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Targeting Protein Tyrosine Kinase 6 in Cancer

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Abstract

Protein tyrosine kinase 6 (PTK6) is the most well studied member of the PTK6 family of intracellular tyrosine kinases. While it is expressed at highest levels in differentiated cells in the regenerating epithelial linings of the gastrointestinal tract and skin, induction and activation of PTK6 is detected in several cancers, including breast and prostate cancer where high PTK6 expression correlates with worse outcome. PTK6 expression is regulated by hypoxia and cell stress, and its kinase activity is induced by several growth factor receptors implicated in cancer including members of the ERBB family, IGFR1 and MET. Activation of PTK6 at the plasma membrane has been associated with the epithelial mesenchymal transition and tumor metastasis. Several lines of evidence indicate that PTK6 has context dependent functions that depend on cell type, intracellular localization and kinase activation. Systemic disruption of PTK6 has been shown to reduce tumorigenesis in mouse models of breast and prostate cancer, and more recently small molecule inhibitors of PTK6 have exhibited efficacy in inhibiting tumor growth in animal models. Here we review data that suggest targeting PTK6 may have beneficial therapeutic outcomes in some cancers.

INTRODUCTION

Protein tyrosine kinase 6 (PTK6), also referred to as BRK (Breast tumor kinase) and SIK (Src-Related Intestinal Kinase), is a nonreceptor tyrosine kinase distantly related to the SRC family, and its family members include FRK (Fyn-related kinase) and SRMS (SRC-related kinase lacking C-terminal regulatory tyrosine and N-terminal myristoylation sites). PTK6 was first identified in cultured human melanocytes (PTK6) [1], and subsequently cloned from human breast cancer cells (BRK) [2] and normal mouse intestinal epithelial cells (SIK) [3, 4]. FRK (formerly called RAK, BSK, GTK, and IYK) was also initially cloned from breast cancer cells [5, 6] and the HEP3B hepatoma cell line [7], and SRMS was cloned from embryonic mouse neural precursor cells [8]. Members of the PTK6 family generally exhibit a more restricted pattern of expression than SRC family kinases and are largely expressed in epithelial tissues and a variety of solid tumors. Although cloned more than 25 years ago,

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functions of the PTK6 family are still being elucidated. Here we focus on summarizing roles of PTK6 and its potential as a therapeutic target in cancers.

The *PTK6* Gene

The genes encoding PTK6 and SRC family members share a common ancestral gene [9], but *PTK6*, *FRK*, and *SRMS* genes share the same gene structure that is different than the structure of genes encoding *SRC* family members, and this differentiates them as a separate family [10]. The *PTK6* gene maps to human chromosome 20q13.3 [11] and is very tightly linked to *SRMS* [10] (Figure 1A). Because of this tight linkage, any copy number alterations would be expected to impact both *PTK6* and *SRMS*. Both *PTK6* and *SRMS* are deleted in 20q13.33 deletion syndrome, associated with a rare developmental psychiatric disorder [12]. Early studies using comparative genomic hybridization analysis revealed amplification of chromosome 20q13 in breast cancers [13] and increased expression of PTK6 [14]. *PTK6* has been reported to be amplified in ovarian tumors [15] and ERBB2 positive breast cancers [16]. Copy number gains of *PTK6* were recently reported in breast cancer metastases compared with primary tumors and correlated with significantly shorter disease specific survival [17].

PTK6 encodes a 451 amino acid protein that consists of SRC homology 3 (SH3), SRC homology 2 (SH2) and tyrosine kinase catalytic domains [2], as well as an alternatively spliced transcript that encodes a 15 kDa protein termed ALT-PTK6 that lacks kinase activity, and consists of a SH3 domain and unique proline rich C-terminus[18](Figure 1B). ALT-PTK6 was originally detected in the T47D human breast cell line and named λm5 [19], but later found in a variety of human cell lines [18]. ALT-PTK6 expression was detected in normal prostate and prostate tumors, with the PTK6/ALT-PTK6 ratio being higher in the cancer tissues [18]. Normal physiological functions for ALT-PTK6 have not been well defined. However, ALT-PTK6 can compete with full length PTK6 for substrate binding, through their shared SH3 domain sequence, and it can act as a PTK6 inhibitor [18, 20].

PTK6 Protein

PTK6 shares structural features with the SRC family kinases and has a short unique amino terminus followed by a SH3, SH2, and kinase domain. While SRC family members share from 54 – 75% amino acid sequence identity, the three PTK6 family members share only 44 – 45% identity with one another and with members of the SRC family. None of the studied PTK6 family members contains a signal for lipid modification such as myristoylation or palmitoylation, found in SRC family members, and all of them have a conserved tyrosine in the kinase catalytic domain that corresponds to tyrosine residue 419 in SRC that is involved in autophosphorylation and activation (Figure 1C). Both PTK6 and FRK have a conserved carboxy-terminal tyrosine that can be phosphorylated to promote intramolecular interactions that negatively regulate kinase activity. SRMS lacks a C-terminal regulatory tyrosine, as does C-terminal SRC kinase CSK [8] (Figure 1D) and it has been suggested that SRMS may be a CSK-like kinase ([21],reviewed in [22]).

No regulatory role for the unique amino terminus of PTK6 that consists of the first 7 amino acids has been demonstrated [23]. The SH3 domain of PTK6 is important for substrate

recognition but is also involved in inhibition of its activity through intramolecular interactions between a hydrophobic patch of the SH3 domain and the linker region located between SH2 and catalytic domains [23–25]. Unique features of the PTK6 SH2 domain are present in the loop regions; the CD loop of PTK6 is shorter while AB loop is longer than those for canonical SH2 domains, which could influence the rearrangement of the central β-barrel and influence ligand specificity [26, 27]. Mutation of R85 and H126, which are conserved in other kinases, is sufficient to disrupt interactions between the SH2 domain and phosphotyrosine containing peptides, thereby mediating SH2 domain-phosphotyrosine binding [27] (Figure 1B).

The PTK6 SH2 domain can bind phospholipids via a distinct lipid binding groove exposed on the surface of the protein that interacts with phosphoinositols with low specificity for different headgroups. Arginine residues 131 and 136 (Figure 1B) are critical for interaction with lipids and mutating them leads to interruption of protein-lipid association [28]. This interaction may be a regulatory step for PTK6 signaling, since it could promote recruitment of PTK6 to the plasma membrane and prime the protein for interaction with binding partners that contain phosphotyrosine binding sites. It could also lead to recruitment of PTK6 to specific membrane locations and compartmentalize the protein. Lipids could also behave like allosteric regulators of the protein [28]. Protein-protein and protein-lipid interactions may cooperate to regulate PTK6 relocalization in prostate cancer.

