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ORIGINAL ARTICLE

PDK1 controls upstream PI3K expression and PIP_3 generation

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The PI3K/PDK1/Akt signaling axis is centrally involved in cellular homeostasis and controls cell growth and proliferation. Due to its key function as regulator of cell survival and metabolism, the dysregulation of this pathway is manifested in several human pathologies including cancers and immunological diseases. Thus, current therapeutic strategies target the components of this signaling cascade. In recent years, numerous feedback loops have been identified that attenuate PI3K/PDK1/Akt-dependent signaling. Here, we report the identification of an additional level of feedback regulation that depends on the negative transcriptional control of phosphatidylinositol 3-kinase (PI3K) class IA subunits. Genetic deletion of 3-phosphoinositide-dependent protein kinase 1 (PDK1) or the pharmacological inhibition of its downstream effectors, that is, Akt and mammalian target of rapamycin (mTOR), relieves this suppression and leads to the upregulation of PI3K subunits, resulting in enhanced generation of phosphatidylinositol-3,4,5-trisphosphate (PIP_3). Apparently, this transcriptional induction is mediated by the concerted action of different transcription factor families, including the transcription factors cAMP-responsive element-binding protein and forkhead box O. Collectively, we propose that PDK1 functions as a cellular sensor that balances basal PIP_3 generation at levels sufficient for survival but below a threshold being harmful to the cell. Our study suggests that the efficiency of therapies targeting the aberrantly activated PI3K/PDK1/Akt pathway might be increased by the parallel blockade of feedback circuits.

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INTRODUCTION

The PI3K/PDK1/Akt pathway regulates various cellular processes, including cell growth, survival and proliferation. Accordingly, the dysregulation of this pathway has been implicated in several human cancers and immunological diseases, and the components of this pathway are attractive targets of current therapeutic strategies (reviewed in references[1–8]).

Phosphatidylinositol 3-kinases (PI3Ks) are intracellular lipid kinases, which are grouped into three classes (I–III). Class I PI3Ks generate phosphatidylinositol-3,4,5-trisphosphate (PIP_3) by phosphorylating phosphatidylinositol-4,5-bisphosphate (PIP_2) at the D3 position of the inositol ring.[9] Class I PI3Ks are heterodimers consisting of a p110 catalytic subunit (p110α, p110β, p110δ and p110γ) and a regulatory subunit. The class IA isoforms p110α, p110β and p110δ pair with the p85 subfamily of regulatory subunits (p85α, p85β, p55α, p50α and p55γ). The class IB p110γ isoform associates with p101 or p84/p87 subunits.[9,10] In turn, PIP_3 levels are negatively regulated by the action of lipid phosphatases, which remove the different phosphate groups from the inositol ring.[11]

Most of the cellular responses to PI3K activation and PIP_3 generation are mediated by the activation of AGC kinases such as Akt (also termed protein kinase B), p70S6K and serum- and glucocorticoid-induced protein kinase (SGK) (reviewed by Mora *et al.*[12] and Pearce *et al.*[13]). For full activation, these kinases have to be phosphorylated both in an activation segment (T-loop) and within a hydrophobic motif.[13] The common upstream activator of these kinases is the AGC kinase 3-phosphoinositide-dependent protein kinase 1 (PDK1), which catalyzes the T-loop phosphorylation.[13–23] PDK1 possesses an N-terminal serine/threonine kinase domain and a C-terminal pleckstrin homology (PH) domain, which binds to PIP_3 and its degradation product phosphatidylinositol-3,4-bisphosphate.[14,15,24,25] The constitutively active PDK1 itself is not stimulated by PI3K.[14] However, the mechanism by which PDK1 activates its substrates is controlled by PIP_3. In case of Akt, PIP_3 induces a conformational change of Akt by binding its N-terminal PH domain, which leads to PDK1-mediated phosphorylation of T308 in the activation segment of Akt.[25] Full Akt activation is achieved by phosphorylation of S473 within the hydrophobic motif via the mammalian target of rapamycin complex 2 (mTORC2).[26,27] In contrast to Akt, the kinases p70S6K and SGK lack a PH domain. They get phosphorylated in the hydrophobic motif by mTORC1 or mTORC2 following PI3K activation.[13,28,29] The hydrophobic motif phosphorylation does not directly activate p70S6K and SGK but regulates their interaction with PDK1.[30,31] PDK1 binds to the phosphorylated hydrophobic motif via its PDK1-interacting fragment pocket and consequently phosphorylates the activation segment and thereby activates these kinases.[13] Upon activation of PI3Ks by insulin, growth factors or antigens, AGC kinases get activated and

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in turn phosphorylate various downstream targets, for example, glycogen synthase kinase 3β (GSK3β), tuberous sclerosis complex 2 (TSC2) or forkhead box O (FOXO) transcription factors.[6,13] Additionally, in recent years, it became evident that PI3K is tightly controlled by its downstream targets, thus providing feedback regulation in response to extracellular signals.[32]

To further characterize the involvement of PDK1 in pro-survival signaling pathways and its contribution to feedback regulation of PI3K, we analyzed the transcriptome in an inducible *PDK1*-knockout system. We show that upon *PDK1*-knockout induction in DT40 B lymphocytes the PI3K class IA subunits p85α, p110β and p110δ are upregulated on both mRNA and protein level. Furthermore, we demonstrate that PDK1 depletion increases the amount of the PI3K product PIP_3 in the plasma membrane. It appears that this PDK1-dependent transcriptional repression of PI3K class IA subunits is mainly mediated via the Akt/mTORC1 signaling axis and members of different transcription factor families, including cAMP-responsive element-binding protein (CREB) and FOXOs. Collectively, we propose a transcriptional level of feedback regulation targeting the PI3K/PDK1/Akt/mTOR signaling pathway. This novel feedback mechanism might further emphasize the need for dual inhibitors of PI3K and mTOR in cancer therapy.

RESULTS

Tonic PDK1 signaling is essential for survival of DT40 B lymphocytes

To gain further insight into the function of PDK1 in survival signaling, we made use of the conditional *PDK1*-knockout chicken DT40 B cell line (*PDK1* − /cond).[33] In these cells, 4-hydroxytamoxifen (OH-TAM)-activated Cre recombinase induces the deletion of the floxed *PDK1* gene. We treated DT40 *PDK1* − /cond cells with OH-TAM and verified the absence of PDK1 expression by immunoblotting. In DT40 wild-type (wt) cells, OH-TAM alone had no effect on PDK1 expression (Figure 1a).

PDK1 plays an important role in survival signaling, and embryos of *Pdk1* − / − mice die at day 9.5.[34] Thus, we analyzed apoptosis in OH-TAM-treated DT40 *PDK1* − /cond and wt cells by measuring hypodiploid nuclei. OH-TAM-treated *PDK1* − /cond cells underwent apoptosis from day 6 of treatment, but not the wt or ethanol (EtOH)-treated cells (Figure 1b). As DT40 cells are immature B lymphocytes and therefore respond with apoptosis induction upon crosslinking of the B-cell antigen receptor (BCR),[35] we investigated if the loss of PDK1 has any effect on BCR-induced apoptosis. We stimulated DT40 *PDK1* − /cond cells on day 4 of OH-TAM treatment, on which the cells were PDK1 negative but still viable (Figures 1a and b), with anti-chicken-IgM antibodies and analyzed apoptosis by measuring hypodiploid nuclei. As control, we used EtOH-treated *PDK1* − /cond and wt cells. Although loss of PDK1 strongly increased BCR-induced apoptosis, wt cells did not display any differences in BCR-induced apoptosis following OH-TAM treatment (Figure 1c).

Taken together, PDK1 is mandatory for the constitutive survival signaling in DT40 cells, and its loss sensitizes these cells to BCR-induced apoptosis.

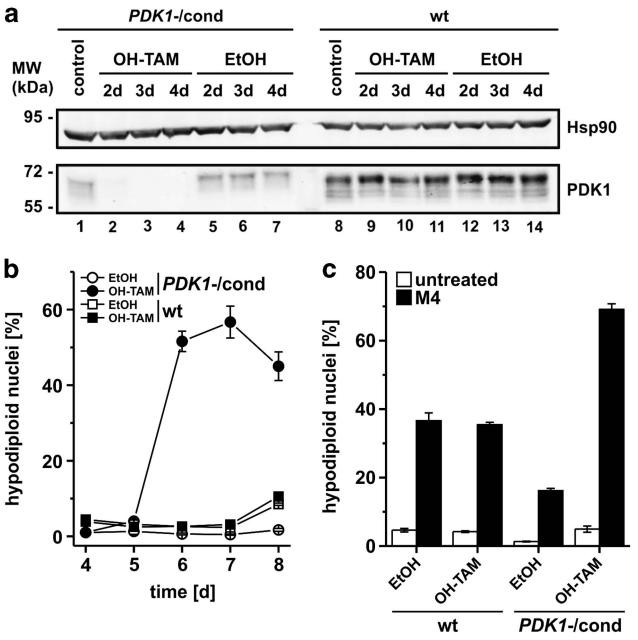


Figure 1. PDK1 is essential for survival of DT40 cells. (**a**) Conditional *PDK1* − /cond chicken DT40 B cells (*PDK1* − /cond) and DT40 wt cells were treated with 0.5 μM OH-TAM or 0.05% EtOH for 48 h or left untreated (control). Then, cells were transferred to normal DT40 medium. On days 2–4 after beginning of OH-TAM/EtOH treatment, cleared cellular lysates were prepared and analyzed for PDK1 and Hsp90 by immunoblotting. Data shown are representative of three independent experiments. (**b**) DT40 *PDK1* − /cond and wt cells were treated with 0.5 μM OH-TAM or 0.05% EtOH for 48 h. Then, cells were transferred to normal DT40 medium. On days 4–8 after beginning of OH-TAM/EtOH treatment, apoptosis was assessed by propidium iodide staining of hypodiploid apoptotic nuclei and flow cytometry. Data shown are mean of triplicates ± s.d. and are representative of three independent experiments. (**c**) DT40 *PDK1* − /cond and wt cells were treated with 0.5 μM OH-TAM or 0.05% EtOH for 48 h. Then, cells were transferred to normal DT40 medium. On day 4 after beginning of OH-TAM/EtOH treatment, cells were stimulated with 10 μg/ml anti-chicken IgM antibody (M4) for 24 h. Apoptosis was assessed by propidium iodide staining of hypodiploid apoptotic nuclei and flow cytometry. Data shown are mean of triplicates ± s.d. and are representative of three independent experiments.

