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ANALYSIS OF 3-PHOSPHOINOSITIDE-DEPENDENT KINASE-1 SIGNALING AND FUNCTION IN ES CELLS

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Abstract

3-Phosphoinositide-dependent kinase-1 (PDK1) phosphorylates and activates several kinases in the cAMP-dependent, cGMP-dependent and protein kinase C(AGC) family. Many putative PDK1 substrates have been identified, but have not been analyzed following transient and specific inhibition of PDK1 activity. Here, we demonstrate that a previously characterized PDK1 inhibitor, BX-795, shows biological effects that are not consistent with PDK1 inhibition. Therefore, we describe the creation and characterization of a PDK1 mutant, L159G, which 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 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 previously assumed PDK1 substrates, but revealed distinct dephosphorylation kinetics. While PDK1 inhibition had little effect on cell growth, it sensitized cells to apoptotic stimuli. Furthermore, PDK1 loss abolished growth of allograft tumors. Taken together we describe a model system that allows for acute and reversible inhibition of PDK1 in cells, to probe biochemical and biological consequences.

Keywords

PDK1; PKB; PI3K; chemical genetics; AGC kinases; apoptosis; teratoma; phosphorylation; BX-795; 1-NM-PP1

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Introduction

Phosphatidylinositol 3-kinase (PI3K) activates a wide variety of cellular protein kinases which coordinate a plethora of processes such as cell growth, proliferation, and survival. Once activated, PI3K synthesizes the lipid second messenger phosphatidylinositol-3,4,5-triphosphate (PIP_3), which in turn acts as a docking site at the plasma membrane that recruits 3-phosphoinositide dependent kinase 1 (PDK1). PDK1 is a serine/threonine kinase that was originally identified as the kinase that phosphorylates the activation loop of protein kinase B (PKB)/Akt (T308) in the presence of PIP_3 [1,2]. PIP_3 recruits PKB/Akt and PDK1 to the membrane by binding their pleckstrin homology (PH) domains. This colocalizes the two enzymes and is thought to lead to a conformational change in PKB/Akt allowing PDK1 to phosphorylate the activation loop of PKB/Akt [3]. PDK1 has subsequently been shown to phosphorylate and activate a whole group of related protein kinases belonging to the AGC (cAMP-dependent, cGMP-dependent, and protein kinase C) kinase family at their activation, or T-loop site. This includes isoforms of p70 ribosomal S6 kinase (S6K) [4,5], p90 ribosomal S6 kinase (RSK) [6], serum- and glucocorticoid-induced kinase (SGK) [7], conventional [8], novel and atypical [9] isoforms of protein kinase C, and PKC related kinases PRK1 and PRK2 [10]. These protein kinases regulate diverse cellular processes such as proliferation, survival, metabolism and translation.

Like other members of the AGC kinase family PDK1 requires phosphorylation of its activation loop site S241 for catalytic activity [11]. Although initially thought to be a constitutively active kinase, more recent evidence suggests that its activity might be regulated by phosphorylation under some circumstances [12,13].

Regulation of PDK1 action instead occurs at the level of PDK1 targets: recruitment of PKB/Akt to the plasma membrane and a subsequent conformational change render PKB/Akt a target for PDK1. Other PDK1 substrates like S6K, SGK, and RSK do not have a PH-domain and do not bind PIP_3, nor is their phosphorylation by PDK1 directly stimulated by PIP_3. 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 hydrophobic motif (HM) site. Phosphorylation of the HM-site by a distinct kinase allows PDK1 to bind to its targets through its specific substrate-docking site [14]. The phosphorylation of the HM in PKCs is more complicated, and may not be required for PDK1 binding – indeed PKCζ, PKCι and PRKs 1&2 have an acidic residue that replaces the HM phosphorylation site. Nevertheless, it seems that for optimal activity all isoforms require phosphorylation at their T-loop site by PDK1 or another kinase [15].

Studies using $PDK1^{+/+}$ and $PDK1^{-/-}$ murine ES cells revealed that PDK1 is absolutely required for the activation of PKB/Akt, S6K, and RSK [16]. Furthermore, stability and phosphorylation of several PKC isoforms and of PRKs are vastly reduced in $PDK1^{-/-}$ ES cells [17]. However, there has been speculation about 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 [18], 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 [16]. 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 [16].

While gene knockout technology, or knockin of an inactive version, can give valuable information about the role of a given protein, the lack of temporal control hampers the study of dynamic processes. Conditional alleles 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 [19]). 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.

We therefore set out to study the biochemical and biological effects of acutely inhibiting PDK1 activity. We initially utilized a recently developed small molecule inhibitor of PDK1, BX-795, which was shown to inhibit PDK1 signaling, cause a cell cycle arrest in G2/M, and inhibit tumor formation [20]. Surprisingly, we noticed that the ability of BX-795 to cause a G2/M arrest was similar in $PDK1^{+/+}$ ES cells compared to $PDK1^{-/-}$ ES cells, suggesting that the cell cycle consequences of this compound were unrelated to PDK1 inhibition. To achieve acute but more specific inhibition of PDK1, we employed a chemical genetic approach, whereby mutation of conserved residue(s) in its ATP binding site confers sensitivity to ATP and inhibitor analogues [21]. We mutated the large hydrophobic amino acid L159, referred to as the gatekeeper residue, to glycine (L159G, hereafter termed LG). This substitution did not dramatically change catalytic activity, but allowed access by inhibitor analogues with bulky constituents. We demonstrate effective inhibition of PDK1 LG by a panel of inhibitor analogues, most of which have no activity against wild type (WT) PDK1. Then, we generated stable cell lines by introducing either PDK1 WT or LG into murine $PDK1^{-/-}$ ES cells. This reconstitutes signaling of PDK1 to its downstream substrates, allows selective inhibition of PDK1 activity, and provides proof of concept that acute inhibition of PDK1 can be used in cells to discern downstream substrates and biological consequences of PDK1 activity. Employing this system, we demonstrate that while PDK1 inhibition barely affects cell growth under regular culture conditions, it sensitizes cells to apoptotic stimuli. Together with our finding that loss of PDK1 hampers the growth of allograft tumors, this suggests that targeting PDK1 by itself or in combination with standard chemotherapeutics could be a beneficial treatment for cancer.

Materials and Methods

Allograft studies

Three to five weeks old female NCr nude outbred mice [(MCr)-$Fox1^{nu}$] (Taconic) were injected subcutaneously in one flank with 5×10^5 cells in 100 µl DPBS. Five mice received $PDK1^{-/-}$ ES cells, another five mice $PDK1^{+/+}$ ES cells. After 75 days allografts were harvested and weighed. Similarly, 24 NCr nude mice were injected subcutaneously in one flank with 1×10^6 $PDK1^{-/-}$ +LG ES cells, in the other flank with 1×10^6 $PDK1^{-/-}$ +WT ES cells, and tumors were excised after 21 days and weighed.

Apoptosis assay

$PDK1^{-/-}$, $PDK1^{+/+}$, $PDK1^{-/-}$ +LG and $PDK1^{-/-}$ +WT ES cells were treated with 10 µM 3,4-DMB-PP1, 1-NM-PP1 or DMSO control for 24 h. Then medium was replaced with fresh medium with or without inhibitor, and with or without 200 nM Actinomycin D or 10 µg/ml Anisomycin (both from Sigma) to induce apoptosis. After 8 h, floating and attached cells were harvested, and apoptosis was measured by assessing Caspase 9 and PARP cleavage by Western blotting.

Cell culture

If not indicated otherwise ES cells were grown on gelatinized dishes in KnockOut Dulbecco's 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.

Cell cycle analysis

Cells were harvested using cell dissociation buffer (Invitrogen) and Dulbecco's phosphate buffered saline (DPBS) (Invitrogen) following 48 h of treatment with either 20 μM 3,4-DMB-PP1, or 1-NM-PP1, or 5 μM BX-795. Cells were fixed in 70% ethanol at 4 °C and resuspended in DPBS containing 10 μg/ml propidium iodide (Roche Applied Sciences) and 1 μg/ml RNase A, incubated for 30 min at room temperature and analyzed using a BD FACS Calibur.