The structure of the PTK6 kinase domain resembles other tyrosine kinases. It consists of two lobes, a β-strand rich N-lobe and an α-helix rich C-lobe. The two lobes are connected by a 9 amino acid long hinge region that binds adenine nucleotide with T264 as the gatekeeper [29]. The gatekeeper threonine residue controls the access of small molecules to the hydrophobic pocket deep in the active site and its mutation can stabilize the active kinase conformation [30]. All other kinase structure motifs are present in the PTK6 kinase domain, including the Pi-loop, αC-helix, hinge, HRD-loop and DFG-loop. The catalytic triad consists of K219, D330 and E235 [29]. Solved crystal structures are in DFG-in and αC-helix-out conformation without activating phosphorylation [29, 31]. Mutation of lysine residue 219 that is involved in ATP binding renders the kinase inactive [32].

PTK6 undergoes activating autophosphorylation at tyrosine residues 342 and 351; tyrosine residue 342 corresponds to the activating phosphorylation site conserved in SRC kinases [33] (Figure 1B). Autophosphorylation was also detected at tyrosine residues 13, 61, 66, and 114, although the MS peaks for peptides around these residues were smaller than those around tyrosine residue 342, which is a major autophosphorylation site. A later study indicated that a peptide corresponding to the carboxy terminus of PTK6 containing the terminal regulatory tyrosine residue at position 447 was also a PTK6 substrate [34]. Using phospho-specific antibodies, we found that expression of full length PTK6 in bacteria results in autophosphorylation at tyrosine residues 342 and 447, indicating that PTK6 may undergo both autoactivation and autoinhibition (Figure 2A). Transient transfection of Myc-tagged wild type PTK6 into LNCaP prostate cancer cell line results in PTK6 phosphorylation at both Y342 and Y447, with peak activating phosphorylation at the earlier 24 hour time point (Figure 2B).

Intramolecular SH2 domain binding with phosphorylated Y447 (PY447) in the C-terminal tail leads to inactivation of PTK6 [27, 33]. The SH3 domain also controls the activity of PTK6 through intramolecular interaction with proline rich linker region. It was demonstrated that amino acids R22, W44, N65, and Y66 bind the linker region that forms a compact hairpin structure. Mutation of any of these residues results in increase kinase activity when compared to wild type protein [24]. The long noncoding RNA (lncRNA) *LINK-A* (long intergenic noncoding RNA for kinase activation) binds the PTK6 SH3 and kinase domains and may cause a conformational change that promotes its recruitment to the membrane [35] (Figure 3). The PTK6 SH2 domain binds the consensus sequence phospho-Y-(D/E)-(D/E)-Y [25]. The consensus substrate sequence phosphorylated by PTK6 X-(E/I/L/N)-Y-(D/E)-(D/E) is also rich in acidic amino acid residues [36, 37].

Cancer-Associated Mutations in *PTK6*

Several cancer-associated somatic mutations that may impact PTK6 activity have been identified, and Tsui et al examined effects of some of these on PTK6 activation and downstream substrate phosphorylation [38]. They found mutations that increase PTK6 activity, include L16F found in clear cell renal cell carcinoma, R131L found in gastric cancer, L343F in cutaneous squamous cell carcinoma, and P450L detected in pancreatic cancer. PTK6 mutations V253M found in head and neck squamous carcinoma and N317S in ovarian carcinoma lead to decreased PTK6 activation [38]. The cBioportal TCGA PanCancer Atlas includes 32 studies with 10,967 samples and PTK6 is mutated in 37 patients [39, 40], while the COSMIC database that includes 38256 unique samples lists 246 unique samples with PTK6 mutations [41].

Regulation of *PTK6* Expression

The *PTK6* promoter region overlaps with the tightly linked *SRMS* gene and factors contributing to its epithelial specific expression have not been identified. Because of their tight linkage, we examined possible coregulation of *Ptk6* and *Srms* in the mouse gastrointestinal tract and skin, and found that *Srms* is also expressed in skin, but not in the gastrointestinal tract, and *Srms* expression was not induced in intestines of *Ptk6*−/− mice (W. Bie and A. L. Tyner, unpublished data). Early studies discovered binding sites for NFkB and SP1 in the promoter sequence [42]. The ribosomal S6 protein kinase RSK2 has also been shown to upregulate *PTK6* mRNA expression through activation of the CREB transcription factor [43]. The bioactive lipid sphingosine 1-phosphate induced *PTK6* mRNA expression in thyroid cancer cells [44].

In triple negative breast cancers, *PTK6* transcription is induced by the hypoxia inducible factors HIF-1α and HIF-2α [45]. A glucocorticoid response element was identified near a hypoxia response element and glucocorticoid receptor (GR) binding was demonstrated by chromatin immunoprecipitation. While HIFs can regulate PTK6 independently of GR, GR requires cooperation with HIFs to upregulate PTK6. [46].*PTK6* may be regulated by estrogen receptor-α (ERα) through estradiol signaling [47], and ERα positive cell lines and tissues express higher protein levels of PTK6 [47, 48].

PTK6 expression is induced by a variety of cellular stresses including DNA damage [49, 50], hypoxia, and reactive oxygen species [45]. PTK6 expression is induced in the proliferating progenitor cell compartments of the intestine and skin after DNA-damage generated by different mechanisms, including gamma [49, 50] and UV [51] irradiation and DNA-damaging drugs [50, 52]. In cancer cells, PTK6 expression increased following treatment with radiation and chemotherapeutic agents, including doxorubicin [50], Taxol, 5' fluorouracil [46], and gemcitabine [53]. Treatment of bladder cancer cells with a PAK4 inhibitor also led to induction of PTK6 expression [54]. Induction of PTK6 in colon cancer cells following irradiation or treatment with doxorubicin was at least partially dependent upon p53 [50].

In breast cancer cells, ERBB receptor ligand mediated activation of PTK6 leads to ERK5 and p38 MAPK activation [55]. Regan Anderson et al. showed that PTK6 activates p38 when cellular stress is present, leading to a positive feedback loop where GR is phosphorylated by p38 and induces *PTK6* expression, resulting in even stronger activation of p38 [46].

Expression of *PTK6* is modulated by several microRNAs, including miR-214 [56], miR-93 [57], miR-187 [58], and miR-17 [44]. MicroRNA-214 is located in the intron of *Dynamin-3* gene on chromosome 1q24.3 and is involved in regulation of signaling networks in normal and cancer tissues in context dependent manner [59]. miR-214 is deregulated in osteosarcoma, melanoma, breast, ovarian, cervical, prostate, gastric, and hepatocellular cancers [56, 59, 60]. It was shown that miR-214 directly targets the *PTK6*-3'UTR by binding to potential binding sites located at positions 28–35 and 201–207 downstream of the stop codon, and this leads to decreased PTK6 expression in several prostate cancer cell lines [56].

miR-93 targets *PTK6* directly and was able to improve barrier disfunction that was caused by the proinflammatory cytokines TNFα/INFγ in mouse colonic epithelial cells [57]. The miR-133a cluster is shown to be downregulated in colorectal cancer [61], and one member of the cluster, miR-187, may target *PTK6* directly. Introduction of miR-187 into colon cancer cells led to decreased PTK6 protein levels [58]. miR-17 binds the *PTK6*-3'UTR and reduces PTK6 levels and inhibits the migration of thyroid follicular carcinoma cells. The expression of miR-17 can be regulated by sphingosine-1-phosphate levels [44].

