Protein Tyrosine Kinase 6 Promotes Oncogenic Signaling at Cell Plasma Membrane in Prostate Cancer

BY

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THESIS

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This thesis is dedicated to my parents Guoguang Zheng and Jianying Tu, without whom it would never have been accomplished.
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LIST OF ABBREVIATIONS

AKT    Ak mouse strain, transforming
ALT-PTK6 Alternatively spliced protein tyrosine kinase
AOM    Azoxymethane
ATP    Adenosine triphosphate
BKS    BRK kinase substrate
BPH    Benign prostatic hyperplasia
BRK    Breast tumor kinase
cDNA   complementary DNA
CSK    C-SRC tyrosine kinase
DAB    3, 3'-diaminobenzidine tetrahydrochloride
DAPI   4, 6'-diamidino-2-phenylindole
DMEM   Dulbecco’s modified eagle medium
DNA    Deoxyribonucleic acid
EGF    Epidermal growth factor
EGFR   Epidermal growth factor receptor
EMT    Epithelial to mesenchymal transition
ERK    Extracellular signal-regulated kinase
FAK    Focal adhesion kinase
FBS    Fetal bovine serum
FITC   Fluorescein isothiocyanate
FRK    Fyn-related kinase
GFP    Green fluorescent protein
GSK3β Glycogen synthase kinase 3β
GST Glutathione S transferase
HA Hemagglutinin
HER2 Human epidermal growth factor receptor 2
HGF Hepatocyte growth factor
IGF Insulin-like growth factor
IgG Immunoglobulin G
IP Immunoprecipitation
IRS Insulin receptor substrate
LC/MS/MS Tandem mass spectrometry
MAPK Mitogen-activated protein kinase
MEF Mouse embryonic fibroblast
MOI Multiplicity of infection
NLS Nuclear localization sequence
p130CAS p130 Crk-associated substrate
Palm Palmitoylation/myristoylation sequence
PBS Phosphate buffered saline
PCNA Proliferating cell nuclear antigen
PCR Polymerase chain reaction
PIN Prostatic intraepithelial neoplasia
PMSF Phenylmethylsulfonyl fluoride
poly-HEMA poly-2-hydroxyethyl methacrylate
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<tr>
<td>PTEN</td>
<td>Phosphatase and tension homolog</td>
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<tr>
<td>PTK6</td>
<td>Protein tyrosine kinase 6</td>
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<td>PY</td>
<td>Phosphotyrosine</td>
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<tr>
<td>RLU</td>
<td>Relative luminescence unit</td>
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<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
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<tr>
<td>Sam68</td>
<td>Src-associated in mitosis, 68kDa</td>
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<tr>
<td>SCID</td>
<td>Severe combined immunodeficiency</td>
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<tr>
<td>S.D.</td>
<td>Standard deviation</td>
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<td>SDS-PAGE</td>
<td>Sodium dodecyl sulfate-polyacrylamide gel electrophoresis</td>
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<tr>
<td>S.E.</td>
<td>Standard error of the mean</td>
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<tr>
<td>SH</td>
<td>SRC-homology domain</td>
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<tr>
<td>shRNA</td>
<td>Small hairpin RNA</td>
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<tr>
<td>Sik</td>
<td>Src-related intestinal kinase</td>
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<tr>
<td>siRNA</td>
<td>Small interfering RNA</td>
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<tr>
<td>SLM</td>
<td>Sam68-like mammalian protein</td>
</tr>
<tr>
<td>SRC</td>
<td>v-src sarcoma</td>
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<tr>
<td>SRMS</td>
<td>SRC-related kinase lacking C-terminal regulatory tyrosine and N-terminal myristoylation sites</td>
</tr>
<tr>
<td>STAT</td>
<td>Signal transducer and activator of transcription</td>
</tr>
<tr>
<td>SV40</td>
<td>Simian vacuolating virus 40</td>
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<tr>
<td>SYF</td>
<td><em>Src-/-, Yes-/-, Fyn-/-</em> MEF</td>
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<td>WT</td>
<td>Wild type</td>
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SUMMARY

Protein tyrosine kinase 6 (PTK6; also called BRK for BReast tumor Kinase or Sik for SRC-related Intestinal Kinase) is an intracellular Src-related tyrosine kinase. Although PTK6 shares overall structural similarity with Src family tyrosine kinases, it lacks an N-terminal myristoylation consensus sequence for membrane targeting. As a consequence, PTK6 is localized to different cellular compartments, including the nucleus. Although PTK6 is not expressed in the normal mammary gland, it is overexpressed in up to 86% of human breast tumors. Increased expression of PTK6 is also detected in other types of cancers, including colon cancer, head and neck cancers, as well as serous carcinoma of ovary. PTK6 is involved in different oncogenic signaling pathways that promote cancer cell proliferation, survival and migration, including Paxillin, p190RhoGAP-A (p190), STAT3, STAT5b, EGFR, HER2, MET and IGF-1R.

PTK6 localizes in the nucleus of epithelial cells of normal human prostate, but it relocates into cytoplasmic compartments as prostate cancer progresses into advanced stage. We discovered that the mRNA level of PTK6 is increased in prostate cancers, especially in metastatic prostate tumor samples. High levels of PTK6 predict low survival and high recurrence of human prostate cancer patients. By using a prostate specific PTEN knockout murine cancer model, we recapitulated the finding of PTK6 translocation from nucleus to cytoplasm in mice. More importantly, we discovered substantial activation of PTK6 at plasma membrane of prostate tumor cells in the knockout mice. We also detected highly active PTK6 at plasma membrane of tumor cells in about 18% (3/16) of human prostate cancer samples. In addition, we found that although the majority of PTK6 localizes in the
cytoplasmic compartments in human prostate hyperplastic or tumor cell lines including BPH1, PC3 and DU145, the pool of active PTK6, which is phosphorylated at tyrosine residue 342, is primarily associated with membrane compartments.

To explore the mechanisms that are involved in prostate tumorigenesis and downstream of PTK6 activation, we tried to identify potential PTK6 direct substrates by a large scale mass spectrometry and available literatures. I showed that PTK6 phosphorylates AKT in a Src family kinase-independent manner. Introduction of PTK6 into Src-/-, Yes-/-, Fyn-/- (SYF) mouse embryonic fibroblasts (MEFs) sensitizes these cells to physiological levels of epidermal growth factor (EGF) and increased AKT activation. Stable introduction of active PTK6 into SYF cells also results in increased proliferation. Knockdown of PTK6 in the BPH-1 human prostate epithelial cell line lead to decreased AKT activation in response to EGF. Our data indicate that in addition to promoting growth factor receptor-mediated activation of AKT, PTK6 can directly activate AKT to promote oncogenic signaling.

I also demonstrated that FAK is a direct substrate of PTK6 in vitro and in vivo. Expression of membrane targeted active PTK6 (Palm-PTK6-YF) in SYF cells induces constitutive activation of FAK and cell morphology changes, which are independent of SRC family kinases. Palm-PTK6-YF expressing SYF cells are transformed and overcome contact inhibition, form colonies in transformation assays, and proliferate when grown in suspension. Expression of FAK and Palm-PTK6-YF in Fak-/- MEFs synergistically activates AKT and protects cells against anoikis. However, expression of Palm-PTK6-YF in Akt1/2/- MEFs fails to protect cells from anoikis, indicating AKT is critical in PTK6 and FAK mediated survival signaling. Knockdown of PTK6 in the PC3 human prostate cancer cell line disrupts
SUMMARY (continued)

FAK and AKT activation and promotes anoikis. Our data reveal important roles for a PTK6-FAK-AKT signaling axis in promoting anchorage-independent cell survival.

p130 CRK-associated substrate (p130CAS) is also identified as a novel direct substrate of PTK6, and it works as a crucial adapter protein in the formation of peripheral adhesion complexes induced by Palm-PTK6-YF in PC3 cells. Activation of ERK5 downstream of p130CAS is also indispensable for this process. Knockdown of endogenous PTK6 lead to reduced cell migration and p130CAS phosphorylation, whereas knockdown of p130CAS attenuates oncogenic signaling induced by membrane-targeted PTK6, including ERK5 and AKT activation. Expression of membrane-targeted PTK6 promotes cell migration, which could be impaired by knockdown of p130CAS or ERK5. This study demonstrated that PTK6-p130CAS-ERK5 signaling cascade plays an important role in prostate cancer cell migration and invasion.

In addition to promoting the formation of peripheral adhesion complexes, expression of Palm-PTK6-YF in PC3 cells causes a cell scattering phenotype, which is coupled with epithelial-mesenchymal transition (EMT). Decreased E-cadherin expression is correlated with increased levels of ZEB1 and Vimentin at both protein and mRNA levels. In the murine prostate cancer model, a group of tumor cells with enhanced activation of PTK6 at the plasma membrane show deregulated E-cadherin expression, indicating an in-vivo EMT. Increased AKT activity is detected in Palm-PTK6-YF expressing PC3 cells, resulting in increased GSK-3β inhibitory phosphorylation, stabilization and nuclear localization of SLUG, an E-cadherin repressor. Knockdown of AKT or inhibition of AKT activity by knockdown of p130CAS partially rescues EMT induced by PTK6. PC3 cells undergoing EMT induced by
SUMMARY (continued)

Palm-PTK6-YF show increased cell migration in vitro and metastases in an ICR-SCID mice model. Knockdown of PTK6 promotes an epithelial phenotype and decreases the tumorigenic and metastatic potential of PC3 cell in vitro and in vivo. This study reveal that aberrant translocation of PTK6 in prostate cancer promotes the EMT, which might be a beneficial therapeutic target in treating prostate cancer metastasis.

In sum, I identified AKT, FAK and p130CAS as novel substrates of PTK6. I demonstrated that PTK6 promotes proliferation, migration, anoikis resistance, EMT, and invasion of prostate cancer cells through phosphorylating these substrates and activating their oncogenic signaling pathways. In human prostate cancer, we observed increased PTK6 expression, translocation of PTK6 from nucleus to cytoplasm, and activation of PTK6 at plasma membrane, all of which might contribute to increased phosphorylation and activation of its substrates AKT, FAK and p130Cas, therefore promoting prostate cancer progression.
I. INTRODUCTION

1. PTK6 family

Human protein tyrosine kinase 6 (PTK6) was identified in cultured human melanocytes and breast tumor cells, and was often referred to as BReast tumor Kinase (BRK) (Lee et al., 1993; Mitchell et al., 1994). Its mouse orthologue was cloned from normal small intestinal epithelial cell RNA in a screen for factors that regulate epithelial cell differentiation, and was thus given the name Src-related Intestinal Kinase (Sik) (Siyanova et al., 1994).

PTK6 belongs to PTK6 family, a family of intracellular non-receptor tyrosine kinases, which also includes Fyn-Related Kinase (FRK, also known as RAK, BSK, Iyk, and Gtk) and Src-Related kinase lacking C-terminal regulatory tyrosine and N-terminal Myristylation Sites (SRMS) [reviewed in (Brauer and Tyner, 2009)]. PTK6 family members share a highly conserved gene structure that is distinct from other intracellular tyrosine kinase families such as Src family kinase (Lee et al., 1998; Serfas and Tyner, 2003).

FRK was originally identified in human melanocytes and breast cancer cells, and also detected in the epithelium of the intestine, kidney, liver, lung and mammary gland [reviewed in (Brauer and Tyner, 2009)]. Overexpression of FRK induces G1 growth arrest of breast cancer cells, which is dependent of Frk kinase activity, but not its interaction to pRb (Craven et al., 1995; Meyer et al., 2003). Recently Yim and colleagues have demonstrated that FRK serves as a novel tumor suppressor by interacting with and phosphorylating PTEN on tyrosine residue 336, leading to decreased binding of PTEN to
its E3 ligase NEDD4-1 and therefore stabilizing PTEN protein levels (Yim et al., 2009).

SRMS was first identified in mouse embryonic neuroepithelial cells. It is a Src-related kinase but lacking an N-terminal glycine residue for myristoylation and a C-terminal regulatory tyrosine. Expression of SRMS is ubiquitous, with highest expression in lung, liver and testis. SRMS knockout mice displayed no apparent phenotype, probably caused by redundant function of other family members (Kohmura et al., 1994).

2. PTK6 structure

The human PTK6 gene maps to human chromosome 20q13.3 (chromosome 2 in the mouse) (Llor et al., 1999; Park et al., 1997) and contains eight exons with its unique intron/exon boundaries (Serfas and Tyner, 2003). It has been reported that there is at least one alternatively spliced transcript of PTK6, which lacks exon 2. Shift of the open reading frame results in early termination of translation, producing a truncated protein that only shares the amino terminus and SH3 domain with full length PTK6 and has a novel carboxy-terminus. This alternative PTK6 transcript (ALT-PTK6), also known as λm5, was originally detected in human breast cancer cells (Easty et al., 1997). Recently, it has been demonstrated that ALT-PTK6 is expressed in prostate cells and is able to negatively regulate growth and modulate PTK6 activity, probably by altering its protein-protein association and/or intracellular localization (Brauer et al., 2011).

PTK6 is a 451 amino acid protein that is structurally similar to Src family tyrosine kinases, but lacks an N-terminal myristoylation consensus sequence, allowing it to localize to different cellular compartments, including the nucleus (Derry et al., 2003). PTK6 shares similar SRC-homology (SH) 3, SH2, tyrosine kinase domain (also referred
to as SH1 domain), and a C-terminal regulatory domain with Src kinase (Fig. 1). Both SH2 and SH3 domains are important for protein-protein interaction and substrate recognition. SH2 domain recognizes tyrosine residue in a specific context while SH3 domain recognizes proline-rich sequence. SH2 and SH3 domain is also known to be involved in regulating the kinase activity (Born et al., 2005; Derry et al., 2000; Qiu and Miller, 2004).

Tyrosine residue 342 is a well conserved site between PTK6 and SRC, and its autophosphorylation causes the activation loop to move out of the substrate-binding site, therefore allowing access to substrates (Qiu and Miller, 2002). Phosphorylated tyrosine 342 serves as a marker of PTK6 activation. Phosphorylation of the conserved tyrosine residue 447 at carboxy terminus results in autoinhibition due to the binding of the carboxy terminus to its own SH2 domain (Qiu and Miller, 2002). Mutation of tyrosine residue 447 to phenylalanine (PTK6-YF) largely increases its kinase activity, since it cannot be autoinhibited. The kinase responsible for phosphorylating the C-terminal regulatory tyrosine residue of PTK6 is still unknown. C-Src tyrosine kinase (CSK), which is able to phosphorylate the Src C-terminal tyrosine and therefore inactivate Src, does not phosphorylate PTK6 (Qiu and Miller, 2002).

3. PTK6 substrates

A variety of proteins have been identified as PTK6 substrates, including the RNA binding proteins Sam68 (Derry, Richard et al. 2000), Sam68 like mammalian protein 1 (SLM-1), SLM-2 (Haegebarth et al., 2004), Polypyrimidine tract-binding protein-
Fig. 1. Comparison of the structure of Src and PTK6

SRC and PTK6 proteins shares SH 3, SH2, tyrosine kinase (SH1) and C-terminal regulatory domains. Lysine residue 219 and tyrosine residues 342 and 447 are conserved in PTK6 and Src family kinase, all of which plays important roles in regulating the kinase activity. Unlike Src, PTK6 lacks an N-terminal domain which contains consensus sequence for myristoylation.
associated splicing factor (PSF) (Lukong et al., 2009), the transcription factors β-catenin (Palka-Hamblin et al., 2010), signal transducers and activators of transcription 3 (STAT3) (Liu et al., 2006), STAT5b (Weaver and Silva, 2007), scaffold proteins paxillin (Chen et al., 2004), signal transducing adapter protein 2 (STAP-2, also known as BKS) (Mitchell et al., 2000), a membrane receptor epidermal growth factor receptor (EGFR) (Li et al.), and a GTPase activating protein p190RhoGAP (Shen et al., 2008).

A. RNA binding proteins

Sam68, SLM-1 and SLM-2 are nuclear RNA binding proteins. PTK6 associates with and phosphorylates them in the nucleus, and inhibits of their RNA-binding activities which affects different aspects of RNA metabolism including stability, translation and transport (Derry et al., 2000; Haegebarth et al., 2004). Interestingly, Sam68 promotes growth and survival of human prostate tumor cells and its level increases in prostate tumor, suggesting a potential oncogenic role of Sam68 in prostate cancer (Busa et al., 2007). PTK6 also interacts with and phosphorylates PSF, promoting the cytoplasmic relocalization of PSF, therefore impairing its binding to polypyrimidine RNA and causing cell cycle arrest (Lukong et al., 2009).

B. Transcription factors

Our group identified β-catenin as a direct substrate of PTK6 (Palka-Hamblin et al., 2010). PTK6 directly phosphorylates β-catenin at multiple tyrosine residues including Tyr64, Tyr142, Tyr331 and/or Tyr333, with the primary target site being Tyr64. Interestingly, different intracellular localizations of PTK6 appear to regulate the
transcriptional activity of β-catenin in distinct manners. When PTK6 is targeted at nucleus, it negatively regulates endogenous beta-catenin/TCF transcriptional activity, whereas membrane-targeted PTK6 enhances it (Palka-Hamblin et al., 2010).

STAT family is a family of transcription factors that were originally identified as key mediators of cytokine signaling, and some of them can be activated by growth factors such as EGF and PDGF. PTK6 specifically phosphorylates and activates STAT3 and STAT5b to promote breast cancer cell proliferation, while the other members do not seem to be responsive to PTK6 expression (Liu et al., 2006; Weaver and Silva, 2007).

C. Scaffold proteins

STAP-2 is an adaptor protein that contains pleckstrin homology (PH) and SH2-like domains, as well as a STAT3-binding motif in its C-terminus. Tyr250 in STAP-2 is a major site that is targeted by PTK6. STAP-2 interacts with PTK6, STAT3 and STAT5b, and modulates their activities through its PH domain. Knockdown of STAP-2 in T47D cells impairs PTK6 mediated STAT3 activation, leading to decreased cell proliferation (Ikeda et al., 2009; Ikeda et al., 2010).

Paxillin, a multidomain protein, works as a molecular scaffold that provides multiple docking sites at the plasma membrane. PTK6 phosphorylates paxillin at Tyr31 and Tyr118 upon EGF stimulation, leading to the activation of small GTPase Rac1, therefore promoting cell migration and invasion (Chen et al., 2004).

D. EGFR/ErbB family
ErbB receptor family signaling also plays important roles in the etiology of human cancer. ErbB receptor family includes EGFR, ErbB2 (HER2/Neu), ErbB3 and ErbB4. PTK6 co-precipitates with ErbB family members HER2/Neu, ErbB3, ErbB4 in human breast cancer cell line T47D (Aubele et al., 2008). Overexpression of PTK6 promotes breast cancer cell growth through stimulating ErbB receptor family signaling and PI3K/AKT signaling (Harvey and Crompton, 2003; Kamalati et al., 2000; Kamalati et al., 1996). Recently it is demonstrated that PTK6 can sustain EGFR signaling by phosphorylating EGFR and inhibiting its downregulation (Li et al., 2012).

**E. Others**

PTK6 can also phosphorylate p190RhoGAP-A and stimulate its activity, leading to RhoA inactivation and Ras activation. p190RhoGAP-A phosphorylation plays essential roles in both migratory and proliferative effects of PTK6 in breast cancer cells (Shen et al., 2008).

ARAP1 has been identified as a novel PTK6 substrate from an in-vitro co-immunoprecipitation followed by MALDI-TOF mass analysis. PTK6 phosphorylates ARAP1 on Tyr231, inhibiting EGFR downregulation and thereby enhancing EGF/EGFR signaling in breast cancer cell lines (Kang et al., 2010).

Interestingly, some of these proteins are also the substrates of Src kinase, some of which have exact the same tyrosine residues that can be targeted by Src and PTK6. In fact, PTK6 and Src share a similar optimal substrate sequences that include the following residues X-2I/E-1Y0D/E1D/E2, although Src prefers acidic amino acids at -3 and -4
positions, while PTK6 shows strong acidic preference at +1 and +2 positions (Kreegipuu et al., 1998; Shin et al., 2008; Songyang and Cantley, 1995).

4. PTK6 in normal tissue

In normal tissues, expression of PTK6 is highest in the non-dividing, differentiating epithelial cells of the gastrointestinal tract (Llor et al., 1999; Vasioukhin et al., 1995). PTK6 is also detected in other differentiated epithelial cells including prostate (Derry et al., 2003), oral cavity (Petro et al., 2004) and skin (Vasioukhin et al., 1995; Vasioukhin and Tyner, 1997).

