Analysis of 3-phosphoinositide dependent kinase 1 signaling and function in murine embryonic stem cells

vorgelegt von
Tanja Tamgüney
aus Schweinfurt
Als Dissertation genehmigt von den Naturwissenschaftlichen Fakultäten der Universität Erlangen-Nürnberg.

Tag der mündlichen Prüfung: 16.06.2008

Vorsitzender der Promotionskommission: Prof. Dr. Eberhard Bänsch

Erstberichterstatter: Prof. Dr. Hans-Martin Jäck

Zweitberichterstatter: Prof. Dr. Thomas Winkler

Drittberichterstatter: Prof. Dr. David Stokoe
Contents

1. Summary ........................................................................................................... 5

2. Zusammenfassung ............................................................................................ 6

3. Introduction ...................................................................................................... 7-25
   3.1 The PI3K/mTOR pathway: signaling downstream of PDK1, PKB and mTOR ................................................................. 7-14
   3.2 PDK1 – A master regulator of AGC kinases ............................................. 14-19
   3.3 The PI3K/PDK1/PKB/mTOR pathway in cancer ...................................... 19-21
   3.4 Inhibiting PDK1 with conventional inhibitors or a chemical genetic approach ................................................................. 21-25

4. Aims .................................................................................................................... 26

5. Results .............................................................................................................. 27-73
   5.1 Analysis of PDK1 signaling in ES cells .................................................... 27-58
      5.1.1 The effect of BX-795 on G2/M arrest does not require PDK1 ...... 28-31
      5.1.2 Identification of inhibitor analogues to block the genetically modified PDK1, PDK1 LG, in vitro and in vivo ............. 32-43
      5.1.3 Examination of phosphorylation of PDK1 targets following long term inhibition of PDK1 activity ......................................................... 44-53
      5.1.4 Generation and characterization of BX-795-based allele-specific PDK1 inhibitors ................................................................. 53-58
   5.2 Effects of PDK1 inhibition or loss on physiological parameters and tumor growth ......................................................... 58-73
      5.2.1 Specific inhibition of PDK1 does not cause cell cycle arrest and has little effect on cell proliferation and viability .......... 58-59
      5.2.2 Loss of PDK1 and specific inhibition of PDK1 sensitize to apoptosis ................................................................. 59-65
      5.2.3 PDK1 contributes to tumor growth and teratoma differentiation ...... 65-73

6. Discussion ......................................................................................................... 74-82
   6.1 Analysis of PDK1 signaling in ES cells .................................................... 74-80
      6.1.1 BX-795 as a PDK1 inhibitor ............................................................... 74-75
      6.1.2 Chemical genetic approach to inhibit PDK1 and biochemical consequences of PDK1 inhibition ............................................. 75-80
6.2 Biological roles of PDK1 ................................................................. 80-82

7. Experimental procedures ................................................................. 83-88

7.1 Allograft studies
7.2 Apoptosis assay
7.3 Cell culture
7.4 Cell cycle analysis
7.5 Cell proliferation and viability assay
7.6 Construction of a PDK1 variant, PDK1 L159G, and generation of stable ES cell lines.
7.7 IC₅₀ determination
7.8 In vitro PDK1 kinase assay
7.9 Sequence alignment
7.10 Synthesis of purine analogues
7.11 Western blotting

8. Abbreviations ..................................................................................... 89-91

9. References ......................................................................................... 92-102

10. Attachments ...................................................................................... 103-106

10.1 Own publications
10.2 Curriculum Vitae
10.3 Acknowledgements
1. **Summary**

The interaction of insulin and growth factors with their receptors leads to the activation of the phosphatidylinositol 3-kinase (PI3K) pathway, which regulates a plethora of events including proliferation, growth, survival, motility and metabolism. Many of the downstream effects of PI3K are mediated by the activation of a subgroup of the cAMP-dependent, cGMP-dependent, and protein kinase C (AGC) family of protein kinases, which comprises protein kinase B (PKB, also known as Akt), p70 ribosomal S6 kinase (S6K), p90 ribosomal S6 kinase (RSK), serum- and glucocorticoid-induced kinase (SGK), and protein kinase C (PKC). Genetic evidence indicates that 3-phosphoinositide dependent kinase 1 (PDK1) is critical for activation and stability of these AGC kinases. However, relatively little is known about the dynamics of signaling downstream of PDK1 and its biological functions.

Thus, in the first part of the work presented here, consequences of acute PDK1 inhibition on downstream signaling in murine embryonic stem (ES) cells were analyzed. Initially, a recently characterized PDK1 inhibitor, BX-795, was used; however this approach revealed biological effects that were not consistent with PDK1 inhibition. In an attempt to achieve transient and more specific inhibition of PDK1, a chemical genetic approach was used. Therefore, a PDK1 mutant, L159G, was generated and characterized. This mutant can bind inhibitor analogues containing bulky groups that hinder access to the ATP-binding pocket of wild type (WT) kinases. When expressed in PDK1−/− ES cells, PDK1 L159G restored the phosphorylation of PDK1 targets known to be hypophosphorylated in these cells. Screening of multiple inhibitor analogues showed that 1-NM-PP1 and 3,4-DMB-PP1 optimally inhibited the phosphorylation of PDK1 targets in PDK1−/− ES cells expressing PDK1 L159G but not WT PDK1. These compounds confirmed the identity of previously assumed PDK1 substrates, but revealed also distinct kinetics of dephosphorylation for individual targets. For example, the PDK1 target PKB T308 was rapidly dephosphorylated within one hour following PDK1 inhibition, whereas significant dephosphorylation of the analogous site in RSK occurs only after several hours. These inhibitors also exposed a novel role for RSK in response to osmotic shock, and indicated that glycogen synthase kinase 3 (GSK3) α/β may be phosphorylated by kinases other than PKB, RSK, and S6K. However, use of this model system in combination with PDK1−/− and PDK1−/− ES cells that have been reconstituted with WT PDK1 also uncovered complications that may occur with this methodology: 1-NM-PP1 and 3,4-DMB-PP1 at concentrations required to efficiently inhibit PDK1 downstream signaling in PDK1 L159G expressing cells, also had a surprisingly clear effect on the phosphorylation of ribosomal protein S6 S235/S236 in WT PDK1 cells. This highlights the importance of appropriate controls and caution in interpreting results from such experiments.

In the second part of this work biological roles of PDK1 were assessed. This revealed that while PDK1 inhibition had little effect on cell growth under regular conditions, it sensitized cells to apoptotic stimuli. Loss of PDK1 also abolished growth of allograft tumors, underpinning the notion that PDK1 may be a valuable drug target for cancer therapy.
2. Zusammenfassung

Die Interaktion von Insulin und Wachstumsfaktoren mit ihren Rezeptoren führt zur Aktivierung der Phosphatidylinositol 3-Kinase (PI3K) Signaltransduktionskette, welche zahlreiche zelluläre Ereignisse reguliert, darunter Proliferation, Wachstum, Überleben, Motilität und Metabolismus. Viele der Effekte unterhalb von PI3K werden von durch Aktivierung einer Untergruppe der cAMP-, cGMP-abhängigen und Protein Kinase C (AGC) Familie von Kinasen vermittelt. Zu dieser Familie gehören Protein Kinase B (PKB, auch Akt), p70 ribosomale S6 Kinase (S6K), p90 ribosomale S6 Kinase (RSK), Serum- und Glucocorticoid-induzierte Kinase (SGK) und Protein Kinase C. Genetische Daten deuten darauf hin, dass die 3-Phosphoinositid-abhängige Kinase (PDK1) wichtig für die Aktivierung und Stabilität dieser AGC Kinasen ist. Allerdings ist noch relativ wenig über die Dynamik der durch PDK1 ausgelösten Signaltransduktion bekannt, und auch die biologische Rolle von PDK1 ist noch wenig erforscht.

Im ersten Teil dieser Arbeit wurden daher die Folgen akuter PDK1 Inhibierung auf die Signaltransduktion in murinen embryonalen Stamm (ES) -zellen untersucht. Anfänglich wurde ein kürzlich charakterisierter PDK1 Inhibitor, BX-795, benutzt. Dieser hatte aber biologische Auswirkungen, die nicht mit einer PDK1 Inhibierung im Einklang standen. Aus diesem Grund wurde eine PDK1 Mutante, PDK1 L159G (LG), erzeugt und charakterisiert, welche Purin-Analoge mit sperrigen Seitenketten binden kann; diese Seitenketten erschweren den Zugang dieser Inhibitoren zur ATP-Bindungstasche von Wildtyp (WT) Kinasen. Expression dieser Mutante in PDK1-/- ES Zellen (PDK1-/- +LG ES Zellen) stellte die Phosphorylierung von Proteinen wieder her, die in PDK1-/- ES Zellen bekannterweiser unterphosphoryliert sind. Eine Analyse mehrerer Inhibitoren zeigte dass 3,4-DMB-PP1 und 1-NM-PP1 optimal die Phosphorylierung von PDK1 Substraten in PDK1-/- +LG ES Zellen, nicht aber PDK1 WT exprimierenden (PDK1-/- +WT) ES Zellen inhibierten. Diese Inhibitoren bestätigten die Identität mutmaßlicher PDK1 Substrate, zeigten aber unterschiedliche Kinetiken für einzelne Substrate. So ist z.B. das PDK1 Substrat PKB T308 schnell, innerhalb einer Stunde nach PDK1 Inhibierung maximal dephosphoryliert, wohingegen eine signifikante Dephosphorylierung von RSK erst nach mehreren Stunden festzustellen ist. Des weiteren legten Versuche mit diesen Verbindungen eine neue Rolle für RSK in der Antwort auf osmotischen Schock offen, und weisen darauf hin, dass Glykogen Synthase Kinase 3 (GSK3) α/β außer von PKB, RSK und S6K auch von einer oder mehreren PDK1-unabhängigen Kinasen phosphoryliert werden kann. Allerdings zeigte der Gebrauch dieses Modellsystems im Zusammenhang mit PDK1-/- und PDK1-/- +WT ES Zellen auch Komplikationen auf, die mit dieser Methode auftreten können: 3,4-DMB-PP1 und 1-NM-PP1 Konzentrationen die nötig waren, um PDK1 Signalgebung in PDK1-/- +LG ES Zellen effizient zu unterbinden, hatten einen überraschend grossen Effekt auf die Phosphorylierung des ribosomalen Proteins S6 an S235/S236. Dies hebt die Bedeutung geeigneter Kontrollen hervor und zeigt, dass Ergebnisse solcher Versuche mit grosser Sorgfalt und Vorsicht interpretiert werden müssen.

Im zweiten Teil dieser Arbeit wurden die biologischen Rollen von PDK1 untersucht. Während PDK1-Inhibierung unter normalen Kulturbedingungen wenig Einfluß auf Zellwachstum hatte, sensibilisierte es Zellen für apoptotische Stimuli. Des weiteren zeigten PDK1-/- Zellen drastisch eingeschränktes Tumorwachstum, was darauf hindeutet, dass PDK1 ein geeignetes Ziel für einen therapeutischen Eingriff in der Behandlung von Krebs sein könnte.
3. Introduction

3.1 The PI3K/mTOR pathway: signaling downstream of PDK1, PKB and mTOR

The class I phosphoinositide 3-kinase (PI3K) pathway is one of the most important signaling transduction cascades used by cell-surface receptors to control intracellular events. The receptors signaling to PI3K include those that recognize growth factors (GF), hormones, adhesion molecules, antigens, and inflammatory stimuli (Figure 1). PI3Ks are heterodimeric enzymes composed of a regulatory p85 and a catalytic p110 subunit, for each of which several isoforms exist. Direct interaction of the regulatory PI3K subunit p85 with phosphotyrosines of activated receptor tyrosine kinases (RTKs) or adaptor proteins, like insulin receptor substrate (IRS), results in activation of PI3K. Similarly, PI3K can be stimulated by cell adhesion and by G protein-coupled receptors. Furthermore, direct binding of the PI3K catalytic subunit p110 to activated Ras protein, which is also induced by growth factors, stimulates PI3K activity as well (reviewed in Cantley, 2002).

PI3K activates a wide variety of downstream protein kinases and thereby coordinates a plethora of cellular events including cell growth, proliferation, survival, and motility. Notably, many of the events downstream of PI3K involve two central players, namely protein kinase B (PKB), also known as Akt, and/or the Ser/Thr kinase mammalian Target Of Rapamycin (mTOR), which is part of two structurally and functionally distinct complexes (mTORC1 and mTORC2).

**PDK1 and PKB.** Once activated, PI3K converts the lipid second messenger phosphatidylinositol-4,5-bisphosphate (PIP$_2$) into phosphatidylinositol-3,4,5-triphosphate (PIP$_3$) (Cantley, 2002); this action is reversed by the tumor suppressor phosphatase and tensin homologue deleted on chromosome ten (PTEN) (Maehama et al., 2001). PIP$_3$ in turn acts as a docking site at the plasma membrane that recruits pleckstrin homology (PH) domain-containing proteins like 3-phosphoinositide dependent kinase 1 (PDK1). PDK1 is a
The PI3K/mTOR pathway is a major regulator of cell growth with mTORC1 controlling ribosome biogenesis, protein synthesis, cell size, transcription and autophagy.

Binding of growth factors (GFs), insulin, hormones, etc. to their respective receptors leads to activation of phosphoinositide 3-kinase (PI3K). This can occur either via direct interaction of the p85 regulatory subunit of PI3K with phosphotyrosine residues of activated receptor tyrosine kinases, its interaction with adaptor proteins like insulin receptor substrate (IRS), or via interaction of the catalytic PI3K subunit p110 with activated Ras.

Once activated, PI3K synthesizes the second lipid messenger phosphatidylinositol-3,4,5-triphosphate (PIP₃) from phosphatidylinositol-4,5-bisphosphate (PIP₂). This action is antagonized by the phosphatase and tensin homologue deleted on chromosome ten (PTEN). Experimentally, the production of PIP₃ can be blocked with the PI3K inhibitor LY294002 (LY). PIP₃ recruits protein kinase B (PKB) and 3-phosphoinositide dependent kinase 1 (PDK1) to the membrane by binding their PH domain, colocalizes these two enzymes and allows PDK1 to phosphorylate T308 of PKB.

The mTOR kinase is the catalytic component of two distinct multirportein complexes called mTORC1 and mTORC2. Mammalian Target Of Rapamycin Complex 2 (mTORC2), consisting of mTOR, mLST8, SIN1 and Rictor, phosphorylates the S473 of PKB. Activated PKB phosphorylates tuberous sclerosis complex 2 (TSC2) within the TSC1-TSC2 complex at multiple sites, thereby blocking the ability of TSC2 to act...
as a GTPase–activating protein for the small GTPase Rheb (Ras homolog enriched in brain). Rheb-GTP activates mTORC1, containing mTOR, mLST8 and Raptor. Many diverse positive signals, such as nutrients, and negative signals like stress influence the activity of mTORC1. Activity of mTORC1, but generally not mTORC2, can be inhibited with Rapamycin. Proline-rich Akt substrate 40 kDa (PRAS40), which is phosphorylated and activated by PKB, also negatively regulates mTORC1 activity. Eukaryotic initiation factor 4E binding protein (4E-BP1) and p70 S6 kinase (S6K) are the best characterized mTORC1 targets. Phosphorylation of 4E-BP1 by mTORC1 releases it from inhibiting the elongation initiation factor 4E (eIF4E), thus promoting protein translation. S6K is also involved in translation regulation, and requires phosphorylation of both T389 by mTORC1 as well as T229 by PDK1 for its activity. Activated PKB phosphorylates proline-rich Akt substrate 40 kDa (PRAS40), which negatively regulates mTORC1 activity.
serine/threonine kinase that was originally identified as the kinase that phosphorylates the activation loop, T308, of PKB in the presence of PIP₃ (Alessi et al., 1997 a,b; Stokoe et al., 1997). PIP₃ recruits PKB and PDK1 to the membrane by binding their PH domains. This colocalizes the two enzymes and is thought to lead to a conformational change in PKB allowing PDK1 to phosphorylate the activation, or T-loop of PKB (Calleja et al., 2007) and thereby leading to its activation.

**mTOR.** Binding of growth factors, hormones and other ligands to their cognate cell surface receptors also activates the mammalian Target Of Rapamycin Complex 2 (mTORC2) by a currently unknown mechanism.

mTORC2 phosphorylates PKB at S473 within the hydrophobic motif (HM) S473 (Frias et al., 2006). PKB in turn activates another mTOR containing complex, mTORC1, which has a central role in translation regulation: PKB phosphorylates tuberous sclerosis complex 2 (TSC2) within the TSC1-TSC2 complex at multiple sites, thereby blocking the ability of TSC2 to act as a GTPase–activating protein for the small GTPase Rheb (Ras homolog enriched in brain) (Dan et al., 2002; Inoki et al., 2002; Potter et al., 2002; Tee et al., 2002). Rheb-GTP activates mTORC1, which in turn phosphorylates downstream targets such as eukaryotic initiation factor 4E (eIF4E) binding protein (4E-BP1) and the hydrophobic motif on p70 S6 kinases (S6K), T389 on S6K1 (Gingras et al., 1998; Lekmine et al., 2003; Hay & Sonenberg, 2004). The key regulatory role mTORC1 has in ribosomal biogenesis, protein synthesis and cell growth is largely mediated by these two bonafide targets, 4E-BP and S6K.

Phosphorylation of 4E-BPs, the best characterized of which is 4E-BP1, on several sites relieves the inhibitory effect of this translational repressor, and promotes cap-dependent translation (Gingras et al., 1998; Lekmine et al., 2003).

Phosphorylation of S6Ks at their HM site by mTORC1 contributes to their activation (Lekmine et al., 2003; Hay & Sonenberg, 2004). Importantly, PDK1 phosphorylates the activation loop on the S6Ks, T229 on S6K1, and S6Ks...
require phosphorylation on both the T-loop as well as the HM site for full activity. S6K stimulates translation by phosphorylating several substrates involved in protein synthesis such as eIF4B and eukaryotic elongation factor 2 kinase (eEF2K) (Ruvinsky and Meyuhas, 2006). The S6K target ribosomal protein S6 is the major determinant of cell size downstream of mTORC1 and PDK1 (Zhang et al., 2000; Stocker et al., 2003; Murakami et al., 2004; Lawlor et al., 2002; McManus et al., 2004; Ruvinsky et al., 2005; Wullschleger et al., 2006). S6 is phosphorylated at multiple clustered residues, at S235/S236, at S240/S244 and S247 (Krieg et al., 1998). S235/236 and S240/S244 are both phosphorylated by p70 S6K, but depending on the stimulus, S235/236 can also be phosphorylated by p90 ribosomal S6 kinase (RSK).

