Oncogene-specific activation of tyrosine kinase networks during prostate cancer progression

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Dominant mutations or DNA amplification of tyrosine kinases are rare among the oncogenic alterations implicated in prostate cancer. We demonstrate that castration-resistant prostate cancer (CRPC) in men exhibits increased tyrosine phosphorylation, raising the question of whether enhanced tyrosine kinase activity is observed in prostate cancer in the absence of specific tyrosine kinase mutation or DNA amplification. We generated a mouse model of prostate cancer progression using commonly perturbed non-tyrosine kinase oncogenes and pathways and detected a significant up-regulation of tyrosine phosphorylation at the carcinoma stage. Phosphotyrosine peptide enrichment and quantitative mass spectrometry identified oncogene-specific tyrosine kinase signatures, including activation of EGFR, ephrin type-A receptor 2 (EPHA2), and JAK2. Kinase:substrate relationship analysis of the phosphopeptides also revealed ABL1 and SRC tyrosine kinase activation. The observation of elevated tyrosine kinase signaling in advanced prostate cancer and identification of specific tyrosine kinase pathways from genetically defined tumor models point to unique therapeutic approaches using tyrosine kinase inhibitors for advanced prostate cancer.

AKT | androgen receptor | ERG | K-RAS | bioinformatics

The future of effective cancer treatment is based on the emerging concept of personalized therapy, which requires detailed analysis of the oncogenic lesions that drive disease. One prominent oncogenic change seen in many cancers is somatic-activating mutations of tyrosine kinases, including BCR-ABL in chronic myelogenous leukemia (CML), mast/stem cell growth factor receptor (SCFR or KIT) in gastrointestinal stromal tumors (GIST), and EGFR in lung cancer (1–3). The dependency on tyrosine kinase activation in these tumors has led to successful clinical treatment with tyrosine kinase inhibitors (4). In prostate cancer, great progress has been made in identifying the genetic determinants of disease progression such as increased expression of androgen receptor (AR) and myeloidomatosis oncogene cellular homolog (MYC), phosphatase and tensin homologue deleted on chromosome 10 (PTEN) deletion, and erythroblast transformation specific (ETS) family gene fusions (7–11). However, recent large-scale cancer genome studies show that activating somatic mutations or DNA amplification of tyrosine kinase genes are rare in prostate cancer (8). This reveals why clinical administration of tyrosine kinase inhibitors for the treatment of advanced prostate cancer has been less effective and strongly implies that more complete understanding of the tyrosine kinases that contribute to this disease is warranted (12, 13).

Despite the paucity of activating somatic mutations in tyrosine kinases, recent evidence suggests that tyrosine kinase phosphorylation in prostate cancer contributes to disease progression. In androgen-depleted conditions, tyrosine kinase, non-receptor, 2 (TNK2 or ACK1), SRC, and erythroblastic leukemia viral oncogene homolog 2 [E8RB2 (HER-2/neu)] tyrosine kinase activity can restore AR function in prostate cancer cells (14–17). Increased expression of the tyrosine kinase SRC and AR can synergistically drive frank carcinoma of the mouse prostate (18). This relationship results in robust activation of SRC tyrosine kinase and MAPK signaling (18). SRC activity was also observed in a subset of castration-resistant prostate cancer (CRPC) patients, which correlated with lower overall survival and increased metastatic disease (19). These data support the idea that tyrosine kinase activity may play a prominent role in prostate cancer progression in the absence of activating mutations.

Nearly 50% of tyrosine kinases are thought to contribute to human cancers, yet tyrosine phosphorylation represents less than 1% of the phosphoproteome (20). Sensitive and specific methods capable of enriching tyrosine phosphorylated peptides via antibody binding followed by quantitative mass spectrometry (MS) identification has become useful for the elucidation of tyrosine kinase signaling pathways, nodes, and negative feedback mechanisms in different cancer types (21–23). The ability to sensitively characterize pathway alterations in the presence of activated tyrosine kinases or tyrosine kinase inhibitors can allow for the identification of new potential drug targets (21, 24). We use this approach to identify and characterize tyrosine kinase signaling networks in transformed tissues that do not express mutated tyrosine kinases.

Global tyrosine phosphorylation in clinical prostate cancer samples was measured by immunohistochemistry (IHC) and showed a substantial increase in tyrosine phosphorylation in late-stage disease. To study this in a controlled manner, we evaluated tyrosine phosphorylation in a mouse model of prostate cancer progression using oncogenes common to prostate tumorigenesis and observed robust tyrosine phosphorylation in the advanced tumor phenotypes. Unbiased phosphotyrosine proteomics was used to investigate the specific tyrosine kinase signaling pathways activated by each of the nontyrosine kinase oncogenes. Analysis of the tyrosine phosphoproteome of these tumors revealed oncogene-specific tyrosine kinase activation including EGFR, ephrin type A receptor 2 (EPHA2), JAK2, ABL1, and SRC.