A variety of studies suggest that PTK6 negatively regulates proliferation and promotes the differentiation of epithelial cells. In the cultured human keratinocyte cell line HaCaT and embryonic mouse keratinocyte cell line EMK, addition of calcium promotes differentiation, which is accompanied by increased PTK6 expression and activation. Overexpression of PTK6 elevates the epidermal differentiation marker keratin-10 in HaCaT cells and filaggrin in EMK cells (Vasioukhin and Tyner, 1997; Wang et al., 2005). PTK6 expression is increased during differentiation of Caco-2 cells, a colon adenocarcinoma cell line (Llor et al., 1999). Studies in a PTK6-deficient mouse model demonstrate the role of PTK6 in promoting cell cycle exit and differentiation in normal intestinal epithelial cells in vivo. PTK6-deficient mice show longer villi with expanded proliferation zone, accompanied by delayed enterocyte differentiation (Haegebarth et al., 2006).
PTK6 also plays important roles in regulating the survival of normal cell in response to a variety of apoptotic stimuli. In a immortalized nontransformed Rat1A cell line, expression of PTK6 sensitize these cells to apoptosis induced by serum deprivation and UV irradiation (Haegebarth et al., 2005). Further in-vivo mice study reveals the underlying mechanisms that PTK6 mediates apoptosis by inhibiting prosurvival signaling including AKT and Erk1/2 (Haegebarth et al., 2009).

5. PTK6 in cancer

Although PTK6 is not expressed in the normal mammary gland, it is overexpressed in up to 86% of human breast tumors, with highest levels in advanced tumors (Barker et al., 1997; Lofgren et al., 2011; Mitchell et al., 1994; Xiang et al., 2008). It has been demonstrated that PTK6 is able to promote breast cancer cell proliferation and migration through multiple mechanisms including activation of the STAT family of transcription factors, EGFR/ErbB receptors and p190RhoGAP-A. Recently PTK6 was shown to play crucial roles in regulating cell survival. PTK6 was identified in an siRNA screen as an important regulator of IGF-1 mediated anchorage-independent breast tumor cell survival (Irie et al., 2010). In a PTK6 transgenic mouse model, mouse mammary gland with PTK6 expression show delayed involution, probably caused by activation of a p38 MAPK pro-survival signaling pathway (Lofgren et al., 2011).

Expression of PTK6 is also upregulated in other types of cancer including colon cancer (Llor et al., 1999), ovarian cancer (Schmandt et al., 2006), head and neck cancer (Lin et al., 2004), and metastatic melanoma cells (Easty et al., 1997). Using a carcinogen induced colon cancer model, our group demonstrated that disruption of Ptk6 is able to
prevent STAT3 activation and decreased AOM induced colon tumorigenesis (Gierut et al., 2011).

In human prostate cancer, aberrant translocation of PTK6 from nucleus to cytoplasm is observed (Derry et al., 2003). PTK6 localizes in the nucleus of normal prostate epithelial cells. However, nuclear localization is lost as prostate cancer progresses from benign hyperplasia to PIN and high grade prostate tumor (Derry et al., 2003). Moreover, cytoplasmic retention of PTK6 promotes growth of prostate cancer cells, while expression of nuclear-targeted PTK6 significantly decreases cell proliferation (Brauer et al., 2010). These findings led to my hypothesis that the relocation of PTK6 from nucleus to cytoplasm in prostate epithelial cells promotes prostate tumorigenesis, and this may be achieved by the access to its novel substrates which are not in nucleus.
II. MATERIALS AND METHODS

1. Plasmids and siRNAs

Full-length human PTK6, PTK6-YF and PTK6-KM in the pcDNA3 vector containing a Myc epitope tag were described in a previous study (Palka-Hamblin et al., 2010). Full-length mouse PTK6, PTK6-YF and PTK6-KM constructs in the pLXSN vector were described previously (Vasioukhin and Tyner, 1997). PTK6-YF has a mutation of the regulatory tyrosine (Y) at position 447 of wild-type PTK6 to phenylalanine (F), resulting in a constitutively active mutant of the kinase. PTK6-KM has a mutation of a critical lysine (K219) in the ATP binding site of wild-type PTK6 to methionine (M), resulting in a kinase-dead mutant. Coding sequences from the pLXSN constructs were subcloned into the pBABE-puro vector (Cell Biolabs, Inc., San Diego, CA). Myc-tagged NLS-PTK6 and Palm-PTK6 constructs in the pcDNA4-TO vector have been described previously (Palka-Hamblin et al., 2010). Coding sequences from the pcDNA3 and pcDNA4-TO constructs were subcloned into the pBABE-puro vector or pBABE-hygro vector (Cell Biolabs, Inc., San Diego, CA). The GST-PTK6 (mouse) constructs were previously described (Vasioukhin and Tyner, 1997). Coding sequences from pcDNA3-human PTK6 were subcloned into the green fluorescent protein (GFP) vector pEGFP-C1 (Clontech, Palo Alto, CA).

Full-length wild-type HA-tagged mouse AKT and mAKT, in the mammalian expression vector pcDNA3, were kindly provided by Nissim Hay (University of Illinois at Chicago, Chicago, IL) (Eves et al., 1998). The AKT mutants with mutations at tyrosine residues 215, 315, 326, and 474 and at proline residues 424, 427, 467, and 470 were
created using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) as described by the manufacturer with pcDNA3 HA-AKT plasmid as a template. Mutagenic primers used for mouse AKT included Y215F (forward, 5’-CTTACGCGCCCTCAAGGCTTCATTCCAGGACCAGG-3’; reverse, 5’-CGTGGGTCTGAAATGAGAACTTWAGGCGGCTAAGG-3’), Y315F (forward, 5’-CTGCGGAAACGCCGAGGATCTGTCAGGAGGT-3’; reverse, 5’-CACCTCAGGGCGCAAGACTCGCGGTTCGCAC-3’), Y326F (forward, 5’-GCTGGAGGAAAGCAGACTCTCGGAGGGCCGTACG-3’; reverse, 5’-CTTCCTTCAAGGCTTCTGCTCCAGACAG-3’), Y315E/Y326E (forward, 5’-GAACGCCGAGTTCCTGAGGACACGTGGAGGGACGG-3’; reverse, 5’-CTTCCTTCAAGGCTTCTGCTCCAGACAG-3’), and P467A/P470A (forward, 5’-GTAGGGAGAACTGGCAGGGAAACTGGCAGGG-3’; reverse, 5’-GAGGTGACCTGCGGCTTGAGGCTCAGA-3’). All constructs were sequenced to verify point mutations. Coding sequences from wild-type AKT and the
AKT Y315F/Y326F mutant were subcloned into the bacterial expression vector pGEX-2TK (GE Healthcare Bio-Sciences, Piscataway, NJ).

A pcDNA3-HA-FAK (mouse) plasmid was a gift from Dr. David Schlaepfer (UCSD, San Diego, CA). Coding sequences of mouse FAK were amplified by PCR and subcloned into the pBABE-puro vector. The pEBG-p130CAS plasmid (plasmid 15001; (Zvara et al., 2001)) was obtained from Addgene (Cambridge, MA).

MISSION TRC PLKO-1 Palmid containing PTK6 short hairpin RNA (shRNA) was purchased from Sigma-Aldrich, St.Louis, MO (TRCN0000021552). Compatible packaging plasmids HIV-trans and VSVG were provided by Bin He [University of Illinois at Chicago, Chicago, IL]).

The siRNA against p130CAS and AKT was purchased from Dharmacon (Lafayette, CO). The sequence against p130CAS has been reported previously (Sanders and Basson, 2005): 5’-GGTCGACAGTGGTGTGTAT-3’ and the sequence against AKT was described in (Catalucci et al., 2009): 5’-TGCCCTTCTACAAACCAGGATT-3’. Dicer-substrate siRNAs against ERK5, FAK, and PTK6 were purchased from the Integrated DNA Technologies predesigned DsiRNA library (Coralville, IA). The sequence for Dsi-PTK6 is 5’-AGGTTCACAAATGTGGAGTGTCTGC-3’. The sequences for Dsi-ERK5 are 5’-GCAGCTATCTAAGTCACAGGTGGAG-3’ and 5’-ACTAGTGTCTACAGTGACATGACT-3’. The sequence for Dsi-FAK is 5’-GCAATGGAGCGAGGTATTAAGGACT-3’.
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2. **Cell culture**

The human embryonic kidney cell line 293 (HEK-293) (ATCC CRL-1573), the mouse embryonic fibroblast cell line SYF (ATCC CRL-2459), the \textit{Fak-/-} mouse embryonic fibroblast cell line (ATCC CRL-2644), the human colon cancer cell line SW620 (ATCC CCL-227), the human prostate cancer cell lines PC3 (ATCC CRL-1435), DU145 (ATCC HTB-81) and LNCaP (ATCC CRL-1740), and the human breast cancer cell lines T47D (ATCC HTB-133) and MDA-MB-231 (ATCC HTB-26) were cultured according to ATCC guidelines in the recommended medium. The benign prostatic hyperplasia epithelial cell line BPH-1 (kindly provided by Simon Hayward [Vanderbilt University, Nashville, TN]) was cultured in RPMI 1640 containing 5\% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin (Hayward et al., 1995). \textit{Akt1/2-/-} mouse embryonic fibroblast cells immortalized by DN-p53 (kindly provided by Dr. Nissim Hay, University of Illinois at Chicago, Chicago, IL) were cultured in DMEM containing 10\% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin (Nogueira et al., 2008). Transfections of SYF cells were performed using the Lipofectamine transfection reagent in combination with PLUS reagent (Invitrogen, Carlsbad, CA) as per the manufacturer’s instructions. Transfections of other cells were performed using the Lipofectamine 2000 transfection reagent (Invitrogen) as per the manufacturer’s instructions.

3. **Antibodies**

Anti-human PTK6 (C-18, G-6), anti-mouse PTK6 (C-17), anti-FAK (C-20), anti-FAK (C-20), anti-phosphotyrosine (PY20), anti-SP1 (PEP2), E-cadherin (H-108), ZEB1 (H-102), p63(4A4) and anti-HER2/Neu (C-18) antibodies were purchased from Santa
Cruz Biotechnology (Santa Cruz, CA). Anti-phosphotyrosine (clone 4G10) and anti-P-PTK6 (Tyr342) antibodies were purchased from Millipore (Bedford, MA). Antibodies directed against AKT, P-AKT (Thr308), P-AKT (Ser473), P-FAK (Tyr925), P-FAK (Tyr576/Tyr577), ERK1/2, P-ERK1/2 (Thr202/Tyr204), ERK5, P-ERK5 (Thr218/Tyr220), P-p130CAS (Tyr165), P-GSK3β (Ser9), cleaved caspase-3 (Asp175), SLUG, HA-tag (6E2) and Myc tag (9B11) were obtained from Cell Signaling Technology (Danvers, MA). Antibodies directed against p27, paxillin, p130CAS, and β-catenin were obtained from BD Pharmingen (San Diego, CA). Anti-P-paxillin (Tyr118) antibodies were obtained from Invitrogen. Anti-β-actin (AC-15), α-tubulin (T-9026), vimentin and vinculin antibodies were purchased from Sigma-Aldrich. Anti-rat P-p130CAS (Tyr-762) antibody, which recognizes human P-p130CAS Tyr-664, and anti-Ki67 antibody were purchased from Abcam (Cambridge, MA). Anti-glutathione S-transferase (GST) tag and hemagglutinin (HA) tag antibodies were purchased from Covance (Cumberland, VA). Anti-GFP tag antibody was purchased from Roche (Indianapolis, IN). Anti-CK5 antibody was provided by Dr. Gian Paolo Dotto (MGH, MA). Donkey anti-rabbit or sheep anti-mouse antibodies conjugated to horseradish peroxidase were used as secondary antibodies (Amersham Biosciences, Piscataway, NJ) and detected by chemiluminescence with SuperSignal West Dura extended duration substrate from Pierce (Rockford, IL).

4. Protein lysates, Immunoprecipitation (IP) and GST pull down assay

Cells were lysed in 1% Triton X-100 lysis buffer (1% Triton X-100, 20 mM HEPES, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 10 mM Na-pyrophosphate, 100 mM NaF, 5 mM iodoacetic acid, 0.2 mM phenylmethylsulfonly fluoride (PMSF),
protease inhibitor cocktail (Roche). Cell fractionation of prostate and breast cancer cell lines was performed using the ProteoExtract subcellular proteome extraction kit from Calbiochem according to the manufacturer’s instructions. One tenth volume of each fraction was subjected to SDS-PAGE and transferred onto Immobilon-P membranes for immunoblotting.

Immunoprecipitation was performed with 500 µg of total cell lysates and 0.5 µg of specific antibodies under overnight incubation at 4 °C. 30 ml of protein A-Sepharose CL-4B beads (GE Healthcare) was added and incubated for 1 hour after which the beads were washed four times in wash buffer (1% Triton X-100, 20mM HEPES, pH7.4, 150mM NaCl, 1mM EDTA, 1mM EGTA, 10mM sodium pyrophosphate). After removing the supernatant in the final wash, samples were resuspended in 30 µl of 2X reducing Laemmli sample buffer and boiled for 5 min. The proteins retained on the protein A beads were subjected to SDS-PAGE on 8% gels and transferred onto Immobilon-P membranes for immunoblotting.

For the GST pulldown assay, the GST tag, GST-PTK6FL (full-length), GST-PTK6-SH2, GST-PTK6-SH3, and GST-PTK6-SH2/SH3 fusion proteins were expressed in BL21 cells (Stratagene). 6 µg of the purified GST fusion proteins GST-PTK6FL, GST-PTK6-SH2, GST-PTK6-SH3, GST-PTK6-SH2/SH3 were incubated with 25 µl Glutathione Sepharose 4B beads for 30 minutes and then incubated with 500 µg of lysates over-expressing AKT alone or in combination with PTK6-YF overnight at 4°C, following which the beads were washed four times in wash buffer (1% Triton X-100, 20 mM Hepes, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 10 mM Na-pyrophosphate). After removing the supernatant in the final wash, samples were
resuspended in 30 µl of 2X Laemmli sample buffer and boiled for 5 minutes. The proteins retained on the GST beads were resolved by SDS-PAGE. Binding was compared with that of 10% of the lysates added to the pulldown reactions.

5. **Immunostaining**

Cells were seeded in 4- or 8-well chamber slides that were uncoated or coated with collagen I (Sigma-Aldrich). Cells were washed with PBS, fixed in Carnoy’s solution (6:3:1 ethanol : chloroform : acetic acid), then blocked with 3% BSA for 1 h, and incubated with primary antibodies overnight. After washing, samples were incubated with biotinylated anti-rabbit or anti-mouse secondary antibodies and then incubated with fluorescein isothiocyanate (FITC)-conjugated avidin (Vector Laboratories, Burlingame, CA). For double staining, FITC-conjugated anti-mouse secondary antibodies (Sigma-Aldrich) were used to detect primary antibodies made in mouse (green), and biotinylated anti-rabbit secondary antibodies (Vector Laboratories) were used and then incubated with rhodamine-conjugated avidin to detect primary antibodies made in rabbit (red). Slides were mounted in Vectashield fluorescent mounting medium containing 4, 6-diamidino-2-phenylindole (DAPI) (Vector Laboratories). Cells were then observed using standard UV, rhodamine, or FITC filters under 40X and 63X differential interference contrast oil immersion objectives using a Zeiss LSM 5 PASCAL confocal microscope. Images were obtained with an Axiocam HRc color digital camera and LSM 5 PASCAL software (Zeiss, Jena, Germany).

6. **In vitro kinase assays**

Kinase assays were performed with 50 ng recombinant human PTK6 (Invitrogen) and 1 µg recombinant human AKT (Millipore), or 250 ng recombinant human FAK, or 1
μg of recombinant human p130CAS (Novus Biologicals, Littleton, CO) in 30 μl kinase buffer (10 mM HEPES, pH 7.5, 150 mM NaCl, 2.5 mM dithiothreitol (DTT), 0.01% Triton X-100, 10 mM MnCl$_2$) with or without 200 μM ATP for 10 min at 30°C. The reaction was stopped by adding 30 μl of 2X reducing Laemmli sample buffer and boiling. Samples were subjected to SDS-PAGE and transferred onto Immobilon-P membranes for immunoblotting.

7. **Tandem mass spectrometry (LC/MS/MS)**

Samples were subjected to in-gel digestion using sequencing grade trypsin followed by C18 reversed-phase microcapillary liquid chromatography tandem mass spectrometry using a hybrid linear ion trap – Orbitrap XL mass spectrometer (Thermo Scientific) in positive-ion data-dependent acquisition mode. MS/MS spectra were searched against the reversed and concatenated Swiss-Prot protein database using the Sequest algorithm (Proteomics browser; Thermo Scientific) with differential modifications for STY phosphorylation (79.97) and methionine oxidation (15.99). Phosphorylation sites were identified if they initially passed the following Sequest scoring thresholds: 2 ions, Xcorr ≥ 1.90, Sf ≥ 0.4, P > 0; 3 ions, Xcorr ≥ 2.55, Sf ≥ 0.5, P > 0 against the forward database. Peptides with gas phase charges of 1 and 4 were generally not accepted due to difficulty of interpretation. Passing MS/MS spectra were then manually validated to ensure that all b- and y- fragment ions aligned with the assigned modified protein sequence. Determination of the exact sites of phosphorylation was aided using GraphMod software (Proteomics Browser Software, Thermo Scientific), resulting in a false-positive identification rate of less than 1.2%.
Tyrosine phosphorylation at Y326 was detected from a tryptic digestion from recombinant AKT using a targeted-ion LC/MS/MS (TIMM) experiment with a high-resolution/mass accuracy hybrid LTQ-Orbitrap XL mass spectrometer (Thermo Scientific) by setting the ion trap to filter for the phosphorylated Y326 peptide, a method that enhances sensitivity of detection. Validation of the site was similar to the method described above in addition to the added criteria that the identified phosphopeptide be present within a ±5-ppm mass accurate window.

8. Retrovirus and lentivirus production and transduction

For retrovirus production, pBABE plasmids were transfected into Phoenix Eco cells (ecotropic) or Phoenix Amho cells (amphotropic) by Lipofectamine 2000, and DMEM with 10% fetal bovine serum (FBS) was changed 24 hours after transfection. Retrovirus was collected 48 hours and 72 hours after transfection. For lentivirus production, PLKO-1 vector were co-transfected with compatible packaging plasmids HIV-trans and VSVG in the HEK-293FT packaging cell line. Fresh complete DMEM was changed 24 hours after transfection, and lentivirus was collected 48 hours and 72 hours after transfection.

Different cell lines were infected by retrovirus or lentivirus in growth media containing 5 μg/ml polybrene at a multiplicity of infection (MOI) of 500 for 24 hours. Stable cell lines were selected in complete growth media containing 2 μg/ml puromycin or 100 μg/ml hygromycin B for a week.

9. Proliferation, colony formation and soft agar assays

For proliferation assays, subconfluent cells were seeded in triplicate for each time point at a density of 2 x 10³ per well in 48-well plates. The fold increase in cell number
was measured by the CellTiter-Glo luminescent cell viability assay (Promega). For colony formation assays, cells were seeded in triplicate at a density of $1 \times 10^3$ per well of six-well plates 24 hours after transfection, and grown for 14 days before fixing and staining with crystal violet (Sigma-Aldrich). For soft agar assays, $1.5 \times 10^3$ cells were seeded in triplicate on the top layer of six-well plates, which contained 0.35% agar in growth medium containing 10% FBS. The bottom layer of soft agar contained 0.7% agar in growth medium containing 10% FBS. Cells were fed twice a week, and colonies were counted at three weeks post-plating.

10. **Wound healing assays, migration chamber assays and invasion assays**

For the wound healing assays, cells were grown to confluence in 6-well plates and serum-starved for 24 hours. Wounds were carefully made across the cell monolayer, and the medium was replaced by fresh complete growth medium. Cell migration was monitored for 48 hours.

For migration assays, cells were transfected with siRNAs for 24 hours if needed and then serum-starved for another 24 hours. $5 \times 10^4$ cells were plated in the top chamber of a Transwell (24-well insert; pore size, 8 μm; Corning) and incubated with 1% FBS containing medium. 20% FBS containing medium was added to the lower chamber as a chemoattractant. After 18 hours, cells that did not migrate through the pores were removed by a cotton swab, and the cells on the lower surface of the membrane were stained by crystal violet.

BD BioCoat™ Matrigel Invasion Chambers (BD Pharmingen) were used for invasion assays, which were performed in a similar way to migration chamber assays, except that 50 ng/ml hepatocyte growth factor (HGF) was used as a chemoattractant and
the incubation time was 24 hours. Images were taken under the phase-contrast microscope using 10X magnification.

11. **Anoikis assays**

MEFs were trypsinized and plated onto poly-HEMA-coated 10 cm plates in growth medium to prohibit attachment. PC3 cells were transfected with control or PTK6 siRNA for 24 hours, and then seeded at poly-HEMA-coated plates in growth medium. Viability of the cells was measured every other day by the CellTiter-Glo luminescent cell viability assay (Promega). The cells were harvested with Triton X-100 lysis buffer at indicated time.

12. **Transformation assays**

5 x 10^3 cells of the line to be tested were mixed with 1 x 10^5 untransformed parental SYF cells, plated into six well dishes, maintained at confluence and scored for foci after 10 days.

