universität wien

MASTERARBEIT

Titel der Masterarbeit

Mono-ubiquitination of phosphoinositide-dependent-kinase 1 (PDK1)

angestrebter akademischer Grad

Master of Science (MSc)

Verfasserin / Verfasser:	Thomas List
Studienrichtung (lt. Studienblatt):	Molekulare Mikrobiologie und Immunbiologie
Betreuerin / Betreuer:	Univ.Prof.Dr.Thomas Decker

Wien, 2010

Acknowledgements

I want to thank Sebastian Nijman for his scientific guidance, advice and inspiring pep talks. Roberto Sacco for his advice on tandem affinity purification and for being ever helpful in providing materials and discussing approaches. André Müller for mass spectrometry analysis and Iris Uras for help with the RNAi screen and PDK1 mutants. I also want to thank Mathew Sloane, Nils Craig-Müller and Markus Müllner for their help, advice and friendship. Finally I would like to thank my family and Stanzi for their constant support.

1 Introduction 7
1.1 The PI3K/AKT pathway 7
1.2 PDK1, a master regulator of the PI3K/AKT pathway 10
1.2.1 Regulation of AKT by PDK1 11
1.2.2 Other PDK1 substrates 12
1.3 Oncogenic pathway alterations 14
1.3.1 PTEN in cancer 15
1.3.2 PI3K in cancer 16
1.3.3 AKT in cancer 16
1.3.4 PDK1 in cancer 17
1.4 The ubiquitin system and DUBs 18
1.4.1 The post translational modifier Ubiquitin 18
1.4.1 Non-degradative ubiquitination 20
1.4.2 DUBs antagonize E3 ligases 21
1.4.3 DUBs can be onco-proteins or tumor suppressors 22
1.5 Therapeutic aspects in cancer 23
1.5.1 Molecular targets in cancer therapy 24
1.5.1.1 Targeting the PI3K/AKT pathway 26
1.5.1.1.1 Targeting PI3K 26
1.5.1.1.2 Targeting mTOR 28
1.5.1.1.3 Targeting AKT 28
1.5.1.1.4 Targeting PDK1 28
1.5.1.2 Targeting the ubiquitin system 30
1.5.1.2.1 Proteasome 31
1.5.1.2.2 E3 ligases 31
1.5.1.2.3 DUBs 32

2 Results 32
2.1 PDK1 is mono-ubiquitinated 32
2.2 Mapping of PDK1 mono-ubiquitination 35
2.3 Affinity purification and LC-ESI-MS/MS 39
2.4 Loss of function RNAi screen for a DUB acting on PDK1 46
2.5 Functional characterization of PDK1 mono-ubiquitination 48
3 Discussion 52
3.1 Mapping the lysine residue 52
3.2 MS/MS: potential and limitations 53
3.3 Finding the DUB 55
3.4 Function of PDK1 mono-ubiquitination 57
3.5 Open questions and outlook 58
4 Conclusions 60
5 Materials and Methods 61
5.1 Mass Spectrometry, an emerging tool in ubiquitin studies 62
5.2 RNAi 64
5.3 shRNA DUB screen 65
5.4 Tissue culture 67
5.1 Fractionaton 69
5.2 PAGE and Western blotting 69
5.3 Affinity Purification 69
5.4 Sequencing 70
5.5 Gateway cloning 70
5.6 PDK1 lysine mutants 74
5.7 PDK1 Truncation mutants 76
5.8 Immunofluorescence 81

6 References 82

7 Appendix 95

7.1 Abbreviations used 95

7.2 Table of Figures 97

7.3 Zusammenfassung 98

7.4 Abstract 100

7.5 Curriculum Vitae 101

1 Introduction

1.1 The PI3K/AKT pathway

Kinases of the PI3K family are activated in a receptor tyrosine kinase (RTK) dependent manner. When bound to ligands such as insulin or insulin-like growth factor 1 (IGF1), RTKs undergo autophosphorylation and PI3K is recruited to the membrane and activated, either by directly binding phosphorylated tyrosine residues on the activated receptor with two SH2 domains in the regulatory subunit or indirectly, by interaction with receptor associated adaptor molecules like insulin-receptor-substrate (IRS). Alternatively, PI3K can be activated by G-protein coupled receptors (GPCRs), by interaction with G-proteins such as Ras or tyrosine kinases like Src which phosphorylate their regulatory subunit [1-2]. Upon activation, PI3K phosphorylates membrane bound phosphatidylinositol-4,5-bisphosphate (PI[4,5]P_2 or PIP_2) to phosphatidylinositol-3,4,5-triphosphate (PI[3,4,5]P_3 or PIP_3) which then functions as a second messenger (Figure 1). Downstream molecules such as AKT and PDK1 are recruited to the plasma membrane (PM) by binding the second messenger PIP_3 via their pleckstrin homology (PH) domain [3]. PI3K activity is associated with inhibition of apoptosis, cell cycle progression, DNA repair, metabolism, cell growth and translation. These diverse signals are mediated mainly by a subgroup of the cAMP-dependent, cGMP-dependent, protein kinase C (AGC) kinase family like AKT, which is activated in a PIP_3 dependent manner [4]. The phosphatase and tensin homologue on chromosome ten (PTEN) dephosphorylates PIP_3 to PIP_2 and thereby counteracts PI3K downstream activating function, thus acting as a tumor suppressor, i.e. a gene or gene product responsible for constraining cell proliferation and whose inactivation can contribute to cancer development. PI3K complexes are typically heterodimers, consisting of the p85 regulatory and the p110 catalytic subunits [5]. The regulatory p85 subunit mediates binding of PI3K to phosphotyrosine motifs on activated transmembrane receptors (e.g. PDGFR, EGFR, HER2) or to receptor associated adaptor proteins (e.g. Ras, IRS1), activation and localization of the enzyme. The catalytic subunit p110 is essential for generating PIP_3 by phosphorylating the 3'OH group on the inositol ring of inositol phospholipids (Figure 1) [6].

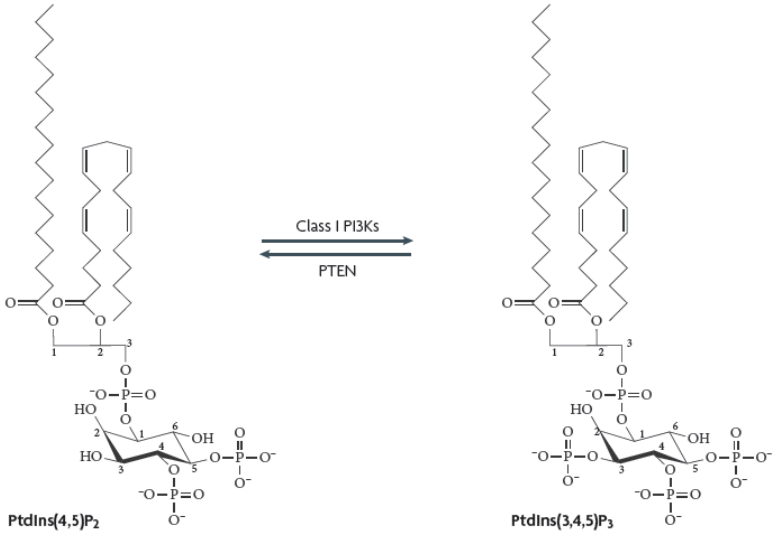


Figure 1 – Generation of PIP_3 by PI3K and dephosphorylation to PIP_2 by PTEN [1]

There are 3 classes of PI3Ks, according to their structure and substrate specificity. Class I is further sub-categorized in class IA, the most relevant for cancer, which is activated as described above and class IB which is activated by GPCRs exclusively [1]. Class II PI3Ks have a single sub-unit and bind phosphoinositides (PI) or phosphatidylinositol-phosphates (PIP), however their cellular function is still unclear. Class III PI3Ks also consist of a single sub-unit (vPS34) and have been shown to be involved in membrane trafficking, autophagy and cell growth through activation of mammalian target of rapamycin (mTOR) in a nutrient dependent manner [7]. The *PI3KCA* gene encodes the p110α subunit isoform, which is associated with the most commonly known functions of PI3K downstream signaling. One of the most prominent downstream targets of PI3K is AKT, a 65 kDa serine-threonine Kinase and a pivotal component of the PI3K/AKT pathway. In the human genome, three genes are known to encode AKT-Family proteins, namely *AKT1*, *AKT2* and *AKT3*. Unlike other members of the AGC kinase family, all AKT isoforms have an N terminal PH domain that mediates binding to PIs and a C-terminal catalytic domain with kinase activity.

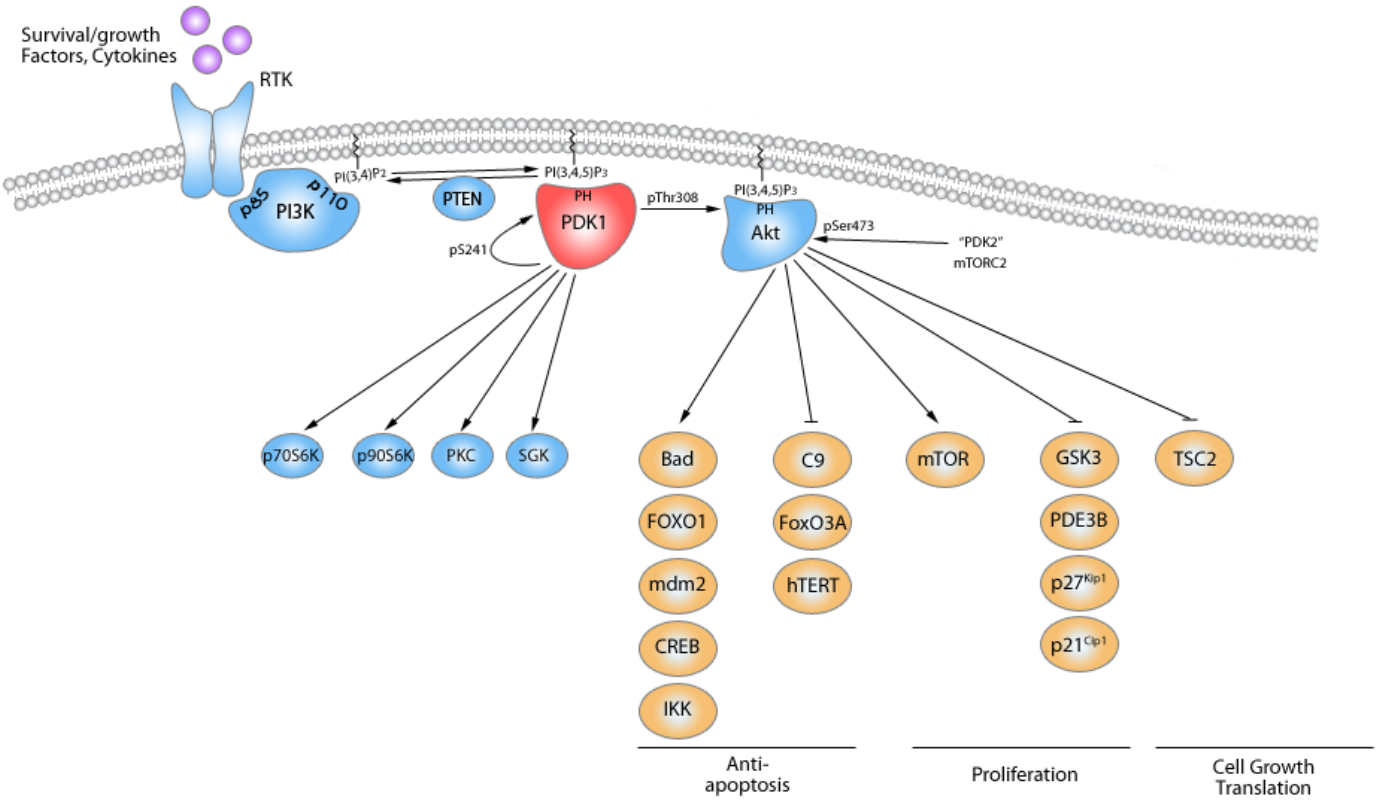


Figure 2 – The PI3K/AKT Pathway and downstream targets.

PI3K is activated by ligand-bound RTKs directly or by a number of RTK-coupled kinases (e.g. Ras, Src, IRS) and phosphorylates PI[4,5]P_2 to PI[3,4,5]P_3. AKT binds PIP_3 and is recruited to the plasma membrane which allows PDK1 to phosphorylate AKT on T308, which is essential for activation. Shown in yellow are some of the known targets of AKT.

AKT is recruited to the plasma membrane following generation of the second messenger molecule PIP_3 by PI3K and is then phosphorylated by PDK1 at Thr308 which is essential for AKT activation [8]. However, full activity of AKT also requires a second phosphorylation at the C-terminal Ser473, which is thought to be carried out by a second PIP-dependent kinase – referred to as "PDK2" [9] - and likely to be mTOR kinase in the mTORC2/MIP1/SIN1 context [10-11]. AKT is a central platform for signal transduction and plays a role in inhibition of mitochondria-mediated and Fas-L mediated apoptosis (e.g. by inhibiting BAD, GSK3), cell cycle regulation (e.g. by inhibiting FoxO-3a, p53), promoting transcription and translation (e.g. by inhibiting TSC1/2 and activating NFκB). AKT activates or inhibits a variety of substrates and downstream signals, the most important of which are listed in Table 1.

Protein	Effect of phosphorylation	Biological effect	Reference
BAD	inhibition	Anti apoptotic	[12]
Caspase 9	inhibition	Anti apoptotic	[6]
FOXO1	inhibition	Anti apoptotic	[12]
Mdm2	activation	Anti apoptotic	[5]
IκB kinase (IKK)	activation	Anti apoptotic	[12]
CREB	activation	Anti apoptotic	[12]
FOXO4	inhibition of $p27^{Kip1}$	Anti apoptotic	[5]
FOXO3A	inhibition	Anti apoptotic	[5]
hTERT	activation	Anti apoptotic	[12]
GSK3beta	inhibition	Proliferation	[12]
$P21^{Cip}$	inhibition	Proliferation	[12]
mTOR	activation	Proliferation	[6]
PDE3B	inhibition	Proliferation	[6]
$p27^{Kip1}$	inhibition	Proliferation	[13]
TSC2	Inhibition	Cell growth	[5]

Table 1 – List of important published AKT substrates involved in cell survival, proliferation and growth.

1.2 PDK1, a master regulator of the PI3K/AKT pathway

PDK1 was first identified in 1997 as the activator of AKT in the presence of PIP_3 [9]. PDK1 is a 556 amino acid, 68kDa serine/threonine kinase of the AGC protein kinase family, and responsible for phosphorylation and activation of several other AGC kinases, the most important of which are p70 ribosomal S6 kinase (S6K), p90 ribosomal S6 kinase (RSK), serum and glucocorticoid induced kinase (SGK), protein kinase C (PKC) isoforms and AKT. Regulation of PDK1 is not well understood, however it is likely that PDK1 is not directly activated by insulin stimulation [14] but is constitutively active in mammalian cells [4] owing to its ability to autophosphorylate its T-loop at Ser241 *in trans* in humans [15]. More recent data suggests that Ser241 phosphorylation alone is not enough for full activity, but that phosphorylation at three more tyrosines (Tyr9, Tyr373, Tyr376) - likely by v-Src - is needed for activity and recruitment to the plasma membrane [16]. Additionally, stabilization of PDK1 by the molecular chaperone HSP90 is essential for PDK1 activity because it protects it from proteasomal degradation. HSP90 inhibitors (e.g. geldanamycin, radicicol) disrupt the PDK1-HSP90

interaction leading to proteasomal degradation of PDK1 and to AKT dephosphorylation [17]. It has been reported that phosphorylated Tyr9 is the residue responsible for HSP90 dependent stabilization [18]. Evidence that PDK1 is an important activator of downstream AGC kinases comes from the fact that in PDK1 knock out cells, S6K, SGK, AKT [14], RSK and PKC [19] fail to be activated and AKT substrate levels are altered. Tumor suppressor $p27^{Kip}$ levels for instance are markedly increased and levels of cyclin D1 are decreased, which leads to G2-M arrest [20]. Furthermore PDK1 knock out is embryonic lethal in mice and embryos show multiple developmental abnormalities in brain formation [21]. It also leads to pancreatic hypoplasia in mice when locally knocked out in pancreatic cells [22] and to cardiac arrest after 5-11 weeks of age by sensitizing cardiomyocytes to hypoxia [23]. Mice expressing hypomorphic PDK1, leading to a reduction of PDK1 levels by 90%, are viable but 40-50% smaller than PDK1 wild type mice which was linked to a decrease of cell volume in hypomorphic mice rather than cell number [21]. This establishes PDK1 as an important regulator of effector AGC kinases in the PI3K pathway, responsible for cell growth, proliferation and survival.

1.2.1 Regulation of AKT by PDK1

AGC kinases are typically activated by phosphorylation at two sites. One is located in the activation loop (t-loop) the other is typically localized in a C terminal hydrophobic motif (H-motif) [4]. PDK1 exerts its growth and survival promoting function via AKT by phosphorylating the Thr308 residue in the AKT t-loop. We will discuss the current model for this mechanism below. PDK1 contains two domains, an N-terminal kinase domain (amino acids 70-359) and a PH domain (amino acids 459 - 550), the latter interacts with PIP_3. It is currently not known what function the PDK1 PH domain plays in activating substrates, as PDK1 binding to PIP_3 seems not to be required for its catalytic activity [24]. PIP_3 binding has an enhancing effect on AKT activation by increasing co-localization with AKT and thus bringing enzyme and substrate into close enough contact for PDK1 to phosphorylate AKT and activate it at a higher rate. This is supported by the fact that AKT phosphorylation levels are highest when incubated with phospholipid vesicles containing PIP_3 at concentrations of 10µM *in vitro* [9]. Whether PDK1 and AKT localize to the plasma membrane only following PI3K stimulation is controversial. Some studies suggest that PDK1 and AKT translocate to the plasma membrane following PI3K stimulation [25-26], while other studies seem to suggest that a pool of PDK1 – which has a PH domain with higher affinity to PIP_3 than AKT – must be bound

constitutively to the plasma membrane [24] and only recruitment of its substrate, AKT, allows activating phosphorylation to occur. Evidence for the latter scenario comes from experiments where AKT was forced to localize to the PM in unstimulated cells, by fusion with a membrane targeting motif, which led to full Thr308 phosphorylation of AKT [27]. While the PDK1/PIP_3 interaction seems to play a minor role, binding of AKT to PIP_3 via its PH domain is essential for its activation. PDK1 is unable to phosphorylate wild type AKT at Thr308 in the absence of PIP_3 but can readily phosphorylate an AKT PH domain deletion mutant [28]. This data is supported by structural analysis that showed that the PH domain of AKT undergoes conformational change when bound to PIP_3 allwoing PDK1 to phosphorylate Thr308 [29]. The details of how this conformational change allows phosphorylation are not clear, but it could be that the AKT activation loop is exposed or that a docking site for PDK1 is revealed. Recently there has been an attempt to challenge this idea, with studies using PDK1 and AKT fused to complementary moieties to induce complex formation in the presence of various PI3K inhibitors. These showed that PDK1-AKT complex formation is sufficient for AKT activation independently of membrane localization and PIP_3 [30]. This suggests once again that in endogenous conditions, PDK1/PIP_3 interaction could play an enhancing role by increasing PDK1/AKT co-localization. A possible limitation of this study however, is that the artificial AKT-PDK1 complexes may behave differently than its endogenous counterpart. The complementary moieties may influence the secondary structure such that an open conformation is mimicked and the PDK1 phosphorylation site exposed. AKT stands out from the other AGC kinases because - like PDK1 - it has a PH domain which plays a role in its activation, a feature absent in other kinases of this family. However, just like other AGC kinases it does have a C-terminal hydrophobic motif which needs to be phosphorylated at Ser473 by mTORC2 to allow AKT to be fully activated.

1.2.2 Other PDK1 substrates

Beyond activating AKT, PDK1 is also responsible for activation of SGK, PKC, RSK, and S6K which are agonists of cell growth and translation. SGK is involved in regulation of ion transport and S6K controls protein synthesis and amino acid storage. PKC-α is activated in a diacylglycerol dependent manner and activates gene transcription via the IKK/NFκB, Fos/Jun/AP1 and MAPK pathways [31]. RSK is involved cell growth and proliferation via the MAPK/ERK pathway [32]. Since these kinases lack a PH domain, the mechanism by which PDK1 activates them is PIP_3 independent,

somewhat different from AKT and takes place in the cytoplasm. These substrates have Ser/Thr residues in their t-loop and C-terminal hydrophobic motives, and phosphorylation of both residues is essential for their kinase activity as well [4]. The catalytic domain of PDK1 contains a hydrophobic pocket - called PKC related kinase 2 (PRK2) interacting fragment pocket ("PIF" pocket), and first identified as binding to PRK2 - that interacts with high affinity with phosphorylated hydrophobic motifs [33]. This first phosphorylation step on AGC kinases is carried out by as of yet unidentified H-motif kinases in response to PI3K activation. When residues in the hydrophobic motif of SGK [34] and S6K [35] are mutated to acidic amino acids, mimicking phosphorylated residues, phosphorylation of their t-loop becomes uncoupled from PI3K activity, suggesting that H-motif kinases are PI3K dependent. Once the H-motif is phosphorylated, PDK1 is able to bind these AGC kinases and phosphorylate their t-loop: this second phosphorylation is the final step that leads to the formation of a small phosphate binding pocket in the AGC kinase domain, leading to a conformation change and intrinsic binding of the phosphorylated H-motif by the pocket, which stabilizes the AGC kinase in an active state [4]. PDK1 activates AKT in a PIP_3 dependent manner at the plasma membrane and its other substrates in a PIP_3 independent manner in the cytosol. Although the mechanisms PDK1 dependent AKT activation differs from the other PDK1 substrates, in both cases insulin and growth factor signaling does not directly stimulate PDK1 activity but rather affects its ability to bind substrates, via a preliminary phosphorylation step [36]. This unusual substrate-dependent activation mechanism shows that PDK1 is both an essential activator and dependent on either a second messenger or priming of its substrates by H-motif kinases, to create a docking site.

1.3 Oncogenic pathway alterations

Components of the PI3K pathway are often mutated, amplified or silenced in somatic and germ line cells, and are thought to be causal in cancer development [1, 37]. Table 2 gives an overview of reported alterations of the most important nodes of the PI3K pathway in a wide range of cancers.

Oncogene	**Alteration**	**Cancer Type**	**Incidence %**
PTEN	Loss of heterozygosity	Gastric	25,3
	Loss of heterozygosity	Breast	24,9
	Loss of heterozygosity	Melanoma	37
	Loss of heterozygosity	Prostate	30
	Loss of heterozygosity	Glioblastoma	28
	Mutation (Loss of function)	Endometrial	38
	Mutation (Loss of function)	Brain	21
	Mutation (Loss of function)	Skin	17
	Mutation (Loss of function)	Prostate	14
	Mutation (Loss of function)	Colon	13
	Mutation (Loss of function)	Ovary	9
	Mutation (Loss of function)	Breast	6
	Mutation (Loss of function)	Hematopoietic and lymphoid tissue	6
	Mutation (Loss of function)	Stomach	6
	Mutation (Loss of function)	Liver	5
	Mutation (Loss of function)	Kidney	5
	Mutation (Loss of function)	Vulva	65
	Mutation (Loss of function)	Urinary tract	9
	Mutation (Loss of function)	Thyroid	5
	Mutation (Loss of function)	Lung	9
PI3K p110α	Mutation	Breast	27
	Mutation	Endometrial	24
	Mutation	Colon	15
	Mutation	Upper digestive tract	11
	Mutation	Gastric	8
	Mutation	Pancreas	8
	Mutation	Ovarian	8
	Mutation	Liver	6
	Mutation	Brain	5,9

	Mutation	Oesophageal	5
	Mutation	Lung	3
	Mutation	Melanoma	9
	Mutation	Urinary tract	17
	Mutation	Prostate	2
	Mutation	Thyroid	2
PI3K p85a	Mutation	Glioblastoma	9
	Mutation	Ovarian	4
	Mutation	Colon	2
AKT1	Mutation	Breast	3,7
	Mutation	Colon	2,8
	Mutation	Ovarian	2
	Mutation	Lung	1,9
	Amplification	Gastric	20
AKT2	Amplification	Ovarian	14,1
	Amplification	Pancreas	20
	Amplification	Head and neck	30
	Amplification	Breast	3
AKT3	Amplification	Skin	1,5
	Amplification	Glioblastoma	2
PDK1	Amplification and overexpression	Breast	20

Table 2 – Incidence of genetic alteration in the PI3K pathway. Adapted from Liu et al. [1]

1.3.1 PTEN in cancer

PTEN is frequently mutated in human cancers [38]. The PI3K pathway was first linked to cancer in the 1990s, when PTEN was discovered to be a phosphatase with substrate specificity for PI3K products, and antagonizing PI3K function [1]. By reversing PI3K function it also inhibits activation of PIP_3 dependent and cell cycle promoting kinases such as PDK1 and AKT, and thus acts as a tumor suppressor. Transcription of PTEN is under the control of pro-apoptotic transcription factor and master tumor suppressor p53, which therefore functions as a negative regulator of the entire PI3K signaling pathway synergistically with PTEN [39-40]. Microsatellite analyses showed that PTEN is mutated in 25-50% of glioblastomas [41], in 40-55% of sporadic prostate cancers [42] and in

35% of endometrial carcinomas [43-44]. These statistics illustrate how widespread PTEN alterations are in carcinogenesis.

1.3.2 PI3K in cancer

Oncogenic PI3K mutations are exclusively somatic and result in constitutive activity of the kinase [45]. Missense mutations at two hotspot regions of the *PIK3CA* locus, exons 9 and 20, of the *PI3KCA* locus are the most commonly occurring PI3K mutations in cancer [46]. These mutations are located at the interfaces between p110 and p85 domains and may increase enzymatic activity by disrupting inter-domain interactions, resulting in changes in the kinase domain [1]. This in turn increases PIP_3 levels, and leads to PDK1 dependent AKT activation that induces cellular transformation. Studies using comparative genomic hybridization have shown that the chromosome region 3q26 which contains *PIK3CA*, is amplified in approximately 40% of ovarian carcinomas [47-48]. In contrast, the *PIK3CB* gene which encodes the p110β isoform of the PI3K catalytic domain has not been found to be mutated in human cancers.