Cell proliferation assay

Cells were seeded into gelatinized 96 well plates at 5000 to 10000 cells per well. 12 h after seeding cells were treated in sets of five with either 10 μM 3,4-DMB-PP1, or 1-NM-PP1, or 5 μM BX-795. Medium was replaced every 24 h. After 72 h cell proliferation was assessed using the CellTiter96® AQ_{ueous} One Solution kit (Promega).

Generation of stable cell lines

PDK1 WT cDNA was cloned into pcDNA3 with a 5' Myc-tag. PCR mutagenesis with primers f-CCATTTTTGGCATAACTACCGCCGAAATAC and r-GAAGCTGTATTTCGGCGGTAGTTATGCCAA gave the mutant encoding PDK1 L159G. Both constructs were electroporated into $PDK1^{-/-}$ ES cells (electroporator and reagents from Amaxa), 24 hours after electroporation cells were selected with 250 μg/ml geneticin (Invitrogen) and pools of cells stably expressing PDK1 WT or LG were expanded.

IC_{50} 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 CPAc-BX), 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.hih.gov/nih-image/), curves were fitted and IC_{50} values were generated with SigmaPlot. Several exposures of the HRP generated ECL films were analyzed to generate the semi-quantitative graphs shown in the figures. Heatmaps were generated with the help of Java TreeView.

In vitro PDK1 kinase assay

PDK1 kinase assays were performed with recombinant proteins purified from Sf9 cells. Both the PDK1 and ΔPH-PKB proteins were N-terminally glu-glu-tagged, and were purified using a glu-glu antibody generated from mouse ascites, and eluted using an EYMPME peptide [22]. 150 ng of WT PDK1 or 500 ng PDK1 L159G were used. ΔPH-PKB/ Akt was used as a substrate at 210 ng. These amounts of kinase and substrate generated linear reaction conditions under the time points analyzed. Inhibitors were used at varying final concentrations from 1 to 50 μM. The reactions were done in 10 μl kinase buffer (20 mM Tris·Cl pH 7.5, 1 mM EDTA, 75 mM NaCl, 5 mM $MgCl_2$, 1 mM DTT) containing 20 μM ATP and 5 μCi of [γ^{32}P]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). Incorporated ^{32}P-radioactivity was assessed using a STORM PhosphoImager (Amersham), and quantitated using ImageQuant5.2.

Sequence analysis

Human and murine AGC kinase T-loop sequences were taken from NCBI and Ensembl databases, 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).

Western blotting

Antibodies against β-Actin and β-Tubulin were from Sigma, against 4E-BP1, phospho-4E-BP1 S65, phospho-4E-BP1 S37/S46, phospho-GSK3 S21/S9, phospho-MSK1 S376, phospho-MSK1 T581, phospho-p38 T180/Y182, phospho-PDK1 S241, phospho-PKA T197, phospho-PKB/Akt T308, phospho-PKC pan, phospho-PKCδ T505, phospho-PKCθ T538, 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 orhovanadate, 5 mM sodium pyrophosphate, 0.27 M sucrose, 1 μM microcystin-LR (Calbiochem), and one complete mini protease inhibitor pill (Roche) per 10 ml. 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 h at room temperature. Proteins were detected by ECL (Amersham Biosciences). Densitometric analysis of the bands was carried out using the NIH ImageJ software.

Results

The effect of BX-795 on G2/M arrest is mostly PDK1 independent

BX-795 is a recently developed aminopyrimidine-based inhibitor of PDK1, which potently inhibits PDK1 activity in vitro (IC_{50} of 6 nM) and reduces phosphorylation of PKB/Akt on T308 in cells with an IC_{50} of 300 nM [20]. We assessed the ability of this compound to inhibit PDK1 signaling in mouse ES cells, and compared this to the signaling in $PDK1^{-/-}$ mouse ES cells. Consistent with the previous report, BX-795 strongly inhibited the phosphorylation of PKB/Akt T308, while having little effect on phosphorylation of S473 (Fig. 1A), which is phosphorylated by mammalian Target Of Rapamycin Complex 2 (mTORC2) [23]. BX-795 also inhibited the phosphorylation of PKB/Akt substrates such as Glycogen Synthase Kinase 3 (GSK3) α/β S21/S9 and 40 kDa Proline-Rich Akt Substrate (PRAS40) T246, as well as S6 S235/S236, 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 – this 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 [24,25].

We next examined the effects of BX-795 on the cell cycle of $PDK1^{+/+}$ and $PDK1^{-/-}$ ES cells. 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 [26]. Nevertheless, a G2/M arrest could clearly be seen when $PDK1^{+/+}$ ES cells were incubated with BX-795, which was also observed using

Nocodazole, a positive control (Fig. 1B). Surprisingly, an increase in G2/M arrested cells was also apparent in PDK1$^{-/-}$ ES cells treated with BX-795, which was almost as great as that seen in PDK1$^{+/+}$ ES cells (32% increase vs 42%). This suggested that BX-795 might be inhibiting additional protein kinases that could contribute to this observed G2/M arrest. Profiling BX-795 against 211 protein kinases 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 cell cycle such as Cdk1, Cdk2, Aurora A, Aurora B, and Aurora C. A recent report that profiled BX-795 against 72 protein kinases also showed inhibition of Aurora kinases and Cdk2, as well as ERK8, MNK2, MARK3 and IKKε [27]. Therefore, it seems likely that one of these is the relevant target responsible for G2/M arrest, and not PDK1.

Identification of inhibitor analogues for PDK1 LG inhibition in vitro and in vivo

Because of the apparent non-specific effects of BX-795, we attempted to develop a system to inhibit PDK1 activity more specifically in ES cells. Mutation of L159G in PDK1 creates an enlarged ATP binding site, potentially allowing inhibition by compounds unable to bind WT kinases. This approach has been successfully applied to many protein kinases (reviewed in [21]), although it is not universally tolerated [28,29]. First we tested the activity of this mutant and its ability to be inhibited *in vitro* by previously described analogues of PP1, a general kinase inhibitor. Fig. 2 shows that PDK1 LG is only slightly compromised compared to WT PDK1 in its to phosphorylate ΔPH-PKB/Akt, a PH domain deleted mutant of PKB/Akt 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 WT PDK1 was not inhibited, or only inhibited ~50%.

We next established a cell based system to analyze the ability of PP1 analogues to inhibit PDK1 LG. PDK1$^{-/-}$ ES cells have previously been shown to lack phosphorylation and activation of a number of PDK1 substrates [16]. 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. We therefore expressed WT and PDK1 LG in PDK1$^{-/-}$ ES cells, generating pools of stable cells by electroporation and stable selection. Although PDK1 overexpression may not be identical in terms of overall cellular consequences due to its docking functions, this completely recovered the signaling defects seen in the knockout cells, as judged by restoration of IGF1 inducible phosphorylation of PKB/Akt on T308 (Fig. 3A). PKB/Akt S473 phosphorylation is less affected by loss of PDK1, as previously shown [16,25]. In addition, the inducible phosphorylation of the downstream PKB/Akt substrates GSK3 and PRAS40 was also fully restored following expression of WT or PDK1 LG (Fig. 3A). 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 [30]. 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. 3A, and [25]). Nevertheless, S6K T389 phosphorylation was restored upon re-expression of either WT or PDK1 LG (Fig. 3A). Furthermore, the cell size defect seen in PDK1$^{-/-}$ relative to PDK1$^{+/+}$ ES cells ([31] and Fig. 3B) was also partially reversed upon expression of either PDK1 allele (Fig. 3B).

We then tested the PP1 analogues shown in Fig. 2, as well as additional ones shown in Fig. 4A for their ability to inhibit PDK1 signaling in the WT and LG reconstituted ES cells. 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. A one hour incubation with these compounds inhibited IGF1 stimulated phosphorylation of PKB T308 in PDK1$^{-/-}$ +LG ES cells. Phosphorylation of PKB/Akt targets GSK3 S9/S21, and PRAS40 T246 was equally inhibited

(Fig. 4B and C, and Supplemental Fig. 1A and B). These compounds had minimal effects on any of these 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 we tested did show some degree of PDK1 inhibition in PDK1$^{-/-}$ +WT ES cells in addition to PDK1$^{-/-}$ +LG ES cells. Moreover, we noticed that S6K T389 and S6 S235/S236 phosphorylation were sensitive to many of these PP1 analogues, even in WT PDK1 ES cells (Fig. 4C). We also analyzed 4E-BP1 phosphorylation in WT PDK1 ES cells in response to these inhibitors. 4E-BP1 phosphorylation was rarely affected in either cell line (Fig. 5E and data not shown), suggesting that mTORC1 is probably not the target and that S6K itself might be particularly susceptible to this class of PP1 analogues. Fig. 4C summarizes the in cell IC_{50} values for all compounds and phosphorylation sites tested, and Supplemental Fig. 1 shows representative Western blots from which these data were calculated.