The macrolide Rapamycin inhibits mTORC1 (but generally not mTORC2) activity and leads to reduced cell size. Mice lacking the mTORC1 target S6K1 are born 20% smaller than their wildtype (WT) littermates due to reduced cell size (Shima et al., 1998). Furthermore, mouse embryonic fibroblasts (MEFs) in which all five S6 phosphorylation sites were mutated to unphosphorylatable residues were 24% smaller than WT MEFs and Rapamycin did not further decrease their cell size (Ruvinsky et al., 2005), again indicating that S6 is the major component downstream of mTORC1 controlling cell size.

mTORC1 integrates not only signals from growth factor receptors, but also nutrient availability and cellular stress to regulate ribosome biogenesis and protein synthesis by phosphorylation of 4E-BP1 and S6K. mTORC1 is also involved in transcriptional regulation and blockade of autophagy (reviewed in Wullschleger et al., 2006 and Bashkar & Hay, 2007).

Aside from phosphorylating and inhibiting TSC2, PKB additionally activates mTORC1 by phosphorylating the proline-rich Akt substrate 40 kDa (PRAS40) at T246, thereby relieving the PRAS40-mediated inhibition of mTORC1 (Sancak et al., 2007; Vander Haar et al., 2007).

**PKB.** PKB is a major effector of PI3K signaling. It not only regulates translation and cell growth via activation of mTORC1, but it also controls proliferation, survival and metabolism (Figure 2). PKB, sometimes also referred to as survival kinase, promotes cell survival by negatively regulating
Upon binding of growth factors (GFs), insulin, hormones, etc. to their respective receptors, phosphoinositide 3-kinase becomes activated and phosphorylates phosphatidylinositol-4,5-bisphosphate (PIP₂) to generate the second messenger phosphatidylinositol-3,4,5-triphosphate (PIP₃) (top left). PIP₃ recruits proteins such as 3-phosphoinositide dependent kinase 1 (PDK1) and protein kinase B (PKB, also known as Akt) to the plasma membrane by virtue of their pleckstrin homology (PH) domain. Binding of PDK1 and PKB to the membrane colocalizes these two enzymes and allows PDK1 to phosphorylate the activation, or T-loop site T308, of PKB1. PDK1 also phosphorylates the analogous site in p90 S6 ribosomal S6 kinase (RSK) (RSK1 S221, RSK2 S227), p70 S6 kinase (S6K) (S6K1 T229), serum and glucocorticoid-induced kinase (SGK), and protein kinase C (PKC) isoforms, as well as PKC related kinases. For maximal activity, PKB, RSK, S6K, SGK and most PKCs need to be phosphorylated by PDK1 at the activation loop site (indicated by an orange circle), as well as a residue called the hydrophobic motif (HM) site (yellow circle) by other, different kinases. Once activated, PKB phosphorylates a plethora of targets (top to bottom right) including tuberous sclerosis complex 2 (TSC2), proline-rich Akt substrate 40 kDa (PRAS40) (T246), glycogen synthase kinase 3 α/β (GSK3α S21, GSK3β S9), Bad, p27 and transcription factors of the Forkhead box O (FOXO) family. Together these targets are involved in the regulation of proliferation, survival, and metabolism. Activated RSK isoforms regulate transcription, proliferation and survival. RSK and S6K isoforms together control translation, cell size, growth, and amino acid storage. S235/S236 of S6 is a common target of RSK and S6K, whereas S240/S244 is solely phosphorylated by S6K. SGKs are involved in transcriptional regulation, and the different PKC isoforms mediate many diverse, sometimes opposing effects, which are therefore not further detailed here.
All phosphorylations in this figure are illustrated by an arrow, whether they have an activating or inhibiting effect on the downstream target. The phosphorylation of PKB, RSK, S6K, SGK and PKCs by PDK1 is activating, as is the phosphorylation of PRAS40 by PKB and S6 by RSK and S6K. The phosphorylation of TSC2, GSK3, Bad, p27 and FOXO by PKB inhibits their activity.
the function or expression of several proapoptotic proteins. For instance, PKB directly phosphorylates and inhibits the proapoptotic protein BAD (Bcl-2/Bcl-XL-antagonist of cell death) (Datta, 1997; del Peso, 1997, Datta 2002). Additionally, PKB phosphorylates the Forkhead box O family (FOXO1, FOXO3a, FOXO4) of transcription factors which results in the block of FOXO-mediated transcription of genes that promote apoptosis, cell-cycle arrest and metabolic processes (reviewed in Tran, 2003). PKB activation can also stimulate proliferation through multiple targets impinging on cell cycle regulation, such as p27<sub>KIP1</sub>. Phosphorylation of the cyclin-dependent kinase inhibitor p27<sub>KIP1</sub> by PKB sequesters p27<sub>KIP1</sub> in the cytosol and thereby attenuates its cell-cycle inhibitory effects (Liang et al, 2002; Shin et al, 2002; Viglietto et al., 2002). Furthermore PKB phosphorylates glycogen synthase kinases (GSK) 3 α and β at S21 and S9 respectively (Cross, DA 1995). This inhibits GSK3 (Cross, DA 1995) and results in the dephosphorylation of GSK3 substrates, including glycogen synthase, thereby promoting glycogen synthesis. Apart from its long established function in glycogen metabolism, GSK3 also has a key role in growth factor signaling, cell division, apoptosis, microtubule function and specification of cell fates during embryonic development by phosphorylating and regulating additional targets including cyclin D1, c-myc, tau, axin, adenomatous polyposis coli (APC) protein and β-catenin (reviewed in Cohen and Frame, 2001).

Taken together, the PI3K downstream signaling mediated by PDK1, PKB, and mTORC1 controls many fundamental aspects of cell growth, proliferation, survival and metabolism.

3.2 PDK1 – A master regulator of AGC kinases

Identification of PDK1 as PKB T308 Kinase. PDK1 was first purified from tissue extracts as an enzyme that could phosphorylate the activation loop of PKBα, T308, in the presence of PIP<sub>3</sub> (Alessi et al., 1997 a,b; Stokoe et al., 1997). Further characterization revealed that PDK1 is a 556 amino acid protein containing a kinase domain at its N-terminus and a PH domain at its
C-terminus, which interacted with high affinity with PIP$_3$ and with lower affinity with one of its immediate breakdown products, phosphatidylinositol-3,4-bisphosphate, also a signaling molecule (Stephens et al., 1998; Currie et al., 1999). PDK1 as well as the PKB isoforms, PKB$\alpha$, PKB$\beta$, and PKB$\gamma$ (Akt1, Akt2, Akt3) are members of the AGC (cAMP-dependent, cGMP-dependent, and protein kinase C) family of kinases, but differ from the other members of that family by containing a PH domain. The mutual ability of PDK1 and PKB to interact with PIP$_3$ via their PH domains is an important factor for co-localizing these enzymes at the plasma membrane and allowing PDK1 to phosphorylate, and activate PKB. The evidence for this is based on the observation that PDK1 can only phosphorylate PKB efficiently \textit{in vitro} in the presence of lipid vesicles containing PIP$_3$ or phosphatidylinositol-3,4-bisphosphate (Alessi et al., 1997 a,b; Stokoe et al., 1997), hence the name 3-phosphoinositide dependent kinase 1. The binding of PKB to PIP$_3$ is thought to induce a conformational change in PKB that greatly enhances phosphorylation by PDK1. This is supported by the fact that in the absence of 3-phosphoinositides, PDK1 can not phosphorylate wildtype PKB, but efficiently phosphorylates a mutant form of PKB that lacks the PH domain, $\Delta$PH-PKB (Alessi et al., 1997 b; Stephens et al., 1998).

\textbf{PDK1 targets in addition to PKB.} Shortly after identification and characterization of PDK1 as the kinase that phosphorylates PKB at the activation loop site T308 in a PIP$_3$ -dependent manner, PDK1 has subsequently been shown to phosphorylate and activate a whole group of related protein kinases belonging to the AGC kinase family at their activation loop site (Figure 3). This includes isoforms of p70 ribosomal S6 kinase (Pullen et al., 1998), p90 ribosomal S6 kinase (Jensen et al., 1999), serum- and glucocorticoid-induced kinase (SGK) (Kobayashi and Cohen, 1999), conventional (Dutil et al., 1998), novel and atypical (Le Good et al., 1998) isoforms of protein kinase C (PKC), and PKC related kinases PRK1 and PRK2 (Flynn et al., 2000). Given the multitude of PDK1 downstream targets in the AGC kinase family, PDK1 has been called a ‘master regulator of AGC kinases’ (Mora et al., 2004). These target protein kinases in turn regulate diverse cellular processes such as proliferation, survival, metabolism and
A considerable part of growth factor signal transduction is mediated by structurally related enzymes belonging to the family of AGC (cAMP-dependent, cGMP-dependent, and protein kinase C) kinases. This includes protein kinase B (PKB, also called Akt), p70 ribosomal S6 kinase (S6K), p90 ribosomal S6 kinase (RSK), serum- and glucocorticoid-induced kinase (SGK), conventional, novel and atypical isoforms of protein kinase C, and PKC related kinases PRK1 and PRK2. These kinases have two regulatory features in common that are critical for activation. First, they all require phosphorylation of a serine or threonine residue in a conserved tail region C-terminal to the kinase domain called the hydrophobic motif (HM) (F-X-X-F-S/T-F/Y). The identity of the hydrophobic motif kinase(s) differ(s) from AGC kinase to kinase and is not clear in all cases (indicated by kinases X, Y, Z). The kinase phosphorylating the hydrophobic motif of S6K, T389, however is known to be mammalian Target Of Rapamycin Complex 1 (mTORC1) and for PKB the hydrophobic motif kinase phosphorylating S473 is mTORC2. PRKs and atypical PKC kinases are exceptions in this regard because they contain a negatively charged amino acid at this site, aspartic or glutamic acid, that mimicks the phospho-Serine or -Threonine.

Second, they all are phosphorylated by 3-phosphoinositide-dependent kinase 1 (PDK1) at a site within the kinase domain called the activation, or T-loop site. The HM can function as docking site for PDK1, recruiting PDK1 to phosphorylate the T-loop site.

Both the T-loop and the HM site phosphorylation (or acid amino acid for PRKs and atypical PKCs) are required for maximal activity of these kinases.

Since PDK1 controls the activity of many different AGC kinases mediating divergent downstream signaling cascades, it has been attributed the term ‘master regulator of AGC kinases’.

PDK1 itself is also a member of the AGC family of kinases, and also requires phosphorylation at its T-loop site S241 for activity. Phosphorylation at this site is thought to occur by autophosphorylation. PDK1 differs from other members of the family in that it does not have a corresponding HM site.
translation that place PDK1 in a central position controlling these events (Figure 2). As detailed above, S6K1 and S6K2 are regulators of translation, cell growth, and amino acid storage (Wullschleger et al., 2006). The four isoforms of RSK are involved in the regulation of transcription, proliferation, and survival (Frodin and Gammeltoft, 1999; Dummler et al., 2005). SGK plays important roles in controlling ion transport (Lang et al., 2006; Tessier and Woodgett, 2006). The PKC family consists of at least 12 such kinases with distinct and in some cases opposing roles in cell proliferation, differentiation, apoptosis and angiogenesis (Mackay and Twelve, 2007). PRK1 and PRK2 (also called PKNα and PKNγ, respectively) are Ser/Thr kinases with a catalytic domain homologous to PKCs, which control cytoskeletal rearrangements, cell adhesion, and vesicle transport, amongst other processes (Mukai, 2003).

**Regulation of PDK1.** Like other members of the AGC kinase family PDK1 requires phosphorylation of its activation loop site S241 for catalytic activity (Casamayor et al., 1999). The fact that bacterially expressed PDK1 was found stoichiometrically phosphorylated and fully active indicated that PDK1 possesses the intrinsic ability to phosphorylate its own T-loop residue. This PDK1 autophosphorylation at S241 was subsequently shown to be mediated by an intermolecular (trans) reaction, rather than an intramolecular (cis) reaction (Wick et al., 2003).

Even though some reports indicate that PDK1 activity can be modulated by (auto)phosphorylation under certain circumstances (Park et al., 2001; Riojas et al., 2006), it is generally believed that PDK1 activity itself is not critically regulated but is constitutive (Casamayor et al., 1999).

Regulation of PDK1 action instead occurs at the level of PDK1 targets: recruitment of PKB to the plasma membrane and a subsequent conformational change render PKB a target for PDK1. Other PDK1 substrates like S6K, SGK, and RSK do not have a PH-domain and do not bind PIP₃, nor is their phosphorylation by PDK1 directly stimulated by PIP₃. Instead, the phosphorylation of their T-loop by PDK1 seems to be dependent on the phosphorylation of these enzymes at a C-terminal Ser/Thr residue termed the
HM site (Figure 3). Phosphorylation of the HM-site by a distinct kinase allows PDK1 to bind to its targets through its specific substrate-docking site called the PDK1-interacting fragment (PIF)-pocket (Biondi et al., 2000, 2001; Collins et al., 2003). For PKCs and PRKs some debate remains about the order and necessity of HM and T-loop phosphorylation, however, it seems that for optimal activity all isoforms require phosphorylation at their T-loop site by PDK1 or another kinase (Parker and Murray-Rust, 2004).

**PDK1-deficient cells and mice.** Studies using PDK1\(^{+/+}\) and PDK1\(^{-/-}\) murine embryonic stem (ES) cells revealed that PDK1 is absolutely required for the activation of PKB, S6K, and RSK (Williams et al., 2000). Furthermore, stability and phosphorylation of several PKC isoforms and of PRKs are vastly reduced in PDK1\(^{-/-}\) ES cells (Balendran et al., 2000). However, there has been speculation whether other related members of the AGC kinase family are also PDK1 targets. cAMP-dependent protein kinase (PKA) for example was shown to be an *in vitro* substrate for PDK1 (Cheng et al., 1998), but phosphorylation of T197, the T-loop site of PKA, as well as PKA activity were found to be similar in PDK1\(^{-/-}\) and PDK1\(^{+/+}\) ES cells (Williams et al., 2000). Additionally, mitogen- and stress activated protein kinase (MSK) 1 also possesses a potential PDK1 target T-loop motif, but MSK1 activity was comparable in PDK1\(^{-/-}\) and PDK1\(^{+/+}\) ES cells (Williams et al., 2000).

Just as PDK1\(^{-/-}\) ES cells are a helpful tool to study PDK1 targets, PDK1-deficient mice are useful to obtain further clues about the biological role of PDK1. PDK1\(^{-/-}\) mice highlight the requirement of PDK1 for normal embryonic development, as these animals die at day E9.5, displaying multiple abnormalities, including lack of forebrain, heart, dorsal root ganglia, and somites (Lawlor et al., 2002).

Additionally, PDK1 hypomorphic mice that express only 10 to 20% of WT PDK1 levels have been generated (Lawlor et al., 2002). These mice were viable and fertile, but were 30 to 40% smaller than control animals, and their organ volumes were proportionally reduced. The reduction in animal size was due to smaller cells rather than a reduction in cell number, indicating that PDK1 and the kinases it controls play crucial roles in regulating cell volume.
Notably, activation of PKB and S6K by insulin in these hypomorphic mice was not disturbed, demonstrating that regulation of cell size by PDK1 is independent of insulin’s ability to activate PKB and S6K. Importantly, this observation also emphasizes that even a reduction of PDK1 expression to 10 to 20% of its WT levels still allows apparently normal signaling towards PKB and S6K.

3.3 The PI3K/mTOR pathway and cancer

*Aberrant PI3K/mTOR signaling in cancer.* The PI3K pathway has been implicated in cancer ever since PI3K was discovered almost two decades ago as an enzymatic activity associated with viral oncoproteins (Whitman et al., 1998; Serunian et al., 1990). Meanwhile it has become evident that the PI3K/mTOR cascade is one of the most frequently targeted pathways in all sporadic tumors, with estimates suggesting that mutation in one or another PI3K pathway component accounts for up to 30% of all human cancers (Luo et al., 2003). For instance, the genes encoding the catalytic subunit p110α of PI3K and PKB are amplified in subsets of human cancers (Vivanco and Sawyers, 2002), and point mutations in p110α were found in 20-30% of breast, colon, brain and gastric tumors (Samuels et al., 2004). Ras, which binds and activates the catalytic subunit of PI3K, is activated in ~30% of epithelial tumors (Downward, 2003). Loss of function of the tumor suppressor PTEN, which counteracts PI3K action, occurs nearly as frequently as that of p53 (Cantley and Neel, 1999). Germline mutations of PTEN have been found in a number of dominantly inherited cancer syndromes including Cowden’s disease and Bannayan-Riley-Ruvalcaba syndrome (Nelen et al., 1996; Marsh et al., 1997). Furthermore, mutations in either TSC1 or TSC2 lead to tuberous sclerosis, a hamartoma syndrome associated with a predisposition to malignancy (Jones et al., 1999).

All the described aberrations affect mTORC1 activity, suggesting that the activation of mTORC1 contributes to cancer. Indeed, PKB cannot promote oncogenic transformation when mTORC1 activity is reduced (Skeen et al., 2006).
**Targeting the PI3K/mTOR pathway in cancer therapy.** Given the frequency of lesions in the PI3K/mTOR pathway, therapeutically targeting this pathway to treat cancer patients seems attractive.

Consistent with this hypothesis, the Rapamycin derivative CCI-779 was shown to inhibit the hyperproliferation of PTEN-deficient cells (Neshat et al., 2001), and to attenuate tumor development in PTEN heterozygous mice (Podsypanina et al., 2001). Consequently, Rapamycin-based mTOR inhibitors have been introduced into several clinical trials in the past couple years (Granville et al., 2006). However, even though tumorigenesis driven by a hyperactive PI3K-PKB pathway seems to require mTORC1 activity, Rapamycin analogues have not shown effective anti-tumor activity against such tumors (Galanis et al., 2005; Chan et al., 2005). This is likely due to the existence of an S6K-based feedback loop, whereby activation of S6K leads to phosphorylation and inhibition of IRS1 and IRS2, and consequently to a reduced ability to activate PI3K. Additionally, IRS1 and IRS2 mRNA levels are downregulated after extended mTOR activation. As a consequence, prolonged treatment with Rapamycin or its analogues could lead to enhanced PI3K-PKB signaling in some tumors (O'Reilly et al., 2006). Depending on other mutations in the tumor, this hyperactivation of PI3K-PKB could make the tumor more aggressive (Cloughesy et al., 2008).