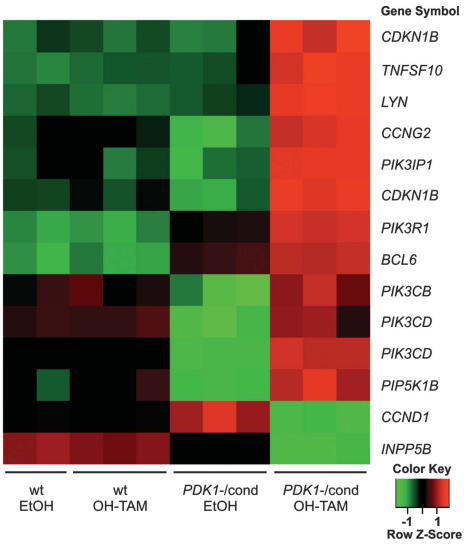
Microarray analysis of *PDK1* − /cond cells

Next, we addressed the question whether PDK1 also regulates long-term transcription dynamics. To investigate this, we performed microarray analyses using the Affymetrix GeneChip Chicken Genome Array. We treated DT40 *PDK1* − /cond cells with OH-TAM or EtOH to identify PDK1-dependent target genes. Additionally, we treated DT40 wt cells with OH-TAM or EtOH to exclude OH-TAM-dependent effects. Successful depletion of PDK1 in *PDK1* − /cond cells used for microarray analyses was confirmed by immunoblotting and real-time reverse transcription (RT)–PCR (Supplementary Figures 1A and B). Only transcripts that were regulated with ⩾2-fold changes were considered as relevant. When comparing the transcripts of *PDK1* − /cond and wt cells both treated with OH-TAM, we identified 1696 relevant transcripts. In turn, the comparison of *PDK1* − /cond cells treated with OH-TAM or EtOH revealed 764 relevant transcripts. Altogether 503 transcripts were regulated in both entity lists (Supplementary Figure 1C). Next, we performed global function and pathway analyses of the gene products of the 503 transcripts using Ingenuity Pathway Analysis Software (Supplementary Figure 1D). We observed that the 503 gene products play key roles in cellular homeostasis and immune responses. Due to the fact that PDK1 deletion is lethal, we were mostly interested in regulated genes of survival signaling pathways. It has been previously reported that transcription factors of the FOXO family are regulated via the PI3K/PDK1/Akt signaling pathway.[36,37] In our microarray analysis, several apoptosis-relevant FOXO target genes such as *TNFSF10 (TRAIL)*, *CDKN1B* and *BCL6* were PDK1-dependently regulated (Figure 2a). Strikingly, our microarray results revealed PDK1-dependent regulation of gene products that regulate the signaling pathway upstream of PDK1. Among these gene products were the

a

gene symbol (protein name)	regulation
known targets of PDK1	
TNFSF10 **(Tumor necrosis factor ligand superfamily member 10; synonym: TRAIL)**	↑
CDKN1B **(cyclin-dependent kinase inhibitor 1B; synonyms: p27, Kip1)**	↑
BCL6 **(B-cell lymphoma 6 protein)**	↑
CCNG2 **(cyclin G2)**	↑
CCND1 **(cyclin D1)**	↓
new targets of PDK1	
PIK3CB **(phosphatidylinositol 4,5-bisphosphate 3-kinase, catalytic subunit beta; PI3K p110β)**	↑
PIK3CD **(phosphatidylinositol 4,5-bisphosphate 3-kinase, catalytic subunit delta; PI3K p110δ)**	↑
PIK3R1 **(phosphoinositide-3-kinase, regulatory subunit 1 (alpha); PI3K p85α)**	↑
INPP5B **(inositol polyphosphate-5-phosphatase)**	↓
PIP5K1B **(phosphatidylinositol-4-phosphate 5-kinase, type I, beta)**	↑
PIK3IP1 **(phosphoinositide-3-kinase interacting protein 1)**	↑
LYN **(v-yes-1 Yamaguchi sarcoma viral related oncogene homolog; synonym: Lyn)**	↑

b



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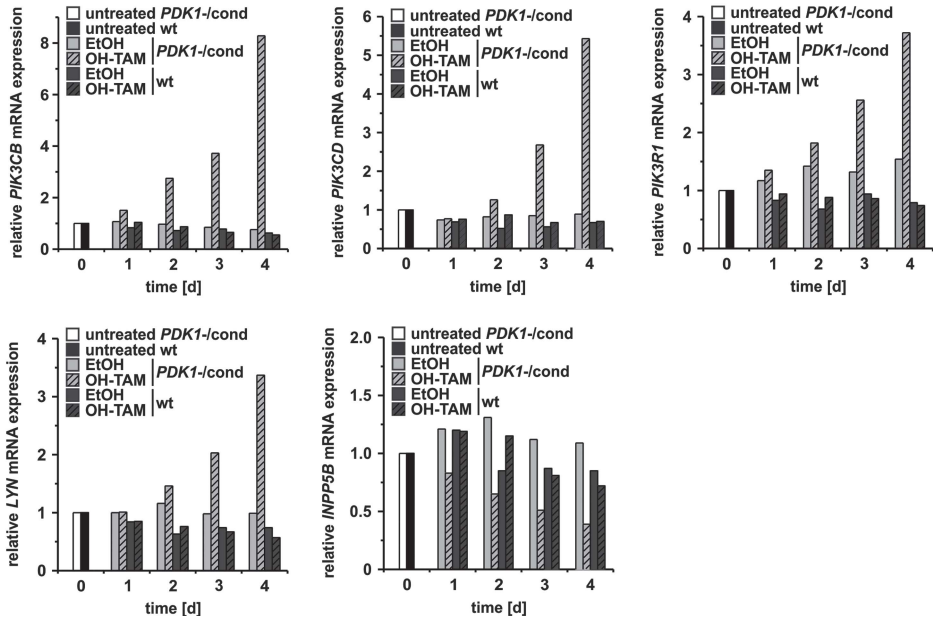

Figure 2. PDK1 negatively regulates transcription of PI3K subunits. (**a**) The table lists known and newly identified target genes of PDK1 and their regulation in *PDK1* − /cond cells compared with that in control cells. (**b**) Cluster analysis for selected probe sets was performed using the statistics software R 2.15.1. Signal intensities were scaled and centered, and the distance between two expression profiles was calculated using Manhattan distance measure. Hierarchical cluster analysis was performed with average linkage. Heatmaps were generated with Bioconductor package geneplotter. (**c**) DT40 *PDK1* − /cond and wt cells were treated with 0.5 μM OH-TAM or 0.05% EtOH for 48 h. Then, cells were transferred to normal DT40 medium. At the indicated time points, the relative mRNA expression of *PIK3CB* (PI3K p110β), *PIK3CD* (PI3K p110δ), *PIK3R1* (PI3K p85α), *LYN* (Lyn) and *INPP5B* was analyzed by quantitative real-time RT–PCR.

PI3K class IA subunits p110β and p110δ (catalytic subunits), p85α (regulatory subunit), additional phosphoinositide-modifying enzymes (INPP5B, PIP5K1-β), the PI3K-interacting protein 1 and the B-cell-specific Src family tyrosine kinase Lyn (Figures 2a and b). Lyn links the BCR to the activation of PI3K/PDK1/Akt signaling in B lymphocytes, as it directly phosphorylates CD19 and contributes to the activation of the tyrosine kinases Syk and Btk, which both phosphorylate the B-cell adapter for PI3K.[38] Tyrosine-phosphorylated CD19 and/or B-cell adapter for PI3K then recruit the regulatory PI3K subunit p85α.[38]

Next, we performed quantitative real-time RT–PCRs of the *PIK3CB* (PI3K p110β subunit), *PIK3CD* (PI3K p110δ subunit), *PIK3R1* (PI3K p85α subunit), *LYN* (Lyn) and *INPP5B* (phosphoinositide 5-phosphatase) genes to confirm the microarray results. We treated DT40 *PDK1* − /cond and wt cells with OH-TAM or EtOH and determined the mRNA level at the indicated time points (Figure 2c). In accordance with the microarray results, *PIK3CB*, *PIK3CD, PIK3R1* and *LYN* were upregulated, and *INPP5B* was downregulated.

Taken together, we showed that knockout of PDK1 indirectly alters the PI3K/PDK1 signaling axis by regulating gene transcription of Lyn, PI3K subunits and phosphoinositide-modifying enzymes.

PDK1 negatively regulates expression of PI3K subunits

To confirm the results obtained by microarray analysis and RT–PCR on the protein level, we used immunoblotting to analyze the

expression of the PI3K subunits p110β, p110δ and p85α, Lyn and INPP5B in DT40 *PDK1* − /cond cells treated with OH-TAM or EtOH. We observed an increase of protein expression of all three PI3K subunits (Figure 3a). Similarly, protein levels of the Src family kinase Lyn were elevated upon PDK1 depletion (Figure 3a). In contrast and confirming the microarray and RT–PCR results, deletion of PDK1 resulted in a downregulation of INPP5B (Figure 3b).

To analyze whether known downstream signaling pathways are affected by the loss of PDK1, we analyzed the phosphorylation status of different PDK1 effector proteins following OH-TAM treatment, such as Akt (T308), GSK3β (S9), FOXO1 (S256) and TSC2 (S939). With loss of PDK1, T308 phosphorylation of Akt is strongly reduced in PDK1-deleted cells (Figure 3c). Additionally, phosphorylation of the Akt substrates GSK3β, FOXO1 and TSC2 was significantly decreased, whereas total expression levels of these proteins remained largely unaffected (Figure 3c). All results obtained by the OH-TAM-induced PDK1 deletion were compared with the effect of the PDK1 inhibitor BX-795.[39] BX-795 essentially phenocopied the effect of the induced PDK1 deletion, that is, reduced phosphorylation of Akt and its substrates, upregulation of the PI3K subunits p110β and p110δ and downregulation of INPP5B (Figure 3c).

To prove the PDK1-mediated regulation of PI3K subunit expression in alternative cell models, we treated human DG75 and Ramos B cells with the PDK1 inhibitor BX-795 and analyzed p110β, p110δ and p85α expression. A significant upregulation of the PI3K subunits was detectable in both cell lines (Supplementary Figures 2A and B). Furthermore, we were interested whether the observed transcriptional control is valid for cells derived from solid tumors. Accordingly, we treated prostate carcinoma PC-3 cells with BX-795 for 2 days. The efficacy of BX-795 in this cell line was confirmed by analyzing the phosphorylation of Akt, GSK3β, TSC2 and FOXO1, respectively. Similar to the B cell lines described above, the expression of the PI3K subunits p110β and p110δ increased upon BX-795 treatment (Supplementary Figure 2C). Collectively, these findings indicate that the PDK1-dependent control of PI3K subunit expression also exists in mammals and non-lymphoid cells.

To ultimately confirm that the above-described effects can be attributed to the loss of PDK1, we reconstituted DT40 *PDK1* − /cond cells with chicken *PDK1* complementary DNA (cDNA). Expression of chicken PDK1 in knockout cells was able to restore the wt phenotype and prevented the decrease of GSK3β phosphorylation and the upregulation of the PI3K subunits following OH-TAM treatment (Supplementary Figure 3A). Furthermore, exogenous PDK1 expression completely inhibited apoptosis induced by PDK1 knockout (Supplementary Figure 3B).

In summary, the conditional knockout of PDK1 leads to upregulation of the upstream PI3K subunits p110β, p110δ and p85α in different cellular model systems. This effect can be prevented by the exogenous expression of PDK1.