PTK6 expression is also regulated at the level of protein stability. PTK6 binds the molecular chaperone HSP90 in a kinase-independent manner. Treatment of breast cancer cells with the HSP90 inhibitor geldanamycin led to proteosomal degradation of PTK6 but not the related kinase SRC [62]. PTK6 was shown to be ubiquitylated in an oxygen-dependent manner in breast cancer cells, and hypoxia promoted rapid PTK6 protein stabilization prior to induction of PTK6 mRNA expression by HIF-1α [63]. PTK6 can also be targeted for degradation by calpain-1, a calcium regulated cysteine protease that is negatively regulated by ERBB2 [64].

Regulation of PTK6 Activity

PTK6 activity is regulated by phosphorylation on tyrosine residues, and the protein tyrosine phosphatase 1B (PTP1B) negatively regulates PTK6 activity in ovarian cancer cells [65]. PTP1B directly dephosphorylates tyrosine residue 342 in PTK6 inhibiting its kinase activity, while MSI-1436, a small molecule inhibitor of PTP1B promoted PTK6 activation [21]. The tumor suppressor protein PTEN, which has both lipid and protein phosphatase activities, also directly dephosphorylates PTK6 at tyrosine residue 342 to inhibit its oncogenic activities [66]. Loss of PTEN in the mouse prostate leads to activation of PTK6 at the plasma membrane which promotes the epithelial mesenchymal transition (EMT) [67] and prostate tumorigenesis *in vivo* [66].

CSK phosphorylates the carboxy terminal tyrosine in SRC family kinases to induce an inactive conformation, but it does not target PTK6 [33]. The PTK6 family member SRMS shares structural similarity to CSK and different substrate specificity than PTK6 and has been proposed to be the carboxy-terminal PTK6 kinase [21]. However, no *in vivo* studies examining the impact of endogenous SRMS on PTK6 Y447 phosphorylation and kinase activity have been performed, and most SRMS functional studies have relied on tagged overexpressed SRMS protein. PTK6 may also autoregulate itself, as PY447 was detected in bacterially expressed PTK6, and PY447 levels increase with time in mammalian cells ectopically expressing PTK6 (Figure 2).

Ligands for the ERBB family of growth factor receptors activate PTK6. EGF promoted activation of ectopically expressed PTK6 in cell lines, leading to paxillin phosphorylation and Rac1 activation, thereby promoting cell migration and invasion [68]. The ERBB ligand heregulin induced PTK6 activation to positively regulate Rac1, p38 MAPK and ERK5 and cell migration in breast cancer cells [55]. PTK6 promoted activation of AKT induced by physiological levels of EGF in mouse embryonic fibroblasts lacking the SRC-family kinases SRC, YES and FYN [69]. PTK6 kinase activation was promoted by calcium in a keratinocyte differentiation model [70], and osteopontin in breast cancer cells [71].

Intracellular localization plays an important role in regulating PTK6 activities, but mechanisms regulating its intracellular localization are not fully understood. While PTK6 can be found in all cellular compartments, it lacks both membrane and nuclear targeting signals. Targeting active PTK6 YF to either the membrane or nucleus had opposite effects in prostate [67, 72, 73] and colon cancer [74] cells, with membrane-targeted PTK6 being oncogenic and nuclear PTK6 growth suppressive. The SH2 domain of PTK6 may bind to phosphorylated tyrosine residues in membrane associated proteins such as activated growth factor receptors, as well as to membrane lipids, to facilitate its membrane association [28]. In triple negative breast cancer cells, the lncRNA *LINK-A* was found to mediate recruitment of PTK6 to EGFR and transmembrane glycoprotein NMB (GPNMB) in response to HB-EGF stimulation [35].

Nuclear localization of PTK6 is lost in prostate cancer [75], and targeting PTK6 to the nucleus by addition of a nuclear localization signal is growth inhibitory [72, 76]. Inhibition of Crm1/exportin-1 mediated nuclear export did not restore PTK6 nuclear localization [72]. In addition, overexpression of the nuclear RNA-binding proteins SAM68, which interacts

with the SH3 and SH2 domains of PTK6 [25], was not sufficient to promote PTK6 nuclear localization [72]. Recently, the nuclear paraspeckle protein PSPC1 was reported to be able sequester PTK6 in the nucleus of liver cancer cells, to inhibit its cancer promoting activities [77, 78].

PTK6 Substrates and Interacting Proteins

At the plasma membrane, PTK6 acts downstream of several growth factor receptors including members of the ERBB family [16, 32, 79–81], IGFR1 [82], and MET [83, 84] (Figure 3). PTK6 enhanced ERBB3 tyrosine phosphorylation and activation of PI3K and AKT in the presence of EGF [80]. PTK6 can promote epidermal growth factor receptor (EGFR) activation by directly phosphorylating it and inhibiting its downregulation upon activation [81]. Membrane targeted PTK6 also phosphorylates the EGFR pathway substrate Eps8, contributing to oncogenic signaling in breast cancer cells [85].

PTK6 binds and phosphorylates RNA binding proteins SAM68 [25, 79, 86], SLM1 and SLM2 [87]. PTK6-mediated phosphorylation of these proteins inhibits RNA binding, which can affect stability, translation and transport of RNAs [25, 87, 88]. The RNA splicing factor PSF is a substrate of PTK6, and its phosphorylation leads to its relocalization to the cytoplasm and cell cycle arrest [89]. PTK6 also disrupts nuclear localization of kinesin associated protein 3A (KAP3A) through its phosphorylation of tyrosine residues in the KAP3A carboxy terminus [90].

PTK6 phosphorylates several transcription factors including STAT3 [91], STAT5b [92], β-catenin [74], HIF-1α [35] and parafibromin [93]. PTK6 phosphorylates STAT3 and STAT5b to promote their activation and proliferation of breast cancer cells [91, 92]. In *Ptk6* knockout mice, STAT3 activation is impaired in colon [52] and breast cancer [94] models. PTK6 phosphorylates β-catenin at several different tyrosine residues, but the effect it has is dependent on PTK6 intracellular localization [74]. Nuclear targeted PTK6 inhibits β-catenin/TCF transcriptional activity, while PTK6 phosphorylation of β-catenin at the plasma membrane enhances Wnt signaling [74]. HB-EGF promotes PTK6 activation and phosphorylation of HIF-1α on conserved tyrosine residue 565, which inhibits proline hydroxylation and stabilizes HIF-1α under normoxia [35]. Parafibromin, encoded by the HRPT2 gene and part of the RNA-polymerase-associated factor complex, is phosphorylated by PTK6, which blocks its scaffolding functions and interactions with β-catenin and Gli-1 [95]. PTK6-mediated phosphorylation of parafibromin leads to selective activation of YAP and transcription of TEAD target genes [93].