Before examining any potential biological consequences of PDK1 inhibition, we tested whether these compounds were able to durably inhibit PDK1 activity. Supplemental Fig. 2 shows that at 24 h following administration PDK1 downstream signaling remained inhibited, as measured by PKB/Akt T308, GSK3 S9/S21, and S6 S235/S236 phosphorylation. Interestingly, BX-795 actually reproducibly caused increased T389 phosphorylation at later time points. The reason for this is not clear but could represent effects of additional targets of BX-795.

Phosphorylation of known and potential PDK1 targets following long term inhibition of PDK1

Next, we analyzed the phosphorylation state of additional known and potential PDK1 targets in the AGC kinase family. Confirming previous reports, multiple AGC kinases showed defects in activation loop phosphorylation in PDK1$^{-/-}$ ES cells, including p90RSK, PRK1/2, and some isoforms of PKC relative to PDK1$^{-/-}$ +LG ES cells (Fig. 5A). Phosphorylation of PKA T197 relative to total PKA was also slightly decreased in PDK1$^{-/-}$ ES cells to PDK1$^{-/-}$ +LG ES cells. Total levels of various PKC isoforms were also increased following expression of PDK1 L159G, consistent with previous reports [32]. We then analyzed phosphorylation of PDK1 substrates following incubation with the PP1 analogues 1-NM-PP1 and 3,4-DMB-PP1 in PDK1$^{-/-}$ +LG cells. As members of this group include protein kinases activated by stimuli other than IGF1, we also included TPA, forskolin, and sorbitol in this analysis. To analyze the effects of basal as well as stimulated phosphorylation, inhibitors were added 23.5 h prior to cell stimulation in these experiments. Again, 3,4-DMB-PP1 and 1-NM-PP1 inhibited PKB/Akt T308 phosphorylation in response to IGF1 (Fig. 5B). Moreover, basal as well as stimulated phosphorylation of GSK3 and PRAS40 at PKB/Akt sites were inhibited by 3,4-DMB-PP1 and 1-NM-PP1. Interestingly, sorbitol-induced GSK3 phosphorylation appears to be somewhat resistant to PDK1 inhibition, and instead is inhibited by U0126 and SB203580, suggesting that GSK3 is phosphorylated by kinases in addition to PKB/Akt in response to osmotic stress.

Phosphorylation of the p90RSK N-terminal kinase domain activation loop is highly dependent on PDK1 activity, with 3,4-DMB-PP1 and 1-NM-PP1 showing strong inhibition of both basal and TPA stimulated phosphorylation of S221/S227, which are activation loop sites of RSK1 and RSK2 respectively (Fig. 5C). 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, is unaffected by 3,4-DMB-PP1 or 1-NM-PP1. Notably, an only one hour inhibition of PDK1 barely affects phosphorylation at RSK1/2 S221/S227 (data not shown). PRK1/2 have been shown to be phosphorylated by PDK1 at their activation loop in vitro and following transient transfection [10]. Surprisingly, we saw very little to no effect of PDK1 inhibition on the phosphorylation of PRK1/2 under the conditions tested. 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. Neither of these represented PKCδ or PKCθ, as determined with isoform-specific phosphor-antibodies (data not shown). 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. Phosphorylation of PKA at T197 was in some experiments very slightly decreased following treatment with 3,4-DMB-PP1 and 1-NM-PP1. 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. 5C).

MSK1/2 show a similar two kinase domain structure and activation profile to p90RSK, however, their activation by UV or TPA was similar in PDK1$^{-/-}$ and PDK1$^{+/+}$ or PDK1$^{-/-}$ +LG ES cells (Fig. 5A and [16]). Given the high homology between the RSK and MSK activation loop sequences (Supplemental Fig. 3), we wanted to assess whether under certain conditions MSK might also be a target for PDK1. Initial experiments indicated that phosphorylation of the activation loop site in the MSK1 N-terminal kinase domain in response to sorbitol was sensitive to PDK1 inhibition (Fig. 5D). However, subsequent experiments showed that 3,4-DMB-PP1 or 1-NM-PP1 also inhibited sorbitol-induced phosphorylation of MSK1 at S581 by ERK/p38 MAPK and ERK/p38 dependent autophosphorylation at S376 (Fig. 5D). Moreover we also observed inhibition of p38 MAPK phosphorylation itself by these compounds. Therefore, the inhibition of the activation loop phosphorylation of MSK1/2 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 [16,33].

We were also interested in the effect of 3,4-DMB-PP1 and 1-NM-PP1 on the T-loop phosphorylation of S6K (T229). However, none of the available phospho-specific antibodies worked reliably enough to obtain interpretable results. We therefore assessed S6K activity indirectly by analyzing its phosphorylation at T389 as well as phosphorylation of S6 at S6K specific sites, namely S240/S244 [34]. We also further analyzed mTORC1 activity by assessing phosphorylation of 4E-BP1 at the mTORC1 sites S37/S46 and S65 [35]. Selective inhibition of S6 S240/S244 by 3,4-DMB-PP1 or 1-NM-PP1 was observed, confirming the inhibition of S6K activity in PDK1$^{-/-}$ +LG ES cells. We did not observe any reduction in phosphorylation of 4E-BP1 at any of the mTORC1 sites, confirming that mTORC1 activity is not affected following inhibition of PDK1 and PKB/Akt activity in ES cells. Interestingly, for 24 h treatments, 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 h at high concentrations of these drugs, even though S240/S244 phosphorylation was unaltered (Fig. 5E). The temporal effect of inhibiting PDK1 on the phosphorylation of its direct downstream substrates is summarized in Table 1.

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 represent useful probes to analyze the effects of specifically inhibiting PDK1 activity, they suffer from drawbacks, namely lack of potency (IC_{50} of 1–5 μM at inhibiting PKB/Akt T308 phosphorylation in cells), lack of selectivity (inhibition of p38 MAPK and S6 phosphorylation in PDK1$^{-/-}$ +WT ES cells) and growth inhibitory properties (Fig. 7B and data not shown). 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 ([20,27] 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 shows 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. We therefore made the compounds shown in Supplemental Fig. 4A and tested them for their ability to inhibit phosphorylation of PKB/Akt T308 in $PDK1^{-/-}$ +LG and $PDK1^{-/-}$ +WT ES cells (Supplemental Fig. 4B). CPAc-BX potently inhibits the phosphorylation of PKB/Akt T308 in $PDK1^{-/-}$ +LG ES cells, and does not inhibit this site in $PDK1^{-/-}$ +WT ES cells (Supplemental Fig. 4B). We therefore extended the analysis of CPAc-BX to additional PDK1-dependent targets and confirmed that the potency of CPAc-BX was indeed enhanced on GSK3 and PRAS40 phosphorylation (Supplemental Fig. 4C,D). However, non-specific effects on S6 phosphorylation at higher CPAc-BX concentrations were apparent, similar to those seen with 3,4-DMB-PP1 and 1-NM-PP1 (Supplemental Fig. 4C,D). The in cell IC_{50} values of CPAc-BX towards PKB/Akt T308 and S6 235/236 phosphorylation are summarized in Supplemental Fig. 4E.

Specific inhibition of PDK1 sensitizes cells to apoptosis

In addition to the biochemical effects of PDK1 inhibition, we were also interested in biological consequences. Since the BX-795 derivatives did not have a considerably? improved specificity window towards S6 S235/S236 than 3,4-DMB-PP1 and 1-NM-PP1, we decided to continue using the latter compounds, always with appropriate controls to check for the specificity of the effects seen.