13. **Primers and quantitative RT-PCR**

Total RNA was extracted using TRIzol reagent (Invitrogen). After DNase I digestion (Promega), 500 ng of RNA was used to generate cDNA using a cDNA synthesis kit (Bio-Rad, Richmond, CA). RT-PCR was performed using the following mixture: 1 × iQ SYBR Green supermix (Bio-Rad), 100 nM of each primers and 1 μl of cDNA in a 25 μl total volume. Reactions were amplified and analyzed in triplicate using a MyiQ single-color real-time PCR detection system (Bio-Rad). The following primers were used: human cyclophilin: (F) 5’-GCAGACAAGGTCCCCAAAGACAG-3’ and (R) 5’-CACCCTGACACATAAACCCTGG-3; human E-cadherin: (F) 5’-ATGCTGATGCCCCCACATACC-3’ and (R) 5’-TCCAAGCCCTTTGCTGTTC-3’;
human vimentin: (F) 5’-TTGACACATGCCTCTGGGCAC-3’ and (R) 5’-CCTGGATTCTCGTGGAGGAGG-3’; human ZEB1: (F) 5’-AACGCTTTTCCCATTCTGGG-3’ and (R) 5’-GAGATGCTTGAGGCTCTTTG-3’; human SLUG: (F) 5’-GCTAGAAAGCCCCATTAGTATG-3’ and (R) 5’-GCCAGCCAGAAAAGTTGAGAAG-3’; human Twist: (F) 5’-GTCCGCAGTCTTCGAGGAGGAG-3’ and (R) 5’-C454CAGCTTCGAGGCTCTGAATC-3’; human PTK6: (F) 5’-GCTATGTGCCCCACACACT-3’ and (R) 5’-CCTGCAGGCGGACTCC-3’; and human β-actin: (F) 5’-AAAATCTG
GCACCACACCTTCTAC-3’ and (R) 5’-TAGCAGCCTGGATAGCAACG-3’.

14. Xenograft model

Cells were trypsinized and resuspended in 10^7 cells/ml in PBS. Male SCID Mice (IcrTac: ICR-Prkdscid, Taconic, Germantown, NY), 6 weeks of age, were anesthetized with a ketamine/xylazine mix and injected intracardiacally with 100 μl of the cell suspension. Mice were sacrificed 10 weeks after injection, and internal organs were formalin-fixed and paraffin-embedded. PTK6 immunohistochemistry was performed using the anti-PTK6 monoclonal antibody G-6 at a 1:100 dilution followed by use of the Mouse on Mouse (M.O.M.) kit (Vector Laboratories) according to manufacturer’s instructions. Reactions were visualized with DAB (Sigma-Aldrich) and counterstained with hematoxylin (Vector Laboratories). Controls were performed with normal mouse IgG antibodies.

To lively monitor in-vivo metastases, cells were infected with lentivirus made from pFU-L2G plasmids (Liu et al., 2010), and then selected through anti-GFP flow cytometry. Cells were trypsinized and resuspended in 10^7 cells/ml in PBS, and injected
intravenously with 100 μl of the cell suspension into male SCID mice at the age of 6 weeks. Metastases were monitored under IVIS spectrum live imaging system every week. Mice were sacrificed 8 weeks after injection, and internal organs and bones were formalin-fixed and paraffin-embedded.

15. **Murine prostate cancer model and immunohistochemistry**

C57BL/6J PTENfl/fox PB-Cre fixed mouse tissues were provided by Dr. Nissim Hay (Trotman et al., 2003). Mice were sacrificed at 6 months and prostates were formalin-fixed and paraffin-embedded. Antigen retrieval was performed in 10 mM sodium citrate buffer on heat plate with a temperature above 90°C for 20 minutes. Immunohistochemistry was performed using the VECTASTAIN Elite ABC Kit (rabbit IgG) as per manufacturer’s instructions. Reactions were visualized with FITC or rhodamine-conjugated avidin. Slides were mounted in Vectashield fluorescent mount media containing DAPI (Vector Laboratories). Tissues were then observed using standard UV, rhodamine, or FITC filters under 40 X water-immersion objectives by the Carl Zeiss LSM 700 laser scanning microscope. Images were obtained with Axiocam HRc Color Digital Camera and LSM 700’s ZEN software (Zeiss, Jena, Germany).

16. **Statistics**

A dataset containing 363 primary prostate cancer samples and the patient information was extracted from Oncomine database (Setlur Prostate Dataset, NCBI dataset GSE8402). In this study, the survival follow-up time and survival status was available. Patients were categorized into “PTK6 high”, “PTK6 median” and “PTK6 low” groups according to their PTK6 expression level. PTK6 high group represents the top 10% of the patients with high PTK6 expression. PTK6 low represents the bottom 25% of the
patients with low PTK6 expression. PTK6 median represents the rest of the patients with PTK6 expression in between. The survival curve was estimated using the Kaplan-Meier method and the differences among three groups was tested using the log-rank test. The analysis was performed using SAS 9.2.

A dataset containing 140 prostate carcinoma samples and the patient information was extracted from Oncomine database to analyze the recurrence rate (Taylor prostate dataset, NCBI dataset GSE21035). Similar categorization was set according to their PTK6 expression level. PTK6 high group represents the top 10% of the patients with high PTK6 expression. PTK6 low represents the bottom 25% of the patients with low PTK6 expression. PTK6 median represents the rest of the patients with PTK6 expression in between. The recurrence rate was estimated using the Kaplan-Meier method and the differences among three groups was tested using the log-rank test. The analysis was performed using SAS 9.2.

PTK6 mRNA levels were analyzed in a NCBI human genome microarray dataset GDS2545, which contains 171 human prostate samples including normal prostate tissue, normal tissue adjacent to the primary tumor, primary tumor and metastatic tumors. Results are shown as the mean ± S.E.

For all the other cell studies, data represent the mean of at least three independent experiments ± S.D. p values were determined using the one-tailed Student’s t test (Microsoft Excel 2010). A difference was considered statistically significant if the p value was equal to or less than 0.05. Quantitative analyses of immunoblots were performed using the NIH ImageJ program.
III. RESULTS

1. Aberrant Expression, Translocation and Activation of PTK6 in Prostate Cancer

A. High levels of PTK6 predict low survival rate and high recurrence rate of human prostate cancer patients

PTK6 mRNA levels were found to be significantly higher in prostate tumor samples, especially in metastatic prostate tumor samples compared with normal prostate tissue and normal tissue adjacent to the tumor, by analyzing a NCBI human genome microarray dataset GDS2545 that contains 171 samples (Yu et al., 2004), indicating an oncogenic role for PTK6 in prostate tumorigenesis and metastasis (Fig. 2A).

To further explore the clinical significance of PTK6 overexpression, a dataset containing 363 primary prostate cancer samples and the patient information was extracted from Oncomine database (Setlur et al., 2008). Patients were categorized into “PTK6 high”, “PTK6 median” and “PTK6 low” groups according to their relative PTK6 mRNA level. Kaplan-Meier survival curve indicated that patients with higher PTK6 mRNA level have significantly poorer survival outcome and lower PTK6 expression was associated with better overall survival (p<0.005) (Fig. 2B). In addition, another dataset containing 140 prostate carcinoma samples with recurrence information was also extracted and analyzed using Kaplan-Meier method (Taylor et al., 2010). Higher PTK6 expression was associated with the earlier recurrence (p<0.05) (Fig. 2C). These findings suggested that PTK6 expression level has clinical implications and higher PTK6 is related to poor prognosis of prostate cancer patients.
**Fig. 2.** High levels of PTK6 predict low survival rate and high recurrence rate of human prostate cancer patients.

A) Increased mRNA levels of PTK6 were detected in human metastatic prostate cancer samples by analyzing the NCBI human genome microarray dataset GDS2545. *, P<0.05; **, P<0.01. B) Kaplan-Meier survival curves of patients with low, median and high PTK6 mRNA expression showed significantly difference in survival proportion. (n = 36 for high PTK6; n=254 for median PTK6; n=73 for low PTK6 for arms; Log-rank test p<0.005; Wilcoxon test p<0.005). C) Kaplan-Meier curves for recurrence-free proportion of patients with low, median and high PTK6 mRNA expression showed significantly difference in recurrence-free proportion. (n = 14 for high PTK6; n=88 for median PTK6; n=38 for low PTK6 for arms; Log-rank test p<0.05; Wilcoxon test p<0.01).
B. PTK6 translocates from nucleus to cytoplasm and is highly active at cell
plasma membrane in a murine cancer model

PTK6 relocalizes from nucleus to cytoplasm in prostate epithelial cells, as human
prostate cancer progresses (Derry et al., 2003). However, the significance of this
relocalization is still not clear. We first tried to utilize a murine prostate cancer model to
recapitulate the finding made in human. Compared with wild type control mice, PB-Cre,
PTENflox/flox mice formed abnormally enlarged anterior prostate (AP) at the age of 8
months (Fig. 3A). Consistent with previous reports, loss of both PTEN allele results in
early murine PIN formation that can progress to adenocarcinoma (Wang et al., 2003).
Pre-existing prostatic ductules and acini in PB-Cre, PTENflox/flox mice were filled with
cells deriving from stratification of the epithelial layer, while a single layer of epithelial
cells was present in the control mice (Fig. 3B). Knockout of PTEN and activation of
AKT were observed in the prostate epithelial cells in PB-Cre, PTENflox/flox mice (Fig. 3B).
As expected, PTK6 localizes at the nucleus of normal prostate epithelium in wildtype
mice, but it relocates to cytoplasmic compartments of murine prostate tumor cells in the
PTEN knockout mice (Fig. 3C, PTK6). More interestingly, we detected substantial
activation of PTK6 at the plasma membrane in a group of prostate tumor cells in the
knockout mice, using an antibody that specifically recognized phosphorylated tyrosine
residue 342 of PTK6 (Fig. 3C, P-PTK6). These data suggested that relocation of PTK6
from nucleus to cytoplasm may promote the activation of PTK6 near the plasma
membrane.
**Fig. 3.** PTK6 translocates from nucleus to cytoplasm and is highly active at cell plasma membrane in a murine cancer model.

A) Enlarged anterior prostate was observed in PB-Cre, PTEN<sup>flox/flox</sup> mice at the age of 6 months. B) PTEN is absent and AKT is activated in prostate epithelial tumor cells in PB-Cre, PTEN<sup>flox/flox</sup> mice. Immunohistochemistry was performed with anti-PTEN and P-AKT (Ser473) antibodies, and samples were counterstained with DAPI (blue). The size bar denotes 20 μm. C) Aberrant localization and activation of PTK6 was observed in prostate epithelial tumor cells in PB-Cre, PTEN<sup>flox/flox</sup> mice. Immunohistochemistry was performed with anti-PTK6 and P-PTK6 (PY342) antibodies, and samples were counterstained with DAPI (blue). The size bar denotes 50 μm and 20 μm, respectively.
C. Active PTK6 is associated with plasma membrane in human prostate tumor tissue and cell lines

We next checked whether PTK6 is activated in human prostate cancer samples. Using a human prostate tumor tissue array which contains 16 prostate tissue samples from human patients, we discovered that PTK6 is highly activated in about 18% (3/16) of human prostate cancer samples (Fig. 4A). Consistent with mouse data, active PTK6 is primarily localized at cell plasma membrane (Fig. 4A). We also examined the localization of PTK6 and active PTK6 in different cancer cell lines. Cells were fractionated into cytoplasmic, membrane/organelles and nuclear compartments. In all three prostate cell lines PC3 (androgen receptor negative), LNCaP (androgen receptor positive) and BPH1 (benign prostatic hyperplasia) (Hayward et al., 1995), PTK6 is primarily localized in the cytoplasmic compartments (Fig. 4B). Similar results were obtained in the MDA-MB-231 and T47D breast cancer cell lines (Fig. 4B). Although only a small pool of PTK6 localized at the membrane compartments, they are highly phosphorylated at tyrosine residue 342, which is a marker of kinase activation, while a large amount of cytoplasmic PTK6 are not phosphorylated (Fig. 4B, C). These data indicates that membrane localization of PTK6 is critical for its activation. Together, we hypothesized that the translocation of PTK6 from nucleus to cytoplasm in prostate cancer may promote its activation and create access to its substrates, which might be the potential mechanisms involved in prostate tumorigenesis and metastasis.
**Fig. 4. Active PTK6 is associated with plasma membrane in human prostate tumor tissue and cell lines**

A) Highly active PTK6 was detected at plasma membrane of tumor cells in about 18% of human prostate cancer samples. Immunohistochemistry was performed in a human prostate tumor tissue array with anti-P-PTK6 (PY342) antibodies, and samples were counterstained with DAPI (blue). The size bar denotes 20 μm. B) Intracellular localization of PTK6 was examined in prostate cells (PC3, LNCaP, BPH1), and breast cancer cells (MDA-MB-231, T47D). Cells were fractionated into three cellular compartments including cytoplasm [C], membrane/organelle [M], and nucleus [N]. AKT, Her2 and Sp1 were used as loading controls for C, M, N compartments respectively. C) The membrane pool of PTK6 is the active pool. PC3, DU145 and BPH1 cells were fractionated into three cellular compartments including cytoplasm, membrane/organelle and nucleus. Immunoblot analysis were performed with anti-P-PTK6 (PY342), AKT, and β-catenin antibodies.
A

B

C

35
2. **PTK6 Directly Phosphorylates AKT and Promotes AKT Activation in Response to Epidermal Growth Factor**

**A. Background**

AKT (also called Protein kinase B) is a serine threonine kinase that is activated downstream of growth factor receptors (Manning and Cantley, 2007). It is a key player in signaling pathways that regulate cell survival, proliferation, cell growth (size) and apoptosis (Bellacosa et al., 2005). Aberrant activation of AKT through diverse mechanisms has been discovered in different cancers (Altomare et al., 2005). AKT activation requires phosphorylation of AKT on threonine residue 308 and serine residue 473. The significance of phosphorylation of AKT on tyrosine residues is less well understood. Src has been shown to phosphorylate AKT on conserved tyrosine residues 315 and 326 near the activation loop (Chen et al., 2001). Substitution of these two tyrosine residues with phenylalanine abolished AKT kinase activity stimulated by EGF (Chen et al., 2001). Use of the Src family inhibitor PP2 impaired AKT activation following IGF-1 stimulation of oligodendrocytes (Cui et al., 2005). The RET/PTC receptor tyrosine kinase that responds to glial cell-line-derived neurotrophic factor also phosphorylated AKT tyrosine residue 315 promoting activation of AKT (Jung et al., 2005). AKT tyrosine residue 474 was phosphorylated when cells were treated with the tyrosine phosphatase inhibitor pervanadate, and phosphorylation of tyrosine 474 contributed to full activation of AKT (Conus et al., 2002). Recently the nonreceptor tyrosine kinase Ack1 was shown to regulate AKT tyrosine phosphorylation and activation (Mahajan et al.).
B. Active PTK6 induces tyrosine phosphorylation of AKT

Previous studies have demonstrated that PTK6 is involved in regulating AKT (Haegebarth et al., 2006; Haegebarth et al., 2009; Kamalati et al., 2000; Zhang et al., 2005). To explore the relationship between PTK6 and AKT, HEK-293 cells were cotransfected with increasing amounts (1, 2, and 3 µg) of Myc-PTK6-YF plasmid and 1 µg HA-AKT or HA-mAKT plasmids. mAKT (myristoylated AKT) represents an active form of AKT that is targeted to the cellular membrane and activated independently of phosphatidylinositol 3-kinase (PI 3-kinase) activation (Eves et al., 1998). PTK6-YF has a substitution of the PTK6 negative regulatory carboxy terminal tyrosine at position 447 to phenylalanine, and is a constitutively active form of the kinase (Derry et al., 2000; Qiu and Miller, 2002). The expression of active forms of PTK6 leads to a dramatic increase in tyrosine phosphorylated proteins as assessed by immunoblotting total cell lysates with anti-phosphotyrosine (PY) antibodies (Fig. 5A, PY). Immunoblot analysis of immunoprecipitated AKT showed that both AKT and mAKT were phosphorylated on tyrosine residues in cells expressing active forms of PTK6, and that the level of phosphorylation is dependent on the level of PTK6 expression (Fig. 5B, PY(LE, long exposure), upper band). However, mAKT shows less tyrosine phosphorylation compared with AKT (Fig. 5B, PY (SE, short exposure), upper band). Probing the AKT immunoprecipitates with an anti-Myc-tag antibody revealed that both AKT and mAKT associated with active PTK6, but there is less PTK6 associating with mAKT than with AKT (Fig. 5B, Myc-tag). In addition, immunoprecipitation using anti-phosphotyrosine antibodies was performed to monitor AKT tyrosine phosphorylation in a reverse manner. Although there was an increasing amount of AKT and mAKT in the immunoprecipitated
**Fig. 5. PTK6 induces phosphorylation of AKT on tyrosine residues.**

A) Immunoblot analysis of total cell lysates of HEK-293 cells coexpressing increasing amounts (1, 2, and 3 µg) of Myc-PTK6-YF plasmid and 1µg HA-AKT or HA-mAKT plasmid. Untreated (UN) HEK-293 cells served as a control. Total cell lysates were analyzed by immunoblotting with anti-phosphotyrosine (PY), PTK6, and AKT antibodies. 

B) Immunoblot analysis of AKT immunoprecipitated (IP) from lysates of HEK-293 cells coexpressing AKT or mAKT and PTK6-YF. AKT tyrosine phosphorylation was analyzed using anti-PY antibodies. Both short exposure (SE) and long exposures (LE) are shown. Arrows point at tyrosine phosphorylated AKT and PTK6 respectively. The membrane was reprobed with antibodies directed against AKT and Myc-tagged PTK6.

C) Immunoblot analysis of phosphotyrosine containing proteins that immunoprecipitated from lysates of HEK-293 cells coexpressing AKT or mAKT and PTK6-YF. The levels of AKT and PTK6 were analyzed using anti-AKT and anti-PTK6 antibodies.
phosphotyrosine protein complex, as the expression of active PTK6 increased, the level of mAKT was much less than AKT (Fig. 5C, AKT), which is probably due to less tyrosine phosphorylation on mAKT.

We hypothesized that the reduced level of mAKT phosphorylation on tyrosine compared with AKT might be due to differences in intracellular localization of PTK6-YF, mAKT, and AKT. This led us to examine the localization of these proteins in HEK-293 cells following transfection using immunofluorescence staining. PTK6 YF (green, FITC) and AKT (red, rhodamine) were predominantly colocalized in the cytoplasm (Fig. 6, left panel). In contrast, much of the mAKT (red, rhodamine) was at the cellular membrane (Fig. 6, right panel) and did not colocalize with PTK6-YF. Views shown depict cells expressing both PTK6 and AKT, and cells that were not cotransfected with both constructs. Overexpression of mAKT was not sufficient to bring PTK6 to the cell membrane.

C. PTK6 directly phosphorylates AKT in vitro

To address whether PTK6 directly phosphorylates AKT, in vitro kinase assays were performed using active recombinant human PTK6 and recombinant human AKT. Reactions were performed in the presence of ATP for 10 minutes at 30°C. Activated PTK6 was detected using anti-phosphotyrosine antibodies (Fig. 7A, PY, lower band). Tyrosine phosphorylated wild type AKT was detected only in the presence of PTK6 (Fig. 7A, PY, upper band). Mass spectrometry was utilized to identify the specific tyrosine site(s) targeted by PTK6, and tyrosine phosphorylation events at AKT tyrosine residues 215 (Y215) and 326 (Y326) were identified. The MS/MS spectrum for the AKT peptide containing phosphorytrosine at residue 326 is shown (Fig. 7B). Tyrosine 215 is a novel
Fig. 6. PTK6 colocalizes with AKT in transfected HEK-293 cells.

Intracellular localization of AKT, mAKT and PTK6 was examined by indirect immunofluorescence in HEK-293 cells coexpressing PTK6-YF and AKT or mAKT. Myc-tagged PTK6 immunoreactivity is visualized with FITC (green), while HA-tagged AKT and mAKT are detected with rhodamine (red). Cells were counterstained with DAPI (blue). Transfected PTK6 and AKT colocalize in the cytoplasm (left panels) whereas mAKT is localized at membrane (right panels). Size bar denotes 10 μm.
site, while tyrosine 326 was previously reported to be phosphorylated by Src (Chen, Kim et al. 2001). Phosphorylation on tyrosine 315 (Y315), another tyrosine residue targeted by Src (Chen et al., 2001), and tyrosine 474 (Y474), which is phosphorylated after treatment of cells with the phosphatase inhibitor pervanadate (Conus et al., 2002), was not detected by mass spectrometry. However, we determined the tyrosine residue 315 is also targeted by PTK6 (see below). A schematic diagram of AKT shows tyrosine residues targeted by PTK6 (Fig. 7E).

