**HIF1α – independent hypoxia-induced rapid PTK6 stabilisation is associated with increased motility and invasion**

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Abbreviations: PTK6 (Protein Tyrosine Kinase 6), Brk (Breast tumour Kinase), HIF (hypoxia-inducible transcription factor), EGF (epidermal growth factor), IGF (Insulin-like Growth Factor), HGF (Hepatocyte Growth Factor), OPN (osteopontin), ALLN (Ac-LLnL-CHO), GLUT1 (Glucose Transporter-1), ALDOA (Aldolase A), BNIP3 (BCL2/adenovirus E1B 19kDa interacting protein 3), RTCA (Real Time Cell Analyser), c-Cbl (Casitas B-Lineage Lymphoma Proto-Oncogene), CHIP (C terminus of Hsc70-interacting protein) and SOCS3 (suppressor of cytokine signalling 3)
Abstract

PTK6/Brk is a non-receptor tyrosine kinase over-expressed in cancer. Here we demonstrate that cytosolic PTK6 is rapidly and robustly induced in response to hypoxic conditions in a HIF-1 independent manner. Furthermore, a proportion of hypoxic PTK6 subsequently re-localised to the cell membrane. We observed that the rapid stabilisation of PTK6 is associated with a decrease in PTK6 ubiquitylation and we have identified c-Cbl as a putative PTK6 E3 ligase in normoxia. The consequences of hypoxia-induced PTK6 stabilisation and subcellular re-localisation to the plasma membrane include increased cell motility and invasion, suggesting PTK6 targeting as a therapeutic approach to reduce hypoxia-regulated metastatic potential. This could have particular significance for breast cancer patients with triple negative disease.
INTRODUCTION

PTK6 (Protein Tyrosine Kinase 6) or Brk (Breast tumour Kinase) is a cytoplasmic non-receptor tyrosine kinase which has been shown to be highly expressed in various tumour types including breast carcinomas (85% of samples), as well as colorectal, prostate, lung, head and neck carcinomas and B- and T-lymphomas. In normal tissues, PTK6 expression is restricted to the differentiated epithelium of the skin and gut, whilst in tumours the highest levels of PTK6 expression correlate with higher tumour grade, larger size, metastasis and consequently a poorer prognosis.

PTK6 has specific functions in different tissue types, including regulating differentiation in normal tissues and promoting proliferation and cell survival in tumours, brought about by variations in cellular localisation. PTK6 is activated by a number of different ligands, as well as displaying a small amount of basal auto-phosphorylation in in vitro kinase assays. EGF (epidermal growth factor) and IGF (Insulin-like Growth Factor) induced signalling have been shown to activate PTK6, as have HGF (Hepatocyte Growth Factor) and osteopontin (OPN). Radiation treatment has also been reported to lead to the induction of PTK6 in both mouse intestine epithelial cells and human colorectal cancer cells, however, little is known about the mechanisms that regulate the de novo expression of Brk in tumours.

The importance of the microenvironment, particularly hypoxia, for tumour establishment and metastasis is well characterised. Tumour hypoxia arises as a consequence of high metabolic demand for oxygen caused by rapid tumour growth and the inefficiency of the tumour vasculature. Many studies have shown that tumour hypoxia is significant as hypoxic tumours are associated with increased invasion, metastasis, poor patient survival and increased resistance to therapy. One of the key regulators of the hypoxic response is the
hypoxia-inducible transcription factor 1 (HIF1). Hypoxia-inducible genes regulate many biological processes including cell proliferation, angiogenesis, metabolism, apoptosis, immortalisation and migration.\textsuperscript{21} Exposure to hypoxic conditions was recently shown to induce PTK6 in a HIF dependent manner in breast cancer cell lines.\textsuperscript{22} However, PTK6 has not previously been identified as a HIF target, since no HIF binding to PTK6 promoter was identified in larger genome-wide ChIP-seq studies.\textsuperscript{23,24} This indicated that there might be other parallel mechanisms for hypoxia-mediated PTK6 induction.