Apart from Rapamycin analogues, inhibitors of the PI3K catalytic subunit p110 are also currently being tested in clinical trials. The applicability of these inhibitors though may be complicated by target-directed side effects such as impaired immune function caused by loss of p110δ function as well as problems in insulin response due to inhibition of p110α (Knight et al., 2006; Knight and Shokat, 2007 b).

**PDK1 as a drug target?** Seeking other, suitable drug targets within the PI3K/mTOR pathway, we were particularly interested in determining if PDK1 inhibition would represent an effective and safe therapeutic strategy. The rational behind this is several fold.

First, PDK1 is a key regulator of many AGC kinases that together control a plethora of events, such as cell growth, proliferation, survival, and metabolism.
(Figure 2), all of which play central roles in tumorigenesis. It is conceivable that simultaneous inhibition of several crucial AGC kinases at once could be more effective than, i.e. Rapamycin or PI3K inhibitors, by themselves. For example, PDK1 inhibition may block the pro-survival effects of PKB, as well as the cell growth and proliferation mediated by S6K and RSK. Besides, with PDK1 inhibition, S6K blockade and disruption of the feedback loop mediated by S6K should not lead to enhanced PKB activation as in the case of Rapamycin. Additionally, unlike Rapamycin, PI3K or PKB inhibitors, an efficient PDK1 inhibitor can be expected to also hamper signaling of at least some PKC isoforms. Given that PKC isozymes are activated by tumor promoting phorbol esters (Castagna et al., 1982) suggesting a role for PKC in tumor promotion and progression, this may provide an additional benefit for the treatment of cancer with PDK1 inhibitors.

Second, as mentioned above, PDK1 hypomorphic mice that express only ~10 to 20% of WT PDK1 levels are 30 to 40% smaller than WT littermates, but are fertile and healthy, indicating that a reduction in PDK1 activity does not have any major detrimental effects in an adult organism (Lawlor et al., 2002). Furthermore, since a great deal of knowledge about PDK1 function was gained using gene knockout or knockin technology, we were also highly interested in studying biochemical and biological consequences of acute PDK1 inhibition.

### 3.4 Inhibiting PDK1 with conventional inhibitors or a chemical genetic approach

While gene knockout technology, or knockin of an inactive mutant, can give valuable information about the role of a given protein, the lack of temporal control hampers the study of dynamic processes. Conditional alleles and RNA-based approaches (antisense and RNAi) overcome this limitation to some extent, but it generally requires several hours to change the protein levels in the cell. Moreover, the deletion of the entire protein of interest can often have effects that are different to merely inhibiting their catalytic activity. Compensation by other related proteins can mask events that are usually
mediated by the protein of interest, or changes in the levels of other proteins can give rise to additional unexpected phenotypes (reviewed in Knight and Shokat, 2007 a). On the other hand, small molecules can temporally and reversibly inhibit catalytic activity without affecting total protein levels or interacting proteins, and are thus more suitable to dissect dynamic cellular events.

UCN-01 (7-hydroxystaurosporine) and cyclooxygenase inhibitor analogues have been shown to inhibit PDK1, but these compounds are not very specific and are, therefore, not optimal to discern PDK1 function (Sato et al., 2002; Komander et al., 2003; Facchinetti et al., 2004; Arico et al., 2002; Ding et al., 2004; Karaman et al 2008). A very recent publication describes a set of ATP competitive PDK1 inhibitors, BX-795, BX-912, and BX-320, which, when tested against a small set of 10 other kinases *in vitro*, showed appreciable specificity for PDK1 over the kinases tested (Table 1) (Feldman et al., 2005). However, protein kinases are a class of proteins that are difficult to study because the human genome encodes over 500 kinases, all of which have highly homologous active sites. Studying kinases using classical genetics is especially complicated due to their redundancy and functional compensation in gene knockout experiments. For the same reasons, it is equally challenging to find mono-selective inhibitors for kinases that allow for specific inhibition of a single kinase in whole cells and animals.

To overcome these limitations of classical reverse genetics and conventional kinase inhibitors, Shokat and his group have devised a strategy that employs a specific mutation in the protein of interest to confer selectivity to a small molecule, an approach referred to as chemical genetics (Bishop et al., 2000 b; Knight and Shokat, 2007 a). A single residue in the ATP-binding pocket of kinases, termed the gatekeeper, has been shown to control sensitivity to a wide range of small molecule inhibitors (Bishop et al., 2000 a). In most kinases this gatekeeper residue is a conserved large hydrophobic amino acid, and mutation of this residue to alanine or glycine creates a novel pocket that can be uniquely accessed by an inhibitor analogue carrying a bulky side chain (Figure 4). The bulky side chain should prevent binding to and inhibition of
Table 1  Potencies of BX compounds for inhibition of PDK1 and other kinases in vitro

To address the selectivity of the compounds BX-912, BX-795, and BX-320 for PDK1, Feldman et al. tested their effect on ten different Ser/Thr kinases in vitro. This included the related AGC kinases PKA and PKCα. This table shows that BX-912, BX-795, and BX-320 are greater than 20-fold selective for PDK1 over other kinases in this panel, except for BX-912 which was 9-fold selective for PDK1 relative to PKA.
Figure 4  The chemical genetic approach

This scheme illustrates (on the left) the specificity problems that occur using general kinase inhibitors. Since kinase catalytic domains are highly conserved, a majority of potent inhibitors block the activity of closely related kinases and broadly hamper pathways mediated by kinase activity. The selective protein kinase inhibition approach, depicted on the right, can overcome such specificity problems. A space-creating mutation is introduced into the ATP-binding site of the kinase of interest, in our case PDK1. Replacement of a large amino acid by alanine or glycine enlarges the ATP-binding pocket such that a rationally designed small molecule inhibitor that carries a bulky side chain can fit in there, but should not fit into any WT kinase’s pocket. The design of complementary kinase–inhibitor pairs should permit highly specific inhibition of the kinase of choice.
wildtype (WT) kinases, and the mutation of the gatekeeper residue typically does not impair the mutated kinase. Hence, in a model system in which the WT allele of the kinase of interest has been replaced with the drug-sensitive allele, the effects of selective pharmacological inhibition of this engineered kinase can be explored (Bishop et al., 2000 b; Knight and Shokat, 2007 a).

Of note, even though not in the focus of the work described in the following, a slight modification of this strategy can also be employed to directly identify new kinase substrates. In this case, a designed ATP analogue interacts uniquely with the engineered mutant kinase of interest. When a radiolabeled $[^{32}\text{P}]$ ATP analogue is added to cell extracts, or a mix of mutant kinase with putative targets, the radiolabel is transferred only to direct substrates of the mutant kinase, which can then be identified. This strategy has been applied, for example, to study the direct targets of c-Jun amino-terminal kinase (JNK) (Habelhah et al., 2001), cyclin-dependent kinase 2 (CDK2) (Polson et al., 2001), and v-Src (Shah et al., 2002). Very recently, this protocol was improved to allow for substrate labeling in cells, or even mice, derived from a ‘gene knock-in’ allele sensitive (AS) kinase, as opposed to in vitro extracts, and, to allow for affinity purification of the kinase substrates (Allen et al., 2007). For this protocol, an AS kinase uses an N6-alkylated ATPγS to thiophosphorylate its substrates. The thiophosphorylated residue can then be alkylated and the target protein purified with thiophosphate ester-specific antibodies (Allen et al., 2007).

The work described in the following employed three strategies to study PDK1 function: 1.) conventional knockout technology involving the comparison of PDK1$^{-/-}$ and WT ES cells, 2.) a purely chemical approach using the small molecule inhibitor BX-795, and, 3.) most extensively, the chemical genetic methodology.
4. Aims

Binding of hormones and growth factors to their respective receptors leads to the activation of phosphatidylinositol 3-kinase (PI3K), which regulates a multitude of events including cell growth, proliferation, survival and motility. 3-phosphoinositide dependent kinase 1 (PDK1) has a central role in relaying the signal from PI3K to downstream effectors by phosphorylating the activation loop of several kinases leading to their activation and/or contributing to their stability. While genetic evidence indicates that protein kinase B (PKB, also known as Akt), p70 ribosomal S6 kinase (S6K), p90 ribosomal S6 kinase (RSK), serum- and glucocorticoid-induced kinase (SGK), and protein kinase C (PKC) are PDK1 targets, relatively little is known about the kinetics of signaling downstream of PDK1. Furthermore, the biological role of PDK1 is not yet fully elucidated and the consequences of specific acute PDK1 inhibition are not clear. Specifically, given PDK1’s central position in the PI3K signaling pathway, it is of great interest to determine the effects of PDK1 inhibition on cell survival, growth and tumorigenesis.

1. While knockout or conditional alleles of PDK1 have greatly aided the understanding of PDK1 function, they cannot provide insight into the dynamics of PDK1 downstream signaling. No highly specific inhibitor of PDK1 has been available which complicated studies of acute and transient inhibition. Very recently though, a novel compound, BX-795, was reported to be a highly specific inhibitor for this kinase. The first goal of this study was to investigate the effects of acute PDK1 inhibition on its downstream signaling. Therefore, first the specificity of BX-795 should be tested employing cells lacking PDK1. Any effects observed in PDK1-ablated cells would be due to off-target effects of BX-795. Should this reveal insufficient specificity of BX-795, then a model system should be generated that would allow a more specific inhibition of PDK1. To achieve this, a PDK1 mutant, PDK1 L159G (LG), should be generated that has an enlarged ATP-binding pocket and thus can be inhibited by purine analogues carrying bulky side chains that would not fit into wildtype (WT) kinase pockets. This mutant and the effect of several purine analogue inhibitors should first be characterized in vitro, and in vivo, and then this model system should be used to determine the biochemical consequences of PDK1 inhibition on downstream signaling.

2. The second aim of this work was to investigate biological roles of PDK1 employing both acute inhibition of PDK1 as well as cells lacking PDK1. Specifically, the involvement of PDK1 in cell growth, viability and apoptosis should be assessed. Furthermore, the teratoma growth and differentiation status of PDK1+/− embryonic stem (ES) cells should be compared to PDK1+/− ES cells, and PDK1−/− ES cells reconstituted with PDK1.
5. Results

5.1 Analysis of PDK1 signaling in ES cells

Much of the knowledge about PDK1 is derived from biochemical studies, the comparison of PDK1\(^{-/-}\) and PDK1\(^{+/-}\) ES cells (Williams et al., 2000), as well as mouse knockout and knockin studies (Lawlor et al., 2002; Collins et al., 2003; McMAnus et al., 2004; Collins et al., 2005). While these studies established PDK1 as a master controller of AGC kinases, a regulator of SGK, PKCs and PRKs and absolutely required for the activity of PKB, S6K and RSK, other biochemical and even more so biological effects of acute PDK1 inhibition are less clear. Thus, the major goal of this thesis was to address the in vivo effects of PDK1 inhibition. Acute inhibition using small molecule inhibitors for a protein of interest oftentimes provides more insight into a protein’s function than gene knockout or RNAi approaches. Since its discovery in 1997 (Alessi et al., 1997 a,b; Stokoe et al., 1997) PDK1 has been an extensively studied kinase. However, its characterization has been complicated by the fact that no highly specific kinase inhibitor for PDK1 has been available. Generally, it is difficult to find an ATP competitive inhibitor specific for a single kinase since there are over 500 kinases in the human genome, all of which share a highly homologous catalytic center.

Recently however, a set of PDK1 inhibitors was described which showed considerable specificity for PDK1 when tested \textit{in vitro} against ten other kinases (Feldman et al., 2005). To utilize PDK1 inhibition for future in vivo studies, the specificity of one of the inhibitors, BX-795, was first assessed in PDK1\(^{-/-}\) and PDK1\(^{+/-}\) ES cells. Since BX-795 affected also PDK1-deficient cells, another system, referred to as ‘chemical genetics’ was used in the second part of the present study to achieve more specific inhibition of PDK1. For this, a mutation was introduced into the ATP-binding pocket of PDK1, which enlarged the pocket and rendered PDK1 susceptible to inhibition by purine analogues carrying a bulky side chain. Using this mutant, PDK1 LG, the effect of a set of more than twenty purine analogue inhibitors on its activity was tested at first \textit{in vitro} and then in PDK1\(^{+/-}\) ES cells expressing the mutant.
kinase, or, as a control wildtype PDK1. Thereafter, some of the more potent and specific inhibitors were used to address the effect of PDK1 LG inhibition on cell cycle and cell growth. Furthermore the effect of short and long term inhibition of PDK1 LG on known and potential PDK1 targets was tested. It was noted that all purine analogues tested commonly seemed to have a non-specific effect on phosphorylation of S235/S236 of S6. To achieve more specific inhibition of PDK1 LG, derivatives of the semi-specific PDK1 WT inhibitor BX-795 were designed and synthesized to generate allele-specific inhibitors with less, or at least different, off-target effects.

5.1.1 The effect of BX-795 on G2/M arrest does not require PDK1

BX-795 is a recently developed aminopyrimidine-based inhibitor of PDK1, which potently inhibited PDK1 activity \textit{in vitro} (IC$_{50}$ = 6 nM) and reduces phosphorylation of PKB on T308 in cultured PC3 cancer cells with an IC$_{50}$ of 300 nM (Feldman et al., 2005). The ability of this compound to inhibit PDK1 signaling in mouse ES cells should now be assessed, and compared to the signaling in PDK1$^{-/-}$ mouse ES cells. Basal phosphorylation of PKB T308 in cells that are continuously grown in the presence of growth factors is sometimes too low to detect by Western blotting and its phosphorylation as well as the phosphorylation of other proteins is variable, depending on the nature, concentration and stability of growth factors present in the medium. In order to test the effects of BX-795 (and other inhibitors described in the following) on phosphorylation of PKB T308 and the phosphorylation of other proteins and to obtain reproducible results, the following experimental setup was used: cells were first starved of growth factors for 3 hours, and then treated with BX-795 for 30 minutes and another 30 minutes with BX-795 together with a distinct concentration of a defined growth factor (IGF1, 100 ng/ml), known to induce PI3K signaling, including phosphorylation of PKB T308. Consistent with the previous report, BX-795 strongly inhibited the IGF1-inducec phosphorylation of PKB T308, while having little effect on phosphorylation of PKB S473 (Fig. 5A), which is controlled by mTORC2 (Sarbassov et al., 2005). As expected, BX-795 also inhibited the IGF1-
Figure 5  Effects of BX-795 on signaling and cell cycle distribution in PDK1+/+ and PDK1+/− ES cells.

A, PDK1+/+ and PDK1+/− ES cells were starved of SR for 3 h, then treated with indicated concentrations of BX-795, 10 μM LY294004, or DMSO as a control for 30 min. Then medium was replaced with fresh medium containing BX-795 plus 100 ng/ml IGF1 or control. Cells were lysed after 30 min and subject to immunoblotting using the indicated antibodies.
Figure 5  Effects of BX-795 on signaling and cell cycle distribution in PDK1^{+/+} and PDK1^{-/-} ES cells.

B. PDK1^{+/+} and PDK1^{-/-} ES cells were cultured in KO-DMEM, 15% SR, 1000 U/ml LIF and treated with 5 μM BX-795 or DMSO as a control for 48 h, replacing medium with inhibitor or DMSO after 24 hours. The positive control for G2/M arrest was treated with 100 ng/ml Nocodazole 24 hours before harvest. Cells were subject to propidium iodide (PI) staining and cell cycle distribution was analyzed using a BD FACS Calibur. Shown is a representative experiment of four performed in triplicates, standard deviations of which are indicated. One exemplary result of three is shown for PDK1^{-/-} ES cells treated with DMSO or BX-795, whereby PI staining intensity, cell number and cell cycle distribution are indicated.
induced phosphorylation of PKB substrates such as GSK3α/β S21/S9 and PRAS40 T246. Furthermore BX-795 inhibited the IGF-induced phosphorylation of S6 S235/S236, two residues which are phosphorylated by S6K, a target of PDK1. In contrast to the previous report, S6K T389 phosphorylation was only slightly inhibited by BX-795, which could reflect differences in the regulation of mTORC1 activity in PC3 cancer cells versus ES cells. Consistent with this, previous reports have shown little alterations in mTORC1 activity in ES cells lacking PDK1 (Wang et al., 2001; Tominaga et al., 2005).

Next, the effects of BX-795 on the cell cycle of PDK1+/+ and PDK1−/− ES cells was examined. If BX-795 is a specific inhibitor for PDK1, the addition of this inhibitor to cultures should not affect the cell cycle distribution of ES cells lacking PDK1. To address this point, the frequency of cell in individual cell cycle stages was determined in PDK1+/+ and PDK1−/− ES cell cultures in the presence and absence of BX-795. ES cells have an unusually rapid cell cycle, with a large S phase population, and are refractory to many normal aspects of cell cycle control (Burdon et al., 2002). Nevertheless, an increase in the frequency of cells in the G2/M phase could clearly be detected in PDK1+/+ ES cells cultured in the presence BX-795, which was also observed using Nocodazole (Noc), a substance known to induce G2 arrest (Fig. 5B). Surprisingly, a similar increase in G2/M arrested cells was also apparent in PDK1−/− ES cells treated with BX-795. This suggested that BX-795 induces G2/M arrest by inhibiting protein kinases other than PDK1. Profiling BX-795 inhibitory activity against 211 protein kinases, in vitro kinase assays with purified proteins, showed that several protein kinases in addition to PDK1 were strongly inhibited by 1 μM BX-795 (data not shown). Among these were protein kinases that influence the cell cycle such as Cdk1, Cdk2, Aurora A, Aurora B, and Aurora C, all of which influence the cell cycle. Therefore, it is likely that one of these is the relevant target responsible for G2/M arrest, and not PDK1. In summary, BX-795 cannot be used to specifically block PDK1 activity and assess its physiologic functions in vivo.
5.1.2 Identification of inhibitor analogues to block the genetically modified PDK1, PDK1 LG, \textit{in vitro} and \textit{in vivo}

Because of the apparent non-specific effects of BX-795, a system should be developed to inhibit PDK1 activity more specifically in ES cells. Mutation of the leucine residue at position 159 to glycine (L159G) in PDK1 creates an enlarged ATP-binding site, potentially allowing inhibition by compounds unable to bind wildtype kinases, since they have a smaller ATP-binding site (Figures 4 and 6). This approach has been successfully applied to other protein kinases (reviewed in Bishop et al., 2001), although it is not universally tolerated (Niswender et al., 2002; Kenski et al., 2005).