Promoter analysis of *PDK1* − /cond cells

As FOXOs are known downstream targets of the PI3K/PDK1/Akt signaling pathway and we observed reduced FOXO1 phosphorylation upon induced PDK1 depletion or BX-795 treatment, we were next interested in the common transcriptional regulation of these genes. Thus, we analyzed the promoter regions of the genes encoding the PI3K subunits p110β, p110δ and p85α; the phosphoinositide-modifying enzymes (INPP5B, PIP5K1-β); the PI3K-interacting protein and Lyn by performing transcription factor mapping using Genomatix Software. Within the promoter regions of these regulated genes, we mainly identified binding sites for

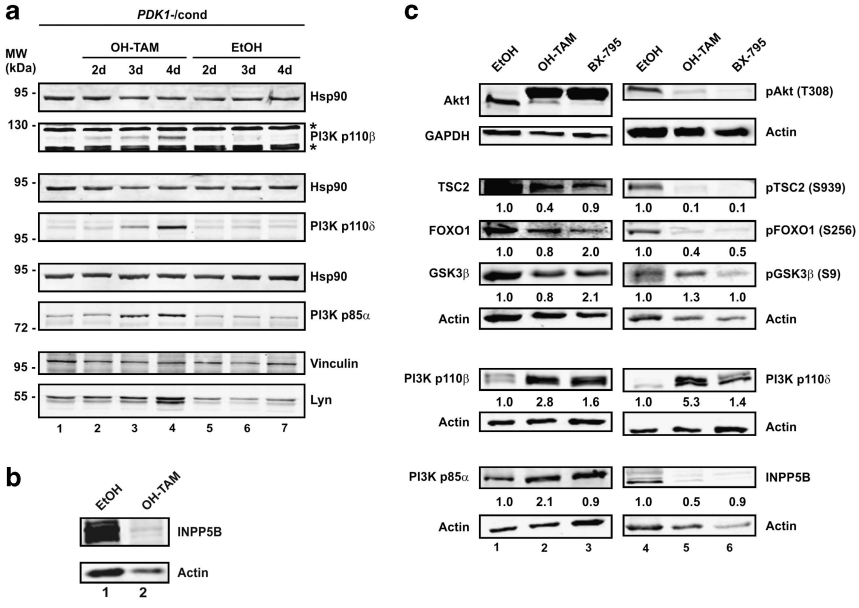

Figure 3. PDK1 negatively regulates expression of PI3K subunits. (**a**) DT40 *PDK1* − /cond and DT40 wt cells were treated with 0.5 μM OH-TAM or 0.05% EtOH for 48 h or left untreated. Then, cells were transferred to normal DT40 medium. On days 2–4 after beginning of OH-TAM/EtOH treatment, cleared cellular lysates were prepared and analyzed for PI3K p110β, PI3K p110δ, PI3K p85α, Lyn, Hsp90 and vinculin by immunoblotting. Data shown are representative of three independent experiments. * Indicates unspecific background bands. (**b**) DT40 *PDK1* − /cond cells were treated with 0.05% EtOH or 0.5 μM OH-TAM for 48 h. Then, cells were transferred to normal DT40 medium and incubated for additional 48 h. Cleared cellular lysates were prepared and analyzed for INPP5B and actin by immunoblotting. Data shown are representative of three independent experiments. (**c**) DT40 *PDK1* − /cond cells were treated with 0.05% EtOH or 0.5 μM OH-TAM for 48 h. Then, cells were transferred to normal DT40 medium and incubated for additional 48 h. Alternatively, DT40 *PDK1* − /cond cells were treated with 10 μM of the PDK1 inhibitor BX-795 for 48 h. Cleared cellular lysates were prepared and analyzed for Akt1, phospho-Akt (T308), TSC2, phospho-TSC2 (S939), FOXO1, phospho-FOXO1 (S256), GSK3β, phospho-GSK3β (S9), PI3K p110β, PI3K p110δ, PI3K p85α, INPP5B, GAPDH and actin by immunoblotting. Protein/loading control ratios were calculated and normalized to EtOH-treated controls. Data shown are representative of three independent experiments.

E-twenty six factors (ETSF), sex/testis-determining and related HMG box factors (SORY), forkhead domain factors (FKHD), heat-shock factors (HEAT) and cAMP response element-binding proteins (CREB) (Figure 4a). Next to FOXOs, which belong to the forkhead domain family, and CREB, the transcription factor families nuclear factor-κB and p53 have previously been shown to be regulated by the PDK1/Akt axis.[6,40,41] However, binding sites for both transcription factor families were not significantly enriched in the promoter regions of the analyzed transcripts (Figure 4a), and functional p53 is absent in DT40 cells.[42] Accordingly, we concentrated on the known Akt-regulated transcription factors FOXO and CREB. First, we employed the FOXO1 inhibitor AS1842856 (ref. 43) and analyzed its effect on the transcriptional upregulation of PI3K subunits upon OH-TAM-induced PDK1 depletion or BX-795-mediated PDK1 inhibition, respectively. Notably, the upregulation of the PI3K subunits p110β, p110δ and p85α was strongly blocked by the simultaneous inhibition of FOXO1, albeit not completely (Figure 4b). In turn, induction of CREB with forskolin (FSK) or CREB inhibition with the CBP–CREB interaction inhibitor resulted in the up- or downregulation of Lyn in DT40 cells (Figure 4c). Additionally, BX-795 treatment led to the induction of CREB-dependent transcription in Jurkat T lymphocytes as detected by a reporter gene assay (Figure 4d). Collectively, these experiments indicate that the CREB transcription factor (family) might also contribute to the transcriptional regulation of the PI3K/PDK1/Akt signaling axis. To further validate these results, we performed an upstream regulator analysis using Ingenuity Pathway Analysis Software with the two complete entity lists obtained by the microarray analysis. Indeed, both transcription factor families were identified as significant upstream regulators (Supplementary Figure 4).

It appears that the transcriptional regulation of Lyn, PI3K subunits and phosphoinositide-modifying enzymes in response to lack of active PDK1 is mainly mediated by transcription factors of the FOXO and CREB family, although the contribution of additional transcription factor families such as ETS has to be investigated in future studies.

PDK1 deficiency leads to increased PIP_3 levels in the plasma membrane

As PI3K class I enzymes produce PIP_3 and INPP5B can degrade PIP_3, we asked if the abundance of this lipid is affected upon upregulation of the PI3K subunits and downregulation of INPP5B following induction of PDK1 knockout. To address this question, we treated DT40 *PDK1* − /cond and wt cells with OH-TAM or EtOH and analyzed the amount of PIP_3 in these cells by a time-resolved

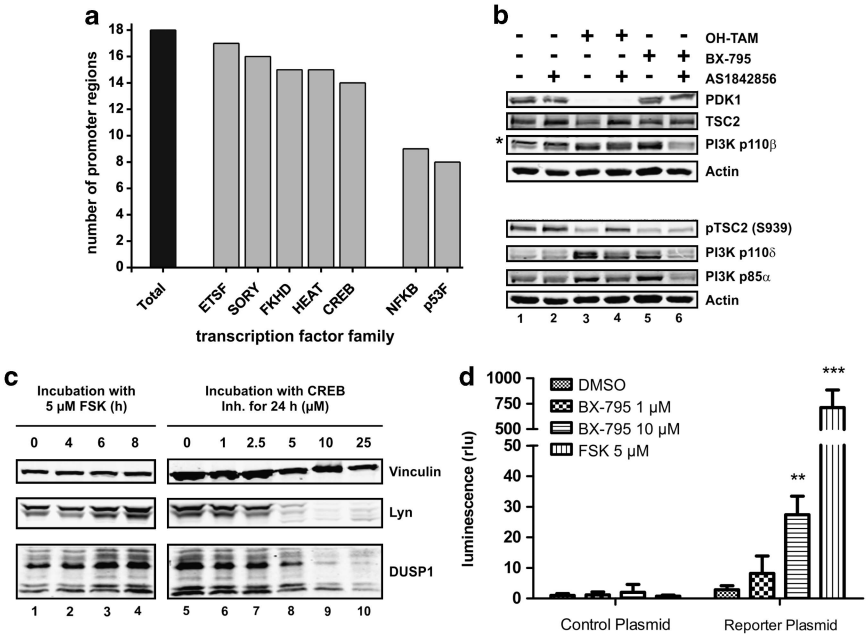

Figure 4. Promoter analysis of PDK1-regulated genes. (**a**) The promoter regions of the genes encoding the PI3K subunits p110β, p110δ and p85α; the phosphoinositide-modifying enzymes (INPP5B, PIP5K1-β); the PI3K-interacting protein and Lyn were analyzed by performing transcription factor mapping using Genomatix Software. The total number of identified promoter regions for these genes is 18 (black bar). The number of promoter regions containing binding sites for the following transcription factors are depicted (gray bars): E-twenty six factors (ETSF), sex/testis-determining and related HMG box factors (SORY), forkhead domain factors (FKHD), heat-shock factors (HEAT), cAMP response element-binding proteins (CREB), nuclear factor-κB (NFKB) and p53 family (p53F). (**b**) DT40 *PDK1* − /cond cells were treated with 0.05% EtOH or 0.5 μM OH-TAM for 48 h. Then, cells were transferred to normal DT40 medium and incubated for 24 h. Either the PDK1 inhibitor BX-795 or the FOXO1 inhibitor AS1842856 or both were added to a final concentration of 10 μM, and 0.1% v/v dimethylsulfoxide (DMSO) was used as control. After another 24 h, cleared cellular lysates were prepared and analyzed for PDK1, TSC2, phospho-TSC2 (S939), PI3K p110β, PI3K p110δ, PI3K p85α and actin by immunoblotting. Data shown are representative of three independent experiments. (**c**) DT40 *PDK1* − /cond cells were incubated with 5 μM forskolin (FSK) for the indicated times or the CREB–CBP interaction inhibitor for 24 h with the indicated concentrations. Cleared cellular lysates were prepared and analyzed for Lyn, the known CREB-regulated protein DUSP1 and vinculin by immunoblotting. Data shown are representative of three independent experiments. (**d**) Jurkat T lymphocytes were transfected with a CREB reporter plasmid or a control plasmid (CRE/CREB Reporter Assay Kit, BPS Bioscience) as described in the Materials and methods section. A plasmid containing a constitutively expressed Renilla luciferase gene served as a control of transfection efficiency. The PDK1 inhibitor BX-795 was added to a final concentration of 1 or 10 μM, and 0.1% DMSO was used as a negative control. The cells were incubated for 24 h. Cells treated with 5 μM of the CREB-inducer FSK for 6 h were used as positive control. Firefly luciferase activity was measured using a Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer's instructions. The firefly luminescence value of each well was divided by the respective Renilla luminescence value. Each column was individually compared with the negative control by unpaired two-tailed Student's *t*-test. Data shown are mean of three independent experiments ± s.d. $^{**}P<0.01$, $^{***}P<0.001$.

fluorescence resonance energy transfer assay.[44] As controls, we treated DT40 *PDK1* − /cond cells with H_2O_2, which activates PI3K, or with the broad-band PI3K inhibitor LY294002. Strikingly, the amount of PIP_3 was significantly increased in the *PDK1* − /cond cells, but not in the control cells (Figure 5a). Furthermore, it appears that incubation with LY294002 for 30 min is not potent enough to completely block the increase of PIP_3 upon PI3K subunit upregulation, whereas it is sufficient for control cells.

Subsequently, we asked if increased PIP_3 levels at the plasma membrane can be detected *in vivo* by exogenous expression of the PDK1 PH domain fused to enhanced green fluorescent protein (EGFP). For that, we generated DT40 *PDK1* − /cond cells stably expressing fluorescently labeled chicken PDK1 PH domain (EGFP-PH_{chPDK1}) or a mutant version (EGFP-$PH_{chPDK1\text{-}K468E}$), which cannot bind to PIP_3.[45] We induced the deletion of PDK1 in these cells and analyzed the recruitment of the EGFP-tagged PH domain by confocal microscopy. Indeed, the PDK1 knockout led to increased binding of EGFP-PH_{chPDK1} to the plasma membrane compared with that in EtOH-treated cells (Figure 5b). Next, we wanted to investigate whether the recruitment could be inhibited by the addition of different PI3K inhibitors. As we observed that incubation with LY294002 for 30 min could not completely block PIP_3 generation (Figure 5a), we made use of a more selective PI3K class I inhibitor (GDC-0941) and treated the cells for 12 h. The translocation of EGFP-PH_{chPDK1} to the plasma membrane was entirely blocked by the simultaneous treatment with this pan-specific PI3K class I inhibitor. In contrast, application of the p110β- and p110δ-specific inhibitors TGX-221 and IC87114 (for 24 h) only resulted in a partially blocked recruitment, suggesting that the two catalytic PI3K subunits fulfill partially redundant functions. In cells expressing the mutant PH domain, no recruitment was detectable, confirming that PH domain translocation depends on PIP_3 in the plasma membrane (Figure 5b).