In this study, we showed that PTK6 was rapidly stabilised in hypoxic conditions in a post-translational HIF1-independent manner in both breast and colorectal cancer cell lines. Specifically, we demonstrated that, in normoxic conditions PTK6 was targeted to the proteasome and that this process was inhibited in hypoxia. Hypoxia-mediated PTK6 induction was associated with increased hypoxia-dependent migration and invasion. These findings are significant as they point to an additional mechanism of PTK6-induction by hypoxia in human cancers.
RESULTS

Hypoxia induces a rapid and robust stabilisation of PTK6

Hypoxia is a known driver of many key aspects of tumour development. Recently, PTK6 has been shown to be hypoxia-inducible in triple negative breast cancer cell lines. In order to confirm whether PTK6 is hypoxia-inducible in different cancer cell types, the hypoxia-mediated induction of HIF1α and PTK6 at both the mRNA and protein levels was examined in the MDA-MB-231 (breast) and RKO (colorectal) cell lines. When protein levels were examined, a rapid and robust induction of PTK6 at the protein level in both cell lines was observed, as early as 5 min after exposure to hypoxia (Figure 1A). PTK6 protein levels were induced in response to hypoxia prior to HIF1α upregulation (Figure 1A) in contrast to previous studies. In MDA-MB-231 cells, PTK6 mRNA increased after 18 h exposure to hypoxia, but not at earlier timepoints (6h), whereas no significant increase of PTK6 mRNA levels was observed for RKO cells (Figure 1B) at any of the time points studied. Due to the observed rapid PTK6 protein level induction kinetics in hypoxia, preceding HIF1α induction, it could be questioned whether PTK6 could in turn affect HIF1α stabilisation and/or activity. To address this, PTK6 was suppressed by RNA interference in RKO and MDA-MB-231 cells, which were then exposed to hypoxia (2% O₂). HIF-1α induction in hypoxia was unaltered by the presence/absence of PTK6 (Figure S1A-B). Similarly, no effect of PTK6 depletion was observed for the transcript levels of three well-characterised HIF-1 targets (Figure S1C), indicating that hypoxic PTK6 does not affect HIF stability and function.

PTK6 is ubiquitylated in an oxygen-dependent manner

Due to its rapid kinetics, it was plausible that the transcription-independent PTK6 protein stabilisation in hypoxia depicted in Figure 1 could occur via post-translational turnover in the
ubiquitin-proteasome system (UPS). In order to investigate this hypothesis, MDA-MB-231 cells were exposed to proteasome inhibitors MG132, ALLN, Lactacystin, and Bortezomib for 6 hours in normoxia (20% O₂). Increased PTK6 levels were observed after treatment with these inhibitors, suggesting that, in normoxic conditions, PTK6 is actively degraded via the UPS (Figure 2A). To investigate the role of direct protein ubiquitylation in PTK6 stabilisation in hypoxia, constructs containing either tagged PTK6 (Flag-PTK6) and/or ubiquitin (HA-Ub) were transfected into HEK293T cells to ensure high levels of expression. The cells were then exposed to normoxia or hypoxia (2% O₂) for 6 hours and Flag-PTK6 was immunoprecipitated (Figure 2B). Higher molecular weight forms of PTK6 were detected in normoxic conditions when both constructs were present. This suggested that, under these conditions, PTK6 was ubiquitylated. The presence of these higher molecular weight forms of PTK6 was decreased in hypoxic conditions, indicating that the level of PTK6 ubiquitylation was lower in hypoxia than in normoxic conditions (Figure 2B). These data imply that an E3 ligase could control the level of PTK6 protein in an oxygen-dependent manner. The role of the known tyrosine kinase E3 ligase c-Cbl was investigated in this context. MDA-MB-231 (breast) and RKO and HCT116 (colorectal) cell lines were transfected with c-Cbl siRNA. In MDA-MB-231 cells, suppression of c-Cbl led to a statistically significant increase in PTK6 levels (Figure 2C, S2A). This effect was also evident (albeit not significant) to a lesser extent in the colorectal cell lines, including RKO (Figure 2C, S2A). To support this finding the levels of c-Cbl and PTK6 were determined in a range of cancer cell lines by Western blotting (Figure 2D). This analysis demonstrated a reciprocal relationship between the levels of PTK6 and c-Cbl; that is, when one was relatively highly expressed the other was relatively low (Figure 2D-E, S2B). Altogether, these data support the hypothesis that PTK6 is ubiquitylated and degraded via the UPS in normoxic conditions and that this degradation is decreased in response to hypoxia, thereby allowing the protein to accumulate.
Hypoxia-induced PTK6 promotes cell motility and invasion