First, the activity of this mutant and its ability to be inhibited \textit{in vitro} by previously described analogues of PP1, a general kinase inhibitor was tested. For this, wildtype PDK1 (PDK1 WT) and PDK1 with the L159G mutation (PDK1 LG) were purified from insect cells. The kinase activity of both PDK1 variants was tested by analyzing the radioactivity transferred from $\gamma^{32}$P-ATP to the PDK1 substrate PKB in the presence of various kinase inhibitors. The results are summarized in Fig. 7, which shows that PDK1 LG retains similar activity to WT PDK1 as measured by its ability to phosphorylate $\Delta$PH-PKB, a PH-domain-deleted mutant of PKB that can be phosphorylated by PDK1 in the absence of PIP$_3$. Importantly, all the analogues tested strongly inhibited the activity of PDK1 LG, whereas PDK1 WT was not or only partially inhibited as, for example, observed with 4-MeOB-PP1 (abbreviated as 4-Me-OB in Fig. 7).

Next, a cell-based system to analyze the ability of analogues of the PP1 kinase inhibitor to interfere with PDK1 LG was established. PDK1$^{-/-}$ ES cells have previously been shown to lack basal as well as IGF1-induced phosphorylation and activation of a number of PDK1 substrates (Williams et al., 2000). However, it is possible that the long term lack of PDK1 protein has resulted in compensatory phosphorylation of certain substrates by other protein kinases, or that additional secondary events have changed the properties of these cells relative to PDK1$^{+/+}$ ES cells. Therefore, PDK1$^{-/-}$ ES
Results

Figure 6  Schematic illustration of PDK1 protein structure

The PDK1 gene is located on human chromosome 16p 13.3 and is expressed ubiquitously in human tissues. PDK1 exist as a single isoform and no splicing variants are known. Human PDK1 (PDPK1_Human, accession numbers: O15530; CAG38755; NP_002604) is a 556 amino acid long protein, the schematic illustration of which is shown here. Both mouse PDK1 (PDPK1_MOUSE, Q9Z2A0) and rat PDK1 (PDPK1_RAT, O55173) are 559 amino acid long polypeptides, with a single amino acid insertion at residue 43 and a two amino acid insertion at residue 67 compared to the human sequence. PDK1 contains few characteristic domains (http://smart.embl-heidelberg.de/smart/show_motifs.pl). Shown here is a schematic of the human PDK1 protein structure, including its Ser/Thr kinase (amino acids 82-342) as well as the C-terminal pleckstrin homology (PH) domain (459-542) mediating protein-lipid interaction (Alessi et al., 1997). Highlighted in red is L159 (L162 for mouse), the gatekeeper leucine that was replaced by a glycine to generate an inhibitable mutant of PDK1, L159G. To generate this mutant, human PDK1 cDNA was cloned and PCR mutagenesis was employed to introduce a two base pair mutation, replacing leucine 159 with glycine.
Figure 7  Inhibition of PDK1 L159G by PP1 analogues *in vitro*.

The effects of several purine analogues on the activity of WT PDK1 and on PDK1 L159G was compared by *in vitro* kinase assay. The assay was carried out with ΔPH-PKB (210 ng/µl) as a substrate and either WT (150 ng/µl) or mutant PDK1 (500 ng/µl) as kinase. All reactions were done in duplicates in kinase buffer, 20 µM ATP and 5 µCi of [γ-32P]ATP. All inhibitors were used at 50 µM. After 15 min incubation at 30°C the reactions were stopped, and separated on 12% Tris-glycine gels. A phosphorimage screen was put onto the gels and exposed for 30 min. Incorporated
$^{32}$P-activity was assessed by scanning the screen with a STORM PhosphorImager. A, shows the scan obtained in one of three similar experiments whereby black bands represent phosphorylated proteins, either autophosphorylated PDK1 or PKB phosphorylated by WT or mutant PDK1. B, displays the quantification of measured radioactivity and illustrates the structure of the purine analogues used.
Results

cells were transfected by electroporation with PDK1 WT and the PDK1 LG variant and stable pools termed PDK1\(^{-/-}\)+WT and PDK1\(^{-/-}\)+LG cells were established by drug selection. This completely recovered the signaling defects seen in the knockout cells, as judged by restoration of IGF1-inducible phosphorylation of PKB on T308 (Fig. 8A). PKB S473 phosphorylation was minimally affected by loss of PDK1, as previously shown (Williams et al., 2000). In addition, the inducible phosphorylation of the downstream PKB substrates GSK3 and PRAS40 was also fully restored following expression of WT or PDK1 LG (Fig. 8A). Phosphorylation of S6 is completely abolished in PDK1\(^{-/-}\) ES cells, due to the defective phosphorylation of S6K on both the activation loop site T229, which is a direct target of PDK1, as well as the HM site T389, a direct target of mTORC1 (Burnett et al., 1998). While this latter observation might implicate defective mTORC1 activity in PDK1\(^{-/-}\) ES cells, this does not appear to be the case as 4E-BP1 phosphorylation is unaffected (Fig. 8A, and Tominaga et al., 2005). Nevertheless, S6K T389 phosphorylation was restored upon re-expression of either WT or PDK1 LG (Fig. 8A). Furthermore, the cell size defect seen in PDK1\(^{-/-}\) relative to PDK1\(^{+/+}\) ES cells (Lawlor et al., 2002 and Fig. 8B) was also partially reversed upon expression of either PDK1 allele (Fig. 8B). The protein levels of the phosphoproteins assessed were also determined as far as commercial antibodies were available. Of the proteins studied a difference in overall levels in PDK1\(^{-/-}\) compared to PDK1\(^{+/+}\) and PDK1\(^{-/-}\)+LG and PDK1\(^{-/-}\)+WT, only some PKC isoforms displayed reduced levels, as determined with an antibody recognizing all PKC isoforms, consistent with previous reports (Balendran et al., 2000; Dutil et al., 1998).

Then the PP1 analogues shown in Fig. 7, as well as additional ones shown in Fig. 9A were tested for their ability to inhibit PDK1 signaling in ES cells reconstituted with PDK1 WT or PDK1 LG by determining the phosphorylation pattern of established PDK1 substrates in the presence of IGF1. Two compounds, 3,4-DMB-PP1 and 1-NM-PP1, emerged as being quite potent and selective for PDK1\(^{-/-}\)+LG over PDK1\(^{-/-}\)+WT ES cells (Fig. 9B and C respectively). A short one hour incubation with these compounds inhibited IGF1 stimulated phosphorylation of PKB T308 in PDK1\(^{-/-}\)+LG ES cells.
Figure 8  Reconstitution of WT and L159G PDK1 into PDK1<sup>−/−</sup> ES cells.

**A**, PDK1<sup>+/+</sup>, PDK1<sup>−/−</sup>, and PDK1<sup>−/−</sup> ES cells stably expressing either PDK1 WT (PDK1<sup>−/−</sup> +WT) or PDK1 L159G (PDK1<sup>−/−</sup> +LG) were starved of SR for 3 hours and then stimulated for 30 min with 100 ng/ml IGF1 before lysis. Proteins were immunoblotted using the indicated antibodies as described in Materials and Methods. **B**, PDK1<sup>+/+</sup>, PDK1<sup>−/−</sup> +WT and PDK1<sup>−/−</sup> +LG ES cells were grown in KO-DMEM with 15% SR and 1000 U/ml LIF. Size of cells in G1 was analyzed using BD FACS Calibur. Mean forward scatter values were determined for each cell line. Results are displayed relative to PDK1<sup>+/+</sup> cell size.
Results

A

2.5-DMB-PP1  1,3-DMB-PP1  3.4-DMB-PP1  2.3-DMB-PP1

1-NM-PP1  2-NM-PP1

B

<table>
<thead>
<tr>
<th>Inhibitor [µM]</th>
<th>PDK1&lt;sup&gt;-/-&lt;/sup&gt;</th>
<th>PDK1&lt;sup&gt;-/-&lt;/sup&gt; +LG</th>
<th>PDK1&lt;sup&gt;-/-&lt;/sup&gt; +WT</th>
</tr>
</thead>
<tbody>
<tr>
<td>IGF1</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>3,4-DMB-PP1</td>
<td>-</td>
<td>-</td>
<td>5 10 20 50</td>
</tr>
<tr>
<td>PKB (T308)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PKB (S473)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GSK3α/β (S21/S9)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S6K (T389)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S6 (S235/S236)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PRAS40 (T246)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Actin</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

C

<table>
<thead>
<tr>
<th>Inhibitor [µM]</th>
<th>PDK1&lt;sup&gt;-/-&lt;/sup&gt;</th>
<th>PDK1&lt;sup&gt;-/-&lt;/sup&gt; +LG</th>
<th>PDK1&lt;sup&gt;-/-&lt;/sup&gt; +WT</th>
</tr>
</thead>
<tbody>
<tr>
<td>IGF1</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>1-NM-PP1</td>
<td>-</td>
<td>5 10 20 50</td>
<td>-</td>
</tr>
<tr>
<td>PKB (T308)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PKB (S473)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GSK3α/β (S21/S9)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S6K (T389)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S6 (S235/S236)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PRAS40 (T246)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Actin</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Figure 9  Effects of PP1 analogues on PDK1 inhibition in ES cells.

A. Additional PP1 analogues used for in cell culture studies. B-D. PDK1<sup>+/+</sup>, PDK1<sup>-/-</sup>, PDK1<sup>-/-</sup> +WT, and PDK1<sup>-/-</sup> +LG ES cells were starved of SR for 3 h, then treated with indicated concentrations of 3,4-DMB-PP1 (B), 1-NM-PP1 (C), or DMSO as a control for 30 min. Then medium was replaced with fresh medium containing respective inhibitor plus 100 ng/ml IGF1 or control. Cells were lysed after 30 min and subject to immunoblotting using the indicated antibodies. D. Quantitation of effects of 3,4-DMB-PP1 and 1-NM-PP1 on phosphorylation of various proteins in PDK1<sup>-/-</sup> +WT and PDK1<sup>-/-</sup> +LG ES cells. Western blots shown in (B) and (C) were quantified using NIH ImageJ and SigmaPlot software was employed to fit curves.
Phosphorylation of PKB targets GSK3α/β S9/S21, and PRAS40 T246, as well as S6 S235/S236 was equally inhibited (Fig. 9B, C and D). 3,4-DMB-PP1 and 1-NM-PP1 had a smaller but reproducible effect on S6K T389 and PKB S473 phosphorylation. These compounds had minimal effects on PKB T308, GSK3α/β S9/S21, and PRAS40 T246 phosphorylation sites in PDK1+/−+WT ES cells at concentrations effective in PDK1+/−+LG ES cells. In contrast to 3,4-DMB-PP1 and 1-NM-PP1, many of the other PP1 analogues that were tested did show some degree of PDK1 inhibition in PDK1+/−+WT ES cells in addition to PDK1+/−+LG ES cells (Fig. 10A and B). Moreover, it was noticed that S6 phosphorylation in particular was sensitive to many of these PP1 analogues, even in PDK1+/−+WT ES cells (Fig. 10A and B) and PDK1+/−+ES cells (data not shown). To distinguish between an effect of these inhibitors on mTORC1 activity or an upstream kinase versus an effect on S6K activity itself, 4E-BP1 phosphorylation in WT PDK1 ES cells was analyzed in response to these inhibitors. 4E-BP1 phosphorylation was rarely affected (Fig. 13E and data not shown), suggesting that mTORC1 is not the target and that S6K itself might be particularly susceptible to this class of PP1 analogues. Figures 10A and B summarize the in cell IC50 values for all compounds and phosphorylation sites tested.

Before examining further biochemical and any potential biological consequences of PDK1 inhibition, these compounds were tested for their ability to durably inhibit PDK1 activity. Most assays for biological roles of PDK1 will require inhibition of PDK1 activity for more than one hour, so it is important to determine how long PDK1 activity stays suppressed for after addition of inhibitor. When cells are cultured in the presence of 15% serum replacement (SR), PKB phosphorylation at T308, the most important readout, is barely or not detectable at all in the ES cells used (data not shown). Therefore, ES cells need to be stimulated with a growth factor, for example IGF1, shortly before harvesting to enhance activation of the PI3K/PDK1 pathway. Thus, to address the duration of PDK1 inhibition, PDK1+/−+LG ES cells were starved for 3 hours of SR, then treated with inhibitor or DMSO as a negative control for 23.5 hoursl, and 30 min before harvest, IGF1 was added to the medium (in contrast to the experiments described prior, where medium
**Figure 10**  Quantitative comparison of effects of PP1 analogues in ES cells.  

**A**, IC$_{50}$ values of a panel of PP1 analogues and BX-795 of inhibition of phosphorylation of several signaling proteins in PDK1$^{-/-}$+WT and PDK1$^{-/-}$+LG ES cells. PDK1$^{-/-}$+WT and PDK1$^{-/-}$+LG ES cells were treated with increasing concentrations of PP1 analogues or BX-795. Western blots were quantified using NIH ImageJ and SigmaPlot software was employed to determine IC$_{50}$ concentrations for each individual inhibitor, cell line, and all phosphoproteins assessed. PP1 analogues shown in shades of red inhibit PDK1 signaling in PDK1$^{-/-}$+LG but not PDK1$^{-/-}$+WT ES cells. Compounds shown in black symbols have no or relatively little effect on phosphorylation of the assessed proteins. Green symbols represent compounds with higher nonspecific effects.  

**B**, IC$_{50}$ data visualized as a heatmap generated with Java Treeview.
Results

Figure 11  **PDK1 LG remains inhibited in cell culture 24 hours after drug addition.**

PDK1\(^{-/-}\) +LG ES cells were starved of SR for 3 h, then treated with 20 μM 3,4-DMB-PP1, 20 μM 1-NM-PP1, 5 μM BX795, 10 μM LY294004, or DMSO as a control for 23.5 hours. Then IGF1 was added to the medium to yield a final concentration of 100 ng/ml. Cells were lysed after 30 min and subject to Western blotting.
was replaced 30 min before harvest) to activate PI3K signaling. Fig. 11 shows that PDK1 downstream signaling as measured by PKB T308, GSK3α/β S9/S21, and S6 S235/S236 phosphorylation, remained inhibited 24 hours after the addition of the inhibitor. Interestingly for 3,4-DMB-PP1 and 1-NM-PP1, S6K T389 phosphorylation seemed more dramatically inhibited at these later time points relative to the 1 hour time point examined in Fig. 9. In contrast, BX-795 actually caused increased T389 phosphorylation. The reason for this discrepancy is not clear, but could represent effects of caused by as yet unknown targets of BX-795.
5.1.3 Examination of phosphorylation of PDK1 targets following long term inhibition of PDK1 activity

Next, the phosphorylation state of additional known and potential PDK1 targets within the AGC kinase family was analyzed. An alignment of T-loop sequences of several AGC kinases was established using the ClustalW algorithm, illustrating the homology of the 20 amino acids surrounding the phosphorylatable serine or threonine T-loop site (Fig. 12). The effects of PDK1 inhibition on phosphorylation of some of these sites was addressed using commercial phospho-specific antibodies.

Confirming previous reports, several AGC kinases showed strongly reduced to absent phosphorylation of their activation loops in PDK1\(^{-/-}\) ES cells, including RSK, PRK1/2, and some isoforms of PKC. As expected, phosphorylation was restored in PDK1\(^{-/-}\) ES that were complemented with PDK1 LG (PDK1\(^{-/-}\) +LG ES cells) (Fig. 13A). Phosphorylation of PKA T197 relative to total PKA was also slightly decreased in PDK1\(^{-/-}\) ES cells when compared to PDK1\(^{-/-}\) +LG ES cells. Next, the phosphorylation on these sites following incubation with the PP1 analogues 1-NM-PP1 and 3,4-DMB-PP1 in PDK1\(^{-/-}\) +LG cells were analyzed. As members of this group include protein kinases activated by stimuli other than IGF1, we also included 12-O-tetradecanoyl-phorbol-13-acetate (TPA), forskolin, and sorbitol as protein kinase activators in this analysis. TPA is a potent activator of PKCs (and tumor promoter); forskolin activates the enzyme adenylyl cyclase, which increases the intracellular levels of cyclic adenosine monophosphate (cAMP) and thereby activates PKA. Sorbitol at concentrations ≥200 mM is frequently used to induce osmotic shock, which is known to – amongst other cascades – activate the p38/MSK pathway (Han et al., 1994; Deak et al., 1998).

To analyze the effects of basal as well as activator-induced phosphorylation, inhibitors were added 23.5 hours prior to cell stimulation in these experiments. Again, 3,4-DMB-PP1 and 1-NM-PP1 inhibited PKB T308 phosphorylation in response to IGF1 (Fig. 13B). Moreover, basal as well as activator-induced phosphorylation of GSK3 and PRAS40 at PKB sites were inhibited by 3,4-DMB-PP1 and 1-NM-PP1. Interestingly, sorbitol-induced GSK3
Figure 12  Phylogenetic tree of several AGC kinase T-loop sequences.

Human and murine AGC kinase T-loop sequences were taken from NCBI and Ensembl databases. A phylogenetic tree was built using the EBI ClustalW algorithm. Shown are human sequences; mouse sequences are identical unless marked with an asterisk. The phosphorylateable T-loop threonine or serine is displayed in bold and marked by an arrow. For reference, the bona fide PDK1 substrate PKB/Akt T308 is highlighted yellow.

(*Mouse sequence differs one to three amino acids. "All PKA isoforms have identical T-loop sequences. SGK2 T193 represents α, T253 the β isoform; RSK2 S218 for isoform a, S226 for isoform b.)
Results

Figure 13  PDK1 LG inhibition differently effects T-loop phosphorylation of PDK1 targets.

Figure 13 A-E legends can be found on page 48
Figure 13  
PDK1 LG inhibition differently effects T-loop phosphorylation of PDK1 targets.

Figure 13 A-E legends can be found on the following page.
Figure 13  PDK1 LG inhibition differently effects T-loop phosphorylation of PDK1 targets.