Neither 3,4-DMB-PP1 nor 1-NM-PP1 caused any effects on cell cycle distribution in $PDK1^{-/-}$ +LG ES cells at 20 μM (Fig. 6A), a concentration that achieved comparable biochemical knockdown of PDK1 activity as 5 μM BX-795 as judged by PKB/Akt T308 phosphorylation. This is consistent with the similar cell cycle profile between $PDK1^{+/+}$ and $PDK1^{-/-}$ ES cells (Fig. 6B). BX-795 on the other hand still caused a G2/M arrest in these cells.

We also analyzed the consequences of 3,4-DMB-PP1 and 1-NM-PP1 on the proliferation and viability of $PDK1^{-/-}$ +LG and $PDK1^{-/-}$ +WT ES cells. When cultured in high serum (15% serum replacement (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. 6B). Next, we analyzed if PDK1 inhibition had an effect on apoptosis following induction of cellular stresses. First, we showed 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, as assessed by cleavage of Caspase 9 and its target poly (ADP-ribose) polymerase (PARP). Both Caspase 9 and PARP are cleaved to a much bigger extent in $PDK1^{-/-}$ ES cells as in cells containing PDK1 (Fig. 6C). Furthermore, specific inhibition of PDK1 reproduced the effect of loss of PDK1 on apoptosis sensitization. A representative experiment shown in Figure 6D and 6E demonstrates that PDK1 inhibition sensitizes to apoptosis induction by Actinomycin D, albeit not to the full extent seen in $PDK1^{-/-}$ ES cells.

To determine whether increased sensitivity of cells lacking PDK1 to apoptotic stimuli may play a role in vivo, we assessed the role of PDK1 in tumor growth. When $PDK1^{+/+}$ and $PDK1^{-/-}$ ES cells were allografted into the flanks of nude mice, 4/5 mice injected with $PDK1^{+/+}$ ES cells grew easily detectable teratoma tumors, whereas only 1/5 mice injected with $PDK1^{-/-}$ ES cells displayed one small tumor (Fig. 6F). Re-expression of WT or LG PDK1 in $PDK1^{-/-}$ ES cells, restored the ability of these cells to rapidly form tumors in all cases (Fig. 6F), demonstrating that the observed differences between $PDK1^{+/+}$ and $PDK1^{-/-}$ ES cells are indeed due to PDK1.

Discussion

In this study we have examined the effects of transiently inhibiting PDK1 activity in murine ES cells. While we initially used BX-795, a previously characterized PDK1 inhibitor, the use

of isogenic PDK1$^{+/+}$ and PDK1$^{-/-}$ ES cells demonstrated that the observed G2/M arrest was due to off-target effects, possibly due to inhibition of Cdks or Aurora kinases. Therefore, we 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 [36], ERK2 [37] and Cdk7 [38].

The effect of PDK1 loss on downstream targets has been extensively profiled in PDK1$^{+/+}$ vs PDK1$^{-/-}$ ES cells by Alessi and colleagues [16,17,39]. The conclusions from these experiments were that AGC kinases of the p90RSK, S6K, PKB/Akt, 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. Our approach allowed a temporal dissection of these events, which led to slightly different conclusions. T-loop phosphorylation of PKB/Akt was dramatically reduced after both 1 h and 24 h inhibition of PDK1 activity. On the other hand, p90RSK phosphorylation at the activation loop site was only slightly reduced after 1 h but was almost completely abolished by 24 h inhibition of PDK1 activity. The phosphorylation of putative PKC isoforms was also reduced following inhibition of PDK1, although the exact identity of different PKC isoforms was not established. However, while the phosphorylation of PRK1/2 was dramatically reduced in the PDK1$^{-/-}$ ES cells, phosphorylation was not affected following 24 h 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 [10]. 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/Akt and PKC isoforms [40,41]. Given the large disparity seen here for dephosphorylation of different activation loop residues, further work in this area is warranted.

Our experiments employing acute PDK1 inhibition in conjunction with various stimuli also revealed that T-loop phosphorylation of p90RSK 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 [42], p90RSK is normally not thought to participate in this response [43]. 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. To our knowledge GSK3 has not been implicated in the response to osmotic stress, and our results suggest that a PDK1-independent kinase, i.e. not PKB, nor S6K, nor RSK, is responsible for phosphorylation of these sites under these conditions.

The allele independent effects of 3,4-DMB-PP1 and 1-NM-PP1 observed in these studies were unexpected, as previous reports using these and similar compounds have not demonstrated many off-target effects (although it is not clear to what extent this has been examined). There are at least three potential explanations for these results. Firstly, these compounds could inhibit the activity of an endogenous S6 kinase, such as p90RSK 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 p90RSK (data not shown). A second possibility is that these agents cause some kind of stress to these cells, 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, we did not see strong effects on direct mTORC1 targets such as S6K T389 or 4E-BP1 phosphorylation. 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 [34] 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.

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 [44–46], and a hypomorphic germline mutation also results in smaller animals [31]. 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 [20]. 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 (data not shown). This observation was also made by another group [27]. Therefore, the G2/M arrest seen in these studies, as well as at least part of the antitumor activity demonstrated in allograft 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 rather causes a profound sensitization to apoptosis induced by cellular stress. As actinomycin D and similar compounds are utilized in the clinical arena, this has implications for the use of PDK1 inhibitors as chemosensitizing agents. Moreover, we demonstrate that cells lacking PDK1 are strongly defective for tumor formation, suggesting that tumor growth *in vivo* encounters similar stresses that PDK1 activity protects against.

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, we show 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, we demonstrate that inhibition of PDK1 activity results in sensitization to cellular stresses and decreased tumor formation, which reinforces the concept of PDK1 as an attractive oncology drug target.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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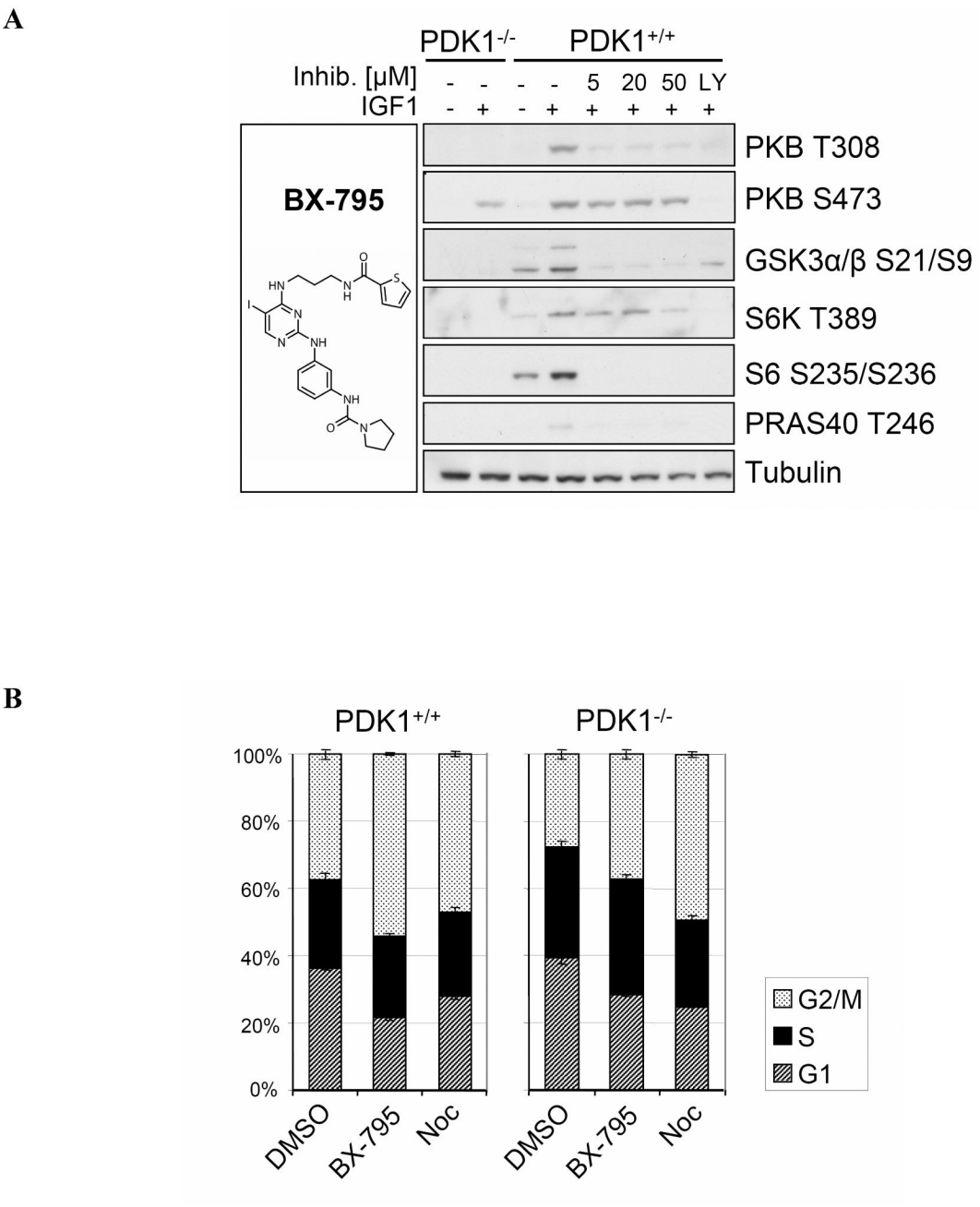