While mass spectrometry is a powerful analytical tool with high precision and sensitivity, it still has its limitations in identifying target sites in low abundance peptides. Therefore, to determine which tyrosine residues in AKT are physiologically relevant targets of PTK6, we examined significance of all four candidate tyrosine residues on AKT, and converted individual and combinations of tyrosine residues (Y215, Y315, Y326, Y474, Y315/Y326, Y215/Y474) to phenylalanine using site directed mutagenesis. HEK-293 cells were cotransfected with increasing amounts (0µg, 1µg, 3µg) of Myc-PTK6-YF plasmid and 1µg of wild type HA-AKT plasmid or mutant plasmids with single tyrosine mutation (Y215F) or double tyrosine mutations (Y315F/Y326F). Immunoblot analysis of immunoprecipitated AKT showed that the AKT Y215F mutant was still phosphorylated by active PTK6, like wild type AKT (Fig. 7C, PY, upper band, lane 2, 3, 8). When both tyrosine residues 315 and 326 were mutated to phenylalanine, the level of tyrosine phosphorylation of the AKT double mutant Y315F/Y326F was greatly reduced (Fig. 7C, PY, lane 5, 6), indicating tyrosine 315 and tyrosine 326 are the major sites phosphorylated by PTK6. We also performed in vitro kinase assay experiments using wild type GST-AKT or mutant AKT (Y315F/Y326F) fusion proteins.
Fig. 7. PTK6 directly phosphorylates AKT on tyrosine residues 315 and 326.

A) PTK6 directly phosphorylates AKT in an in vitro kinase reaction. Recombinant human PTK6 and human AKT were incubated in kinase buffer in the presence of ATP for 10 minutes at 30°C and then subjected to SDS-PAGE and immunoblot analysis with anti-PY, AKT and PTK6 antibodies. B) The MS/MS spectrum of the AKT peptide TFCGTPEYLAPEVLEDNDpYGR showing phosphorylation at Tyr326. The fragment ions at b9 and y11 minus phosphate prove that phosphorylation is present on the tyrosine residue on the C-terminal end of the peptide. C) Immunoblot analysis of AKT immunoprecipitated (IP) from lysates of HEK-293 cells coexpressing PTK6-YF and wild type AKT or AKT mutants (Y215F, Y315F/Y326F). AKT tyrosine phosphorylation was analyzed using anti-PY antibodies. Both short exposure (SE) and long exposures (LE) are shown. Arrows point at tyrosine phosphorylated AKT and PTK6 respectively. The membrane was reprobed with an antibody directed against the HA-tag. Total cell lysates were analyzed by immunoblotting with anti-AKT, PTK6, and α-tubulin antibodies as input. D) Reduced phosphorylation of GST-AKT Y315F/Y326F in an in vitro kinase reaction. Recombinant human PTK6 and purified GST-tagged wild type AKT or AKT Y315F/Y326F mutant were incubated in kinase buffer in presence of ATP for 10 minutes at 30°C and subjected to SDS-PAGE and immunoblot analysis with anti-PY, GST-tag and PTK6 antibodies. E) Schematic representation of tyrosine phosphorylated AKT. Tyrosine residues 215 and 326 were identified as sites phosphorylated by PTK6 using tandem mass spectrometry (solid arrow). Tyrosine residue 315 was found to be targeted by PTK6 through point mutation studies (dashed arrow). Phosphorylation on tyrosine residue 474 was reported in previous publications.
as substrates of active PTK6, and much less tyrosine phosphorylation was detected on the GST-AKT Y315F/Y326F mutant protein (Fig. 7D, PY, upper band). These data confirm that AKT tyrosine residues 315 and 326 are primary sites phosphorylated by PTK6.

**D. The SH2 and SH3 domains of PTK6 are involved in mediating association between PTK6 and AKT**

In previous experiments, we found that less association between PTK6 and AKT is coupled with less tyrosine phosphorylation on AKT (Fig. 7C, PY(SE), lower band, lane 2, 3, 5, 6). These data led us to hypothesize that tyrosine phosphorylation is critical for interaction between PTK6 and AKT. To test this, HEK-293 cells were cotransfected with Myc-PTK6-YF and HA-tagged wild type AKT or AKT mutants (Y315F, Y326F, Y315F/Y326F, Y315E/Y326E), and PTK6 immunoprecipitations were performed. An anti-HA-tag immunoblot showed that the interaction between PTK6 and AKT is slightly decreased when a single tyrosine residue 315 or 326 was mutated to phenylalanine, but largely decreased when both sites were mutated to phenylalanine (Fig. 8A, HA-tag). AKT Y315E/Y326E served as a tyrosine phosphomimetic form, and the interaction between PTK6 and this AKT form is relatively higher than AKT Y315F/Y326F (Fig. 8A, HA-tag, lane 5, 6), suggesting tyrosine phosphorylation promotes the association of PTK6 and AKT. The column graph shows the level of AKT binding to PTK6 after normalization to the total amount of immunoprecipitated PTK6 (Fig. 8B). The reverse immunoprecipitation was also performed using an anti-AKT antibody. Consistent with previous results, there was a slight reduction in the amount of PTK6 that coimmunoprecipitated with AKT when a single tyrosine residue 315 or 326 was mutated to phenylalanine, and a striking reduction when both tyrosine residues were mutated to
phenylalanine (Fig. 8C, Myc-tag). The interaction between PTK6 and the AKT Y315E/Y326E phosphomimetic form was comparable to WT AKT (Fig. 8C, Myc-tag). In addition, probing blots with anti-phosphotyrosine antibodies showed that tyrosine phosphorylation of AKT (Fig. 8C, PY, upper band) correlated well with the association of AKT with PTK6 (Fig. 8A, HA-tag; Fig. 8C, Myc-tag), further indicating the importance of AKT tyrosine phosphorylation in regulating the association of these two proteins.

We hypothesized that the PTK6 SH2 domain might recognize phosphorylated tyrosine residues on AKT, and thus regulate the association between PTK6 and AKT. To test this, GST tag, GST-PTK6FL (full length), GST-PTK6-SH2, GST-PTK6-SH3 and GST-PTK6-SH2/SH3 fusion proteins were prepared and GST pull down assays were performed using HEK-293 cell lysates expressing AKT alone or in combination with active PTK6. GST pull down assays using cell lysate lacking active PTK6 showed that the SH3 domain is important for PTK6 to interact with AKT, while the SH2 domain interacted weakly with AKT (Fig. 8D, AKT, lane 1, 3, 5, 7, 9). Reduced association of the PTK6 SH3 domain with tyrosine phosphorylated AKT in lysates with overexpression of active PTK6 might be caused by conformational change of AKT (Fig. 8D, AKT, lane 5, 6). In contrast, SH2 domain containing GST fusion proteins (GST-PTK6-SH2, GST-PTK6-SH2/SH3, GST-PTK6FL) are all able to pull down more tyrosine phosphorylated AKT (Fig. 8D, AKT, compare lanes 3 and 4; 7 and 8; 9 and 10). These data indicate that the PTK6 SH2 domain is able to recognize tyrosine phosphorylation on AKT induced by PTK6-YF, promoting a more stable association between AKT and PTK6.
Fig. 8. Interactions between PTK6 and AKT are mediated by the PTK6 SH2 and SH3 domains.

A) Immunoblot analysis of PTK6 immunoprecipitated (IP) from lysates of HEK-293 cells coexpressing PTK6-YF and wild type AKT or AKT mutants (Y315F, Y326F, Y315F/Y326F, Y315E/Y326E). AKT association was analyzed using an anti-HA-tag antibody. The membrane was stripped and reprobed with an antibody directed against Myc-tag. Total cell lysates were analyzed by immunoblotting with anti-AKT, PTK6, and α-tubulin antibodies as input. B) A column graph showing the ratio of AKT (HA-tag) over PTK6 (Myc-tag) band density quantified by NIH ImageJ software. C) Immunoblot analysis of AKT immunoprecipitated (IP) from lysates of HEK-293 cells coexpressing PTK6-YF and wild type AKT or AKT mutants (Y315F, Y326F, Y315F/Y326F, Y315E/Y326E). The levels of tyrosine phosphorylated AKT and PTK6 (pY-AKT and pY-PTK6) were analyzed using anti-PY antibodies (arrow). The level of PTK6 that interacts with AKT was analyzed using an anti-Myc-tag antibody. The membrane was reprobed with an antibody directed against AKT. D) GST pulldown assay using lysates of HEK-293 cells expressing AKT alone or in combination with PTK6-YF. Glutathione Sepharose 4B beads which bind purified GST tag, GST-PTK6, GST-SH2, GST-SH3 or GST-SH2/SH3 protein, were used to pull down AKT from HEK-293 lysates. Levels of bound AKT were analyzed using an anti-AKT antibody. 10% of the lysates added to the pull down reaction served as input. E) Immunoblot analysis of HA-tag IP from lysates of HEK-293 cells coexpressing GFP-PTK6 and wild type AKT or AKT mutants (P424A/P427A, P467A/P470A). PTK6 association was analyzed using an anti-GFP antibody. The membrane was stripped and reprobed with an antibody directed against HA-tag. Total cell lysates were analyzed by immunoblotting with anti-GFP, HA-tag and β-actin antibodies as input.
To investigate the role of PTK6 SH3 domain involved in the association between PTK6 and AKT, two proline-rich motifs PXXP at the C-terminus of AKT were mutated. Wild type HA-tagged AKT or mutant AKT P424A/427A, P467A/P470A were coexpressed with GFP-PTK6 in HEK-293 cells, and HA-tag immunoprecipitation were performed to detect PTK6 association. A dramatic decrease in PTK6 binding was observed when proline 424 and 427 were mutated to alanine, while mutation of proline 467 and 470 to alanine did not affect interaction with PTK6. Consistent with pull down data (Fig. 8D), these data suggests an important role of PTK6 SH3 domain in regulating the association between PTK6 and AKT, which recognizes the proline-rich sequence P424XXP427 at AKT C-terminus.

E. Active PTK6 induces tyrosine phosphorylation of AKT independent of Src in SYF cells

Tyrosine residues 315 and 326 are also phosphorylated by Src, suggesting possible redundancy between PTK6 and Src (Chen et al., 2001). Although we showed that PTK6 directly phosphorylates AKT, it is also possible that PTK6 promotes Src-mediated phosphorylation of AKT on tyrosine. To exclude the effect for Src in AKT tyrosine phosphorylation in cells, MEFs from mouse embryos harboring functionally null mutations in both alleles encoding the Src family protein tyrosine kinases, Src, Yes, and Fyn were utilized (SYF cells) (Klinghoffer et al., 1999). In these cells, AKT tyrosine phosphorylation can be examined in a background without Src, Yes, and Fyn activity. Src expression is absent in SYF cells as expected, while present in wild type MEF cells (Fig. 9A). To detect whether PTK6-YF expression in SYF cells is able to induce tyrosine phosphorylation on AKT, SYF cells were transfected with different combinations of HA-
AKT and Myc-PTK6-YF plasmids. Protein expression was examined by immunoblotting using anti-Myc-tag and AKT antibodies (Fig. 9B). Probing blots with anti-phosphotyrosine antibodies showed that phosphotyrosine signaling is induced in the presence of active PTK6 (Fig. 9B). AKT was then immunoprecipitated from those cell lysates and immunoblotting was performed with anti-phosphotyrosine antibodies. Both endogenous and exogenous AKT protein was phosphorylated on tyrosine residues when active PTK6 is present, indicating that PTK6 is able to induce tyrosine phosphorylation of AKT in the absence of Src family kinases (Fig. 9C, PY, upper band).

**F. Ectopic PTK6 sensitizes SYF cells to physiological levels of EGF and stimulates SYF cell proliferation**

Others previously showed that Src mediated phosphorylation of AKT tyrosine residues 315 and 326 played a role in AKT activation by EGF (Chen et al., 2001). Since our data shows that PTK6 also targets AKT on tyrosine residues 315 and 326, we hypothesized that PTK6 might also regulate AKT signaling in response to growth factors. AKT is activated through phosphorylation of Thr-308 in the activation loop of the kinase domain by PDK1 (Alessi et al., 1996), and Ser-473 within the hydrophobic motif of the regulatory domain by mammalian target of rapamycin (mTOR) in a rapamycin-insensitive complex with Rictor and Sin1 (Jacinto et al., 2006; Sarbassov et al., 2005). Therefore, we examined phosphorylation on Thr-308 and Ser-473 as markers for AKT activation. To exclude potential interference caused by Src, we utilized SYF cells to construct stable cell lines expressing PTK6-YF or empty vector. SYF stable cell lines were serum starved for 48 hours and then stimulated with different concentrations of
Fig. 9. PTK6 induces Src-family independent phosphorylation of AKT on tyrosine residues.

A) Immunoblotting demonstrates the absence of Src expression in SYF cells. Wild type MEFs express high levels of Src Kinase. B) Immunoblot analysis of the lysates from SYF cells cotransfected with PTK6-YF and AKT plasmids. Total cell lysates were analyzed using anti-phosphotyrosine (PY), AKT, Myc-tag, and α-tubulin antibodies. C) Immunoblot analysis of AKT immunoprecipitated (IP) from lysates of SYF cells cotransfected with PTK6-YF and AKT plasmids. Tyrosine phosphorylation of AKT was analyzed using anti-PY antibodies (arrow). The membrane was reprobed with an antibody directed against AKT.
EGF (0.1 ng/ml, 1 ng/ml, 10 ng/ml) for 5 or 15 minutes. Immunoblot analysis showed that the AKT phosphorylation on Thr-308 and Ser-473 peaks after 5 minutes of EGF stimulation in all conditions (Fig. 10A). Interestingly, AKT is able to achieve higher activation in the presence of PTK6-YF when stimulated by physiological levels of EGF (0.1 ng/ml, 1 ng/ml), but not higher concentrations of EGF (10 ng/ml) (Fig. 10A). To confirm this result, we treated both PTK6-YF expressing SYF cells and control cells with 0.1 ng/ml EGF for 5 minutes in triplicate. Anti-Thr-308 and Ser-473 immunoblotting showed that AKT achieves higher activation when PTK6-YF is present, while the phosphorylation of p44/p42 MAPK upon EGF stimulation is not affected, indicating that PTK6 specifically regulates AKT signaling (Fig. 10B). AKT phosphorylation on Thr-308 and Ser-473 was further normalized by total AKT level, and two column graphs were created to show the differences in AKT activation, with P values of 0.031 and 0.0007 respectively (Fig. 10C). Also, increased AKT activation correlated with increased tyrosine phosphorylation of AKT, as is shown in Fig. 10D. There is higher AKT tyrosine phosphorylation in PTK6-YF expressing SYF cells after 5 minutes of 0.1 ng/ml EGF treatment, compared with SYF vector cells (Fig. 10D).

To investigate whether the regulation of AKT activation by PTK6 is dependent on PTK6 kinase activity, SYF cell lines stably expressing PTK6-WT (wild type), PTK6-YF (constitutively active), PTK6-KM (kinase dead) or empty vector were serum starved for 48 hours, and then stimulated by 0.1 ng/ml EGF for 5 minutes. Immunoblot analysis showed that AKT achieves higher activation in the presence of PTK6-WT or PTK6-YF, but not PTK6-KM or empty vector (Fig. 10E), indicating the regulation of AKT activation is PTK6 kinase activity dependent.
We further delineated the role of PTK6 in regulating SYF cells proliferation. As shown in Fig. 10F, SYF cells expressing PTK6-KM or empty vector proliferated similarly with a doubling time around 29.0 ± 1.0 hours; while PTK6-WT or PTK6-YF expressing SYF cells had a higher proliferation rate, which reduced the doubling time to 25.8 ± 1.1 hours and 24.0 ± 0.4 hours, respectively. SYF cells growth curves clearly show that PTK6 is able to boost the SYF cells proliferation rate, and this is dependent on PTK6 kinase activity.

G. Active PTK6 promotes AKT activation through phosphorylation on tyrosine residues 315 and 326

To confirm that AKT activation induced by a low dose of EGF in the presence of PTK6 is correlated with tyrosine phosphorylation of AKT induced by PTK6, we transiently transfected PTK6-YF plasmids into both HEK-293 cells and SYF cells, which significantly increases intracellular phosphotyrosine signaling compared with stable PTK6-YF expressing cell line. Twenty four hours post transfection, cells were serum starved for 24 hours and then stimulated by 0.1 ng/ml EGF for 5 minutes. Twenty four hour serum starvation is able to reduce the phosphotyrosine background induced by active PTK6 in SYF cells, and EGF stimulation dramatically induced phosphotyrosine signaling only in the presence of active PTK6 (Fig. 11A, PY, right panel). Phosphotyrosine signaling in HEK-293 cells is more resistant to 24 hours serum starvation, but differences under unstimulated (0’) and stimulated (5’) conditions could still be observed in the presence of active PTK6 (Fig. 11A, PY, left panel). To examine AKT tyrosine phosphorylation, AKT protein was immunoprecipitated from cell lysates in panel A, and immunoblotting was performed with anti-phosphotyrosine antibodies.
**Fig. 10.** PTK6 positively regulates AKT activation stimulated by physiological levels of EGF in SYF cells.

A) Increased activating phosphorylation of AKT on Thr-308 and Ser-473 was detected in SYF cells expressing PTK6-YF following addition of 0.1 ng/ml EGF. SYF cells stably expressing PTK6-YF or empty vector were serum starved for 48 hours and then stimulated with different concentrations of EGF (0.1 ng/ml, 1 ng/ml, 10 ng/ml) for 5 or 15 minutes. Cell lysates were analyzed by immunoblotting with anti-AKT phospho-Thr-308, AKT phospho-Ser-473, AKT, mouse PTK6, and β-actin antibodies. B) SYF cells stably expressing PTK6-YF or empty vector were serum starved for 48 hours and then stimulated by 0.1 ng/ml EGF for 5 minutes. Samples were in triplicate. Cell lysates were analyzed by immunoblotting with anti-AKT phospho-Thr-308, AKT phospho-Ser-473, AKT, mouse PTK6, phospho-p42/p44 and α-tubulin antibodies. C) The ratio of AKT Thr-308 phosphorylation and Ser-473 phosphorylation band density over total AKT band density was quantified with NIH ImageJ software. Results were formatted as means ± standard deviations from three independent experiments. (*P-value = 0.031, **P-value=0.0007). D) Immunoblot analysis of AKT immunoprecipitated (IP) from SYF cells lysates described in B. Tyrosine phosphorylation of AKT was analyzed using anti-PY antibodies. E) SYF cells stably expressing PTK6-YF, PTK6-KM, PTK6-WT or empty vector were serum starved for 48 hours and then stimulated with 0.1 ng/ml EGF for 5 minutes. Cell lysates were analyzed by immunoblotting with anti-AKT phospho-Thr-308, AKT phospho-Ser-473, AKT, mouse PTK6, and β-actin antibodies. Relative band densities were quantified with NIH ImageJ Software and are indicated under corresponding bands. F) Growth curves of SYF cells stably expressing empty vector, PTK6-YF, PTK6-KM, or PTK6-WT.
### A

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**IB:**
- p-AKT (Thr-308)
- p-AKT (Ser-473)
- AKT
- PTK6
- β-actin

### B

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**IB:**
- p-AKT (Thr-308)
- p-AKT (Ser-473)
- AKT
- PTK6
- p-p42/p44
- α-tubulin

### C

**C1:**
- Threonine 308/Tot-AKT
- Serine 473/Tot-AKT

### D

**IP:**
- AKT
- PT6-YF
- AKT

### E

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**IB:**
- p-AKT (Thr-308)
- p-AKT (Ser-473)
- AKT
- PTK6
- β-actin

### F

**Graph:**
- Cell Number vs. Days
- YF
- WT
- KM
- vec

---

57
In both HEK-293 cells and SYF cells, tyrosine phosphorylation on AKT is dramatically increased in response to EGF stimulation only in the presence of active PTK6 (Fig. 11B, PY). In empty vector transfected control cells, there is no change in AKT tyrosine phosphorylation before and after EGF stimulation (Fig. 11B, PY). AKT activation was also examined in SYF cells. After EGF treatment, AKT phosphorylation on Thr-308 and Ser-473 is increased in the presence of PTK6-YF (Fig. 11C). These data confirm the positive correlation between AKT tyrosine phosphorylation and AKT activation induced by PTK6-YF overexpression, in both stable SYF cell lines (Fig. 10) and transiently transfected SYF cells (Fig. 11).

To further address whether PTK6 directly promotes AKT activation through phosphorylation of tyrosine residues 315 and 326, PTK6-YF was coexpressed with HA-tagged wild type AKT or AKT Y315F/Y326F mutant in SYF cells. Exogenous AKT was immunoprecipitated using anti-HA-tag antibody and immunoblotting was performed to detect AKT activation with anti-phospho-Thr-308 antibody. Wild type AKT is more activated in the presence of active PTK6, while the AKT Y315F/Y326F mutant has much less phosphorylation on Thr-308, and active PTK6 does not promote activation of the AKT Y315F/Y326F mutant (Fig. 11D). These data indicated that PTK6 positively regulates AKT signaling by directly targeting AKT on tyrosine residues 315 and 326.

**H. Knockdown of PTK6 in BPH-1 cells impairs AKT activation in response to EGF**

PTK6 is expressed in prostate epithelial cells (Derry et al., 2003). BPH-1 is a benign prostatic hyperplasia cell line expressing PTK6 (Hayward, Dahiya et al. 1995).
Fig. 11. Active PTK6 promotes AKT activation through phosphorylation on tyrosine residues 315 and 326.