A, Comparison of PDK1−/− and PDK1−/− +LG ES cells. Cells were starved of SR for 3 h, then treated for 30 min with 200 μM TPA or control before harvesting and submitting to Western blot analysis using the indicated antibodies. B-D, PDK1−/− +LG ES cells were starved of SR for 3 h, then treated with 5 μM 3,4-DMB-PP1, 5 μM 1-NM-PP1, 10 μM SB203580 and 10 μM UO126, or DMSO as a control for 23.5 h. Then medium was replaced with fresh medium containing respective inhibitor plus 100 ng/ml IGF1, 200 μM TPA, 200 μM Forskolin, or 480 mM sorbitol, or no stimulus. After 30 min cells were lysed and subject to Western blotting. Shown are effects of PDK1 LG inhibition on (B) PKB/Akt T308 phosphorylation and PKB/Akt downstream signaling (GSK3 S9/S21, PRAS40 T246, and S6 S235/S236 phosphorylation), (C) T-loop phosphorylation of RSK1/2 S221/S227, PKC isoforms, PRK1/2 T774/T816, PKA T197, and PDK1 S241 and the hydrophobic motif site of RSK1 S380, (D) T-loop phosphorylation of MSK1 S212, the ERK/p38 MAPK phosphorylation sites S360 and T581, total MSK1, phosphorylated ERK1/2 T202/Y204, and phosphorylated p38 MAPK T180/Y182. E, Effect of PDK1 LG inhibition on S6K and mTOR signaling towards S6K T389, S6 S235/S236 and S240/S244, 4E-BP1 S65 and S37/S46. PDK1−/− +LG and +WT ES cells were starved of SR for 3 h, then treated with 5 μM 3,4-DMB-PP1, 5 μM 1-NM-PP1, or 100 nM Rapamycin for 23.5 h, then medium was replaced with fresh medium containing respective inhibitor plus 100 ng/ml IGF1 for 30 min before lysis. T-loop sites are indicated by an asterisk, dark arrowheads indicate phospho-MSK1, the open arrowhead marks a crossreactive phospho-RSK band.
phosphorylation appears to be somewhat resistant to PDK1 inhibition, and instead is inhibited by U0126, a MEK1/2 inhibitor, and SB203580, a p38 MAPK inhibitor, suggesting that GSK3 is phosphorylated by kinases in addition to PKB in response to osmotic stress. Phosphorylation of S6 S235/S236 is inhibited by 3,4-DMB-PP1 and 1-NM-PP1 as expected, but is also strongly inhibited by U0126 and SB203580, suggesting an input to these sites from MAP kinases in these cells.

Next, phosphorylation of the N-terminal kinase domain of RSK was addressed. There are four isoforms of RSK: RSK1, RSK2 and RSK3 are 75 to 80% identical at the amino acid level, whereas the more recently discovered RSK4 is more distantly related. The regulation of RSK isoforms is complex: RSK is composed of two functional kinase domains that are activated in a sequential manner by a series of phosphorylations. Phosphorylation of RSK by ERK1/2 at several sites leads to activation of the C-terminal kinase domain which autophosphorylates a site in the linker region between the two kinase domains. PDK1 can dock at this phosphorylated residue S380, which is the HM site for the N-terminal kinase domain, and phosphorylates the activation loop site of the N-terminal kinase domain, S221 and S227 of RSK1 and RSK2 respectively (for RSK4 the PDK1 dependency is less clear). This in turn activates the N-terminal kinase domain, which then phosphorylates RSK targets (Frodin and Gammeltoft, 1999; Dummler et al., 2005).

Phosphorylation of the N-terminal kinase domain activation loop of RSK1 and RSK2 is highly dependent on PDK1 activity. As expected, 3,4-DMB-PP1 and 1-NM-PP1 therefore show strong inhibition of both basal and TPA-stimulated phosphorylation of S221 and S227, which are activation loop sites of RSK1 and RSK2 respectively (Fig. 13C). In contrast, phosphorylation of the hydrophobic motif site S380, which is phosphorylated by the RSK C-terminal kinase domain following phosphorylation and activation by MAPKs, was unaffected by 3,4-DMB-PP1 or 1-NM-PP1.

PRK1/2 have been shown to be phosphorylated by PDK1 at their activation loop in vitro and in transiently transfected cells (Flynn et al., 2000).
Surprisingly, we saw very little to no effect of PDK1 inhibition on the phosphorylation of PRK1/2 under the conditions tested (Fig. 13C).

Analysis of multiple PKC isoforms using an antibody that recognizes phosphorylated PKC activation loops showed that only two putative PKC isoforms were sensitive to PDK1 inhibition. Experiments with isoform-specific phospho-antibodies revealed that neither of these represented PKCγ or PKCθ (data not shown). Therefore, it is still unclear which PKC isoforms are the targets of PDK1-mediated phosphorylation, and which are independent of PDK1 in these cells.

Phosphorylation of PKA at T197 was in some experiments very slightly decreased following treatment with 3,4-DMB-PP1 and 1-NM-PP1 and phosphorylation of PDK1 itself on its autophosphorylation site S241 was also slightly but consistently decreased following addition of 3,4-DMB-PP1 or 1-NM-PP1 (Fig. 13C).

MSK1 and 2 share a similar two kinase domain structure and activation profile with RSK. Unlike the RSK C-terminal kinase domain of RSK, which can be activated only by the ERK branch of MAPK kinase signaling, the C-terminal kinase domain of MSK can be activated by both phosphorylation by ERK as well as p38 MAPK. Despite the similarity of RSK and MSK, the activation of MSK by UV or TPA was similar in PDK1−/− and PDK1+/+ ES cells, unlike that of RSK in response to appropriate stimuli (Fig. 13 and Williams et al., 2000). Given the high analogy between the RSK and MSK activation loop sequences (Fig. 12), it should be assessed whether MSK might under certain conditions also be a target for PDK1. Initial experiments indicated that phosphorylation of the activation loop site in the N-terminal kinase domain of MSK1 in response to sorbitol (Fig. 13D) and MSK1 activity itself (data not shown) were sensitive to PDK1 inhibition. However, subsequent experiments showed that 3,4-DMB-PP1 or 1-NM-PP1 also inhibited sorbitol-induced and ERK/p38 MAPK-mediated phosphorylation of MSK1 at S581 as well as ERK/p38 MAPK-dependent autophosphorylation at S376 (Fig. 13D). Moreover, inhibition of p38 MAPK phosphorylation itself by these compounds was also observed. Therefore, the inhibition of the activation loop phosphorylation and activity of
MSK1 as well as MSK2 by 3,4-DMB-PP1 or 1-NM-PP1 is likely a secondary event due to non-specific inhibition of the priming site phosphorylation. These results therefore indicate that phosphorylation of the N-terminal kinase domain activation loop site in MSK1 occurs independently of PDK1, which is consistent with previous observations (McCoy et al 2005; Williams et al., 2000).

Additionally, the effect of 3,4-DMB-PP1 and 1-NM-PP1 on the T-loop phosphorylation of S6K (T229) was assessed. However, none of the available phospho-specific antibodies worked reliably enough to obtain interpretable results. Therefore, S6K activity was assessed indirectly by analyzing its phosphorylation at T389 as well as phosphorylation of S6 at S6K specific sites, namely S240/S244 (Pende et al., 2004). Also, mTORC1 activity was analyzed further by assessing mTORC1-mediated and -specific phosphorylation of 4E-BP1 at S37/S46 and S65 (McMahon et al., 2002). The 24 hour inhibition of PDK1 activity resulted in a larger decrease of S6K T389 phosphorylation than a 1 hour incubation (Fig. 9B,C). This suggests that T229 dephosphorylation and the effect of T229 dephosphorylation on the stability of T389 phosphorylation occurs between 1 hour and 24 hours of PDK1 inhibition. Selective inhibition of S6 S240/S244 by 3,4-DMB-PP1 or 1-NM-PP1 was also seen, confirming the inhibition of S6K activity in PDK1-/- +LG ES cells (Fig. 13E). In contrast, a reduction in phosphorylation of 4E-BP1 at any of the mTORC1 sites was not observed, confirming that mTORC1 activity is not affected following inhibition of PDK1 and PKB activities in ES cells. Interestingly, in 24 hour experiments, inhibition of S6 S235/S236 phosphorylation by 3,4-DMB-PP1 and 1-NM-PP1 was also apparent in PDK1+/+ +WT ES cells, similar to the effects seen after 1 hour at high concentrations of these drugs, even though S240/S244 phosphorylation was unaltered (Fig. 13E).

The temporal effect of inhibiting PDK1 on the phosphorylation of its direct downstream substrates is summarized in Table 2.

Taken together, the experiments involving short -1 hour- and longer -24 hours- of PDK1 inhibition demonstrate, that different PDK1 targets are affected by PDK1 inhibition with different kinetics: While PKB T308
### Table 2 Effects of 3,4-DMB-PP1 and 1-NM-PP1 on T-loop phosphorylation of assessed AGC kinases.

Summary of effects on T-loop phosphorylation after 1 hour (blots not shown) and 24 hours (blots shown in figures 13B-D) inhibitor treatment. ‘–’ indicates no inhibition, ‘+’ / ‘++’ / ‘+++’ degrees of inhibition; ‘- / ++’ for phospho pan PKC reflects different effects on different PKC isoforms.

<table>
<thead>
<tr>
<th>target</th>
<th>Inhibition by 3,4-DMB and 1-NM</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1 h</td>
<td>24 h</td>
</tr>
<tr>
<td>PKB T308*</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>RSK1/2 S221/S227*</td>
<td>-</td>
<td>+++</td>
</tr>
<tr>
<td>Phospho pan PKC*</td>
<td>- / ++</td>
<td>- / ++</td>
</tr>
<tr>
<td>PRK1/2 T774/T816*</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>PKA T197*</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>PDK1 S241*</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>

Table 2 Effects of 3,4-DMB-PP1 and 1-NM-PP1 on T-loop phosphorylation of assessed AGC kinases.
phosphorylation is already maximally inhibited after 1 hour of PDK1 inhibition and still inhibited after 24 hours inhibitor treatment, phosphorylation of RSK1/2 S221/S227 is barely affected after 1 hour, but strongly reduced after 24 hours of PDK1 inhibition. Furthermore, the experiments of 24 hours PDK1 inhibition also demonstrate that T-loop phosphorylation of some PKC isoforms is dependent on PDK1, whereas T-loop phosphorylation of other isoforms seems to be completely independent of PDK1. No effect of PDK1 inhibition was seen on the T-loop phosphorylation of PRK1 and 2, which was unexpected as previous reports indicated that PRK1 and 2 were PDK1 substrates. The T-loop phosphorylation of PKA at T197 was also not at all or only very slightly affected by PDK1 inhibition for 1 hour or 24 hours, suggesting that PDK1 is by far not the major kinase phosphorylating this site in ES cells, but may make a minor contribution to its phosphorylation. The T-loop phosphorylation of PDK1 itself at S241 was modestly but consistently reduced after 24 hours (but not 1 hour) of PDK1 inhibition, indicating that this S241 may indeed be a target for autophosphorylation. Overall, the method of acutely inhibiting PDK1 LG does not, in contrast to other reports (Flynn et al., 2000) indicate that PRK1 and 2 are PDK1 targets, at least not in ES cells. The data obtained so far does not fully clarify the question whether PKA T197 is a PDK1 target or not, but clearly confirms the previously reported PDK1 targets PKB T308 (Alessi et al., 1997; Stokoe et al., 1997), RSK1/2 S221/S227 (Williams et al., 1998) and PDK1 S241 (Casamayor et al., 1999; Wick et al., 2003), and beyond these reports, gives clues about the kinetics of dephosphorylation of these targets.

5.1.4 Generation and characterization of BX-795-based allele-specific PDK1 inhibitors

While 3,4-DMB-PP1 and 1-NM-PP1 in combination with PDK1 LG and PDK1 WT as a control may represent useful probes to analyze the effects of specifically inhibiting PDK1 activity, they suffer from lack of potency (IC$_{50}$ of 1-5 μM at inhibiting PKB T308 phosphorylation in cells), lack of selectivity (inhibition of PDK1-independent phosphorylation of p38 MAPK and S6
Results

phosphorylation in PDK1^−/− +WT ES cells) and PDK1-independent growth inhibition (decrease in cell viability in PDK1^−/− +WT ES cells). Therefore, we sought to improve upon the initial design of adding chemical groups onto the generic protein kinase inhibitor PP1, to modifying BX-795, a potent inhibitor of PDK1 that also inhibits a smaller number of additional protein kinases (Feldman et al., 2005, and our unpublished results). We reasoned that using a completely different chemical scaffold which was more specific to PDK1 would reduce the off-target effects that all the pyrazolopyrimidines seemed to commonly have. Modeling of BX-795 in the active site of PDK1 revealed that the Iodo group lies ~3 Å from the side chain of L159, suggesting that modifications at this group may potently and specifically inhibit PDK1. Therefore, the compounds shown in Fig. 14A were made and tested for their ability to inhibit phosphorylation of PKB T308 in PDK1^−/− +LG and PDK1^−/− +WT ES cells. CPAc-BX potently inhibited the phosphorylation of PKB T308 in PDK1^−/− +LG ES cells but not in PDK1^−/− +WT ES cells (Fig. 14B), suggesting that this inhibitor blocks PDK1 LG but not PDK1 WT. Therefore, the analysis of CPAc-BX was extended to additional PDK1-dependent targets, which confirmed that the potency of CPAc-BX was indeed enhanced on GSK3α/β S21/9 and PRAS40 T246 phosphorylation (Fig. 14C and D). However, non-specific effects on S6 S235/S236 phosphorylation at higher CPAc-BX concentrations were apparent, similar to those seen with 3,4-DMB-PP1 and 1-NM-PP1. The in cell IC50 values of CPAc-BX towards PKB T308 and S6 S235/236 phosphorylation are illustrated in Fig. 14E.

Taken together, CPAc-BX is a significantly more potent inhibitor of PDK1 LG than 1-NM-PP1 or 3,4-DMB-PP1. However, it also nonspecifically inhibits phosphorylation of S235/S236 of S6. As for 1-NM-PP1 and 3,4-DMB-PP1 the concentrations of CPAAs-BX needed to achieve 50% inhibition of PKB phosphorylation on T308 are close to those inhibiting phosphorylation of S6 S235/S236 nonspecifically. Therefore, CPAc-BX does not provide an enlarged specificity-window, and is thus not superior to 1-NM-PP1 and 3,4-DMB-PP1 in terms of selectivity. This indicates that 1-NM-PP1, 3,4-DMB-PP1 and CPAc-BX in conjunction with PDK1 LG can only be used with great
Figure 14  PDK1 LG inhibition differently effects T-loop phosphorylation of PDK1 targets.

Figure 14 A-E legends can be found on page 57
Figure 14  BX-795 analogues as PDK1 LG inhibitors.

Figure 14 A-E legends can be found on the following page.
Results

Figure 14  BX-795 analogues as PDK1 LG inhibitors.

A, Structure of BX-795 and its derivatives. B, C, PDK1^-/- +LG and PDK1^-/- +WT ES cells were starved of SR for 3 h, then treated with indicated concentrations of BX-795, VN-BX, CPAc-BX, Ph-BX, 3-MeOH-BX, or 3,4-DMB-PP1 for 30 min. Then medium was replaced with fresh medium containing respective inhibitor plus 100 ng/ml IGF1 for another 30 min before lysis. Western blotting was performed to analyze (B) the effects of BX-795, VN-BX, CPAc-BX, Ph-BX, 3-MeOH-BX, and 3,4-DMB-PP1 on PKB T308 phosphorylation and (C) the consequences of CPAc-BX treatment on additional phospho-proteins and 4E-BP1. D, Blots shown in (C) were quantified with NIH ImageJ, and curves fitted with SigmaPlot. E, Heatmap illustrating the IC50 concentrations of 3,4-DMB-PP1, 1-NM-PP1, BX-795, and CPAc-BX for phosphorylation of PKB T308 and S6 S235/S236 for both cell lines used. Bright red indicates potent inhibition, darker shades indicate weaker to no inhibition. Heatmap was generated with the help of Java TreeView.
caution and with the appropriate PDK1 WT control to study biological effects of PDK1 inhibition.

5.2 Effects of PDK1 inhibition or loss on physiological parameters and tumor growth

After having studied the biochemical effects of PDK1 inhibition, the biological consequences of PDK1 inhibition should be assessed. As none of the BX-795 derivatives displayed a significantly improved specificity window towards S6 S235/S236 when compared to the two inhibitors 3,4-DMB-PP1 and 1-NM-PP1 (see 5.1.4), the latter compounds were used to assess biological consequences of PDK1 inhibition, such as effects on cell cycle, cell proliferation and viability, as well as sensitivity to apoptotic stimuli. Furthermore, transplantation of wildtype PDK1 ES cells as well as PDK1\(^{-/-}\) ES cells and PDK1\(^{-/-}\) ES cells stably expressing PDK1 LG (PDK1\(^{-/-}\) +LG ES cells) or PDK1 WT (PDK1\(^{-/-}\) +WT ES cells) into appropriate mouse strains was used to assess the effects of PDK1 on tumor growth and differentiation status of teratomas.

5.2.1 Specific inhibition of PDK1 does not cause cell cycle arrest and has little effect on cell proliferation and viability

The experiments described in Fig. 9-11 showed that in ES cells, PDK1 LG can be durably (>24 hours) inhibited by 3,4-DMB-PP1 and 1-NM-PP1 at concentrations that do not affect PDK1 WT. Even though nonspecific effects on the phosphorylation of S6 S235/S236 and p38 MAPK were observed with both of these two inhibitors in PDK1\(^{-/-}\) +WT ES cells, the use of these inhibitors in PDK1\(^{-/-}\) +LG ES cells and PDK1\(^{-/-}\) +WT ES cells in parallel should still allow to test for biological functions of PDK1: Effects of 3,4-DMB-PP1 and 1-NM-PP1 that occur in both cell lines are due to off-target effects of these compounds, whereas consequences of inhibitor treatment that are only found in the PDK1\(^{-/-}\) +LG ES cells can be attributed to inhibition of PDK1 LG.
Therefore, 3,4-DMB-PP1 and 1-NM-PP1 could be used to assess the role of PDK1 in the regulation of cell cycle and cell viability. As summarized in Fig. 15A, neither 3,4-DMB-PP1 nor 1-NM-PP1 caused any effects on cell cycle distribution in PDK1\(^{+/−}\) +LG ES cells at 20 μM, a concentration that achieved comparable biochemical knockdown of PDK1 activity as 5 μM BX-795 as judged by PKB T308 phosphorylation (Fig. 5A and Fig. 9B-D). This is consistent with the similar cell cycle profile between PDK1\(^{+/+}\) and PDK1\(^{−/−}\) ES cells (Fig. 5B). BX-795 on the other hand still caused a G2/M arrest in these cells. Thus, the PDK1 pathway does not play a role in cell cycle control of ES cells.

Furthermore, the consequences of 3,4-DMB-PP1 and 1-NM-PP1 on cell proliferation and viability of PDK1\(^{+/−}\) +LG and PDK1\(^{+/−}\) +WT ES cells were analyzed. When cultured in high serum (15% SR), these compounds had only minor effects on cell viability that were not different in the two cell lines, in contrast to BX-795 which strongly inhibited viability (Fig. 15B). When cultured in low serum (1.5% SR), a mild specific inhibition of cell viability in PDK1\(^{+/−}\) +LG cells was apparent, but this was almost obscured by a similar inhibition in PDK1\(^{−/−}\) +WT ES cells (Fig. 15C). In summary, the PDK1 pathway does not play a role in the control of cell cycle and viability in ES cells.