1.3.3 AKT in cancer

AKT has been linked to diverse diseases, including diabetes, and neurodegenerative disorders [12]. In cancer, AKT activation has been shown to correlate with advanced disease and poor prognosis and has three consequences: increased survival via anti-apoptotic signals, increased cell proliferation and increased cell growth. Isoform AKT1 is hyperactive in carcinomas, glioblastomas and various hematological malignancies. Amplification of AKT1 is frequent in gastric [49], prostate, breast and ovary carcinomas [50] and overexpression of AKT has *in vitro* transforming activity [50]. Furthermore it was shown that the gene encoding the AKT2 isoform is amplified in breast, ovarian and pancreatic cancers [50-54]. A constitutively membrane bound, phosphorylated and active form of AKT has been found in cells from a breast cancer patient, bearing an E17K mutation in the PH domain and showing higher levels of activating K63 ubiquitination - as discussed later - than wild type [55-56]. Additionally, levels of activated AKT (Ser473 phosphorylation) have been found to be increased by 43% in alveolar rhabdomyosarcomas and by 55% in embryonal

rhabdomyosarcomas [57]. Table 3 summarizes tumors that commonly show activated AKT. This underlines the oncogenic potential of AKT, its central role in disease progression and the importance of getting a grip on its aberrant activity in order to inhibit cancer cell proliferation.

Tumor type	**% tumors with active AKT**
Glioma	55
Thyroid carcinoma	80
Breast carcinoma	37,5
Lung carcinoma	60
Gastric	80
Gastrointestinal stromal	30
Pancreatic carcinoma	50
Bile duct	85
Ovarian carcinoma	55
Endometrial	35
Prostate	50
Renal	40
Large cell Lymphoma	100
AML	70
Multiple myeloma	90
Malignant mesothelioma	65
Malignant melanoma	55

Table 3 – Tumors that commonly show aberrant AKT activation. Adapted from Altomare et al. [52]

1.3.4 PDK1 in cancer

Despite not being a hot spot for alteration itself, PDK1 is the master regulator of the AKT oncoprotein and essential for its activation. As discussed, PDK1 is constitutively active and mediates one out of two required phosphorylation events to activate the proto-oncoprotein AKT. Thus PDK1 is located at a crucial position, acting as a switch and providing a rationale to target it in order to silence the aberrantly active PI3K/AKT pathway in transformed cells. An example of how PDK1 adds to the oncogenic signal is the activating E17K mutation in the AKT PH domain, which is likely to allow PDK1 to phosphorylate AKT even in the absence of extracellular stimulus and at low PIP_3 levels. The role of PDK1 in cancer can therefore be thought of as a constitutively active and essential activator of the AKT onco-protein with the potential of being a limiting factor in proliferative signal

transduction. Evidence that PDK1 indeed makes for a potentially interesting target comes from several studies which suggest its causal role in proliferation and transformation. For example, antisense depletion of PDK1 in $PTEN^{-/-}$ glioblastomas reduces cell proliferation *in vitro* [58] and PDK1 overexpression in mammary epithelial cells induces their transformation and adhesion independent growth in soft agar [59]. $PTEN^{-/-}$ cell progenitors cannot transform or develop into invasive and fatal T lymphoma without PDK1 in mice [60], which supports the role of PDK1 as a limiting factor. Furthermore, heterozygous PTEN+/- mice, which are normally predisposed to develop PTEN deficient tumors through LOH, but expressing reduced PDK1 levels are less likely to develop these neoplasms [61]. It has also been suggested that PDK1 is overexpressed and amplified in 20% of human breast carcinomas [1, 62]. More recently, it was shown that human cancer cells and breast cancer cells with low AKT levels are dependent on the PDK1 substrate SGK for proliferation [63].

1.4 The ubiquitin system and DUBs

1.4.1 The post translational modifier Ubiquitin

Ubiquitin is a 76 amino acid (8.5 kDa) small modifier protein present in eukaryotic cells that is covalently attached to proteins via an isopeptide bond. It is covalently attached in a three step process called ubiquitination that requires activation of the ubiquitin molecule by an activating enzyme (E1), transfer of the activated ubiquitin to a conjugating enzyme (E2) and the presence of a ubiquitin ligase (E3) that binds the E2 and the target protein and forms an isopeptide bond between the ε-amino group of a lysine on the target substrate and the C-terminal glycine 76 (G76) on ubiquitin [64]. Ubiquitination can be reversed by de-ubiquitinating enzymes (DUBs) which are proteases able to cleave the isopeptide bond between ubiquitin and the covalently bound protein [65]. The human proteome comprises two known E1 ubiquitin activating enzymes, approximately 40 E2 ligases, 600 E3 ligases and approximately 90 DUBs. Ubiquitin itself contains 7 lysines (K6, K11, K27, K29, K33, K48, K63) which can be linked to further ubiquitin molecules, thus yielding diverse poly-ubiquitin chains with different non-proteolytic roles in the cell.

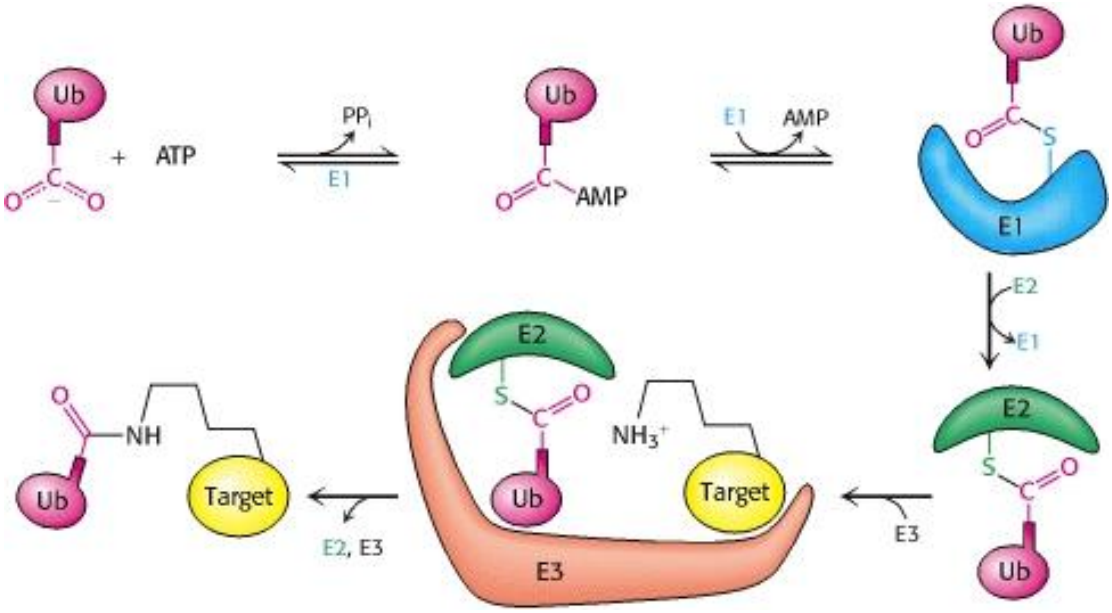


Figure 3 – Ubiquitin conjugation steps. Image taken from Stryer Biochemitry 5th Edition [64]

The synthesis of specifically linked poly-ubiquitin chains seems to depend on the E2 ligase catalyzing the reaction or on E2-E3 combinations [66-67]. For example, Cdc34 (E2) generates mainly K48-linked poly-ubiquitin, the Mms2/Ubc13 complex (E2) catalyzes the formation of K63 poly-ubiquitin chains and the APC(E3)/UbcH10(E2) complex forms K11-poly ubiquitin chains [68]. Branching poly-ubiquitin polymers using two different lysine residues (e.g. K6 and K11) have also been reported [69], yet their physiological function remains unclear. The mono-, oligo- or poly-ubiquitin epitope on a modified protein can be recognized by at least 20 reported ubiquitin binding domains (UBDs) on specific effector proteins and thus affect localization or degradation of the target [65]. However the roles of most of the different topologies of the ubiquitin epitopes and the cellular mechanisms they control are still poorly understood. The implication of various types of protein ubiquitination in cellular processes suggests the notion of a ubiquitin code that regulates protein stability, localization and activity.

1.4.1 Non-degradative ubiquitination

The only two well understood types of ubiquitination are the canonical K48-linked poly-ubiquitin chains which targets conjugated proteins for degradation by the 26S proteasome [70] and K63-linked poly-ubiquitination which serves non-degradative roles. K29-linked and K11-linked chains also lead to degradation by the proteasome [71-72] or endoplasmatic reticulum associated degradation (ERAD) [68]. It is currently not known why different types of poly-ubiquitin chains are used for seemingly redundant functions. Non-degradative forms of ubiquitination such as mono-ubiquitin, multi-mono-ubiquitin - i.e. the attachment of several single ubiquitin molecules on a target protein - or poly-ubiquitin chains linked via K63 of an enzyme can affect protein localization, recruitment of binding partners in DNA damage repair, inflammation, signal transduction, endocytosis and enzymatic activation. An example for K63 dependent activation are TNF-receptor-associated-factor 2 and 6 (TRAF 2 and TRAF6) which require K63-poly ubiquitination in order to further ubiquitinate the inhibitor of IκB complex (IKK), targeting IKK for degradation and thus de-repressing NFκB transcriptional activity [73]. Furthermore it has recently been shown that the kinase AKT is K63-ubiquitinated by E3 ligase TRAF6 and that, upon growth-factor stimulation, this modification is important for AKT membrane translocation which enhances AKT activity [55]. Both K27 and K33 linkages may be a result of stress response [74] and K6 ubiquitination catalyzed by BRCA1 (E3) is involved in DNA repair [75]. The function of mono-ubiquitination, is currently poorly explored. Yet there are some examples that hint at different roles that it plays in endocytosis, auto-inhibition, protein localization and transcriptional regulation. Following ligand binding, EGFR and PDGFR are poly-mono-ubiquitinated at the membrane leading to receptor internalization and lysosomal degradation, preventing their recycling to the plasma membrane [76]. This mechanism allows fine-tuning of receptor activity and is mediated by the endocytic proteins Sts1, Sts2, Eps15 and Hrs. These regulators of endocytosis are recruited to activated RTKs and bind the mono-ubiquitinated receptors via their UBA-type UBDs [77]. Recently it has been shown that surface receptor ErbB4 is also internalized after mono-ubiquitination by E3 ligase Itch [78]. It has also been suggested that mono-ubiquitination is required for cleavage and activation of Notch transcription factor by γ-secretase and subsequent downstream signal transduction [79]. Furthermore, in mammalian cells, several ion channels undergo internalization in endosomes following mono-ubiquitination in response to extra cellular signaling [80]. Interestingly, UBDs often mediate mono-ubiquitination of proteins that contain them [81]. This is the case for Sts1, Sts2, Eps15 and Hrs,

which undergo conformational re-arrangement when mono-ubiquitinated, following stimulation of RTKs. Their UBD binds their mono-ubiquitinated site in *cis*, thus inhibiting their own ubiquitin binding function in *trans* [77], in a negative feedback loop that leads to auto-inhibition. Mono-ubiquitination can also affect the post translational modification state of histones as well as transcriptional activity. Nucleosomes are hetero-octamers containing histones H2A, H2B, H3 and H4, which are post translationally modified in eukaryotic cells. It was shown that H2B mono-ubiquitination is required for H3K4 and H3K79 tri-methylation - a transcription de-repressing post translational modification - in a process known as histone cross-talk [82]. Furthermore TGF-β dependent SMAD4 mono-ubiquitination mediated by p300 was shown to disrupt the SMAD4-SMAD2 transcription factor complex and lead to relocalization of SMAD4 to the cytoplasm [83]. Proteins can be modified by different types of ubiquitination as is illustrated by the differential regulation of the tumor suppressor p53, where mono- and poly-ubiquitination serve different roles. Mono-ubiquitination on its C terminus exposes a nuclear export sequence which allows p53 to relocate to the cytosol whereas K48 poly-ubiquitination of p53 by HDM2 leads to its proteasomal degradation [84]. Another example is PTEN, which needs to be mono-ubiquitinated to translocate to the nucleus but is degraded upon poly-ubiquitination, which affects its activity as a tumor suppressor [85].

1.4.2 DUBs antagonize E3 ligases

The process of ubiquitination can be reversed by isopeptidases which hydrolyze the isopeptide bonds between ubiquitin and substrate, thus cleaving off ubiquitin moieties. These deubiquitinating enzymes (DUBs) can rescue proteins from proteasomal degradation, relocalize proteins to sites of activity from which they have been sequestered or revert conformational changes that have been induced by ubiquitination [86]. The human genome encodes 79 functional proteases with DUB activities, which are cysteine- or metallo-proteases and can be classified into five families (Figure 4) [87]. Ubiquitin specific proteases (USP) are the most abundant and contain well conserved motifs called cysteine and histidine “boxes” in their catalytic domain. The other families are ovarian tumor proteases (OTU), Jamm motif proteases (JAMM), Machado-Joseph disease protein domain proteases (MJD) and ubiquitin C-terminal hydrolases (UCH) which are thought to play a role in rescuing ubiquitin falsely conjugated to intracellular molecules like glutathione or polyamines and to process newly synthesized ubiquitin polymer precursors into

single ubiquitin molecules [87]. As opposed to proteases, which are often translated as inactive precursors and get activated by post translational modification or changes in pH, DUBs are produced as active enzymes [87].

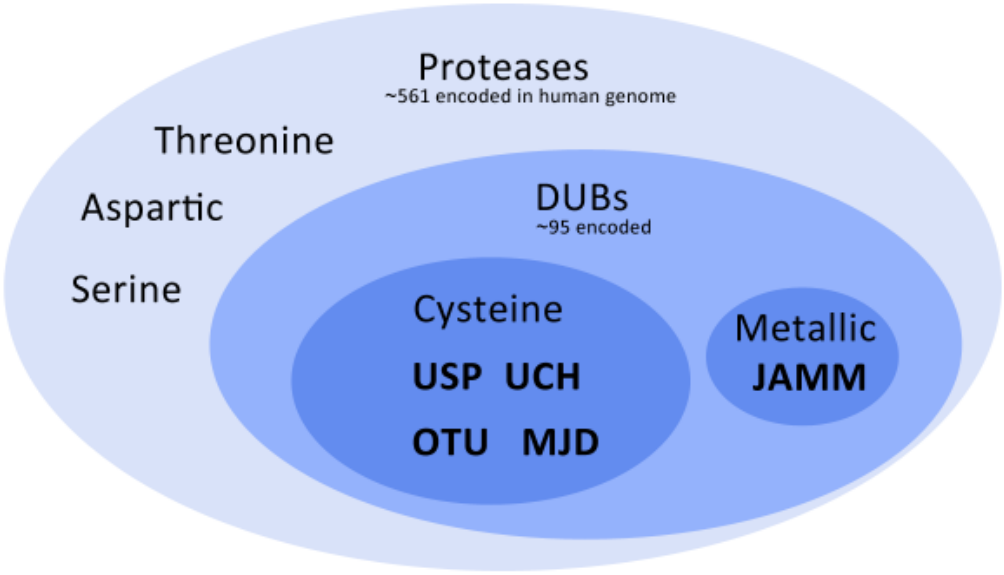


Figure 4 – The five DUB Families.

1.4.3 DUBs can be onco-proteins or tumor suppressors

There is increasing evidence that deregulation of the ubiquitin system can lead to cancer [88]. Phosphorylation and ubiquitination are critical in cell proliferation, however ubiquitination mediated events are far from being as well understood and documented as phosphorylation. More work is required to understand the mechanistic details of ubiquitin dependent regulation of important cancer related proteins like AKT, of the catalytic activity, regulation and substrate specificity of DUBs, all of which are important for the development of targeted drugs. Since ubiquitination is involved in the regulation of many cell signaling processes and cell cycle progression in a timed and specific manner, it is assumed that removal of ubiquitin molecules plays an equally important role in these processes. Interest in DUBs has grown as they have been reported to be mutated in human cancers, supporting their role as oncogenes or tumor suppressors [86], and

because they are enzymes which could, if suitable inhibitors are found, be targets in cancer therapy. The DUB TRE2 for instance cleaves ubiquitin from proteasomal degradation remnants and is tumorigenic when overexpressed [89]. Another example for the oncogenic potential of DUBs is USP9x, which stabilizes MCL1 by deubiquitinating it and preventing its proteasomal degradation, thus promoting tumor survival [90]. Of clinical relevance is USP1 which reverses ubiquitination of Fanconi Anemia Complementation Group D2 protein (FANCD2), a modification essential for DNA damage repair, for maintaining genomic stability and avoiding malignant transformation [91]. Fanconi anemia (FA) is characterized by skeletal anomalies, bone marrow failure, cellular hypersensitivity to DNA damage and increased incidence of solid tumors and leukemia [91]. In normal cells FANCD2 localizes to sites of DNA damage in the nucleus following mono-ubiquitination by E3-ligase FANCL, and participates in DNA repair by homologous recombination [92]. Since USP1 reverses ubiquitination and thus activity of FANCD2, it can be hypothesized that loss of USP1 is a reason for hypersensitivity of FA patients to DNA damage and ultimately for malignant transformation. Therefore overexpression or hyperactivity of USP1 could promote genomic instability and ultimately transformation. Examples of DUBs that act as tumor suppressors are CYLD and HAUSP. Patients with familial cylindromatosis carry a mutated or truncated allele of the gene encoding CYLD, which predisposes them to develop skin neoplasms [93-94]. In normal cells CYLD binds the IKK complex and de-ubiquitinates the adaptor protein TRAF2 thus effectively blocking NFκB signaling [95], a process that is deregulated in cells that have undergone LOH for CYLD. A20 is another remarkable DUB, as it is both an OTU type DUB for RIP and a Zn finger E3-ligase that attaches K48 ubiquitin chains to RIP [96]. DUBs like CYLD, USP6 and A20 can be thought of as true oncogenes or tumor suppressors [86]. HAUSP antagonizes the E2-ligase responsible for p53 K48 poly-ubiquitination and degradation, and thus stabilizes p53 [97]. Currently there is no DUB known to act on the K63 poly-ubiquitinated AKT proto-oncogene or mono-ubiquitinated PDK1; however it is reasonable to assume that one exists, as ubiquitination is a dynamic and reversible process.

1.5 Therapeutic aspects in cancer

Cancer is a disease characterized by the uncontrolled proliferation of a group of cells that metastasize. Cancer therapy therefore focuses on halting or reversing proliferation and metastasis formation. Since the 1970s this has been attempted with partial success using chemotherapy and

radiotherapy [31]. The aim of these treatments is the killing of rapidly dividing cells by interfering with mitosis, while non-dividing tissues in the body are left as unharmed as possible. In chemotherapy, cytostatic agents such as DNA alkylating agents which cross-link nucleotides and prevent DNA replication, anti-metabolites which stall DNA elongation, topoisomerase inhibitors (etoposide, doxorubicin) and DNA damaging agents (cisplatin) have been used. The rationale being that cancer cells often lack G2/M checkpoint components (CyclinB/CDK1) and therefore proceed into mitosis without repair of DNA damage [31]. Over several days, this results in what has been called a mitotic catastrophe, i.e. aneuploidy, accumulating DNA damage, formation of many micro-nucleoli and eventually cell death driven by apoptosis [98]. These unspecific approaches have however many caveats and patients often suffer from adverse side effectssuch as somatitis (mucosa inflammation), alopecia (hair loss), nausea, diarrhea, bone marrow suppression (neutropenia, thrombocytopenia), nephrotoxcitiy, hepatotoxicity, and neurotoxicity [99]. Over the last thirty years, our understanding of the molecular basis of cancer has greatly increased, and it has become apparent that cancer is a set of diseases with a highly heterogeneous genetic background [100]. Cancer types are no longer classified according to their tissue of origin, but according to their genetic profile. This has prompted the search for more targeted therapeutic approaches, which are oncoprotein and patient specific. Inhibiting cancer type specific oncogenes could be a way to increase efficiency in killing tumor cells, while eliminating the adverse effects of chemotherapy.

1.5.1 Molecular targets in cancer therapy

Efficient relapse free cancer therapy is hampered by the fact that cancer cells have a wide range of genetic and epigenetic variation across patients, while exhibiting very little characteristic surface markers, which makes them difficult to distinguish from wild-type cells and causes them to respond differently to drugs. Despite this high genetic variability, studies in mice [101], human cancer cell lines [102] and cancer patients [103-104] have shown that cancer cell survival can sometimes be greatly reduced by inhibition of only a single oncogene [105]. For instance, siRNA silencing or inhibition of SYK, a tyrosine kinase linked to hematopoietic malignancies, leads to differentiation of acute myeloid leukaemia (AML) cells and enhanced survival of mice with AML [106]. Dependence on a single oncogene is a phenomenon known as “oncogene addiction” [107] and is sought to be exploited in the current development of targeted therapy strategies. This poses two problems for

the development of efficient therapies. First, the problem of specificity. At a cellular level, we want therapy to stop cancer cells from proliferating, for example by killing them, without harming normal cells of the organism. At a molecular level, this generally means inhibiting oncogenic protein as specifically as possible without interfering with other proteins, thus avoiding off-target effects, which could cause unpredicted and potentially harmful side-effects. Moreover, although a great number of altered proteins participating in carcinogenesis are known, only a subset is easily targetable. It is generally easier to inhibit an onco-protein, than to restore full function of a defective or deleted tumor suppressor. This has established the concept of “druggability” of protein targets. A protein is considered to be druggable if it has a well defined function and a catalytic grove in which a low molecular weight compound can bind, thus masking several amino acid residues, and leading to catalytic inactivation [31]. The problem of specificity must be addressed by developing drugs that inhibit druggable targets while remaining harmless for other proteins with similar drug binding properties. Second, the problem of delivery. A therapeutic agent usually needs to reach every cancer cell to the last in order to achieve complete remission in the patient. Killing off only the bulk of cancer cells in a tumor, will eventually lead to relapse after a period of "dormancy" and formation of new tumors due to metastases spreading throughout the body which are virtually impossible to eradicate with broadband approaches targeting dividing cells only [99]. But the delivery problem also extends to more targeted approaches. For instance, many small molecule drugs are prone to poor diffusion in solid tumors because of the high interstitial pressure and the resulting sub-optimal drug concentrations within the tumor mass [108]. Targeted cancer therapy distinguishes two types of drugs used for targeting oncoproteins specifically: macromolecules and small molecule inhibitors (molecules smaller than 800 Daltons). Monoclonal antibodies are part of the repertoire of macromolecules used to bind cancer antigens that are normally not present in normal cells (e.g. fetal antigens) or present in aberrantly high number on the cell surface of transformed cells (e.g. cell surface receptors). Trastuzumab (Herceptin) is an antibody against the EGF surface receptor HER2/Neu which is overexpressed due to amplification of the *Erbb2* gene in a quarter of breast cancers [109]. It is one of the earliest examples of targeted therapy and of a drug whose efficiency relies on oncogene addiction [107]. The goal of monoclonal antibodies directed against surface onco-proteins such as surface receptors, is to stimulate an immune response towards cancer cells or inhibit downstream cell cycle activating pathways which are driven by the targeted surface receptors (e.g. VEGF, PDGFR, HER2). Small molecule inhibitors on the other hand are used to bind to and sterically inhibit mainly intra-cellular onco-proteins. The most promising targets to date are

protein kinases involved in signal transduction, such as serine/threonine kinases, cyclin dependent kinases (CDKs), DNA damage checkpoint kinases and Aurora kinases [110] due to their involvement in cell cycle regulation and their druggability. Kinase activity is relatively easy to inhibit with ATP analogues which competitively bind the ATP binding grove. The most widely known example of successful targeted therapy by a small molecule inhibitor is imatinib (Gleevec), which inhibits kinase activity of the constitutively active fusion protein BCR/ABL, found in of CML patients as the result of aberrant recombination of chromosome 9 and chromosome 22 forming the so-called "Philadelphia chromosome". Despite being a breakthrough in treatment of non-solid tumors and making BCR/ABL positive CML a manageable condition, the BCR/ABL onco-protein can acquire mutations rendering it resistant to imatinib, and leading to relapse in patients. This has been countered by the design of an improved imatinib derivative, dasatinib, with inhibitory function against most of the BCR/ABL mutants [111]. Other competitive inhibitors have been designed to bind molecular cavities, and inhibit functions other than ATP binding. For instance, nutlins block the p53 binding pocket of HDM2, thus preventing protein-protein interaction and subsequent p53 degradation. More recently, small molecule inhibitors of poly(ADP-ribose) polymerases (PARP) have been developed, which compete with NAD+ for binding and inhibit PARP to increase sensitivity to DNA damage from alkylating agents [112]. There is a wide variety of possible targets for small molecule inhibitors, so we will limit our discussion to the most promising components of the PI3K/AKT pathway and selected examples of the ubiquitin system as they are most relevant here.

1.5.1.1 Targeting the PI3K/AKT pathway

1.5.1.1.1 Targeting PI3K

Wortmannin and LY294002 both inhibit PI3K however they are isoform unspecific and have shown toxicity in animals [113]. In fact, broad spectrum PI3K inhibition is likely to result in high cytotoxicity due to involvement of PI3K in many cellular processes. For instance, the p110α isoform of the PI3K catalytic subunit is largely attributed to functions such as insulin signal transduction and AKT activation [114]. The p110β isoform was thought to have redundant function to p110α but it was shown that p110α and p110β seem to have distinct roles in cellular activity. Although involved in metabolic pathways, p110β plays a role in inflammation, vascular trafficking [115] and platelet

formation [116]. Furthermore, cells of the immune system are more dependent on the p110γ and p110δ isoforms rather than p110α [117]. Thus targeting the p110α isoform specifically could impede tumor cell growth with reduced toxicity to the immune system. It is therefore desirable to achieve isoform specificity with inhibitors, as this may help reduce unwanted side effects, and so, more specific inhibitors are being developed and are currently in phase I and II clinical trials. PI-103 for example inhibits the p110α subunit of PI3K which is essential for insulin signaling, and also inhibits mTOR as a convenient off target effect [118]. In fact, dual target PI3K inhibitors are currently entering clinical Phase I trials. Another example for such an inhibitor is BEZ235 that inhibits class I PI3K isoforms and mTOR by binding to the ATP‐binding grove [119].