The role of hypoxia in regulating the ability of cancer cells to disseminate and proliferate to secondary sites clearly contributes for the metastatic process. Furthermore, PTK6 has been reported to regulate a number of processes that are central for cellular proliferation and metastatic spread, when associated with different membrane subcellular fractions. The subcellular localisation of normoxic and hypoxic PTK6 was investigated by biochemical and immunofluorescence approaches, where a clear increase in cytoplasmic hypoxic PTK6 was observed (Figure S3). Although most PTK6 protein in hypoxic MDA-MB-231 cells remained cytoplasmic, a fraction of it translocated to the cell membrane and co-localised with F-actin (Figure S3), indicating a potential role in cell motility under hypoxic conditions. In order to investigate this, scratch wound assays were performed in both MDA-MB-231 and RKO cells transfected with Scr (scramble) or PTK6 siRNA and exposed to hypoxic conditions (2% O$_2$) (Figure 3A-B). Wound closure was significantly delayed in the absence of PTK6 in hypoxic conditions for both cell lines (Figure 3A-B). The xCELLigence real-time cell analyser (RTCA) system was used to allow real-time kinetic analysis of early motility events. This system allows the differentiation between early motility and proliferation events. Real-time hypoxic cell motility of MDA-MB-231 cells was decreased in the absence of PTK6 (Figure 3C). Although PTK6 knockdown did not affect cell proliferation and clonogenic survival in hypoxia using 2D models (Figure S4), its potential role in 3D growth in hypoxic conditions was investigated using the mammosphere system. This allows the evaluation of the ability of breast cancer cells to survive and proliferate in an ECM-like substrate. Control Scr treated MDA-MB-231 cells formed larger mammospheres in hypoxic conditions (2% O$_2$) when compared with normoxia (Figures 3D, S5). However, mammospheres were significantly smaller in the absence of PTK6 (Figure 3D), indicating a role for PTK6 in anchorage-independent 3D cell growth. Interestingly, mammospheres formed from the cells
lacking PTK6 were predominantly smooth, whereas PTK6-expressing mammospheres presented a spiky/invasive appearance (Figure 3E). This phenotype has been previously associated with increased invasive and tumorigenic ability. The decreased invasive phenotype in the absence of PTK6 was further tested using conventional transwell assays, where both MDA-MB-231 and RKO cells had a lower invasive ability in the absence of PTK6 (Figures 3F, S6). This was reflected by an invasion index below 1 (0.46 for MDA-MB-231 and 0.16 for RKO). These results indicate that PTK6 is important for hypoxia-mediated cellular motility and invasion, which are key factors in metastasis.

**PTK6 expression is linked to distant metastasis-free survival**

To determine whether our in vitro data suggesting that PTK6 expression is linked to metastatic potential correlated with findings in human tumours we examined the effects of PTK6 expression on distant metastasis-free survival (DMFS) in 1609 breast cancer patients using data from Györffy and colleagues (2010). High Brk expression was correlated with a reduced metastasis-free survival ($P=0.0017$) (Figure 4A).

Given our findings in the triple-negative breast cancer cell line, MDA-MB-231, we determined whether PTK6 expression was particularly linked with DMFS in the 220 patients from the Györffy data set with triple-negative (basal-like) breast tumours. The Kaplan Meier plot in Figure 4B shows that, as with the overall cohort, PTK6 expression is a poor prognostic indicator for DMFS in patients with basal-like breast cancer. Notably, there was a greater difference between the probabilities for basal-like breast cancers than was observed for the overall patient group.