Results Tyrosine Phosphorylation Is Increased in Clinical Castration-Resistant Prostate Cancer Samples. We performed IHC staining of prostate cancer tissue microarrays with the tyrosine phosphorylation—


The authors declare no conflict of interest.

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Data deposition: MS2 spectra for all phosphopeptides reported in this paper have been deposited in the PRIDE database, http://www.ebi.ac.uk/pride (accession nos. 20879–20889).

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specific antibody 4G10 to evaluate phosphotyrosine expression during disease progression. CRPC (androgen independent) exhibited a robust increase in phosphotyrosine staining intensity compared with benign prostate, the precursor lesion high-grade prostatic intraepithelial neoplasia (HGPIN), or hormone naïve (androgen dependent) prostate cancer (HNPC) (Fig. L4). Analysis of these tissue microarray samples indicated that 44% of CRPC specimens stain for phosphotyrosine at moderate to high levels (staining intensity 2–3), whereas only 11% of normal, 2% of HGPIN, and 2% of HNPC tissues stain at this intensity (Fig. 1B). Further, the average staining intensity of all of the CRPC tissue samples was significantly increased by over twofold compared with the other clinical phenotypes (Fig. 1C). These data reveal that tyrosine phosphorylation is present and elevated in CRPC and raise the notion that systemic treatment of patients with this disease may induce this response.

Tyrosine Phosphorylation Is Robust in Mouse Models of Advanced Prostate Cancer. The observation of increased tyrosine phosphorylation in late-stage prostate cancer specimens raises the question of whether tyrosine kinase activity is evident in prostate cancer models that do not express mutated or amplified tyrosine kinases. We recapitulated different stages of prostate cancer ranging from prostate intraepithelial neoplasia (PIN) to adenocarcinoma using the prostate in vivo regeneration model system (25, 26). We chose four of the most commonly perturbed oncogenes in prostate cancer, both in androgen-dependent and -independent states: activated AKT (myristoylated AKT, resembling PTEN deletion, ~40–70% of prostate cancers), AR amplification (~20–60% of prostate cancers), ERG rearrangements (~40–70% of prostate cancers), and activated K-RAS (K-RASG12V, resembling RAS/RAF pathway activation, observed in ~40–50% of prostate cancers) (7, 8, 11, 27–30).

We infected total mouse prostate cells with AKT alone or in combination with each respective oncogene using a lentiviral vector delivery system (Fig. 2A) and evaluated the histological phenotype of the resulting tumors after 12 wk. These tumors displayed histological characteristics of PIN (AKT), well differentiated and less aggressive cancer (AKT/ERG), or adenocarcinoma (AKT/AR and AKT/K-RASG12V) (Fig. 2B). IHC and Western blot analysis confirmed ectopic expression of each oncogene (Fig. S1A and B). IHC staining and Western blot analyses displayed a gradient of phosphotyrosine expression in these tumors ranging from low to undetectable levels of tyrosine phosphorylation in the normal and indolent lesions (mouse prostate, AKT, or AKT/ERG) to very high levels in the more advanced tumors (AKT/AR and AKT/K-RASG12V) (Fig. 2B and Fig. S2A and B).

Phosphoproteomic Profiling Identifies Oncogene-Dependent Tyrosine Phosphorylation of Kinases and Phosphatases. We enriched for tyrosine phosphorylated peptides and performed quantitative label-free MS to identify phosphopeptides that contribute to this increased tyrosine phosphorylation (21, 31). We identified 139 phosphopeptides corresponding to 102 proteins (Dataset S1). Statistical analysis (ANOVA, 0.2 cutoff) revealed differential phosphorylation of 116 phosphopeptides corresponding to 87 proteins across all of the tumor phenotypes. Unsupervised hierarchical clustering analysis identified unique and overlapping patterns of tyrosine phosphorylated peptides for each tumor type, with an increased abundance of tyrosine phosphorylation events observed in the more advanced tumors (AKT/AR and AKT/K-RASG12V) (Fig. 3A and Fig. S3). These data demonstrate oncogene-specific signatures of phosphotyrosine activation across the spectrum of prostate cancer progression.

