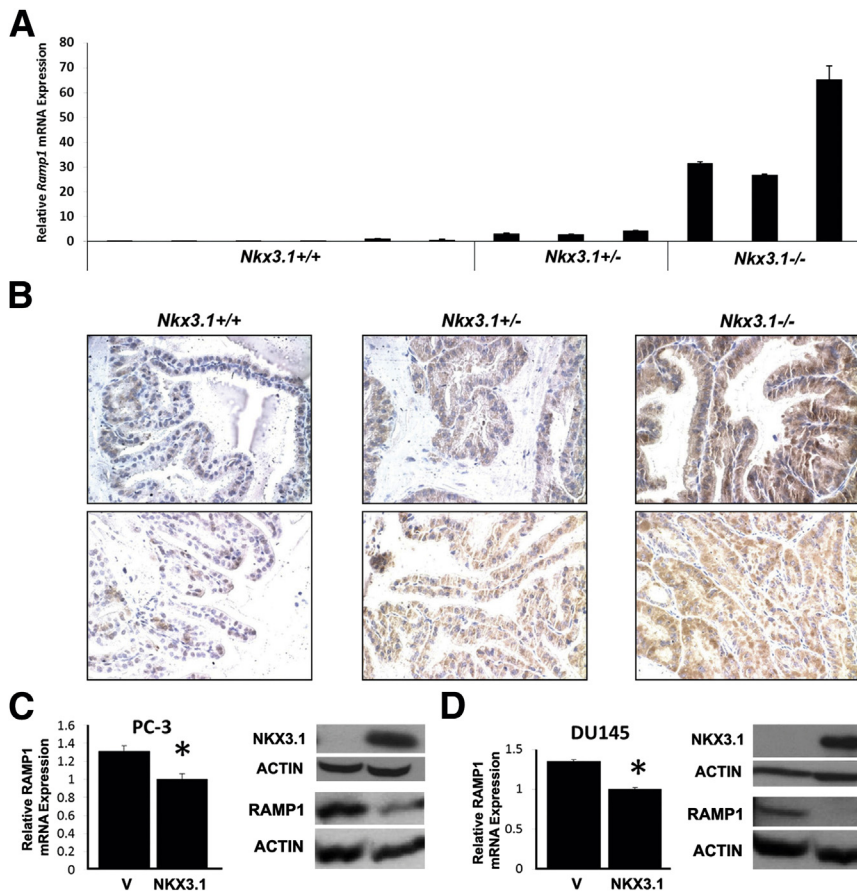
RNA isolation, reverse transcription, and subsequent RT-qPCR (TaqMan; Applied Biosystems) using SYBR Green dye were performed as described previously.<sup>33</sup> All RT-qPCRs were performed in triplicate. The following primers were used: *18S* forward, 5'-CGCCGCTAGAGGTGAAATTCT-3'; *18S* reverse, 5'-CGAACCTCCGACTTTCGTTCT-3'; *Ramp1* forward, 5'-CGCACACGATTGGCTGTTT-3'; *Ramp1* reverse, 5'-TGGACAGCGATGAAGAATCTGT-3'; *GAPDH* forward, 5'-ATGGAAATCCCATCACCATCTT-3'; *GAPDH* reverse, 5'-CGCCCCACTTGATTTTGG-3'; *RAMP1* forward, 5'-CCTCACCCAGTTCAGGTAG-3'; *RAMP1* reverse, 5'-CATGTGCCAGGTGCAGTC-3'; *RAMP2* forward, 5'-CCTTATAGCACCCTGCGAGATT-3'; *RAMP2* reverse, 5'-GGGAAGCCCAGGTCAAACA-3'; *RAMP3* forward, 5'-GGAAGGCTTTCGAGACATG-3'; *RAMP3* reverse, 5'-CGGACAGTTGCACCACTT-3'; *MAP2K1* forward, 5'-TTCTTGCTGGGCATACTTTCTCT-3'; and *MAP2K1* reverse, 5'-CATGCACTGCCTGTGAAGGA-3'.

### Western Blot Analyses

Western blot analysis was performed, as described previously,<sup>34</sup> using the following antibodies: anti-Ramp1 (rabbit, 1:1000; Santa Cruz Biotechnology), anti- $\beta$ -actin (goat, 1:1000; Santa Cruz Biotechnology), anti-Nkx3.1 (rabbit, 1:2000; provided by Dr. Charles Bieberich, University of Maryland, Baltimore County, Baltimore), and anti-phosphorylated-extracellular signal regulated kinase (ERK) 1/2 (rabbit, 1:2000; Cell Signaling, Boston, MA).

### IHC and Immunocytochemistry

Immunohistochemistry (IHC) was performed as previously described.<sup>35</sup> For antigen retrieval, slides were steamed for 15 minutes in Tris-EDTA buffer (pH 9.0). Slides were incubated overnight at 4°C with the following antibodies: anti-RAMP1 (rabbit, 1:150; Santa Cruz Biotechnology) for mouse tissue or 1:40 for human prostate TMAs; anti-Ki-67 (rabbit, 1:1000; Abcam, Cambridge, MA), phosphorylated-