PTK6 binds and phosphorylates several adaptor proteins including STAP2 [96, 97], paxillin [68], BCAR1 [98], and Dok1 [34, 99]. STAP-2 interacts with STAT3 and STAT5b and modulates their activity through its PH domain. Knockdown of STAP-2 disrupts PTK6 activation of STAT3 and STAT5 [97, 100, 101]. Ectopically expressed PTK6 promoted phosphorylation of paxillin, a molecular scaffold protein localized at the plasma membrane leading to activation of Rac1 to promote cell migration and activation [68].

The Cdk-Cyclin assembly factor/inhibitor p27Kip1 is a protein substrate of PTK6. The SH3 domain of PTK6 binds to p27 and PTK6 phosphorylates p27 at tyrosine residue 88, thereby

promoting activation of the CDK4-Cyclin complex [20]. PTK6-mediated phosphorylation of p27 can be inhibited by ectopic expression of the alternative PTK6 splice product ALT-PTK6, which competes for p27 binding with the full-length PTK6 [18, 20]. It has been suggested that inhibition of PTK6-mediated p27 tyrosine phosphorylation may have advantages in breast cancer patients sensitive to CDK4/6 inhibition [102].

Membrane targeted active PTK6 promotes ERK1/2 activation [103]. This could occur through growth factor receptor activation (i.e. EGFR) [81]. Alternatively, PTK6 binds and phosphorylates p190RhoGAP to promote inactivation of RHO and activation of RAS (Figure 3) contributing to proliferation and cell migration [104]. Cisplatin treatment of testicular cancer was reported to epigenetically impact the “PTK6 -> RHO GTPase ->RAS GTPase -> MAPK” reactome [105].

In several instances, activities of PTK6 are kinase-independent and may be attributed to adaptor/scaffolding functions mediated by its SH3 and SH2 domains. Association with certain substrates such as SAM68 and ERBB3 does not require kinase activity [25, 80, 106]. While the PTK6 SH2 and SH3 domains regulate its association with IRS-4, a member of the insulin receptor substrate family, IRS-4 did not appear to be a PTK6 substrate [107]. Kinase-dead PTK6 was shown to promote proliferation of the T47D breast cancer cell line [108]. PTK6 kinase activity was not required for PTK6-dependent HGF induced cell migration or for PTK6-ERK5 interaction and ERK5 activation [83]. PTK6 was able to promote epithelial characteristics in colon cancer cell lines independent of kinase activity [109]. These data suggest that PTK6 has both kinase-dependent and -independent functions in cancer.

PTK6 in Normal Tissues

PTK6 is expressed in differentiated epithelial cells of the gastrointestinal tract [3, 4, 110–112], skin [51, 70, 113], and prostate [66, 75, 114]. PTK6 was detected in nuclei of normal prostate epithelial cells [75], and in T cells upon activation [115]. PTK6 expression was not detected in the normal mouse mammary gland [110, 116], but was detected in noncancerous human breast biopsy tissues [117]. Expression of an inducible PTK6 transgene in mammary glands of mice forced to wean litters led to delayed involution and activation of p38 MAPK signaling [116].

Expression of *Ptk6* is developmentally regulated and was first detected late in gestation at mouse embryonic day 15.5 in the differentiating suprabasal layers of the skin, and later in the small intestine as the crypt-villus axis develops near the end of gestation [4]. PTK6 was activated in a calcium induced mouse embryonic keratinocyte differentiation model, and its expression promoted keratinocyte differentiation marker expression [70].

PTK6 promotes epithelial cell differentiation during normal turnover of the intestinal epithelium. Systemic disruption of the *Ptk6* gene led to increased epithelial proliferation and impaired differentiation in the mouse small intestine [112]. This was correlated with increased β-catenin activation and MYC expression in intestinal crypts. Later studies demonstrated that disruption of *Ptk6* led to increased expression of a β-catenin regulated LacZ reporter transgene in the mouse intestine [74], suggesting that PTK6 inhibits β-catenin transcription in the intestine during normal homeostasis. In addition, studies with cultured

Ptk6 null young adult mouse colon cells suggested that PTK6 may play roles in regulating epithelial barrier function [57, 118].

Although primarily studied in epithelial cells, PTK6 expression has been reported in normal activated T cells [115] and endothelial cells [119]. PTK6 expression was not detected in normal resting peripheral blood mononuclear cells (PBMCs). Stimulation of PBMCs with the mitogen phytohemagglutinin or CD3/CD28, which promote T-cell activation, induced expression and activation of PTK6 [115]. PTK6 was detected in normal primary mouse microvascular endothelial cell cultures, where it was shown to contribute to TNFα induced barrier dysfunction [119].

PTK6 sensitized nontransformed Rat1a fibroblasts to inducers of apoptosis such as starvation and UV irradiation [120]. In the mouse, DNA-damage induced by total body irradiation led to induction of PTK6 expression in proliferating progenitor cells of intestinal crypts. Systemic disruption of *Ptk6* impaired DNA-damage induced apoptosis in the small intestine [49]. PTK6 expression was also induced in the normal colon by the carcinogen azoxymethane [52].

PTK6 in Cancer

Breast Cancer: PTK6 has been most well studied in breast cancer and increased expression has been identified in the majority of breast cancers including ER positive, ERBB2 positive and triple negative breast cancers [16, 48, 55, 82, 121–123] reviewed in [124, 125]. PTK6 expression has been shown to contribute to breast cancer cell migration, invasion and metastases [45, 63, 83, 126]. Several studies indicate PTK6 promotes breast cancer cell survival. PTK6 regulates IGF-1 mediated anoikis resistance in breast and ovarian cancer cell lines [82]. Knockdown of PTK6 promoted apoptosis of Lapatinib-resistant ERBB2 positive breast cancer cells [127]. PTK6 may also protect breast cancer cells from autophagic cell death [128].

While PTK6 can be detected at low levels in normal human breast tissue, no signal for active PTK6 phosphorylated at tyrosine residue 342 (PY342) could be detected in the normal mammary gland [117]. The pool of active PTK6 is at the plasma membrane in breast cancer cells, but a small percentage of the active protein is also found in the cytoplasm [117]. PTK6 expression was induced in all mammary gland tumors originating in mice, including spontaneous tumors and those promoted by activated ERBB2 and H-Ras [129].

Mammary gland specific transgenic expression of PTK6 promoted tumorigenesis in transgenic mice [116, 129]. PTK6 expression also enhanced MET driven mammary gland tumorigenesis in transgenic mice [45]. Systemic disruption of *Ptk6* in mice expressing activated ERBB2 in the mammary gland prolonged survival and delayed tumor formation and reduced lung metastases [94]. PTK6 can promote mammary gland tumorigenesis through activation of STAT3 [91] and STAT5b [92]. Reduced STAT3 activation was detected in *Ptk6*−/− ERBB2 positive mouse mammary glands [94].