From the MS data, the activation sites of several tyrosine kinases and protein phosphatases were identified in the specific tumor

![Fig. 1. Robust phosphotyrosine expression is observed in castration-resistant prostate cancer (CRPC) specimens. (A) Representative image of immunohistochemical staining using the phosphotyrosine-specific antibody, 4G10, of prostate specimens ranging from normal to CRPC. Tissue spots from patients with CRPC show high levels of phosphotyrosine expression in the epithelial compartment. (B) Increased tyrosine phosphorylation is observed in CRPC, with nearly 50% of the patients displaying high-intensity staining (2, 3) compared with normal, HGPIN, or HNPC tissues. (C) Average staining intensity of all of the tissues clearly show a significant increase of tyrosine phosphorylation in CRPC patients. HGPIN, high-grade prostatic intraepithelial neoplasia; HNPC, hormone naïve prostate cancer; HRPC, hormone refractory prostate cancer. ***P < 0.001, one-way ANOVA. (Scale bar, 200 μm.)](#)

![Fig. 2. Phosphotyrosine expression is increased during prostate cancer progression. (A) Lentiviral vector diagram displaying the organization of oncogene and fluorescent marker expression used in these tumors. (B) Gross and histological morphology of each tumor type after 12-wk engraftment in SCID mice using the prostate regeneration protocol. Fluorescence corresponds to expression of a particular oncogene. IHC staining of progressive mouse tumor phenotypes reveals an increasing gradient of phosphotyrosine expression with more aggressive tumors expressing higher levels than indolent tumors. Tt, transillumination; H&E, hematoxylin and eosin; pY, phosphotyrosine. (Scale bars, 50 μm.)](#)
types (Table 1 and Figs. S3 and S4) (32). Consistent with these findings, Western blotting confirmed high levels of a second EGFR phosphorylation site (Y1172) and PTPN11 (SHP-2) Y584 in AKT/ERG tumors (Fig. 3B). Activation of the JAK/STAT pathway was also revealed in AKT/AR tumors as high levels of phosphorylation of STAT3 Y705 were observed. Western blotting confirmed activation of the upstream kinase JAK2 Y1007/08 and STAT3 Y705 in this tumor type (Fig. 3B). We additionally identified an increase in phosphorylation of PTK2B/PYK2/FAK2 Y579 and Y849 in AKT/K-RASG12V tumors and Western blot confirmed the phosphorylation of the activation site Y402 of PTK2B (Fig. 3B). Together, these data demonstrate that each combination of prostate cancer oncogenes generates distinct patterns of tyrosine kinase and phosphatase activity.

**Bioinformatic Inference of Tyrosine Kinase Activity Reveals Enrichment of Dasatinib Targets in AKT/AR Tumors.** In addition to direct observation of phosphorylated tyrosine kinases and phosphatases by MS, we sought to use the phosphotyrosine peptide data to infer kinase activities specific to each tumor type. We predicted the activated kinases directly upstream for each observed phosphorylation site using known relationships from PhosphoSite (32), kinase motifs from PhosphoMotif Finder (33) and Phosida (34), and predictions from NetworKin (35). We then performed an

**Table 1. Oncogene-specific phosphoactivation of tyrosine kinases and phosphatases**

<table>
<thead>
<tr>
<th>Oncogene combination</th>
<th>Tyrosine kinase (phosphoresidue)</th>
<th>Tyrosine phosphatase (phosphoresidue)</th>
<th>Functional significance*</th>
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<tbody>
<tr>
<td>AKT/ERG</td>
<td>EGFR (Y1172)</td>
<td>PTPN11 (Y584)</td>
<td>Enzymatic activation</td>
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<td></td>
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<td>PTPRA (Y620)</td>
<td>Enzymatic activation</td>
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<td></td>
<td></td>
<td>INPPL1 (Y1136)</td>
<td>Unknown</td>
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<tr>
<td>AKT/AR</td>
<td>JAK2 (Y1007/08)†</td>
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<td>AKT/K-RASG12V</td>
<td>EPHA2 (Y595)</td>
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<td>LYN (Y508)</td>
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<td>FER (Y402)</td>
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<td>AKT/ERG</td>
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<td>AKT/AR</td>
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<td>AKT/K-RASG12V</td>
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*All measured phosphorylation events are relative to AKT-only lesions.

†JAK2 was not identified by MS, but inferred on the basis of high STAT3 Y705 phosphorylation observed in AKT/AR tumors.
enrichment analysis of kinase-associated phosphorylation targets (Materials and Methods) to determine which kinase activities were predicted to be highly active in each tumor type.

Using this unbiased bioinformatic approach, we identified a statistically significant enrichment of the EGFR kinase substrate (D[E)pY in AKT/ERG but not in AKT/AR or AKT/K-RASG12V tumors (Fig. S5 and Dataset S2). Notably, this bioinformatic prediction was in direct agreement with our phosphoproteomic and Western blot data (Fig. 3B). Inference of kinase activity in AKT/K-RASG12V tumors further revealed an enrichment of ERK1/2 and MEK1/2 substrates, consistent with direct activation of MAPK signaling by the K-RASG12V oncogene (Fig. 4B and Fig. S4 and Dataset S2) (36).