**Figure 3** NKX3.1 represses RAMP1 expression in mouse and human prostate cells. **A:** Real-time RT-qPCR analysis of Ramp1 expression in the *Nkx3.1*-deficient mouse prostate. Total RNA was isolated from anterior prostates of 16-week-old *Nkx3.1*<sup>+/+</sup> ( $n = 6$ ), *Nkx3.1*<sup>+/-</sup> ( $n = 3$ ), and *Nkx3.1*<sup>-/-</sup> ( $n = 3$ ) mice. Expression levels were normalized to 18S. The results shown are representative of two independent experiments performed in triplicate. Data are reported as means  $\pm$  SD. **B:** Immunostaining for anti-Ramp1 in the anterior prostate of 16-week-old *Nkx3.1*<sup>+/+</sup>, *Nkx3.1*<sup>+/-</sup>, and *Nkx3.1*<sup>-/-</sup> mice shows up-regulation of Ramp1 (brown) in *Nkx3.1*<sup>+/-</sup> and *Nkx3.1*<sup>-/-</sup> mouse prostates. Images from two different mice are shown for each genotype. Original magnification,  $\times 40$ . **C** and **D:** Lentiviral-mediated expression of Nkx3.1 represses RAMP1 mRNA and protein levels in PC-3 and DU145 cells. RT-qPCR for RAMP1 and Western blot analyses for NKX3.1, RAMP1, and ACTIN control are shown. Results are representative of two independent experiments. RT-qPCR data are reported as means  $\pm$  SD, with RAMP1 expression levels normalized to *GAPDH*. \* $P < 0.05$ .

histone H3 (pHH3; rabbit, 1:500; Millipore), and activated caspase-3 (rabbit, 1:100; Cell Signaling). Slides were stained with 3,3'-diaminobenzidine tablets (Sigma) and counterstained with hematoxylin. For immunocytochemistry, cells were plated on coverslips and incubated overnight at 4°C with anti-RAMP1 (1:50), pHH3 (rabbit, 1:500; Millipore), and activated caspase-3 (rabbit, 1:100; Cell Signaling). Slides were counterstained with DAPI (Vector Labs, Burlingame, CA). The number of cells staining positive for pHH3 and caspase-3, of a total of 500 cells in three independent fields, was recorded. For cell block preparation, cells were grown to 80% confluency, harvested, and washed thoroughly with PBS. Cells were fixed for 5 minutes in 10% neutral-buffered formalin. Cell block was processed by the Vanderbilt Translational Pathology Shared Resource, and slides were stained for anti-RAMP1 (1:50).

### Cell Growth Assay

Cell growth was determined by cell count. A total of  $1 \times 10^4$  cells were plated in a 6-well plate in triplicate in complete medium and counted at the indicated time points.

### Soft Agar Assay

A soft agar colony formation assay was performed as previously described.<sup>36</sup> Briefly,  $1.5 \times 10^4$  PC-3 or LNCaP

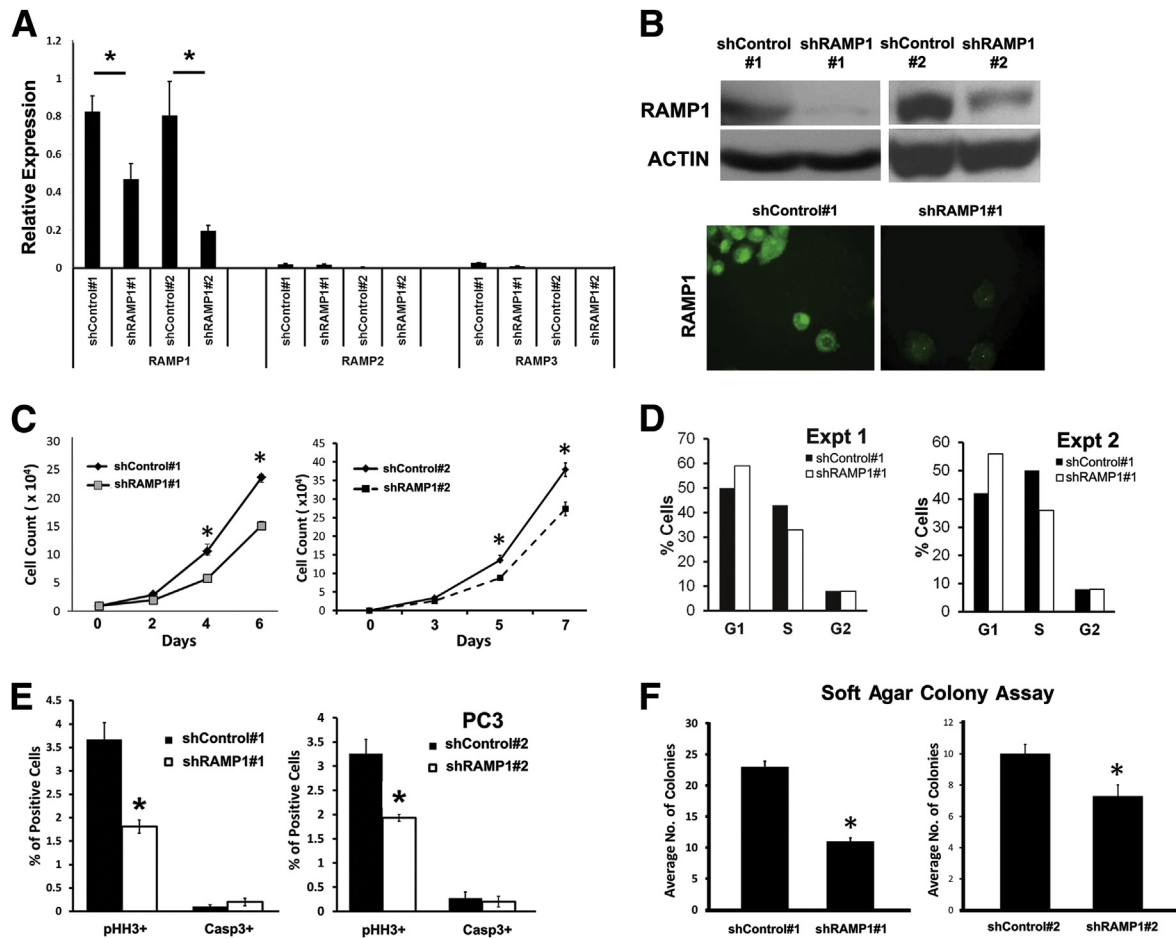
cells, stably expressing shControl or shRAMP1, were mixed with 0.35% soft agar and plated on top of a 0.5% bottom agar in a 6-well plate. Cells were incubated at 37°C for 2 to 3 weeks to allow colony formation. Each cell line was plated in triplicate. Three random low-power view fields were chosen, and the total number of colonies (cutoff sizes or more) was counted.