Prostate Cancer: High PTK6 mRNA levels correlate with poor prognosis in prostate cancer, and recurrent and androgen independent prostate cancers have higher levels of PTK6

mRNA when compared to normal or adjacent tissue [67, 130]. PTK6 is expressed in normal prostate tissue and its localization is generally nuclear [75]. In prostate cancers, PTK6 is localized in the cytoplasm [75], and active PTK6 is found at the membrane [66, 67, 75]. Targeted expression of PTK6 to the nucleus inhibited cell proliferation [72]. Translocation of PTK6 from the nuclear to cytoplasmic compartment allows it to phosphorylate substrates involved in promoting cell proliferation, survival and migration [37]. Membrane localized active PTK6 promoted the EMT and prostate tumor cell metastasis in xenograft models [67].

Systemic disruption of *Ptk6* in mice with conditional disruption of *Pten* in the prostate led to decreased development of invasive prostate adenocarcinomas and urethral carcinomas [66]. An inverse correlation between PTEN expression and PTK6 activation and expression was demonstrated in patient samples in a prostate tumor tissue microarray. In addition, higher *PTK6*expression coincided with decreased *PTEN* expression in different prostate cancer datasets including TCGA.

Loss of the tumor suppressor PTEN in prostate cancers leads to PTK6 activation, since PTEN negatively regulates PTK6 activity by dephosphorylating it at Y342 [66]. PTK6 can then directly phosphorylate BCAR1 (p130CAS) [98] as well as FAK [73] and AKT [69], thereby promoting their activation in prostate cancer cells [73]. Membrane associated PTK6 promoted resistance to anoikis and cell survival through enhanced activation of AKT in prostate cancer cells [73].

Colon Cancer: PTK6 is expressed throughout the normal human gastrointestinal tract. Early work showed that *PTK6* RNA expression was slightly increased in a small sample of colon tumors compared with adjacent normal tissue when normalized to keratin expression [110]. However, later studies which included tumor tissue microarray staining, showed that PTK6 expression is highest in normal differentiated colon epithelial cells and decreases during colon tumor progression [109]. In addition, analysis of several datasets including TCGA Colorectal Cancer do not reveal increased PTK6 expression in colorectal cancers [109].

Active PTK6 PY342 is not readily detected in human colon cancer cell lines, where PTK6 appears to function in maintaining the epithelial phenotype independent of PTK6 kinase activity, similar to its role the normal intestinal tract. PTK6 was shown to suppress tumorigenesis by opposing the EMT in colon cancer cell line models [109]. While PTK6 is most often reported as being oncogenic, it has been shown to have tumor suppressive roles in a few cancers including esophageal cancer [131, 132] and laryngeal squamous cell carcinomas [133, 134].

Systemic disruption of the *Ptk6* gene did not lead to spontaneous tumor development in the gastrointestinal tract [112]. However, impaired tumorigenesis was detected in *Ptk6*−/− mice in an azoxymethane/dextran sodium sulfate mouse model of colon cancer. This was initially surprising since PTK6 has a growth inhibitory role during normal homeostasis in the normal intestine, and we had predicted that disruption of *Ptk6* might lead to increased tumor formation [112]. However, in the azoxymethane model, carcinogen driven induction of PTK6 promoted STAT3 activation in the crypts to promote survival and proliferation of

damaged cells and colon tumorigenesis [52]. PTK6 promoted resistance to DNA-damage induced apoptosis through activating STAT3 in mice and in human colon cancer cell lines [50, 52].

Other Cancers: PTK6 is not expressed in normal ovarian tissue but is detected in ovarian cancer tissues and cell lines [15, 65]. mRNA and protein levels of PTK6 are higher in nonsmall cell lung cancers (NSCLC) than in normal tissues [135, 136], and increased *PTK6* mRNA levels are associated with decreased survival [136]. PTK6 is expressed in a number of other tumor types including skin [51], head and neck [111, 137], thyroid [44, 138], pancreatic [139], hepatocellular [77, 140], cervical [141] and bladder [142] [54] cancers. Systemic disruption of *Ptk6* reduced skin tumor formation caused by UV-radiation in mice [51]. One study reported PTK6 expression in cutaneous T-cell lymphomas and transformed B and T cells, and knockdown of PTK6 led to increased apoptosis and reduced proliferation in the murine BaF3 lymphocyte cell line [115]. Ectopic expression of wild type but not kinase dead PTK6 restored proliferation of BaF3 cells following withdrawal of IL-3 [115]. Several earlier comprehensive reviews go into depth about the roles of PTK6 in signaling in cancer [37, 84, 106, 114, 124, 125].

Targeting PTK6 in Cancer

Increased PTK6 expression has been reported in several different types of cancer, and often patients with high levels of PTK6 expression exhibit reduced periods of disease remission and survival. No PTK6 specific inhibitors are currently used in the clinic. However, multiple compounds have been identified that display activity against PTK6 and these are summarized in Table 1.

The ability of vemurafenib and the structurally related compound PLX4720 to inhibit PTK6 has been demonstrated *in vitro* and *in vivo* [103]. Both vemurafenib and PLX4720 were developed as small molecule inhibitors of BRAF V600E, but they also inhibit PTK6 as an off-target [143, 144]. Using a quantitative competitive binding assay, Vin et. al. calculated IC50s for a panel of kinases and showed that PTK6 is inhibited by vemurafenib and PLX4720 with efficacy comparable to inhibition of BRAF V600E [145]. Calculated IC50s for vemurafenib were 64.78 nM for BRAF(V600E) and 68.04 nM PTK6. The calculated IC50s for PLX4720 were 32.04 nM for BRAF(V600E) and 30.38 nM for PTK6. Vemurafenib inhibited PTK6 activity and downstream signaling in prostate cancer cell lines, and treatment with PLX4720 reduced xenograft prostate tumor growth in mice [103]. Vemurafenib targets the PTK6 catalytic domain and interacts with PTK6 through sulfonamide and propyl groups with minimal interaction with azaindole part of the molecule [103].

Jiang et al. performed high-throughput screening of a compound library designed to target ATP binding sites of kinases and identified and further developed a novel small molecule XMU-MP-2 that acts as a potent inhibitor of PTK6 with low toxicity [146]. XMu-MP-2 exhibited an IC50 of 29.7 nmol/L in BRK transformed Ba/F3 cells and was shown to bind PTK6 and inhibit its activity. The drug suppressed growth of tumors induced by PTK6

transformed cells in xenograft models and showed synergistic effects with a type I ERBB2 inhibitor and tamoxifen [146].

Qiu et al. identified novel small molecule Type I and Type II PTK6 inhibitors that were used to inhibit PTK6 in breast cancer cell lines with endogenous PTK6 or engineered to overexpress wild type PTK6, and it was concluded that PTK6 inhibition did not significantly impact tumor cell growth [147]. A caveat with the cell culture experiments is that only cell lines with very low endogenous PTK6 activity were targeted in vitro; PTK6 activation was not detected by immunoblotting and cells were not stimulated to promote kinase activation prior to the treatment with inhibitors. In addition, the influence of possible inhibitory phosphorylation at the terminal carboxy tyrosine residue was not taken into account in the experiments with ectopic overexpression of wild type PTK6 (Figure 2B). PTK6 expression does not correlate with its activation, and it is important to take contributions of environmental cues and signaling pathways that activate PTK6 into consideration when designing assays to test the efficacy of PTK6 inhibitors.