Collectively, these results indicate that PDK1 negatively regulates the amount of PIP_3 in the plasma membrane via the negative transcriptional regulation of PI3K subunits. This feedback suppression is apparently relieved upon depletion of PDK1.

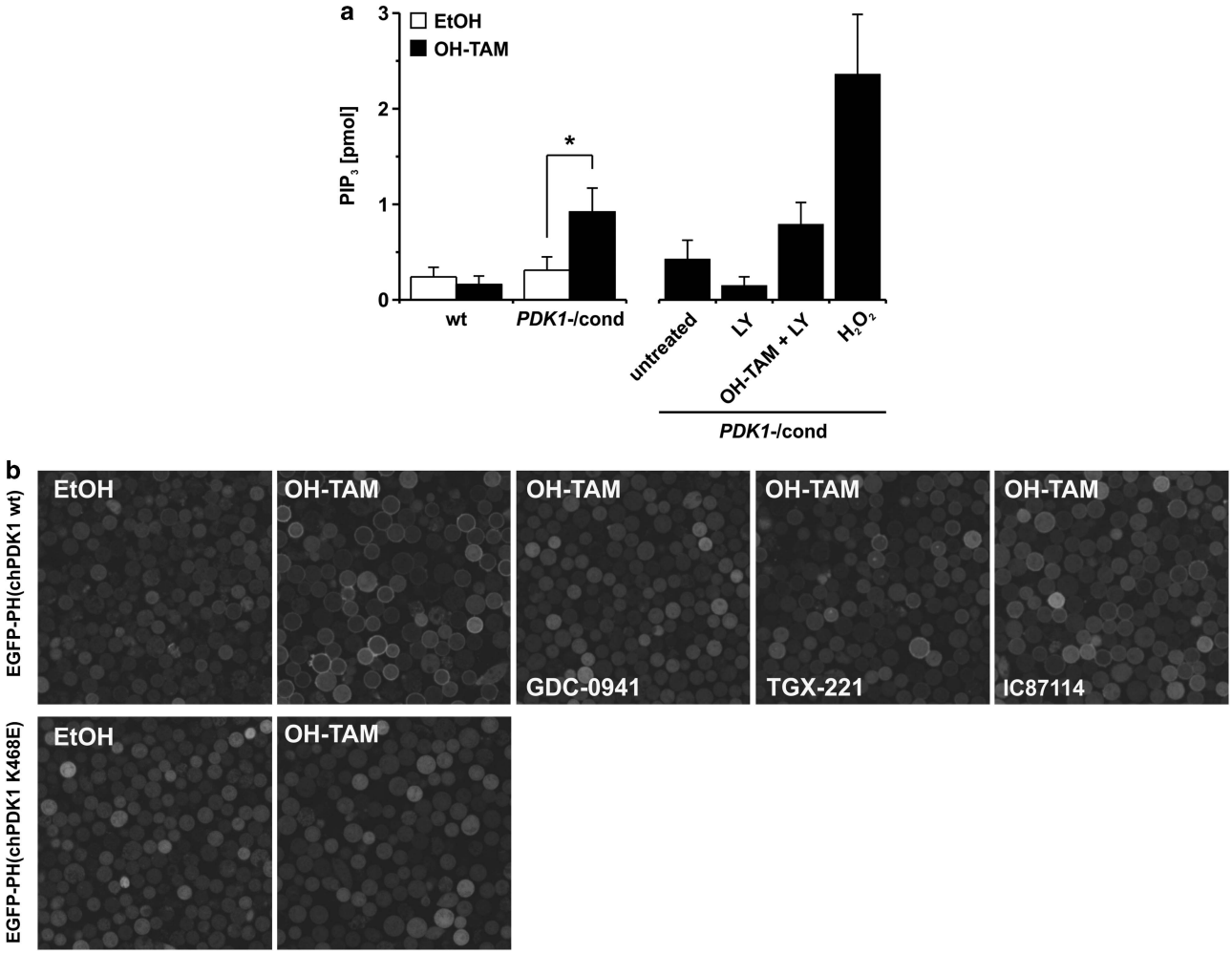

Figure 5. PDK1 deficiency increases PIP_3 levels in the plasma membrane. (**a**) DT40 *PDK1* − /cond and wt cells were treated with 0.5 μM OH-TAM or 0.05% EtOH for 48 h or left untreated. Then, cells were transferred to normal DT40 medium. On day 4 after beginning of OH-TAM treatment, control cells were treated with 10 μM LY294002 (LY) for 30 min (cells treated with OH-TAM or left untreated), 5 mM H_2O_2 for 2 min or left untreated. PIP_3 levels were measured using a time-resolved fluorescent resonance energy transfer displacement assay as described in detail in the Materials and methods section. Data shown are mean of triplicates ± s.d. and are representative of three independent experiments. $^*P<0.014$ (two-tailed paired *t*-test). (**b**) DT40 *PDK1* − /cond cells, retrovirally transfected with EGFP-PH(chPDK1 wt) or EGFP-PH(chPDK1 K468E) cDNAs, were treated with 0.5 μM OH-TAM or 0.05% EtOH for 48 h. Then, cells were transferred to normal DT40 medium and incubated for additional 48 h. Alternatively, OH-TAM-treated DT40 *PDK1* − /cond cells expressing EGFP-PH(chPDK1 wt) were incubated with 5 μM GDC-0941 (for 12 h prior to analysis), 10 μM TGX-221 (for 24 h prior to analysis) or 10 μM IC87114 (for 24 h prior to analysis). Cells were analyzed by confocal laser scanning microscopy. Data shown are representative of three independent experiments.

Akt and mTOR are important for the regulation of PI3K expression

PDK1 is an upstream activator of at least 23 different AGC family protein kinases including Akt, SGK, p70S6K, ribosomal S6 kinase, protein kinase C and protein kinase N.[12,13] The recruitment and activation of Akt can be blocked by mutating K465 in the PH domain of human PDK1 to glutamate.[45] In contrast, mutation of L155 to glutamate in the PDK1-interacting fragment pocket of human PDK1 reduces phosphorylation of SGK and p70S6K.[30,31] To address the relative contribution of the two different branches of PDK1 signaling to the negative transcriptional regulation of PI3K subunits, we mutated the corresponding amino acids in chicken PDK1 (L158 and K468) to glutamate, expressed these mutants in DT40 *PDK1* − /cond cells and analyzed the ability of both mutations to retain the knockout phenotype. Upon deletion of endogenous PDK1, PI3K subunit expression was increased in both cell lines expressing the single-mutated PDK1 variants, but not to the extent as in completely PDK1-depleted cells (Figure 6a). In contrast, cells expressing the double-mutated PDK1 version retained the knockout phenotype, indicating that both branches of PDK1 signaling are involved in the regulation of PI3K expression (Figure 6a). Next, we directly inhibited Akt using the inhibitor Akti-1/2/3 (MK-2206).[46,47] This led to a strong increase of the expression of the PI3K subunits p110δ and p85α and to a lesser extent p110β (Figure 6b). Similarly, treatment of human Jurkat T lymphocytes, DG75 B lymphocytes or Nalm-6 B lymphocytes with Akti-1/2/3 resulted in increased p110δ levels (Supplementary Figure 5A).

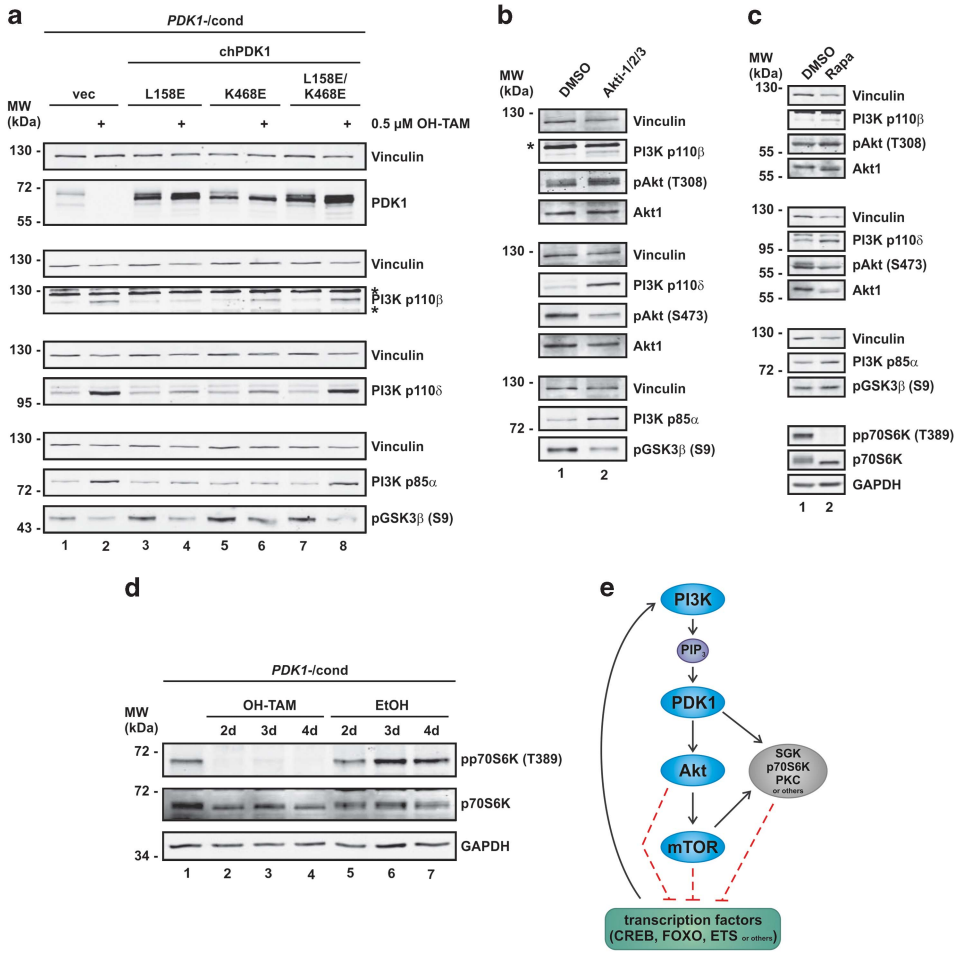

Figure 6. The PDK1 effector proteins Akt and mTOR are involved in the suppression of PI3K subunits. (**a**) DT40 *PDK1* − /cond cells, retrovirally transfected with chicken PDK1-L158E cDNA (L158E), chicken PDK1-K468E cDNA (K468E), chicken PDK1-L158E/K468E cDNA (L158E/K468E) or an empty vector (vec) were treated with 0.5 μM OH-TAM or 0.05% EtOH for 48 h. Then, cells were transferred to normal DT40 medium. On day 4 after beginning of OH-TAM/EtOH treatment, cleared cellular lysates were prepared and analyzed for PDK1, PI3K p110β, PI3K p110δ, PI3K p85α, phospho-GSK3β (S9) and vinculin by immunoblotting. Data shown are representative of three independent experiments. * Indicates unspecific background bands. (**b**) DT40 *PDK1* − /cond cells were treated with 1 μM Akti-1/2/3 for 48 h. Cleared cellular lysates were prepared and analyzed for PI3K p110β, PI3K p110δ, PI3K p85α, phospho-Akt (T308 and S473), Akt1, phospho-GSK3β (S9) and vinculin by immunoblotting. Data shown are representative of three independent experiments. * Indicates unspecific background band. (**c**) DT40 *PDK1* − /cond cells were treated with 100 nM rapamycin (Rapa) for 48 h. Cleared cellular lysates were prepared and analyzed for PI3K p110β, PI3K p110δ, PI3K p85α, phospho-Akt (T308 and S473), Akt1, phospho-GSK3β (S9), p70S6K, phospho-p70S6K (T389), vinculin and GAPDH by immunoblotting. Data shown are representative of three independent experiments. (**d**) DT40 *PDK1* − /cond cells were treated with 0.5 μM OH-TAM or 0.05% EtOH for 48 h or left untreated. Then, cells were transferred to normal DT40 medium. On day 4 after beginning of OH-TAM/EtOH treatment, cleared cellular lysates were prepared and analyzed for p70S6K, phospho-p70S6K (T389) and GAPDH by immunoblotting. Data shown are representative of three independent experiments. (**e**) Schematic diagram of the negative feedback regulation of the PI3K/PDK1/Akt signaling pathway. PDK1 activates Akt and other AGC kinases such as SGK, p70S6K or PKC. These kinases together with their downstream effectors (e.g., mTOR) in turn inhibit the transcription of PI3K subunits via the regulation of transcription factors of the CREB, FOXO, ETS and/or other families. By this means, cellular PIP_3 amounts are fine-tuned to levels that are sufficient for cell survival.