### Xenograft Studies

Xenograft studies were performed as previously described.<sup>37</sup> Male nude (nu/nu) mice were obtained from Charles River Laboratories (Wilmington, MA). A total of  $8 \times 10^6$  PC-3 cells stably expressing shControl or shRAMP1 were injected s.c. in both flanks of nude mice. Tumor volumes were calculated as previously described.<sup>37</sup> Animal care and experiments were performed according to protocols approved by the Institutional Animal Care and Use Committee at Vanderbilt University.

### Gene Expression Profiling and Analysis

Total RNA was extracted from PC-3 cells stably expressing shControl or shRAMP1, according to the TRIzol manufacturer's protocol (Invitrogen, Grand Island, NY). Three independent sample preparations for shControl and shRAMP1 cells were performed. RNA was treated with DNase I (Qiagen,



**Figure 4** RAMP1 promotes prostate cancer cell proliferation and tumorigenicity in PC-3 cells. **A:** RT-qPCR analysis of *RAMP1*, *RAMP2*, and *RAMP3* expression in PC-3 cells stably expressing shControl and shRAMP1 confirms specificity of RAMP1 knockdown. **B:** Western blot (top panel) and immunocytochemical (bottom panel) analyses confirm knockdown of RAMP1 in PC-3 cells. **C:** Cell growth was determined by cell count. **D:** Cell cycle analysis of RAMP1 knockdown PC-3 cells by flow cytometry. **E:** Quantitation of proliferation and apoptosis in shControl and shRAMP1 cells after staining for pHH3 and caspase 3, respectively. Data are reported as percentage of cells positive for the marker. **F:** Analysis of *in vitro* tumorigenicity by soft agar assay. Results are representative of at least two independent experiments performed in triplicate. Data are reported as means  $\pm$  SEM. \* $P < 0.05$ .

Gaithersburg, MD), according to the manufacturer's protocol, followed by purification using the RNA Clean Up protocol from the RNeasy Mini Kit (Qiagen). RNA processing and microarray analysis were performed by the Vanderbilt Genome Sciences Resource Core, as previously described.<sup>38</sup> cDNA was hybridized to the GeneChip PrimeView Human Gene Expression Array (Affymetrix, Santa Clara, CA). CEL files were imported to R, version 2.15.0 (<http://www.r-project.org>), for quality control and preprocessing. Quality control was implemented in Bioconductor with affyPLM, version 1.32, and simpleaffy, version 2.32, packages. Arrays for three independent shControl and shRAMP1 samples passed quality control tests. By using the Affy package, version 1.34.0,<sup>39</sup> raw intensity scores were normalized by quartiles, background corrected with robust multi-array average,<sup>40</sup> and summarized by mean polish using perfect match-only probes. The C2 (curated) and C6 (oncogenic signatures) gene sets of Molecular Signatures Database, version 3.1 (Broad Institute, Cambridge, MA), were queried using Gene Set Enrichment Analysis (GSEA) (Broad Institute), version 2.07,<sup>41</sup> to test for

differences between shControl and shRAMP1 cells. All microarray and GSEA analyses were performed on a node running Debian Linux Squeeze, version 6.0.6 (<http://www.debian.org/releases>).