Evaluating kinase activity from AKT/AR phosphopeptides revealed statistically significant enrichment of two motifs associated with ABL1 and SRC kinases [EXIpYXXP and (I|V)|L(S) XpYXX(L|I)], respectively (37). Because these kinases are both targets of the tyrosine kinase inhibitor, dasatinib, we combined these motifs into a “dasatinib target” group and found enrichment of predicted ABL1 and SRC substrates in AKT/AR tumors (Fig. 4A and Dataset S2). AKT/K-RASG12V and AKT/ERG tumors demonstrated modest and no enrichment of these motifs, respectively. Western blotting and IHC validated this bioinformatic prediction, as both SRC Y416 and ABL1 Y245 were phosphorylated in AKT/AR tumors but not in AKT/ERG or AKT/K-RASG12V tumors. Enrichment scores for all kinase motifs are shown in Dataset S2.

Further, the identification of SRC and ABL1 substrates Y705 of STAT3, Y14 of caveolin-1 (Cavin-1), and Y1007/1008 of JAK2 with binding partners vinculin (VCL) Y822, Paxillin (PAXN) Y118, CTNNB1 Y136, and PTPN11 Y62, suggest strong association and activation of the EGFR tyrosine kinase pathway (Fig. 5). In AKT/AR tumors, detection of elevated SRC and ABL1 activity prompted us to investigate other substrates and binding partners of these kinases within our phosphoproteomic data. The identification of SRC and ABL1 substrates Y771 of PTPN11 and CTNNB1 Y96, PTPN11 Y62, and PTPRA, suggest strong association and activation of the EGFR tyrosine kinase pathway.

Discussion

Many studies have linked the aberrant activation of tyrosine kinases by somatic mutation or DNA amplification to a wide array of cancers (39, 40). We demonstrate oncogenic-specific signatures of global phosphotyrosine activity without ectopic expression of mutant tyrosine kinases in a mouse model of prostate cancer progression. The activation of tyrosine kinase signaling suggests...
Tyrosine kinase activation offers therapeutic opportunities following the emerging successes of tyrosine kinase inhibitor therapies (5, 50). Our observation of SRC activity supports previous work that this kinase synergizes with other genes, including AR, to contribute to prostate adenocarcinoma (18, 51). SRC has also been shown to interact with the intracellular region of ERBB2 (HER-2), supporting the notion that SRC may be an important node for targeted therapy in advanced prostate cancer (17, 52). In support of these data, the SRC and ABL1 tyrosine kinase inhibitor dasatinib in combination with docetaxel is currently in phase III clinical trials for advanced prostate cancer and has shown modest phase II trial results in overall patient survival (53). Due to the heterogeneity of prostate cancer, this modest effect may be a result of the general administration of dasatinib without stratification of patients on the basis of SRC and ABL1 activity.

Strong activation of the EGFR pathway was observed in AKT/ERG-expressing mouse prostate tumors. Roughly half of all prostate cancer patients display the TMPRSS2-ERG translocation, a gene rearrangement fusing the androgen-regulated promoter of TMPRSS2 with the ETS transcription factor ERG, which is considered to be a marker for prostate cancer progression from PIN to adenocarcinoma (54). The product of the TMPRSS2-ERG translocation was shown to interact with the enzyme poly(ADP ribose) polymerase 1 (PARP1), and inhibition of this enzyme abrogates growth of prostate cancer xenografts that ectopically express ERG (55). PARP1 inhibition represents a promising treatment option for patients with TMPRSS2-ERG translocations. Our data suggest that ERG activity level is another candidate target in patients with TMPRSS2-ERG translocations. This result is in agreement with recent reports of SPINK1+/ETS prostate cancers where SPINK1-mediated growth occurs via EGFR signaling, demonstrating alternative pathways to activate EGFR (56). It will be important to further evaluate the relationship between EGFR activity and ERG clinically.

Our data suggest the molecular stratification of patients to target prostate cancer with tyrosine kinase inhibitors even in tumors without obvious tyrosine kinase mutations. Future work will extend this approach to prostate cancer patients to match tyrosine kinase inhibitor therapies with signaling activation patterns for targeted treatment of this disease.

**Materials and Methods**

**Clinical Prostate Tissue Microarrays, Lentiviral Vector Construction, Prostate Regeneration and Prostate Epithelial Viral Infections, and Western Blot and Immunohistochemistry** can be found in SI Materials and Methods.