Several feedback signaling loops targeting the PI3K/PDK1/Akt pathway are controlled by mTOR. Thus, we analyzed the contribution of mTOR to the negative transcriptional regulation of the PI3K subunits. Inhibition of mTORC1 with rapamycin resulted in a similar effect as observed for Akt inhibition, that is, distinctly increased expression of p85α and p110δ and slightly increased expression of p110β (Figure 6c). Additionally, we observed that PDK1 depletion results in a complete inhibition of mTORC1 activity, as detected by phospho-p70S6K immunoblotting (Figure 6d). The rapamycin-mediated increase of PI3K subunit expression could also be demonstrated in Jurkat T lymphocytes (Supplementary Figure 5B). Collectively, we conclude that the Akt/mTORC1 axis is mainly responsible for the negative regulation of PI3K subunit expression downstream of PDK1. Additionally, the above-described feedback suppression appears to exist in different species and cell types.

DISCUSSION

The PI3K/PDK1/Akt signaling pathway plays a central role in cellular homeostasis by producing specific 'zip codes' at the inner leaflet of the plasma membrane. The PI3K product PIP_3 serves as an anchor for the recruitment of downstream effectors, including PDK1 and Akt. It has been confirmed that PIP_3 is the essential component of PI3K-mediated oncogenesis,[48] and accordingly increased levels of PIP_3 caused by either activation of PI3K or inactivation of phosphatase and tensin homolog (PTEN) are frequently found in tumor cells.[1–7] Concomitantly, aberrantly activated downstream effectors are frequently detectable in these cells. Generally, PI3K and PTEN activity is tightly controlled by a complex network of feedback regulations to maintain basal PIP_3 levels below a threshold for signaling activation. In this study, we identified an additional level of feedback control, that is, the PDK1-dependent transcriptional regulation of PI3K subunits and additional phosphoinositide-modifying enzymes. We showed that depletion of PDK1 or inactivation of its downstream effectors Akt or mTOR leads to the transcriptional upregulation of enzymes able to elevate PIP_3 levels in the plasma membrane and the concomitant downregulation of a PIP_3-degrading phosphatase. This transcriptional control is at least in part mediated by transcription factors of the FOXO and/or CREB family, although we cannot exclude the contribution of additional transcription factors (families) at this stage. Our observations strongly support a model of PDK1-dependent negative feedback regulation and indicate that the PIP_3-binding kinase PDK1 is a central negative regulator of PI3K expression. In this scenario, PDK1 functions as a sensor for PIP_3 levels and orchestrates pro-survival signals via the direct regulation of the activation threshold and signaling strength of Akt and mTOR.

It appears that the sole upregulation of PI3K subunits is sufficient to increase PIP_3 levels in DT40 B cells and that no additional activating stimulus is necessary. Along these lines, it has been previously reported that the overexpression of wt subunits p110β and p110δ is sufficient to induce an oncogenic phenotype in cultured chicken embryonic fibroblasts[49] and that p110β and p110δ subunits can lead to basal PI3K activity in PTEN-deficient human prostate cancer cells independently of receptor tyrosine kinase activation.[50]

Multiple branches of PI3K-dependent signal transduction converge on the level of PDK1.[12,13] The model of PDK1 as a gatekeeper of PI3K expression levels is attractive, as (1) PDK1 itself is constitutively active and (2) it regulates the activation of several downstream kinases that depend on the PI3K product PIP_3. This PIP_3 dependency might be either direct, for example, by PH domain-mediated recruitment in the case of Akt, or indirect, for example, by PIP_3-induced signaling cascades leading to the phosphorylation of the hydrophobic motif in the case of p70S6K or SGK.[13] According to our mutational analyses, both branches of PDK1 signaling participate in the negative regulation of PI3K subunit expression. It is tempting to speculate that the different branches of PDK1 signaling differently affect the expression of PI3K subunits. Indeed, the usage of Akti-1/2/3 and rapamycin leads to an upregulation of p110δ and p85α, whereas the p110β increase is rather moderate. This would suggest that the indirect PIP_3 signaling axis regulates p110β expression. Interestingly, the PH domain mutant in turn does not preferentially regulate the levels of p110δ and p85α, which might be explained by the fact that this mutation does not completely abolish Akt T308 phosphorylation.[45] Furthermore, it has recently been published that Akt might also be activated by a PDK1-interacting fragment pocket-dependent mechanism.[51] Accordingly, our data obtained with the pharmacological inhibition of Akt or mTORC1 in different cell lines indicate that this axis is centrally involved in the transcriptional regulation of PI3K expression.

Next to the PI3K catalytic subunits, PIP_3 levels are regulated by additional enzymatic activities. Interestingly, our microarray results revealed that non-PI3K PIP_3-regulating proteins are also regulated on a transcriptional level. These targets include the lipid phosphatase INPP5B, the phosphatidylinositol kinase PIP5K1-β and the PI3K-interacting protein 1. Of note, the transcriptional regulation of INPP5B and PIP5K1-β follows the ultimate goal to increase cellular PIP_3 levels: INPP5B, which accepts PIP_3 as substrate and has been implicated in the systemic dephosphorylation of PIP_3 to PI-3-P,[52] is downregulated upon PDK1 depletion, whereas PIP5K1-β, which participates in the synthesis of the PI3K substrate phosphatidylinositol-4,5-bisphosphate,[53] is upregulated.

At first glance, it seems paradoxical that Akt should contribute to the negative regulation of PIP_3 levels, as PIP_3 itself is mandatory for Akt activation and many tumorigenic effects of increased PIP_3 concentrations are mediated by Akt. However, it appears comprehensive that PIP_3 levels are tightly kept at a certain level sufficient for Akt-dependent pro-survival signaling. In this regard, one might speculate that 'basally active' PDK1/Akt adjust PIP_3 levels to a value that is sufficient for their own activation and the generation of survival signals but does not exceed a harmful threshold leading to the demise of the cell. Indeed, it has been reported that chronic Akt activation leads to apoptosis and that Akt sensitizes cells to reactive oxygen species-induced apoptosis.[40,41,54] Given the existence of this 'dark side' of Akt together with the observation that Akt is the only critical target activated by increased PIP_3 concentrations due to loss of PTEN (at least in *Drosophila*),[55] it readily makes sense that PI3K expression levels are adjusted to a certain level. As soon as one interferes with these PI3K/PIP_3 levels necessary for the mere pro-survival machinery, for example, by PDK1 depletion or Akt inhibition, this repression is relieved and PIP_3 generation is increased.

Major classes of transcription factors are controlled by Akt, such as the FOXO family of transcription factors, CREB, nuclear factor-κB or p53.[6,56,57] Akt-mediated phosphorylation of CREB enables the binding of accessory proteins that are necessary for the transcription of pro-survival genes such as *BCL2* and *MCL1*.[6,58,59] According to our data, it appears that either the deletion of PDK1 or the inhibition of Akt might positively affect CREB-dependent transcription. The second major component that participates in the transcriptional control of PI3K subunits and PIP_3-modifying enzymes are members of the FOXO famiy of transcription factors. Akt-catalyzed phosphorylation of FOXO1, FOXO3a and FOXO4 transcription factors leads to their binding to 14-3-3 proteins and their retention in the cytoplasm. This in turn prevents the transcription of pro-apoptotic target genes such as the BH3-only family proteins and the Fas ligand.[6,36] Accordingly, so far CREB and FOXOs have been essentially placed downstream of the PI3K/PDK1/Akt signaling cascade. Although our data suggest that the CREB and FOXO transcription factors mediate the observed

upregulation of Lyn, PI3K subunits and phosphoinositide subunits, we cannot exclude the involvement of additional transcription factor families. Our *in silico* analysis revealed that binding sites for family members of the ETS transcription factor exist in most promoter regions of the relevant genes, and different reports describe the Akt-mediated control of ETS family members or even the cooperativity between CREB and c-Ets1.[60–62] Additionally, a highly conserved transcription factor-binding cluster containing an ETS-binding sequence has been identified for the murine and human *PIK3CD* gene encoding p110δ.[63] Nevertheless, further experiments are required to elucidate the exact mechanistic details. This is also underlined by the observation that the Akt effector protein mTOR participates in the described regulation. The kinase mTOR has been shown to be involved in the regulation of gene transcription.[64] Gene expression-profiling experiments have shown that ~5% of the transcriptome are differentially expressed in response to rapamycin-mediated mTOR inhibition.[65] Accordingly, we cannot exclude that mTOR also directly regulates the expression of PI3K subunits and PIP_3-modifying enzymes. The antidromic regulation of PIP_3-generating kinases and the PIP_3-degrading phosphatase INPP5B already indicates that additional factors are necessary for this transcriptional regulation. It is tempting to speculate that different Akt effectors (CREB, FOXOs and mTOR) control the overall signaling strength of the upstream tyrosine kinase/PI3K/PDK1/Akt signaling pathway, for example, CREB-mediated control of Lyn or FOXO-mediated control of PI3K subunits.

In summary, we identified a novel negative feedback regulation of the PI3K/PDK1/Akt pathway. This feedback depends on the PDK1-mediated suppression of regulatory and catalytic PI3K subunits. Upon PDK1 deletion or Akt inhibition, this suppression is relieved and the corresponding mRNAs are transcribed under the control of CREB/FOXO transcription factors (Figure 6e). This transcriptional feedback regulation adds to the existing 'catalog' of feedback mechanisms that attenuate PI3K/PDK1/Akt signaling. These feedback loops include the mTORC1-dependent transcriptional downregulation or post-translational inhibition of insulin receptor substrate-1/2, insulin-like growth factor-1 or insulin-like growth factor-1R or the recently reported Akt-dependent suppression of receptor tyrosine kinase expression and activity.[32,66] Collectively, all these regulatory feedback loops underscore the necessity of novel therapeutic strategies based upon combinatorial approaches. In recent years, mTOR inhibitors have been developed as potential therapeutics. However, one might hypothesize that the efficiency of therapies that are mainly aiming at mTOR inhibition could be further improved by the parallel inhibition of PI3K, PIP_3, PDK1 or Akt, depending on the malignancy-dependent existence or emergence of feedback signaling cascades.