**DISCUSSION**
The role of hypoxia in tumour development and spread is well characterised. However, although many key players in this process have been identified, many others remain uncharacterised. Tyner and co-workers identified hypoxia to be an inducer of the non-receptor tyrosine kinase PTK6 in normal and neoplastic cells and that the induction was dependent on HIF1α. In our study we describe the rapid, post-translational induction of PTK6 protein levels in response to hypoxia in both breast and colorectal cancer cell lines (Figure 1) in a much shorter time frame than previously reported. PTK6 induction occurred prior to HIF1α stabilisation, implying that there is an additional HIF-independent mechanism mediating PTK6 protein level increase in hypoxic conditions. We did observe an increase of PTK6 mRNA after prolonged exposures to hypoxia for the breast cancer cell line MDA-MB-231, similarly to the published findings by Tyner and co-workers. However, as we observed no PTK6 transcript upregulation on shorter exposures to hypoxia in this cell line model, our data suggest that PTK6 induction could initially be independent of mRNA expression in breast cancer cells. Importantly, there was no hypoxia-dependent transcriptional upregulation of PTK6 in the RKO colorectal cancer cell line model, indicating the hypoxia-mediated transcriptional upregulation of PTK6 could be cancer type dependent. To confirm that PTK6 induction in hypoxia could be independent of HIF and transcriptional mechanisms we showed that, in response to hypoxia, proteasomal-mediated degradation of PTK6 is reduced and that this correlated with decreased ubiquitylation of PTK6 (Figure 2). A candidate-based approach was used to investigate the possible role of specific E3 ligases in PTK6 stabilisation. c-Cbl was prioritised as it is known to have substrates including both receptor and non-receptor tyrosine kinases. It appears that c-Cbl could, in part, be responsible for regulating PTK6 levels during early hypoxia, independently of HIF, although how c-Cbl is itself regulated in an oxygen-dependent manner to effect PTK6 levels is still unclear. Recently, PTK6 has been reported to promote the ubiquitylation and degradation of
c-Cbl through targeted phosphorylation, which raises the intriguing possibility that a reciprocal feedback loop exists between c-Cbl and PTK6. Furthermore, other E3 ligases, namely CHIP (C terminus of Hsc70-interacting protein) and SOCS3 (suppressor of cytokine signalling 3) were recently reported to enhance the proteasomal degradation of PTK6.

These data add further support to our finding that PTK6 levels are regulated by the proteasome and the regulation of E3 ligases in hypoxia warrants further investigation. This study also demonstrates that hypoxic PTK6 has a role in regulating cellular invasion and migration (Figure 3). Importantly, this is associated with a relocalisation to the cell membrane, a process that is reported to be essential for PTK6’s role in oncogenesis.

Finally we show in a large patient cohort (1609 samples) that high PTK6 expression is correlated with reduced metastasis-free survival (Figure 4A) across all tumour subtypes, supporting our previous findings that elevated PTK expression is associated with breast tumours that are either invasive, more likely to metastasise, as well as data from other studies using much smaller sample sizes (less than 300). Aubele and colleagues reported that high PLA signals, indicating a physical interaction between PTK6 and HER2, correlated with reduced metastasis-free survival, although their earlier findings in 193 invasive breast cancers suggest that PTK6 may be a positive prognostic indicator. This discrepancy has been discussed elsewhere and does highlight the difference between expression at the mRNA level compared to protein-based studies.