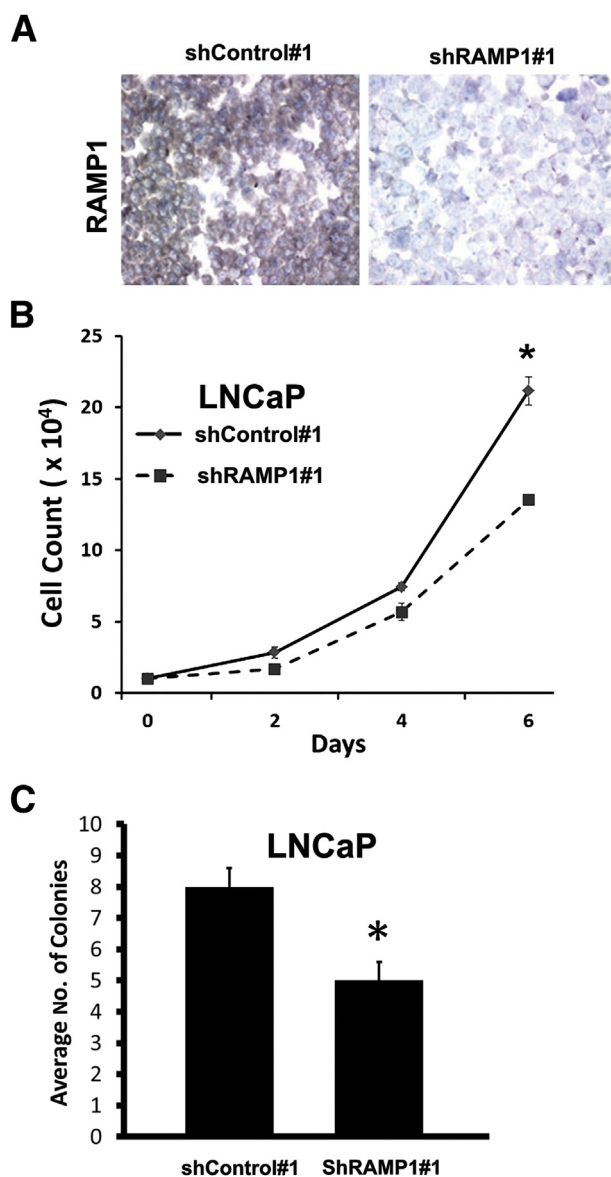
### Statistical Analysis

To compare groups, we used the unpaired Student's *t*-test. Data are expressed as means  $\pm$  SD or SEM for each group. Data were considered statistically significant at  $P < 0.05$ .

## Results

### RAMP1 Is Overexpressed in Human Prostate Cancer

A previous study identified RAMP1 mRNA as one of several mRNAs with potential to be a biomarker in prostate cancer.<sup>8</sup> To determine whether RAMP1 is up-regulated in prostate or other cancers, we queried the Oncomine database for a comprehensive evaluation of RAMP1 expression in human cancers.



**Figure 5** Knockdown of RAMP1 in LNCaP cells decreases cell proliferation and tumorigenicity *in vitro*. **A:** IHC analysis of cell blocks shows reduced RAMP1 expression (reddish brown staining) in LNCaP cells expressing shRAMP1 compared with shControl cells. **B:** Cell growth was determined by cell count. **C:** Analysis of *in vitro* tumorigenicity by soft agar assay. Results are representative of at least two independent experiments performed in triplicate. Data are reported as means  $\pm$  SEM. \* $P < 0.05$ .

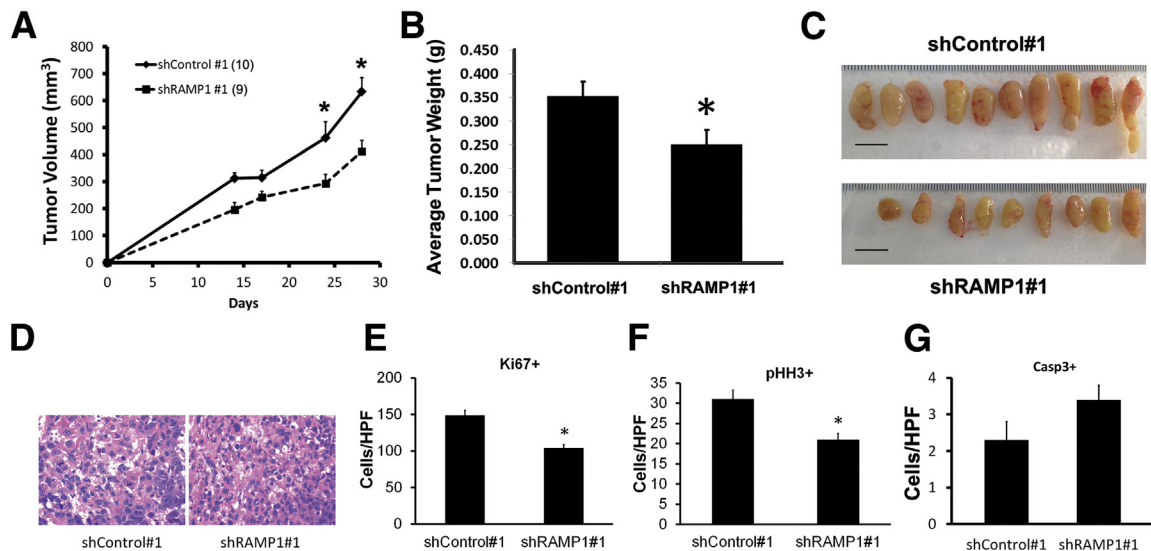
Oncomine is a cancer-specific database that identifies genes, pathways, and networks deregulated across gene expression microarrays.<sup>31</sup> Notably, *RAMP1* was specifically up-regulated in prostate cancer ( $P = 6.36 \times 10^{-47}$ ) compared with other human cancers that include bladder, breast, colorectal, kidney, liver, lung, ovarian, and pancreatic in the Su Multicancer data set<sup>42</sup> (Figure 1A). Next, we examined the expression of RAMP1 in normal prostate versus prostate carcinoma. *RAMP1* was significantly up-regulated in prostate carcinoma compared with normal prostate in multiple data sets<sup>43–46</sup> (Figure 1B). To determine whether RAMP1 protein expression is also altered during prostate tumorigenesis, we performed RAMP1 IHC on

two independent human prostate cancer TMAs. RAMP1 expression was detected in the cytoplasm and membrane of prostatic epithelial cells with limited expression in the stroma (Figure 1C). RAMP1 protein expression was also significantly up-regulated in human prostate cancer compared with benign glands (Figure 1D). Overall, these findings show that RAMP1 is specifically up-regulated in prostate cancer and may be relevant for prostate tumorigenesis.