**Quantitative Analysis of Phosphorysine Peptides by Mass Spectrometry.** A total of 300–500 mg of frozen tumor mass was homogenized and sonicated in urea lysis buffer (20 mM Hepes pH 8.0, 9 M urea, 2.5 mM sodium pyrophosphate, 1.0 mM β-glycerophosphate, 1% N-octyl glycoside, 2 mM sodium orthovanadate). A total of 35 mg of total protein was used for phosphoproteomics profiling and bioinformatics delineates distinct tyrosine kinase signaling pathways in an oncogene-specific manner. Selected substrate and interaction pathways from each tyrosine kinase were generated from a combination of our phosphoproteomics dataset and the HPRD and Phosphosite databases. An elevated phosphorylation event identified by MS is indicated by a phosphorylation residue depicted above the protein and color coded. Solid arrow, protein is a direct substrate of the upstream kinase at that site. Dashed arrow, protein interacts directly with the upstream kinase/protein. Dotted arrow, protein is found within the pathway of the upstream kinase/protein.

The presence of alternative mechanisms regulating tyrosine kinase activity not related to activating mutations (18, 21, 22). These include but are not limited to loss of negative feedback mechanisms (e.g., increased or decreased phosphatase activity), transcriptional up-regulation of kinases, or increased stabilization of tyrosine kinases through decreased protein degradation (22, 41, 42). Our data suggest that some of these mechanisms may control tyrosine kinase signaling in our mouse model of prostate cancer.

Tyrosine phosphorylation of the protein tyrosine phosphatase PTPN11 may contribute to the phosphotyrosine signatures observed in our tumors. Activity of this phosphatase is often associated with increased signaling activity (43, 44). This phosphatase was highly phosphorylated on Y526 and Y530 in AKT/ERG and AKT/AR tumors, respectively. In EGFR-expressing fibroblasts, epidermal growth factor (EGF) stimulation resulted in Y526 phosphorylation of PTPN11 leading to RAF/ERK pathway activation (45). This supports our findings that Y526 of PTPN11 is highly phosphorylated in AKT/ERG tumors and suggests receptor tyrosine kinase pathway-mediated activation of PTPN11. PTPN11 inhibition leads to decreased xenograft growth of lung and prostate tumors and reduced activity of numerous tyrosine kinases, including SRC (46). PTPN11 Y526,530 activation results in tyrosine dephosphorylation of the inactive site of SRC Y530 by regulation of the Csk regulator PAG/Cbp, indicating that SRC activity in AKT/AR tumors may be dependent on PTPN11 activation (43, 46).

Transcriptional up-regulation of tyrosine kinases may also enhance tyrosine kinase activity as suggested by the phosphorylation of EPHA2 at Y505 in the AKT/K-RASG12V tumors. EPHA2 was shown to be a transcriptional target of the RAS–MAPK pathway and ligand-stimulated EPHA2 negatively regulates RAS activity (47). Constitutive activation of RAS through mutation bypasses the negative regulation of EPHA2 and results in increased MAPK activation, which is in direct agreement with our phosphoproteomic data. RAS activation may reveal why high expression levels of EPHA2 protein are observed in breast and prostate cancer and support further clinical investigation of the connection between RAS mutation and EPHA2 status in these diseases (48, 49).
Enrichment Analysis of Kinase Activity. Phosphotyrosine peptides were ranked by the signal-to-noise ratio observed for a given perturbation (e.g., AKT/AR tumors compared with AKT alone). Having annotated the phosphopeptides with their predicted upstream kinases, we calculated a Kolmogorov-Smirnov statistic against the expected distribution for each upstream kinase. The statistical significance of enrichment was then determined by permutation analysis. This approach is analogous to the normalized enrichment score of gene set enrichment analysis (59). The enrichment scores for all putative upstream kinases are shown in Dataset S2. Additional details can be found in the SI Methods and Materials.

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SI Materials and Methods

Clinical Prostate Tissue Microarrays (TMA). The prostate TMA was constructed as previously described (1). Briefly, 75 prostatectomy specimens from patients never treated with hormonal therapy were reviewed and areas of normal prostate, high-grade prostatic intraepithelial neoplasia (HPIN), and hormone-naive prostate cancer (HNPC) were marked for sampling. Tumors ranged from Gleason patterns 2-5. Two to three cores per sample, measuring 0.6 mm in diameter, were obtained from selected regions in each donor paraffin block and transferred to a recipient paraffin block and the resulting block contained a total of 200 cores. A section was obtained from the TMA for H&E staining as quality control and unstained sections were used for immunohistochemical staining.