FIGURE 1. Effects of BX-795 on signaling and cell cycle distribution in PDK1$^{+/+}$ and PDK1$^{-/-}$ ES cells

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. *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 h. The positive control for G2/M arrest was treated with 100 ng/ml Nocodazole 24 h before harvest. Cells were subject to propidium iodide staining and cell cycle distribution

was analyzed using a BD FACS Calibur. Shown is a representative experiment performed in triplicates, standard deviations of which are indicated.

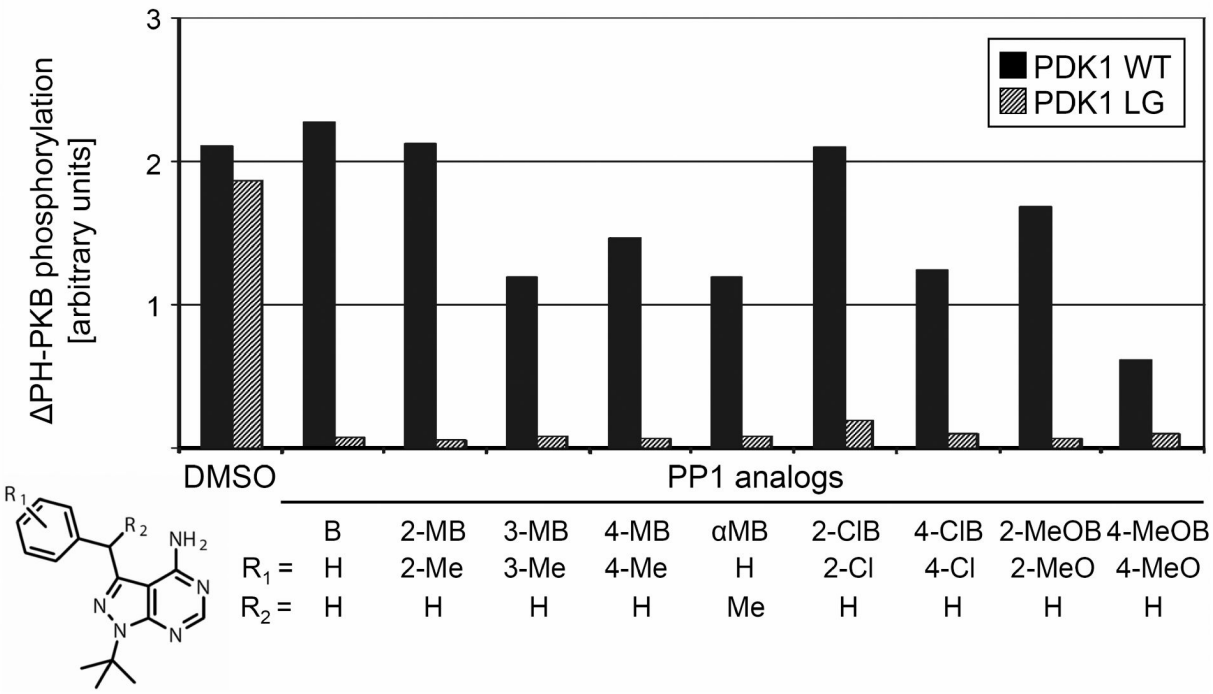


FIGURE 2. Inhibition of PDK1 L159G by PP1 analogues

Structure of purine analogues and effect on activity of PDK1 WT and PDK1 LG. *In vitro* kinase assays were carried out with ΔPH-PKB/Akt as a substrate and either WT or mutant PDK1 as kinase. All reactions were done in duplicates in kinase buffer, 20 μM ATP and 5 μCi of [γ-^{32}P]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.

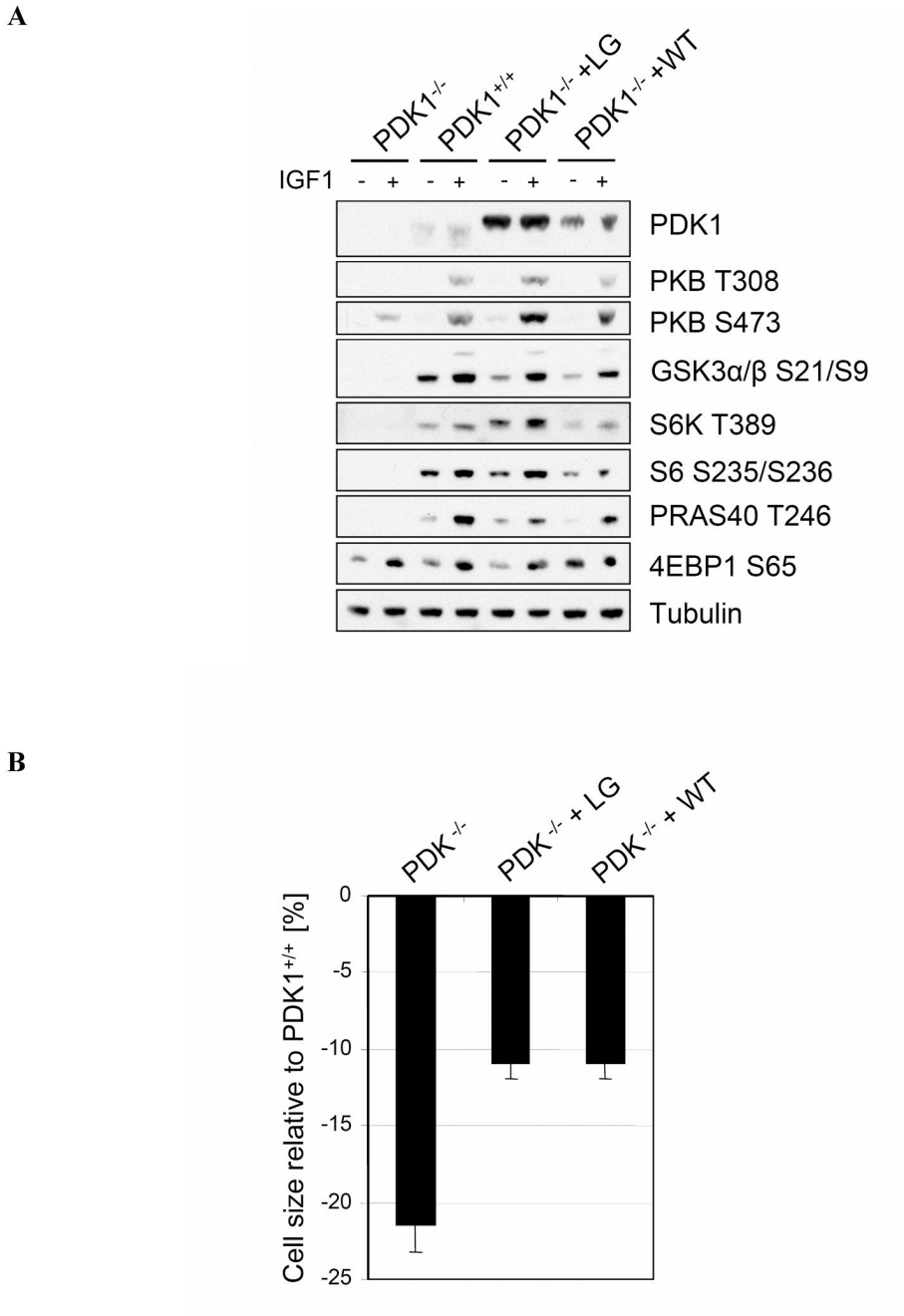
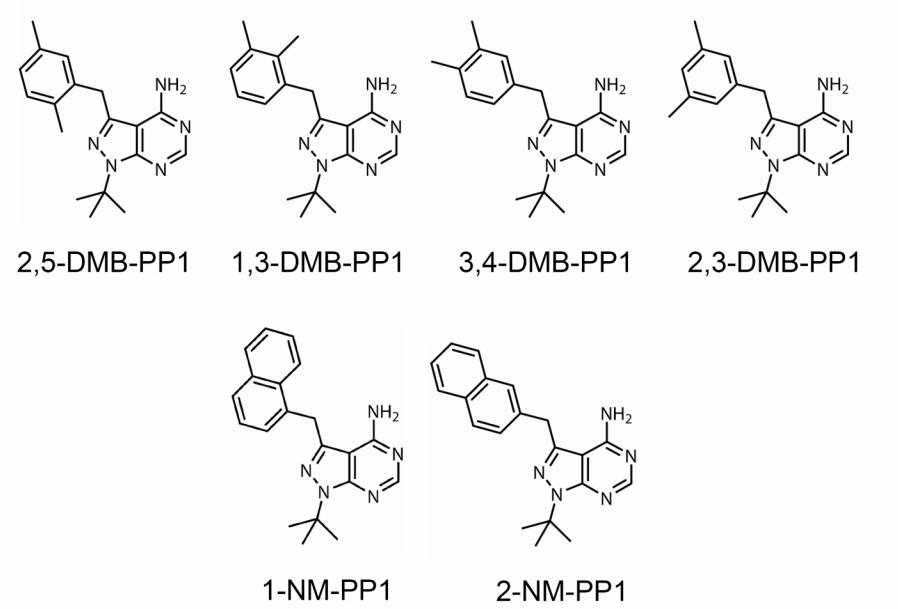