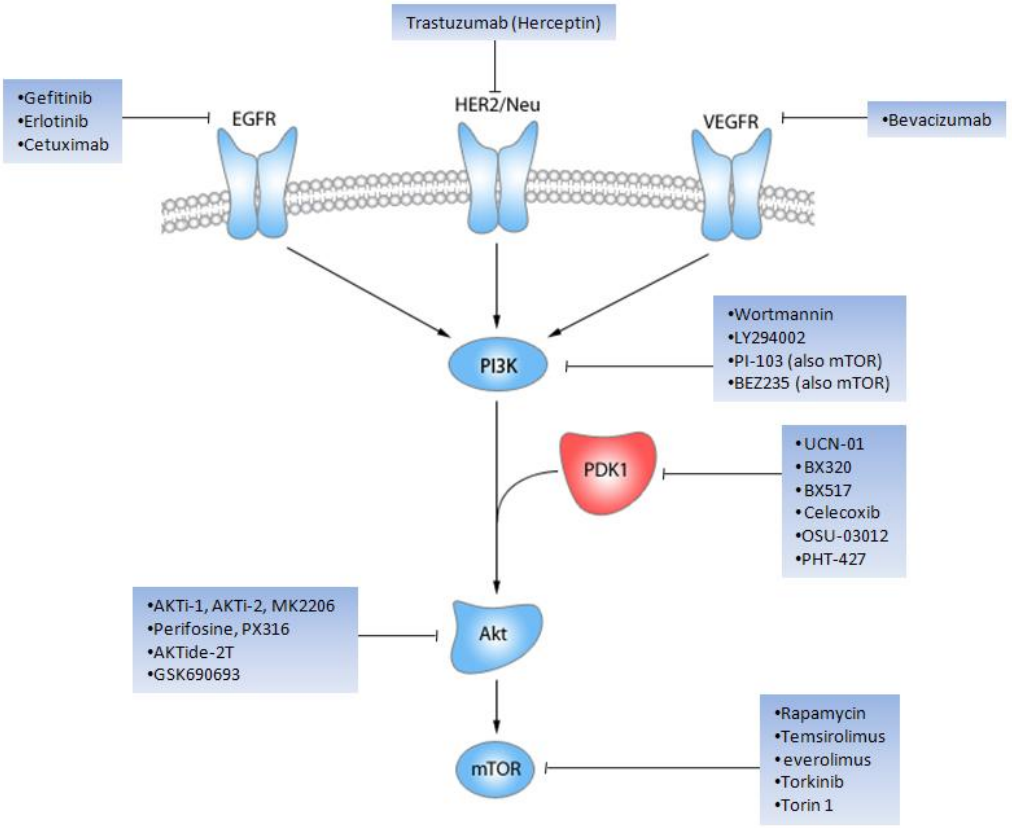


Figure 5 – Target nodes of the PI3K pathway with examples of drugs in use or in clinical development.

1.5.1.1.2 Targeting mTOR

Mammalian target of rapamycin (mTOR) is a serine/threonine kinase that exists in the form of two complexes: mTORC1 and mTORC2. mTORC1 can be activated by AKT and acts on p70S6K and 4EBP1 to promote protein synthesis while mTORC2 phosphorylates AKT on Ser473 and leads to its activation [1]. Rapamycin was the first inhibitor for mTORC1 and rapamycin analogues temsirolimus and everolimus have since been developed, with better pharmacological properties [120]. They have improved survival in patients with renal carcinoma, but low response rate in patients with breast cancer and glioma [121]. More recently the ATP competitors torkinib and torin 1 have been shown to inhibit both mTORC1 and mTORC2, and thus block the PI3K pathway more efficiently [1].

1.5.1.1.3 Targeting AKT

Inhibiting AKT, because of its role in cell proliferation, growth and anti-apoptotic signaling could impede tumor growth by induction of apoptosis or by sensitizing tumors to cytotoxic agents. A variety of agents have been successfully used to inhibit AKT *in vitro* and *in vivo*. These include small molecule ATP competitive inhibitors (e.g. GSK690693, in phase I clinical trials [1]), allosteric inhibitors (e.g. AKTi-1, AKTi-2 and MK2206 show isoform specificity for AKT1 and AKT2 and are currently in phase I against metastatic solid tumors [1]), phosphoinositide (PI) analogs aiming at inhibiting the AKT PH domain (e.g. PX316 in phase I and perifosine currently in phase II have shown inhibitory effects in tumors with high AKT activity [122]), single chain variable fragment synthetic antibodies (scFv) and pseudosubstrates (e.g. 14-mer peptide AKTide-2T which binds to the substrate binding site of the AKT1 isoform [123]) [124]. Despite partially promising results in patients, none of the compounds discussed above have completed clinical trials or have achieved complete remission in patients to this day, thus justifying the search for an alternative approach.

1.5.1.1.4 Targeting PDK1

The attractiveness of PDK1 as a potential anticancer target is linked to its ability to phosphorylate and activate a diverse set of AGC kinase members, in particular the AKT isoform 1.

But despite its importance as an essential activator of AKT, only few studies have considered PDK1 as a potential target for cancer therapy compared with studies of AKT1. Yet there is evidence that PDK1 inhibition can sensitize cells for treatment with cytostatic drugs more efficiently than inhibition of other members of the pathway and may thus be an interesting target. Tamoxifen is a prodrug that is metabolized into 4-hydroxy-tamoxifen in the liver and competes with estrogen for estrogen receptor (ER) binding [125]. It is one of the most used small molecule drugs against ER positive breast cancers, but tumors can become resistant, which leads to relapse in metastatic and some non-metastatic patients. It has been reported that RNAi mediated PDK1 knock down sensitizes MCF7 breast cancer cells to tamoxifen and to ICI 182780 *in vitro*, a drug that leads to ER degradation [126]. Furthermore, mouse mammary cells overexpressing PDK1 are more resistant to gemcitabine than cells overexpressing AKT, and human breast cancer cells are rendered more resistant to gemcitabine treatment if they have high levels of phosphorylated PDK1 as compared to high levels of phosphorylated AKT [127]. PDK1 knock down in PTEN defective glioblastoma cells reduces levels of activated AKT and inhibition of cell proliferation *in vitro* [58]. The fact that the allosteric AKT inhibitors Akti-1 and Akti-2, which rely on preventing AKT phosphorylation by PDK1, have shown success and are in clinical trials [128-129] also argues for PDK1 as a master activator and important target. Thus, PDK1 may be a superior inhibition target to AKT for sensitizing cells to treatment with cytostatic drugs like gemcitabine. Targeting PDK1 and thus indirectly curbing AKT activity may also help reducing selective pressure on tumors and thus reduce tumor resistance in transformed cells with oncogenic AKT, as opposed to targeting AKT directly. In addition, the fact that PDK1 hypomorphic mice expressing low levels of PDK1 are viable suggests that PDK1 inhibition could suffer less from side effects and toxicity [119]. Several classes of structurally diverse PDK1 inhibitors, including tetracyclics, tricyclics, azaindoles, indazoles, and indenylpyrazoles have already been identified [130]. UCN-01 is a staurosporine derivative kinase inhibitor which inhibits PDK1 activity with an IC_{50}=5nM [131] and is currently in phase I clinical trials with good results [119] but UCN-01 is a non-specific kinase inhibitor, which has the drawback of being prone to off target effects. A caveat of inhibiting the PI3K/AKT pathway, is that it can cause insulin resistance - insensitivity to insulin signals - in normal cells and lead to weight loss, increased blood sugar levels and even type 2 diabetes [119]. BX320 and BX517 are ATP competitive binders that inhibit PDK1 with better selectivity than UCN-01 and are also currently in clinical trials [119]. Celecoxib can block AKT activation in several cancers by inhibiting PDK1 kinase activity with IC_{50}=30μM [132] and is currently in phase II/III trials as a single compound or in combination with others [119]. OSU-03012 is a

derivative of celecoxib based on structural design optimization with slightly better anti-proliferative activity [133]. PHT-427 is a PH domain inhibitor originally designed to inhibit AKT PH domain but which was reported to also inhibit the PH domain of PDK1 and lead to reduction in xenograft size with minimal toxicity [134].

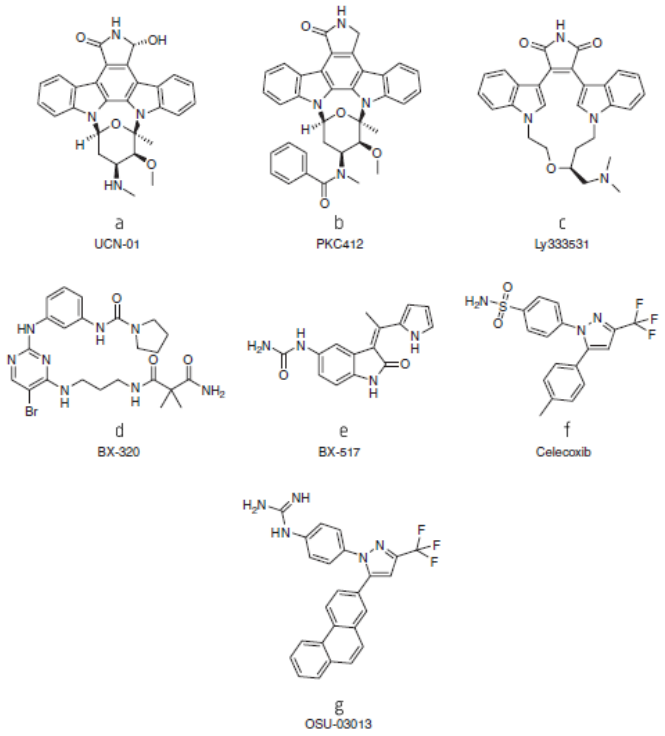


Figure 6 – Small molecule inhibitors of PDK1. Adapted from Garcia-Echeverria et al. 2008 [119]

1.5.1.2 Targeting the ubiquitin system

The ubiquitin system is involved in regulation of stability, localization and activity of many proteins by proteolytic and non-proteolytic ubiquitination and thus plays an indirect role in controlling cancer relevant cellular mechanisms like cell cycle and apoptosis. Therefore interfering with the ubiquitination state of cancer relevant proteins is considered a possible new strategy for cancer therapy.

1.5.1.2.1 Proteasome

Although proteasome malfunction itself has not been linked to cancer, it is considered a target for inhibition. The most prominent example being bortezomib (Velcade), a small molecule inhibitor of the active sites of the 20s proteasome [135]. This prevents degradation of IκB, a negative regulator of the proliferation promoting transcription factor NFκB. p53 is also stabilized by bortezomib because it cannot be degraded following poly-ubiquitination by HDM2. New proteasome inhibitors like carfilzomib, NPI-0052, CEP-18770 and argyrin A aim at blocking the proteasome in a pathway dependent manner [136]. For instance, NPI-0052 is dependent on FADD-Caspase8 cell death signaling for inhibition [137].

1.5.1.2.2 E3 ligases

Inhibiting the active site of ubiquitin ligases was initially thought to be a strategy to selectively inhibit them [136]. For example, Inhibiting overactive HDM2 could be a way of restoring p53 activity in tumors or inhibiting SKP2, a member of the SCF ligase complex responsible for ubiquitin dependent p21 and p27 degradation [138-139] could prevent G1-S transition in tumors with low p27. Another example is inhibition of E3-ligase TRAF6 in tumors with high AKT activity, as TRAF6 depleted cells with overactive AKT injected into mice have reduced tumorigenic potential [55]. Nutlins were the first drugs designed to competitively inhibit activity of a specific E3-ligase, by blocking the substrate binding grove of HDM2, and thus preventing it from ubiquitinating p53 and targeting it for degradation [140]. But nutlins have the caveat of requiring a wild type copy of p53 to be present in the cancer cell. Additionally, nutlins have off-target effects, as shown by their ability to induce cell cycle arrest despite a $p53^{-/-}$ background, in lung carcinoma cells [141]. The reverse strategy of inhibiting substrates of E3 ligases, for instance with the small molecular inhibitor RITA targeting p53 and preventing its association with HDM2, suffer from low specificity [142]. Enhancing E3 ligase activity on the other hand, despite being a much more challenging approach, could potentially be the more interesting one. For instance, enhancing Fbxwd7 and pVHL activity using agonists rather than inhibitors, would improve inhibition of cyclin E and HIF1a respectively, preventing cell proliferation and tumor vascularization. Unfortunately none of these approaches have so far yielded any satisfying results in terms of specificity and off target effects.

1.5.1.2.3 DUBs

The role of DUBs in regulating many cell cycle relevant processes makes them potential targets for therapy. However there are currently no active compounds being clinically used as drugs that selectively act on them. But examples exist showing that in principle DUBs can be targeted in cancer. One such example is UCH-L1, a DUB that has been linked to Parkinson's disease [143-144] and cancer as it is expressed in many primary lung tumors but is absent from normal lung tissue [145]. A small molecule inhibitor (an o-acyl oxime derivative of isatins) against UCH-L1 has been shown to selectively kill tumor cells *in vitro* [146-147]. However, much better understanding of DUB catalytic activity, regulation and substrate specificity, as well as large scale mapping of DUB substrates will be required before DUB inhibitors or agonists will enter clinical use.

2 Results

We have discussed the involvement of the PI3K/AKT pathway in cancer as well as the importance of the ubiquitin system as a regulator of cellular processes and as a therapy target. Here we show that PDK1, a kinase crucial for AKT activation in normal and transformed cells, is endogenously mono-ubiquitinated and perform experiments to better characterize this post translational modification. We attempt to map the site of ubiquitination on PDK1, to identify a DUB that reverses this conjugation and to describe possible functional roles of PDK1 ubiquitination. Identifying the site, role and interactors responsible for PDK1 ubiquitination will lead to a better understanding of PDK1 activity and its regulation and may yield a potential new therapeutic target upstream of AKT, in cancers with an overactive PI3K/AKT pathway.

2.1 PDK1 is mono-ubiquitinated

While performing an unrelated screen for DUBs, we have serendipitously observed a western blot signal suggesting that PDK1 may be conjugated to a ubiquitin-like modifier (Ubl) when ubiquitin was overexpressed. The main downstream target of PDK1, the serine/threonine

kinase AKT, has been shown to be K63 poly-ubiquitinated, a process required for its translocation to the plasma membrane (PM) and subsequent activation [55]. Ubiquitination of PDK1 on the other hand has not been described to our knowledge, but could potentially play a role in PDK1 regulation. In order to establish whether this post translational modification occurred endogenously, we immunoprecipitated endogenous PDK1 without overexpressing ubiquitin. As control an IgG antibody was used. Immunoprecipitation of endogenous PDK1 in HEK293T cells reproducibly co-precipitated a band running approximately 10kDa higher than endogenous PDK1, at the predicted height of mono-ubiquitinated PDK1 (Figure 7a). We were able to reproduce this result in the U2OS osteosarcoma cell line (Figure 7b). When overexpressing His-tagged PDK1 and affinity purifying it, we could enrich for both the PDK1 and ubiquitinated PDK1 forms (Figure 7c). The same holds true when only ubiquitin is overexpressed in HEK293 cells, where we observed an increase in the signal intensity of the ubiquitinated form of PDK1 (Figure 7d). Three isoforms of PDK1 exist – isoforms 2 and 3 are catalytically inactive - but interestingly we observed only one PDK1-ubiquitin band approximately 10kDa above the triple PDK1 signal, suggesting that only the largest PDK1 isoform is ubiquitinated in HEK293T and U2OS cell lines. Furthermore we were able to immunoprecipitate mono-ubiquitinated PDK1 in 6 out of 9 tested cell lines with no PDK1 or ubiquitin overexpressed (Figure 7e). These data suggest that endogenous PDK1 is indeed mono-ubiquitinated. We therefore wanted to further characterize this post translational modification by identifying the site of conjugation and elucidate the cellular function of PDK1 mono-ubiquitination.

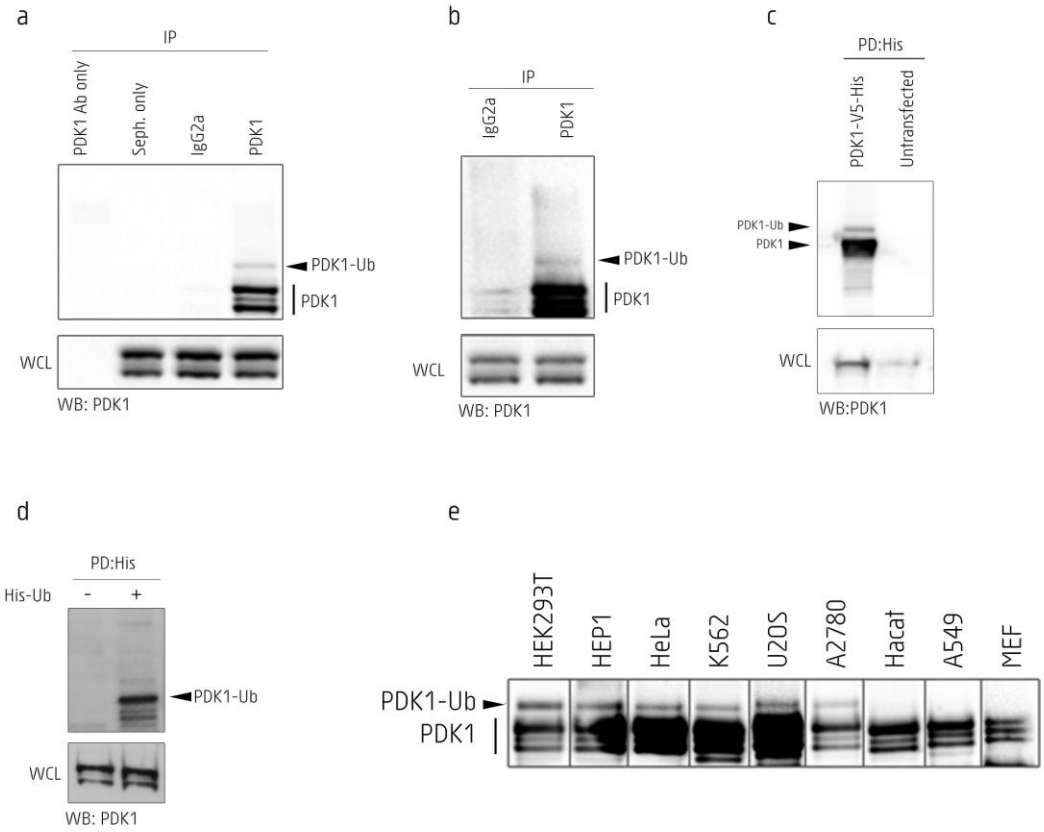


Figure 7 – PDK1 is mono-ubiquitinated.

a. Western blot showing endogenous PDK1 signal following PDK1 IP in HEK293T cells using PDK1 antibody only (lane 1), sepharose beads only (lane 2), igG2a control antibody with beads (lane 3) or PDK1 antibody with beads (lane 4). **b.** Western blot showing endogenous PDK1 signal following PDK1 IP in U2OS cells using IgG2a control antibody (lane 1) or PDK1 antibody (lane 2) **c.** Western blot showing PDK1 signal following His AP from untransfected HEK293T or HEK293T transfected with His-V5 tagged PDK1. **d.** Western blot showing PDK1 signal following His AP from untransfected HEK293T or HEK293T transfected with His- tagged ubiquitin. **e.** Western blot showing endogenous PDK1 signal following PDK1 IP from various cell lines. HEK293T (human embryonic kidney cells), HEP1 (human hepatoma), HeLa (human cervix carcinoma), K562 (human AML), U2OS (human osteocarcinoma), A2780 (human ovarian carcinoma), Hacat (human keratinocytes), A549 (alveolar basal epithelial carcinoma), MEF (mouse embryonic fibroblasts).

2.2 Mapping of PDK1 mono-ubiquitination

Mono-ubiquitination is known to regulate processes like translocation from the nucleus to the cytoplasm of transcription factors or endocytosis of membrane bound receptors and could therefore be a previously undescribed regulating modification of PDK1. PDK1 contains 38 lysine residues, each of which could potentially form an isopeptide bond with G76 on the ubiquitin molecule. Inter-species sequence comparison of PDK1 using the ClustalW algorithm shows that 12 of these lysines are conserved (Figure 8).

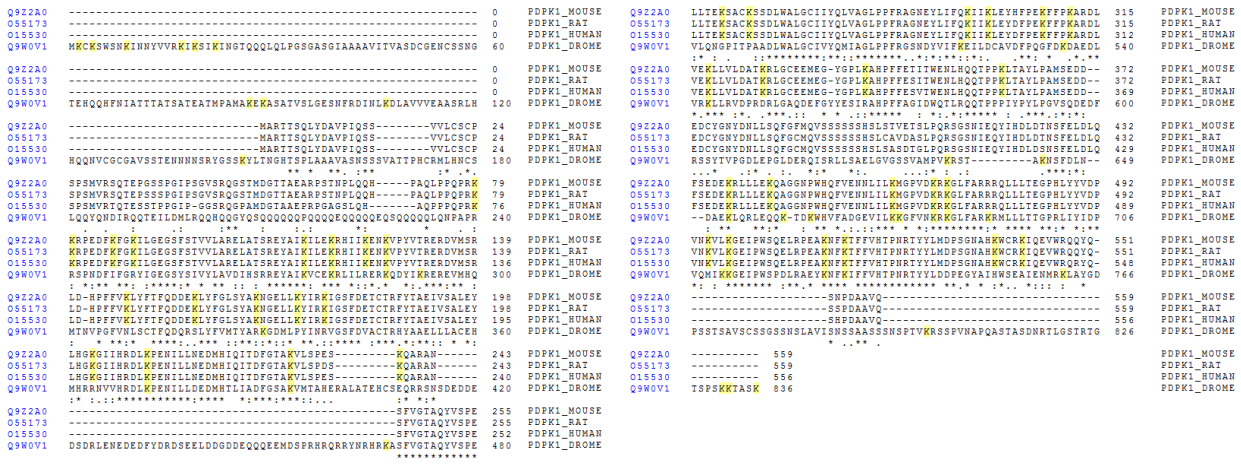


Figure 8 – Inter species sequence alignment of PDK1.

Alignment was done using the ClustalW algorithm in mouse, rat, human and fruit fly. Sequence information was obtained from UniProt. Lysine residues are highlighted in yellow. Lysines were counted as conserved when they were present in all four organisms.

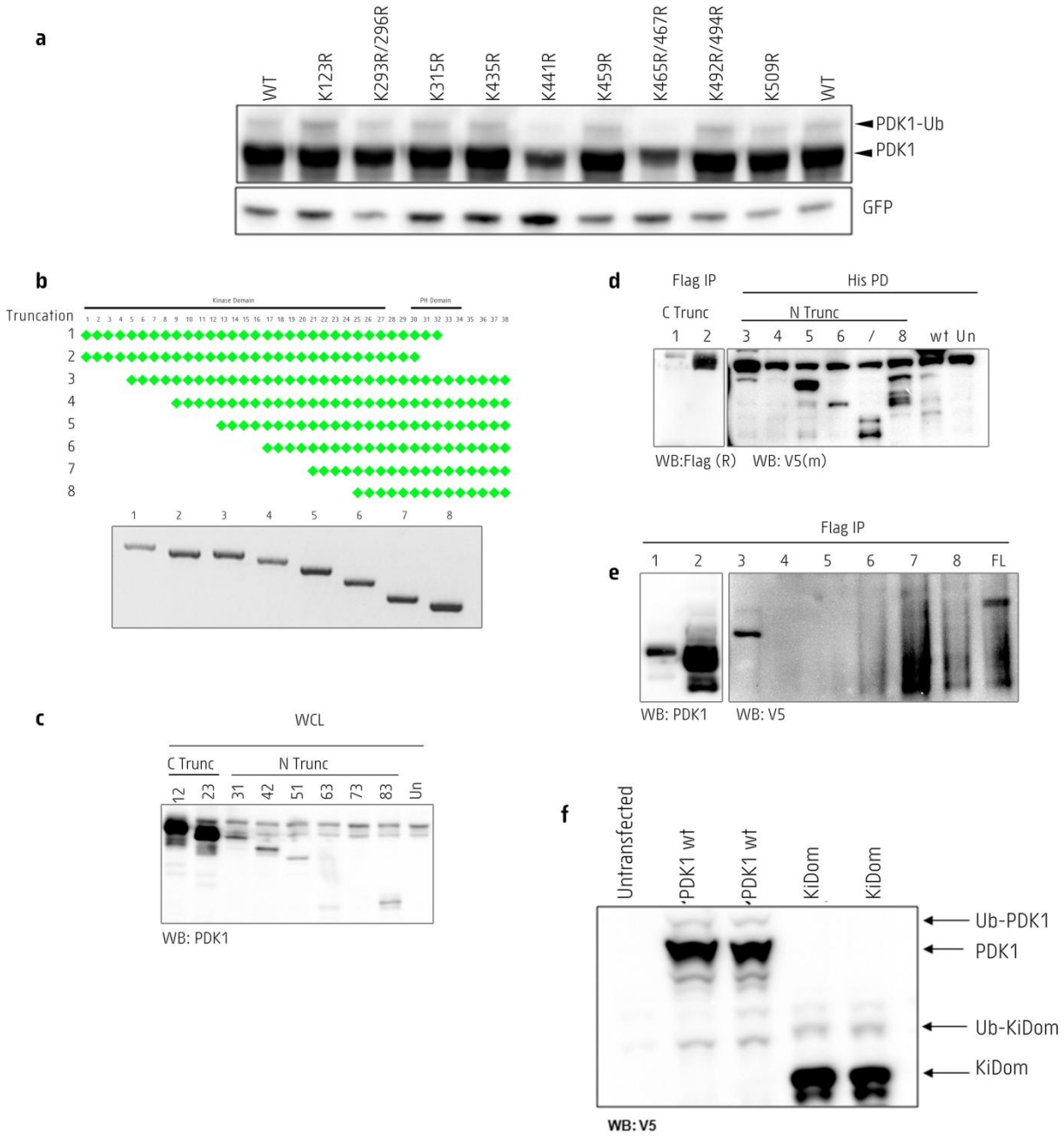


Figure 9 – Mapping the mono-ubiquitinated lysine residue.

a. Mutated conserved lysine residues of PDK1. Western blot showing PDK1 signal from HEK293T cells overexpressing PDK1 lysine mutants. GFP is co-transfected as transfection control. **b.** Truncation mutants of PDK1 (top), showing only the lysines (green) and agarose gel picture of truncated PDK1 PCR products prior to ligation into expression vectors. **c.** Whole cell lysate from HEK293T cells transfected with expression vectors encoding the PDK1 C and N terminal truncations. **d.** Western blot V5 (PDK1) and Flag (PDK1) signals after affinity purification of overexpressed truncations from HEK293T cells. **e.** Western blot showing PDK1 signal after Flag-ubiquitin affinity purification from HEK293T cells overexpressing truncations 1

and 2 and Flag-ubiquitin (lane 1 and 2). V5 signal (PDK1-Ub) from HEK293T cells transfected with truncations 3-8 and full length (FL) V5 tagged PDK1 and Flag-ubiquitin (lane 3-9). **f.** Western blot showing V5 signal (PDK1-Ub) from HEK293T cells transfected with truncations 3-7 V5 tagged PDK1 and Flag-ubiquitin. **g.** WCL western blot showing V5 signal from HEK293T cells ectopically expressing PDK1wt and PDK1 kinase domain (KiDom).