MATERIALS AND METHODS

Cell culture

Chicken DT40 wt B cells, human DG75 B cells, human Nalm-6 B cells, human Ramos B cells, human Jurkat T cells and human prostate carcinoma PC-3 cells were obtained from DSMZ (Braunschweig, Germany). Chicken DT40 conditional *PDK1* − /cond cells (DT40 *PDK1* − /cond) have been previously described[33] and were kindly provided by Tomohiro Kurosaki (RIKEN Research Center for Allergy and Immunology, Yokohama, Japan). All cell lines were maintained in 5% CO_2 at 37 °C. All chicken DT40 cell lines were cultivated in RPMI 1640 (Lonza, Cologne, Germany) supplemented with 10% fetal calf serum, 1% chicken serum, 3 mM L-glutamine, 50 μM β-mercaptoethanol, 50 U/ml penicillin and 50 μg/ml streptomycin. Human DG75 and Nalm-6 B cells and human PC-3 prostate carcinoma cells were grown in RPMI 1640 supplemented with 10% fetal calf serum, 50 U/ml penicillin and 50 μg/ml streptomycin. Human Jurkat T cells and human Ramos B cells were cultivated in RPMI 1640 supplemented with 10% fetal calf serum, 10 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), 50 U/ml penicillin and 50 μg/ml streptomycin.

Antibodies and reagents

Anti-Akt, anti-phospho-Akt (T308 and S473), anti-FOXO1, anti-phospho-FOXO1 (S256), anti-phospho-GSK3β (S9) and anti-GSK3β antibodies were purchased from Cell Signaling Technology (New England Biolabs, Frankfurt, Germany), anti-Akt1, anti-PI3K p110δ, anti-Lyn and anti-GAPDH (glyceraldehyde 3-phosphate dehydrogenase) antibodies from Abcam (Cambridge, UK), anti-PDK1 and anti-PI3K p85α antibodies from Epitomics (Abcam), anti-PI3K p110β from Santa Cruz Biotechnology (Heidelberg, Germany), anti-Hsp90 from BD Biosciences (Heidelberg, Germany), anti-INPP5B from GeneTex (Irvine, CA, USA), anti-DUSP1 from Novus Biologicals (Acris Antibodies, Herford, Germany) and anti-actin and anti-vinculin from Sigma-Aldrich (Schnelldorf, Germany). OH-TAM was purchased from Sigma-Aldrich and the broad-band PI3K inhibitor LY294002 from Cell Signaling Technology. The pan-PI3K inhibitor GDC-0941, the PI3K p110β inhibitor TGX-221 and the PI3K p110δ inhibitor IC-87114 were purchased from Selleckchem (ICS International Clinical Service GmbH, Munich, Germany). FSK, the CBP–CREB interaction inhibitor, and the FOXO1 inhibitor AS1842856 were purchased from Calbiochem (Merck KGaA, Darmstadt, Germany). Anti-chicken-IgM (M4) was obtained from Southern Biotech (Biozol, Eching, Germany). Puromycin was purchased from InvivoGen (Toulouse, France). BX-795 was purchased from Axon Medchem (Groningen, The Netherlands). Akti-1/2/3 (MK-2206) was kindly provided by Dario R Alessi.

Expression constructs and retroviral infection

Wild-type chicken *PDK1* cDNA was cloned into pMSCVpuro (Clontech, Takara, Saint-Germain-en-Laye, France). cDNA encoding the chicken PDK1 PH domain (codons 409–557) was cloned into pEGFP-C1 (Clontech). pMSCVpuro-EGFP-PH_{chPDK1} was generated by cloning of *EGFP-PH*$_{chPDK1}$ cDNA into pMSCVpuro. Substitutions of leucine 158 and lysine 468 to glutamate were generated by site-directed mutagenesis to create a PDK1-interacting fragment-binding pocket mutant, PH domain mutant or double-mutant chicken PDK1. For retroviral gene transfer, Plat-E cells were transfected with pMSCV-based vectors using FuGENE 6 transfection reagent (Roche, Mannheim, Germany). The MMLV was pseudotyped with VSV-G. DT40 *PDK1* − /cond cells were incubated with retroviral supernatant containing 3 μg/ml Polybrene (Sigma-Aldrich) and selected in a medium containing 0.5 μg/ml puromycin.

Reporter gene assay

A number of 4×10^6 Jurkat cells was harvested by centrifugation at $600 \times g$ for 5 min at 4 °C, washed with phosphate-buffered saline and transfected with 300 ng of either CREB reporter plasmid or control plasmid (CRE/CREB Reporter Assay Kit, BPS Bioscience, San Diego, CA, USA) using an Amaxa Nucleofector I device with program K-25 and Solution T (Lonza) according to the manufacturer's instructions. After transfection, the cells were incubated at room temperature for 10 min and then transferred to a prewarmed medium and incubated overnight. BX-795 was added to a final concentration of 1 or 10 μM, and 0.1% dimethylsulfoxide was used as a negative control. After 24 h of incubation, FSK was added to previously untreated cells to a final concentration of 5 μM. The cells were transferred to a white opaque 96-well plate and incubated for another 6 h. The plate was then centrifuged at $600 \times g$ for 5 min at 4 °C. The cells were washed with phosphate-buffered saline, and firefly luciferase activity was measured using a Dual-Luciferase Reporter Assay System (Promega, Mannheim, Germany) according to the manufacturer's instructions, using a Synergy MX multiwell reader with dispenser (BioTek, Bad Friedrichshall, Germany).

Measurement of apoptosis

DT40 cells were cultivated in a medium containing 0.5 μM OH-TAM or 0.05% EtOH as control. After 48 h, OH-TAM-treated cells were transferred to a normal medium. Either the leakage of fragmented DNA was measured 4–8 days after beginning of OH-TAM treatment or on day 4 cells were stimulated with 10 μg/ml anti-chicken-IgM (M4) for 24 h and apoptosis was measured. Nuclei were prepared by lysing cells in hypotonic lysis buffer (1% sodium citrate, 0.1% Triton X-100 and 50 μg/ml propidium iodide) and subsequently analyzed by flow cytometry. Nuclei to the left of the 2N peak were considered as apoptotic. Flow cytometric analyses were performed on FACSCalibur (BD Biosciences) using CellQuest software or on LSRFortessa (BD Biosciences) using FACSDiva software.

Cell extracts and immunoblotting

DT40 cells were incubated in a medium containing 0.5 μM OH-TAM or 0.05% EtOH as control. After 48 h, OH-TAM-treated cells were transferred to a normal medium and harvested at the indicated time points. Following treatment of DT40, DG75, Nalm-6 or Jurkat cells with the different compounds at the indicated concentrations for the indicated times, cells were lysed in lysis buffer (20 mM Tris–HCl, pH 7.5, 150 mM NaCl, 0.5 mM EDTA, 1% Triton X-100, 10 mM NaF, 2.5 mM NaPP, 10 μM Na_2MoO_4, 1 mM Na_3VO_4 and protease inhibitors (P2714, Sigma-Aldrich)), and lysates were clarified by centrifugation at 16 000 × *g* for 10 min. Equal total protein amounts, as determined by the Bradford method, were separated on 8% or 10% SDS–polyacrylamide gels and transferred to PVDF membrane (Merck, Millipore, Darmstadt, Germany). Immunoblot analysis was performed using the indicated primary antibodies and appropriate IRDye 800- or IRDye 680-conjugated secondary antibodies (LI-COR Biosciences, Bad Homburg, Germany). Signals were detected with an Odyssey Infrared Imaging system (LI-COR Biosciences).

Analysis of PIP_3 levels

DT40 cells were incubated in a medium containing 0.5 μM OH-TAM or 0.05% EtOH as control. After 48 h, OH-TAM-treated cells were transferred to a normal medium. On day 4, control cells were treated with 10 μM LY294002 (LY) for 30 min (cells treated with OH-TAM or left untreated), 5 mM H_2O_2 for 2 min or left untreated. Subsequently, cells were harvested and the mass of PIP_3 was determined. Cells were precipitated by the addition of 0.5 ml ice-cold 0.5 M trichloroacetic acid and kept on ice for 5 min. The precipitated cells were collected by centrifugation, trichloroacetic acid was aspirated and the pellet was immediately frozen on dry ice until lipids were extracted. The mass of PIP_3 was estimated using a time-resolved fluorescence resonance energy transfer displacement assay as described previously.[44]

Quantitative real-time RT–PCR

Quantitative real-time RT–PCR analysis was performed using the ABI Prism 7000 Sequence Detection System (Applied Biosystems, Darmstadt, Germany) and Maxima qPCR Master Mix (Fermentas, Thermo Scientific, St Leon-Rot, Germany). Total RNA from 1×10^6 cells was isolated using the NucleoSpin RNA II-Kit (Macherey–Nagel, Düren, Germany). cDNA was generated from 1 μg of total RNA with 200 U RevertAid H Minus reverse transcriptase, 50 μM random hexamers, 400 μM dNTPs and 1.6 U/μl RiboLock RNase inhibitor (all from Fermentas), according to the manufacturer's recommendations. A portion of 50 ng of the resulting cDNA was applied to quantitative RT–PCR analyses (20 μl final volume) and amplified in the presence of 200 nM primers and 100 nM probe or 300 nM primers in the case of SYBR Green detection with the standard temperature profile (2 min 50 °C, 10 min 95 °C, 40 cycles 15 s 95 °C and 1 min 60 °C). Relative quantification was performed employing the standard curve method. The results were normalized on the reference gene *18S ribosomal RNA*. The cell populations on day 0 of OH-TAM treatment were used as calibrator.