FIGURE 3. Reconstitution of WT and L159G PDK1 into PDK1$^{-/-}$ ES cells

A, PDK1$^{+/+}$, PDK1$^{-/-}$, and PDK1$^{-/-}$ ES cells stably expressing either PDK1 WT (PDK1$^{-/-}$ +WT) or PDK1 L159G (PDK1$^{-/-}$ +LG) were starved of SR for 3 h 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. Exogenous PDK1 has a glu-glu tag and thus runs slightly higher. *B*, PDK1$^{+/+}$, PDK1$^{-/-}$, PDK1$^{-/-}$ +WT and PDK1$^{-/-}$ +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$^{+/+}$ cell size. Shown is a representative experiment performed in triplicates; standard deviations are indicated.

A



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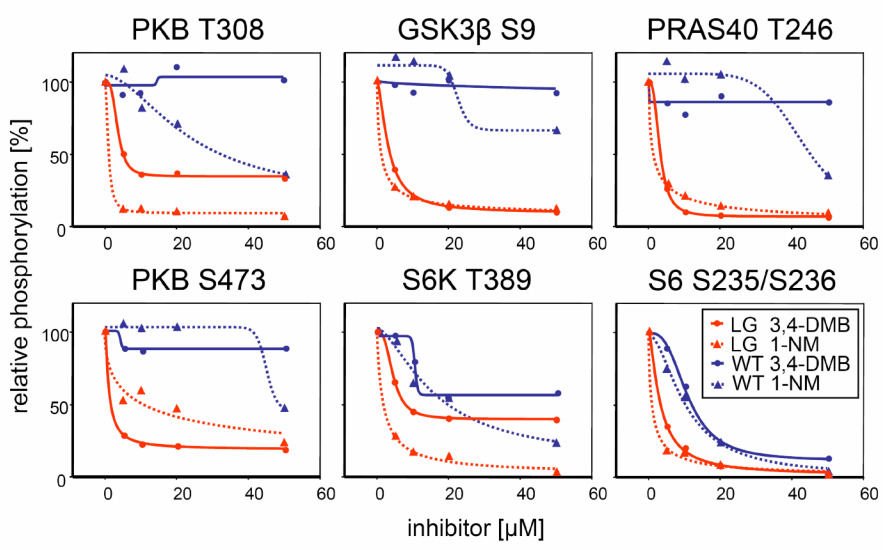


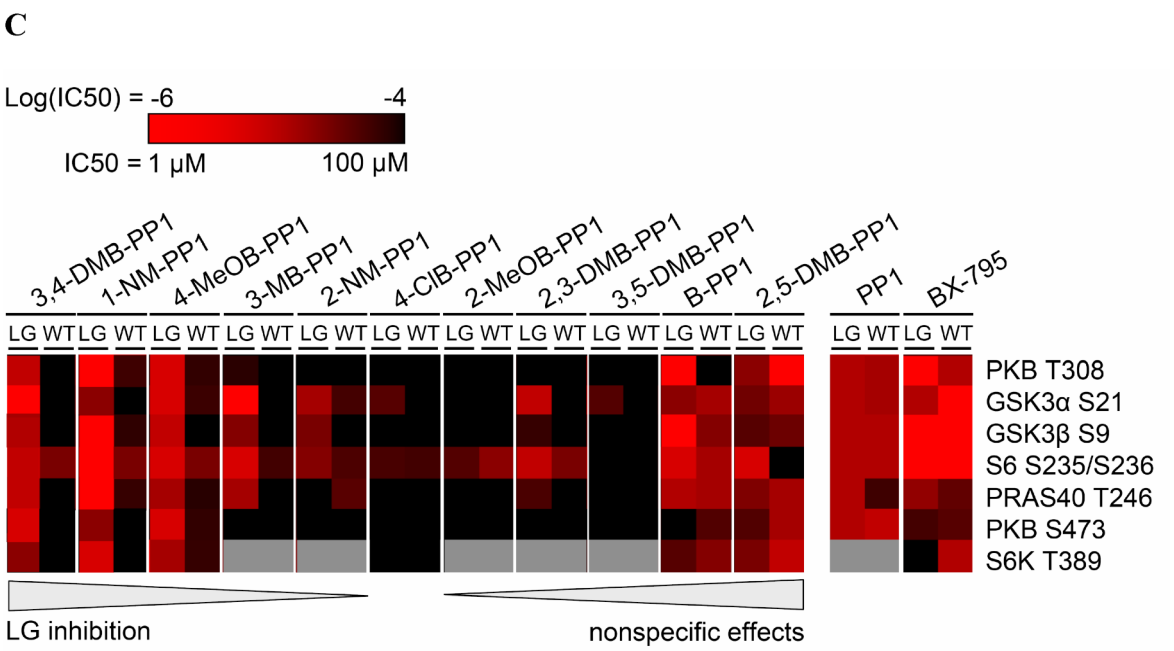
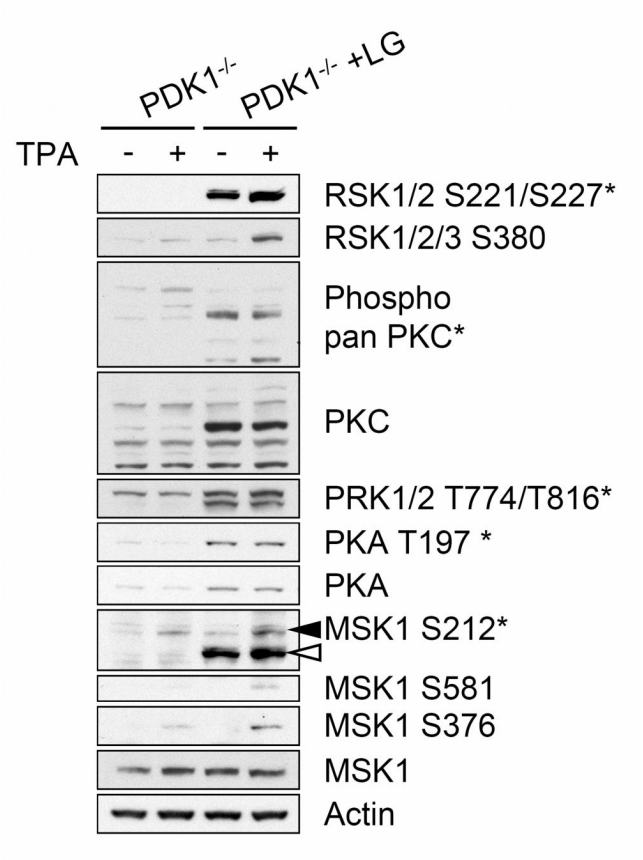