Another TMA was constructed from transurethral resection tissue blocks from 20 patients who failed hormonal therapy (cancer-regeneration process, lentivirus preparation, titering, and infection of dissociated prostate cells). The tumors, FU-mAKT-CRW (MOI ∼ 40) for mAKT/K-RASG12V tumors, FU-mAKT-CGW (MOI ∼ 40) for mAKT/K-RASG12V tumors. Infected cells were stained with hematoxylin and eosin for representative histology. Tissue sections were heated at 65 °C for 1 h to melt the paraffin followed by rehydration. Antigen retrieval was performed using citric acid buffer and visualization was performed using EnVision® system (Dako). The same primary antibodies from Western blots were used, unless explicitly stated, and diluted as follows: 4G10 (1:1,000), pSRC Y416 (1:50), pSTAT3 Y105 (1:200), Cell Signaling), STAT3 (1:1,000; Cell Signaling), AKT (1:1,000; Santa Cruz), AR (1:1,000; Santa Cruz), K-RAS (1:250; Calbiochem), 4G10 (1:500; Millipore), pEGFR Y1068 (1:1,000; Cell Signaling), EGFR (1:100; Cell Signaling), pPTPN11 Y528 (1:100; Cell Signaling), PTEN (1:1,000; Cell Signaling), pSTAT3 Y705 (1:2,000; Cell Signaling), STAT3 (1:1,000; Cell Signaling), pJAK2 Y1007/1008 (1:500; Cell Signaling), PAK2 (1:1,000; Cell Signaling), ERLK1/2 (1:1,000; Cell Signaling), pPTK2B Y492 (1:1,000; Cell Signaling), pSRC Y416 (1:1,000; Cell Signaling), pERK1/2 (1:5,000; Cell Signaling), ABL1 (1:5,000; Witte Laboratory) (8), and pABL1 Y254 (1:500; Cell Signaling). ECL substrate (Millipore) was used for detection and development on GE/Amersham film. For IHC, sections were stained with hematoxylin and eosin for representative histology. Tissue sections were heated at 65 °C for 1 h to melt the paraffin followed by rehydration. Antigen retrieval was performed using citric acid buffer and visualization was performed using EnVision® system (Dako). The same primary antibodies from Western blots were used, unless explicitly stated, and diluted as follows: 4G10 (1:1,000), pSRC Y416 (1:50), pSTAT3 Y105 (1:200), AKT (1:300; Cell Signaling), AR (1:200), and ERG (1:50). All primary antibodies recognizing tyrosine phosphorylated motifs were diluted in commercial antibody diluent (PAA).

Lentiviral Vector Construction. Construction of the plasmids carrying the oncogenes myristoylated AKT (mAKT), AR, AKT-ERG are described previously (2-4). The plasmid 12544 carrying K-RASG12V DNA was purchased from Addgene (5). The ORF of K-RASG12V was amplified by PCR using the following primers: forward primer, 5′-CATCATACTAGGCCACGATCACTATAAG-3′; and reverse primer, 5′-CATATGTTACCATACATACTTTAGCTG-3′. The PCR product was digested with Spel and HpaI endonuclease, whereas the lentiviral vector FC-CRW-Cre was digested with the same enzyme that released the Cre gene and generated the lentiviral backbone with Spel and HpaI cohesive ends. The K-RASG12V fragment was ligated into the lentiviral backbone and is designated as FU-K-RASG12V-CRW.

Prostate Regeneration and Prostate Epithelial Viral Infections. The regeneration process, lentivirus preparation, titration, and infection of dissociated prostate cells were performed as described previously under University of California, Los Angeles (UCLA) safety regulations for lentivirus use (6). Housing, maintenance, and all surgical and experimental procedures were undertaken in compliance with the regulations of the division of Laboratory Animal Medicine of the UCLA. Prostate regeneration was adapted from previous reports (7). Dissociated prostate cell suspension was prepared from 6- to 10-wk-old B6 mice. The dissociated cells were infected with lentivirus to generate the following tumors: FU-mAKT-CRW (multiplicity of infection, MOI ∼ 60) for mAKT tumors, FU-mAKT-RES-ERG-CRW (MOI ∼ 50) for mAKT/ERG tumors, FU-mAKT-CRW (MOI ∼ 40) and FU-AR-CGW (MOI ∼ 40) for mAKT/AR tumors, and FU-mAKT-CGW (MOI ∼ 40) and K-RASG12V-CRW (MOI ∼ 40) for mAKT/K-RASG12V tumors. Infected cells (2 × 10⁵) were mixed with urogenital sinus mesenchyme (UGSM) (2 × 10⁵). Grfts were implanted under the kidney capsule in SCID mice the following morning and allowed to regenerate for 12 wk.