Confocal laser scanning microscopy

Cells were resuspended in Krebs–Ringer solution (10 mM HEPES, pH 7.0, 140 mM NaCl, 4 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$ and 10 mM glucose) and seeded onto chambered cover glasses (Nunc, Thermo Scientific, Langenselbold, Germany). After 20 min, cells were analyzed on a Leica TCS SP II confocal laser scanning microscope (Leica, Wetzlar, Germany). EGFP was excited at 488 nm.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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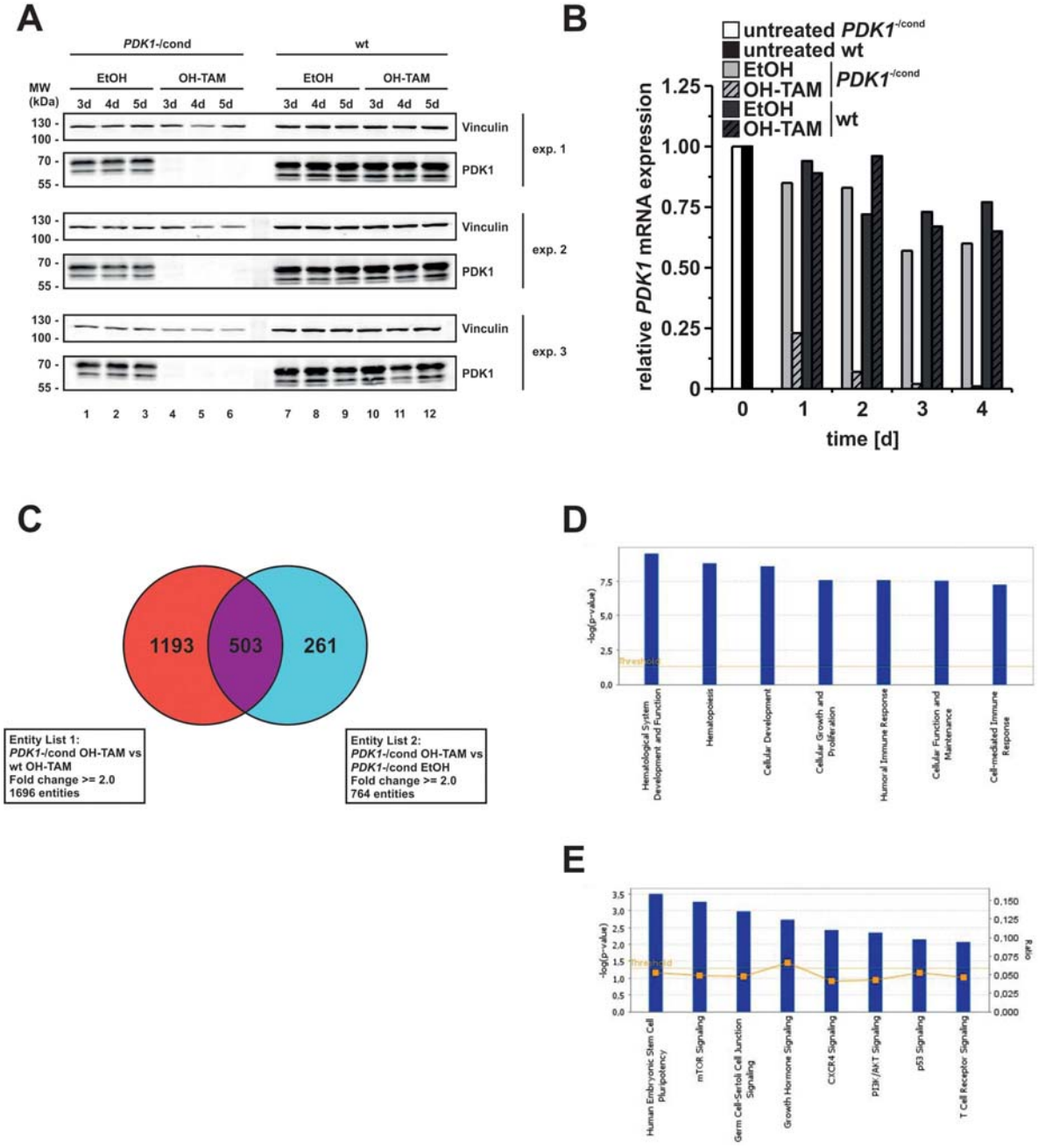
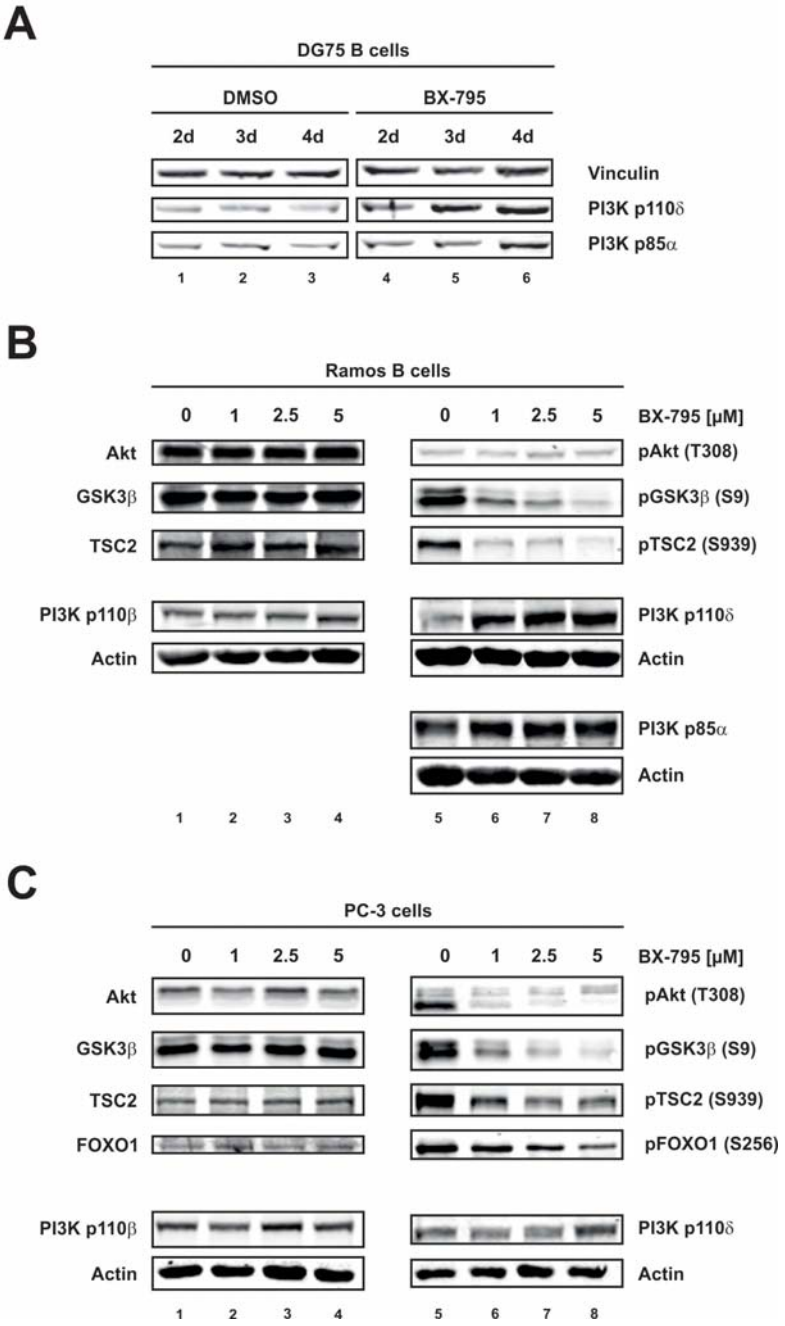
Supplementary Information accompanies this paper on the Oncogene website (http://www.nature.com/onc)