### 5.2.2 Loss of PDK1 and specific inhibition of PDK1 sensitize to apoptosis

After studying the effect of PDK1 inhibition on cell proliferation and viability under ‘regular’ ES cell culture conditions as well as low serum concentrations, the next step was to assay if PDK1 inhibition had an effect on cell viability after apoptotic challenges. To address this question, several means of apoptosis induction were tested to identify such conditions that would induce detectable apoptosis in all four ES cell lines used for this assay, PDK1\(^{+/+}\), PDK1\(^{+/−}\), PDK1\(^{−/−}\) +LG, PDK1\(^{−/−}\) +WT ES cells, within 24 hours or less so that allele-specific inhibitors would not have to be replaced during the course of the experiment. Cell death induction by anoikis was tested by growth on Poly 2-hydroxyethyl methacrylate (Poly HEMA), which prevents attachment of cells
Figure 15  Effect of PDK1 inhibition on cell cycle, viability and proliferation.

A, PDK1\textsuperscript{-/-} +LG ES cells were cultured in KO-DMEM, 15% SR, 1000 U/ml LIF and 20 μM 3,4-DMB-PP1, 20 μM 1-NM-PP1, 5 μM BX-795, or DMSO as a control for 48 h,
Results

replacing medium with inhibitor after 24 h. Cells were subject to propidium iodide staining and cell cycle distribution was analyzed using BD FACS Calibur. B, C, PDK1+/− +WT and PDK1+/− +LG ES cells were seeded in 6 well plates at 10^5 cells/well and cultured in the presence of 15% SR (B) or 1.5% SR (C). 12 hours after seeding cells were treated with 10 μM 3,4-DMB-PP1, or 1-NM-PP1, or 5 μM BX-795, or DMSO control. Medium was replaced every 24 h. After 72 hours cell proliferation was assessed using the CellTiter96® AQueous One Solution assay (Promega). The graph represents the mean and standard deviation of one representative experiment (n=5) out of four.
Results to the culture dish. Furthermore taxol, doxorubicin, anisomycin and actinomycin D, all known inducers of apoptosis in eukaryotic cells, were tested in several concentrations and for various incubation periods (data not shown). As readout for apoptosis induction, cleavage of caspase 9 and its target poly(ADP-ribose) polymerase (PARP) was evaluated by Western blotting. This revealed that anisomycin at 10 \( \mu \text{g/ml} \) and actinomycin at 200 nM resulted in apoptosis induction within six to twelve hours in these cells (data not shown). Both, anisomycin as well as actinomycin D, are antibiotics derived from Streptomyces species. While anisomycin hampers protein synthesis by impeding the peptidyl transferase in eukaryotic ribosomes, actinomycin D inhibits transcription and is used in the treatment of cancer, specifically several types of sarcomas, carcinomas and adenocarcinomas (http://www.nlm.nih.gov/medlineplus/druginfo/medmaster/a682224.html). Thus it is particularly interesting to test if PDK1 inhibition results in an enhanced apoptosis induction by actinomycin D because this may have potential implications for the treatment of cancer.

Figure 16A illustrates that PDK1\(^{-/-}\) ES cells are much more sensitive than PDK1\(^{+/+}\), PDK1\(^{-/-}\) +LG, and PDK1\(^{-/-}\) +WT ES cells to induction of apoptosis by anisomycin and actinomycin D. Apoptosis was assessed in this assay by cleavage of caspase 9 and its target PARP. Both caspase 9 and PARP are cleaved to a much bigger extend in PDK1\(^{-/-}\) ES cells than in cells expressing PDK1.

Next, it was assessed whether specific inhibition of PDK1 could reproduce the effect of PDK1 loss on apoptosis sensitization. For this, PDK1\(^{-/-}\), PDK1\(^{+/+}\), PDK1\(^{-/-}\) +LG, and PDK1\(^{-/-}\) +WT ES cells were treated for 24 hours with 3,4-DMB-PP1 or 1-NM-PP1 or DMSO control. (It should be mentioned that under these conditions, the biochemical knockdown of PDK1 remains and cell cycle and viability are not affected.) Medium was replaced 24 hours after the addition of the PDK1 inhibitor with medium containing the respective inhibitor or DMSO control with or without anisomycin or actinomycin D and the cells were cultured for another eight hours. Cultures without the inhibitor and the apoptotic inducer serve as controls. A representative result of three independent experiments is shown in Figure 16B. As seen before in Fig. 16A,
Figure 16  Loss of PDK1 and inhibition of PDK1 sensitize to apoptosis.

A, PDK1+/+, PDK1+/+, PDK1+/+ +LG, and PDK1+/+ +WT ES cells were treated with 10 μg/ml anisomycin or 200 nM actinomycin D for eight hours. Then floating and attached cells were harvested for Western blotting. The results of one of two
representative experiments are shown. B, PDK1+/+, PDK1+/-, PDK1-/- +LG, and PDK1-/- +WT ES cells were treated for 24 hours with 3,4-DMB-PP1 or 1-NM-PP1 or DMSO control, then medium was replaced with medium containing the respective inhibitor or DMSO control with or without 10 μg/ml anisomycin or 200 nM actinomycin D for eight hours. Cells were lysed, and caspase 9 and poly (ADP-ribose) polymerase (PARP) cleavage analyzed by Western blotting. Full size caspase 9 and PARP proteins are marked with a black arrow, the biggest cleavage product for caspase 9 and PARP are indicated by a white arrow. Phosphorylation of GSK3α/β is shown to indirectly confirm activity of PDK1 inhibitors. Western blots were quantified with NIH ImageJ software, and the ratio of cleaved caspase 9 to uncleaved caspase 9 is shown, as well the relative cleavage of PARP. The result of one of three comparable experiments is shown. Both cleavage of caspase 9 as well as cleavage of its target PARP are indicators for induction of apoptosis.
the 8 hour treatment with actinomycin D induces cleavage of both caspase 9 and PARP in both cell lines used, PDK1\(^{-/-}\) +LG and PDK1\(^{-/-}\) +WT ES cells. The pretreatment of PDK1\(^{-/-}\) +LG but not PDK1\(^{-/-}\) +WT ES cells with 3,4-DMB-PP1 or 1-NM-PP1 lead to a significant increase in both caspase 9 cleavage as well as PARP cleavage, as indicated by a decrease of the full sized proteins (upper band marked with black arrow in Western blot) and an increase in cleavage products (marked with white arrow). Specifically, quantification of the Western blots for caspase 9 and PARP showed that 1-NM-PP1 resulted in a ~3 fold increase in caspase 9 cleavage and a ~5 fold enhancement in PARP cleavage induced by actinomycin D. Similarly, 3,4-DMB-PP1 lead to a ~3 fold augmentation in caspase 9 cleavage, and a ~17 fold increase in PARP cleavage. These results demonstrate that PDK1 inhibition sensitizes to apoptosis induction by actinomycin D, albeit not to the full extent seen in PDK1\(^{-/-}\) ES cells. The same experiment performed with a 1 hour pretreatment with PDK1 inhibitor (data not shown) also rendered cells more susceptible to apoptosis, but to a smaller extent, indicating that transcriptional and translational events are likely involved in the sensitization effects observed.

5.2.3 PDK1 contributes to tumor growth and teratoma differentiation

PDK1 is a cardinal nodal point in the signaling downstream of PI3K signaling activated by GFs, hormones, cytokines and Ras (Fig. 2 and 3). It critically regulates members of the AGC family of kinases including PKB, RSK, S6K, SGK, PKCs and PRKs, and is even absolutely required for the activation of PKB, S6K and RSK (at least RSK isoforms 1-3). Together these AGC kinases mediate a plethora of events such as translation, growth, proliferation and survival, altogether processes relevant for tumorigenesis (Fig. 3).

Thus, a reasonable question to ask is whether inhibition of the master regulator PDK1, which should simultaneously hit several pro-tumorigenic pathways, would be an effective and safe cancer therapeutic strategy.

PDK1\(^{-/-}\) mice die at embryonic day E9.5 displaying multiple developmental defects (Lawlor et al., 2002). At the time the studies described here were
initiated, no genetic mouse model was described that addressed the role of PDK1 in tumorigenesis. Studying the involvement of PDK1 in tumorigenesis is further complicated by the fact that knockdown of even ~80-90% of PDK1 protein levels does apparently not affect signaling towards PKB and S6K (Lawlor et al., 2002). However, several small molecule inhibitors that, amongst other targets also inhibit PDK1, have been shown to exert anti-proliferative and anti-tumorigenic properties.

For example, the multiple kinase inhibitor UCN-01 (7-hydroxysporine) has been shown to directly bind to and inhibit PDK1 (Komander et al., 2003) and to have anti-proliferative effects in several *in vitro* and *in vivo* cancer models (Fujita et al., 2003; Sato et al., 2002). Some of these effects of UCN-01 have been shown to be mediated by inhibition of CDKs (Dai et al., 2004), MAPK mediated induction of p27 (Facchinetti et al., 2004), and disruption of PKB signaling (Sato et al., 2002; Fujita et al., 2003).

While the latter studies make inhibition of PDK1-PKB seem like a good therapeutic strategy, the fact that UCN-01 has so many targets renders interpretation of these results difficult.

In order to address the role and the requirement for PDK1 in tumorigenesis in a more specific way, first the ability of PDK1−/− and PDK1+/+ ES cells to form allograft tumors in nude mice should be analyzed. Additionally, the growth of PDK1−/− +LG and PDK1−/− +WT ES cell allografts in the presence or absence of allele-specific inhibitor should be examined. Second, the differentiation status of the tumors with or without PDK1 expression/activity should be compared.

5.2.3.1 PDK1−/− ES cells form fewer and smaller teratomas than PDK1+/+ ES cells

Embryonic stem cells have the remarkable ability to undergo both self renewal and differentiation into different lineages. ES cells are derived from the inner cell mass of a developing embryo and they have the potential to differentiate into any cell type of an adult organism. The techniques for isolating and culturing mouse embryonic stem (mES) cells from blastocysts were first reported in 1981 independently by two groups (Evans and Kaufman, 1981;...
Results

Martin et al., 1981). Shortly thereafter, they were shown to be capable of contributing to many different tissues in chimeras, including the germline, when injected into host blastocysts and returned to foster mothers (Bradley et al., 1984). When grafted into isogenic or immune compromised mice these cells form teratomas. Teratomas also arise spontaneously in humans and mice, mostly in testis or ovaries. A teratoma is a tumor derived from pluripotential cells and made up of elements of different types of tissue from one or more of the three germ cell layers, endoderm, ectoderm and mesoderm. Teratomas vary considerably in the differentiation and maturation of its involved tissues; they have been reported to contain hair, teeth, bone and very rarely even more complex structures, hence the name teratoma, which is Greek for ‘monstrous tumor’. Typically however, a teratoma will contain no organs but rather one or more tissues normally found in adult organs such as brain, thyroid, muscle, cartilage and lung.

In order to address the effect PD K1 loss on tumor formation, PDK1\(^{+/+}\) and PDK1\(^{-/-}\) ES cells were compared in their ability to form teratomas in nude mice. Therefore five mice were injected subcutaneously in their flanks with 5x10\(^5\) PDK1\(^{+/+}\) ES cells, another five mice received PDK1\(^{-/-}\) ES cells, and the growth of tumors was monitored. After 75 days 4 out of 5 mice that had received PDK1\(^{+/+}\) ES cells had a detectable tumor, whereas only 1 out of 5 mice injected with PDK1\(^{+/+}\) ES cells had a small tumor (Figure 17).

When PDK1\(^{-/-}\) +LG and PDK1\(^{-/-}\) +WT ES cells were injected into nude mice, for both cell lines the tumor take rate was 100% and tumors were comparable in size or larger than tumors derived from PDK1\(^{+/+}\) ES cells (Figure 18). This indicates that the decreased ability of PDK1\(^{-/-}\) ES cells to form teratomas owes to the loss of PDK1 activity and is not simply due to a clonal effect of this particular ES cell line.

Moreover, it was interesting to assess whether PDK1 inhibition can hamper or even reverse the growth of already established tumors. To investigate this, I first wanted to test biochemically if the allele-specific inhibitors could reach and effectively inhibit PDK1 signaling in the tumors in the teratoma model used. Hence, 24 mice with already established three week old tumors from
Results

Figure 17  Tumor growth of PDK1\(+/+\) and PDK1\(-/-\) ES cells in nude mice.

NCr nude mice were injected subcutaneously in one flank with 5x10\(^6\) cells. Five mice received PDK1\(+/-\) ES cells and five mice received PDK1\(+/-\) ES cells. After 75 days, 4 out of 5 mice injected with PDK1\(+/-\) ES cells had easily detectable tumors. Only 1 out of 5 mice that received PDK1\(+/-\) ES cells displayed a small tumor.
Results

Figure 18  Tumor growth of PDK1⁺⁻ +LG and PDK1⁺⁻ +WT ES cells in nude mice.

24 NCr nude mice were injected subcutaneously in one flank with 1x10⁶ PDK1⁺⁻ +LG ES cells, in the other flank with 1x10⁶ PDK1⁺⁻ +WT ES cells. All mice grew tumors in both flanks. Tumors were taken out after 21 days; the size of tumors at this time is displayed here.
Results

PDK1+/- +LG ES cells in one flank, and PDK1+/- +WT ES cells in the other flank, were injected intraperitoneally with either 4-MeOB-PP1 or 1-NM-PP1 at 10 mg/kg or DMSO control, and tumors were collected 3 or 20 hours after injection. Tumors were snapfrozen immediately to maintain the phosphorylation status of proteins to be analyzed; frozen tumors were pulverized by grinding in liquid nitrogen, and further processed for Western blotting to assess phosphorylation of PKB T308, PRAS T246, and S6 S235/S236. No significant reduction of PDK1 downstream signaling could be observed under the conditions tested, thus no tumor regression study was performed.

5.2.3.2 PDK1+/- ES cells form less differentiated teratomas

Using ES cell derived teratomas as a tumor model also provided the ability to assess the role of PDK1 in differentiation. When ES cells are grown as embryoid bodies in cell culture or implanted into an animal, where they can grow as teratomas, they can recapitulate many of the events occurring in an early embryo. Under these conditions, appropriately cultured WT ES cells have the potential to differentiate into all three embryonic germ layers. Teratoma formation can be used as a simple and reliable method of analyzing the differentiation potency of ES cells.

Thus, histology of PDK1+/-, PDK1+/+, PDK1+/- +LG and PDK1+/- +WT ES cell derived teratomas was analyzed to get clues about the involvement of PDK1 in differentiation. Figure 19A illustrates that PDK1+/- ES cells form teratomas with very elaborate tissue architecture, for example well differentiated tumors with cysts, glandular structures, various epithelia including simple squamous, cuboidal and columnar, as well as stratified and bronchiolar epithelium with ciliated cells. One of four PDK1+/- teratomas also contained immature parts characterized by neurofibroma-like rosettes and pseudo-rosettes, and a high nucleo-cytoplasmic ratio, indicative of a malignant tumor. Teratomas derived from PDK1+/- +LG and PDK1+/- +WT ES cells displayed similar features (data not shown).
Figure 19A  Histology of PDK1<sup>+/+</sup> teratomas.

a) Overview. Well differentiated tumor with cysts, glandular structures (Gl), various epithelia including simple squamous, cuboidal and columnar, as well as stratified and bronchiolar epithelium (BE). b) Broncholar epithelium (BE) with ciliated cells (Ci) and underlying smooth muscle cells (SmM). c) Cysts lined with simple columnar and cuboidal, as well as stratified, including bronchiolar epithelia. StM: Striated Muscle. d) Overview. Tumor characterized by large cysts, simple squamous epithelia and abundance of collagen fibrils (Co). e) Broncholar epithelium (BE) and simple squamous epithelium (SSE). f) Fat cells (F) and striated muscle (StM) interspersed in connective tissue. g) Overview. Tumor with mature, e.g. cartilage (C), and immature parts. Immature parts characterized by neurofibrom-like rosettes (R) and pseudo-rosettes, and high nucleo-cytoplasmic (N/C) ratio. h) Simple epithelium with transition from normal, well ordered, single cell layered, to abnormal, disordered epithelium with high N/C ratio. i) Loose connective tissue. j) Well differentiated bronchiolar epithelium (BE) with Goblet cells (G) and ciliated cells (Ci).
Figure 19B  Histology of PDK1-/- teratoma.

a) Overview. b) and c) Higher magnification. Simple tissue organization with connective tissue, collagen fibrils, and few cysts lined with simple squamous epithelium.
The single tumor derived from PDK1−/− ES cells (Figure 19B) however displayed only simple tissue organization with connective tissue, collagen fibrils, and few cysts lined with simple squamous epithelium. This indicates that PDK1−/− ES are not only compromised in their ability to form teratomas, but also in their capability to differentiate, either generally, or into specific cell types.
6. Discussion

6.1 Analysis of PDK1 signaling in ES cells

The work presented here examined biochemical and biological functions of PDK1 employing conventional gene knockout technology, inhibition by small-molecule inhibitors, and, most extensively, a chemical genetic approach. Moreover, it describes previously under-appreciated caviats associated with the latter methodology.

6.1.1 BX-795 as a PDK1 inhibitor

Initially, the effects of transiently inhibiting PDK1 activity in murine ES cells were studied using BX-795, a previously characterized PDK1 inhibitor (Feldman et al., 2005). However, the use of isogenic PDK1+/+ and PDK1−/− ES cells demonstrated that the G2/M arrest observed with BX-795 was due to off-target effects, since BX-795 caused a similar G2/M arrest in PDK1−/− ES as in PDK1+/+ ES cells. This illustrates that gene knockout technology can be helpful to verify specificity of biochemical inhibition. As profiling of BX-795 against a set of kinases showed that it affected the activity of Aurora A, Aurora B, Aurora C, Cdk1 and Cdk2 the observed cell cycle block in G2/M is likely due to inhibition of these known cell cycle regulators, or other proteins, but not PDK1. A recent publication also reported inhibition of Aurora kinases by BX-795 (Bain et al., 2007). This also challenges the statement that PDK1 inhibition by BX-795 or BX-320 results in inhibition of tumor growth (Feldman et al., 2005); given the data presented here and the work by Bain and coworkers (Bain et al., 2007) it is unclear, if the reported growth inhibitory effect is due to PDK1 inhibition or the non-specific cell cycle arrest, or even another non-specific effect.