Dasatinib is a small molecule inhibitor of BCR-ABL and SRC family tyrosine kinases used for treatment of several types of leukemias [148, 149]. PTK6 is one of the off-target tyrosine kinases inhibited by dasatinib [150]. Using *in vitro* kinase assays, Anastassiadis et al. demonstrated that dasatinib reduces PTK6 activity to 4.7% when normalized to the uninhibited reaction [151]. Dasatinib binds to the PTK6 ATP binding pocket [31] in manner similar to how it interacts with ABL [152], BMX [153], and BTK [154]. Lee and colleagues performed chemical library screens to identify potential PTK6 inhibitors [155, 156] and determined that the SRC-family inhibitors PP1 and PP2, and a LCK inhibitor have some selectivity against PTK6 [157].

Several natural products and their derivatives have been described in literature as potential PTK6 inhibitors, but mechanisms of action and efficacy *in vivo* have not been extensively studied. It was shown that treating breast cancer cell line MDA-MB-231 with *Hwanggeumchal sorghum* leads to downregulation of PTK6 expression, although the mechanism was not elucidated [158].

Novel synthesized 4-anilino-α-carboline derivatives were screened *in silico* as potential PTK6 inhibitors. Molecular docking was performed with PTK6 homology model and IC50 were determined for all the analogues [159], and two derivatives, MK138 and MK150 were used for further *in vitro* studies. Two derivatives were shown to target PTK6 and reduce activation of STAT3 in cell lines [160]. Bioactive sipholane triterpene from Red Sea sponge *Callyspongia siphonellai* was used as a starting point for development of novel molecules that could potentially exibit inhibitory effect on some enzymes. Ester derivatives showed the best results *in vitro* so they were used in KINOMEscan where PTK6 was detected as a major target among 451 human protein kinases. Derivatives of this molecule reduce invasiveness and migration of MDA-MB-231 cells [161].

Some compounds have been shown to promote a decrease in activating Y342 phosphorylation or an increase in inhibitory Y447 phosphorylation. Marine natural products were used as a starting material for synthesis of phenylmethylene hydantoins which

inhibited PTK6 phosphorylation in MDA-MB-231 cell lines and in a murine xenograft model [162]. Oleanolic acid derivatives that inhibited breast cancer cell line migration and invasion also inhibited PTK6 phosphorylation [163]. These last compounds require further characterization to understand their value because specific phosphorylation sites of PTK6 that were affected were not identified and PTK6 kinase activity was not measured. Using a deep phosphotyrosine proteomics approach Abe et. al. reported increase in Y447 phosphorylation upon treatment with erlotinib [164].

PTK6 expression is induced by chemotherapeutic agents and radiation, and it may be particularly useful to target PTK6 when administering combination chemotherapy. Induction of PTK6 was important for survival of breast cancer cells following Taxol treatment [46]. Treatment of pancreatic cancer cell lines with gemcitabine led to induction of PTK6 [53]. The PTK6 inhibitor XMU-MP-2 synergized with tamoxifen inhibiting growth of ER positive MCF7 cells grown in the absence of estrogen [146]. PTK6 expression/activation was found to be part of the kinome reprogramming that occurred with acquisition of lapatinib resistance in ERBB2 positive breast cancer cells [165]. PTK6 expression increased in bladder cancer cells that were treated with a PAK4 inhibitor and targeting both PTK6 and PAK4 reduced viability of the bladder cancer cells better than targeting either kinase alone [54].

SUMMARY

PTK6 has been described as an “effective collaborative oncogene” [82]. It is capable of promoting oncogenic signaling from a variety of growth factor receptors including members of the ERBB family, IGF1R, and MET. In addition, PTK6 regulates a number of intracellular signaling pathways and substrates (see Figure 3), which can be differentially impacted by cell type specific factors and intracellular localization of the PTK6.

A variety of data indicates that targeting PTK6 may have therapeutic benefits in specific cancers, particularly those that express high levels of PTK6 and/or maintain PTK6 activation at the plasma membrane. These would include cancers with amplification of specific growth factor receptors or loss of inhibitors such as the tumor suppressor PTEN. In addition, PTK6 expression and activity may be induced by cancer treatment regimens. Multiple genetic studies have demonstrated instrumental roles for PTK6 in breast and prostate cancer. Genetic ablation of *Ptk6* has been shown to impact breast, prostate, skin, and colon tumorigenesis *in vivo*. In normal tissues where PTK6 is expressed at highest levels, such as the intestine and skin, PTK6 induction in progenitor cells following DNA-damage may also promote tumorigenesis by enhancing cell survival. PTK6 activation can be regulated by environmental signals, intracellular substrates and associated proteins, including other kinases and phosphatases, and intracellular localization. It is important to take into account the activation status, localization and accessibility of PTK6 when evaluating the functionality of novel PTK6 inhibitors in cancer cells and tumor tissues.

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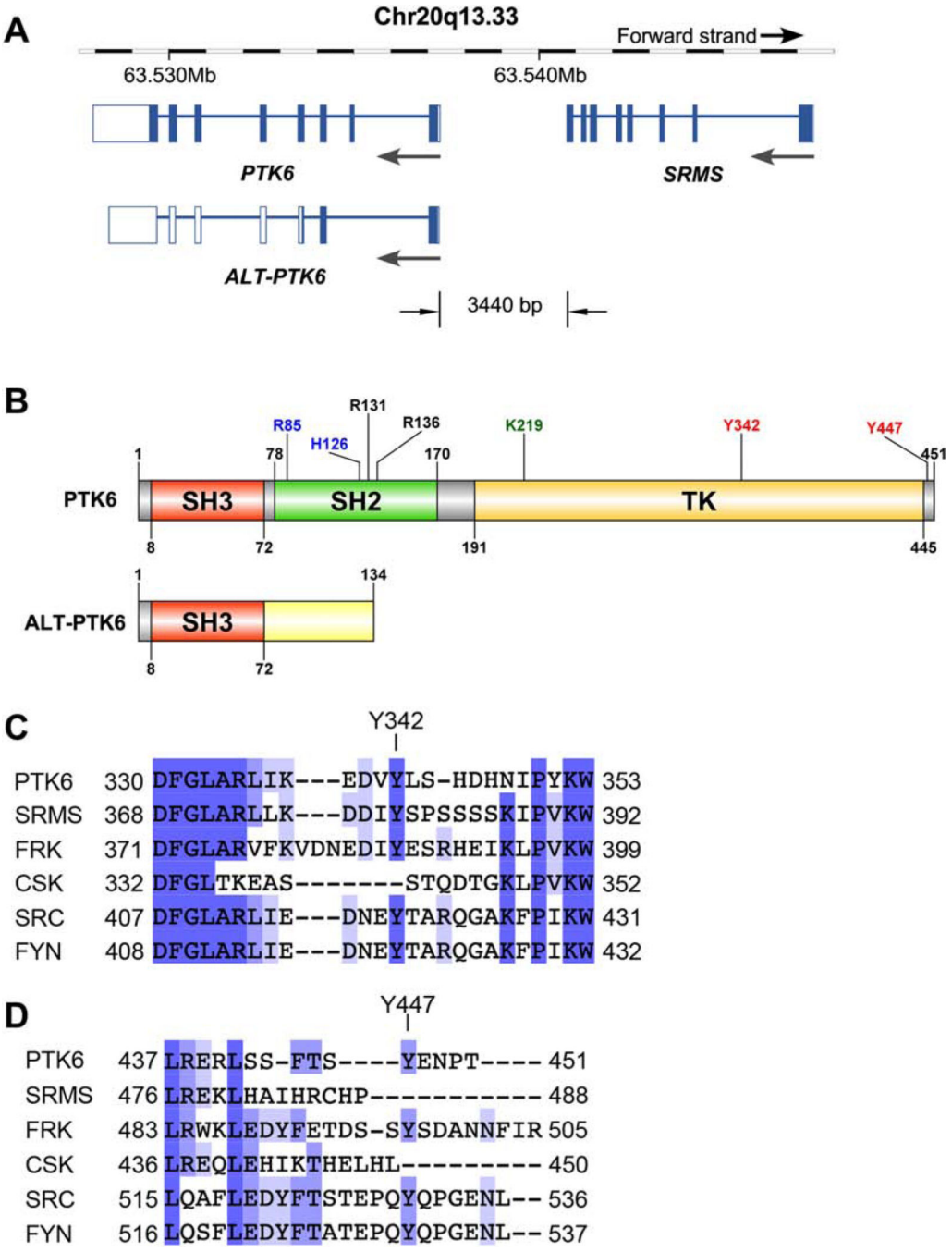