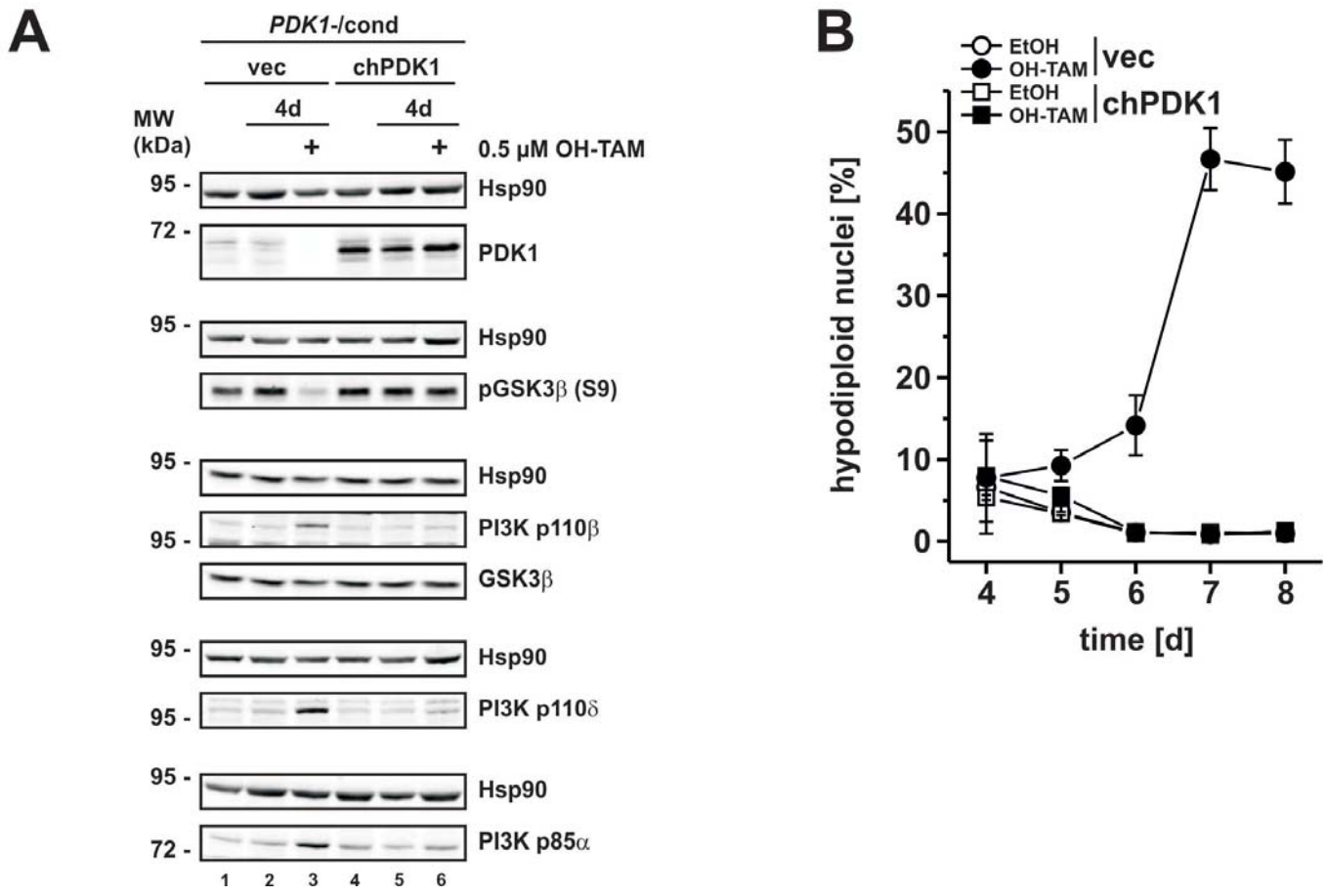
Supplementary Figure 1



Supplementary Figure 2



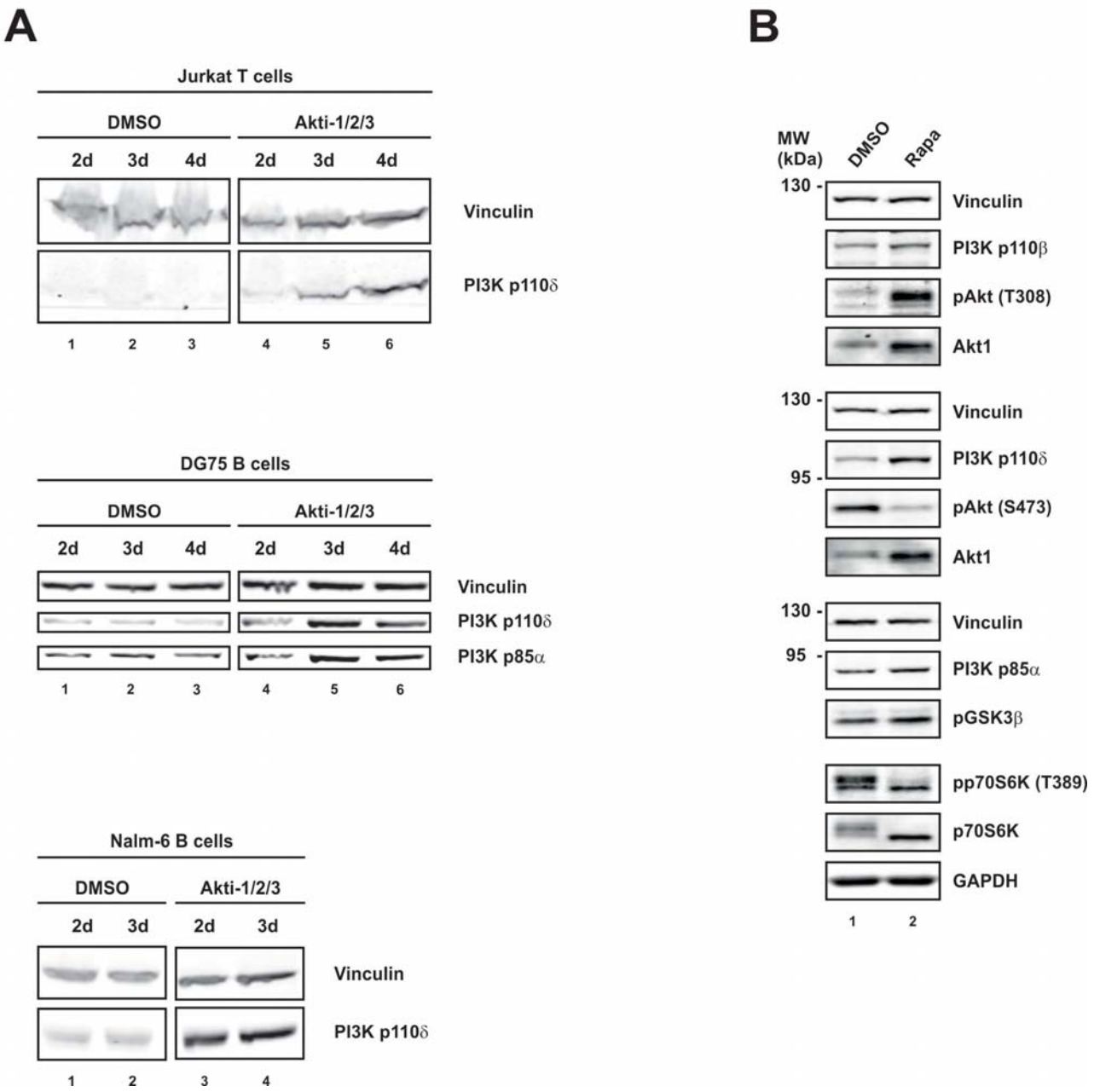
Supplementary Figure 3



Supplementary Figure 4

Upstream regulator	*PDK1-/cond OH-TAM vs wt OH-TAM*		*PDK1-/cond OH-TAM vs PDK1-/cond EtOH*	
	p-value	**known target molecules in dataset**	**p-value**	**Target molecules in dataset**
FOXO1			1.14×10^{-4}	ADIPOQ,AICDA,CCND1,CCNG2,CDKN1B,DAPK1, GADD45B,HPD,KLF2,MVD,SCD,SPC25,TNFSF10
FOXO4	6.03×10^{-6}	BCL6,CDKN1B,DHCR24,FDFT1, GADD45A,GADD45B,IDI1,MMP9,SCD, SLC2A1	5.97×10^{-7}	BCL6,CCND1,CCNG2,CDKN1B,DHCR24,GADD45B, IDI1,SCD
FOXC1	2.7×10^{-6}	CXCR4,DLL1,FOXO1,LFNG,MMP9, NOTCH1,PLOD1		
CREB1	9.15×10^{-5}	ACHE,BCL2,CCNA1,CDC37,CPT1A, CSRNP1,DUSP14,ERRFI1,GADD45A, GADD45B,HERPUD1,HMOX1,ID1,IDI1, LDHA,LGALS8,LSS,NFIL3,NOS2,RGS2, SCD,SLC2A3,TIPARP	8.49×10^{-4}	CCNA1,CCND1,CSRNP1,GADD45B,HMOX1,ID1, IDI1,LSS,MEST,PTGS2,SCD,TIPARP

Supplementary Figure 5



Supplementary Information

Supplementary Figure Legends

Supplementary Figure 1

Characterization of probe sets used for Affymetrix microarray. (A) Conditional *PDK1*-/cond chicken DT40 B cells (*PDK1*-/cond) and DT40 wt cells were treated with 0.5 µM 4-hydroxytamoxifen (OH-TAM) or 0.05% EtOH for 48 hrs. Then cells were transferred to normal DT40 medium. On days three to five after beginning of OH-TAM/EtOH treatment cleared cellular lysates were prepared and analyzed for PDK1 and vinculin by immunoblotting. Triplicate probe sets were analyzed in parallel; total RNA isolated on day four was used for microarray analyses. (B) DT40 *PDK1*-/cond and wt cells were left untreated or were treated with 0.5 µM OH-TAM or 0.05% EtOH for 48 hrs. Then cells were transferred to normal DT40 medium. At the indicated time points relative mRNA expression of *PDK1* was analyzed by quantitative real-time RT-PCR. (C) The two sets of regulated transcripts (entity list 1: red circle, entity list 2: blue circle) were displayed in a Venn diagram. The 503 transcripts regulated in both entity lists are displayed as the overlap (purple) of the red and blue circles. (D, E) With the 503 transcripts, global function and pathway analyses were performed using Ingenuity Pathway Analysis Software. Depicted are the best hits of the function (D) and pathway groups (E) with a threshold of p-value 0.05.

Supplementary Figure 2

PDK1 inhibition leads to PI3K subunit up-regulation in human DG75 and Ramos B lymphocytes and in prostate carcinoma PC-3 cells. (A) DG75 B lymphocytes were treated with 10 µM BX-795 or 0.1% DMSO for 48, 72 or 96 hrs. Cleared cellular lysates were

prepared and analyzed for PI3K p110δ, PI3K p85α and vinculin by immunoblotting. (B) Ramos B lymphocytes were incubated with the indicated concentrations of BX-795 or with DMSO (0.1% v/v) for 48 hrs. Cleared cellular lysates were prepared and analyzed for Akt, phospho-Akt (T308), GSK3β, phospho-GSK3β (S9), TSC2, phospho-TSC2 (S939), PI3K p110β, PI3K p110δ, PI3K p85α and actin by immunoblotting. Data shown are representative of three independent experiments. (C) Prostate carcinoma PC-3 cells were incubated with the indicated concentrations of BX-795 or with DMSO (0.1% v/v) for 48 hrs. Cleared cellular lysates were prepared and analyzed for Akt, phospho-Akt (T308), GSK3β, phospho-GSK3β (S9), TSC2, phospho-TSC2 (S939), FOXO1, phospho-FOXO1 (S256), PI3K p110β, PI3K p110δ and actin by immunoblotting. Data shown are representative of three independent experiments.

Supplementary Figure 3

Expression of chicken PDK1 reconstitutes wildtype phenotype in DT40 PDK1ko cells. (A) DT40 *PDK1*-/cond cells, retrovirally transfected with chicken PDK1 cDNA, were treated with 0.5 µM OH-TAM or 0.05% EtOH for 48 hrs or left untreated. Then cells were transferred to normal DT40 medium. On day four after beginning of OH-TAM/EtOH treatment cleared cellular lysates were prepared and analyzed for PDK1, PI3K p110β, PI3K p110δ, PI3K p85α, phospho-GSK3β (S9), GSK3β and Hsp90 by immunoblotting. Data shown are representative of three independent experiments. (B) Cells described in (A) were treated with 0.5 µM OH-TAM or 0.05% EtOH for 48 hrs. Then cells were transferred to normal DT40 medium. On days four to eight after beginning of OH-TAM/EtOH treatment apoptosis was assessed by propidium iodide staining of hypodiploid apoptotic nuclei and flow cytometry. Data shown are mean of triplicates ±SD and are representative of three independent experiments.

Supplementary Figure 4

Members of the FOXO and CREB transcription factor families are upstream regulators of regulated transcripts. (A) The two complete sets of regulated transcripts were analysed using Ingenuity Pathway Analysis (IPA) Software. Ingenuity's Upstream Regulator Analysis in IPA is a tool that predicts upstream regulators from gene expression data based on the literature and compiled in the Ingenuity® Knowledge Base. A Fisher's Exact Test p-value is calculated to assess the significance of enrichment of the gene expression data for the genes downstream of an upstream regulator.

Supplementary Figure 5

Akt and mTORC1 inhibition lead to up-regulation of PI3K subunits in different human cell lines. (A) Jurkat T lymphocytes, DG75 B lymphocytes and Nalm-6 B lymphocytes were treated with 10 µM Akti-1/2/3 or 0.1% DMSO for 48, 72 or 96 hrs (DG75 and Jurkat only). Cleared cellular lysates were prepared and analyzed for PI3K p110δ, PI3K p85α (DG75 only) and vinculin by immunoblotting. (B) Jurkat T lymphocytes were treated with 100 nM rapamycin (Rapa) for 72 hrs. Cleared cellular lysates were prepared and analyzed for PI3K p110β, PI3K p110δ, PI3K p85α, phospho-Akt (T308 and S473), Akt1, phospho-GSK3β (S9), p70S6K, phospho-p70S6K (T389), vinculin, and GAPDH by immunoblotting.

Supplementary Materials & Methods

Microarray analysis

DT40 cells were incubated in medium containing 0.5 µM 4-hydroxytamoxifen (OH-TAM) or 0.05% EtOH as control. After 48 hrs OH-TAM treated cells were transferred to normal medium. On day four after beginning of OH-TAM/EtOH treatment total RNA was extracted using the RNeasy Micro Kit (Qiagen) according to manufacturer's instructions for tissues. QIAshredder mini-spin columns (Qiagen) as well as needle and syringe homogenization were applied. RNA quality was observed with Agilent 2100 Bioanalyzer using RNA 6000 Nano LabChip Kit (Agilent Technologies, Böblingen, Germany) following the manufacturer's instructions. Microarray experiments were performed using the Affymetrix™ platform according to manufacturer's instructions. Fragmented and labeled cRNA were hybridized on Affymetrix™ GeneChip Chicken Genome Arrays. A probe-level summary was determined using the Affymetrix™ GeneChip Operating Software using RMA algorithm. Normalization of raw data was performed by the Array Assist™ Software 4.0 (Stratagene, La Jolla, Canada), applying a GC-robust multichip average (RMA) algorithm. Significance was calculated using a t-test without multiple testing correction (Array Assist™ Software), selecting all transcripts with a minimum change in expression level of 2-fold together with a p-value less than 0.05.

Gene regulation networks

Gene regulation networks were generated by Ingenuity Pathways Analysis 3.1 (http://www.ingenuity.com). For that purpose, data sets containing orthologs of gene identifiers and the corresponding expression and significance values were uploaded into the application. These genes, called Focus Genes, were overlaid onto a global molecular network developed from information contained in the Ingenuity Pathways Knowledge Base. Networks of these Focus Genes were then algorithmically generated based on their connectivity.

Functional classification of differentially expressed genes

Functional Analysis was used to determine the biological functions that were most significant to the data set. Ortholog genes from the data set that met the negative logarithmic significance cut-off of two or higher, and were associated with biological functions in Ingenuity Pathways Knowledge Base were considered for the analysis. Fisher's exact test was used to calculate a p-value determining the probability that each biological function assigned to that data set is due to chance alone.

Genomatix-based promoter analyses

The promoter regions of the genes encoding the PI3K subunits p110β, p110δ and p85α, the phosphoinositide-modifying enzymes (INPP5B, PIP5K1-β), the PI3K interacting protein, and Lyn were analysed by performing transcription factor mapping using the Genomatix Software Suite v2.7 (http://www.genomatix.de). The promoters of the genes listed above were directly extracted from ElDorado database. Transcription factor binding sites were identified using MatInspector (Release professional 8.06, August 2012) with the library "MatInspector Release professional 8.06, August 2012". Default parameters were used for the initial analyses in all programs, if not indicated otherwise.

Upstream Regulator Analysis

The two complete sets of regulated transcripts were analysed using Ingenuity Pathway Analysis (IPA) Software. Ingenuity's Upstream Regulator Analysis in IPA is a tool that predicts upstream regulators from gene expression data based on the literature and compiled in the Ingenuity® Knowledge Base. A Fisher's Exact Test p-value is calculated to assess the significance of enrichment of the gene expression data for the genes downstream of an upstream regulator.