Additionally, given that the reduction in metastasis free survival with high PTK6 expression appeared to be more marked in the 220 patients with triple-negative or basal-like breast cancer (Figure 4B), it is possible that, in the absence of other prognostic factors such as HER2, ER and PR, PTK6 levels become more important in predicting prognosis.
It has been suggested that inhibition of PTK6 would be an effective therapeutic approach. However, the lack of commercially available specific inhibitors has not allowed for further investigations. Our *in vitro* results in both breast and colorectal cell line models show that PTK6 induction in hypoxia can be regulated by HIF-independent mechanisms, such as post-translational modifications. Combined with the findings in breast cancers, our data add to the wealth of information describing the role of hypoxia in driving cell motility and invasion, indicating that targeting of PTK6 through the development of pharmacological inhibitors could potentially be used to decrease tumour metastatic potential and that this may be of particular benefit to patients with basal-like/triple negative breast cancers.
MATERIALS AND METHODS

Cell lines, hypoxia, drug treatment and siRNA transfections. MDA-MB-231, MDA-MB-453, MDA-MB-468, MDA-MB-361, BT474, T47D, MCF-7 and SKBR3 (breast), RKO, HCT116 and DLD1 (colorectal) and RT112, VmCuB1, T24 and 253J (bladder) cancer cell lines were grown in DMEM or RPMI-1640 (Sigma, USA) with 10% FBS. HEK293T (kidney) cells were grown in DMEM/10% FBS. All cell lines were purchased from ATCC or ECCAC and routinely tested as negative for mycoplasma. Hypoxia treatments were carried out in an In vivo 400 (Ruskinn, Bridgend, UK) or Heracell incubator (ThermoFisher, UK). For experiments at <0.1% O₂, cells were plated in glass dishes and placed in a Bactron II anaerobic chamber (Shell labs, USA). MG132 (Sigma, USA), ALLN (Ac-LLnL-CHO, Sigma, USA), Lactacystin (Merck Millipore, USA) and Bortezomib (Selleck Chemicals, USA) stocks were prepared in dimethyl sulfoxide (DMSO, Sigma, USA). Cells were transfected with PTK6 siRNA (GGUGAUUUCUCGAGACAAC dTdT)⁸ or scramble siRNA (Life Technologies, UK) using DharmaFECT1 (Thermo Scientific, UK). Knockdown was obtained after double transfection over 48h. Transfection with Flag-PTK6 and HA-Ub (gift from Jason Parsons) was done using PEI (Polyethylenimine, Polysciences, USA).

Cell lysis and Western blotting. For whole cell extract (WCE) preparation, cells were lysed in UTB (9 M urea, 75 mM Tris-HCl pH 7.5 and 0.15 M β-mercaptoethanol) and immunoblotted as previously described.³⁹ Antibodies used were PTK6 (ICR-100)¹², Hif1α and GAPDH (BD Biosciences, USA), c-Cbl, EGFR and α-tubulin (Cell Signaling Technology, USA), HA-tag (Abcam, UK) and β-actin (Santa Cruz, USA). The Odyssey infrared imaging technology was used for protein detection (LI-COR Biosciences, USA). Densitometry was done using ImageJ software (NIH, USA).
Immunoprecipitation (IP). Cells were lysed in IP lysis buffer: 150 mM NaCl, 20 mM Hepes pH 7.5, 0.5 mM EDTA, 0.5% NP40, 1X Complete protease inhibitor cocktail and 1X PhosStop phosphatase inhibitor cocktail (Roche, UK). Flag-PTK6 was immunoprecipitated using Flag-M2 agarose (Sigma, USA).

Quantitative real time PCR. Quantitative real time PCR (qRT-PCR) was performed using Thermo Scientific Verso™ QRT-PCR (Thermo Scientific, UK) as previously described. The qPCR reaction was carried out using the 7500 Fast Real Time PCR System (Life Technologies, UK). PTK6 and GLUT-1 expression levels were normalised to 18S rRNA. Primer sequences are available in the Supplementary Material (Table S1).

Scratch wound assay and measurement of cellular invasion. Scratch wound assays were carried out as described previously. For cellular invasion assays, cells were plated in control or Matrigel invasion chambers with 8 μm pore size (BD Biosciences, USA). Cells were allowed to invade for 18 h before fixing and staining with DAPI to visualise nuclei.