Furthermore, long term inhibition of PDK1 LG with both 3,4-DMB-PP1 as well as 1-NM-PP1 resulted in suppression of S6K T389 phosphorylation, whereas BX-795 always led to a profound increase of phosphorylation of S6K T389
under the same conditions, suggesting that this observation too might reflect an off-target effect of BX-795.

Small cell-permeable inhibitors of protein kinases have become invaluable tools for investigating the biochemical and physiological roles of these enzymes, because they can readily be used to quickly block endogenous kinase activity in cells, tissues and organisms. However, the conclusions that can be drawn from such experiments are only as good as the effectiveness and specificity of the compound used. The results presented here further emphasize the need for considerable caution when employing small molecule inhibitors of protein kinases to assess their biological function.

6.1.2 Chemical genetic approach to inhibit PDK1 and biochemical consequences of PDK1 inhibition

As apparent non-specific effects of BX-795 were observed, a different approach employing the combination of genetics and chemistry was explored to investigate the consequences of acutely inhibiting PDK1 function. Therefore, I characterized the ability of forms of PDK1 with mutations in the ATP-binding pocket to be inhibited by purine based inhibitors containing bulky groups. This chemical genetic approach has been used for several kinases to identify substrates, for example with JNK (Habelhah et al., 2001), ERK2 (Eblen et al., 2003) and Cdk7 (Larochelle et al., 2006).

Of all PP1 analogues tested 3,4-DMB-PP1 and 1-NM-PP1 seemed the best at inhibiting PDK L159G, but not PDK1 WT. In trying to improve upon the specificity and potency of these PP1 derived inhibitors, derivatives of BX-795, which displayed a higher potency for PDK1 inhibition in vitro, were generated in an attempt to obtain novel PDK1 LG allele-specific inhibitors. The idea for this approach arose from the observation that in a co-crystal of BX-795 with WT PDK1 one arm of BX-795, carrying an easily substitutable iodo-group, pointed directly towards the gatekeeper residue L159 (Feldman et al., 2005). Hence, different bulky substituents were added to BX-795, replacing the iodo-
group. Of the four BX-795 derivatives tested, only one, CPAc-BX, had PDK1 LG allele-specific features, as determined by PKB T308 phosphorylation. In comparison to BX-795 the other three inhibitors displayed less to no inhibition of both LG and WT PDK1, suggesting that the bulky side groups added to BX-795 sterically or energetically hamper binding to both alleles, and that even the enlarged LG pocket is not sufficient to accommodate them. Surprisingly, VN-BX seemed to inhibit WT PDK1 better than PDK1 LG, which might reflect an interaction of the bulky side chain of VN-BX with the ATP-binding pocket of WT PDK1 that is disrupted by the LG mutation.

The effect of PDK1 loss on downstream targets has been extensively profiled in PDK1+/+ versus PDK1−/− ES cells by Alessi and colleagues (Williams et al., 2000; Balendran et al. 2000; Collins et al., 2003). The conclusions from these experiments were that AGC kinases of the RSK, S6K, PKB, SGK, PRK, and PKC families are all either fully or partially (for PRK2) dependent on PDK1 for phosphorylation at their T-loop site and activity. However, these experiments were all performed under conditions of chronic lack of PDK1 protein. The chemical genetic approach used here allowed a temporal dissection of these events, which led to slightly different conclusions. T-loop phosphorylation of PKB was dramatically reduced after both 1 hour and 24 hours inhibition of PDK1 activity. On the other hand, RSK phosphorylation at the activation loop site was only slightly reduced after 1 hour but was almost completely abolished by 24 hours inhibition of PDK1 activity, indicating significantly different dephosphorylation kinetics for PDK1 downstream targets, which is a novel finding.

A pan-PKC phospho-T-loop antibody indicated that only two putative PKC isoforms were sensitive to PDK1 inhibition. Isoform-specific antibodies revealed that neither of these represented PKCδ and PKCθ. Therefore, it is still unclear which PKC isoforms are the most sensitive to PDK1 mediated phosphorylation, and which are independent of PDK1 in these cells. Furthermore, no changes in total PKC levels (including the two putative PKC isoforms that displayed reduced phosphorylation) were observed after 24 hours of PDK1 inhibition. Previous studies showed the levels of PKCα, PKCβ1,
PKCy, and PKCε to be strikingly reduced in PDK1−/− ES cells, and PKCδ levels were also decreased. Additionally, T-loop phosphorylation of most isoforms was abolished, except for PKCδ. It has been proposed that the T-loop phosphorylation of PKC isoforms by PDK1 enables PKCs to achieve catalytic maturity and to stabilize the proteins (Balendran et al., 2000; Dutil et al., 1998). It may well be that PDK1 would have to be inhibited for longer than 24 hours to show effects on phosphorylation and stability of most PKC isoforms, and that this may be an explanation for the seemingly conflicting observations. It has also been proposed though, that PKCδ and PKCθ can autophosphorylate at their T-loop sites (Czerwinski et al., 2005), which may explain why PKCδ is still phosphorylated in PDK1−/− ES cells. Moreover, a recent report indicates that phosphorylation of protein kinase D, a fairly well characterized target of novel PKC isoforms (δ, ε, η, θ) is not significantly compromised in PDK1−/− ES cells, and is still sensitive to the PKC inhibitor GF109203X (Wood et al., 2007). Taken together, the direct role for PDK1 in the regulation of activity and stability of different PKC isoforms is still controversial, and should be addressed further. The generation of reliable PKC phospho- as well as isoform-specific antibodies and the identification of PKC isoform-exclusive targets would greatly aid such studies.

Similarly, while the phosphorylation of PRK1/2 was dramatically reduced in the PDK1−/− ES cells, phosphorylation was not affected following 24 hours incubation with PDK1 inhibitors. This could reflect a structural role of PDK1 protein in the maintenance of these phosphorylation sites. This hypothesis is supported by the demonstration of direct binding of PDK1 to PRK1 and PRK2 (Flynn et al., 2000). However, it could also reflect differences in the activities of, or accessibilities by various phosphatases to the different activation loops. Surprisingly little is known about phosphatases which act on the activation loop residues of AGC kinases, with limited evidence implicating protein phosphatase 2A (PP2A) for PKB and PKC isoforms (Yamada et al., 2001; Hansra et al., 1996). Given the large disparity seen here for dephosphorylation of different activation loop residues, further work in this area is warranted.
The experiments employing acute PDK1 inhibition in conjunction with various stimuli also revealed that T-loop phosphorylation of RSK by PDK1 is strongly induced following sorbitol treatment, which suggests a previously underappreciated role of this pathway in osmotic stress response. This occurred concomitant with an increase in phosphorylation of the ERK-dependent phosphorylation site S380 of RSK as well as an increase in ERK phosphorylation. Although ERK has previously been shown to be phosphorylated in response to osmotic shock in some cells (Kayali et al., 2000), RSK is normally not thought to participate in this response (Smith et al., 2000). This may therefore represent a cell type specific response to ES cells and it will be interesting to determine the significance of this.

Induction of osmotic stress also led to an increase in S21/S9 phosphorylation of GSK3α/β that was not blocked by PDK1 inhibition. GSK3 has not been implicated in the response to osmotic stress, and the results presented here suggest that a PDK1-independent kinase, i.e. not PKB, nor S6K, nor RSK, is responsible for phosphorylation of these sites under these conditions. In addition to PKB, some reports had implicated RSK or S6K as GSK3 kinases (Cross et al., 1994; Sutherland et al., 1993); since PKB, RSK (at least RSK1, RSK2 and RSK3) and S6K activities are strictly dependent on PDK1, the data presented here points towards a novel kinase phosphorylating GSK3.

The allele independent effects of 3,4-DMB-PP1 and 1-NM-PP1 observed in these experiments were unexpected, as previous studies using these and similar compounds have not reported many off-target effects. There are at least three potential explanations for these results. Firstly, these compounds could inhibit the activity of an endogenous S6 kinase, such as RSK or S6K. Although possible, this seems unlikely due to the fact that a large number of different side groups are able to cause these effects, including completely unrelated compounds such as the BX-795 analogues and many PP1 analogues. In addition, when 1-Na-PP1 was profiled against multiple protein WT kinases, it did not show significant activity against either S6K or RSK (data not shown). A second possibility is that these agents cause some kind of cellular stress, which is reflected in decreased S6 phosphorylation.
Although it is tempting to implicate mTORC1 activity in the response to this stress, as mTORC1 has been shown to act as a sensor for various cellular insults (Reiling and Sabatini, 2006), no strong effects on direct mTORC1 targets such as S6K T389 or 4E-BP1 phosphorylation were seen. Nor is it clear whether S6K is responsible for the effects seen on S6 S235/S236 phosphorylation, as measurement of more specific sites of S6K phosphorylation, namely S6 S240/S244 (Pende et al., 2004) showed that these sites were not affected by 3,4-DMB-PP1 or 1-NM-PP1 in PDK1^{-/} +WT ES cells. A third possibility is that the bulky analogues inhibit WT PDK1 to a small extent, and that S6 phosphorylation is a very sensitive readout for this minor inhibition. Independent of the cause, these results stress the importance of appropriate controls such as the parallel use of WT and allele sensitive kinases, as well as active and inactive versions of inhibitor analogues, in all experiments.

Consistent with these results, Bain and coworkers, who profiled 1-NM-PP1 against a set of more than 70 protein kinases in vitro, (at concentrations required to inhibit the gatekeeper mutants of JNK,) found it to inhibit receptor interacting protein 2 (RIP2), cyclin G associated kinase (GAK), casein kinase (CK1) and p38 MAPK, Src, lymphocyte cell specific protein tyrosine kinase (Lck) and C-terminal Src kinase (Csk), similar to PP1. Additionally, 1-NM-PP1 inhibited PKD1, mammalian homologue Ste20-like kinase (MST2) and PKA (Bain et al., 2007).

Taken together, these findings emphasize that caution needs to be taken when interpreting results from experiments with cells and animals expressing the gatekeeper mutants. Even control experiments employing cells or tissues from WT or knockout mice might not reveal the full extent of off-target effects, since sometimes two different signaling pathways need to be inhibited to block phosphorylation of a particular downstream protein or biological process.

Nonetheless, the chemical genetics methodology is a powerful tool that, in conjunction with others, can be used to elucidate protein kinase signaling. Used in combination with other methods, such as conventional inhibitors,
gene knockout, conditional knockout, dominant negative versions, and siRNA, chemical genetic experiments can be interpreted with more confidence. Just recently this methodology has been made applicable to lipid kinases such as PI3K (Alaimo et al., 2005), and GTPases like H-Ras (Vincent et al., 2007), and it will be exciting to see the novel insights into signaling and biology of these enzyme classes this may reveal.

6.2 Biological roles of PDK1

Information on the biological role of PDK1 remains limited. Total lack of PDK1 during embryogenesis is not tolerated, with death occurring at E9.5 due to multiple developmental abnormalities. Targeted deletion of PDK1 generally results in smaller organ size (Mora et al., 2003; Hashimoto et al., 2006; Hinton et al., 2004), and a hypomorphic germline mutation also results in smaller animals (Lawlor et al., 2002). However, the exact mechanisms leading to these size defects have not been worked out. A recent report suggested that inhibition of PDK1 activity using novel PDK1 inhibitors, BX-795 and analogues, caused a cell cycle block at the G2/M phase of the cell cycle in breast cancer cells (Feldman et al., 2005). While we were also able to demonstrate a G2/M arrest in ES cells using these inhibitors, this was not seen when specifically inhibiting PDK1 activity in the PDK1 LG expressing cells with PP1 analogues, despite similar inhibition of PDK1 activity. We have profiled BX-795 against a large number of protein kinases, and noticed that in addition to PDK1, it also inhibits Cdk1, Cdk2, and Aurora A, B and C with similar potencies. This observation was also made by another group (Bain et al., 2007). Therefore, the G2/M arrest seen in these studies, as well as at least part of the antitumor activity demonstrated in xenograft models, is likely due to either Aurora/Cdk inhibition, combined PDK1/Aurora/Cdk inhibition, or an additional target not yet elucidated. Similarly, BX-795 was effective at reducing the viability of ES cells growing in high serum, whereas allele-specific inhibitors were not. In contrast, we show that specific inhibition of PDK1 does not affect intrinsic cell viability when cells are grown in high serum, but mildly reduces viability and proliferation under low serum
Discussion

conditions and causes a rather profound sensitization to apoptosis induced by cellular stress.

Moreover, the work described here demonstrates that cells lacking PDK1 are strongly defective for tumor formation, suggesting that tumor growth in vivo encounters similar stresses that PDK1 activity protects against, and maintains enthusiasm for PDK1 as an oncology drug target.

This is consistent with a recent report showing that reduced expression of PDK1 in PTEN+/- mice markedly protects these animals from developing a wide range of tumors (Bayascas et al., 2005). This finding suggests that PDK1 could be an attractive drug target in tumors that possess elevated PIP₃ levels, such as those with loss of PTEN, mutations and amplifications in PI3K or upstream RTKs, like epidermal growth factor receptor (EGFR) for example, or the GTPase Ras. The important contribution of PI3K downstream signaling in Ras-mediated tumorigenesis was recently demonstrated by Downward’s laboratory. The group generated mice with mutations in the gene encoding the catalytic p110α isoform (PIK3CA) that block its interaction with Ras. Cells from these mice show proliferative defects and selective disruption of signaling from growth factors to PI3K, and, most importantly, they are highly resistant to endogenous Ras oncogene-induced tumorigenesis. The interaction of Ras with p110α is thus required in vivo for certain normal growth factor signaling and for Ras-driven tumor formation (Gupta et al., 2007). Hence, one can speculate, that also an only partial disruption of PI3K downstream signaling by PDK1 inhibition may alleviate Ras-driven tumorigenesis. Moreover, PDK1 inhibition will likely be beneficial in cases with overexpression or overactivation of PKB or S6K.

Besides, patients treated with rapamycin analogues may also benefit from an additional treatment with a PDK1 inhibitor. Inhibition of mTOR can arrest tumors in model systems, but shows only limited anti-tumor activity in patients because mTOR inhibition induces the expression of IRS1, which in turn upregulates PKB activity and reduces or negates the effects of mTOR inhibition (O’Reilly et al., 2006). This feedback mechanism could potentially be
Discussion

Disrupted by blocking PDK1, which may potentiate the effects of mTOR inhibition.

Thus, PDK1 inhibition may beneficial for a wide variety of tumors with various genetic and epigenetic aberrations, including loss of PTEN, overexpression or overactivation of Ras, RTKs, PI3K, and PKB. This is especially important since these alterations are among the most frequent ones in human tumors: for example, activating mutations in the PI3K p110α catalytic subunit (PIK3CA) is found in 30% of cancers of the breast, colon and endometrium, and in 5–15% of the liver, ovary, stomach, esophagus and pancreas (Vogt et al., 2007). Also, hyperactivation of PKB is observed in 25-75% of breast and lung tumors (David et al., 2004). And a very recent publication reports a newly identified mutational activation of PKB, a single amino acid change, E17K, in the PH domain of PKB1, which has transforming properties and was found in 8% of analyzed breast, 6% colorectal, and 2% ovarian cancers (Brugge et al., 2007; Carpten et al., 2007).

Moreover, the observation that PDK1 inhibition sensitizes to apoptosis induction by actinomycin D suggests that PDK1 inhibition in combination with standard chemotherapeutics may also be a favorable treatment of cancers with genetic aberrations not directly impacting on the PI3K pathway.

In sum, these experiments show for the first time the ability to reconstitute PDK1 signaling in PDK1–/– ES cells, using either WT or LG forms of PDK1. This allows the ability to determine the consequences of specifically inhibiting PDK1 activity in a temporal and reversible manner. Using this approach, this work shows that the previously determined G2/M arrest seen with BX-795 is unlikely to be due to PDK1 inhibition, and that discrete PDK1 targets respond differently following short term inhibition of PDK1 activity. Furthermore, this work demonstrates that inhibition of PDK1 activity results in sensitization to cellular stresses and decreased tumor formation, which reinforces the concept of PDK1 as an attractive drug target for cancer therapy.
7. Experimental procedures

7.1 Allograft studies.
Three to five weeks old female NCr nude outbred mice [(NCr)-Fox1\textsuperscript{nu}] (Taconic) were anesthetized with O\textsubscript{2}/isoflurane and injected subcutaneously in one flank with 5x10\textsuperscript{5} cells in 100 μl DPBS. Five mice received PDK1\textsuperscript{-/-} ES cells and five mice received PDK1\textsuperscript{+/-} ES cells. Mice were housed according to the University of California San Francisco (USCF) standards and allograft growth was monitored regularly. 75 days after injection mice were euthanized with CO\textsubscript{2} and cervical dislocation, allografts were harvested and weighed. Tissue sections of the allografts were prepared and stained with Hematoxylin-Eosin (H&E). Similarly, 24 NCr nude mice were injected subcutaneously in one flank with 1x10\textsuperscript{6} PDK1\textsuperscript{-/-} +LG ES cells, in the other flank with 1x10\textsuperscript{6} PDK1\textsuperscript{-/-} WT ES cells, and tumors were taken out after 21 days, weighed and H&E stained. Tumors intended for Western blotting were immediately snapfrozen in liquid nitrogen.

7.2 Apoptosis assay.
Per well 1.5 x10\textsuperscript{6} PDK1\textsuperscript{-/-}, PDK1\textsuperscript{+/-}, PDK1\textsuperscript{-/-} +LG and PDK1\textsuperscript{-/-} +WT ES cells were seeded into 6 well plates. 24 hours later cells were treated with 10 μM 3,4-DMB-PP1, 1-NM-PP1 or DMSO control in medium containing 15% SR for 24 hours. Then, medium was replaced with fresh medium containing SR with or without inhibitor, and with or without 200 nM actinomycin D or 10 μg/ml anisomycin (both from Sigma) to induce apoptosis. After eight hours, floating and attached cells were harvested, and apoptosis was measured by assessing caspase 9 and PARP cleavage by Western blotting.

7.3 Cell culture.
If not indicated otherwise, ES cells were grown on gelatinized dishes in KnockOut Dulbecco modified Eagle medium (DMEM) (Invitrogen) supplemented with 15% KnockOut serum replacement (SR) (Invitrogen), 0.1 mM nonessential amino acids, 2 mM L-glutamine, 0.1 mM 2-mercaptoethanol, and 1000 U/ml LIF (Chemicon). Cells were treated with insulin-like growth
factor (IGF1), Forskolin, sorbitol, SB203580 (all from Sigma), LY294002 (Calbiochem), 12-O-Tetradecanoyl-phorbol 13-acetate (TPA) and UO126 (Cell Signaling) as indicated.