Western Blot and Immunohistochemistry. Tumors were either flash frozen (Western analysis) or fixed in 10% buffered formalin overnight, embedded in paraffin, and sectioned at 4 μm (IHC analysis). For Westerns, equal protein amounts of urea lysates were used from tissues prepared as described in Quantitative Analysis of Phosphotyrosine Peptides by Mass Spectrometry. Antibodies were diluted as follows: AKT (1:1,000; Santa Cruz), pAKT S473 (1:2,000; Cell Signaling), ERG (1:500; Epitomics), AR (1:1,000; Santa Cruz), K-RAS (1:250; Calbiochem), 4G10 (1:500; Millipore), pEGFR Y1068 (1:1,000; Cell Signaling), EGFR (1:100; Cell Signaling), pPTPN11 Y528 (1:100; Cell Signaling), PTEN (1:1,000; Cell Signaling), pSTAT3 Y705 (1:2,000; Cell Signaling), STAT3 (1:1,000; Cell Signaling), pJAK2 Y1007/1008 (1:500; Cell Signaling), PAK2 (1:1,000; Cell Signaling), ERLK1/2 (1:1,000; Cell Signaling), pPTK2B Y492 (1:1,000; Cell Signaling), pSRC Y416 (1:1,000; Cell Signaling), pERK1/2 (1:5,000; Cell Signaling), ABL1 (1:5,000; Witte Laboratory) (8), and pABL1 Y254 (1:500; Cell Signaling). ECL substrate (Millipore) was used for detection and development on GE/Amersham film. For IHC, sections were stained with hematoxylin and eosin for representative histology. Tissue sections were heated at 65 °C for 1 h to melt the paraffin followed by rehydration. Antigen retrieval was performed using citric acid buffer and visualization was performed using EnVision® system (Dako). The same primary antibodies from Western blots were used, unless explicitly stated, and diluted as follows: 4G10 (1:1,000), pSRC Y416 (1:50), pSTAT3 Y105 (1:200), AKT (1:300; Cell Signaling), AR (1:200), and ERG (1:50). All primary antibodies recognizing tyrosine phosphorylated motifs were diluted in commercial antibody diluent (PAA).

Quantitative Analysis of Phosphotyrosine Peptides by Mass Spectrometry. The hybridoma was purchased from The Developmental Studies Hybridoma Bank, University of Iowa, and purified antibody (clone 4G10) was then chemically conjugated to protein G beads using dimethyl pimelimidate (DMP) as described (CASH Protocols; doi:10.1101/pdb.prot4303). Phosphorysine peptide immunoprecipitation was performed with pan-specific antiphosphotyrosine antibodies (clone 4G10,) using 35 mg of total protein isolated from 300 to 500 mg frozen tumor mass as previously described (9, 10). Phosphorylated peptides were analyzed by liquid chromatography tandem mass spectrometry (LC-MS/MS) using an Eksigent autosampler coupled with Nano2DLC pump (Eksigent) and LTQ-Orbitrap (Thermo Fisher Scientific). The samples were loaded onto an analytical column (10 cm × 75 μm i.d.) packed with 5 μm Integralt Proteopep2 300 Å C18 (New Objective). Peptides were eluted into the mass spectrometer using a HPLC gradient of 5–40% Buffer B in 45 min followed by a quick gradient of 40–90% Buffer B in 10 min, where Buffer A contains 0.1% formic acid in water and Buffer B contains 0.1% formic acid in acetonitrile. All HPLC solvents were Ultima Gold quality (Fisher Scientific). Mass spectra were collected in positive ion mode using the Orbitrap for parent mass determination and the LTQ for data-dependent MS/MS acquisition of the top five most abundant peptides. Each sample was analyzed twice (replicate runs) and in each run one-half of the sample was injected. MS/MS fragmentation spectra were searched using SEQUEST (version v.27, rev. 12, Thermo Fisher Scientific) against a database containing the combined human-mouse IPI protein database (downloaded December 2006 from ftp.ebi.ac.uk). Search parameters included carbamidomethyl cysteine (C) as a static modification. Dynamic modifications included phosphorylated tyrosine, serine, or threonine (pY, pS, pT, respectively) and oxidized methionine (M). Results derived from database searching were filtered using the following criteria: Xcorr >1.0(+1), 1.5 (+2), 2(+3); peptide probability score <0.001; dCn >0.1; and mass accuracy <25 ppm (parts per million) with Bioworks version.
3.2 (Thermo Electron). We estimated the false-positive rate of sequence assignments at 0.5% on the basis of a composite target-reversed decoy database search strategy (11). AScore was used to more accurately localize the phosphate on the peptide (12). Peptide peaks sequenced in experimental tumors and control lines but not others were located in the remaining samples by aligning the chromatogram elution profiles using a dynamic warping algorithm (9, 10). Relative amounts of the same phosphopeptide across samples run together were determined using custom software to integrate the area under the unfragmented (MS1) monoisotopic peptide peak (10, 13). All peaks corresponding to phosphosites were inspected manually and any errors in the automated quantitation were corrected. MS2 spectra for all reported phosphopeptides are available under the PRIDE accession numbers 20879–20889 (14).