Measurement of cellular proliferation and motility using the xCelligence system. Experiments were carried out using the xCelligence Real Time Cell Analyser (RTCA) DP instrument (Cambridge Biosciences, UK). Cell migration was assessed using 16-well CIM-plates 16 as described. DMEM/10% FBS was added to the lower chamber as chemottractant and cells were seeded into the upper chamber at 40,000/well in serum free medium.

Mammosphere formation assay. Matrigel (BD Biosciences, USA) diluted 1:1 in serum free medium was added to 24-well plates. Cells were seeded at 2500 cells/well and allowed to adhere for 6h before exposure to hypoxia (2% O₂). After 24 h cells were returned to normal culture conditions. Medium was changed every 2-3 days. After 10 days the mammospheres were imaged using an Eclipse SE2000-E microscope (Nikon, UK). Images were analysed...
using ImageJ software (NIH, USA). At least 150 mammospheres were measured per
condition.

Breast cancer patient distant metastasis-free survival analysis. Kaplan-Meier curves for
distant metastasis-free survival (DMFS) were generated using the KM-plotter on-line tool,
(http://kmplot.com/analysis), which used microarray data for over 20,000 genes for 1609
breast cancer patients.\(^{30}\) Analysis of PTK6 expression (Affymetrix ID 206482_at) was
performed for 1609 breast cancer patient samples and a subset of 220 triple negative (basal-
like) breast cancer patients. Both analyses were performed regardless of lymph node status.
Patients were grouped as having high or low PTK6 expression, and median expression was
used as the cut-off.

Statistical analysis. Statistical significance was determined using Student’s \( t \)-test and error
bars represent +/- SEM.
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FIGURE LEGENDS

Figure 1. Hypoxia induces the rapid stabilisation of PTK6

(A) RKO colorectal and MDA-MB-231 breast cancer cells were exposed to hypoxia (2% O$_2$) for the periods indicated. Cells were lysed and PTK6 and HIF1α levels were determined by western blotting. (B) RKO and MDA-MB-231 cells were exposed to hypoxia (2% O$_2$) for the periods indicated. PTK6 and GLUT-1 expression levels were determined by qRT-PCR.

Figure 2. PTK6 is ubiquitylated in an oxygen-dependent manner

(A) MDA-MB-231 cells were treated for 6h with either vehicle (DMSO), 10 μM MG132, 50 μM ALLN, 5 μM Lactacystin or 50 nM Bortezomib. PTK6 levels were determined by Western blotting. HIF1α was used as a positive control for proteasomal inhibition in normoxic conditions and GAPDH as a loading control. (B) HEK293T cells were transfected with constructs expressing either Flag-PTK6, HA-Ub or both and exposed to normoxia (Norm) or Hypoxia 2% O$_2$ (Hyp) for 6h in the presence of 10 μM MG132. Flag-PTK6 was immunoprecipitated (IP) and analysed by Western blotting for the presence of ubiquitinated PTK6 (indicated by arrows). Whole cell extracts (WCE) were analysed for the presence of PTK6 and GAPDH (loading control). Endogenous and ectopically expressed PTK6 in WCE are indicated as * and **, respectively. (C) MDA-MB-231 (breast), RKO and HCT116 (colorectal) cells were transfected with either Scr (scramble) or c-Cbl siRNA for 72h. Western blotting was carried out to detect the endogenous levels of c-Cbl and PTK6. (D) Whole cell extracts were prepared from the breast, colorectal and bladder cancer cell lines indicated and Western blotting was performed for PTK6 and c-Cbl. B-actin was used as a loading control. (E) Histogram represents PTK6/c-Cbl ratios from panel in (D). Quantification values are depicted in Supplementary Figure S2B. (a.u.= arbitrary units of fold increase relative to β-actin).

Figure 3 Hypoxia-induced PTK6 promotes cell motility and invasion

(A) MDA-MB-231 and RKO cells were treated with Scr (scramble) or PTK6 siRNA. Graph represents the percentage of wound closure after 18 h in 2% O$_2$. Graphs represent the mean of
n=3 independent experiments. (B) Representative images of scratch wound assays for (A).