Figure 1. The *PTK6* gene and PTK6 proteins.

A) Organization of *PTK6* and *SRMS* genes on human chromosome 20q13.33. Exons are represented by rectangles, with protein coding in blue, while introns are represented by a line. The distance between *PTK6* and *SRMS* genes is 3440 bp. Information on genes was retrieved from Ensembl [166]. **B)** Structure and key amino acid residues in PTK6. Full length PTK6 contains conserved phosphorylation sites that correspond with its activation (Y342) and inhibition (Y447). In addition, sequences have been identified in the SH2 domain that mediate phospho-tyrosine (R85, H126) and lipid binding (R131, R136) [28].

Mutation of K219 in the ATP binding site abolishes kinase activity [32]. The schematic protein diagram was drawn using DOG 2.0 software [167]. **C and D)** Sequences surrounding the activating (Y342) **(C)** and inhibitory (Y447) **(D)** regulatory phosphorylation sites in human PTK6 are not well conserved, allowing for the production and use of phospho-specific antibodies with specificity for PTK6 PY342 or PTK6 PY447 (see Figure 2). However, specificity of commercially available anti-PY342 and PY447 PTK6 polyclonal antibodies may vary from lot to lot and each lot should be validated. Primary sequences were aligned using Clustal Omega [168] and visualized in Jalview [169].

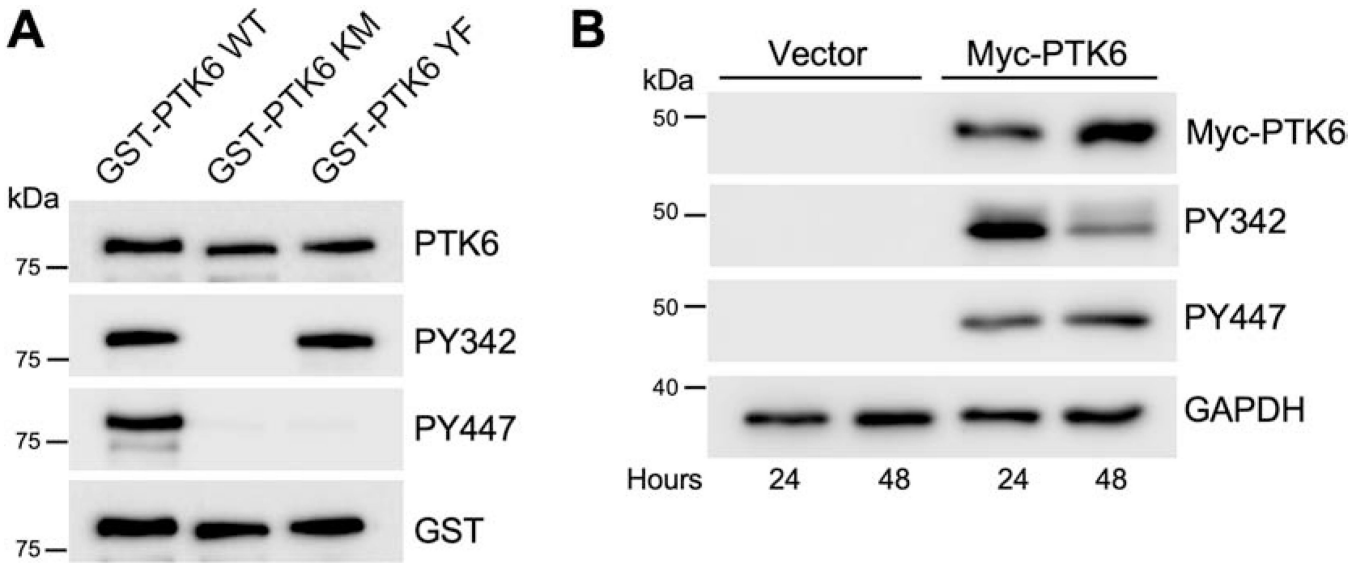

Figure 2. Autophosphorylation of PTK6 at the regulatory tyrosine residues Y342 and Y447.

A) The cDNAs encoding wild type, kinase dead (KM) and active (YF) PTK6 were cloned into the pGEX-KG vector and expressed as GST fusion proteins in the Novagen Rosetta(DE3)pLysS bacterial strain (Millipore Sigma, Burlington, MA) at 16°C in the presence of 1 mM IPTG for 16 hours. Proteins were purified using glutathione sepharose beads and subjected to immunoblotting as previously described [69]. Anti-GST antibodies (#2622) were purchased from Cell Signaling Technology (Danvers, MA). Mouse monoclonal anti-human PTK6 (G6, sc-166171) was purchased from Santa Cruz Biotechnology (Dallas, TX). Rabbit polyclonal antibodies against the PTK6 activating phosphorylation site PY342 (09–144) were purchased from Millipore Sigma. Rabbit polyclonal antibodies against PTK6 phospho-Y447 (ab138368) were purchased from abcam (Cambridge, MA). Expression of wild type PTK6 (WT) in bacteria leads to autophosphorylation of PTK6 at residues Y342 and Y447. The constitutively active PTK6 mutant PTK6 YF contains a mutation of the negative regulatory tyrosine at amino acid position 447 to phenylalanine and we detect phosphorylation only at Y342. Catalytically dead PTK6 KM lacks kinase activity and no tyrosine phosphorylation can be detected, underscoring the requirement for kinase active PTK6 for PTK6 Y342 and Y447 phosphorylation. **B)** Transfection of Myc-tagged wild type PTK6 into the LNCaP prostate cancer cell line (ATCC CRL-1740) leads to peak activation (PY342) of ectopic PTK6 at 24 hours post transfection. While expression of Myc-tagged wild type PTK6 increases at 48 hours post transfection, activating phosphorylation (PY342) decreases and inhibitory phosphorylation (PY447) increases or is maintained. Cells were harvested 24 and 48 hours post transfection, and cells were stimulated with fresh media 10 minutes prior to lysis. GAPDH (14C10, #2118), and Myc-tag (9B11, #2276) antibodies were purchased from Cell Signaling Technology.