**A)** HEK-293 and SYF cells were transiently transfected with PTK6-YF or empty vector for 24 hours and serum starved for another 24 hours, and then stimulated with a low dose of EGF (0.1 ng/ml) for 5 minutes. Cell lysates were analyzed by immunoblotting with anti-PY, Myc-tag, and β-catenin antibodies. β-catenin served as a loading control. **B)** Immunoblot analysis of AKT immunoprecipitated (IP) from lysates of HEK-293 cells and SYF cells previously described in A. Tyrosine phosphorylation of AKT was analyzed using anti-PY antibodies. The membrane was reprobed with an antibody directed against AKT. **C)** SYF cell lysates described in A were analyzed by immunoblotting with anti-phospho-Thr-308, phospho-Ser-473, total AKT, Myc-tag, β-catenin and β-actin antibodies. **D)** Immunoblot analysis of HA-tag immunoprecipitated (IP) from lysates of SYF cells coexpressing PTK6-YF and wild type AKT or AKT Y315F/Y326F mutant. AKT activation was analyzed using anti-phospho-Thr-308 antibodies. Both short exposure (SE) and long exposures (LE) are shown. The membrane was reprobed with an antibody directed against the HA-tag. Relative band densities were quantified with NIH ImageJ software and are indicated under corresponding bands. Total cell lysates were analyzed by immunoblotting with anti-phospho-Thr-308, HA-tag, Myc-tag, β-catenin and β-actin antibodies as input. β-catenin and β-actin served as loading controls.
BPH-1 cells also express high levels of EGFR, and EGF is able to induce strong tyrosine phosphorylation in this cell line (El Sheikh et al., 2004). Therefore the BPH-1 cell line was utilized to investigate whether loss of PTK6 is able to affect AKT activation in response to EGF. Both BPH-1 PTK6 knockdown and vector cells were serum starved for 48 hours and stimulated by 0.1 ng/ml or 1 ng/ml EGF for 5 minutes. Probing blots with anti-Thr-308 and anti-Ser-473 antibodies showed impaired AKT activation when PTK6 is absent (Fig. 12A). AKT immunoprecipitation followed by immunoblotting was performed to detect AKT tyrosine phosphorylation in BPH-1 cells stably expressing PTK6 shRNA or empty vector. The level of AKT tyrosine phosphorylation in response to 1 ng/ml EGF is dramatically decreased in BPH-1 cells with knockdown of PTK6, compared with vector cells (Fig. 12B). The correlation of decreased AKT activation and decreased AKT tyrosine phosphorylation in PTK6 knockdown cells further supports a positive role for PTK6 in regulating AKT activation.
Fig. 12. Knockdown of PTK6 in BPH-1 cells impairs AKT activation in response to EGF.

A) BPH-1 cells stably expressing PLKO-1 vector or PTK6 shRNA were serum starved for 48 hours and then stimulated with 0.1 ng/ml or 1 ng/ml EGF for 5 minutes. Cell lysates were analyzed by immunoblotting with anti-phospho-Thr-308, phospho-Ser-473, total AKT, PTK6, β-catenin and β-actin antibodies. β-catenin and β-actin served as loading controls. B) Immunoblot analysis of AKT immunoprecipitated (IP) from BPH-1 cell lysates previously described in A. Tyrosine phosphorylation of AKT was analyzed using anti-PY antibodies. The membrane was reprobed with an antibody directed against AKT.
3. **PTK6 Protects Cells from Anoikis by Directly Phosphorylating FAK and Activating AKT**

**A. Background**

Focal adhesion kinase (FAK) was identified as a highly phosphorylated protein localized in focal adhesion contacts of normal cells [reviewed in (Parsons, 2003)]. Its activity is regulated by integrin mediated cell adhesion as well as activation of growth factor receptor and G-protein linked receptor signaling [reviewed in (Schaller, 2001)]. FAK is involved in various cell functions including proliferation, survival, motility and invasion [reviewed in (Schaller, 2001; Schlaepfer and Mitra, 2004)]. It regulates cell survival and apoptosis through several different mechanisms. FAK is able to activate PI3K/AKT signaling to promote survival of fibroblasts (Xia et al., 2004). Depending upon the cellular context, FAK forms signaling complexes with Paxillin or p130CAS to transmit distinct survival signals to fibroblasts and epithelial cells (Zouq et al., 2009). Recently, nuclear FAK was reported to promote cell survival by facilitating p53 turnover via enhanced Mdm2-dependent p53 ubiquitination (Lim et al., 2008).

AKT, a serine threonine protein kinase that plays critical roles in cell proliferation and survival, is frequently activated in different types of human cancers [reviewed in (Song et al., 2005; Zhang et al.)]. AKT regulates cell survival through multiple mechanisms including direct inhibition of pro-apoptotic signals such as Bad and FoxOs, and activation of pro-survival genes through the IKK/NF-κB signaling pathway (Datta et al., 1997; Kane et al., 1999; Zhang et al.). AKT regulates cell cycle and proliferation through direct phosphorylation of p21WAF1/CIP1 and p27KIP1 and indirect transcriptional regulation by inhibition of FoxO transcription factors (Liang et al., 2002;
Rossig et al., 2001). AKT itself is sufficient to suppress anoikis, a form of programmed cell death induced by the loss of cell-matrix interactions (Khwaja et al., 1997).

**B. PTK6 directly phosphorylates FAK independent of SRC family kinases**

FAK plays crucial roles in suppressing anoikis and promoting anchorage-independent survival in a variety of cell types (Derry et al., 2000; Frisch et al., 1996; Lim et al., 2008; Xia et al., 2004; Xu et al., 2000; Zouq et al., 2009). Since phosphorylation of FAK at several tyrosine residues is important for its activation and downstream signaling, we investigated if FAK is a direct substrate of PTK6. In an in-vitro kinase assay, tyrosine phosphorylation of FAK is increased in the presence of PTK6 and ATP (Fig. 13A). We detected phosphorylation of FAK tyrosine residue 861 in vitro using LC/MS/MS (Fig. 13B). Expression of active PTK6 in HEK-293 and SW620 cells induces the tyrosine phosphorylation of FAK, as evident by FAK immunoprecipitation followed by immunoblot analysis with anti-phosphotyrosine antibodies (Fig. 13C). In addition, a GST pull down assay using a series of GST-PTK6 fusion proteins showed that PTK6 interacts with FAK primarily through its SH2 domain, which recognizes phosphorylated tyrosine residues on FAK (Fig. 13D). Since FAK can be phosphorylated by SRC kinase (Calalb et al., 1995), SYF cells were used to exclude the interference by SRC family kinases. Following serum stimulation, higher tyrosine phosphorylation of FAK was observed in SYF cells stably expressing PTK6-YF or PTK6-WT, compared with cells expressing kinase dead PTK6-KM or vector controls (Fig. 13E, FAK IP; 9F). This was also observed using phosphorylation-specific FAK antibodies PY925 or PY576/Y577 (Fig. 13E). Interestingly, FAK reaches the peak of tyrosine phosphorylation at the 10-minute time point in PTK6-WT or PTK6-YF expressing cells, correlating with the activation of
Fig. 13. PTK6 phosphorylates FAK independent of SRC family kinase. A) FAK is a direct PTK6 substrate. Tyrosine phosphorylation of FAK is induced by active PTK6 in the presence of ATP in an in-vitro kinase assay. Immunoblot analysis of kinase reactions was performed using anti-PY, FAK and PTK6 antibodies. B) PTK6 phosphorylates FAK at tyrosine residue 861. The LC/MS/MS tandem mass spectrum of the triply charged tryptic peptide GSIDREDGSLQDEAMGPIGNQHIPYQDEAMPVGBK PDPAAPPK acquired via CID using a hybrid linear ion trap-orbitrap mass spectrometer show the phosphorylation site Y861. C) PTK6 phosphorylates FAK in cells. HEK293 and SW620 cells were transiently transfected with PCDNA3-PTK6-YF or empty vector, and total cell lysates were harvested 24 hour after transfection. HA-tag-IP or FAK-IP was performed and tyrosine phosphorylation of FAK was detected by immunoblotting with anti-PY antibodies. Immunoblot analysis of total cell lysates was performed using anti-FAK and PTK6 antibodies as input. D) PTK6 interacts with FAK through its SH2 domain. Glutathione-Sepharose CL-4B beads binding with GST fusion proteins were used to pull down FAK from HEK-293 cell lysates. Bound FAK was analyzed by immunoblotting with anti-FAK antibodies. 10% of the lysates added to the pulldown reaction served as input. E) PTK6 induces tyrosine phosphorylation of FAK independent of SRC family kinases. SYF cells stably expressing vector, PTK6-WT, PTK6-KM or PTK6-YF were serum starved for 48 hours and stimulated by 20% FBS for 10 or 30 minutes. FAK IP was performed and tyrosine phosphorylation of FAK was detected by immunoblotting with anti-PY antibodies. Immunoblot analysis of total cell lysates was performed using anti-FAK, P-FAK (Tyr576/Tyr577), P-FAK (Tyr925), PTK6, P-PTK6 (Tyr-342), and β-catenin antibodies. F) PTK6 induced tyrosine phosphorylation of FAK is kinase dependent. The relative band density of PY blot in the FAK IP was normalized by the density of FAK using NIH ImageJ software.
PTK6, monitored by phosphorylation of PTK6 tyrosine residue 342 (Fig. 13E, P-PTK6). These data suggest that PTK6 induced tyrosine phosphorylation on FAK is dependent on the kinase activity of PTK6, and independent of SRC family kinases.

C. Membrane targeted active PTK6 induces constitutive FAK activation and transformation of SYF fibroblasts

Although PTK6 is primarily cytoplasmic in the PC3 prostate cancer cell line, the active pool of PTK6 that is phosphorylated at tyrosine 342 is associated with membrane compartments (Zheng et al.). SYF cells stably expressing membrane targeted active PTK6 (Palm-PTK6-YF) have much higher PTK6 activity than cells expressing the nontargeted active PTK6-YF construct (Fig. 14A, P-PTK6). Palm-PTK6-YF is constitutively active even after 48-hour serum starvation, resulting in constitutive phosphorylation of FAK at tyrosine residues 576, 577 and 925 (Fig. 14A). Immunostaining of SYF cells shows that PTK6-YF induces formation of focal adhesions, as evident by induced tyrosine phosphorylated FAK and Paxillin signaling around cell plasma membrane (Fig. 14B, YF). Expression of Palm-PTK6-YF dramatically changed cell morphology, and led to formation of numerous thin long actin-bundle protrusions around plasma membrane, with tyrosine phosphorylated FAK and Paxillin at the tip of each protrusion (Fig. 14B, Palm-YF).

Next, we investigated the behavior of SYF cells with constitutive PTK6 and FAK activation. While wild type SYF cells form a single cell layer due to contact inhibition after confluence, cells with Palm-YF expression overcome contact inhibition and continue to grow after confluence. Cells expressing nontargeted PTK6-YF are more densely packed than control cells, but they do not lose contact inhibition (Fig. 15A).
Fig. 14. Membrane associated active PTK6 induces constitutive activation of FAK in SYF cells.

A) Membrane targeted PTK6 (Palm-PTK6-YF) is constitutively active. SYF cells stably expressing vector, PTK6-YF or Palm-PTK6-YF were serum starved for 48 hours and stimulated by 20% FBS for 10 or 30 minutes. Immunoblot analysis of total cell lysates was performed using anti-FAK, P-FAK (Tyr576/Tyr577), P-FAK (Tyr925), PTK6, P-PTK6 (Tyr342), and β-catenin antibodies. B) Membrane targeted PTK6 dramatically changes the morphology of SYF cells. SYF cells expressing vector, PTK6-YF or Palm-PTK6-YF were grown at 8-well chamber slides, and indirect immunofluorescence was performed using anti-Myc-tag, P-FAK (Tyr576/Tyr577), PY and P-Paxillin (Tyr118) antibodies. Rhodamine-conjugated phalloidin was used to selectively stain F-actin. Cells were counterstained with DAPI (blue). The size bar denotes 20 μm.
This observation is consistent with the growth curve data, showing that Palm-YF expressing cells continue proliferating after cells reach confluence at day 5, while growth rates under preconfluent conditions are almost the same for the three SYF cell lines (Fig. 15B). Anchorage independent growth of SYF cells in soft agar assays is augmented when Palm-YF is expressed and to a lesser extent when PTK6-YF is present (Fig. 15C; D). Foci-forming ability was also measured by performing transformation assays. Only Palm-YF expressing SYF cells acquired the capacity to form foci on plates when mixed with the non-tumorigenic parental SYF cells (Fig. 15E). Resistance to anoikis is another trait of transformed cells. SYF cells expressing Palm-YF overcome anoikis and continue to proliferate in suspension. Significantly larger and more cell spheres were observed in Palm-YF expressing cells at 6 days after seeding on Poly-HEMA coated plates (Fig. 15F). Immunoblotting of lysates prepared from cells following 72 hours of growth in suspension showed highly activated PTK6 (P-PTK6) and FAK (PY567/Y577, PY925) in Palm-YF expressing cells, accompanied by increased AKT activation (Thr-308, Ser-473) and decreased levels of cleaved caspase 3 (CC3) and p27, which are markers of apoptosis and cell cycle arrest respectively (Fig. 15G). Taken together, our data indicate that SYF cells can be transformed by Palm-YF, due to the constitutive activation of a signaling cascade including PTK6, FAK and AKT.

D. PTK6 and FAK provide synergistic protection against anoikis

To further characterize the roles of FAK and AKT in PTK6 mediated resistance to anoikis, Fak/-/- MEFS and Akt1/2/-/- MEFS were utilized. The absence of endogenous FAK or AKT was shown by immunoblot analysis (Fig. 16A). While expression of Palm-YF or FAK alone protects Fak/-/- MEFS against anoikis, co-expression of Palm-YF and
Fig. 15. Membrane associated active PTK6 transforms SYF MEFs.

A) SYF cells stably expressing Palm-PTK6-YF are able to overcome contact inhibition. SYF cells stably expressing vector, PTK6-YF or Palm-PTK6-YF were seeded at complete growth medium, and images were taken 5 days after confluence. B) Growth curve of SYF cells as measured by CellTiter-Glo luminescent cell viability assay demonstrates the ability of Palm-PTK6-YF expressing cells to continue growing after confluence. Y axis is the fold change of relative luminescence unit (RLU), with day 1 set to 1. C) SYF cells expressing PTK6-YF or Palm-PTK6-YF show increased anchorage independent growth in soft agar assay. D) A bar graph illustrates the number of colonies formed in soft agar assay in C (**, p < 0.01; ***, p < 0.001). E) Transformation (foci-forming) assays show increased foci-forming ability of SYF cells expressing Palm-PTK6-YF. F) Palm-PTK6-YF expressing SYF cells are able to proliferate under suspended growth condition. Anoikis assays were performed on poly-HEMA coated plates for 6 days. G) Palm-PTK6-YF activates FAK and AKT survival signaling pathways. Palm-PTK6-YF expressing SYF cells were seeded on poly-HEMA coated plates for 72 hours. Immunoblot analysis was performed with anti-P-PTK6 (Tyr342), PTK6, P-FAK (Tyr576/Tyr577), P-FAK (Tyr925), FAK, P-AKT (Thr308), P-AKT (Ser473), AKT, cleaved caspase 3 (CC3) and p27 antibodies.
FAK exerts synergistic effects in protecting cells from anoikis, leading to increased viability and formation of larger spheres after 6 days of growth on Poly-HEMA coated plates (Fig. 16B; C). Immunoblotting of lysates prepared from cells following 72 hours of growth in suspension showed that co-expression of Palm-YF and FAK induces higher FAK activation (PY576/Y577) even under suspended condition, resulting in substantially higher levels of AKT activation (Thr-308, Ser-473) and lower levels of cleaved caspase 3 (CC3) and p27 (Fig. 16D).

AKT plays important roles in regulating cell survival and growth [reviewed in (Song et al., 2005)]. Expression of Palm-YF in Akt1/2/-/- MEFs fails to protect them from anoikis (Fig. 16E). Both control Akt1/2/-/- MEFs and Palm-YF expressing Akt1/2/-/- MEFs undergo apoptosis when grown under suspended condition, as measured by a cell viability assay (Fig. 16E). This is consistent with immunoblot analysis showing the same level of cleaved caspase 3 (CC3) induction after 24-hour suspension in both MEFs, although increased FAK phosphorylation (PY576/Y577, PY925) was observed in Palm-YF expressing MEFs (Fig. 16F). Our data suggest AKT is a critical downstream mediator of PTK6-FAK survival signaling that protects cells from anoikis.

E. Knockdown of PTK6 in prostate cancer cells promotes anoikis

In a PTENflox/flox, Probasin (PB)-Cre transgenic murine prostate cancer model, we discovered highly induced PTK6 activation in a group of prostate tumor cells which possesses enlarged nuclei and substantial cytoplasm (Fig. 17A). Activated P-FAK signaling was also observed in the same group of cells, providing in-vivo evidence of the tight relationship between PTK6 and FAK signaling in prostate tumor cells (Fig. 17A).
Fig. 16. Membrane associated active PTK6 mediates protection against anoikis through activation of FAK and AKT.

A) Immunoblot analysis of lysates of Fak-/- MEFs and Akt1/2/-/- MEFs demonstrates the absence of FAK in Fak-/- MEFs, and no expression of AKT in Akt1/2/-/- MEFs. B) Expression of FAK and Palm-PTK6-YF in Fak-/- MEFs shows synergistic protection against anoikis. Fak-/- MEFs stably expressing vector, FAK, Palm-YF, or both FAK and Palm-YF were seeded on poly-HEMA plates for 6 days. The size bar denotes 200 µm. C) Anoikis assays of Fak-/- MEFs were performed for 5 days and the cell viability was measured by CellTiter-Glo luminescent assay (**, p < 0.01; ***, p < 0.001). D) Expression of FAK and Palm-PTK6-YF synergistically activate AKT survival signaling in Fak-/- MEFs. Fak-/- MEFs stably expressing vector, FAK, Palm-YF, or both FAK and Palm-YF were incubated on poly-HEMA coated plates for 72 hours. Immunoblot analysis was performed with anti-P-FAK (Tyr576/Tyr577), HA-tag, PTK6, P-AKT (Thr308), P-AKT (Ser473), AKT, cleaved caspase 3 (CC3), p27 and β-actin antibodies. E) Expression of Palm-PTK6-YF in Akt1/2/-/- MEFs fails to protect cells against anoikis. The viability of Akt1/2/-/- MEFs was measured for 7 days by CellTiter-Glo luminescent assay. F) Palm-PTK6-YF induced FAK activation in Akt1/2/-/- MEFs fails to protect cells against anoikis. Akt1/2/-/- MEFs expressing vector or Palm-PTK6-YF were incubated on plastic plates or poly-HEMA coated plates for 24 hours. Immunoblot analysis was performed with anti-P-FAK (Tyr576/Tyr577), P-FAK (Tyr925), FAK, PTK6, cleaved caspase 3 (CC3), p27 and β-actin antibodies.
Next we investigated the role of PTK6 in protecting prostate cancer cells from anoikis. In the PC3 prostate cancer cell line, endogenous PTK6 was knocked down using siRNAs against PTK6 (Fig. 17B, PTK6). Although knockdown of PTK6 does not affect FAK activation when cells are grown under adherent conditions, decreased FAK activation was observed in knockdown cells grown under suspended conditions, indicating that the primary role of PTK6 is to maintain the level of FAK activation when cells are detached from the extracellular matrix (Fig. 17B, PY576/Y577, PY925). Decreased FAK activation is accompanied by impaired AKT activation and increased expression of cleaved caspase 3 (Fig. 17B, Thr-308, Ser-473, CC3). The population of PC3 cells treated with scrambled siRNA is able to reach a balance between proliferation, cell cycle arrest and cell death under suspended growth conditions, as measured by a viability assays performed from day 1 to day 8 (Fig. 17C). Knockdown of PTK6 triggers apoptosis, and only 20% cells are viable after 8 days of suspension growth (Fig. 17C).
**Fig. 17. Knockdown of PTK6 in prostate cancer cells promotes anoikis.**

A) A group of prostate tumor cells have highly activated PTK6 and FAK at plasma membrane in a murine prostate tumor model (PTEN$^{flox/flox}$, PB-Cre). Immunohistochemistry was performed with anti-P-PTK6 (Tyr342) and P-FAK (Tyr576/Tyr577) antibodies. The size bar denotes 20 μm. B) Knockdown of PTK6 in PC3 cells impairs FAK and AKT survival signaling under suspended condition. PC3 cells were transfected with siRNA against PTK6 or control siRNA for 24 hours and then seeded on plastic plates or poly-HEMA coated plates for 24 hours. Immunoblot analysis was performed with anti-PTK6, P-FAK (Tyr576/Tyr577), P-FAK (Tyr925), FAK, P-AKT (Ser473), P-AKT (Thr308), AKT, cleaved caspase 3 (CC3), p27 and β-catenin antibodies. C) Knockdown of PTK6 promotes PC3 cells to undergo anoikis. PC3 cells transfected with siRNA against PTK6 or control were seeded on poly-HEMA coated plates for indicated periods. Cell viability was measured by CellTiter-Glo luminescent assays.
A

B

C

si-PTK6

Plate  HEMA
24h     24h

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Fold Change of RLU

- Si-Control
- Si-PTK6

* * *
4. **PTK6 Promotes Peripheral Adhesion Complex Formation and Cell Migration by Phosphorylating p130CAS**

**A. Background**

p130 Crk-associated substrate (p130CAS) was first identified as a hyperphosphorylated protein in v-Crk and v-Src transformed cells (Kanner et al., 1991; Reynolds et al., 1989). The human gene encoding p130CAS, BCAR1 (breast cancer resistance 1), was identified in a retroviral insertion screen for genes that promote resistance to the antiestrogen tamoxifen (Brinkman et al., 2000). p130CAS is concentrated at focal adhesions (Petch et al., 1995). Following activation of integrin signaling, focal adhesion kinase (FAK) and Src phosphorylate p130CAS at several tyrosine residues, which provide binding sites for the adaptor protein Crk. This leads to activation of the small GTPase RAC, inducing membrane ruffling, cytoskeleton remodeling, and promoting cell migration (Smith et al., 2008; Tachibana et al., 1997; tSakai et al., 1994). p130CAS is essential for Src-induced transformation of primary fibroblasts (Honda et al., 1998). Overexpression of p130CAS in MMTV-HER2/Neu mice results in multifocal mammary tumors with significantly reduced latency (Cabodi et al., 2006). A human breast cancer study showed that tumor levels of p130CAS were inversely correlated with relapse-free survival and overall survival time (Dorssers et al., 2004). Higher p130CAS expression was also detected in metastatic prostate cancer, compared with localized prostate lesions, which correlated with EGFR expression (Fromont et al., 2007).