(C) MDA-MB-231 cells were treated with Scr or PTK6 siRNA. Kinetic real-time migration assays were performed at 3% O₂ using the xCelligence Real Time Cell Analyser (RTCA) DP instrument. The graph depicts changes in the cell index (CI). Graph represents n=2 experiments. (D) MDA-MB-231 cells were treated with Scr or PTK6 siRNA. Mammospheres were established and exposed to normoxia (Norm) or 2% O₂ (Hyp) for 24 h. The graph represents a quantification of the average size of at least 150 mammospheres per condition. Data represent n=6 individual experiments. (E) MDA-MB-231 mammospheres were generated from cells transfected with Scr or PTK6 siRNA and treated as described in (D). Mammospheres were scored according to their morphological phenotype as non-invasive or invasive. Representative images of both phenotypes depicted in inset. Graph represents the percentage of different morphologies under each condition. Data represent n=6 individual experiments. (F) MDA-MB-231 and RKO cells were transfected with Scr or PTK6 siRNA as before. Cells were seeded in control (uncoated) or matrigel coated Transwell inserts with 8 μm pore size and allowed to invade for 18 h at 2% O₂. Invasion index = % invasion PTK6 siRNA/% invasion Scr. Results are representative of n=3 individual experiments. * p<0.05; ** p<0.005; ***p<0.0001

Figure 4. High PTK6 expression is associated with decreased distant metastasis-free survival in breast cancer patients

(A-B) Kaplan-Meyer curves depicting the effect of PTK6 expression in distant metastasis-free survival (DMFS) in 1609 breast cancer patients (A) and a subset of 220 triple negative (basal-like) patients (B). Kaplan-Meyer curves were generated using the KMplot online tool. Median expression was used as a cut-off for grouping into low (black) or high (grey) PTK6 expression. HR – Hazard ratio
Figure 2

A

B

C

D

E

PTK6/Cbl ratio (a.u.)

0.5 1.5 2.5 3.5 4.0

MDA-MB-453  MDAMB-231  MCF-7  MDAMB-361  BT474  T47D  OHL1  HCT116  RKO  T24  VM-29  T253  RT112

MDA-MB-453  MDAMB-231  MCF-7  MDAMB-361  BT474  T47D  OHL1  HCT116  RKO  T24  VM-29  T253  RT112
A

All patients

HR = 1.38 (1.3 - 1.69)
logrank P = 0.0017

B

Triple negative (basal-like) patients

HR = 1.8 (1.07 - 3.04)
logrank P = 0.026

Numbers at risk

Low PTK6 | High PTK6
---|---

Low PTK6
- 804
- 603
- 309
- 110
- 17
- 2
- 0

High PTK6
- 805
- 554
- 281
- 55
- 10
- 0
- 0

Low PTK6
- 804
- 603
- 309
- 110
- 17
- 2
- 0

High PTK6
- 805
- 554
- 281
- 55
- 10
- 0
- 0

Pires et al. Figure 4
Figure S1. Hypoxic PTK6 does not affect HIF function

(A-B) RKO (A) and MDA-MB-231 (B) cells were transfected with Scr or PTK6 siRNA and exposed to hypoxia (2% O₂) at increasing times and harvested. PTK6, HIF1α and GAPDH protein levels were detected by western blotting. (C) MDA-MB-231 cells were treated with Scr or PTK6 siRNA and exposed to hypoxia (2% O₂) at increasing times and harvested. The levels of HIF1α target genes GLUT1, ALDOA and BNIP3 were determined by qRT-PCR and normalized to 18S rRNA. Primer sequences available on Table S1.
**S2: Quantification of the relative levels of PTK6 and c-Cbl**

(A) Extracts were prepared from the cancer cell lines indicated, treated or not with Scr or c-Cbl siRNA. Western blotting was performed for PTK6, c-Cbl and to β-actin. (A) Histogram represents PTK6/β-actin ratios from semi-quantitative analysis of PTK6 protein levels regarding Scr or c-Cbl siRNA transfected MDA-MB-231, RKO and HCT116 cells (representative blot example in Figure 2C). Results are representative of n=3 individual experiments. * p<0.05 (B) Semi-quantitative analysis of Western blots in Figure 2D-E. (a.u.= arbitrary units of fold increase relative to β-actin).
Figure S3: Endogenous and ectopically expressed cytoplasmic PTK6 is induced by hypoxia and localizes to F-actin ruffles.