We hypothesized that if mono-ubiquitination is involved in regulating PDK1, this post translational modification is likely to be present in different species and therefore possibly located at a conserved lysine residue. We proceeded to mutate 12 conserved lysines to arginines individually and to screen for a loss of ubiquitination signal. Arginines, while having similar electrostatic properties as lysine (basic side chain), are not ubiquitin acceptor sites, and thus substitution prevents ubiquitination while minimizing disruption of protein folding or charge repartition. For lysines #293, #296 and #492, #494 which are located close together, we created double mutants to eliminate the possibility of these mutants being ubiquitinated at either residue randomly, thus effectively cutting down the number of total mutants to 10. PDK1 mutants were transfected in HEK293T cells and whole cell lysates were analyzed by western blot. Membranes were probed with anti-PDK1 antibody. None of the mutants showed a loss of ubiquitination signal, suggesting that despite being conserved, none of these residues are ubiquitinated in HEK293T cells. The K441R and K465R/K467R mutants seemed to show a loss of mono-ubiquitin signal, however they also show a weaker total PDK1 signal indicating that these mutants are not stable in the cell (Figure 9a). It is therefore unlikely that the loss of upper band signal in these mutants is due to a loss in mono-ubiquitination. As an alternative approach we created C and N terminal truncation mutants of PDK1 (Figure 9b), reasoning that eliminating lysines successively and screen for a loss of ubiquitination would allow us to narrow down the ubiquitin-conjugated lysine residue. The C-terminal and N-terminal truncation mutants were Flag and His-V5 tagged respectively, transfected in HEK293T cells and immunoprecipitated. Both C-terminal truncations were expressed, detectable and showed ubiquitination signal (Figure 9c). Of the N-terminal truncations, only the truncations #3, #5, #6 and #7 seemed to be stably expressed and detectable after pull down (Figure 9d). For reasons we could not determine, a ubiquitination signal could not be detected for these truncations except for the smallest truncation #7. We next co-overexpressed Flag-tagged ubiquitin with the truncations in HEK293T cells, immunoprecipitated with Flag antibody and probed for V5 tagged PDK1 (Figure 9e). We reasoned that overexpressing ubiquitin increases ubiquitin signal and may facilitate detection of ubiquitination on PDK1 truncations or the absence thereof. However we could only

detect full length PDK1 and N-terminal truncations #3, #5 and #7. Again, truncation #7 seemed to be ubiquitinated, despite lacking 24 of the N-terminal lysines and despite the fact that longer truncations did not appear ubiquitinated. These results do not allow a clear conclusion on the modified site on PDK1. Since truncation mutants containing large parts of PDK1 domains seemed unstable in the cell, we decided to try an alternative truncation approach, by overexpressing only the intact His tagged kinase domain or the intact PH domain in HEK293T cells. We reasoned that if domain integrity is a criterion for peptide stability, full length domains expressed separately should be more likely to be stable than mutants containing partial domains. The kinase domain however was stably expressed and showed ubiquitination signal after affinity purification (Figure 9f) yet the PH domain alone was not stably expressed. Although the lack of an expressed PH domain as control does not allow us to conclude with absolute certainty that PDK1 is ubiquitinated in the kinase domain, this result hints at the fact that it may be the case. We conclude that the modified lysine residue can be narrowed down with reasonable probability to one of the 27 lysines of the kinase domain. In order to improve mapping accuracy we next applied a tandem affinity purification/mass spectrometry (TAP-MS) approach.

2.3 Affinity purification and LC-ESI-MS/MS

As opposed to truncation experiments, mass spectrometry (MS) is a more powerful tool that offers the potential to pin-point the ubiquitinated lysine by identifying the m/z fingerprint of a peptide conjugated to a double glycine remnant (GG). Finding the ubiquitinated lysine is crucial since it will allow us to investigate the role of PDK1 mono-ubiquitination in a targeted manner. As a proof of principle, we analyzed highly purified di-ubiquitin by LC-ESI-MS/MS and were able to detect ubiquitin and the attached GG remnant (data not shown); indicating that in principle mono-ubiquitination and its site of conjugation can be detected. However, the bottleneck to a conclusive MS result when analyzing complex mixtures such as whole cell lysates is the preliminary step of affinity purification. The ubiquitinated protein must be purified as stringently as possible to avoid contamination by other peptides and thus reduce noise in subsequent fragment ion detection. In order to find an affinity purification protocol that would allow us to efficiently purify the ubiquitinated form of PDK1, we chose to use three different approaches to isolate ubiquitinated PDK1 from whole cell lysate (Figure 10). Samples from all three affinity purification approaches were verified via western blot, to assess whether they could be used for silver staining, band excision, in gel tryptic digestion and mass spectrometry analysis. In our first approach (Figure 10a) we created a plasmid vector to overexpress His-tagged PDK1 in HEK293T cells, which we affinity purified with Ni coated agarose beads, and were able to detect in its ubiquitinated form in the eluate via western blot (Figure 11a). Unfortunately this approach did not allow a clear detection and excision of the PDK1-Ubiquitin band by silver stain. This may be due to the lack of specificity of the approach. Western blot allows very specific detection of proteins in complex mixtures by use of specific antibodies, but whole protein staining methods like silver staining require a much higher level of preliminary separation to unambiguously visualize individual bands of interest.

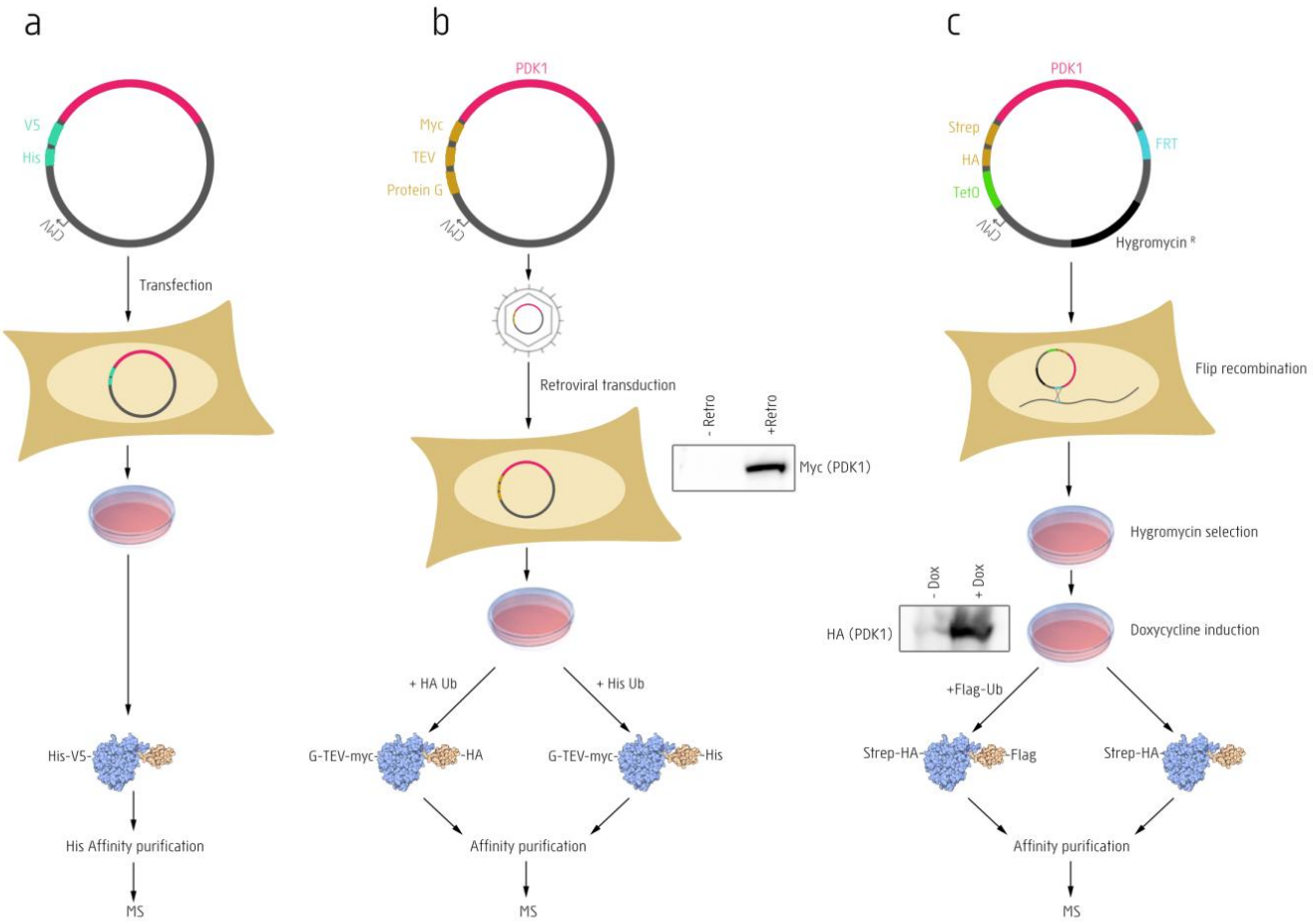


Figure 10 – Affinity purification (AP) approaches to isolate PDK1-Ubiquitin for MS/MS.

a. His-tagged PDK1 is transfecfted in HEK293T cells for AP. **b.** Protein G and Myc tagged PDK1 is retrovirally introduced (infection) in HEK293T cells. Cells are then transfected with His or HA tagged ubiquitin before AP. Panel on the right is western blot showing Myc signal from cells infected and uninfected with retrovirus vector. **c.** Flp-In™ T-REx™ 293 Cells were flip recombined with PDK1-SH construct to create HEK293TSH cell line expressing PDK1-SH in a Dox inducible manner. AP was then done using a two step approach (Strep-tactin coated beads followed by HA-IP) or a three step approach using Strep-Tactin/HA/Flag AP.

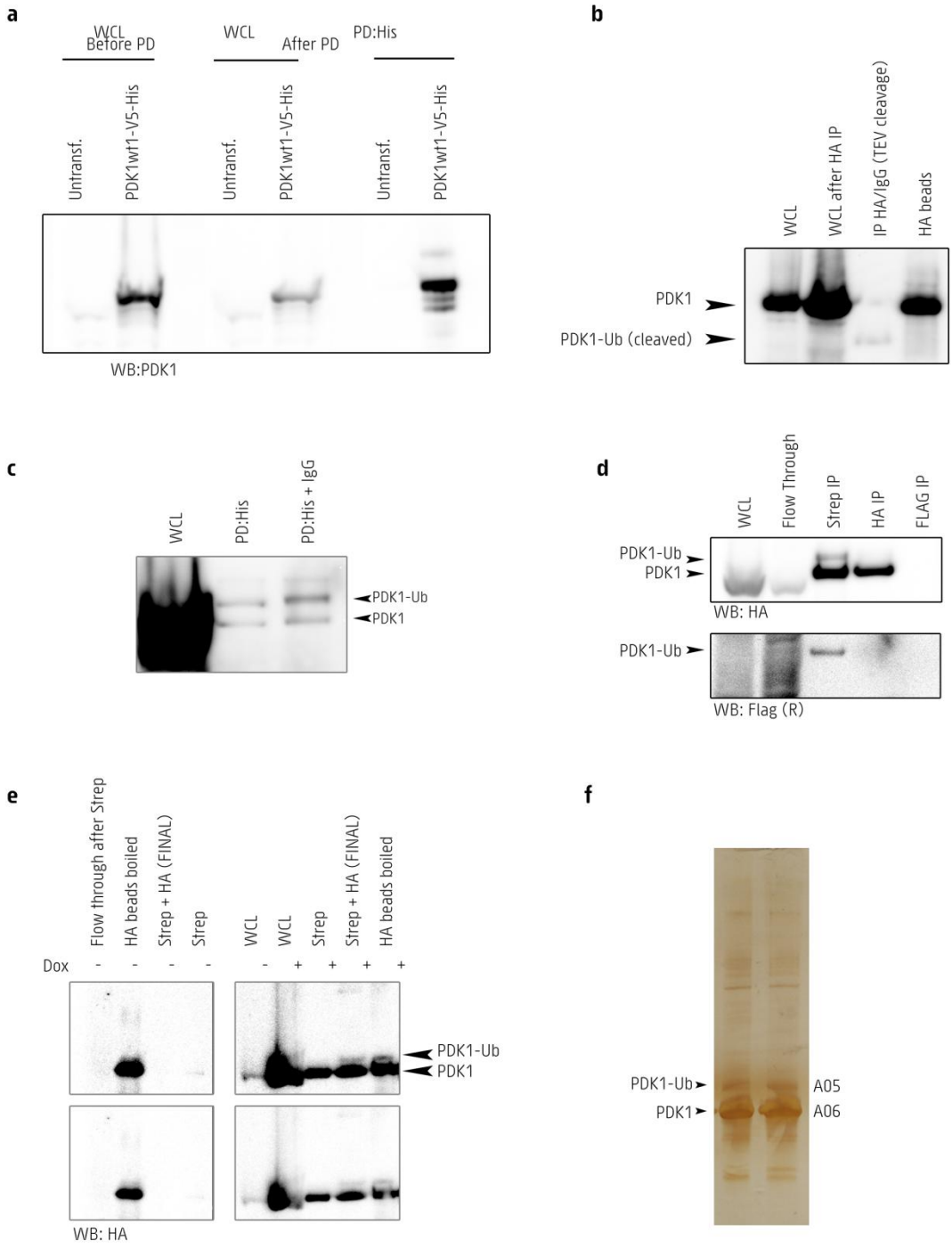


Figure 11 – Affinity purification of ubiquitinated PDK1 and silver staining.

a. Western blot PDK1 signal from the first AP approach. Cells are transfected with His tagged PDK1 and AP is done with Ni-coated beads with affinity to the His-tag. WCL, whole cell lysate, Untransf, untransfected control cells. **b.** PDK1 signal from TAP followed by TEV cleavage. First AP is an immunoprecipitation of HA-tagged ubiquitin. Second AP is an immunoprecipitation of Protein G. Lane 1 and 2 show PDK1 signal from the WCL before and after HA immunoprecipitation.

Lane 3 shows cleaved PDK1 band running lower due to the loss of its protein G tag. Lane 4 is the PDK1 signal from boiled HA-antibody coated beads after TEV cleavage. **c.** Western blot PDK1 signal from second TAP approach. First step is a His AP followed by immunoprecipitation of Protein G **d.** Western blot HA (PDK1) and Flag (Ub-PDK1) signals from Triple-AP in HEK293TSH cells transfected with Flag-Ub. First step is a StrepIII AP (for PDK1), followed HA IP (for PDK1) and Flag IP (for ubiquitin). **e.** Western blot HA (PDK1) signal from Strep-HA AP in HEK293TSH cells overexpressing ubiquitin. **f.** Silver stain gel of lane 9 from (e). Bands A05 (PDK1-ub) and A06 (PDK1) were excised and analyzed by LC-ESI-MS/MS.

To increase pull down specificity we therefore chose a two-step protocol next. Tandem affinity purification (TAP) is a protocol that has been used to isolate proteins and protein complexes for subsequent MS analysis [148] and should therefore be usable for isolation of ubiquitinated PDK1 for MS. In our second approach (Figure 10b) we cloned wild type PDK1 in a TAP vector, under the control of a strong promotor (CMV), in frame with a myc tag, a tobacco etch virus cleavage sequence (TEV), which can selectively be cleaved by a TEV protease (Nuclear Inclusion A protein "NIa"), and a protein G sequence. The PDK1-proteinG construct was stably expressed in HEK293T cells after transduction with a retroviral vector and 72h puromycin selection. We refer to this cell line as HEK-TAP. We then transfected HEK-TAP cells with His or hemagglutinin (HA) tagged ubiquitin. The ubiquitinated fraction of the lysate was then enriched by nickel-affinity purification or using antibody against the HA epitope. In a second step we immunoprecipitated PDK1 from the ubiquitinated fraction with an anti-proteinG antibody. HA/IgG purification allowed PDK1 isolation and subsequent TEV cleavage mediated elution also worked as indicated by the PDK1-Ub band running lower than non-ubiquitinated PDK1 (Figure 11b). However not enough material could be isolated by this method and no PDK1 or ubiquitin peptides were detected by mass spectrometry. Loading the eluted anti-HA antibody coated agarose beads to control for the quality of elution revealed that significant amounts of PDK1 are seemingly aspecifically attached to the beads. Additionally, as lane 2 and 3 suggest, depletion of PDK1 was not efficient. Whole cell lysate (WCL) after anti-HA AP still shows a strong PDK1 signal and the cleaved PDK1-Ubiquitin is faint. Repeating the experiment with a different lysis buffer did not resolve the problem. Replacing the HA immunoprecipitation step by a Ni/His AP (Figure 11c) allowed us to precipitate PDK1 in good amounts but at the cost of specificity, as the isolate did not reveal clean excisable bands after silver staining. As a third approach (Figure 10) we decided to use alternative affinity tags, in order to circumvent the specificity problem while obtaining good enrichment for PDK1-Ubiquitin. To this end

a Flip-in recombination system was used to create a cell line (HEK293TSH) stably and inducibly expressing StrepIII-HA-tagged PDK1. This system has been developed based on standard TAP protocols to increase throughput and yield in large scale proteomics studies [149]. The advantage of this approach is that PDK1 is stably expressed at comparable rates in different experiments after doxycycline induction, allows culture of cells in larger amounts for lysis and that the StrepIII-tag interacts strongly with the streptavidin derivative Strep-tactin [150]. We cloned PDK1 wild type cDNA sequence into an expression vector containing StrepIII and HA sequences using the gateway cloning system, which was co-transfected into HEK293 cells with a plasmid expressing Flip-recombinase. Cells were then transfected with Flag-ubiquitin or left untransfected. Affinity purification was done, in one case, using strep-tactin sepharose followed by immunoprecipitation with HA antibody - thus isolating PDK1 in its ubiquitinated and unubiquitinated form - and subsequent immunoprecipitation with Flag antibody to purify the ubiquitinated fraction of PDK1 from this pool. In the other case, we only precipitated with Strep-tactin and HA antibody, relying on PAGE for separation of unmodified and ubiquitin-conjugated PDK1. The signal from ubiquitinated PDK1 was lost in the StrepIII/HA/Flag after the HA immunoprecipitation step, and PDK1 signal was lost following Flag immunoprecipitation (Figure 11d). It is unclear why the ubiquitinated PDK1 signal was lost after HA precipitation, but it could be due to the insufficient amount of biological starting material. When we repeated the last experiment with double the amount of cells and without Flag immunoprecipitation, we were able to isolate both PDK1 and ubiquitinated PDK1 (Figure 11e). Elution however was not optimal, as can be seen when comparing lane 8 and lane 9, where boiled HA beads gave a stronger PDK1 signal than the eluate. However the overall purification using this protocol returned a silver stain signal that was clean enough for band excision and subsequent MS analysis. Bands A05 and A06 were excised (Figure 11f), digested in gel with trypsin and analyzed by LC-ESI-MS/MS. A05 is predicted to be the ubiquitinated form of ubiquitin and A06 unmodified PDK1. Figure 12 shows the MASCOT readout of the database matched fragment peptides, from two MS runs of each digest, respectively using inclusion list 1 or list 2. The inclusion lists include predicted fragment ions of PDK1, with priority on fragment ions resulting from tryptic cleavage C-terminally of lysines rather than arginines. PDK1 was detected in A05 and A06 and ubiquitin was detected in A05 with both inclusion lists and in A06 with only one inclusion list. Detection of ubiquitin alone is a good indicator that affinity purification worked and further supports our finding that PDK1 is ubiquitinated, but does not allow to conclude on the ubiquitination site of PDK1. In fact, no m/z fingerprint of GG-coupled PDK1 was detected and thus it is not possible to conclude where the

ubiquitin signal stems from or what lysine residue is conjugated to the GG remnant. It may also be that the predicted m/z for GG-bound PDK1 was not detected due to low sequence coverage (highest sequence coverage was 44% for PDK1 and 50% for ubiquitin). If the GG-remnant lies outside the covered sequence, it is not detected in this analysis, requiring more replicates of this assay to be run.

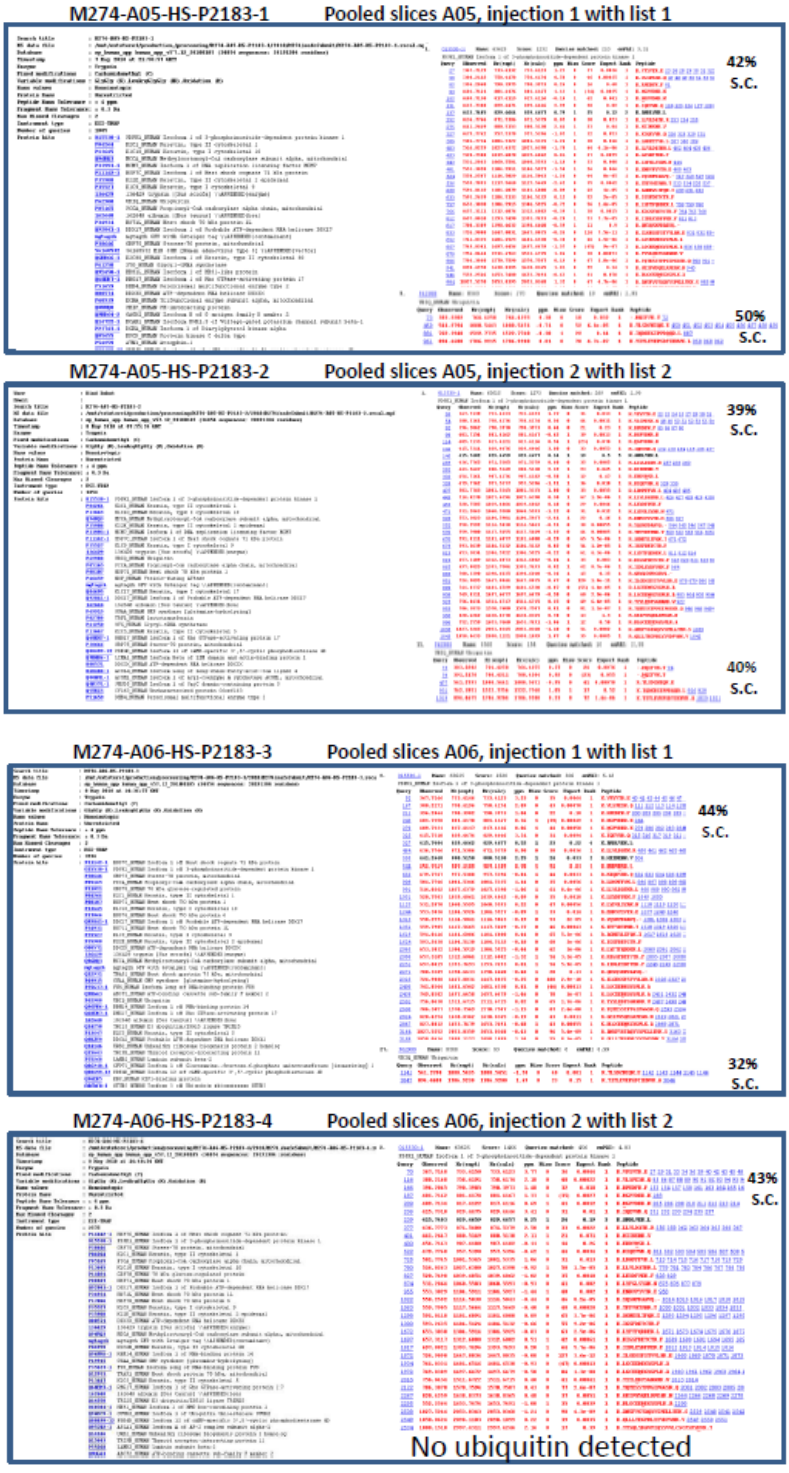


Figure 12 – MASCOT readout from MS/MS analysis of A05 and A06 trypsin digested bands.

Top panel, A05. Bottom panel, A06. S.C., sequence coverage. The left side of each panel shows the MASCOT raw data for potentially identified proteins according to peptide fingerprints. The right side (red) shows peptides that were matches for PDK1 (top) and ubiquitin (bottom).

2.4 Loss of function RNAi screen for a DUB acting on PDK1

We proceeded to screen two libraries of DUBs for a deubiquitinase that would act on PDK1 and reverse its ubiquitination. Identifying interactors of PDK1 that might play a role in its post translational modification - DUBs specifically - may shed light on the role of PDK1 mono-ubiquitination. Additionally, on the background of therapeutically targeting PDK1 in cancers, a DUB could provide a new angle of attack. To perform the loss of function RNAi screen we used a shRNA library mediating stable RNA interference against 50 known DUBs established by T.R. Brummelkamp and S.M. Nijman [95] and an siRNA Smart pool library by Dharmacon targeting 100 genes. The shRNA library consists of the pSUPER vector system, where each vector encodes a synthetic 19bp oligonucleotide under the control of the mammalian RNA Pol III promoter. The shRNA library we used contains pools of 4 knock down vectors against each targeted DUB for increased knock down efficiency. Functionality of this library was verified by RT-PCR for the shCYLD and shUSP1 constructs (Figure 13c) and by western blot (Figure 13e). Functionality of the Dharmacon siRNA library was verified by RT-PCR for the USP3, USP32, USP5, USP7 and USP9x constructs (Figure 13d). HEKTAP cells were seeded in 6 well plates and each well transfected with GFP as a transfection control and a siRNA or shRNA knockdown vector pool directed against one specific DUB. Two days after transfection cells were lysed, protein G tagged PDK1 was immunoprecipitated using anti-G antibody, analyzed by PAGE and western blot and the precipitate was monitored for an increase in the PDK1-ubiquitin signal (Figure 13a). An increase in the ubiquitinated form of PDK1 would suggest that the knocked-down DUB normally acts on PDK1 to deubiquitinate it. A representative example of a western blot read-out is given in (Figure 13b). However no significant increase in the ubiquitinated fraction was detected and thus no DUB was identified for PDK1.