Ectopic expression of active Src in KM12C cells induces the formation of peripheral adhesion complexes, which are focal adhesion-like structures. Vinculin,
paxillin, FAK and integrins were enriched in these discrete structures at the tips of membrane protrusions. Src induced formation of peripheral adhesions relies on integrin αv, β1, FAK and Erk1/2 signaling cascades (Avizienyte et al., 2004; Avizienyte et al., 2002). Src is also a central mediator in forming podosome/invadopodia structures in a wide range of cells, which play critical roles in cell migration and invasion (Ayala et al., 2008). Podosome/invadopodia are dynamic, actin-rich adhesion structures, critically regulated by Src kinase, and also share common molecular components with focal adhesions, including FAK, vinculin and integrins (Weaver, 2006).

**B. Membrane targeted PTK6 induces formation of peripheral adhesion complexes in PC3 cells**

A variety of data indicate that PTK6 may activate oncogenic signaling when localized at the membrane or within the cytoplasm, while it has a growth inhibitory effect when targeted to the nucleus (Brauer et al., 2011; Brauer et al., 2010; Ie Kim and Lee, 2009; Palka-Hamblin et al., 2010). Therefore, we designed PTK6 constructs to target PTK6 to different cellular compartments in PC3 cells. A myristoylation/ palmitoylation sequence (Palm) from the Src family kinase Lyn was added to the N-terminus of PTK6 for membrane targeting, and an SV40 nuclear localization signal (NLS) was added to the N-terminus of PTK6 for nuclear targeting. Levels of ectopic Myc-tagged PTK6 are relatively equal in PC3 stable cell lines (Fig. 18A). Immunofluorescence using anti-Myc-tag antibody shows that Palm-PTK6-YF is localized in membrane and cytoplasmic compartments, while NLS-PTK6-YF is in the nucleus. Non-targeted PTK6-YF is localized both in the cytoplasm and nucleus (Fig. 18B). Overexpression of Palm-PTK6-YF induces striking formation of focal-adhesion like structures around cell membrane
(Fig. 18C), which are similar to the peripheral adhesion complexes induced by Src in KM12C cells (Avizienyte et al., 2002). This is not observed in vector, PTK6-YF, or NLS-PTK6-YF expressing cells, indicating this phenotype is dependent on PTK6 membrane localization. In addition to the dramatic cell morphology change, these cells also show a scattering phenotype with fewer cell-cell contacts (Fig. 18C).

To determine if formation of peripheral adhesion complexes is dependent on PTK6 kinase activity, cell lines stably expressing Palm-PTK6-WT, PTK6-WT, Palm-PTK6-KM, and PTK6-KM (kinase-dead mutant) were constructed. All cell lines express comparable levels of ectopic PTK6 (Fig. 19A). Peripheral adhesion structures form in PC3 cells expressing Palm-PTK6-YF and Palm-PTK6-WT but not kinase-dead Palm-PTK6-KM, indicating that formation of these adhesion structures depends on both PTK6 kinase activity and membrane localization (Fig. 19B). Since PTK6 is a tyrosine kinase, phospho-tyrosine (PY) signaling was checked by immunofluorescence. Only Palm-PTK6-YF and Palm-PTK6-WT expressing PC3 cells show highly induced phospho-tyrosine signaling in peripheral adhesion complexes around the plasma membrane (Fig. 19C).

C. Integrin and growth factor receptor signaling promotes peripheral adhesion complexes formation

Integrin $\alpha_v$ and $\beta_1$ were reported to be the essential components for the formation of peripheral adhesion complexes induced by active Src in KM12C colon cancer cells (Avizienyte et al., 2004; Avizienyte et al., 2002). We next investigated the role of integrin signaling in PTK6 induced peripheral adhesion formation. Collagen I is a ligand of integrin receptor, and is often used to activate integrin signaling. Palm-PTK6-YF
Fig. 18. Membrane targeted PTK6 induces the formation of peripheral adhesion complexes.

A) Immunoblot analysis of total cell lysates of PC3 cells stably expressing vector, PTK6-YF, NLS-PTK-YF or Palm-PTK6-YF was performed using anti-Myc-tag (PTK6) and α-tubulin antibodies. B) Intracellular localization of exogenous PTK6 was examined by indirect immunofluorescence with anti-Myc-tag antibody. Myc tagged PTK6 immunoreactivity is visualized with FITC (green). Cells were counterstained with DAPI (blue). Size bar denotes 20 μm. C) Peripheral adhesion complexes were induced in Palm-PTK6-YF expressing cells. Phase contrast images of PC3 cells stably expressing vector, PTK6-YF, NLS-PTK-YF or Palm-PTK6-YF are shown. Size bar denotes 50 μm.
A

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kDa
-50
-60

B

PTK6-YF  NLS-YF  Palm-YF
Myc-PTK6  Merge

C

Vec  PTK6-YF  NLS-YF  Palm-YF
Fig. 19. The formation of peripheral adhesion complexes depends on PTK6 kinase activity and membrane localization.

A) Immunoblot analysis of total cell lysates of PC3 cells stably expressing vector, Myc-tagged PTK6-KM, PTK6-WT, PTK6-YF, Palm-PTK6-KM, Palm-PTK6-WT, Palm-PTK6-YF or NLS-PTK-YF was performed with anti-Myc-tag and α-tubulin antibodies.

B) Peripheral adhesion complexes only form in Palm-PTK6-YF or Palm-PTK6-WT expressing cells. Phase contrast images of PC3 cells described in A are shown. Size bar denotes 50 μm.

C) Induced phospho-tyrosine signaling in peripheral adhesion complexes was detected in Palm-PTK6-YF or Palm-PTK6-WT expressing cells. PC3 cells were stained with anti-phospho-tyrosine antibodies and visualized with FITC (green). Cells were counterstained with DAPI (blue). Size bar denotes 50 μm.
expressing PC3 cells are able to form more peripheral adhesions on Collagen I coated chamber slides, compared with non-coated chamber slides. Peripheral adhesions were visualized by anti-phospho-tyrosine immunostaining (Fig. 20A). These data indicate that integrin receptors work upstream of PTK6, and activated integrin signaling promotes PTK6 induced peripheral adhesion formation.

PTK6 is involved with several growth factor receptors including EGFR, Her2, IGF-R1 and Met (Castro and Lange, ; Irie et al., ; Kamalati et al., 2000; Xiang et al., 2008). Therefore we checked whether growth factor stimulation is able to promote the formation of peripheral adhesions. Palm-PTK6-YF expressing cells show diminished phospho-tyrosine signaling in peripheral adhesion complexes after 24-hour serum starvation, which can be reactivated following 1-hour 20% FBS stimulation (Fig. 20B), indicating growth factor receptors work upstream of PTK6 in regulating peripheral adhesion formation.

D. Phospho-p130CAS is enriched in peripheral adhesion complexes

To examine components of Palm-PTK6-YF induced peripheral adhesion complexes, PC3 cells stably expressing Palm-PTK6-YF were stained with antibodies against proteins found in focal adhesion complexes including p130CAS, vinculin, paxillin and FAK (Avizienyte et al., 2002; Petch et al., 1995). As evident by immunofluorescence, p130CAS, vinculin, paxillin and FAK were detected in adhesion complexes induced by Palm-PTK6-YF (Fig. 21A, E, H, I). Tyrosine phosphorylated p130CAS was detected by specific antibodies against tyrosine residues 165 in the substrate domain and 664 in the C-terminal domain of p130CAS. Although p130CAS is mainly localized in the cytoplasm, phospho-p130CAS PY165 and PY664 are highly
Fig. 20. Integrin and growth factor signaling promote the formation of peripheral adhesion complexes.

A) Palm-PTK6-YF expressing PC3 cells form more peripheral adhesions on collagen I coated plates than non-coated plates. PC3 cells stably expressing Palm-PTK6-YF or vector were seeded in collagen I coated or non-coated chamber slides for 24 hours. Cells were stained with anti-phospho-tyrosine antibodies (green), and counterstained with DAPI (blue). Size bar denotes 50 μm. B) 1-hour 20% FBS stimulation after 24-hour serum starvation promotes the formation of peripheral adhesion complexes in Palm-PTK6-YF expressing PC3 cells. Peripheral adhesion complexes were visualized by anti-phospho-tyrosine immunostaining (green). Cells were counterstained with DAPI (blue). Size bar denotes 50 μm.
enriched in the peripheral adhesion complexes (Fig. 21A-C). Palm-PTK6-YF-induced phospho-tyrosine signaling colocalizes with FAK in peripheral adhesion complexes (Fig. 21D-F). Although exogenous Palm-PTK6-YF localized in membrane and cytoplasmic compartments (Fig. 18B), phospho-PTK6 (PY342) is specifically concentrated in peripheral adhesion complexes (Fig. 21G), suggesting some components of peripheral adhesion complexes are important for PTK6 activation.

To test whether endogenous PTK6 is involved in focal adhesion formation, collagen I coated plates were utilized to activate integrin signaling, since the fluorescence intensity on non-coated chamber slides is too weak to distinguish focal adhesions. Phospho-tyrosine signaling, Phospho-PTK6 (PY342) and phospho-p130CAS (PY165) were detected in those focal adhesions induced by collagen I (Fig. 21J-L).

**E. p130CAS is a direct substrate of PTK6**

Since p130CAS was reported as a highly tyrosine phosphorylated protein with a large substrate domain targeted by Src family kinase (Fig. 22A) (Reynolds et al., 1989), we examined the possibility that p130CAS is a direct substrate of PTK6. An in-vitro kinase assay was performed with recombinant human PTK6 and p130CAS protein, which demonstrates that p130CAS can be directly phosphorylated by PTK6 in the presence of ATP (Fig. 22B). Tyrosine residues 165 and 664 of p130CAS were both phosphorylated by PTK6 in vitro (Fig. 22C). Further tandem mass spectrometry (LC/MS/MS) of the phospho-p130CAS peptide revealed that eleven tyrosine residues in the substrate domain are phosphorylated by PTK6 (Fig. 22D).

We also examine the ability of PTK6 to phosphorylate p130CAS in HEK-293 cells. Immunoprecipitation of p130CAS protein followed by anti-phospho-tyrosine
Fig. 21. Phospho-p130CAS, vinculin, paxillin, and FAK are enriched in peripheral adhesion complexes.

A-I) Indirect immunofluorescence of PC3 cells stably expressing Palm-PTK6-YF was performed using anti-p130CAS (A), phospho-p130CAS (PY664) (B), phospho-p130CAS (PY165) (C), phospho-tyrosine (D), FAK (E), phospho-PTK6 (PY342) (G), paxillin (H) and vinculin (I) antibodies. (F) is a merge of (D) and (E). J-L) PC3 cells expressing vector controls were grown at collagen I coated chamber slides, and indirect immunofluorescence was performed using anti-phospho-PTK6 (PY342) (J), phospho-p130CAS (PY165) (K), phospho-tyrosine (L). Cells were counterstained with DAPI (blue). Size bar denotes 20 µm.
immunoblotting shows that tyrosine phosphorylation of both endogenous (bottom band) and exogenous (upper band) p130CAS is induced by active PTK6 (Fig. 22E). A molecular weight upper shift was observed in the phospho-p130CAS band in the presence of PTK6, suggesting that PTK6 is able to phosphorylate p130CAS at multiple tyrosine residues in HEK-293 cells (Fig. 22E).

We performed pull down assays with purified GST-PTK6 fusion proteins to identify the domains of PTK6 involved in its interactions with p130CAS. All PTK6 SH2 domain containing fusion proteins (GST-PTK6FL, GST-SH2, GST-SH2+SH3), but not the SH3 domain alone, were able to pull down p130CAS from HEK-293 cell lysates, suggesting that the PTK6 SH2 domain interacts with p130CAS (Fig. 23A). We next investigated the interaction of PTK6 and p130CAS in SYF cells (Src -/-, Yes -/-, Fyn -/- mouse embryonic fibroblasts). Overexpression of PTK6-WT or PTK6-YF induces the phosphorylation of p130CAS on tyrosine residue 165 (Fig. 23B). Immunoprecipitation of PTK6 followed by anti-p130CAS immunoblotting shows that p130CAS interacts with wild type PTK6 and constitutively active PTK6-YF, while the interaction between p130CAS and kinase dead PTK6-KM is markedly attenuated (Fig. 23B). Although PTK6-KM is kinase-defective, p130CAS might be phosphorylated by other tyrosine kinases including FAK and PYK2 (Astier, Manie et al. 1997; Tachibana, Urano et al. 1997), facilitating PTK6-KM-p130CAS interactions. These data suggest PTK6 kinase activity is important for the interaction between p130CAS and PTK6.

F. p130CAS mediates oncogenic signaling induced by Palm-PTK6-YF

Erk1/2 signaling is critical for the assembly of Src-induced peripheral adhesion complexes in colon cells (Avizienyte et al., 2004). PTK6 has been coupled with
Fig. 22. PTK6 directly phosphorylates p130CAS.
A) A schematic structure of p130CAS protein shows that it contains four discrete domains: an SH3 domain, a substrate domain, a 4-helix bundle (4HB), and an evolutionarily conserved C-terminal domain. B) PTK6 directly phosphorylates p130CAS in an in-vitro kinase assay. Recombinant human PTK6 and human p130CAS were incubated in kinase buffer with or without ATP for 10 minutes at 30°C. Immunoblot analysis was performed using anti-phospho-tyrosine, p130CAS and PTK6 antibodies. The black arrow points at phospho-p130CAS, and the white arrow points at phospho-PTK6. C) Immunoblot analysis of an in-vitro kinase assay as described in B was performed using anti-phospho-tyrosine, phospho-p130CAS (Y165), phospho-p130CAS (Y664), p130CAS and PTK6 antibodies. D) Eleven tyrosine residues of p130CAS phosphorylated by PTK6 were identified by microcapillary liquid chromatography tandem mass spectrometry (LC/MS/MS). No phosphorylation was detected in the Y664DYVHL motif, probably due to low peptide sequence coverage of this tyrosine residue. * Tyrosine residues 165 and 664 were shown to be directly targeted by PTK6 by using phospho-p130CAS Y165 and Y664 antibodies. E) Tyrosine phosphorylation of both endogenous p130CAS and exogenous GST-p130CAS were induced in the presence of active PTK6 in HEK-293 cells. p130CAS was immunoprecipitated from lysates of HEK-293 cells co-expressing GST-p130CAS and PTK6-YF. p130CAS tyrosine phosphorylation was analyzed by immunoblotting with anti-phospho-tyrosine and p130CAS antibodies. The black arrow points at exogenous GST-p130CAS, and the white arrow points at endogenous p130CAS.
**A**

A diagram showing the domain structure of p130CAS, including SH3, Pro, Substrate domain, 4HB, and C-terminal domain.

**B**

A table listing tyrosine sites and their sequences for p130CAS and PTK6.

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**C**

A diagram showing the effect of PTK6 and p130CAS on ATP binding and phosphorylation (PY).

**D**

**E**

A diagram showing the interaction of Myc-PTK6-YF and GST-p130CAS with p130CAS and PTK6.
Fig. 23. PTK6 SH2 domain recognizes phosphorylated tyrosine residues of p130CAS.
A) PTK6 interacts with p130CAS through its SH2 domain. Glutathione Sepharose 4B beads binding with GST fusion proteins were used to pull down p130CAS from HEK-293 cell lysates. Bound p130CAS was analyzed by immunoblotting with anti-p130CAS antibody. 10% of the lysates added to the pull down reaction served as input. B) Interaction between p130CAS and PTK6 depends on tyrosine phosphorylation of p130CAS. PTK6 was immunoprecipitated from lysates of SYF cells expressing vector, PTK6-YF, PTK6-KM or PTK6-WT. p130CAS association was analyzed by immunoblotting with anti-p130CAS and PTK6 antibodies. The black arrow points at the band of IgG heavy chain, and the white arrow points at immunoprecipitated PTK6. SYF cell lysates were analyzed by immunoblotting with anti-p130CAS, phospho-p130CAS (Y165) and PTK6 antibodies as input.
Erk5 in regulating breast cancer cell migration following Met receptor activation (Castro and Lange, ; Ostrander et al., 2007). Therefore, we examined whether MAPK/Erk signaling is induced by Palm-PTK6-YF in PC3 cells. Following 20% fetal bovine serum (FBS) stimulation, increased phosphorylation of p130CAS (Y165) was detected in Palm-PTK6-YF expressing PC3 cells, as well as increased Erk5 activation (Fig. 24A, B). Total Erk5 levels were also upregulated. However, Erk1/2 activation upon FBS stimulation was lower in the Palm-PTK6-YF expressing cells (Fig. 24A, B), perhaps due to a compensatory effect among MAPK family members (Castro and Lange). Immunofluorescence showed induction of phospho-Erk5 but not phospho-Erk1/2 in Palm-PTK6-YF-induced peripheral adhesion complexes after FBS stimulation (Fig. 24C). Immunoblotting also showed higher AKT activation in Palm-PTK6-YF expressing cells (Fig. 24A). Based on these data, we hypothesized that membrane targeted PTK6 phosphorylates the scaffold protein p130CAS, creating multiple docking sites for interacting proteins, and therefore promoting MAPK/Erk5 and AKT signaling in peripheral adhesion complexes. To test this, p130CAS was knocked down by siRNAs in Palm-PTK6-YF expressing PC3 cells (Fig. 24D). Both AKT and Erk5 signaling cascades induced by Palm-PTK6-YF were attenuated after p130CAS knockdown (Fig. 24D). These data supported our hypothesis that p130CAS works as a scaffold protein to convey the downstream signaling induced by membrane targeted PTK6.

G. p130CAS and Erk5, but not FAK, are essential for the formation of peripheral adhesion complexes

To investigate the role of p130CAS in the formation of peripheral adhesion complexes, p130CAS targeted siRNAs were used to knockdown p130CAS expression in
Fig. 24. p130CAS mediates oncogenic signaling induced by Palm-PTK6-YF. A) Increased p130CAS tyrosine phosphorylation, Erk5 and AKT activation were detected in Palm-PTK6-YF expressing PC3 cells following FBS stimulation. PC3 cells stably expressing PTK6-Palm-YF or vector were serum starved for 48 hours and stimulated with 20% FBS for 10, 30 or 60 minutes. Immunoblotting was performed with anti-p130CAS, phospho-p130CAS (Y165), Erk5, phospho-Erk5, Erk1/2, phospho-Erk1/2, AKT, phospho-AKT (Thr308), PTK6, phospho-PTK6 (Y342) and β-catenin antibodies. B) Increased Erk5 activation correlates with decreased Erk1/2 activation upon FBS stimulation in Palm-PTK6-YF expressing cells. Relative band densities from A were quantified with NIH ImageJ Software. The density of phospho-Erk5 and phospho-Erk1/2 was normalized by the density of total Erk5 and total Erk1/2, respectively. C) Increased Erk5 activation in Palm-PTK6-YF expressing PC3 cells upon FBS stimulation was detected by indirect immunofluorescence. PC3 cells expressing vector or Palm-PTK6-YF were serum starved for 48 hours and stimulated with 20% FBS for 10 minutes. Phospho-Erk1/2 immunoreactivity is visualized with FITC (green), while phospho-Erk5 is detected with Rhodamine (red). Cells were counterstained with DAPI (blue). Size bar denotes 50 μm. D) Erk5 and AKT activation was diminished upon knockdown of p130CAS. PTK6-Palm-YF expressing PC3 cells were transfected with p130CAS siRNAs or control siRNAs for 24 hours, and then serum starved for 48 hours and stimulated by 20% FBS for 10, 30 or 60 minutes. Total cell lysates were analyzed by immunoblotting with anti-p130CAS, Erk5, phospho-Erk5, AKT, phospho-AKT (Thr308) (Ser473), β-catenin and α-tubulin antibodies.
PC3 cells. p130CAS protein decreased to 20% of the level of control cells 3 days after transfection (Fig. 25A). Formation of peripheral adhesion complexes induced by Palm-PTK6-YF was disrupted upon p130CAS knockdown, and the cells become round-shaped, compared with fully spread control cells with protrusions (Fig. 25B). Induced phospho-tyrosine signaling in peripheral adhesion complexes also dramatically decreased upon p130CAS knockdown, as evident by anti-phospho-tyrosine immunofluorescence (Fig. 25B). Interestingly, 6 days after siRNA transfection, p130CAS protein was re-expressed to 50% of the level of control cells (Fig. 25A), which restores the ability of Palm-PTK6-YF expressing cells to form peripheral adhesion complexes (Fig. 25B); phospho-tyrosine signaling was again induced (Fig. 25B). These data indicate that formation of peripheral adhesion complexes is reversible, which relies on the scaffold protein p130CAS.