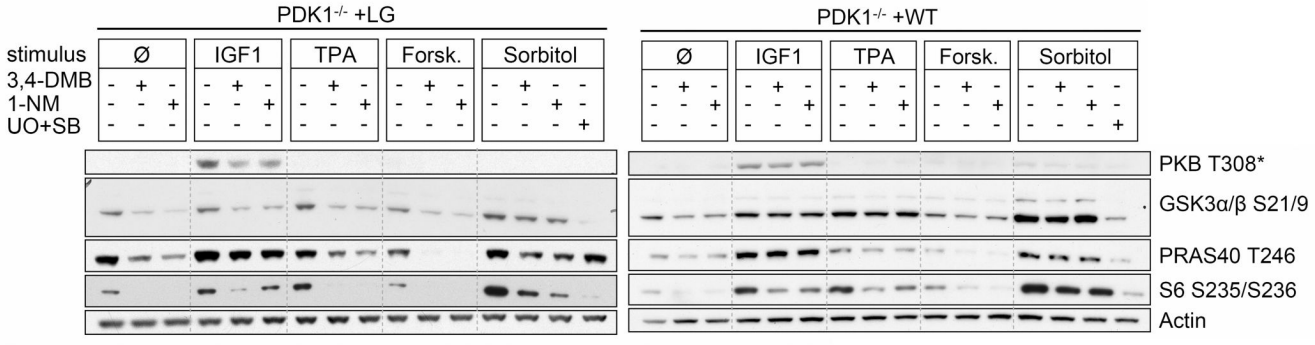
FIGURE 4. Effects of PP1 analogues on PDK1 inhibition in ES cells

A, Additional PP1 analogues used for in cell culture studies. *B–C*, PDK1$^{+/+}$, PDK1$^{-/-}$, PDK1$^{-/-}$ +WT, and PDK1$^{-/-}$ +LG ES cells were starved of SR for 3 h, then treated with indicated concentrations of 3,4-DMB-PP1, 1-NM-PP1, 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. Bands were quantitated using NIH ImageJ software and displayed relative to no inhibitor. *C*, IC_{50} values of a panel of PP1 analogues and BX-795 on 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. The heatmap was generated using Java TreeView. Bright red squares indicate potent inhibition, darker shades of red less potent inhibition; grey signifies not determined.

A



B



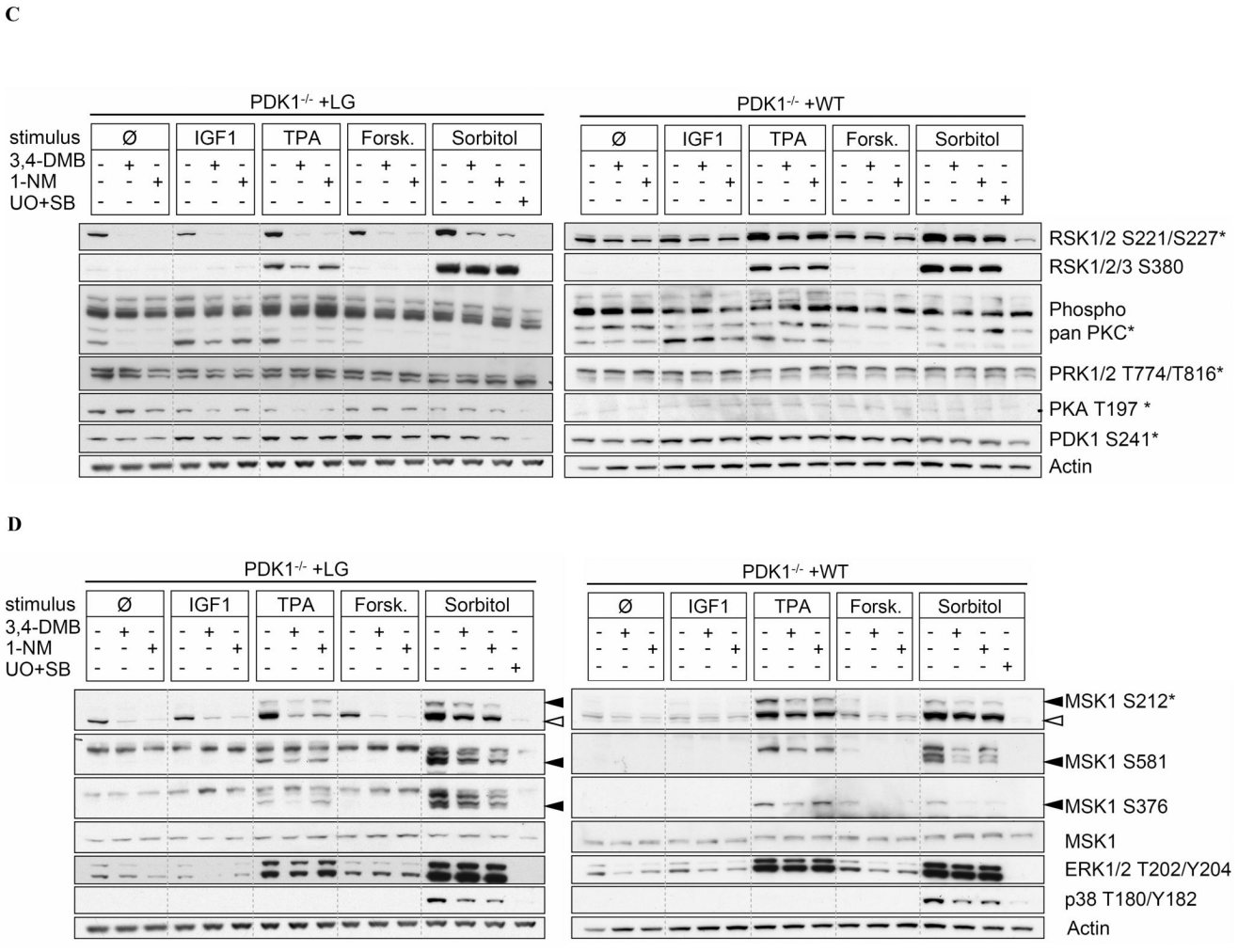
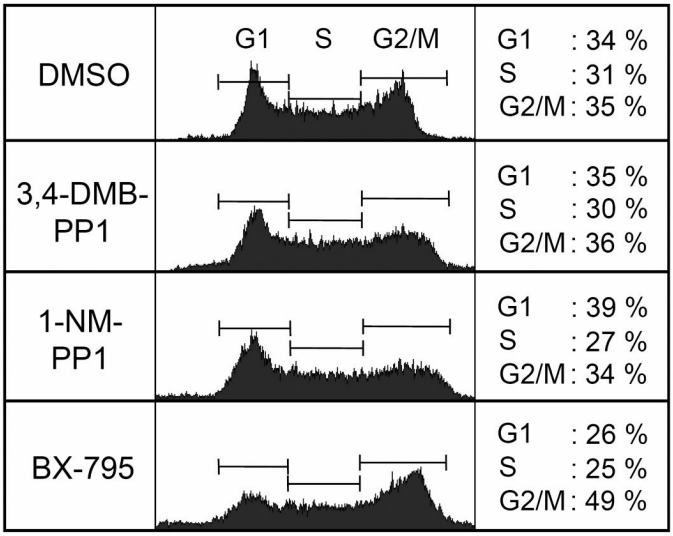


FIGURE 5. PDK1 LG inhibition differently effects T-loop phosphorylation of PDK1 targets

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.