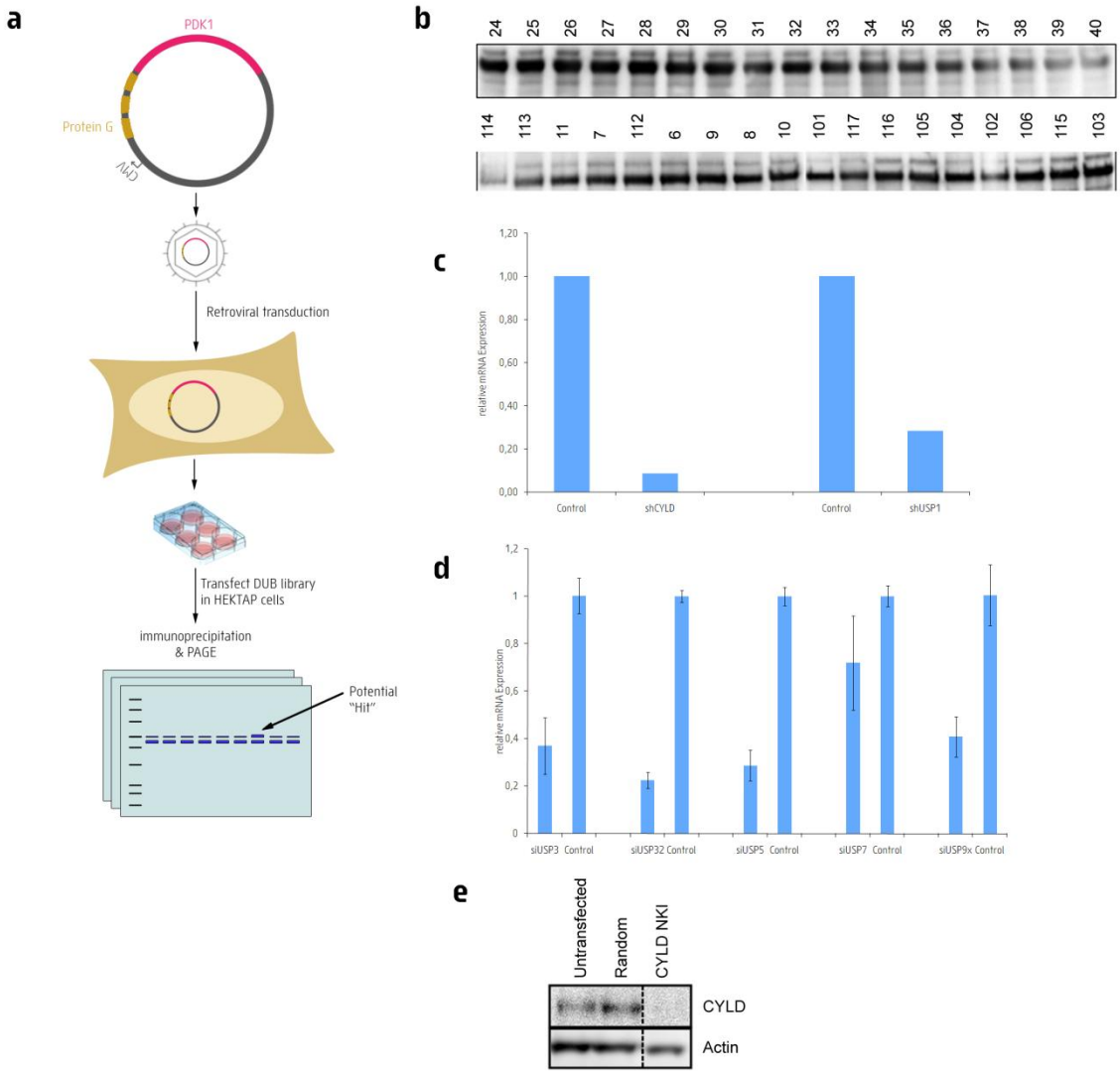


Figure 13 – Loss of function screen for a DUB acting on PDK1.

a. Workflow of loss of function DUB screen. HEK293T cells are infected with a retroviral vector with Protein-G tagged PDK1. Cells are then transfected with shRNA or siRNA libraries against DUBs, and lysates are screened by Western blot **b.** Example of western blot screen showing PDK1 signal after protein G (PDK1) IP from HEKTAP cells transfected with DUB knock-down library. **c.** RT-PCR for CYLD and USP1, in untransfected cells (control) and cells transfected with shRNA. **d.** RT-PCR for USP3, USP32, USP5, USP7 and USP9x in untransfected cells (control) and cells transfected with siRNA. Three technical replicates. **e.** Western blot from cells untransfected, transfected with random shRNA and shRNA against CYLD.

2.5 Functional characterization of PDK1 mono-ubiquitination

Understanding the functional role of PDK1 ubiquitination could yield important insight into its role in regulating PDK1 activity, which may also be used to target PDK1 therapeutically. More work is needed to identify the lysine residue that is modified by ubiquitin and until this information is available it is difficult to accurately generate PDK1 mutants lacking ubiquitination or physiologically correct PDK1-ubiquitin fusion mutants, which are essential to further study the function of ubiquitination. Despite lacking this knowledge, we can nevertheless perform experiments that will give an idea of the role of ubiquitination. We first investigated if attachment of a mono-ubiquitin influenced PDK1 localization. Although to our knowledge there are no cytoplasmic kinases known to relocalize upon mono-ubiquitination, examples exist of other proteins such as the transcription factor p53 which relocalize to the cytoplasm upon mono-ubiquitination. We created a V5 tagged PDK1-ubiquitin fusion protein which we expressed in U2OS cells. We then performed immunostaining with an anti-V5 antibody and a fluorescently labeled secondary antibody, which was analyzed by confocal laser microscopy. Transfection efficiency was controlled by co-transfecting GFP and detection efficiency of our construct was controlled by transfecting V5-labelled PDK1 with no ubiquitin fusion. The fluorescence levels of the PDK1-V5-Ub fusion and PDK1-V5 were comparable, as were transfection levels. We did however not observe any differences in localization to the plasma membrane or the cytoplasm between the two constructs (Figure 14a). Our observation suggests that this type of ubiquitination does not influence localization of PDK1. Ubiquitin binding domains on effector proteins, which are responsible for recognition of ubiquitinated proteins are diverse and specific to different types of ubiquitination [81]. We can therefore not exclude the possibility that the artificial PDK1-Ubiquitin fusion does not accurately represent the normal physiological setup within the cell. The PDK1-ubiquitin fusion may not be recognized by the normal effectors and may thus not show a different repartition within the cell than the control. To circumvent the problem of an artificial PDK1-ubiqutin fusion, we next fractionated the cell lysate from HEK293T cells transfected with PDK1-V5, and immunoprecipitated PDK1-V5 with a V5 antibody. PDK1 was found in all three fractions (cytoplasmic, membrane and nuclear), as was the ubiquitinated form of PDK1 (Figure 14b). This suggests once more that mono-ubiquitinated PDK1 does not show a preference for localization to either one of these cellular compartments. Since we haven't found mono-ubiquitin to influence localization so far, we wanted to investigate whether it played a role in pathway activation. The PI3K/AKT pathway is involved in

insulin signaling, leading to phosphorylation of PDK1, which in turn phosphorylates AKT. We therefore immunoprecipitated endogenous PDK1 from HEK293T cells which were starved over night and then left starving or grown in medium complemented with insulin or with LY294003 - a PI3K inhibitor. Insulin was washed out and cells were harvested at different time points after washout. No difference in PDK1 ubiquitination levels was observed between stimulated and unstimulated cells (Figure 14c and d). This seems to indicate that PDK1 ubiquitination is not affected by activation of the PI3K/AKT pathway and conversely, that ubiquitination does not influence activation of downstream components. As suggested in Figure 9, we suspect that the mono-ubiquitinated lysine is located in the kinase domain of PDK1. To understand if ubiquitination of the kinase domain is important for PDK1 catalytic activity or catalytic activity is a prerequisite for ubiquitination, we created a catalytically inactive K110R mutant of PDK1. Lysine 110 is located in the catalytic center and crucial for wild type PDK1 kinase activity [26]. We overexpressed the K110R mutant and wild type PDK1 as control, in HEK293T cells and immunoprecipitated using a PDK1 specific antibody. Both the catalytically inactive and wild type PDK1 were mono-ubiquitinated (Figure 14e), which suggests this modifications is neither crucial nor dependent on catalytic activity. It has been suggested that phosphorylation of AKT by PDK1 is not strictly dependent on PIP_3 binding via the PH domain of either protein [30], however binding PIP_3 is thought to bring AKT and PDK1 into close enough contact to favor AKT phosphorylation and is therefore one of the regulators of PI3K/AKT pathway activation. We thus wanted to examine if PDK1 mono-ubiquitination played a role in PDK1 binding to PIP_3. We overexpressed wild type PDK1 and the PDK1 K465E PH domain mutant which does not bind PIP_3 [151] in HEK293T cells and affinity purified with PIP_3-coated agarose beads. Non-coated agarose beads were used as control. As expected, the K465E mutant did not bind to PIP_3 beads but wild type PDK1 was precipitated in both its ubiquitinated and non-ubiquitinated forms (Figure 14f). This suggests that mono-ubiquitination is not necessary for PDK1 binding to PIP_3 as levels of ubiquitinated PDK1 are comparable in the whole cell lysate and the precipitate. Conversely it seems that PDK1 ubiquitination does not disrupt its ability to bind to PIP_3. Overall our results suggest that mono-ubiquitination does not affect and is not essential for PDK1 localization, PIP_3 binding or kinase activity and that ubiquitination levels do not change with PI3K/AKT pathway activation.

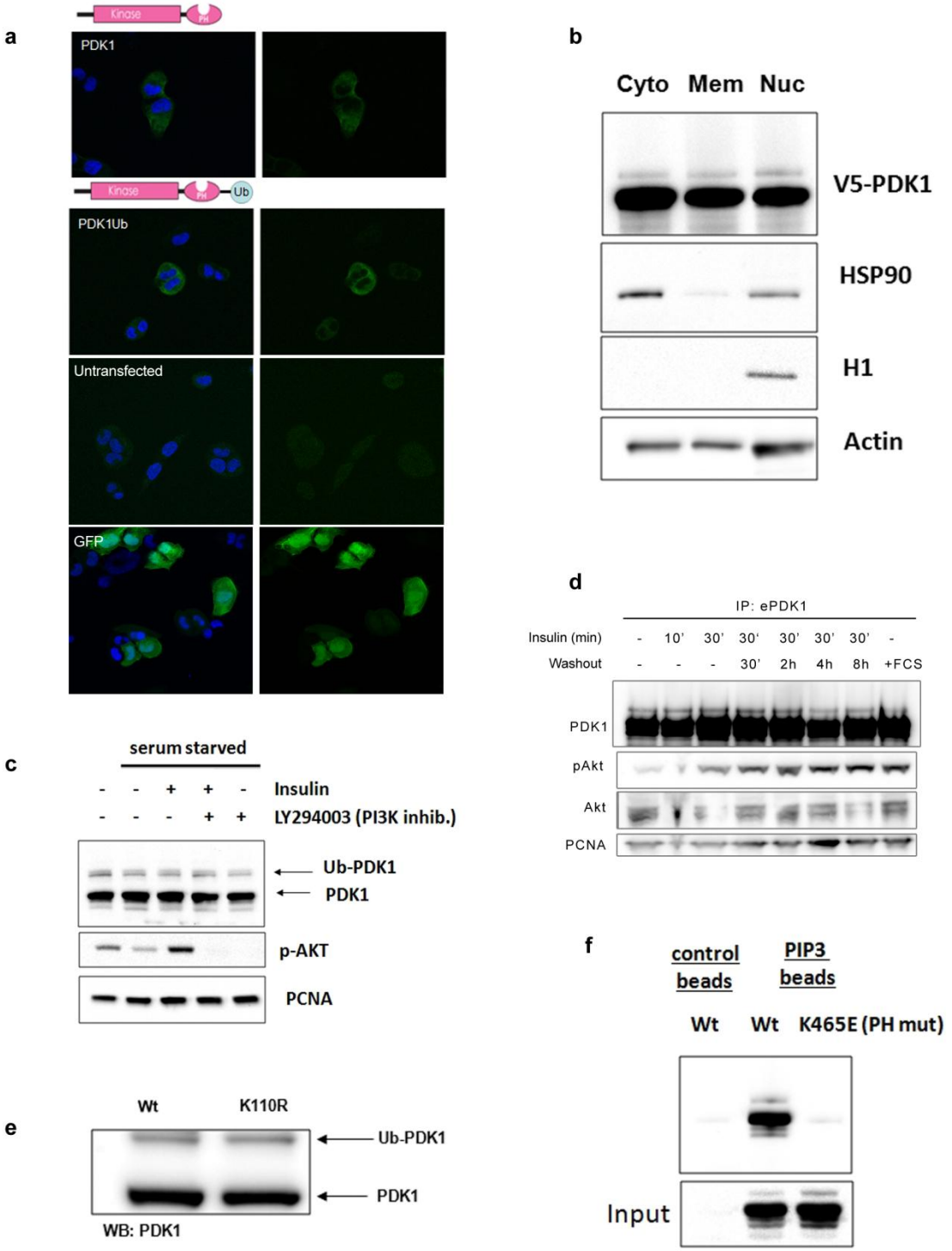


Figure 14 – Functional characterization of PDK1 mono-ubiquitination.

a. Immunofluorescence visualized by confocal laser microscopy. HEK293T cells transfected with PDK1-V5, PDK1-V5-Ubiquitination fusion, or GFP. Cells are stained with DAPI and with V5 antibody coupled to alexa fluor secondary antibody. **b.** Western blot with V5 (PDK1), Histone 1 (H1), HSP90 and actin (loading control) signal from cytoplasm, membrane, nuclear fractions in HEK293T cells. PDK1 was immunoprecipitated with V5 antibody. **c.** Western blot for endogenous immunoprecipitated PDK1, activated AKT (pAKT) and PCNA as loading control in unstimulated HEK293T or HEK293T

stimulated with insulin, with or without LY294003 PI3K inhibitor for 8h. **d.** Time course. Western blot for endogenous immunoprecipitated PDK1, AKT, activated AKT (pAKT) and PCNA as loading control in HEK293T stimulated with insulin. Insulin was washed out and cells were harvested after the times indicated. **e.** Western blot. IP in HEK293T cells ectopically expressing wt or K110R (catalytically inactive) PDK1. **f.** Western blot showing signal from AP using PIP_3 coated beads, in HEK293T cells transfected with wild type PDK1 or PDK1 K465E (PIP_3 binding deficient) mutant.

3 Discussion

3.1 Mapping the lysine residue

We were able to show for the first time that PDK1 is endogenously mono-ubiquitinated in HEK293T cells *in vivo* and were also able to detect this ubiquitination via western blot. Our results suggest that this is also the case in HEP1, HeLa, K562, U2OS and A2780 cells. Our MS data also supports this finding, since ubiquitin peptides were detected in affinity purified PDK1 fraction of HEK293TSH WCL. To perform further functional characterization identifying the ubiquitinated lysine residue is crucial because it will allow experiments in a more focused and targeted manner to elucidate the role of mono-ubiquitination of PDK1. Mutating 14 conserved lysines to arginines did not cause loss of PDK1 mono-ubiquitination, indicating that none of these are likely to be used for mono-ubiquitin conjugation endogenously in HEK293 cells. Efforts to map the post translational modification site by truncating PDK1 did not yield a conclusive result due to the fact that most of the truncations were not stably expressed by the transfected cells. The lack of detectable middle-sized truncations could be due to the fact that most of our truncations form peptides that are labeled as misfolded proteins and degraded (Figure 7b). This hypothesis is supported by the fact that we can detect long truncations such as C-terminal #1, #2 and N-terminal #3, whereas smaller truncations such as #4, #5, #6 are lost to degradation. Lager truncations which lack only a few lysines may resemble full length PDK1 in their secondary structure close enough to pass as well folded proteins in the cell, and thus escape degradation, whilst shorter truncation may be too aberrant to do so. However, the fact that the smallest truncation #7 appears to be well expressed, and what's more, ubiquitinated, challenges the above explanation. This raises two questions: first, why is #7 stably expressed when larger truncations, which should have a less aberrant secondary structure are not? A possible answer to this is the fact that #7 has lost most of its kinase domain, and consists essentially only of a PH domain with a few amino acids reaching into the kinase domain. This may allow #7 to fold in such a way as to leave the peptide catalytically inactive but unscathed by quality control mechanisms. Larger truncations on the other hand, while also containing an intact PH domain, retain much larger portions of their kinase domain sequence. It may form folding intermediates which are recognized by quality control mechanisms such as chaperones and marked for ER-associated degradation (ERAD). However, the fact that we failed to see expression of the PH domain alone, argues against the idea of domain integrity being the crucial factor for overall peptide

stability. Secondly, the above raises the question if we can use this information to narrow down the ubiquitinated lysine. We were able to stably overexpress the kinase domain alone in HEK293T and found it to be ubiquitinated. This supports the fact that mono-ubiquitin may be conjugated to the kinase domain in endogenous PDK1. We furthermore established that ubiquitination does not depend on and is not required for PDK1 kinase activity. We suggest that the kinase domain bears the modification in wild type PDK1, a claim that is also supported by statistical probability, considering that the kinase domain contains 27 lysines versus only 5 in the PH domain and 5 in intermediate regions. Assuming that #7 is indeed ubiquitinated, we have no good explanation as to why we fail to see ubiquitination in larger and stably expressed truncations like #3 as well. A possible solution to circumvent the problem of truncation mutant instability could be to create substitution mutants by starting with a PDK1 mutant lacking all lysines and sequentially mutating arginines back to lysines, instead of eliminating lysines by truncating them off, and monitoring for a return of the ubiquitination signal. Alternatively, one could create lysine to arginine mutants for all the 38 lysines individually and screen for ubiquitination loss, however this would not be cost and time effective. This approach has an additional caveat since it cannot discriminate between situations where conjugation of the mono-ubiquitin occurs interchangeably at two or more lysines. If PDK1 mono-ubiquitination was to occur at two or more different lysines randomly or with certain preference, mutating either lysine residue alone would not allow us to see a loss of ubiquitination even if one of the canonical ubiquitin-accepting sites on PDK1 was mutated.

3.2 MS/MS: potential and limitations

Alternative approaches for identifying the ubiquitin conjugated lysine residue by AP-MS (Figure 10) were performed and we were able to isolate ubiquitinated PDK1 from HEK293SH cells with high enough efficiency and with little enough contamination by other proteins to visualize it by silver staining. MS/MS detected a ubiquitin fingerprint predominantly in the excised bands where expected. However predicted m/z fingerprint scores for GG remnant conjugated PDK1 peptides were not detected, and thus no ubiquitinated lysine residue was identified. A reason for this could be low sequence coverage i.e. “the fraction of the amino acid sequence that is represented by peptides in the peptide mass map” [152]. While low sequence coverage may suffice to identify the overall presence of a given protein with reasonable certainty in a sample, identification of PTMs is

by definition dependent on nearly complete sequence coverage to have a good probability of detection. Only if the site of modification is included in the mass fingerprints obtained by MS/MS, can it be detected and mapped. Several factors can be responsible for low coverage, such as a wide range in concentrations in a mixture of proteins or varying ESI efficiency, resulting in different ion intensities despite comparable concentrations of analytes [153]. Indeed, the PDK1-Ubiquitin band is weaker by a factor of around 50 than non-ubiquitinated PDK1 and thus small amounts of contaminants can create noise that easily impacts MS detection efficiency of the PTM. Another reason for lack of detection could be the choice of our inclusion lists. Because of technical limitations, of 121 peptides predicted *in silico* only 35 and 34 could be used in inclusion lists 1 and 2 respectively, based on priority of lysines located within PDK1 active domains. It can be speculated that a new run of the sample with alternative inclusion lists may shift the sequence coverage to include the modified lysine residue. While AP-MS is currently the most powerful tool for the identification of PTMs, it also suffers from some general caveats and limitations which we will discuss below. First, it has been shown that proteins that are ubiquitinated can also simultaneously be conjugated to other ubiquitin like (Ubl) modifiers, which can leave GG remnants just like ubiquitin after tryptic digestion. Examples for this are HDM2 which is modified by SUMO, ubiquitin and NEDD8 [154]. Indeed NEDD8, Rub1 and ISG15 all leave GG remnants. Since the detection of ubiquitinated residues depends on mass-shift caused by these remnants, loss of modifier identity can be a problem. A solution to this problem is the use of tags for affinity purification on the modifier that is being studied. Strep-HA purification has allowed sufficient enrichment for ubiquitinated PDK1 but none of our approaches that made use of tagged ubiquitin have allowed isolation of a sample analyzable by MS. Although we can be reasonably confident that the modifying moiety is ubiquitin, certainty will be obtained by repeating the assay using a tagged form of ubiquitin for isolation. Another known issue is the use of Iodoacetamide (IA), an alkylating agent used in standard protein digestion protocols to block cysteine residues, forms adducts on peptides which look like GG remnants in MS and thus create false positives [155]. We therefore avoided use of IA in our approaches. Second it has been reported that tagged modifiers have altered conjugation specificity. A study showed that Flag-tagged SUMO for example was conjugated at lower levels to proteins [156]. This could be a reason why we were unable to isolate ubiquitinated PDK1 by using epitope tagged ubiquitin. A solution to this could be the use of several different tags, since it is less likely that all of them would negatively impact conjugation. A quantitative preliminary screen to determine which of such tagged forms of ubiquitin is most readily conjugated may prove

challenging. An ELISA assay detecting ubiquitin levels on immobilized PDK1 could provide quantitative information. Third, Ubl modified proteins can be less abundant than their non-modified counterparts and the turnover can be high, with rapid conjugation and deconjugation [157]. This is the case here, where ubiquitinated PDK1 levels are significantly lower than unmodified PDK1. This makes the window of possible isolation small and limits the maximum possible yield, which is critical for MS based analysis. In the case of poly-ubiquitination, this may be counteracted by the use of proteasome inhibitors such as MG132, chemically modified non-deconjugable Ubls in the lysis buffer [158-159] or co-transfection of ubiquitin mutants such as G76A which forms chains that cannot be proteolyzed and deconjugated by DUBs [160]. In our assays we have used the general protease inhibitor N-ethylmaleimide to reduce the chances of PDK1 de-ubiquitination. A positive hit on the DUB screen that would reveal the identity of a DUB for PDK1 could also positively impact our MS experiments. If a DUB was known, its knock-down could be combined with AP-MS approaches to shift the equilibrium towards an increase of the amount of ubiquitinated PDK1 available for affinity isolation. Finally, MS data analysis algorithms may also form a bottleneck in the identification of ubiquitin modified lysines. Long Ubl tails - which may still be present in our samples from other, aspecifically enriched proteins - can themselves produce fragments by CID and thus create complex overlapping fragmentation patters that cannot be analyzed by conventional search algorithms.

3.3 Finding the DUB

Detecting PDK1 mono-ubiquitination by western blot and the availability of shRNA and siRNA libraries allowed us to mount a loss of function screen to identify a DUB for PDK1. Of all the DUBs screened, none have yielded an increase in the ubiquitinated fraction and thus we have not found any DUB that acts on PDK1. We can therefore only speculate on the reasons. First, a western blot based screen may not be sensitive enough to pick up changes in mono-ubiquitination. Although we have shown that protein-G tagged and ectopically expressed PDK1 is mono-ubiquitinated and can be detected via western blot, there is no guarantee that a DUB will act on it as it would on endogenous PDK1-ubiquitin conjugates. It could be that the protein G-tagged version of PDK1 is either not recognized by the DUB or that, despite DUB activity, an excess of ubiquitinated PDK1 from overexpression makes the increase of ubiquitinated PDK1 signal undetectable due to quenching. This latter possibility means that despite having succeeded in knocking down the responsible DUB

via RNAi, our assay would not have been sensitive enough (i.e. too noisy) to pick up the change. An alternative approach such as an enzyme linked immunoabsorbent assay (ELISA) could be used to obtain a more quantitative signal. The human genome is predicted to encode as many as 95 DUBs based on sequence comparison analysis [87] and the pSUPER and Dharmacon libraries contain oligonucleotides targeting 50 and 100 genes respectively. Thus, the second possible explanation for a lack of detection of a change in ubiquitin signal, may be that the DUB for PDK1 is not yet known, thus not contained within either library and therefore not identifiable with either of them. Here a solution is technically more challenging: one possibility to identify the unknown DUB would be by a proteomics approach. High-throughput tandem affinity purification and mass spectrometry analysis (TAP-MS) is being used to identify protein interactomes in human cells [149]. This approach could be applied to identify novel interactors of PDK1, which would then have to be characterized functionally for de-ubiquitinase activity *in vitro*, for instance by LanthaScreen [161]. This approach comes with the caveat that the DUB-substrate interaction may be transient, which could reduce the yield of co-precipitated DUB available for MS-detection. Chemically cross-linking with reagents such as such as di-N-hydryoxysuccinimide, that crosslinks proteins via lysines can be used to increase to stabilize transient complexes, but the unspecificity of these approaches may cause false positives [162]. Yet such an approach has been reported in combination with TAP [163]. The third possibility is that some of the knock downs in our library may not have effectively silenced their target for technical reasons. Verifying every single shRNA pool is difficult but it may be possible to bypass this, by performing a gain of function (GOF) screen in addition to a loss of function (LOF) screen as was done here, by using a DUB cDNA overexpression library and looking for a loss of PDK1 ubiquitination rather than for an increase. This would reduce the chance that hits that were missed in the LOF screen are missed a second time. The fourth explanation why we failed to pick up an increase in signal could be redundancy in DUB action. If more than one DUB were to act on PDK1, knocking down one would not suffice to allow a change in ubiquitination status to become visible, as the silenced protease's action would be compensated by the other. So far, substrate sharing has not been reported in the literature, however there are examples of functional redundancy – as opposed to substrate redundancy - such as CYLD and A20, two DUBs that both negatively influence the NFκB signaling pathway [95, 164-165]. Last, it could be hypothesized that no DUB was found because no DUB exists that acts on PDK1 in HEK293T cells, making the mono-ubiquitination irreversible. This scenario seems unlikely however, because ubiquitination is likely to be a dynamic process, as suggested by the smaller fraction of PDK1 that is found to be modified. If PDK1 was constantly being

ubiquitinated and this ubiquitination has a non-degradative role – as we assume it does due to its detection on western blot – the proportion of ubiquitinated PDK1 would be dominant in the cell lysate. However this is clearly not the case. We have performed experiments with TS20 cells expressing a temperature sensitive E1 mutant (data not shown) allowing shut down of the ubiquitin machinery at a non permissive temperature [166]. Shutting down ubiquitination while monitoring for a decrease in ubiquitination levels of PDK1 can serve as a control for the existence of an active DUB. However, due to low total PDK1 stability in this cell line, no conclusive result was obtainable. We cannot completely exclude at this point that no DUB exists for PDK1 but it seems reasonable to assume that it does exist.