Since Palm-PTK6-YF expression leads to activation of Erk5 in peripheral adhesion complexes (Fig. 24), we examined whether Erk5 signaling is required for the formation of peripheral adhesion complexes. Erk5 was knocked down to 20% of the level of control cells by two different siRNAs (Fig. 25C). Two Erk5 bands were observed in the control siRNA treated Palm-PTK6-YF expressing cells, of which the top band co-migrates with the phospho-Erk5 band shown in the anti-P-Erk5 immunoblotting (Fig. 25C). Knockdown of Erk5 by both siRNAs abolished peripheral adhesion complex formation, as well as phospho-tyrosine signaling induced by Palm-PTK6-YF (Fig. 25D), suggesting Erk5 is also crucial for peripheral adhesion complex formation. While it has been reported that Src-induced formation of peripheral adhesion complexes depends on Src-induced tyrosine phosphorylation of FAK (Avizienyte et al., 2002), knockdown of
Fig. 25. p130CAS and Erk5, but not FAK, are crucial for forming peripheral adhesion complexes.

A) p130CAS protein is knocked down by siRNAs at day 3 and re-expressed at day 6. PC3 cells were transfected with p130CAS siRNAs or control siRNAs and harvested at 3 days or 6 days. Total cell lysates were analyzed by immunoblotting with anti-p130CAS, Myc-tag (PTK6) and β-catenin antibodies. B) Palm-PTK6-YF-induced peripheral adhesion complexes are disrupted upon p130CAS knockdown (si-CAS), and reassembled once p130CAS is re-expressed at day 6. Phase contrast images and indirect immunofluorescence using anti-phospho-tyrosine antibodies are shown. Size bar denotes 50 μm. C) Erk5 protein is knocked down by two Erk5 siRNAs. PC3 cells were transfected with Erk5 siRNAs or control siRNAs and harvested at 3 days. Total cell lysates were analyzed by immunoblotting with anti-Erk5, phospho-Erk5, Myc-tag and β-catenin antibodies. D) Peripheral adhesion complexes are disrupted upon Erk5 knockdown. Phase contrast images and indirect immunofluorescence using anti-phospho-tyrosine antibodies are shown. Size bars denote 50 μm. E) Immunoblot analysis of total cell lysates of PC3 cells described in A was performed with anti-FAK and β-actin antibodies. F) PC3 cells expressing Palm-PTK6-YF were transfected with FAK siRNAs or control siRNAs for 3 days. Cells were stained with anti-phospho-tyrosine antibodies and visualized with FITC (green). Cells were counterstained with DAPI (blue). Size bar denotes 20 μm. Phase contrast images are also shown. Size bar denotes 50 μm.
A

B

C

D

E

F

103
FAK in PC3 cells did not affect formation of peripheral adhesion complexes induced by Palm-PTK6-YF (Fig. 25 E, F).

**H. PTK6 promotes cell migration through p130CAS and Erk5 signaling**

The p130CAS-Crk signaling complex plays important roles in activating Rac and promoting Rac driven cell migration and invasion (Smith et al., 2008). PTK6 has been shown to be coupled with Erk5 in regulating Met mediated cell migration (Castro and Lange, ; Locatelli et al., 2011). We examined whether PTK6 promotes cell migration through phosphorylation of p130CAS and activation of Erk5. Endogenous PTK6 was knocked down in PC3 cells using siRNA against PTK6, and the effect of knockdown lasts up to 4 days after transfection (Fig. 26B). Upon PTK6 knockdown, the ability of PC3 cells to migrate to the bottom side of the membrane is largely impaired (Fig. 26A). This is associated with a substantial reduction of p130CAS phosphorylation and a modest decrease of Erk5 phosphorylation in PTK6 knocked-down cells (Fig. 26B). There is an about 30% decrease of phospho-Erk5/Erk5 ratio in PTK6 knock down cells at day 2 and day 4, respectively (Fig. 26C).

We examined the migratory ability of Palm-PTK6-YF expressing PC3 cells. In wound healing assays, Palm-PTK6-YF expressing cells were able to close the wound at a much faster rate than vector control expressing cells (Fig. 26D). In trans-well chamber assays, these cells also show a 60% increase in the number of migrating cells when 20% FBS containing medium was used as chemoattractant (Fig. 26E). In Palm-PTK6-YF expressing PC3 cells, knockdown of FAK cause a small but significant reduction of cell migration, while knockdown of p130CAS or Erk5 largely impairs cell migratory ability (Fig. 26E), indicating the essential roles of p130CAS and Erk5 in PTK6 mediated cell...
migration. The oncogenic signaling induced by PTK6 which is critical for cell migration is more dependent on p130CAS and Erk5, rather than FAK.
Fig. 26. PTK6 promotes cell migration through p130CAS and Erk5 signaling.

A) PC3 cell migration is diminished upon PTK6 knockdown in Transwell chamber assays using 20% FBS as a chemoattractant. B) Knockdown of PTK6 by siRNA in PC3 cells lasts 4 days. Cells were transfected with control siRNA or PTK6 siRNA for 24 hours, and harvested at 2 days or 4 days after transfection. Total cell lysates were analyzed by immunoblotting with anti-PTK6, p130CAS, phospho-p130CAS (PY165), Erk5, P-Erk5 and β-actin antibodies. C) Knockdown of PTK6 results in decreased Erk5 phosphorylation in PC3 cells. Relative band densities from B were quantified with NIH ImageJ Software. Data were collected from two individual experiments, and standard deviations were shown. D) PC3 cells stably expressing Palm-PTK6-YF show increased migration in wound healing assays. Representative images at 0 and 48 hour are shown. Size bars denote 50 μm. E) Membrane targeted PTK6 promotes cell migration through phosphorylation of p130CAS and activation of Erk5. PC3 cells stably expressing Palm-PTK6-YF show greater cell migratory ability than control cells in Transwell chamber assays using 20% FBS as a chemoattractant. Knockdown of FAK causes a modest decrease, while knockdown of p130CAS or Erk5 results in a substantial reduction of cell migration induced by Palm-PTK6-YF.
5. **PTK6 Regulates Epithelial-Mesenchymal Transition of Prostate Cancer Cells**

**A. Background**

Elevated expression or activation of tyrosine kinases are often associated with epithelial-mesenchymal transition (EMT), in which loss of the epithelial marker E-cadherin and elevation of the mesenchymal marker vimentin are observed (reviewed in (Thiery, 2002; Thiery and Sleeman, 2006). Activated Src kinase induces disorganization of E-cadherin dependent cell-cell contacts and increased vimentin expression in KM12C colon cancer cells. Deregulation of E-cadherin and formation of peripheral adhesions induced by active Src kinase rely on integrin, FAK and Erk1/2 signaling cascades (Avizienyte et al., 2004; Avizienyte et al., 2002).

**B. Membrane targeted PTK6 diminishes E-Cadherin expression in benign prostate hyperplasia and prostate cancer cells**

When PTK6 is targeted to membrane compartments in PC3 cells, in addition to the formation of peripheral adhesion complexes, we also observed the cell-scattering phenotype, where less cell-cell contacts are built. This is also observed in BPH1 cell line, a benign prostatic hyperplasia cell line (Fig. 27A). Compared with control cells, both PC3 and BPH1 cells expressing Palm-PTK6-YF underwent profound morphological changes, most of whom showed ruffled membrane structure instead of a smooth plasma membrane (Fig. 27A). The ectopic expression of palm-YF PTK6 was confirmed by immunoblotting (Fig. 27B).

Cell-scattering phenotype is often coupled with epithelial-mesenchymal transition. Both BPH1 and PC3 cells are prostate epithelial cell lines that express E-cadherin. Thus we next examine whether E-cadherin expression and localization are changed in cells
Fig. 27. Expression of Palm-PTK6-YF induced cell-scattering phenotype in BPH1 and PC3 cells.

A) Cells expressing Palm-PTK6-YF show the cell-scattering phenotype. Phase contrast images of PC3 and BPH1 cells stably expressing Palm-PTK6-YF or vector are shown. Size bar denotes 50 μm. B) Immunoblot analysis of total cell lysates of PC3 and BPH1 cells stably expressing Palm-PTK6-YF or vector was performed using anti-Myc-tag and β-actin antibodies. C) Loss of E-cadherin at the membrane in PC3 cells stably expressing Palm-PTK6-YF. Cells were co-stained with anti-E-cadherin and phospho-tyrosine antibodies, and counterstained with DAPI (blue). Size bar denotes 20 μm. D) BPH1 cells that form peripheral adhesion complexes show deregulated E-cadherin at cell plasma membrane. Cells were co-stained with anti-E-cadherin and phospho-tyrosine antibodies and counterstained with DAPI (blue). Size bar denotes 20 μm.
expressing Palm-PTK6-YF. Interestingly, reduced E-cadherin membrane staining in Palm-PTK6-YF expressing cells is accompanied by induced phospho-tyrosine signaling in peripheral adhesion complexes (Fig. 27C). This phenomenon was observed in BPH1 cells expressing Palm-PTK6-YF as well. Membrane E-cadherin is lost in the pool of cells which form peripheral adhesion complexes, as confirmed by anti-phospho-tyrosine immunofluorescence, while cells that do not form peripheral adhesion complexes still contain E-cadherin at cell-cell contacts (Fig. 27D). These data suggest that cells expressing Palm-PTK6-YF undergo an EMT transition, and the signaling induced in the peripheral adhesion complexes might be involved in deregulation of E-cadherin.

C. **Active PTK6 at plasma membrane promotes EMT in prostate tumor cells**

Loss of E-cadherin is regarded as one of the universal hallmarks in the process of EMT. Consistent with immunostaining data, the immunoblots showed reduced E-cadherin levels in the presence of Palm-PTK6-YF in PC3 cells (Fig. 28A). We also explore the other EMT markers to confirm that cells expressing Palm-PTK6-YF undergo EMT. The expression of mesenchymal marker vimentin and the E-cadherin transcriptional repressor ZEB1 were both increased (Fig. 28A). Active PTK6 primarily localized at membrane compartments as evident by the subcellular fractionation analysis (Fig. 28B). Exogenous expression of Palm-PTK6-YF largely increased the active pool of PTK6 at cell membrane, which leads to decreased pool of E-cadherin at cell membrane, increased pool of ZEB1 in nucleus, and increased pool of vimentin at cytoplasm and membrane (Fig. 28B). mRNA levels of EMT Markers were also measured by either quantitative real-time PCR or semi-quantitative PCR. Our results showed that the expression of E-cadherin mRNA is decreased, while the levels of vimentin and ZEB1
mRNA are both increased (Fig. 28C, D). In addition, the mRNA expression of another E-cadherin transcriptional repressor SLUG is also substantially increased, while the level of Twist, also a key EMT inducer, is not much changed (Fig. 28D).

In a PTEN knockout murine prostate cancer model, we observed a group of tumor cells with highly activated PTK6 at plasma membrane, which can be recognized by either anti-PY or anti-P-PTK6 (PY342) antibody (Fig. 28E, a). Interestingly, the cells with high phosphor-tyrosine signaling shows decreased E-cadherin at plasma membrane and increased E-cadherin endocytosis, suggesting these cells are undergoing EMT in vivo (Fig. 28E, b-d).

D. PTK6 mediated EMT is partially through increased AKT activity

AKT is reported to be crucial regulator of EMT in squamous cell carcinoma lines (Grille et al., 2003). Increased AKT activation was observed in response to 20% FBS activation in Palm-PTK6-YF expressing cells (Zheng et al.). Therefore, we next examine whether AKT and its downstream signaling are involved in PTK6 mediated EMT in PC3 cells. Thr308 and Ser473 phosphorylation on AKT, which are the markers of its activation, were increased in PC3 cells expressing Palm-PTK6-YF (Fig. 29A). This is correlated with the inhibitory phosphorylation of GSK3β, which is a direct target of AKT (Fig. 29A). GSK3β is reported to directly target Snail (SNAI1) to affect its stability and localization, which shares high homology with its family member SLUG (SNAI2) (Zhou et al., 2004). Our results showed that AKT mediated inhibition of GSK3β might also contribute to the stabilization and nuclear location of SLUG in PC3 cells (Fig. 29A, B). To test whether AKT activation is required in PTK6 induced EMT, we used siRNAs against AKT to knockdown endogenous AKT in PC3 cells. Knockdown of AKT
**Fig. 28. Active PTK6 at plasma membrane promotes EMT in prostate tumor cells.**

A) Immunoblot analysis of total cell lysates of PC3 cells stably expressing Palm-PTK6-YF or vector was performed using anti-E-cadherin, Vimentin, ZEB1, Myc-tag and β-catenin antibodies. B) Subcellular fractionation assay was performed using PC3 cells expressing Palm-PTK6-YF or vector, and immunoblot analysis was performed using anti-E-cadherin, Vimentin, ZEB1, P-PTK6 (PY342) and PTK6 antibodies. Both short and long exposures of PTK6 immunoblot are shown. C) mRNA levels of EMT markers are deregulated in Palm-PTK6-YF expressing PC3 cells. Quantitative RT-PCR was performed, and E-cadherin and vimentin mRNA levels were normalized by cyclophilin mRNA levels. D) Semi-quantitative PCR was performed to monitor the change of mRNA levels of EMT markers including E-cadherin, Vimentin, ZEB1, SLUG and Twist. Cyclophilin served as a loading control. E) In a murine prostate tumor model (PTENflox/flox, PB-Cre), a group of prostate tumor cells with highly activated PTK6 at cell plasma membrane show deregulated E-cadherin levels. Immunohistochemistry was performed with anti-PY, P-PTK6 (PY342) and E-cadherin antibodies. The size bar denotes 20 μm.
is only able to partially revert EMT induced by membrane targeted PTK6 (Fig. 29C).
Levels of E-cadherin in Palm-YF expressing cells treated with AKT siRNA are still lower than vector control cells treated with scrambled siRNA, indicating there are other mechanisms involved in addition to AKT signaling (Fig. 29C). We also used siRNAs to knockdown a scaffold protein p130CAS in PC3 cells, which is crucial for AKT activation (Zheng et al., 2012). In the absence of p130CAS, AKT activity is almost annihilated, while total AKT level is not changed (Fig. 29D). Decreased AKT activity is correlated with decreased GSK3β phosphorylation, increased E-cadherin expression and decreased Vimentin level in both Palm-PTK6-YF and vector control cells (Fig. 29D). However, consistent with AKT siRNA experiment, inhibition of AKT activity by p130CAS knockdown only partially rescues E-cadherin downregulation induced by Palm-PTK6-YF (Fig. 29D).

**E. PC3 cells expressing Palm-PTK6-YF are more tumorigenic and invasive**

We examined the tumorigenic and invasive ability of PC3 cells stably expressing Palm-PTK6-YF in vitro and in vivo. Overexpression of Palm-PTK6-YF promotes anchorage independent growth of PC3 cells (Fig. 30A), while not affecting cell proliferation, suggesting that membrane targeted PTK6 promotes the tumorigenicity of PC3 cells independently of activating cell proliferation pathways. Trans-well chamber assays show that PC3 cells expressing Palm-PTK6-YF have a 60% increase in the number of migrating cells (Fig. 30B). In addition, intracardiac injection of PC3 cells expressing Palm-PTK6-YF or vector was performed to monitor cell metastatic ability in immunodeficient SCID mice. In the group injected with Palm-PTK6-YF expressing cells,
Fig. 29. PTK6 mediated EMT is partially through increased AKT activity.

A) Increased AKT signaling in PC3 cells expressing Palm-PTK6-YF. Immunoblot analysis of total cell lysates of PC3 cells stably expressing Palm-PTK6-YF or vector was performed using anti-AKT, P-AKT (Thr308), P-AKT (Ser473), P-GSK3β (Ser9), SLUG PTK6 and β-catenin antibodies. B) Increased nuclear localization of SLUG in PC3 cells stably expressing Palm-PTK6-YF. Cells were stained with anti-SLUG antibody and counterstained with DAPI (blue). Size bar denotes 50 μm. C) Knockdown of AKT partially rescues Palm-PTK6-YF-induced EMT. PC3 cells expressing Palm-PTK6-YF or vector were transfected with AKT siRNAs or control siRNAs for 3 days. Immunoblotting was performed with anti-AKT, E-cadherin, Vimentin, PTK6 and β-catenin antibodies. D) Knockdown of p130CAS partially rescues Palm-PTK6-YF-induced EMT. PC3 cells expressing Palm-PTK6-YF or vector were transfected with p130CAS siRNAs or control siRNAs for 3 days. Immunoblotting was performed with anti-p130CAS, AKT, P-AKT (Thr308), P-GSK3β (Ser9), E-cadherin, Vimentin, Myc-tag and β-catenin antibodies.
3 mice out of 5 were dead after 8 weeks, while the two surviving mice showed dramatic metastases to internal organs including liver, lung and pancreas after 12 weeks (Fig. 30C). H&E staining of liver and lung tissue showed tumors growing inside the normal tissue, and immunohistochemical staining showed the membrane staining of PTK6 in those tumor tissues, confirming the origin of these tumors (Fig. 30D). In the group injected with control cells, only 1 mouse out of 5 was found dead after 8 weeks, and no metastases were detected in internal organs in the 4 surviving mice. To lively monitor in-vivo metastasis, both Palm-PTK6-YF expressing and control PC3 cells were infected with lentivirus carrying a luciferase gene and a GFP gene, purified by GFP flow cytometry, and then intravenously injected into SCID mice. At day 0, Equal number of cells were injected and trapped in mice lung, as shown in the images of dorsal and ventral view (Fig. 30E). After one week, the mice injected with PC3 cells expressing Palm-PTK6-YF showed much higher signals, indicating better survival ability of these tumor cells in harsh conditions, and this leads to increased metastases to internal organs, and probably jaws and mammary glands at day 50 (Fig. 30E). Further histological analysis will be needed to validate these metastases. These data together demonstrate that membrane targeted active PTK6 further promotes the EMT in PC3 cells, and stimulates anchorage independent growth, migration, and metastatic potential in vivo.

F. PC3 cells are less tumorigenic and invasive after knockdown of PTK6

We next tested whether knockdown of endogenous PTK6 in PC3 cells might reverse the EMT and attenuate tumorigenicity of PC3 cells. PTK6 was knocked down by an siRNA approach in PC3 cells, and the effect of knockdown lasted to day 6 after transfection (Fig. 31A). E-cadherin levels are increased upon PTK6 knockdown, while Vimentin and
Fig. 30. PC3 cells expressing Palm-PTK6-YF are more tumorigenic and invasive.

A) Palm-PTK6-YF expressing cells form increased number of colonies in soft agar. Representative images are shown. B) Palm-PTK6-YF expression promotes cell migration in Transwell chamber assays. Representative images are shown. C) Intracardiac injection of PC3 cells expressing Palm-PTK6-YF results in increased metastases to internal organs in immunodeficient SCID mice after 10 weeks. White arrows point at tumors in liver and pancreas. D) H&E staining was performed with lung and liver tumor sections. Black arrows point at tumors. Size bar denotes 100 μm. Immunohistochemistry using anti-PTK6 antibody shows that tumor cells in lung and liver contain membrane staining of PTK6 (Palm-PTK6-YF). Size bar denotes 20 μm. E) Intravenous injection of PC3 cells expressing Palm-PTK6-YF showed increased metastases in SCID mice. Both control and Palm-PTK6-YF expressing cells stably express luciferase. One million cells were injected intravenously at day 0. Mice were monitored under IVIS spectrum imaging system every week until day 50.
ZEB1 expression is decreased (Fig. 31A). In addition, knockdown of PTK6 decreases proliferation (Fig. 31B), colony formation (Fig. 31C) and anchorage independent growth (Fig. 31D) of PC3 cells. Invasion chamber assays were performed to monitor the invasive capacity of PC3 cells. Upon PTK6 knockdown, the ability of PC3 cells to invade through extracellular matrix layer to the bottom side of the membrane is largely decreased (Fig. 31E).