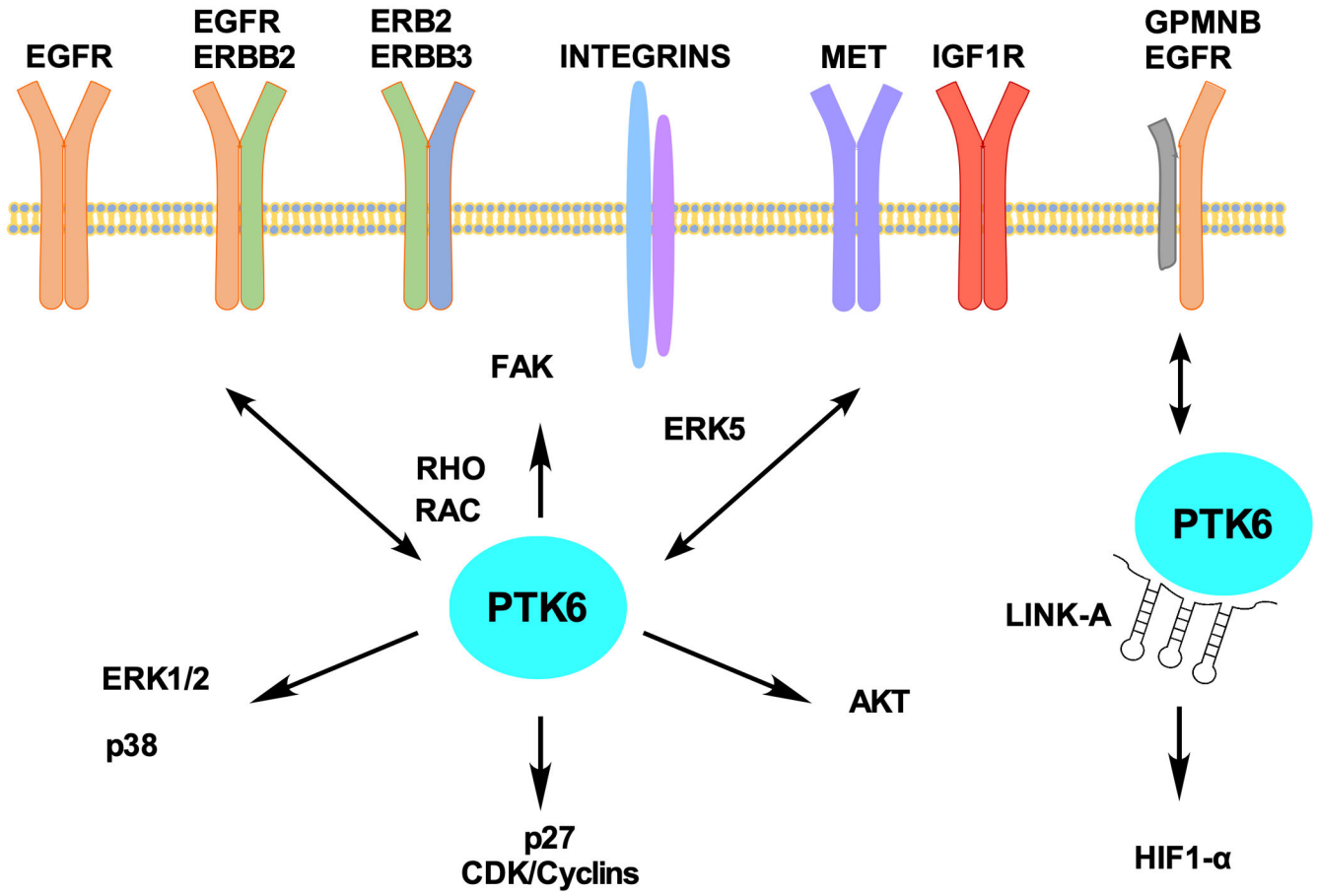

Figure 3. PTK6 contributes to several kinase pathways that can promote cancer.

PTK6 activation occurs in response to activation of different members of the ERBB family including EGFR and ERBB2. Feedback between PTK6 and EGFR also exists, as PTK6 has been reported to phosphorylate EGFR at Y845, promoting its activation. PTK6 is also downstream of the IGF1R and MET receptors, targeting PAK4 led to increased PTK6 expression. While integrins may activate both PTK6 and FAK, FAK is a direct substrate of PTK6. PTK6 has also been shown to regulate activation of RHO, RAC, ERK5, AKT, p38 MAPK, and CDK/CYCLIN complexes (see text for references). In response to HB-EGF, PTK6 is recruited to EGFR and GPNMB by the lncRNA *LINK-A* leading to PTK6 activation and its phosphorylation of HIF-1α resulting in the normoxic stabilization of HIF-1α [35]. Targeting PTK6 in addition to other kinases in combination therapies may have enhanced benefits in cancers with high PTK6 activation and expression.

Table 1.

Compounds that Target PTK6 Activity.

Inhibitor	**Targeted PTK6 *In Vivo***	**References**	**Notes**
Vemurafenib (PLX4032) and PLX4720	Yes	[103, 145]	Related B-RAF(V600E) inhibitors; PTK6 is a selective off-target
Ibrutinib	No	[56]	Bruton's tyrosine kinase (BTK) inhibitor that also inhibits BLK and PTK6
21a, 21c	No	[147]	Type I PTK6 inhibitors
PF-6683324, PF-6689840	No	[147]	Type II PTK6 inhibitors
Erlotinib	No	[164]	EGFR inhibitor; promoted PTK6 Y447 inhibitory phosphorylation
Pyrazolopyrimidines PP1 and PP2	No	[157]	SRC family kinase inhibitors
XMU-MP-2	Yes	[146]	Developed as a specific PTK6 inhibitor
Oleanolic acid derivatives	No	[163]	Analogues of triterpene oleanolic acid extracted from *Terminalia bentzoe* L.
Phenylmethylene hydantoins	Yes	[162]	Derivatives of Z-4-hydroxyphenylmethylene hydantoin (PMH)
Marine triterpene sipholenols derivatives	No	[161]	Derivatives of sipholenol A, a sipholane triterpene isolated from the Red Sea sponge *Callyspongia siphonella*
Dasatinib	No	[29, 150, 151]	Dual inhibitor of SRC/ABL and c-KIT