3.4 Function of PDK1 mono-ubiquitination

Attempts to characterize PDK1 mono-ubiquitination did not reveal a particular functional role; however we were able to exclude several possibilities. Binding of PDK1 to PIP_3 was not dependent on mono-ubiquitination and conversely, binding was not essential for ubiquitination. Second, we showed that PDK1 kinase activity is not required for its ubiquitination. Third, we have created an epitope tagged C-terminal PDK1-ubiquitin fusion which we transfected and visualized by confocal laser microscopy to test for differential localization between ubiquitinated and non-ubiquitinated forms. This construct did not seem to preferentially localize to the membrane, the cytoplasm or the nucleus. It can be argued however that this linear fusion is too artificial and does therefore not accurately mimic mono-ubiquitinated PDK1. This also suggests that, should endogenous mono-ubiquitin affect PDK1 localization, then it is not dependent on the mere presence of a ubiquitin moiety but requires recognition by a ubiquitin binding domain (UBD) that specifically recognizes the mono-ubiquitin epitope conjugated to a particular lysine. Alternatively, the fact that we did not observe a difference in localization of the PDK1-Ub fusion could be due to the fact that its effect on localization is too faint to be observed in confocal laser microscopy. A possible solution here could come from identifying the E3-ligase or E2/E3 complex responsible for mono-ubiquitination of PDK1. Overexpressing the E3 ligase may be an alternative approach to enrich for ubiquitinated PDK1 and possibly observe differential localization. Fractionation of HEK293T cell lysate in membrane, nuclear and cytoplasmic fractions showed that PDK1 was ubiquitinated in all these cellular compartments thus further underlining that ubiquitination does not seem to play a

role in PDK1 localization. Fractionation with higher resolution, such as endosomal isolation, may yet reveal a differential repartition. Pathway activation by PDK1 was measured by the levels of phosphorylated AKT, but PDK1 ubiquitination levels remained constant. We therefore concluded that mono-ubiquitination does not correlate with pathway activity as measured by AKT phosphorylation levels. However, we can speculate that PDK1 may activate other of its downstream targets in a ubiquitin dependent manner. In such a scenario PDK1 mono-ubiquitination could serve to fine tune its activity towards substrates other than AKT. The fact that only a small fraction of PDK1 seems to be ubiquitinated could support this hypothesis. Activation levels of SGK, S6K, RSK and PKC were not measured in this work. A possibility to assay if mono-ubiquitination is involved in steering PDK1 activity towards different targets could be achieved by co-precipitating known substrates with PDK1 and monitoring mono-ubiquitination state of PDK1 in these complexes.

3.5 Open questions and outlook

Another important issue is how to proceed once the mono-ubiquitinated lysine residue is identified. Knowing the modification site is crucial to understand its role. For instance it will allow the creation of non-ubiquitinatable point mutants. Monitoring the activation status of downstream PDK1 substrates in cells overexpressing this mutant, may reveal a differential activation pattern of substrates, indicating that under normal conditions mono-ubiquitin is involved in regulation of a particular PDK1 substrate. Basal levels of endogenously ubiquitinated PDK1 will remain in transfected cells, which will be problematic if indeed mono-ubiquitination is a subtle differential activator that steers a subset of PDK1 molecules towards different substrates. This is difficult to prevent, without knowledge of the ubiquitin ligase. While overexpressing a non-modifiable mutant is easily feasible, but only of limited use, making a constitutively ubiquitinated PDK1 version is more challenging but may prove an informative tool. A possible way to address this problem is by identifying the E3 ligase responsible for PDK1 mono-ubiquitination and overexpressing it. This should allow to shift the equilibrium towards ubiquitination and bolster the levels of modified PDK1. Subsequently this can prove more representative by keeping the ubiquitin epitope endogenous instead of fusing it artificially to the protein. Constitutively highly ubiquitinated PDK1 might also prove valuable to study downstream activation, as it may allow us to identify which of PDK1's substrates are dependent on mono-ubiquitin. This mutant may also be used in combination with the

above discussed point mutant. The E3 ligase specific for PDK1 can in theory be identified by affinity purification of PDK1 with its interactors from cell lysate in non-denaturing conditions, coupled with shotgun mass-spectrometry [167]. We have however not used this approach so far. Our MS/MS (orbitrap) setup described previously, does not identify peptides which are not on the inclusion list. Shotgun mass spectrometry uses scanning analyzers to generate great amounts of spectra from complex mixtures and is therefore suited for interactome studies, with the potential of identifying interactors. Ultimately, the search for PDK1 mono-ubiquitination aims at gaining better functional understanding of this kinase with hopes to offer a targeting strategy for this important kinase in cancers with activated PI3K/AKT. How likely is it, that given the identification of the ubiquitination site and the functional understanding of the modification, PDK1 will be a drug target in cancer? Several factors speak for the attractiveness of PDK1 as a target: it is constitutively active and essential for AKT function and inhibiting PDK1 is a way to reduce AKT activity indirectly, thus potentially being less favorable to development of resistance in the tumor. This raises the question of putative and *in vivo* effectiveness. It is not clear how distant a targeted molecule (node) of the same pathway can be from the actually mutated onco-protein to still be effective in inhibiting it. For instance, mTORC which is a target of AKT also exerts negative feedback on AKT. Thus, treating with mTORC inhibitors in tumors with activated AKT (e.g. with rapamycin and analogues) can worsen the situation. While over active AKT may favor transformation and proliferation, in some cases inhibiting mTORC will remove what little remaining negative control is left on AKT and can even further stimulate tumor growth [168]. This is one of the reasons why double inhibitors of mTORC and other PI3K pathway components are more promising than single components targeting the mTORC hub. Furthermore the cross-talk landscape amongst PI3K and other pathways like β-catenin [169] in tumors is starting to be filled in. It seems that PDK1 is essential enough for activation of AKT and its other substrates to be a solid target, especially in the context of drug combination treatments affecting more than one pathway.

4 Conclusions

In this work we have described for the first time that PDK1 is mono-ubiquitinated *in vivo*, and that this modification occurs in the kinase domain. Furthermore we have established that PDK1 kinase activity is not required for its ubiquitination. We also present evidence that ubiquitination of PDK1 is not affected by insulin stimulus, by PIP_3 binding and that ubiquitination does not affect PDK1's ability to bind to PIP_3.

5 Materials and Methods

Chemicals: Ampicillin (Roth); Big Dye for sequencing kit (AppliedBiosystems); D+Biotin 98% (Alfa Aesar); DAPI (Sigma-Aldrich); Di-ubiquitin (Boston Biochem); DMEM medium (Invitrogen GIBCO®); Fetal calf serum (FCS) (Invitrogen GIBCO®); HA beads (Sigma-Aldrich); LB agar (Roth); LY294003 (Sigma-Aldrich); Lysogeny broth (LB) medium (Roth); Ni beads (Sigma-Aldrich); Nucleotides for PCR (Fermentas); Opti-MEM medium (Invitrogen GIBCO®); Phosphate buffered saline (PBS) (Sigma-Aldrich); PIP_3 beads (Echelon Biosciences); Polybrene (Sigma-Aldrich); Protein G beads (Pierce); Proteinase K (Sigma-Aldrich); Puromycin (Sigma-Aldrich);Restriction enzymes (New England Biolabs); RPMI 1640 medium (Invitrogen GIBCO®); Strep-tactin sepharose 50% suspension (IBA); Tergitol-NP40 (Sigma-Aldrich); triton-x100 (Sigma-Aldrich); Trypsin from (Invitrogen GIBCO®); V5 beads (Sigma-Aldrich); VECTASHIELD (Vector Labs). **Oligonucleotides** were designed by us and and synthesized by Sigma-Aldrich (Germany). **Antibodies**: AKT (Cell Signaling); Alexa Fluor 488 secondary (invitrogen); IgG2a (Abcam); pAKT (Cell Signaling); PDK1 (Cell Signaling); Ubiquitin (P4D1) (Cell Signaling).

10x Hepes Buffered Saline (HBS): 1.4 M NaCl, 250 mM HEPES, 7.5 mM Na2HP04, pH7.5, filter sterilized, stored at -20°C.

SDS reducing sample buffer: 320 mM Tris-HCl pH 6.8, 40% (v/v) Glycerol, 0.008g bromphenol blue, 8% SDS (stock is 20%), 20 % (v/v) beta-mercapto-ethanol

RIPA cell lysis buffer: 25 mM Tris-HCl pH 7.6, 150 mM NaCl, 1 % NP40 or Tergitol, 0.5% Triton X100, 0.1 % SDS

Immunofluorescence blocking buffer: for 12.5ml = 1.25ml 10xPBS, 0.63ml Serum from the same species as the secondary Ab. While stiring add 375µl Triton X-100. Ad 12.5ml dH20

Immunofluorescence Antibody dilution buffer: for 40ml = 4ml 10XPBS + 36ml dH2O. 0.4g BSA, while stiring add 120µl Triton X-100

Agarose gel electrophoresis: Dissolve agarose in TAE (1 g/100ml). Boil mixture in microwave until the agarose is fully dissolved. Add Ethidium bromide 10 µl/100ml.

5.1 Mass Spectrometry, an emerging tool in ubiquitin studies

The ability to use affinity purification with mass spectrometry (AP-MS), to purify protein complexes under near physiological conditions and obtain amino acid sequence information for refined database search, has made AP-MS a method of choice in protein interactome studies [170]. It is also emerging as a tool to identify post translational modification sites on proteins conjugated to ubiquitin and ubiquitin-like modifiers (Ubl). For instance, MS has been employed successfully to identify the poly-ubiquitinated lysine residue on EGFR [171] and mono-ubiquitinated lysine on Sts2 [77]. As discussed in the results section, we have used single epitope affinity purification and tandem affinity purification (TAP) to isolate ubiquitinated PDK1 from cells transfected, retrovirally transduced or Flip-recombined with vectors over-expressing epitope tagged PDK1 and/or ubiquitin. Expression and successful affinity purification of epitope tagged PDK1-ubiquitin conjugates was verified via western blot. PAGE separation and protein silver staining of isolated protein allows the visualization and excision of bands that run at the predicted height of modified PDK1. In order to be analyzed, isolated proteins and protein complexes are digested, for example with trypsin, which cleaves C-terminally of lysines or arginines unless they are followed by a proline. Tryptic digestion leads to smaller peptides, with a double glycine (GG) remnant from digested ubiquitin, covalently bound to the post-translationally modified lysine from the original protein [157]. The fragment peptides are sequence specific and in sum create a peptide mass fingerprint of the digested protein, which allows digest database matchmaking and identification of the protein [172]. Trypsin digestion is not 100% efficient and some lysines may be skipped, leading to so called missed cleavage sites (MCS), which may, in turn, give rise to peptides with more than one GG remnant in proteins conjugated to several Ubls or to heavier LRGG signatures instead of GG [66, 68, 97, 149, 173]. To take miscleavage into account, we used the MASCOT search algorithm allowing a less stringent two missed cleavage sites instead of one, as the bulk of the ubiquitin modifier may further reduce the tryptic digestion efficiency at the mono-ubiquitinated lysine, causing an additional MCS. We analyzed the peptides resulting from affinity purified and trypsin digested proteins by high performance liquid chromatography (LC) in line with ESI and a tandem MS approach - this is known as "hyphenated separation" - commonly annotated LC-ESI-MS/MS . A mass spectrometer consists of an ionizing source, one or more analyzer units and a detector. Mixtures can be separated by LC prior to MS analysis which allows to separate peptides according to their retention time in the column, thus improving analysis sensitivity because different components of the analyzed mixture

enter the mass spectrometer sequentially as narrow focused bands [174]. ESI is a soft ionization method, leading to very little to no further fragmentation and is thus very frequently employed in analysis of biological samples, where the conservation of whole non-covalently bound protein complexes is essential. In ESI, the analyte is injected through a needle and dispersed into charged droplets by a high potential (2.5 - 4 kV) between the needle and a counter-electrode [174]. Ionization of tryptically digested proteins creates protonated and multiply charged peptides of different size and mass. Tandem mass spectrometry (MS/MS) is a process in which two mass spectrometry analyzers (such as ion traps) are coupled in line. In the first MS step, an m/z scan is used to isolate ions with an m/z corresponding to the intact peptide. In the second MS step, the peptide is first fragmented by collision induced dissociation (CID) - analyte ions are made to collide with inert gas particles such as He - and the resulting fragment ions are once again separated according to their m/z. In ion trap analyzers, molecular ions of all m/z ratios take up stable trajectories, and which can be made unstable and ejected sequentially from the trap [174]. In our setup this is done using a linear quadrupole ion trap (LTQ). Ejected ions of interest are determined by an inclusion list that we generated from *in silico* prediction of differentially charged PDK1 fragment ions. The LTQ also acts as a collision chamber for CID [175]. Protonation is important for the way in which peptides fragment during CID. The most abundant fragment ions formed are b_n and y_n ions, where the charge is retained on the N-terminal and C-terminal fragment respectively, and where n indicates the number of amino acid residues from the respective terminus [174]. The specific mass (m) to charge (z) ratio - m/z - of CID fragment ions allows sequence determination of the original peptides. PTM signatures cause mass shifts, in the case of GG remnants +114.04 kDa, that affect the m/z signal. Using search algorithms like MASCOT that perform database matchmaking while taking into account these predicted shifts allow identification of the modified protein, as well as mapping of the site of PTM conjugation [157].

Mass spectrometry setup. Our inclusion lists was generated using the freeware online tool Ubl Finder and encompassed double and triple charged PDK1 fragment ions unmodified and modified by the GG remnant. Additionally the lists include 2 ions of the ubiquitin peptide itself to allow detection of ubiquitin as positive control.

MS analysis on a hybrid LTQ-OrbitrapXL. The analyses were performed in a data dependent acquisition mode using a top 6 collision induced dissociation (CID) method (up to 6 CID spectra were

acquired following each MS scan) and a dynamic exclusion for selected ions of 60s. No lock masses were employed. Maximal ion accumulation time allowed on the LTQ Orbitrap in CID mode was 150 ms for MS ninth eLTQ and 1000ms in the C-trap. Automatic gain control was used to prevent overfilling of the ion traps and was set to 5000 ions in MS*n* mode for the LTQ and 106 ions for a full FTMS scan. Injection wave forms were activated for both LTQ and Orbitrap. Intact peptides were detected in the Orbitrap at 60000 resolution.

MS Data Analysis. The acquired data were processed with Bioworksv3.3.1SP1 (ThermoFisher,Waltham,MA,USA), dta files merged with an internally-developed program, and searched against the human SwissProt database v57.12 (including isoforms as obtained from varsplic.pl) with the search engine MASCOT (MatrixScience,London,UK). Submission to the search engines was via a Perlscript that performs an initial search with relatively broad mass tolerance so n both the precursor and fgmentions (10ppmand0.6Da,respectively). High-confidence peptide identifications are used to recalibrate all precursor and fragment ion masses prior to a second search with narrower mass tolerance (4ppmand0.3DaforCID). Two missed tryptic cleavage site was allowed. Carbamidomethyl was set as fixed modifications, and oxidized methionine, residual ubiquitin-specific GG- and LRGG-signatures were set as a variable modification. To validate the proteins, MASCOT output files were processed by internally developed parsers. For MASCOT, two unique peptides with an ion score > 18 (plus additional peptides from proteins fulfilling the criteria with an ion score > 10) are required. The validated proteins retrieved by the two algorithms are merged, any spectral conflicts discarded and grouped according to shared peptides. A false positive detection rate (FDR) of <0.25% and <0.1% (including the peptides exported with lower scores) was determined for proteins and peptides, respectively, by applying the same procedure against a reversed database.

5.2 RNAi

RNA interference (RNAi) is a powerful tool that can be used to perform loss of function screens, i.e. in *Drosophila melanogaster* [176] and *Caenorhabditis elengans* [177]. Gene silencing by RNAi is a two step process. When entering the cell, dsRNA is recognised in the cytoplasm by the Dicer nuclease and processed into smaller double stranded RNA fragments, known as small interfering

RNAs (siRNAs). These siRNAs are then bound by RNA-induced silencing complex (RISC), which uses the siRNAs as templates to degrade complementary mRNA in the cell, thus silencing gene expression in a sequence dependent manner. In mammalian cells, introduction of long dsRNAs leads to an interferon response that shuts down protein synthesis altoghether. Therefore, small hairpin RNAs (shRNAs) have been developed, that are processed into siRNAs without illiciting an interferon response. The advantage of using shRNAs over siRNAs is that although being slightly less efficient in knocking down the target gene, shRNAs are less transient and therefore knock down the target gene for longer. The synthesized shRNAs recruit the RISC complex, are degraded and used as templates for degradation of specific mRNAs *in vivo*. Berns *et al.* [178] and others have successfully used multiple shRNAs against the same target mRNA, thus leading to efficient knock down, whilst avoiding unspecific cytotoxicity mediated via the interferon pathway in transfected cells. With our current knowledge of human genome sequences, it has been possible to apply this approach to screens in human and other mammalian cells. For example, stable RNA interference has been used to identify new components of the p53 dependent senescence pathway [159], an inventory of essential cancer genes [160], genes involved in metastasis [161] and the deubiquitinating enzyme and tumor suppressor CYLD as a regulator of the NFκB pathway [96].

5.3 shRNA DUB screen

The screen was done by transfecting HEKTAP cells as follows. 2.5µl Silentfect in 100µl Optimem were mixed with 10nM DUB shRNA vector pool in 100µl Optimem and incubated at room temperature for 20'. Then added to 500.000 cells per well in a 6 well plate, and filled to a total volume of 1200µl DMEM.

Dharmacon shRNA library: Dharmacon ON-TARGETplus™ SMARTpool® siRNA Library- Human deubiquitinating Enzymes (stock 1µM).

Dharmacon siRNA DUB screen was done by transfecting HEKTAP cells as follows. 2.5µl Silentfect in 100µl Optimem were mixed with 10nM DUB siRNA in 100µl Optimem and incubated at room temperature for 20'. Then added to 500.000 cells per well in a 6 well plate, and filled to a total volume of 1200µl DMEM.

Transformation of competent XL10Gold bacterial cells. 25µl competent cells are thawed on ice. 1-2µl DNA are added, gently homogenized and and incubated on ice for 30min. Cells are heat shocked 20sec at 42°C and cooled on ice 2min. Cells are then allowed to expand in LB without antibiotic for 30min to 1h at 37°C. Transformed cells are plated on LB agarose plates over night and colonies are picked the following day.

RT-PCR DUB library knock down verification. HEKTAP cells were transfected with shRNA/siRNA targeting DUBs, using Silentfect in 96 well format. After 72h RNA was isolated using RNeasy Minelute kit (Qiagen) according to the manufacturer's protocol. cDNA was synthesized and quantitative RT-PCR was performed. RNA isolated from siRNA samples was treated with TURBO™ DNAse (Applied Biosystems) prior to cDNA synthesis. From samples transfected with shRNA, 500ng RNA were incubated 10min at 70°C, then 2min on ice. For cDNA synthesis, we used 0.5 µg/µl Random Hexamere primer, Dithiothreitol (DTT) 0.1 M, 1mM dNTPs, 5X First-Strand Buffer (250 mM Tris-HCl pH 8.3, 375 mM KCl, 15 mM MgCl2) (Invitrogen), 200u MMLV reverse transcriptase (Invitrogen), 40u RNaseOUT™ Recombinant Ribonuclease Inhibitor (Invitrogen). Synthesis cycle: 5min at 25°C, 45min at 37°C, 15min at 42°C, 20min at 70°C. Samples transfected with siRNA (Dharmacon) were treated with 5µl TURBO DNase 10x buffer + 1µl TURBO DNAse 2u/10µg RNA at 37°C for 30min. After incubation 5µl inactivator is added and sample is incubated 5min at RT. Sample is centrifuged 1.5min at 13.000rpm and supernatant is collected. 500ng DNAse treated RNA is incubated with with 100µM random hexamere primer (fermentas) at 65°C for 5min. cDNA synthesis was done using RevertAid™ Reverse Transcriptase (fermentas) according to manufacturer's protocol using 20u RiboLock™ RNase Inhibitor, 1mM dNTP final concentration, 200u RevertAid reverse transcriptase. Synthesis cycle: 10min at 25°C, 60min at 42°C, 10min at 70°C.

RT-PCR was done using 5µl iTaq Sybr-Green (Biorad), 10 pmol/µl Primer mix, 1µl cDNA in 10µl total reaction volume. Cycle: 2min at 50°C, 10' at 95°C, (15sec at 95, 1min at 60)x40.

Primers for RT-PCR

USP1

Forward 5'TGTTACCATTTGTGGGACTG3'

Reverse 5'CCAGATTTAAAACGGGACA3'

USP3

Forward 5’CATACCACACCAGAGCCAA3’

Reverse5’GGCTGCCTTGCCATAAAGCA3’

USP32

Forward5’TTCATCCGGGAAGTGCTTGG3’

Reverse5’TGCAGCCCTTTGGATGTTCC3’

USP7

Forward5’ACGAGCCGGACGTTTCGA3’

Reverse5’GCGCCACTGTGACAAGCATC3’

USP9x

Forward5’TCAGGATGTGGGTCGTTACA3’

Reverse5’TGTCTGCCAAGCCTTTTCTT3’

5.4 Tissue culture

293T cells were cultured at 37°C, 5% CO_2, in DMEM + 0.5% v/v Gibco® PenStrep (10mg/ml Penicillin and Streptomycin in 0.85% Saline) + 10% v/v heat inactivated FCS (heat inactivation: 30min at 56°C). For K562 cells, RPMI was used instead of DMEM.

HEK 293T cells. Are human embryonic kidney cells transformed with adenovirus 5 DNA and thus rendered oncogenic [179]. 293T cells are derived from 293 cells by insertion and constitutive experession of the SV40 T-antigen, rendering them immortal. 293T cells are characterised by high transfectability.

HEK293SH cell line was created using Flip-In recombination. Flp-In™ T-REx™ 293 Cells (invitrogen) are plated in one well of a 6-well plate (4 ml media/well). Transfect 200ng of LR (pcDNA5/FRT/TO/SH/GW-PDK1) construct+1µg of pOG44 (Recombinase) vector using SilentFect. After transfection cells are split to 10cm dish. Selection: add 10 mL of DMEM to cells (Blasticidin HCl (38µg/mL); Hygromycin B (260µg/mL)). Almost all cells should die in a week. Maintenance: change media every 3 days (Blasticidin HCl (15µg/mL); Hygromycin B (100µg/mL)). Single colonies are confluent enough after 2-3 weeks. Trypsinize the plate, mix well to separate cells and plate the pool

onto another 10cm dish. Keep in selecting medium until next passage, then grow to make stock and to test expression. Induction of expression with 1µg/mL Doxycycline for 24h.

HEKTAP cell line was created by infecting HEK293T cells with a retroviral vector. Retrovirus was made as described in the methods, using pCDNA-Myc-PDK1. Infection was done in 10cm dishes. Replace medium with 5ml DMEM containing 10µg/ml polybrene. Virus is then added 1:30 v/v. Expression on the PDK1-myc by the cells was verified by western blot.

Other cell lines used

Cell line	Cell Type	Organ	Species
Hacat	Keratinocyte	Skin	Human
HeLa	Adenocarcinoma	Cervix	Human
A2780	Carcinoma	Ovaries	Human
HEP1	Hepatoma	Liver	Human
A549	Alveolar basal epithelial carcinoma	Lung	Human
K562	AML	Blood	Human
U2OS	Osteocarcinoma	Bone	Human
MEF	Embryonic	Fibroblast	Mouse

Lipofection. Biorad® SilentFect™ lipid reagent was used to transfect cells with plasmid DNA. Cells are plated to 80% confluency. Medium on cells was replaced ca. 20-60min before transfection. 0.2µg, 1.2µg, 3.2µg DNA are transfected respectively for 96well plates, 6well plates, 10cm tissue culture dishes. Per well, 250µl Gibco® Optimem was mixed separately with 4µl Silentfect, 1.2µg DNA (200ng GFP DNA). Both were then mixed and incubated at room temperature for 20min, and then added to the cells and the plate was incubated for 48h.

Transfection with Calcium Chloride. Cells are plated to 80% confluency. Twenty minutes before transfection medium is replaced with fresh medium. For 10cm dish, 20 ug DNA, are mixed with 950 µl 1x HBS and 50 µl 2.5 M $CaCl_2$ are added under vortexing. Transfection mixture is added to the cells. For 6-well plate 3.2 ug DNA, 158 µl 1x HBS and 8.3 µl 2.5 M $CaCl_2$ are used. One day after transfection medium is replaced.

Retrovirus production: HEK293T producer cells are $CaCl_2$ transfected in 10cm plate format with 0.92µg VSV-G, 7.08 µg pPol, 0.8µg CMV-GFP as transfection control and 1µg vector of interest containing a Puromycin resistance marker. Cells are selected in medium containing 2µg/ml Puromycin for 3-5 days.