7.4 Cell cycle analysis.
Cells were seeded into 6 cm dishes at 4\times10^5 cells per dish. 24 hours after plating, cells were treated with inhibitor or DMSO control as indicated. Cells were harvested using cell dissociation buffer (Invitrogen) and Dulbecco’s phosphate buffered saline (DPBS) (Invitrogen) following 48 hours of treatment with either 20 \mu M 3,4-DMB-PP1, or 1-NM-PP1, or 5 \mu M BX795, or 24 hours of treatment with 100 ng/ml nocodazole. Cells were fixed in 70% ethanol at 4 °C and resuspended in DPBS containing 10 \mu g/ml propidium iodide (Roche Applied Sciences) and 1 \mu g/ml RNase A, incubated for 30 min at room temperature and analyzed by flow cytometry.

7.5 Cell proliferation assay.
Cells were seeded into gelatinized 96 well plates at 5000 to 10000 cells per well. 12 hours after seeding cells were treated in sets of five with either 10 \mu M 3,4-DMB-PP1, or 1-NM-PP1, or 5 \mu M BX795 in medium containing either 15% or 1.5% SR. Medium was replaced every 24 h. Cell proliferation was assessed 72 hours later using the CellTiter96® AQueous One Solution kit (Promega), which indirectly measures cell number as NADH or NADPH reduces a tetrazolium compound into a colored formazan product, the absorbance of which was read at 490 nm.

7.6 Construction of a PDK1 variant, PDK1 L159G, and generation of stable ES cell lines.
PDK1 WT cDNA was cloned into pcDNA3 with a 5’ Myc-tag. PCR mutagenesis with primers f-CCATTTTTTGGCATAACTACCGAAATAC and r-GAAGCTGTATTTCGGCGGTAGTTATGCCAA gave the mutant encoding PDK1 in which leucine at position 159 was replaced with a glycine (PDK1 L159G).
6 μg of both constructs were electroporated into 4 x 10^6 PDK1−/− ES cells (electroporator and reagents from Amaxa; electroporation program A-13), cells were plated in 12 ml medium into 10 cm tissue culture plates. Cells were selected 24 hours after electroporation with 250 μg/ml geneticin (Invitrogen) and pools of cells stably expressing PDK1 WT or LG were expanded (called PDK1−/− +WT and PDK1−/− + LG ES cells respectively).

7.7 IC50 determination.
PDK1−/− +LG and PDK1−/− +WT ES cells were starved for 3 hours, treated for 30 min with increasing concentrations ranging from 0 to 50 μM of inhibitor (PP1, PP1-derivatives, BX-795 or BX-CZ02), then medium was replaced with fresh inhibitor with or without 100 ng/ml IGF1 and cells were lysed 30 min later and subjected to Western blotting. Densitometric analysis of bands was performed with NIH ImageJ software (http://rsb.info.nih.gov/nih-image/), curves were fitted and IC50 values were generated with SigmaPlot. Heatmaps were produced with the help of Java Treeview.

7.8 In vitro PDK1 kinase assay.
PDK1 kinase assays were performed with recombinant proteins purified from SF9 insect cells. The recombinant proteins are Glu-Glu-tagged and were purified using an anti–Glu antibody. As kinase either 150 ng/μl of WT PDK1 or 500 ng/μl PDK1 L159G were used. ΔPH-PKB was used as a substrate at 210
ng/μl. Inhibitors were used at varying final concentrations from 1 to 50 μM. The reactions were done in a 10 μl kinase buffer (20 mM Tris·Cl pH 7.5, 1 mM EDTA, 75 mM NaCl, 5 mM MgCl₂, 1 mM DTT) containing 20 μM ATP and 5 μCi of [γ³²P]ATP. Reactions were incubated at 30 °C for 15 min, terminated by addition of 4x protein sample buffer and separated on 12% Tris-glycine gels (Invitrogen) at 100-120 volts. Incorporated ³²P-radioactivity was assessed using a STORM PhosphoImager (Amersham), and quantitated using ImageQuant5.2.

7.9 **Sequence alignment.**
Human and murine AGC kinase T-loop sequences were retrieved from NCBI and Ensembl databases, and encompassed 21 bases surrounding the phosphorylateable T-loop threonine or serine. A phylogenetic tree was built using the EBI ClustalW algorithm (http://www.ebi.ac.uk/ clustalw / index.html).

7.10 **Synthesis of purine analogues.**
BX-795 was synthesized by Dorothea Fiedler, BX-795 derivatives and purine analogues were synthesized as described before (Bishop et al., 1998; Liu et al., 1998) by Chao Zhang, both at Kevan Shokat’s laboratory, Department of Cellular and Molecular Pharmacology, University of Californian San Francisco.
7.11 **Western blotting.**

Antibodies against β-actin and β-tubulin were from Sigma, against 4E-BP1, phospho-CREB, phospho-4E-BP1 S65, phospho-4E-BP1 S37/S46, phospho-GSK3α/β S21/S9, phospho-MSK1 S376, phospho-MSK1 T581, phospho-p38 T180/Y182, phospho-PDK1 S241, phospho-PKA T197, phospho-PKB T308, phospho-PKC pan, phospho-PRK1/2 T774/T816, phospho-RSK T380, phospho-p38 Y182, phospho-S6K T389, and phospho-S6 S235/S236 from Cell Signaling, against MSK1 and PKC from Santa Cruz Biotechnology, PDK1 from BD Transduction Laboratories, phospho-MSK1 S212 from R&D Systems, phospho-PRAS40 T246 from Biomol, and phospho-RSK1/2 S221/S227 from Biosource. Anti-caspase 9 antibody was from MBL, and anti-PARP from BD Pharmingen. Anti-mouse and -rabbit secondary antibodies were from Amersham Biosciences, anti-goat from Santa Cruz Biotechnology. Cells were lysed at 4 °C in buffer containing 50 mM Tris HCl, pH 7.5, 1 mM EDTA, 1 mM EGTA, 1% Triton-X100, 0.1% β-mercaptoethanol, 50 mM NaF, 10 mM sodium glycerophosphate, 1mM sodium orthovanadate, 5 mM sodium pyrophosphate, 0.27 M sucrose, 1 μM microcystin-LR (Calbiochem), and one complete mini protease inhibitor pill (Roche) per 10 ml. Lysates from tumor tissue were obtained by pulverizing frozen chunks of tissue with a mortar and pestle in liquid nitrogen. Ground tissue was transferred to a microcentrifuge tube and lysis buffer was added. Protein concentrations were determined using the Bio-Rad DC Lowry-based protein assay. Equal amounts (30 to 40 μg) of protein were loaded onto polyacrylamide gels and separated by standard SDS-PAGE. Proteins were transferred to Immobilon-P membrane (Millipore) and blocked with 5% nonfat dry milk in Tris-buffered saline containing 0.1% Tween 20 and incubated with primary antibody overnight at 4°C, followed by incubation with horseradish peroxidase-conjugated secondary antibodies for 1 hour at room temperature. Proteins were detected by ECL (Amersham Biosciences) and exposure to film. To correctly identify the detected proteins their position on the membrane was compared to a molecular size marker (SeeBlue2 from Invitrogen). The schematic below illustrates where in
comparison to this marker the analyzed proteins are found on a 4-12% Bis-Tris gel (Invitrogen).

Densitometric analysis of the bands was carried out using the NIH ImageJ software.
### 8. Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>AGC</td>
<td>cAMP-dependent, cGMP-dependent, and protein kinase C</td>
</tr>
<tr>
<td>Akt</td>
<td>from thymoma in AKR mouse (also called PKB)</td>
</tr>
<tr>
<td>APC</td>
<td>adenomatous polyposis coli</td>
</tr>
<tr>
<td>AS</td>
<td>allele sensitive</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>BAD</td>
<td>Bcl-2/Bcl-XL-antagonist of cell death</td>
</tr>
<tr>
<td>cAMP</td>
<td>cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>CDK2</td>
<td>cyclin-dependent kinase 2</td>
</tr>
<tr>
<td>cGMP</td>
<td>cyclic guanosine monophosphate</td>
</tr>
<tr>
<td>CK1</td>
<td>casein kinase 1</td>
</tr>
<tr>
<td>Csk</td>
<td>C-terminal Src kinase</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethyl sulfoxide</td>
</tr>
<tr>
<td>DPBS</td>
<td>Dulbecco’s phosphate buffered saline</td>
</tr>
<tr>
<td>4E-BP1</td>
<td>eukaryotic initiation factor 4E binding protein 1</td>
</tr>
<tr>
<td>eEF2K</td>
<td>eukaryotic elongation factor 2 kinase</td>
</tr>
<tr>
<td>EGFR</td>
<td>epidermal growth factor receptor</td>
</tr>
<tr>
<td>eIF4E</td>
<td>eukaryotic initiation factor 4E</td>
</tr>
<tr>
<td>ERK1/2</td>
<td>extracellular signal-regulated kinase 1/2</td>
</tr>
<tr>
<td>ES cell</td>
<td>embryonic stem cell</td>
</tr>
<tr>
<td>FOXO</td>
<td>Forkhead box O family</td>
</tr>
<tr>
<td>GAK</td>
<td>cyclin G-associated kinase</td>
</tr>
<tr>
<td>GSK3α/β</td>
<td>glycogen synthase kinase 3 α/β</td>
</tr>
<tr>
<td>GTP</td>
<td>guanosine triphosphate</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Name</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>H&amp;E</td>
<td>Hematoxylin-Eosin</td>
</tr>
<tr>
<td>HM</td>
<td>hydrophobic motif</td>
</tr>
<tr>
<td>IGF1</td>
<td>insulin-like growth factor 1</td>
</tr>
<tr>
<td>IRS1/2</td>
<td>insulin receptor substrate 1/2</td>
</tr>
<tr>
<td>JNK</td>
<td>c-Jun amino-terminal kinase</td>
</tr>
<tr>
<td>Lck</td>
<td>lymphocyte cell-specific protein-tyrosine kinase</td>
</tr>
<tr>
<td>LY</td>
<td>here: LY294002, a PI3K inhibitor</td>
</tr>
<tr>
<td>MAPK</td>
<td>mitogen activated protein kinase</td>
</tr>
<tr>
<td>MEF</td>
<td>mouse embryonic fibroblast</td>
</tr>
<tr>
<td>MSK</td>
<td>mitogen- and stress- activated protein kinase</td>
</tr>
<tr>
<td>MST2</td>
<td>mammalian homologues Ste20-like kinase</td>
</tr>
<tr>
<td>mTOR</td>
<td>mammalian Target Of Rapamycin</td>
</tr>
<tr>
<td>mTORC1/2</td>
<td>mammalian Target Of Rapamycin Complex 1/2</td>
</tr>
<tr>
<td>PARP</td>
<td>poly (ADP-ribose) polymerase</td>
</tr>
<tr>
<td>PI3K</td>
<td>phosphoinositide 3-kinase</td>
</tr>
<tr>
<td>PIP₂</td>
<td>phosphatidylinositol-4,5-bisphosphate</td>
</tr>
<tr>
<td>PIP₃</td>
<td>phosphatidylinositol-3,4,5-triphosphate</td>
</tr>
<tr>
<td>PDK1</td>
<td>3-phosphoinositide dependent kinase 1</td>
</tr>
<tr>
<td>PH</td>
<td>pleckstrin homology</td>
</tr>
<tr>
<td>PKA</td>
<td>cAMP-dependent Protein Kinase</td>
</tr>
<tr>
<td>PKB</td>
<td>protein kinase B (also called Akt)</td>
</tr>
<tr>
<td>PKC</td>
<td>protein kinase C</td>
</tr>
<tr>
<td>PKD</td>
<td>protein kinase D</td>
</tr>
<tr>
<td>PRAS40</td>
<td>proline-rich Akt substrate 40 kDa</td>
</tr>
<tr>
<td>PRK</td>
<td>protein kinase C related kinase</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>PTEN</td>
<td>phosphatase and tensin homologue deleted on chromosome ten</td>
</tr>
<tr>
<td>Raptor</td>
<td>regulatory associated protein of mTOR</td>
</tr>
<tr>
<td>Ras</td>
<td>from: rat adenosarcoma</td>
</tr>
<tr>
<td>Rictor</td>
<td>Rapamycin-insensitive companion of mTOR</td>
</tr>
<tr>
<td>RIP</td>
<td>receptor interacting protein 2</td>
</tr>
<tr>
<td>Rheb</td>
<td>Ras homologue enriched in brain</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>RSK</td>
<td>p90 ribosomal S6 kinase</td>
</tr>
<tr>
<td>RTK</td>
<td>receptor tyrosine kinases</td>
</tr>
<tr>
<td>S</td>
<td>serine</td>
</tr>
<tr>
<td>Ser</td>
<td>serine</td>
</tr>
<tr>
<td>S6K</td>
<td>p70 S6 kinases</td>
</tr>
<tr>
<td>SGK</td>
<td>serum- and glucocorticoid-induced kinase</td>
</tr>
<tr>
<td>SR</td>
<td>serum replacement</td>
</tr>
<tr>
<td>Src</td>
<td>cellular homologue of transforming gene of Rous sarcoma virus</td>
</tr>
<tr>
<td>T</td>
<td>threonine</td>
</tr>
<tr>
<td>Thr</td>
<td>threonine</td>
</tr>
<tr>
<td>TPA</td>
<td>12-O-tetradecanoyl-phorbol-13-acetate</td>
</tr>
<tr>
<td>TSC1/2</td>
<td>tuberous sclerosis complex 1/2</td>
</tr>
<tr>
<td>WT</td>
<td>wild type</td>
</tr>
</tbody>
</table>
9. References


Han J, Lee JD, Bibbs L, Ulevitch RJ. A MAP kinase targeted by endotoxin and hyperosmolarity in mammalian cells. Science 1994;265(5173):808-11.


References


Vanhaesebroeck B, Alessi DR. The PI3K-PDK1 connection: more than just a road to PKB. Biochem J 2000;346 Pt 3:561-76.


10. Attachments

10.1 Own publications

1. Tominaga, Y., Tamgüney, T., Kolesnichenko, M., Bilanges, B., and Stokoe, D.
   \textit{Translational Deregulation in PDK-1\textsuperscript{-/-} Embryonic Stem Cells.}

   \textit{Methylation of the PTEN promoter defines low-grade gliomas and secondary glioblastoma multiforme.}

3. Tamgüney, T., Stokoe, D.
   \textit{New insights into PTEN.}
   Journal of Cell Science, Dec. 2007, 1;120(Pt 23):4071-9

4. Tamgüney, T., Zhang, C., Fiedler, D., Shokat, K., and Stokoe, D.
   \textit{Analysis of 3-phosphoinositide-dependent kinase 1 signaling and function.}
   \textit{Manuscript submitted}

5. Tamgüney, T., Stokoe, D.
   \textit{PDK1 regulates murine ES cell pluripotency and differentiation.}
   \textit{Manuscript in preparation}
10.2 Curriculum Vitae

NAME
Tanja Manuela Tamgüney
Maiden name: Tanja Manuela Meyer
Date of Birth: August 14, 1978
Place of Birth: Schweinfurt, Germany
Address: 301 Carl, Apt#22
San Francisco, CA, 94117
USA
Phone: (415) 682-9982
Email: tmeyer@cc.ucsf.edu
tamguney@gmail.com

EDUCATION
1999 – 2008 Undergraduate and Graduate Program in Molecular Medicine,
Medical School,
Friedrich-Alexander University Erlangen-Nürnberg,
Erlangen, Germany
2002 Vordiplom in Molecular Medicine (Bachelor of Science)
2004 Diplom in Molecular Medicine (Masters of Science)
1998 –1999 Undergraduate Program in Biology,
Bayerische Julius-Maximilians University Würzburg,
Würzburg, Germany

Lab experience
08/2002 - present UCSF Cancer Center, San Francisco, CA, USA,
Internship, Diplom thesis and PhD thesis work in Dr. David Stokoe’s lab.
2001 - 2002 Friedrich-Alexander-University Erlangen-
Nürnberg, Germany
Lab assistant in the lab of Dr. Frank Neipel at the Institute for Clinical and Molecular Virology.
Summer 2000 Loyola University, Chicago, IL, USA
Internship in the lab of Dr. Adam Driks, Department of Microbiology and Immunology.
Spring 2000 Friedrich-Alexander-University Erlangen-
Nürnberg, Germany
Internship in the lab of Dr. Hans-Martin Jäck at the Institute for Experimental Medicine.
Summer 1999 University of Nancy, France
Internship at L.H.R.S.P., a hygiene and water lab.
Spring 1999 Laboklin, Bad Kissingen, Germany
Internship in diagnostic lab.
**FELLOWSHIPS**

2005-2007  
**PhD Fellowship,**  
Ernst Schering Foundation, Berlin, Germany.

**POSTERS & PRESENTATIONS**

1. Meyer T and Stokoe D.
   
   *Investigating the Role of PDK1 in tumorigenesis.*
   
   Poster, Annual Fellows Meeting of the Ernst Schering Foundation,  
   Berlin, Germany, 2005

2. Tamgüney T and Stokoe D.
   
   *Chemical genetic approach to assess the role of PDK1 in tumorigenesis.*
   
   Poster, Cold Spring Harbor Meeting ‘Mouse models of Cancer’,  
   Cold Spring Harbor, NY, 2006

3. Tamgüney T and Stokoe D.
   
   *Chemical genetic approach to assess the role of PDK1 in tumorigenesis.*
   
   Talk, Annual Fellows Meeting of the Ernst Schering Foundation,  
   Berlin, Germany, 2007

**PROFESSIONAL ACTIVITIES**

2007 – present  
Journal of Cell Science, ad hoc reviewer
10.3 Acknowledgements

I owe thanks to several people without whom this work would not have been possible.

I thank Dr. David Stokoe for giving me the opportunity to work in his laboratory and embark on this project. I thank him for the freedom he gave me and all his aid.

Also, I am very grateful to Dr. Frank McCormick for support to finish this work.

Furthermore, I am thankful to Dr. Martin McMahon for all his valuable input and suggestions.

I also thank Dr. Mike Fried for his support and encouragement.

Additionally, I am very grateful for everything my colleague Dr. Benoit Bilanges taught me, for fruitful discussions, and the great atmosphere he created in the lab.

Moreover, I am thankful for all the help I received from Dr. Markus Lacher.

I thank the Ernst Schering Foundation for supporting me financially with a PhD fellowship.

Furthermore, I owe thanks to Dr. Hans-Martin Jäck, who has been my mentor since I started studying at the University of Erlangen, and has been of great help in many respects since then.

I also thank Dr. Thomas Winkler for assessing this thesis.

Last but not least, I am tremendously thankful for everything my parents gave me, and I am deeply grateful for my husband’s patience, encouragements and support.