(A) MDA-MB-231 cells were exposed to either normoxia (20% O\textsubscript{2}) or hypoxia (2% O\textsubscript{2}) for 18 h and harvested for biochemical fractionation. The various fractions and corresponding whole cell extracts were analysed by Western blotting for PTK6. EGFR and \(\alpha\)-tubulin were used as membrane fraction and cytosolic fraction markers, respectively. MDA-MB-231 cells were exposed to either normoxia or hypoxia (2% O\textsubscript{2}) for 18 h, fixed and stained for endogenous PTK6 (B) or transiently expressed Flag-tagged PTK6 (C). Staining was carried out as previously reported. \(^1\) PTK6 was detected using anti-Brk antibody C19 (Santa Cruz, USA). TRITC-Phalloidin was used to stain for F-actin. Cells were visualised using a 90i fluorescence microscope (Nikon).
Figure S4: PTK6 knockdown does not affect cell proliferation and viability in hypoxic conditions

(A) MDA-MB-231 cells were transfected with either Scr (white) or PTK6 (black) siRNA. Kinetic real-time proliferation assays were performed at 3% O₂. Briefly, cell proliferation was assessed using the xCelligence Real Time Cell Analyser (RTCA) DP instrument. Cells were seeded at 40,000/well in DMEM with 10% FBS in 16-well E-plates 16. The graph depicts changes in the cell index (CI). Graph represents n=2 experiments. (B) RKO, HCT116 and MDA-MB-231 cells were treated with Scr (white) or PTK6 (grey) siRNA and exposed to 24h of normoxia (Norm) or 2% O₂ (Hyp). Colony survival assays were carried out. Briefly, cells were seeded into 6 well plates to obtain a minimum of 100 colonies. After treatment colonies were allowed to form for 10-14 days.
Figure S5: Representative fields of the effect of the knockdown of PTK6 on MDA-MB-231 mammosphere formation.

MDA-MB-231 cells were transfected with scramble (scr) or PTK6 siRNA. Cells were then seeded in matrigel-coated wells and exposed to normoxia (Norm) or hypoxia 2% O₂ (Hyp) for 24 hours. After reoxygenation, cells were allowed to grow for 10 days. Bright field images were taken using a Nikon Eclipse SE2000-E microscope (4x objective) Scale bar = 200µm.
Figure S6: Representative fields of Transwell invasion assays

MDA-MB-231 and RKO cells were treated with Scr or PTK6 siRNA. Cells were seeded in control of matrigel coated Transwell inserts and were allowed to invade for 18h at 2% O₂. Figure represents examples of fields of view from experiments depicted in Figure 3-F.
**Table S1 – Sequence of primers used in this study**

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Primer sequences</th>
</tr>
</thead>
</table>
| **PTK6**  | F: CTGCTCCGCGACTCTGATG  
|           | R: GTAATTCTCGGACTGCCAGGTAAC                          |
| **GLUT1** | F: ATACTCATGACCATCGCGCTAG  
|           | R: AAGAAGGCCACAAAGCCAAAG                             |
| **ALDOA** | F: GTGTTGGGCATCAAGGT  
|           | R: CAATCTTCAGCACACAACG                              |
| **BNIP3** | F: GGTGTGGGTATTTTTGAAGGC  
|           | R: AAGGTAATGGTGACAGCAAGG                             |
| **18S rRNA** | F: GCCCGAAGCGTTTTACTTTGA  
|           | R: TCCATTATTTCCTAGCTGCGGTATC                         |