5.1 Fractionaton

HEK293T cells were transfected with 18µg V5-PDK1 and 2µg CMV-GFP. The next day, transfected cells were washed with PBS and collected in eppendorf tube and fractionated using Proteoextract kit (Calbiochem) Cat# 539791 according to manufacturer's protocol. PDK1 was immunoprecipitated from fractions using V5 antibody.

5.2 PAGE and Western blotting

Cells were lysed directly in the 96 well plate, by adding 25µl SDS denaturing buffer buffer (4x stock: 320mM Tris-HCl pH 6.8, 40% v/v Glycerol, 0.008g bromphenol blue, 8% SDS, 20% v/v beta-mercapto Ethanol). 8µl lysate were run on PAGE (Invitrogen® NuPAGE Novex 4-12% Bis-Tris Gels, 1.0 mm) at 150V for approximately 2hours. Blotting was done at 400mA for 1h on polyvinylidenfluoride (PVDF) membranes. Membranes were blocked with Applied Biosystems® 2% (w/v) iBlock™ in PBS with 1% Tween for 30min and incubated with antibody for 4h at room temperature. Washing of the membrane was done with PBS + 1% Tween.

5.3 Affinity Purification

Immunoprecipitation. Lysis was done in 1ml RIPA buffer. Lysate was sonicated 2x6sec on ice, cell debris were pelleted and discarded by spinning at 14.000rpm for 10min at 4°C. Precipitation was done in pre-cleared lysate (with 10µl Protein A coupled agarose) with 20µl Protein A coupled agarose and 5µl antibody for 4h at 10°C. A fraction of the lysate was kept as whole cell lisate control.

Elution was done by boiling the beads for 3min at 95°C in 4x SDS denaturing buffer buffer (described above) and eluate was loaded on PAGE.

Ni/His affinity purification. Lysis was done in 1ml RIPA buffer. Lysate was sonicated 2x6sec on ice, cell debris were pelleted and discarded by spinning at 14.000rpm for 10min at 4°C. Precipitation was done with 30µl Nickel coupled agarose for 4h at 10°C. A fraction of the lysate was kept as whole cell lisate control. Elution was done by boiling the beads for 3min at 95°C in 4x SDS denaturing buffer buffer (described above) and eluate was loaded on PAGE.

Strepavidin/Strep-tactin purification. Lysis was done in 1ml RIPA buffer. Lysate was sonicated 2x6sec on ice, cell debris were pelleted and discarded by spinning at 14.000rpm for 10min at 4°C. Precipitation was done with 400µl Strep-tactin sepharose packed column, washed 2x with lysis buffer. Elution was done using a 2.5mM D+Biotin solution in lysis buffer. Strep-tactin is a streptavidin derivative engineered to bind the 8 amino acid peptide Trp-Ser-His-Pro-Gln-Phe-Glu-Lys (StrepII tag) via its biotin binding pocket [180]. StrepIII tag is sequential arrangement of StrepII tags with a poly-Gly and poly-Ser linker [181].

5.4 Sequencing

BigDye® sequencing was done using 250ng Plasmid, 1µl 5x BigDye buffer provided by the manufacturer, 1µl BigDye 3.1® di-deoxynucleotide mix, 1µl Primer (10mM) in 10µl ddH2O total volume. PCR Program: 96°C 1', 96°C 10", (50°C 5" x26), 60°C 4'. BigDye purification was done using a 250µl G50 sephadex bead (5% in H2O) column. BigDye reaction sample was mixed with 10µl formamide on a 96-well PCR plate. And boiled 2" at 98°C to denature strands prior to sanger sequencing.

5.5 Gateway cloning

Expression vectors used for retroviral transfection (PDK1-TEV-Myc-ProtG) and Flip recombination (PDK1-HA-Strep) were created by Gateway cloning. This site-specific two-step recombination method consists of inserting attB flanked DNA of interest (PCR product) into an entry clone (EC)

vector (BP reaction), which can then be transferred to a destination vector (DV) using recombinases specific for att recombination sites (LR reaction). attB-PDK1-attB was PCR amplified from pcDNA6-PDK1-V5-His using the following primers. Sense primer: 5'GGGGACAAGTTTGTACAAAAAAGCAGGCTCCGCCAGGACCACCAGCCAG3' Antisense primer: 5'GGGGACCACTTTGTACAAGAAAGCTGGGTTCACTGCACAGCGGCGTC3'. BP Reaction: The EC was created by recombining the PCR product with pDONR backbone vector. 1µg pDONR, 3µg PCR DNA product, 2µl BP clonase and 4µl ddH20 were mixed and incubated for 1h at room temperature. To stop the reaction, 1µl Proteinase K was added and samples were incubated 10' at room temperature. Ligation product was then transformed into XL10Gold competent bacteria as described in the methods. Transformed bugs were plated in agarose plates with kanamycin, colonies were picked the next day, grown up in liquid LB medium with kanamycin 30µg/ml. Plasmid DNA preparation was carried out according to protocol, and insert was sequenced by sanger sequencing. LR reaction: 3µg EC, 1µg destination vector, 2µl LR clonase and 4µl ddH20 were mixed and incubated 1h at room temperature. To stop the reaction, 1µl Proteinase K was added and samples were incubated 10' at room temperature.

PDK1 DNA sequence (*H.sapiens*):

ATGGCCAGGACCACCAGCCAGCTGTATGACGCCGTGCCCATCCAGTCCAGCGTGGTGTTATGTTCCTGCCCAT
CCCCATCAATGGTGAGGACCCAGACTGAGTCCAGCACGCCCCCTGGCATTCCTGGTGGCAGCAGGCAGGGCC
CCGCCATGGACGGCACTGCAGCCGAGCCTCGGCCCGGCGCCGGCTCCCTGCAGCATGCCCAGCCTCCGCCGC
AGCCTCGGAAGAAGCGGCCTGAGGACTTCAAGTTTGGGAAAATCCTTGGGGAAGGCTCTTTTTCCACGGTTG
TCCTGGCTCGAGAACTGGCAACCTCCAGAGAATATGCGATTAAAATTCTGGAGAAGCGACATATCATAAAAG
AGAACAAGGTCCCCTATGTAACCAGAGAGCGGGATGTCATGTCGCGCCTGGATCACCCCTTCTTTGTTAAGCT
TTACTTCACATTTCAGGACGACGAGAAGCTGTATTTCGGCCTTAGTTATGCCAAAAATGGAGAACTACTTAAAT
ATATTCGCAAAATCGGTTCATTCGATGAGACCTGTACCCGATTTTACACGGCTGAGATTGTGTCTGCTTTAGAG
TACTTGCACGGCAAGGGCATCATTCACAGGGACCTTAAACCGGAAAACATTTTGTTAAATGAAGATATGCACA
TCCAGATCACAGATTTTGGAACAGCAAAAGTCTTATCCCCAGAGAGCAAACAAGCCAGGGCCAACTCATTCGT
GGGAACAGCGCAGTACGTTTCTCCAGAGCTGCTCACGGAGAAGTCCGCCTGTAAGAGTTCAGACCTTTGGGC
TCTTGGATGCATAATATACCAGCTTGTGGCAGGACTCCCACCATTCCGAGCTGGAAACGAGTATCTTATATTTC
AGAAGATCATTAAGTTGGAATATGACTTTCCAGAAAAATTCTTCCCTAAGGCAAGAGACCTCGTGGAGAAACT
TTTGGTTTTAGATGCCACAAAGCGGTTAGGCTGTGAGGAAATGGAAGGATACGGACCTCTTAAAGCACACCC
GTTCTTCGAGTCCGTCACGTGGGAGAACCTGCACCAGCAGACGCCTCCGAAGCTCACCGCTTACCTGCCGGCT

ATGTCGGAAGACGACGAGGACTGCTATGGCAATTATGACAATCTCCTGAGCCAGTTTGGCTGCATGCAGGTG
TCTTCGTCCTCCTCCTCACACTCCCTGTCAGCCTCCGACACGGGCCTGCCCCAGAGGTCAGGCAGCAACATAGA
GCAGTACATTCACGATCTGGACTCGAACTCCTTTGAACTGGACTTACAGTTTTCCGAAGATGAGAAGAGGTTG
TTGTTGGAGAAGCAGGCTGGCGGAAACCCTTGGCACCAGTTTGTAGAAAATAATTTAATACTAAAGATGGGC
CCAGTGGATAAGCGGAAGGGTTTATTTGCAAGACGACGACAGCTGTTGCTCACAGAAGGACCACATTTATAT
TATGTGGATCCTGTCAACAAAGTTCTGAAAGGTGAAATTCCTTGGTCACAAGAACTTCGACCAGAGGCCAAGA
ATTTTAAAACTTTCTTTGTCCACACGCCTAACAGGACGTATTATCTGATGGACCCCAGCGGGAACGCACACAA
GTGGTGCAGGAAGATCCAGGAGGTTTGGAGGCAGCGATACCAGAGCCACCCGGACGCCGCTGTGCAGTGA

PDK1 amino acid sequence (*H.sapiens*)**:**

MARTTSQLYDAVPIQSSVVLCSCPSPSMVRTQTESSTPPGIPGGSRQGPAMDGTAAEPRPGAGSLQHAQPPPQP
RKKRPEDFKFGKILGEGSFSTVVLARELATSREYAIKILEKRHIIKENKVPYVTRERDVMSRLDHPFFVKLYFTFQDDEK
LYFGLSYAKNGELLKYIRKIGSFDETCTRFYTAEIVSALEYLHGKGIIHRDLKPENILLNEDMHIQITDFGTAKVLSPESK
QARANSFVGTAQYVSPELLTEKSACKSSDLWALGCIIYQLVAGLPPFRAGNEYLIFQKIIKLEYDFPEKFFPKARDLVE
KLLVLDATKRLGCEEMEGYGPLKAHPFFESVTWENLHQQTPPKLTAYLPAMSEDDEDCYGNYDNLLSQFGCMQV
SSSSSSHSLSASDTGLPQRSGSNIEQYIHDLDSNSFELDLQFSEDEKRLLLEKQAGGNPWHQFVENNLILKMGPVDK
RKGLFARRRQLLLTEGPHLYYVDPVNKVLKGEIPWSQELRPEAKNFKTFFVHTPNRTYYLMDPSGNAHKWCRKIQE
VWRQRYQSHPDAAVQ

Ubiquitin B sequence (*H.sapiens*):

ATGGGCGCACCCTGTCTGACTACAACATCCAGAAAGAGTCCACCCTGCACCTGGTGCTCCGTCTCAGAGGTGG
G

GFP sequence:

TTGGGGTTGCGCCTTTTCCAAGGCAGCCCTGGGTTTGCGCAGGGACGCGGCTGCTCTGGGCGTGGTTCCGGG
AAACGCAGCGGCGCCGACCCTGGGTCTCGCACATTCTTCACGTCCGTTCGCAGCGTCACCCGGATCTTCGCCG
CTACCCTTGTGGGCCCCCCGGCGACGC

PDK1 kinase domain (KD) and **PDK1 PH domain (PH) expression vectors** were created by PCR amplifying the coding sequence for the respective domains and cloning them into pcDNA6 (Invitrogen) using EcoRI and XbaI. For KD, forward primer 5'GATCGAATTCCCACCATGGCCAGGACCACCAGC3', reverse primer

5'GATCTCTAGACGGGTGAGCTTCGGAGGCGTC3'.
For PH, forward primer 5'GATCGAATTCATGCAGCAACATAGAGCAGTA3', reverse primer 5'GATCTCTAGACGCTGCACAGCGGCGTCCGG3'.

Ubiquitin expression vector was made by PCR amplifying monomeric human ubiquitin B and cloning it into a pLKO2 (Invitrogen) backbone using restriction sites EcoRI and BstXI. Forward primer: 5'TGGGCGCACCCTGTCTGACT3', reverse primer 5'CCACCTCTGAGACGGAGCACCA3'. This construct was sequence verified by sanger sequencing using CMV promotor specific primer 5'CAAGCGGCCTCTGATAACCA3'.

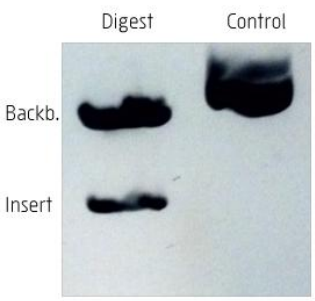
Flag-ubiquitin expression vector, was created by cloning human ubiquitin B into pcDNA3.1 backbone containing a Flag sequence (pVLAG) using restriction sites BamHI and XbaI.

HA-ubiquitin expression vector was ordered from Addgene (pRK5-HA-Ubiquitin-WT)
His-ubiquitin expression vector was created by cloning human ubiquitin B into pcDNA3 backbone using restriction sites BamHI and XbaI.

PDK1-V5-His expression vector was made by PCR amplifying PDK1 wild type sequence (forward primer 5' GAATTC GCCAGGACCACCAGCCAG 3‘, reverse primer 5'TCACTGCACAGCGGCGTCTCTAGA3' containing restriction sites) and cloning it into pcDNA6 eukaryotic expression vector backbone using the EcoRI and XbaI sites.

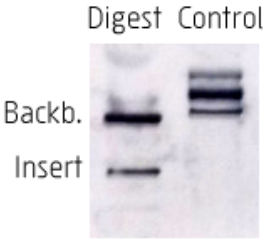
PDK1-Ub fusion expression vector was created by PCR amplifying human ubiquitin B and cloning it 5' of PDK1 in pcDNA6-PDK1-V5-His using KpnI/EcoRI

PDK1-TEV-HA-Protein G TAP expression vector was made by gateway cloning. Entry clone was made by cloning PCR amplified human PDK1 wt sequence into pDONR (Invitrogen) by Gateway cloning BP recombinase. Transfer into expression vector pRVN was done with LR recombinase. Gateway cloning is described in the methods. Primers used for PCR:
Sense primer 5‘ GGGG ACA AGT TTGTAC AAA AAA GCA GGC TCC GCCAGGACCACCAGCCAG 3‘, Antisense primer 5‘ GGGG AC CAC TTT GTA CAA GAA AGC TGG GT TCACTGCACAGCGGCGTC 3‘ .



Construct was verified by test digest using BsrGI restriction enzyme and agarose gel electrophoresis to check for insert size, and by Sanger sequencing.

PDK1-HA-Strep TAP expression vector was created by gateway cloning. Entry clone was made by cloning PCR amplified human PDK1 wt sequence into pDONR (Invitrogen) by Gateway cloning BP recombinase. Transfer into expression clone pcDNA5/FRT/TO/SH/GW [149] was done with LR recombinase. Gateway cloning is described in the methods. Primers used for PCR:



Sense primer 5‘ GGGG ACA AGT TTGTAC AAA AAA GCA GGC TCC GCCAGGACCACCAGCCAG 3‘, Antisense primer 5‘ GGGG AC CAC TTT GTA CAA GAA AGC TGG GT TCACTGCACAGCGGCGTC 3‘ .

Construct was verified by test digest using BsrGI restriction enzyme and agarose gel electrophoresis to check for insert size, and by Sanger sequencing.

5.6 PDK1 lysine mutants

Mutants were created using the Quickchange II kit (Stratagene), according to manufactuerer's protocol and using the following primers. Conserved Lysines (*Homo S., C. Elegans , Drosophila M.*) were mutated to Arginine.

1. K123R

AAG -> AGG

Forward 5'CGACATATCATAAAAGAGAACAGGGTCCCCTATGTAACCAG3'

Reverse 5'CTGGTTACATAGGGGACCCTGTTCTCTTTTATGATATGTCG3'

2. K207R

AAA -> AGA

Forward 5'CATCATTCACAGGGACCTTAGACCGGAAAACATTTTGTTAAATG3'

Reverse 5'CATTTAACAAAATGTTTTCCGGTCTAAGGTCCCTGTGAATGATG3'

3. K293/296R

AAG -> AGG

AAG -> AGG

Forward 5'GGAAACGAGTATCTTATATTTCAGAGGATCATTAGGTTGGAATATGACTTTCCAG3'

Reverse 5'CTGGAAAGTCATATTCCAACCTAATGATCCTCTGAAATATAAGATACTCGTTTCC3'

4. K315R

AAA -> AGA

Forward 5'GAGACCTCGTGGAGAGACTTTTGGTTTTAGATGCC3'

Reverse 5'GGCATCTAAAACCAAAAGTCTCTCCACGAGGTCTC3'

5. K435R

AAG -> AGG

Forward 5'GTTTTCCGAAGATGAGAGGAGGTTGTTGTTGGAGAAG3'

Reverse 5'CTTCTCCAACAACAACCTCCTCTCATCTTCGGAAAAC3'

6. K441R

AAG -> AGG

Forward 5'GTTGTTGTTGGAGAGGCAGGCTGGCGGAAAC3'

Reverse 5'GTTTCCGCCAGCCTGCCTCTCCAACAACAAC3'

7. K459R

AAG -> AGG

Forward 5'GAAAATAATTTAATACTAAGGATGGGCCCAGTGGATAAGCGG3'

Reverse 5'CCGCTTATCCACTGGGCCCATCCTTAGTATTAAATTATTTTC3'

8. K465/467R

AAG -> AGG

AAG -> AGG

Forward 5'GGGCCCAGTGGATAGGCGGAGGGGTTTATTTGCAAG3'

Reverse 5'CTTGCAAATAAACCCCTCCGCCTATCCACTGGGCCC3'

9. K492/494R

AAA -> AGA

AAA -> AGA

Forward 5'GTGGATCCTGTCAACAGAGTTCTGAGAGGTGAAATTCCTTGG3'

Reverse 5'CCAAGGAATTTCACCTCTCAGAACTCTGTTGACAGGATCCAC3'

10. K509R

AAG -> AGG

Forward 5'GAACTTCGACCAGAGGCCAGGAATTTTAAAACTTTCTTTG3'

Reverse 5'CAAAGAAAGTTTTAAAATTCCTGGCCTCTGGTCGAAGTTC3'

5.7 PDK1 Truncation mutants

Truncations were made using the following primers and based on the PDK1 wild type sequence. C terminal truncations were cloned into pcDNA3.1 containing a Flag sequence (pVLAG) using BamHI and XbaI. N terminal truncations were cloned into pcDNA6-V5-His using KpnI and XbaI.

C-terminal truncations forward primer (constant) 5'GATCGGATCCGCCAGGACCACCAGCCAG3'.

C-terminal truncations reverse primers:

5'GATCTCTAGACTAGTTGACAGGATCCACATAATATAAATG3'

5'GATCTCTAGACTAATCCACTGGGCCCATCTTTAG3'

N-terminal truncations reverse primer (constant) 5'GATCTCTAGACTCTGCACAGCGGCGTCCGG3'.

N-terminal forward primers:

5' GATCGGTACCCCACCATGATCCTTGGGGAAGGCTCTTTTTC3'

5'GATCGGTACCCCACCATGGTCCCCTATGTAACCAGAG3'

5'GATCGGTACCCCACCATGTATATTCGCAAAATCGGTTC3'

5'GATCGGTACCCCACCATGTCTTATCCCCAGAGAGCAAAC3'

5'GATCGGTACCCCACCATGATCATTAAGTTGGAATATG3'

5'GATCGGTACCCCACCATGCTTTTGGTTTTAGATGCC 3'

N-terminal PDK1 truncations

(1) 1440bp

GATCGGTACCCCACCATGATCCTTGGGGAAGGCTCTTTTTCCACGGTTGTCCTGGCTCGAGAACTGGCAACCT
CCAGAGAATATGCGATT**AAA**ATTCTGGAG**AAG**CGACATATCATA**AAA**GAGAAC**AAG**GTCCCCTATGTAACCA
GAGAGCGGGATGTCATGTCGCGCCTGGATCACCCCTTCTTTGTT**AAG**CTTTACTTCACATTTCAGGACGACGA
G**AAG**CTGTATTTCGGCCTTAGTTATGCC**AAA**AATGGAGAACTACTT**AAA**TATATTCGC**AAA**ATCGGTTCATTC
GATGAGACCTGTACCCGATTTTACACGGCTGAGATTGTGTCTGCTTTAGAGTACTTGCACGGC**AAG**GGCATCA
TTCACAGGGACCTT**AAA**CCGGAAAACATTTTGTTAAATGAAGATATGCACATCCAGATCACAGATTTTGGAAC

AGCA**AAA**GTCTTATCCCCAGAGAGC**AAA**CAAGCCAGGGCCAACTCATTCGTGGGAACAGCGCAGTACGTTTC
TCCAGAGCTGCTCACGGAG**AAG**TCCGCCTGT**AAG**AGTTCAGACCTTTGGGCTCTTGGATGCATAATATACCAG
CTTGTGGCAGGACTCCCACCATTCCGAGCTGGAAACGAGTATCTTATATTTCAG**AAG**ATCATT**AAG**TTGGAAT
ATGACTTTCCAGAA**AAA**TTCTTCCCT**AAG**GCAAGAGACCTCGTGGAG**AAA**CTTTTGGTTTTAGATGCCACA**AA**
GCGGTTAGGCTGTGAGGAAATGGAAGGATACGGACCTCTT**AAA**GCACACCCGTTCTTCGAGTCCGTCACGTG
GGAGAACCTGCACCAGCAGACGCCTCCG**AAG**CTCACCGCTTACCTGCCGGCTATGTCGGAAGACGACGAGGA
CTGCTATGGCAATTATGACAATCTCCTGAGCCAGTTTGGCTGCATGCAGGTGTCTTCGTCCTCCTCCTCACACT
CCCTGTCAGCCTCCGACACGGGCCTGCCCCAGAGGTCAGGCAGCAACATAGAGCAGTACATTCACGATCTGG
ACTCGAACTCCTTTGAACTGGACTTACAGTTTTCCGAAGATGAG**AAG**AGGTTGTTGTTGGAG**AAG**CAGGCTG
GCGGAAACCCTTGGCACCAGTTTGTAGAAAATAATTTAATACTA**AAG**ATGGGCCCAGTGGAT**AAG**CGG**AAG**
GGTTTATTTGCAAGACGACGACAGCTGTTGCTCACAGAAGGACCACATTTATATTATGTGGATCCTGTCAAC**A**
AAGTTCTG**AAA**GGTGAAATTCCTTGGTCACAAGAACTTCGACCAGAGGCC**AAG**AATTTT**AAA**ACTTTCTTTGT
CCACACGCCTAACAGGACGTATTATCTGATGGACCCCAGCGGGAACGCACAC**AAG**TGGTGCAGG**AAG**ATCCA
GGAGGTTTGGAGGCAGCGATACCAGAGCCACCCGGACGCCGCTGTGCAGAGTCTAGAGATC

(2) 1329bp

GATCGGTACCCCACCATGGTCCCCTATGTAACCAGAGAGCGGGATGTCATGTCGCGCCTGGATCACCCCTTCT
TTGTT**AAG**CTTTACTTCACATTTCAGGACGACGAG**AAG**CTGTATTTCGGCCTTAGTTATGCC**AAA**AATGGAGA
ACTACTT**AAA**TATATTCGC**AAA**ATCGGTTCATTCGATGAGACCTGTACCCGATTTTACACGGCTGAGATTGTGT
CTGCTTTAGAGTACTTGCACGGC**AAG**GGCATCATTCACAGGGACCTT**AAA**CCGGAAAACATTTTGTTAAATGA
AGATATGCACATCCAGATCACAGATTTTGGAACAGCA**AAA**GTCTTATCCCCAGAGAGC**AAA**CAAGCCAGGGC
CAACTCATTCGTGGGAACAGCGCAGTACGTTTCTCCAGAGCTGCTCACGGAG**AAG**TCCGCCTGT**AAG**AGTTC
AGACCTTTGGGCTCTTGGATGCATAATATACCAGCTTGTGGCAGGACTCCCACCATTCCGAGCTGGAAACGAG
TATCTTATATTTCAG**AAG**ATCATT**AAG**TTGGAATATGACTTTCCAGAA**AAA**TTCTTCCCT**AAG**GCAAGAGACCT
CGTGGAG**AAA**CTTTTGGTTTTAGATGCCACA**AAG**CGGTTAGGCTGTGAGGAAATGGAAGGATACGGACCTCT
T**AAA**GCACACCCGTTCTTCGAGTCCGTCACGTGGGAGAACCTGCACCAGCAGACGCCTCCG**AAG**CTCACCGC
TTACCTGCCGGCTATGTCGGAAGACGACGAGGACTGCTATGGCAATTATGACAATCTCCTGAGCCAGTTTGGC
TGCATGCAGGTGTCTTCGTCCTCCTCCTCACACTCCCTGTCAGCCTCCGACACGGGCCTGCCCCAGAGGTCAG
GCAGCAACATAGAGCAGTACATTCACGATCTGGACTCGAACTCCTTTGAACTGGACTTACAGTTTTCCGAAGA
TGAG**AAG**AGGTTGTTGTTGGAG**AAG**CAGGCTGGCGGAAACCCTTGGCACCAGTTTGTAGAAAATAATTTAAT
ACTA**AAG**ATGGGCCCAGTGGAT**AAG**CGG**AAG**GGTTTATTTGCAAGACGACGACAGCTGTTGCTCACAGAAG
GACCACATTTATATTATGTGGATCCTGTCAAC**AAA**GTTCTG**AAA**GGTGAAATTCCTTGGTCACAAGAACTTCG

ACCAGAGGCC**AAG**AATTTT**AAA**ACTTTCTTTGTCCACACGCCTAACAGGACGTATTATCTGATGGACCCCAGC
GGGAACGCACAC**AAG**TGGTGCAGG**AAG**ATCCAGGAGGTTTGGAGGCAGCGATACCAGAGCCACCCGGACG
CCGCTGTGCAGAGTCTAGAGATC