### *RAMP1* Is a Direct NKX3.1 Target Gene

We have previously used ChIP-Seq to identify genomic loci bound by NKX3.1 in the prostate cancer cell line, LNCaP (M.L., P.D.A., and S.A.A., unpublished data) and in the normal mouse prostate.<sup>26</sup> Analysis of data from these ChIP-Seq studies showed that *Nkx3.1* binds *Ramp1* downstream of the transcription start site and within the gene in the mouse prostate (Figure 2A). In LNCaP cells, NKX3.1 binds the *RAMP1* locus at three different sites (Figure 2B). NKX3.1 binding to the *RAMP1* gene was confirmed by ChIP, followed by qPCR, using primers flanking the three NKX3.1 binding sites within the *RAMP1* gene in LNCaP cells (Figure 2C). An androgen receptor promoter region containing a consensus NKX3.1 binding site was used as a positive control. All three binding sites exhibited significant enrichment for NKX3.1 compared with IgG control. These results suggest that the prostate tumor suppressor NKX3.1 might be involved in the regulation of RAMP1 expression in the prostate.

To explore the functional significance of *Nkx3.1* binding to the *Ramp1* gene, we examined the expression of *Ramp1* in *Nkx3.1* knockout mice. RT-qPCR analysis of the prostates of *Nkx3.1* mutant mice showed a dose-dependent increase in *Ramp1* mRNA in *Nkx3.1*<sup>+/-</sup> and *Nkx3.1*<sup>-/-</sup> mouse prostates (Figure 3A). We observed a similar effect of loss of *Nkx3.1* on *Ramp1* protein expression in the prostate by IHC (Figure 3B). *Nkx3.1* knockout mice expressed high levels of *Ramp1* protein in the prostatic epithelium with limited expression in the stroma, consistent with the known cellular localization of *Nkx3.1* protein in the prostate.<sup>12</sup> These results indicate that *Nkx3.1* represses *Ramp1* expression in a dose-dependent manner in the mouse prostate. We extended these mouse results to human prostate cells by assessing the effect of exogenous NKX3.1 expression in PC-3 and DU145 cells on RAMP1 expression. We used lentiviral-mediated gene transfer to express mouse *Nkx3.1* in PC-3 and DU145 cells, which normally lack NKX3.1 expression (Figure 3, C and D). The levels of exogenous *Nkx3.1* expression achieved are comparable to the endogenous NKX3.1 levels in LNCaP cells (data not shown). In both PC-3 and DU145 cells, *Nkx3.1* expression repressed RAMP1 mRNA and protein expression, as determined by RT-qPCR and Western blot analyses, respectively (Figure 3, C and D). The results presented, when taken together, indicate that NKX3.1 binds to and represses the *RAMP1* gene in the prostate.



**Figure 6** Depletion of RAMP1 reduces tumorigenicity and proliferation *in vivo*. **A–C:** Xenografts of shRAMP1 1 PC3 cells grown s.c. in nude mice show reduced tumor growth (**A**) and final tumor weight (**B**) and size (**C**). **D:** Representative images of H&E-stained sections of tumor grafts. **E:** Ki-67 proliferation index. **F:** pHH3, phosphorylated-histone H3 serine 10 index. **G:** Apoptotic index by activated caspase-3 staining. The total number of cells staining positive for either Ki-67, pHH3, or activated caspase-3 in five random high-power field (HPFs) were counted for each graft section. Data are reported as means  $\pm$  SEM. \* $P < 0.05$ .

### Down-Regulation of RAMP1 Suppresses Cell Proliferation and *in Vitro* Tumorigenicity in PC-3 and LNCaP Cells

To examine the functional significance of the RAMP1 up-regulation in human prostate cancer, we stably knocked down RAMP1 in PC-3 cells using two independent shRNAs, shRAMP1 1 and shRAMP1 2 (Figure 4, A and B). Knockdown efficiency was evaluated by RT-qPCR, Western blot, and immunocytochemistry analyses (Figure 4, A and B). To further confirm specificity of the shRAMP1 constructs, we evaluated the expression of RAMP2 and RAMP3 and found these to be expressed at low levels in these cells without being affected by RAMP1 knockdown (Figure 4A). Functionally, knockdown of RAMP1 significantly decreased cell proliferation in both shRAMP1 1 and shRAMP1 2 PC-3 cells, as determined by cell counting and the pHH3 index (Figure 4, C and E). Similarly, we observed a decrease in the percentage of cells in the S phase of the cell cycle in RAMP1-deficient cells compared with shControl cells (Figure 4D). There was no significant change in apoptosis, as determined by staining for activated caspase-3 (Figure 4E). We next examined the effect of RAMP1 depletion in prostate cancer cells on *in vitro* tumorigenicity using the soft agar colony formation assay. Consistent with the proliferation assays, a significant decrease in colony formation was observed in PC-3 cells with stable knockdown of RAMP1 (Figure 4F). These findings were replicated in LNCaP prostate carcinoma cells with stable depletion of RAMP1 by shRNA, which also showed significant reductions in proliferation and tumorigenicity (Figure 5, A–C). These results establish a functional role for RAMP1 in promoting the proliferation and *in vitro* tumorigenicity of prostate cancer cells.