**Data Analysis.** The number of unique phosphorylation sites identified in our experiments was determined by collapsing multiple phosphopeptide ions representing the same phosphorylation site. Multiple detections of the same phosphosite includes phosphopeptides of different ion charge state, modification (e.g., oxidized methionine), and missed trypsin cleavage sites. Multiple detections were compared to ensure no disagreement in trend, and the MS ion with the highest intensity across the samples in an experimental batch was kept as representative for subsequent data analysis. The phosphosite residue numbers listed correspond to the International Protein Index (IPI) accession number in the mouse genome, and any phosphopeptides that did not map to the mouse genome were filtered out. An ANOVA score less than 0.2. Hierarchical clustering was performed using the Cluster program with the Pearson correlation and pairwise complete linkage analysis (15). Java TreeView was used to visualize clustering results (16). Quantitative data for each phosphopeptide can be found in Dataset S1.

**Enrichment Analysis of Kinase Activity.** Permutation analysis was performed by randomly shuffling the peptide ranked list, followed by calculation of the Kolmogorov–Smirnov statistic for this permutation. After 1,000 permutations, the fraction of randomly ranked lists resulting in a Kolmogorov–Smirnov statistic greater than or equal to the observed value was defined as the permutation-based frequency of random occurrence (i.e., the permutation-based P value). To normalize for the different number of predictions for each upstream kinase, we calculated the normalized Kolmogorov–Smirnov score by dividing the observed enrichment score by the mean of the absolute value of all permutation enrichment scores.

Fig. S1. Confirmation of oncogene expression in mouse tumors. (A) Immunohistochemical staining for AKT, androgen receptor (AR), and ERG show expression of these proteins in the respective tumors investigated. ERG staining can also localize to endogenous endothelial cells as shown in AKT/AR and AKT/K-RASG12V tumors. (B) Western blot analysis confirms the expression of each oncogene after lentiviral transduction and tumor formation. Arrows, myristoylated AKT expression is shown at the higher molecular weight. ERG expression from the lentiviral vector. ERG expression at the lower molecular weight is from other cells within the tissue (e.g., endothelial cells). (Scale bars, 50 μm.)
Fig. S2. Analysis of tyrosine phosphorylation in mouse prostate and tumors. (A) IHC analysis reveals little tyrosine phosphorylation in normal mouse prostate, similar to what is observed in indolent prostate cancer. (B) Western blot analysis using the phosphotyrosine-specific antibody 4G10 reveals increased levels of phosphorylation in the more aggressive tumors. (Scale bars, 50 μm; Inset, 100 μm.)
Fig. S3. Global quantitative phosphoproteomics of prostate cancer progression reveals phosphorylation events with distinct oncogene-specific profiles. The phosphoproteomics heatmap of Fig. 4A with the protein and residue identities of the phosphorylation events are listed. Gene symbol, phosphosite residue number; Entrez gene product name, phosphopeptide (charge state of mass spectrometry ion).
Identification of activated tyrosine kinases in non-tyrosine kinase-driven prostate tumors. (A–L) Relative abundance of phosphopeptide levels of tyrosine kinases, substrates, and phosphatases during progression of prostate cancer. Signal-to-noise ratio (SNR) (relative to AKT) was calculated for each phosphopeptide and plotted. Data from the full phosphoprofile dataset are depicted in Fig. 4A. (M) Immunohistochemical staining for tyrosine phosphorylated STAT3 (pSTAT3 Y705) reveals tumor-specific activation of this particular phosphorylation event. (Scale bars, 50 μm.)
Fig. S5. Enrichment of EGFR target substrates in AKT/ERG tumors. A statistical analysis of tyrosine phosphorylated motifs reveals an enrichment of phosphopeptides of the tyrosine kinase EGFR in AKT/ERG tumors. No significant enrichment of these phosphopeptides was observed in either AKT/AR or AKT/K-RASG12V tumors. Enrichment scores for all kinase motifs are shown in Dataset S2.

Other Supporting Information Files

Dataset S1 (XLSX)
Dataset S2 (XLSX)