We also perform xenograft study to monitor in-vivo metastasis in SCID mice. Knockdown of PTK6 by siRNA effectively reduce metastatic ability of PC3 cells, compared with control siRNA treated group (Fig. 32). Eight weeks after injection, two mice injected with PC3 cells treated with control siRNAs were dead, probably due to vital metastasis at crucial internal organs. The surviving three mice in this group all show certain levels of bioluminescence, one of which showed obvious metastases at internal organs (Fig. 32, si-Cont). In the group of mice injected with PC3 cells treated with PTK6 siRNAs, only two mice showed low levels of bioluminescence around lung (Fig. 32, si-PTK6). In-vivo mice study revealed a crucial role of PTK6 in metastasis, which is necessary for tumor cells to survive and then attach to metastatic sites.
Fig. 31. PC3 cells are less tumorigenic and invasive after knockdown of PTK6.
A) E-cadherin is increased upon knockdown of PTK6 in PC3 cells. PC3 cells were transfected with PTK6 siRNAs or control siRNAs for 2, 4 or 6 days. Total cell lysates were analyzed by immunoblotting with anti-E-cadherin, ZEB1, PTK6 and β-actin antibodies. B) A growth curve of PC3 cells transfected with PTK6 siRNAs or control siRNAs shows decreased proliferation from day 1 to day 7 after PTK6 knockdown. Relative Light Units (RLU) were measured by CellTiter-Glo Luminescent Cell Viability Assay. C) The number of colonies that form on plates 2 weeks post-plating is decreased upon PTK6 knockdown. Corresponding images are shown below the graph. D) The number of colonies that form in soft agar 3 weeks post-plating is decreased upon PTK6 knockdown. Representative images are shown. E) Cell invasion is impaired upon PTK6 knockdown in Matrigel invasion chamber assays.
Fig. 32. Knockdown of PTK6 in PC3 cells largely reduce metastases in SCID mice. PC3 cells stably expressing luciferase were transfected with PTK6 siRNA or control siRNA twice before injection. One million cells were injected intravenously at day 0. Mice were monitored under IVIS spectrum imaging system every week until day 56.
IV. DISCUSSION

1. PTK6 Positively Regulates AKT Activity

Similar to Src family kinases, the SH3 and SH2 domains of PTK6 play important roles in substrate recognition (Qiu and Miller, 2002, 2004). Src interacts with AKT through its SH3 domain, which recognizes the proline-rich motif (PXXP) located at the AKT C-terminus (Jiang and Qiu, 2003). Like Src, PTK6 associates with AKT through its SH3 domain (Fig. 8D). In addition, we demonstrated that the SH2 domain of PTK6, which recognizes phosphorylated tyrosine, also plays a role in regulating PTK6-AKT interactions. Mutation of AKT tyrosine residues 315 and 326 to phenylalanine impairs association between PTK6 and AKT (Fig. 8A-C), and active PTK6-YF promotes PTK6-AKT association (Fig. 8D). The tyrosine residues targeted by PTK6 are located in the catalytic domain of AKT, and regulated association of PTK6 with AKT through kinase-independent SH3 and/or kinase-dependent SH2 mediated interactions may have an impact on AKT activity.

While phosphorylation of AKT on Ser-473 and Thr-308 is critical for AKT activation, phosphorylation of AKT on tyrosine residues is also becoming recognized as important for modulation of its activities. Both positive and negative roles for tyrosine phosphorylation in regulating AKT activity have been reported (Chen et al., 2001; Conus et al., 2002; Jung et al., 2005; Lodeiro et al., 2009; Zhang et al., 2005). We demonstrate a role for PTK6 in sensitizing SYF cells to a physiological concentration of EGF (0.1 ng/ml) leading to increased AKT activation and cell proliferation. This oncogenic role for PTK6 is kinase-dependent (Fig. 10). These experiments were performed in a background
without the Src, Yes and Fyn kinases, allowing us to delineate the specific roles of PTK6 more clearly. Our data are consistent with a previous publication that showed phosphorylation of AKT on tyrosine residues 315 and 326 contributes to AKT activation (Chen et al., 2001), and the observation that overexpression of PTK6 promotes breast cancer cell growth through stimulating ErbB receptor family signaling and PI3K/AKT signaling (Harvey and Crompton, 2003; Kamalati et al., 2000; Kamalati et al., 1996).

Although PTK6 appears to enhance oncogenic signaling in breast cancer cells, its activities have been correlated with differentiation and stress induced apoptosis in normal tissues. Disruption of the Ptk6 gene led to enhanced proliferation and delayed enterocyte differentiation in the untreated mouse intestine (Haegebarth et al., 2006). In addition, radiation induced apoptosis was impaired in the Ptk6 null mouse (Haegebarth et al., 2009). Both phenotypes were accompanied by an increase in activation of AKT signaling, leading to the hypothesis that PTK6 acts as an inhibitor of AKT in normal tissues. In addition, PTK6 was found to inhibit AKT in PTK6/AKT complexes in unstimulated cultured cells (Zhang et al., 2005). When these studies were initiated, our goal was to determine how PTK6 inhibited AKT activities in mouse tissues and cells. However, although we determined that AKT is a direct PTK6 substrate, we did not discover a kinase-dependent inhibitory role for PTK6 in AKT regulation. In contrast, we found that PTK6 may stimulate AKT activation and cell proliferation in response to physiological levels of growth factors. The underlying mechanisms for PTK6 mediated inhibition of AKT activation may involve kinase independent association/sequestration of AKT by PTK6 that is induced during differentiation or stress. Recent studies that are underway
suggest that PTK6 may also promote activation of a phosphatase that targets activating phosphorylation of AKT (Perekatt and Tyner, unpublished data).

Like several other regulatory proteins, PTK6 functions appear dependent on cellular context, coexpression of other interacting signaling molecules, and its intracellular localization. A correlation between PTK6 and ErbB2 overexpression was revealed in invasive ductal breast carcinomas (Born et al., 2005; Ostrander et al., 2007), and PTK6 increases the ErbB2-induced activation of Ras/MAPK signaling to induce cell proliferation in breast cancers (Xiang et al., 2008). Taken together, our studies suggest that PTK6 may also promote ErbB receptor signaling by enhancing AKT activation in response to physiological levels of growth factors. These studies provide insight about the potential benefits of targeting PTK6 as part of a therapeutic regime to treat different types of cancer that have up-regulated PTK6 expression and activity.

2. Active PTK6 Protects Cells from Anoikis through Activating FAK and AKT

Mutation of tyrosine residue 447 to phenylalanine (PTK6-YF) increases its kinase activity, due to the lack of autoinhibition (Qiu and Miller, 2002). However, the activity of PTK6-YF can still be down-regulated by serum starvation (Fig. 13E). Interestingly, membrane targeted PTK6 (Palm-PTK6-YF) is not down-regulated by serum starvation (Fig. 14A), and represents an authentic constitutively active form of PTK6. This could also explain the active involvement of PTK6 in multiple receptor-signaling pathways including EGFR, HER2, IGF-1R, MET and integrin (Castro and Lange, ; Irie et al., ; Li et al., ; Xiang et al., 2008; Zheng et al.).
Our data show that constitutive activation of PTK6 transforms SYF MEFs, as manifested by their ability to overcome contact inhibition, resistance to anoikis and anchorage independence, probably through constitutive activation of FAK and AKT (Fig. 15). AKT plays a critical role downstream of PTK6 in survival signaling, since Palm-PTK6-YF fails to protect Akt1/2/- MEFs against anoikis, which show greater sensitivity to apoptosis under suspended growth conditions compared with SYF and Fak/- MEFs (Fig. 16). In Fak/- MEFs, overexpression of FAK is able to provide protection for cells lacking signals from extracellular matrix, while co-expression of FAK and Palm-PTK6-YF induces stronger FAK and AKT activation and synergistically promotes cell survival. Expression of Palm-PTK6-YF alone also shows some protective effect, indicating that the constitutively active PTK6 is able to activate survival signaling independent of FAK (Fig. 16). This is probably through directly phosphorylating AKT at tyrosine residues and thereby lowering its sensitivity to growth receptor signaling (Fig. 33) (Zheng et al., 2010).

Resistance to anoikis is often acquired by metastatic cancer cells to avoid death under adverse conditions. Cancer cells escape anoikis primarily through regulating the extrinsic death receptor pathway and the ECM-integrin mediated cell survival pathway [reviewed in (Sakamoto and Kyprianou)]. Different strategies that target various players in integrin/SRC/FAK/AKT survival signaling have shown promising results in inhibiting tumor growth in-vitro and in-vivo [reviewed in (Desgroisellier and Cheresh, ; Sakamoto and Kyprianou)]. In our study, metastatic PC3 prostate cancer cells display the ability to escape from anoikis, which have a 90% viability rate after 8-day suspended growth on poly-HEMA plates. Apoptosis is not induced, but levels of p27 are substantially increased upon detachment (Fig. 17), contributing to cell cycle arrest and promoting cell
Fig. 33. A proposed model shows how PTK6 regulates cell survival.

When cells detached from extracellular matrix (ECM), membrane associated active PTK6 is able to phosphorylate and activate FAK and following AKT survival signaling. PTK6 is also able to protect cells from anoikis independent of FAK, probably through direct phosphorylating AKT and promoting its activation (Zheng et al., 2010).
survival (Wu et al., 2006). Knockdown of PTK6 induces apoptosis under suspended growth conditions, and cell viability decreases to about 20% (Fig. 17). Our research reveals that PTK6 induced FAK phosphorylation and following AKT activation is critical for the survival of cancer cells in the absence of correct extracellular stimuli, and shows the promise of the combinational cancer therapeutics that target integrins and PTK6 in human prostate cancer.

3. Active PTK6 Promotes Cell Migration through Phosphorylating p130CAS and Activating Erk5

We identified p130CAS as a novel substrate of PTK6. Using phosphorylation specific antibodies, we show that PTK6 phosphorylates p130CAS within its substrate domain, which contains several YxxP motifs, as well at the carboxy terminal Y664DYVHL motif. Using mass spectrometry, we identified eleven tyrosine residues within the p130CAS substrate domain that can be phosphorylated by PTK6 in vitro. While no phosphorylation of the YDYVHL motif was detected using LC/MS/MS, probably due to low abundance of YDYVHL containing peptides, we did detect phosphorylation of this motif with a phospho-specific antibody (Fig. 22). In the canonical model, p130CAS is phosphorylated by FAK at the YDYVHL motif upon integrin activation. Then Src kinase binds to the pYDpYVHL motif through its SH2 domain, and further phosphorylates the p130CAS substrate domain, creating binding sites for Crk (Tachibana et al., 1997). Knockdown of FAK expression did not affect formation of peripheral adhesion complexes induced by Palm-PTK6-YF (Fig. 25), and we hypothesize that PTK6 does not rely on FAK to initiate the phosphorylation at the p130CAS C-
terminal YDYVHL motif. PTK6 might directly target the YDYVHL motif and then further phosphorylate the substrate domain, activating downstream signaling of p130CAS.

PTK6 shares only 44% amino acid identity with Src (Serfas and Tyner, 2003). The structure of the PTK6 SH2 and SH3 domains have unique features that distinguish it from Src-family kinases, which may modulate its recognition of interacting proteins and substrates (Ko et al., 2009; Qiu and Miller, 2004). While, the Src SH3 domain is important for its interaction with and phosphorylation of p130CAS (Pellicena and Miller, 2001), PTK6 interacts with p130CAS through its SH2 domain (Fig. 23). Differential phosphorylation of p130CAS tyrosine residues and distinct domain-domain interactions may lead to the formation of distinct scaffolding complexes at the plasma membrane.

Activating integrin signaling by collagen I is able to stimulate the activity of membrane associated PTK6. In addition, growth factor receptors are also involved in activating PTK6 upon ligand binding (Fig. 20). PTK6 participates in different growth factor receptor signaling pathways including EGFR, Her2, IGF-R1 and Met (Castro and Lange, ; Irie et al., ; Kamalati et al., 2000; Xiang et al., 2008). It will be of great interest to further identify specific integrin receptors and growth factor receptors that are involved in PTK6 induced peripheral adhesion formation.

We show that expression of membrane targeted PTK6 induces the formation of peripheral adhesion complexes at the cell periphery, and this depends on PTK6 kinase activity and membrane localization (Fig. 19). We did not observe the rosette ring structure of f-actin bundles in those peripheral structures by rhodamine conjugated
phalloidin staining, which is commonly observed in Src induced podosome/invadopodia (Seals et al., 2005), suggesting this could be a different adhesion structure.

Like Src induced peripheral adhesions in KM12C cells (Avizienyte et al., 2002), peripheral adhesion complexes induced by Palm-PTK6-YF shared the common components of a mature focal adhesion, including paxillin, vinculin and FAK (Fig. 21). FAK and Erk1/2 signaling cascades are important mediators downstream of Src (Avizienyte et al., 2004; Avizienyte et al., 2002). However, here we did not detect roles for FAK and Erk1/2. Instead, we found that p130CAS and Erk5 serve as important regulators of peripheral adhesion complex formation induced by Palm-PTK6-YF. Phospho-p130CAS and phospho-Erk5 are enriched in peripheral adhesions, and knockdown of either protein disrupts the formation of peripheral adhesion complexes (Fig. 24, 25). Importantly, PTK6 can form a complex with Erk5, and Erk5 is required for the migration of breast cancer cells (Castro and Lange, 2010). It is possible that p130CAS serves as a scaffold to form a functional complex with PTK6 and Erk5, since knockdown of p130CAS impairs Erk5 activation (Fig. 24D).

We previously reported that PTK6 directly phosphorylates AKT at two tyrosine residues, 315 and 326, promoting AKT activation in response to epidermal growth factor (Zheng et al., 2010). Consistent with this, overexpression of Palm-PTK6-YF in PC3 cells promotes serum induced AKT activation. Knockdown of p130CAS protein largely impaired serum induced AKT activation (Fig. 24), suggesting that intact p130CAS scaffold complexes are important for activation of AKT signaling. It has been reported that Erk5 is required for AKT activation and VEGF-mediated survival of microvascular
Integrin and growth factor receptor receptors are upstream of PTK6. PTK6 phosphorylates p130CAS near the plasma membrane and then activates Erk5 signaling, inducing formation of peripheral adhesion complexes and promoting migration. PTK6 has been shown to directly target AKT and promote AKT activation (Zheng et al., 2010). Palm-PTK6-YF expressing PC3 cells show increased AKT activation upon FBS stimulation, which can be impaired by knockdown of p130CAS. AKT was reported to promote migratory and invasive ability of squamous carcinoma cells (Grille et al., 2003). Solid line arrows indicate direct regulation, while dotted line arrows represent proposed or indirect regulation.
endothelial cells (Roberts et al., 2010). Activated Erk5 signaling was also found to promote survival of fibroblasts via AKT-dependent inhibition of FoxO3a (Wang, Finegan et al. 2006). Increased AKT signaling might also contribute to cell migration, since AKT has been reported to regulate the epithelial to mesenchymal transition in squamous cell carcinoma lines and promote migration and invasion (Grille, Bellacosa et al. 2003).

Our studies revealed the underlying mechanism how PTK6 induced migration in prostate cancer cells (Fig. 34). PTK6 might be a beneficial target as part of a therapeutic regimen to treat prostate cancer.

4. **Active PTK6 is Involved in Epithelial-Mesenchymal Transition of Prostate Cancer Cells**

Prostate cancer is the most common cancer and second leading cause of cancer-related deaths in American men. Most prostate cancer related deaths are due to advanced disease, resulting from lymphatic, blood, or contiguous local spread, while there is no effective treatment available nowadays. Two most well recognized markers of EMT are loss of E-cadherin and increased expression of vimentin (Thiery, 2002). A significant inverse correlation between prostate tumor grade and E-cadherin expression has been reported. Patients with normal E-cadherin expression have a significantly higher overall survival rate than patients with low expression (Richmond et al., 1997; Umbas et al., 1994).

Deregulation of E-cadherin could involve several PTK6 downstream players AKT, p130CAS and Erk5. AKT plays an important role in regulating EMT in different cancer cell lines (Grille et al., 2003; Larue and Bellacosa, 2005). By inhibitory phosphorylation
of GSK3-beta, AKT is able to stabilize and promote nuclear localization of snail family proteins, therefore repressing expression of E-cadherin (Zhou et al., 2004). Aberrant expression of p130CAS and downregulation of E-cadherin were detected in hepatocellular carcinoma, and this is correlated with cell invasion and poor prognosis (Guo et al., 2008). Knockdown of p130CAS protein partially rescues E-cadherin downregulation induced by Palm-PTK6-YF, which is possibly through impaired AKT activation (Fig. 29D). Rac1 and PAK, downstream targets of p130CAS signaling, have also been shown to disrupt E-cadherin cell-cell adhesion of keratinocytes (Lozano et al., 2008). In addition, a breast cancer cell study showing that EMT phenotype of MCF-7-MEK5 cells depends on activated MEK5/Erk5 signaling, suggests activated Erk5 might also contribute to the EMT (Zhou et al., 2008).

Cells that have undergone the EMT are generally more migratory and invasive. Here we have shown that overexpression of membrane associated PTK6 induces the EMT in PC3 cancer cells, which correlates with increased cell migration and metastases in vivo. In contrast, knockdown of PTK6 restores E-cadherin levels, impairing PC3 cell migration and invasion in vitro (Fig. 30-32). Recent evidence indicates that EMT of tumor cells is also coupled with increased cell survival and drug resistance [Reviewed in (Singh and Settleman, 2010)]. Our studies suggest that targeting PTK6 might be a beneficial part of a therapeutic regimen to treat metastatic prostate cancer.

5. **PTK6 mediated signaling in normal and cancer cells**

PTK6 plays different roles in normal and cancer cells. PTK6 negatively regulates proliferation, promotes differentiation and mediates apoptosis in normal cells from
intestinal tract and skin (Haegebarth et al., 2005; Haegebarth et al., 2009; Llor et al., 1999; Wang et al., 2005), while it promotes proliferation, migration and survival in various cancer cells from breast, colon, ovarian and prostate tumor (Chen et al., 2004; Gierut et al., 2011; Irie et al., 2010; Shen et al., 2008; Zheng et al., 2012; Zheng et al., 2010).

The obviously opposite roles of PTK6 may be resulted from activation of different signaling pathways in normal and cancer cells. In normal cells, PTK6 is induced and activated during differentiation or stress. Ongoing studies suggest that the underlying mechanism of PTK6 mediated proliferation inhibition and differentiation promotion may be through activation of a phosphatase that targets activating phosphorylation of AKT and Erk1/2 (Perekatt and Tyner, unpublished data). In addition, impaired apoptosis is detected in PTK6-deficient mice intestinal cells, which is also accompanied with decreased activation of AKT and Erk1/2 survival signaling (Haegebarth et al., 2009). On the other hand, the expression of PTK6 is significantly induced in various cancer cells, especially breast cancer and prostate cancer (Xiang et al., 2008; Zheng et al., 2012), where high levels of PTK6 predicts poor prognosis of human patients (Fig. 2) (Irie et al., 2010). In addition to elevated expression levels, activation and translocation of PTK6 in prostate cancer may also provide the possibility for PTK6 to phosphorylate and activate its non-nuclear substrates which PTK6 cannot activate in normal tissue (Fig. 3, 4).

To further investigate the mechanisms of PTK6 mediated signaling in normal and cancer cells, it is necessary to identify more novel substrates of PTK6. Dr. Gierut performed a large scale immunoprecipitation to enrich tyrosine phosphorylated proteins in the presence of active PTK6. Mass spectrometry analysis of these proteins gave rise to
a list of potential substrates of PTK6, from which I validated FAK and p130CAS, and revealed their biological functions in the context of PTK6 regulation. It will be beneficial to continue the validation of those candidate substrates of PTK6 and investigate their roles in normal and cancer cells, which will help us to further understand the complexes of PTK6 mediated signaling.

6. Conclusions

In sum, I demonstrated that expression of PTK6 was significantly increased in prostate cancer, especially in metastatic prostate cancer, which predicts low survival and high recurrence rate of human prostate cancer (Fig. 2). I also observed translocation of PTK6 from nucleus to cytoplasm and activation of PTK6 at cell plasma membrane in mice and human prostate cancer. Although majority of PTK6 localized at cytoplasm, active pool of PTK6 is only associated with plasma membrane in several prostate cancer cell lines and tumor tissues (Fig. 3, 4).

I identified several direct substrates of PTK6, including AKT, FAK and p130CAS. Relocation of PTK6 from nucleus to cytoplasm might facilitate the activation of PTK6 by integrin and growth factor receptor signaling, as well as the access to its cytoplasmic substrates such as AKT, FAK, p130CAS, Paxillin, p190RhoGAP and EGFR. PTK6 phosphorylates these substrates and activates different oncogenic signaling, therefore promoting cancer cell proliferation, migration, survival and EMT, contributing to the progression of human prostate cancer.
V. CITED LITERATURE


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