### Knockdown of RAMP1 Decreases Prostate Cancer Cell Tumorigenicity *in Vivo*

Next, we performed xenograft studies in nude mice using PC-3/shControl 1 and PC-3/shRAMP1 1 cells to access the effect of RAMP1 knockdown *in vivo*. Tumors in mice injected with shRAMP1-expressing cells grew at a slower rate and were smaller in size and weight at the end of the experiment compared with tumors in mice injected with shControl cells (Figure 6, A–C). We analyzed proliferation in these tumors by IHC staining for Ki-67 and pHH3. Proliferation was significantly reduced in RAMP1-depleted tumors relative to the control tumors (Figure 6, D–F). Staining for activated caspase-3 showed a trend for increased apoptosis that did not achieve statistical significance (Figure 6G). These results are consistent with our *in vitro* data showing that RAMP1 knockdown mainly affects prostate cancer cell proliferation.

### Down-Regulation of RAMP1 Modulates MAPK Signaling in Prostate Carcinoma Cells

To explore possible molecular pathways by which RAMP1 down-regulation modulates the tumorigenicity of prostate cancer cells, we performed gene expression profiling using a GeneChip PrimeView Human Gene Expression Array (Affymetrix). We found 936 genes to be significantly altered ( $\alpha = 0.05$ ) before a multiple testing penalty in RAMP1 knockdown cells. By using these genes to interrogate WebGestalt, we identified several significantly altered pathways, including the epidermal growth factor receptor 1 (EGFR1), mitogen-activated protein kinase (MAPK), and IL-6 signaling pathways (Table 1). Notable among the



**Table 1** Pathway Analysis of Genes Dysregulated in RAMP1 Knockdown PC3 Cells

Variable	No. of genes	Expected	Enriched	Adjusted <i>P</i> value
<b>KEGG</b>				
Pathways in cancer	23	5.62	4.09	$1.37 \times 10^{-6}$
MAPK signaling pathway	14	4.58	3.06	0.0016
Endocytosis	16	3.18	5.02	$6.52 \times 10^{-6}$
Spliceosome	11	2.18	5.05	0.0004
Neurotrophin signaling pathway	10	2.15	4.66	0.001
Prostate cancer	8	1.52	5.28	0.001
Small-cell lung cancer	8	1.43	5.59	0.001
Chronic myeloid leukemia	8	1.28	6.26	0.0009
Pancreatic cancer	7	1.23	5.71	0.0016
Glioma	7	1.11	6.32	0.001
<b>WIKI Pathways</b>				
EGFR1 signaling pathway	20	3.03	6.6	$1.95 \times 10^{-9}$
Insulin signaling	10	2.72	3.67	0.0024
B-cell receptor signaling pathway	12	2.69	4.46	0.0002
Regulation of actin cytoskeleton	10	2.43	4.11	0.0013
mRNA processing	11	2.32	4.75	0.0002
Adipogenesis	9	2.26	3.97	0.0027
Androgen receptor signaling pathway	11	1.96	5.62	$8.22 \times 10^{-5}$
IL-6 signaling pathway	10	1.67	5.99	$9.31 \times 10^{-5}$
Senescence and autophagy	10	1.02	9.79	$1.79 \times 10^{-6}$
Id signaling pathway	6	0.87	6.91	0.0013
<b>Pathway Commons</b>				
Glypican pathway	23	7.42	3.1	0.0001
Glypican 1 network	23	6.88	3.34	$4.95 \times 10^{-5}$
IFN- $\gamma$ pathway	19	5.82	3.26	0.0004
TRAIL signaling pathway	17	5.06	3.36	0.0006
Signaling by GPCR	13	3.32	3.92	0.0008
EGFR1	14	2.45	5.71	$2.33 \times 10^{-5}$
Sphingosine 1-phosphate pathway	10	1.92	5.2	0.0007
Signaling by EGFR	10	1.12	8.9	$2.33 \times 10^{-5}$
Synaptic transmission	7	0.92	7.61	0.0008
IL-6-mediated signaling events	7	0.77	9.14	0.0004