A



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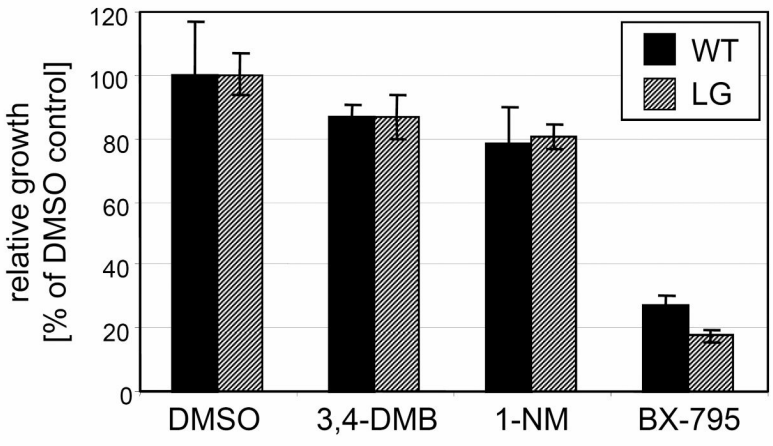

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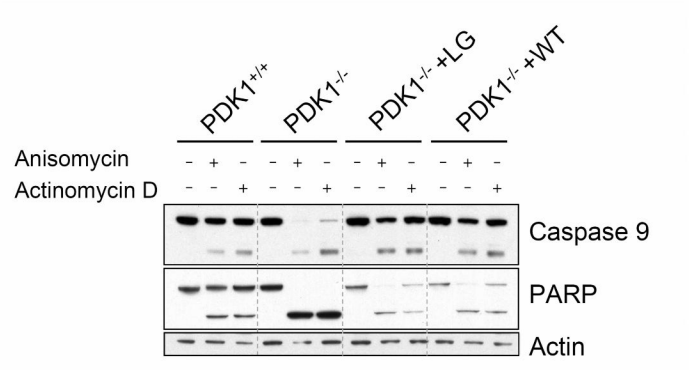

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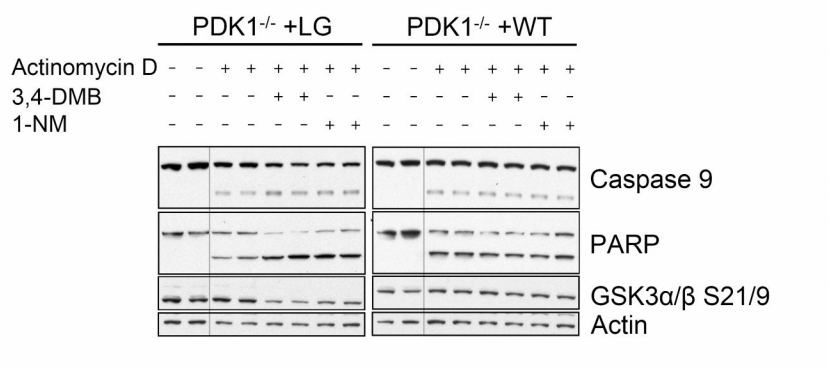

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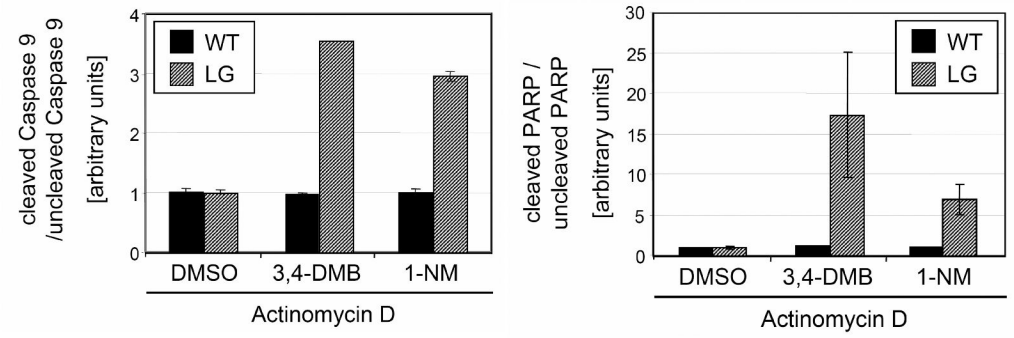

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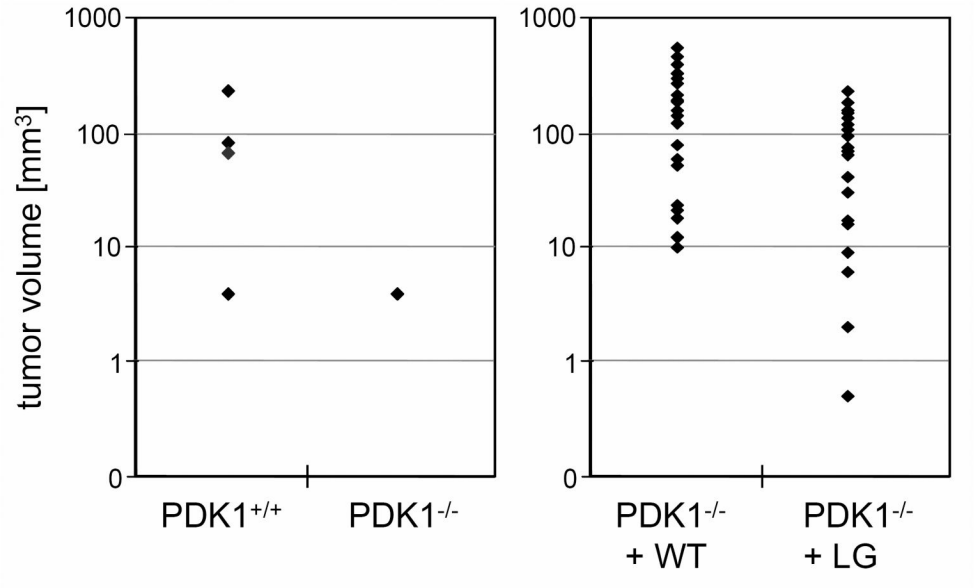

FIGURE 6. Biological consequences of PDK1 inhibition and PDK1 loss

A, Cell cycle. PDK1$^{-/-}$ +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, replacing medium with inhibitor after 24 h. Cells were subject to propidium iodide staining and cell cycle distribution was analyzed using BD FACS Calibur. Shown is a representative experiment performed in triplicates; standard deviations are indicated. *B, Cell viability and proliferation*. PDK1$^{-/-}$ +WT and PDK1$^{-/-}$ +LG ES 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, cell proliferation was assessed after 72 h. The graph represents the mean and standard deviation of one representative experiment (n=5) out of four. *C–E, Apoptosis*. PDK1−/−, PDK1$^{+/+}$, PDK1$^{-/-}$ +LG and PDK1$^{-/-}$ +WT ES cells were treated with 10 μM 3,4-DMB-PP1, 1-NM-PP1 or DMSO control for 24 hours. Then medium was replaced with fresh medium with or without inhibitor, and with or without 200 nM Actinomycin D or 10 μg/ml Anisomycin 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. Densitometric analysis of Western blots was performed using NIH ImageJ. *F, Tumor formation*. PDK1$^{+/+}$ and PDK1$^{-/-}$ ES cells were injected subcutaneously into the flanks of nude mice. After 75 days allografts were harvested and weighed. 4/5 mice injected with PDK1$^{+/+}$ ES cells had easily detectable tumors (teratomas), whereas only 1/5 mice that had received PDK1$^{-/-}$ ES cells had one small tumor. Similarly, 24 NCr nude mice were injected subcutaneously in one flank with 1×10^6 PDK1$^{-/-}$ +LG ES cells, in the other flank with 1×10^6 PDK1$^{-/-}$ +WT ES cells, and tumors were taken out after 21 days and weighed; the tumor take rate was 100% for both these cell lines.

Table 1

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 h (blots not shown) and 24 h (blots shown in figures 5B–D) inhibitor treatment.

target	Inhibition by 3,4-DMB-PP1 and 1-NM-PP1	
	1h	24h
PKB T308	+++	+++
RSK1/2 S221/S227	–	+++
Phospho pan PKC	– / ++	– / ++
PRK1/2 T774/T816	–	–
PKA T197	–	–
PDK1 S241	–	+

‘–‘ indicates no inhibition, ‘+’ / ‘++’ / ‘+++’ degrees of inhibition

‘– / ++’ for phospho pan PKC reflects different effects on different PKC isoforms.