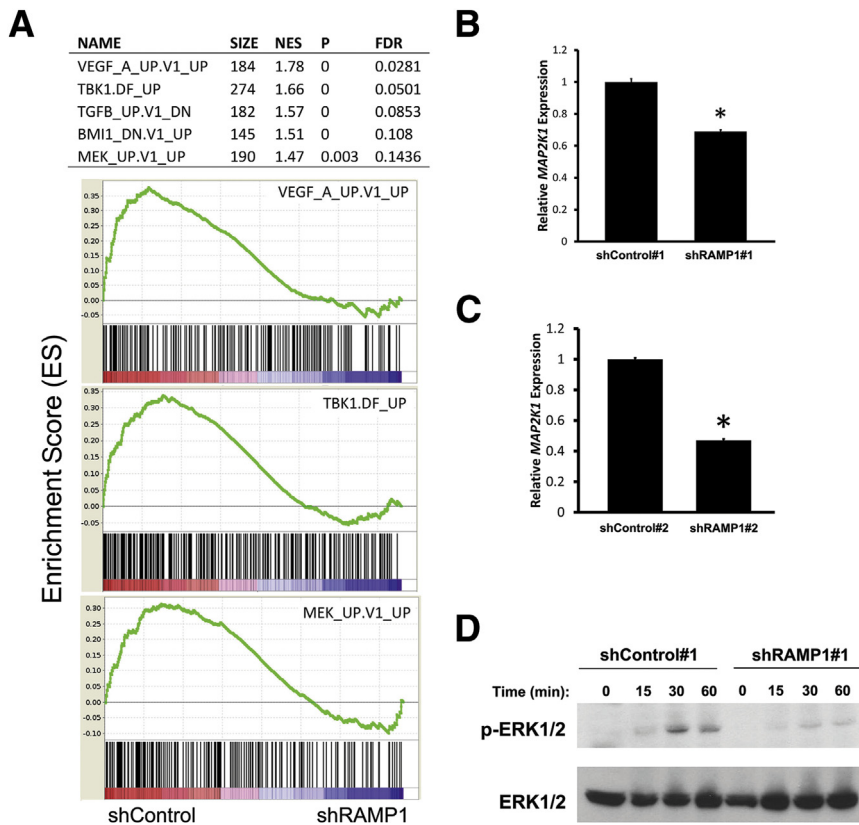
GPCR, G protein-coupled receptor; Id, inhibitor of DNA binding; IFN, interferon; KEGG, Kyoto Encyclopedia of Genes and Genomes; TRAIL, TNF-related apoptosis-inducing ligand.

genes altered in the EGFR1 and MAPK signaling pathways is the MAPK signaling pathway regulator, MAP2K1 (*MEK1*) (Supplemental Table S1). Some of the IL-6 signaling pathway genes identified as altered in RAMP1-depleted cells include *CCND*, *JAK1*, *IL6*, *IL6R*, and *SOS2*.

We next performed GSEA to define gene sets significantly enriched or depleted in RAMP1 knockdown cells. GSEA is a comprehensive database of genes, pathways, and regulatory and functional networks activated and repressed in human cancer.<sup>31,47</sup> We interrogated the curated cancer-relevant database C6 of the Molecular Signatures Database, a collection of gene sets curated from gene expression profiles in cancer-relevant experiments, to identify possible cancer-relevant pathways altered in shRAMP1 cells. Interestingly, among the top gene sets enriched in control relative to shRAMP1 cells is MEK\_UP.V1\_UP, a gene set found to be activated in cells with enhanced MAPK signaling (Figure 7A and Supplemental Table S2). This gene set

consists of genes up-regulated in MCF-7 breast cancer cells stably overexpressing constitutively active MAP2K1 (*MEK1*).<sup>48</sup> Other gene sets identified in this analysis were proangiogenic genes, *VEGF-A* and *TBK1* (Figure 7A). The *VEGF-A* gene set consists of genes that were found to be up-regulated on vascular endothelial growth factor (VEGF)-A treatment, whereas the *TBK1* gene set is composed of genes that were up-regulated after overexpression of the RAS oncogene.

Because *MEK* encodes a protein that phosphorylates and activates ERK1/2, we hypothesized that depletion of RAMP1 may inhibit prostate cell tumorigenicity by suppressing the MAPK signaling pathway. We confirmed reduced expression of MAP2K1 (*MEK1*) in prostate cells depleted of RAMP1 using both shRAMP1 1 and shRAMP1 2 (Figure 7, B and C). Next, we performed Western blot analysis to determine the effect of RAMP1 knockdown on ERK1/2 activation. As expected, *RAMP1* knockdown



**Figure 7** Down-regulation of RAMP1-altered gene expression and MAPK signaling in PC-3 cells. **A:** GSEA shows a list of selected gene sets enriched in shControl relative to shRAMP1 cells (**top panel**). GSEA enrichment score plots for selected gene sets (**bottom panel**). Normalized enrichment score (NES), nominal *P* value (statistical significance of enrichment), and false-discovery rate (FDR) are presented. **B** and **C:** RT-qPCR analysis of *MAP2K1* expression in RAMP1 knockdown cells. Results are representative of at least two independent experiments performed in triplicate. Data are reported as means  $\pm$  SEM. \**P* < 0.05. **D:** Western blot analysis of ERK1/2 activation in shControl and shRAMP1 cells. Cells were serum starved for 24 hours, induced with 10% fetal bovine serum, and harvested at 0-, 15-, 30-, and 60-minute time points. Results are representative of at least two independent experiments.

suppressed activation of ERK1/2 in PC3 cells, as revealed by reduced ERK1/2 phosphorylation (Figure 7D). These results suggest suppression of MAPK signaling as a mechanism for the reduced prostate tumorigenicity observed in RAMP1-deficient cells.

## Discussion

Loss of NKX3.1 expression is often associated with the initiation of prostate tumorigenesis. In this study, we identified *RAMP1* as a direct NKX3.1 target gene involved in promoting prostate tumorigenesis and have, for the first time to our knowledge, reported a functional role for RAMP1 in this disease. NKX3.1 binds multiple sites in the *RAMP1* locus in both the human and mouse genomes. Furthermore, deletion of the *Nkx3.1* gene in mice led to the up-regulation of Ramp1 in a dose-dependent manner, whereas exogenous Nkx3.1 suppressed RAMP1 expression in human prostate cancer cells. Our studies suggest that loss of NKX3.1 during human prostate tumorigenesis may be a key factor in the up-regulation of RAMP1 in tumors.

RAMP1 is well known for regulating the trafficking and pharmacological features of G protein-coupled receptors. In addition, overexpression of RAMP1 has been shown to play a protective role in cardiovascular disease by attenuating angiotensin II-induced hypertension and vascular dysfunction.<sup>49,50</sup> Herein, we described another functionally significant role for RAMP1 in human prostate cancer.

Previously, RAMP1 mRNA was shown to be up-regulated in human prostate tumors, and it was considered to be a novel potential biomarker for prostate cancer.<sup>8</sup> We observed up-regulation of RAMP1 protein in prostate tumors, and that depletion of RAMP1 decreased cell proliferation and tumorigenicity in prostate cancer cells. These findings indicate that RAMP1 may play a pivotal role in prostate tumorigenesis.

RAMP1 has been shown to interact with VIPR1 and CALCRL, two receptors that bind ligands that have been shown to promote tumorigenesis.<sup>1,4</sup> RAMP1 interaction with the CALCRL receptor yields the CGRP receptor, which has high affinity for the neuropeptide, CGRP.<sup>1</sup> CGRP has been shown to increase the invasiveness and migration of human prostate cancer cells, and angiogenesis and tumor growth in a xenograft model.<sup>51–53</sup> Equally important, CGRP has been reported to be elevated in the serum of untreated patients with prostate cancer.<sup>54</sup> It has previously been reported that CGRP can regulate VEGF expression in human HaCaT keratinocytes by activation of ERK1/2.<sup>55</sup> VEGF is a proangiogenic gene that is overexpressed in prostate cancer and in NKX3.1-deficient cells.<sup>56–58</sup> Interestingly, our GSEA analysis shows *VEGF-A* as the top gene altered in RAMP1-deficient cells. TANK-binding kinase-1 (*TBK1*) is another proangiogenic gene that is altered in RAMP1-knockdown cells. TBK1 has been shown to induce cell proliferation and secretion of angiogenesis-associated factors,<sup>59</sup> possibly by mediating insulin and AKT signaling pathways.<sup>60</sup> Taken together, our data suggest that

RAMP1 may regulate tumor growth and tumor-associated angiogenesis by modulating cell survival pathways.

Likewise, VIPR1 binds the neuropeptide, VIP. *In vitro*, VIP induced the malignant transformation of nontumorigenic prostate epithelial cells and increased migration of LNCaP cells, an androgen-sensitive human prostate cancer cell.<sup>52,61</sup>

VIP has also been shown to enhance angiogenesis and tumor growth in *in vivo* models of human prostate cancer.<sup>61–63</sup>

These studies are important considering that the prostate gland contains autonomic and sensory neurons and neuroendocrine cells that secrete these neuropeptides. In fact, it has been reported that, in prostatic adenocarcinomas, neuroendocrine cells tend to occur in close proximity to proliferating cells.<sup>64</sup>

Thus, this may explain why both CGRP and VIP may cause prostate tumor growth. However, it is possible that there are other unknown RAMP1-interacting partners that may also promote prostate tumorigenesis.

Our gene expression profiling data indicated that altered MAPK signaling is one of the functional consequences of RAMP1 depletion. We identified MAP2K1, an upstream kinase in the MAPK signaling pathway as a gene whose expression is impaired in RAMP1-depleted cells. Previous studies have shown that both activation of CGRP and VIP can induce ERK1/2 activation,<sup>55,65</sup> suggesting that lack of RAMP1 leads to reduced MAPK signaling by attenuating signal transduction via RAMP1 coreceptors. Another pathway altered in RAMP1-deficient cells is the EGFR1 signaling pathway. EGFR1 is frequently overexpressed in hormone-refractory and metastatic prostate cancer.<sup>66,67</sup> EGFR1 signaling can also lead to activation of downstream cell survival pathways, MAPK, and phosphatidylinositol 3-kinase.<sup>68</sup> In addition, activation of IL-6 signaling was also suppressed in RAMP1-depleted cells. IL-6 is a pleiotropic cytokine with diverse roles in immune and inflammatory responses and in mediating growth of normal and tumor cells.<sup>69</sup> It mediates its effects through activation of the MAPK, phosphatidylinositol 3-kinase, and Janus activated-kinase/signal transducers and activators of transcription signaling pathways that promote survival of cancer cells.<sup>70–72</sup> Several studies have observed elevated IL-6 in the sera of patients with castration-resistant prostate cancer compared with men with a normal-functioning prostate, benign prostatic hyperplasia, prostatitis, and localized or recurrent disease.<sup>73–76</sup> Also, IL-6 is expressed in metastatic prostate cancer cells, PC-3, which do not express NKX3.1.<sup>71</sup> Interestingly, IL-6 is undetectable in LNCaP cells that express NKX3.1.<sup>71,77</sup> Our studies suggest that loss of NKX3.1 results in dysregulation of RAMP1, which leads to activation of cell survival pathways.

RAMP1 has been proposed as a potential biomarker in prostate cancer.<sup>8</sup> Because RAMP1 presents G protein-coupled receptors that bind ligands known to promote prostate tumorigenesis to the cell membrane, it could potentially be a molecular target for the diagnosis and treatment of prostate cancer. The use of a monoclonal antibody to therapeutically target RAMP1-expressing cells may prove beneficial in treating patients with prostate cancer, or may be ideal for imaging prostate lesions.

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## Supplemental Data

Supplemental material for this article can be found at <http://dx.doi.org/10.1016/j.ajpath.2013.05.021>.

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