(3) 1191bp

GATCGGTACCCCACCATGTATATTCGC**AAA**ATCGGTTCATTCGATGAGACCTGTACCCGATTTTACACGGCTG
AGATTGTGTCTGCTTTAGAGTACTTGCACGGC**AAG**GGCATCATTCACAGGGACCTT**AAA**CCGGAAAACATTTT
GTTAAATGAAGATATGCACATCCAGATCACAGATTTTGGAACAGCA**AAA**GTCTTATCCCCAGAGAGC**AAA**CA
AGCCAGGGCCAACTCATTCGTGGGAACAGCGCAGTACGTTTCTCCAGAGCTGCTCACGGAG**AAG**TCCGCCTG
T**AAG**AGTTCAGACCTTTGGGCTCTTGGATGCATAATATACCAGCTTGTGGCAGGACTCCCACCATTCCGAGCT
GGAAACGAGTATCTTATATTTCAG**AAG**ATCATT**AAG**TTGGAATATGACTTTCCAGAA**AAA**TTCTTCCCT**AAG**G
CAAGAGACCTCGTGGAG**AAA**CTTTTGGTTTTAGATGCCACA**AAG**CGGTTAGGCTGTGAGGAAATGGAAGGA
TACGGACCTCTT**AAA**GCACACCCGTTCTTCGAGTCCGTCACGTGGGAGAACCTGCACCAGCAGACGCCTCCG**A**
AGCTCACCGCTTACCTGCCGGCTATGTCGGAAGACGACGAGGACTGCTATGGCAATTATGACAATCTCCTGAG
CCAGTTTGGCTGCATGCAGGTGTCTTCGTCCTCCTCCTCACACTCCCTGTCAGCCTCCGACACGGGCCTGCCCC
AGAGGTCAGGCAGCAACATAGAGCAGTACATTCACGATCTGGACTCGAACTCCTTTGAACTGGACTTACAGTT
TTCCGAAGATGAG**AAG**AGGTTGTTGTTGGAG**AAG**CAGGCTGGCGGAAACCCTTGGCACCAGTTTGTAGAAA
ATAATTTAATACTA**AAG**ATGGGCCCAGTGGAT**AAG**CGG**AAG**GGTTTATTTGCAAGACGACGACAGCTGTTGC
TCACAGAAGGACCACATTTATATTATGTGGATCCTGTCAAC**AAA**GTTCTG**AAA**GGTGAAATTCCTTGGTCACA
AGAACTTCGACCAGAGGCC**AAG**AATTTT**AAA**ACTTTCTTTGTCCACACGCCTAACAGGACGTATTATCTGATG
GACCCCAGCGGGAACGCACAC**AAG**TGGTGCAGG**AAG**ATCCAGGAGGTTTGGAGGCAGCGATACCAGAGCC
ACCCGGACGCCGCTGTGCAGAGTCTAGAGATC

(4) 1013bp

GATCGGTACCCCACCATGTCTTATCCCCAGAGAGC**AAA**CAAGCCAGGGCCAACTCATTCGTGGGAACAGCGC
AGTACGTTTCTCCAGAGCTGCTCACGGAG**AAG**TCCGCCTGT**AAG**AGTTCAGACCTTTGGGCTCTTGGATGCAT
AATATACCAGCTTGTGGCAGGACTCCCACCATTCCGAGCTGGAAACGAGTATCTTATATTTCAG**AAG**ATCATT
AAGTTGGAATATGACTTTCCAGAA**AAA**TTCTTCCCT**AAG**GCAAGAGACCTCGTGGAG**AAA**CTTTTGGTTTTAG
ATGCCACA**AAG**CGGTTAGGCTGTGAGGAAATGGAAGGATACGGACCTCTT**AAA**GCACACCCGTTCTTCGAGT
CCGTCACGTGGGAGAACCTGCACCAGCAGACGCCTCCG**AAG**CTCACCGCTTACCTGCCGGCTATGTCGGAAG
ACGACGAGGACTGCTATGGCAATTATGACAATCTCCTGAGCCAGTTTGGCTGCATGCAGGTGTCTTCGTCCTC
CTCCTCACACTCCCTGTCAGCCTCCGACACGGGCCTGCCCCAGAGGTCAGGCAGCAACATAGAGCAGTACATT

CACGATCTGGACTCGAACTCCTTTGAACTGGACTTACAGTTTTCCGAAGATGAG**AAG**AGGTTGTTGTTGGAG**A**
AGCAGGCTGGCGGAAACCCTTGGCACCAGTTTGTAGAAAATAATTTAATACTA**AAG**ATGGGCCCAGTGGAT**A**
AGCGG**AAG**GGTTTATTTGCAAGACGACGACAGCTGTTGCTCACAGAAGGACCACATTTATATTATGTGGATC
CTGTCAAC**AAA**GTTCTG**AAA**GGTGAAATTCCTTGGTCACAAGAACTTCGACCAGAGGCC**AAG**AATTTT**AAA**A
CTTTCTTTGTCCACACGCCTAACAGGACGTATTATCTGATGGACCCCAGCGGGAACGCACAC**AAG**TGGTGCAG
G**AAG**ATCCAGGAGGTTTGGAGGCAGCGATACCAGAGCCACCCGGACGCCGCTGTGCAGAGTCTAGAGATC

(5) 819bp

GATCGGTACCCCACCATGATCATT**AAG**TTGGAATATGACTTTCCAGAA**AAA**TTCTTCCCT**AAG**GCAAGAGACC
TCGTGGAG**AAA**CTTTTGGTTTTAGATGCCACA**AAG**CGGTTAGGCTGTGAGGAAATGGAAGGATACGGACCTC
TT**AAA**GCACACCCGTTCTTCGAGTCCGTCACGTGGGAGAACCTGCACCAGCAGACGCCTCCG**AAG**CTCACCG
CTTACCTGCCGGCTATGTCGGAAGACGACGAGGACTGCTATGGCAATTATGACAATCTCCTGAGCCAGTTTGG
CTGCATGCAGGTGTCTTCGTCCTCCTCCTCACACTCCCTGTCAGCCTCCGACACGGGCCTGCCCCAGAGGTCAG
GCAGCAACATAGAGCAGTACATTCACGATCTGGACTCGAACTCCTTTGAACTGGACTTACAGTTTTCCGAAGA
TGAG**AAG**AGGTTGTTGTTGGAG**AAG**CAGGCTGGCGGAAACCCTTGGCACCAGTTTGTAGAAAATAATTTAAT
ACTA**AAG**ATGGGCCCAGTGGAT**AAG**CGG**AAG**GGTTTATTTGCAAGACGACGACAGCTGTTGCTCACAGAAG
GACCACATTTATATTATGTGGATCCTGTCAAC**AAA**GTTCTG**AAA**GGTGAAATTCCTTGGTCACAAGAACTTCG
ACCAGAGGCC**AAG**AATTTT**AAA**ACTTTCTTTGTCCACACGCCTAACAGGACGTATTATCTGATGGACCCCAGC
GGGAACGCACAC**AAG**TGGTGCAGG**AAG**ATCCAGGAGGTTTGGAGGCAGCGATACCAGAGCCACCCGGACG
CCGCTGTGCAGAGTCTAGAGATC

(6) 753bp

GATCGGTACCCCACCATGCTTTTGGTTTTAGATGCCACA**AAG**CGGTTAGGCTGTGAGGAAATGGAAGGATAC
GGACCTCTT**AAA**GCACACCCGTTCTTCGAGTCCGTCACGTGGGAGAACCTGCACCAGCAGACGCCTCCG**AAG**
CTCACCGCTTACCTGCCGGCTATGTCGGAAGACGACGAGGACTGCTATGGCAATTATGACAATCTCCTGAGCC
AGTTTGGCTGCATGCAGGTGTCTTCGTCCTCCTCCTCACACTCCCTGTCAGCCTCCGACACGGGCCTGCCCCAG
AGGTCAGGCAGCAACATAGAGCAGTACATTCACGATCTGGACTCGAACTCCTTTGAACTGGACTTACAGTTTT
CCGAAGATGAG**AAG**AGGTTGTTGTTGGAG**AAG**CAGGCTGGCGGAAACCCTTGGCACCAGTTTGTAGAAAAT
AATTTAATACTA**AAG**ATGGGCCCAGTGGAT**AAG**CGG**AAG**GGTTTATTTGCAAGACGACGACAGCTGTTGCTC
ACAGAAGGACCACATTTATATTATGTGGATCCTGTCAAC**AAA**GTTCTG**AAA**GGTGAAATTCCTTGGTCACAAG
AACTTCGACCAGAGGCC**AAG**AATTTT**AAA**ACTTTCTTTGTCCACACGCCTAACAGGACGTATTATCTGATGGA

CCCCAGCGGGAACGCACAC**AAG**TGGTGCAGG**AAG**ATCCAGGAGGTTTGGAGGCAGCGATACCAGAGCCACC
CGGACGCCGCTGTGCAGAGTCTAGAGATC

C-terminal PDK1 truncations

(7) 1485bp

GGATCCGCCAGGACCACCAGCCAGCTGTATGACGCCGTGCCCATCCAGTCCAGCGTGGTGTTATGTTCCTGCC
CATCCCCATCAATGGTGAGGACCCAGACTGAGTCCAGCACGCCCCCTGGCATTCCTGGTGGCAGCAGGCAGG
GCCCCGCCATGGACGGCACTGCAGCCGAGCCTCGGCCCGGCGCCGGCTCCCTGCAGCATGCCCAGCCTCCGC
CGCAGCCTCGG**AAGAAG**CGGCCTGAGGACTTC**AAG**TTTGGG**AAA**ATCCTTGGGGAAGGCTCTTTTTCCACGG
TTGTCCTGGCTCGAGAACTGGCAACCTCCAGAGAATATGCGATT**AAA**ATTCTGGAG**AAG**CGACATATCATA**A**
AAGAGAAC**AAG**GTCCCCTATGTAACCAGAGAGCGGGATGTCATGTCGCGCCTGGATCACCCCTTCTTTGTT**A**
AGCTTTACTTCACATTTCAGGACGACGAG**AAG**CTGTATTTCGGCCTTAGTTATGCC**AAA**AATGGAGAACTACT
T**AAA**TATATTCGC**AAA**ATCGGTTCATTCGATGAGACCTGTACCCGATTTTACACGGCTGAGATTGTGTCTGCTT
TAGAGTACTTGCACGGC**AAG**GGCATCATTCACAGGGACCTT**AAA**CCGGAAAACATTTTGTTAAATGAAGATA
TGCACATCCAGATCACAGATTTTGGAACAGCA**AAA**GTCTTATCCCCAGAGAGC**AAA**CAAGCCAGGGCCAACT
CATTCGTGGGAACAGCGCAGTACGTTTCTCCAGAGCTGCTCACGGAG**AAG**TCCGCCTGT**AAG**AGTTCAGACC
TTTGGGCTCTTGGATGCATAATATACCAGCTTGTGGCAGGACTCCCACCATTCCGAGCTGGAAACGAGTATCT
TATATTTCAG**AAG**ATCATT**AAG**TTGGAATATGACTTTCCAGAA**AAA**TTCTTCCCT**AAG**GCAAGAGACCTCGTG
GAG**AAA**CTTTTGGTTTTAGATGCCACA**AAG**CGGTTAGGCTGTGAGGAAATGGAAGGATACGGACCTCTT**AAA**
GCACACCCGTTCTTCGAGTCCGTCACGTGGGAGAACCTGCACCAGCAGACGCCTCCG**AAG**CTCACCGCTTACC
TGCCGGCTATGTCGGAAGACGACGAGGACTGCTATGGCAATTATGACAATCTCCTGAGCCAGTTTGGCTGCAT
GCAGGTGTCTTCGTCCTCCTCCTCACACTCCCTGTCAGCCTCCGACACGGGCCTGCCCCAGAGGTCAGGCAGC
AACATAGAGCAGTACATTCACGATCTGGACTCGAACTCCTTTGAACTGGACTTACAGTTTTCCGAAGATGAG**A**
AGAGGTTGTTGTTGGAG**AAG**CAGGCTGGCGGAAACCCTTGGCACCAGTTTGTAGAAAATAATTTAATACTA**A**
AGATGGGCCCAGTGGAT**AAG**CGG**AAG**GGTTTATTTGCAAGACGACGACAGCTGTTGCTCACAGAAGGACCA
CATTTATATTATGTGGATCCTGTCAACTAGTCTAGA

(8) 1404bp

GGATCCGCCAGGACCACCAGCCAGCTGTATGACGCCGTGCCCATCCAGTCCAGCGTGGTGTTATGTTCCTGCC
CATCCCCATCAATGGTGAGGACCCAGACTGAGTCCAGCACGCCCCCTGGCATTCCTGGTGGCAGCAGGCAGG

GCCCCGCCATGGACGGCACTGCAGCCGAGCCTCGGCCCGGCGCCGGCTCCCTGCAGCATGCCCAGCCTCCGC
CGCAGCCTCGG**AAGAAG**CGGCCTGAGGACTTC**AAG**TTTGGG**AAA**ATCCTTGGGGAAGGCTCTTTTTCCACGG
TTGTCCTGGCTCGAGAACTGGCAACCTCCAGAGAATATGCGATT**AAA**ATTCTGGAG**AAG**CGACATATCATA**A**
AAGAGAAC**AAG**GTCCCCTATGTAACCAGAGAGCGGGATGTCATGTCGCGCCTGGATCACCCCTTCTTTGTT**A**
AGCTTTACTTCACATTTCAGGACGACGAG**AAG**CTGTATTTCGGCCTTAGTTATGCC**AAA**AATGGAGAACTACT
T**AAA**TATATTCGC**AAA**ATCGGTTCATTCGATGAGACCTGTACCCGATTTTACACGGCTGAGATTGTGTCTGCTT
TAGAGTACTTGCACGGC**AAG**GGCATCATTCACAGGGACCTT**AAA**CCGGAAAACATTTTGTTAAATGAAGATA
TGCACATCCAGATCACAGATTTTGGAACAGCA**AAA**GTCTTATCCCCAGAGAGC**AAA**CAAGCCAGGGCCAACT
CATTCGTGGGAACAGCGCAGTACGTTTCTCCAGAGCTGCTCACGGAG**AAG**TCCGCCTGT**AAG**AGTTCAGACC
TTTGGGCTCTTGGATGCATAATATACCAGCTTGTGGCAGGACTCCCACCATTCCGAGCTGGAAACGAGTATCT
TATATTTCAG**AAG**ATCATT**AAG**TTGGAATATGACTTTCCAGAA**AAA**TTCTTCCCT**AAG**GCAAGAGACCTCGTG
GAG**AAA**CTTTTGGTTTTAGATGCCACA**AAG**CGGTTAGGCTGTGAGGAAATGGAAGGATACGGACCTCTT**AAA**
GCACACCCGTTCTTCGAGTCCGTCACGTGGGAGAACCTGCACCAGCAGACGCCTCCG**AAG**CTCACCGCTTACC
TGCCGGCTATGTCGGAAGACGACGAGGACTGCTATGGCAATTATGACAATCTCCTGAGCCAGTTTGGCTGCAT
GCAGGTGTCTTCGTCCTCCTCCTCACACTCCCTGTCAGCCTCCGACACGGGCCTGCCCCAGAGGTCAGGCAGC
AACATAGAGCAGTACATTCACGATCTGGACTCGAACTCCTTTGAACTGGACTTACAGTTTTCCGAAGATGAG**A**
AGAGGTTGTTGTTGGAG**AAG**CAGGCTGGCGGAAACCCTTGGCACCAGTTTGTAGAAAATAATTTAATACTA**A**
AGATGGGCCCAGTGGATTAGTCTAGA

5.8 Immunofluorescence

U2OS cells were transfected using SilentFect with wild type PDK1-V5, PDK1-V5-Ub fusion or GFP (transfection control) and seeded in chambered slides. Cells were washed with 1x PBS for 5’ and fixation was done by incubating with Formaldehyde 4% (MeOH free) for 15min at room temperature. The slide was then washed 3x 5’ with PBS at room temperature, incubated 10min at -20°C covered in 3mm ice-cold MeOH and 5min with PBS at room temperature. Samples were then blocked with blocking buffer (as described in the methods) for 60min at room temperature. Incubation with primary (anti-V5) antibody (1:200 in Antibody dilution buffer) was done for 3h at room temperature. Cells were then washed 3x 5min with PBS and incubated 1h at room temperature with secondary fluorophore labelled antibody (Alexa Fluor 488 from invitrogen) at 1:4000 in antibody dilution buffer (incubation is carried out in the dark). For DAPI staining, the secondary antibody is washed away with PBS and DAPI is applied for 3min at room temperature at

1:50.000 dilution in antibody dilution buffer. Samples are washed 3x 5min with PBS and the cover is mounted with VECTASHIELD mounting medium. Slides can be kept for weeks at 4°C in the dark.

Software. Illustrations were created with Adobe Photoshop CS4. Vector NTI was used for reading sequencer output and for plasmid/vector design and drawing.

6 References

1. Liu, P., et al., *Targeting the phosphoinositide 3-kinase pathway in cancer.* Nat Rev Drug Discov, 2009. **8**(8): p. 627-44.
2. Katso, R., et al., *Cellular function of phosphoinositide 3-kinases: implications for development, homeostasis, and cancer.* Annu Rev Cell Dev Biol, 2001. **17**: p. 615-75.
3. Isakoff, S.J., et al., *Identification and analysis of PH domain-containing targets of phosphatidylinositol 3-kinase using a novel in vivo assay in yeast.* EMBO J, 1998. **17**(18): p. 5374-87.
4. Mora, A., et al., *PDK1, the master regulator of AGC kinase signal transduction.* Semin Cell Dev Biol, 2004. **15**(2): p. 161-70.
5. Duronio, V., *The life of a cell: apoptosis regulation by the PI3K/PKB pathway.* Biochem J, 2008. **415**(3): p. 333-44.
6. Vanhaesebroeck, B. and D.R. Alessi, *The PI3K-PDK1 connection: more than just a road to PKB.* Biochem J, 2000. **346 Pt 3**: p. 561-76.
7. Engelman, J.A., J. Luo, and L.C. Cantley, *The evolution of phosphatidylinositol 3-kinases as regulators of growth and metabolism.* Nat Rev Genet, 2006. **7**(8): p. 606-19.
8. Stokoe, D., et al., *Dual role of phosphatidylinositol-3,4,5-trisphosphate in the activation of protein kinase B.* Science, 1997. **277**(5325): p. 567-70.
9. Alessi, D.R., et al., *Characterization of a 3-phosphoinositide-dependent protein kinase which phosphorylates and activates protein kinase Balpha.* Curr Biol, 1997. **7**(4): p. 261-9.
10. Jacinto, E., et al., *SIN1/MIP1 maintains rictor-mTOR complex integrity and regulates Akt phosphorylation and substrate specificity.* Cell, 2006. **127**(1): p. 125-37.
11. Hresko, R.C. and M. Mueckler, *mTOR.RICTOR is the Ser473 kinase for Akt/protein kinase B in 3T3-L1 adipocytes.* J Biol Chem, 2005. **280**(49): p. 40406-16.

12. Brazil, D.P. and B.A. Hemmings, *Ten years of protein kinase B signalling: a hard Akt to follow.* Trends Biochem Sci, 2001. **26**(11): p. 657-64.
13. Fresno Vara, J.A., et al., *PI3K/Akt signalling pathway and cancer.* Cancer Treat Rev, 2004. **30**(2): p. 193-204.
14. Alessi, D.R., et al., *3-Phosphoinositide-dependent protein kinase-1 (PDK1): structural and functional homology with the Drosophila DSTPK61 kinase.* Curr Biol, 1997. **7**(10): p. 776-89.
15. Casamayor, A., N.A. Morrice, and D.R. Alessi, *Phosphorylation of Ser-241 is essential for the activity of 3-phosphoinositide-dependent protein kinase-1: identification of five sites of phosphorylation in vivo.* Biochem J, 1999. **342 (Pt 2)**: p. 287-92.
16. Park, J., et al., *Identification of tyrosine phosphorylation sites on 3-phosphoinositide-dependent protein kinase-1 and their role in regulating kinase activity.* J Biol Chem, 2001. **276**(40): p. 37459-71.
17. Fujita, N., et al., *Involvement of Hsp90 in signaling and stability of 3-phosphoinositide-dependent kinase-1.* J Biol Chem, 2002. **277**(12): p. 10346-53.
18. Yang, K.J., et al., *Regulation of 3-phosphoinositide-dependent protein kinase-1 (PDK1) by Src involves tyrosine phosphorylation of PDK1 and Src homology 2 domain binding.* J Biol Chem, 2008. **283**(3): p. 1480-91.
19. Balendran, A., et al., *Further evidence that 3-phosphoinositide-dependent protein kinase-1 (PDK1) is required for the stability and phosphorylation of protein kinase C (PKC) isoforms.* FEBS Lett, 2000. **484**(3): p. 217-23.
20. Nakamura, K., et al., *PDK1 regulates cell proliferation and cell cycle progression through control of cyclin D1 and p27Kip1 expression.* J Biol Chem, 2008. **283**(25): p. 17702-11.
21. Lawlor, M.A., et al., *Essential role of PDK1 in regulating cell size and development in mice.* EMBO J, 2002. **21**(14): p. 3728-38.
22. Westmoreland, J.J., et al., *Pdk1 activity controls proliferation, survival, and growth of developing pancreatic cells.* Dev Biol, 2009. **334**(1): p. 285-98.
23. Mora, A., et al., *Deficiency of PDK1 in cardiac muscle results in heart failure and increased sensitivity to hypoxia.* EMBO J, 2003. **22**(18): p. 4666-76.
24. Currie, R.A., et al., *Role of phosphatidylinositol 3,4,5-trisphosphate in regulating the activity and localization of 3-phosphoinositide-dependent protein kinase-1.* Biochem J, 1999. **337 (Pt 3)**: p. 575-83.

25. Anderson, K.E., et al., *Translocation of PDK-1 to the plasma membrane is important in allowing PDK-1 to activate protein kinase B.* Curr Biol, 1998. **8**(12): p. 684-91.
26. Filippa, N., et al., *Effect of phosphoinositide-dependent kinase 1 on protein kinase B translocation and its subsequent activation.* Mol Cell Biol, 2000. **20**(15): p. 5712-21.
27. Andjelkovic, M., et al., *Role of translocation in the activation and function of protein kinase B.* J Biol Chem, 1997. **272**(50): p. 31515-24.
28. Stephens, L., et al., *Protein kinase B kinases that mediate phosphatidylinositol 3,4,5-trisphosphate-dependent activation of protein kinase B.* Science, 1998. **279**(5351): p. 710-4.
29. Milburn, C.C., et al., *Binding of phosphatidylinositol 3,4,5-trisphosphate to the pleckstrin homology domain of protein kinase B induces a conformational change.* Biochem J, 2003. **375**(Pt 3): p. 531-8.
30. Ding, Z., et al., *Physical association of PDK1 with AKT1 is sufficient for pathway activation independent of membrane localization and phosphatidylinositol 3 kinase.* PLoS One, 2010. **5**(3): p. e9910.
31. Weinberg, R.A., *The biology of cancer*. 2007, New York: Garland Science. 1 v. (various pagings).
32. Roux, P.P., S.A. Richards, and J. Blenis, *Phosphorylation of p90 ribosomal S6 kinase (RSK) regulates extracellular signal-regulated kinase docking and RSK activity.* Mol Cell Biol, 2003. **23**(14): p. 4796-804.
33. Biondi, R.M., et al., *The PIF-binding pocket in PDK1 is essential for activation of S6K and SGK, but not PKB.* EMBO J, 2001. **20**(16): p. 4380-90.
34. Kobayashi, T. and P. Cohen, *Activation of serum- and glucocorticoid-regulated protein kinase by agonists that activate phosphatidylinositide 3-kinase is mediated by 3-phosphoinositide-dependent protein kinase-1 (PDK1) and PDK2.* Biochem J, 1999. **339 (Pt 2)**: p. 319-28.
35. Pullen, N., et al., *Phosphorylation and activation of p70s6k by PDK1.* Science, 1998. **279**(5351): p. 707-10.
36. Komander, D., et al., *Structural insights into the regulation of PDK1 by phosphoinositides and inositol phosphates.* EMBO J, 2004. **23**(20): p. 3918-28.
37. Vivanco, I. and C.L. Sawyers, *The phosphatidylinositol 3-Kinase AKT pathway in human cancer.* Nat Rev Cancer, 2002. **2**(7): p. 489-501.
38. Cully, M., et al., *Beyond PTEN mutations: the PI3K pathway as an integrator of multiple inputs during tumorigenesis.* Nat Rev Cancer, 2006. **6**(3): p. 184-92.

39. Stambolic, V., et al., *Regulation of PTEN transcription by p53.* Mol Cell, 2001. **8**(2): p. 317-25.
40. Inoki, K., T. Zhu, and K.L. Guan, *TSC2 mediates cellular energy response to control cell growth and survival.* Cell, 2003. **115**(5): p. 577-90.
41. Liu, W., et al., *PTEN/MMAC1 mutations and EGFR amplification in glioblastomas.* Cancer Res, 1997. **57**(23): p. 5254-7.
42. Cairns, P., et al., *Frequent inactivation of PTEN/MMAC1 in primary prostate cancer.* Cancer Res, 1997. **57**(22): p. 4997-5000.
43. Risinger, J.I., et al., *PTEN/MMAC1 mutations in endometrial cancers.* Cancer Res, 1997. **57**(21): p. 4736-8.
44. Sun, H., et al., *Mutational analysis of the PTEN gene in endometrial carcinoma and hyperplasia.* Am J Clin Pathol, 2001. **115**(1): p. 32-8.
45. Zhao, J.J., et al., *The oncogenic properties of mutant p110alpha and p110beta phosphatidylinositol 3-kinases in human mammary epithelial cells.* Proc Natl Acad Sci U S A, 2005. **102**(51): p. 18443-8.
46. Samuels, Y., et al., *Mutant PIK3CA promotes cell growth and invasion of human cancer cells.* Cancer Cell, 2005. **7**(6): p. 561-73.
47. Iwabuchi, H., et al., *Genetic analysis of benign, low-grade, and high-grade ovarian tumors.* Cancer Res, 1995. **55**(24): p. 6172-80.
48. Shayesteh, L., et al., *PIK3CA is implicated as an oncogene in ovarian cancer.* Nat Genet, 1999. **21**(1): p. 99-102.
49. Staal, S.P., *Molecular cloning of the akt oncogene and its human homologues AKT1 and AKT2: amplification of AKT1 in a primary human gastric adenocarcinoma.* Proc Natl Acad Sci U S A, 1987. **84**(14): p. 5034-7.
50. Sun, M., et al., *AKT1/PKBalpha kinase is frequently elevated in human cancers and its constitutive activation is required for oncogenic transformation in NIH3T3 cells.* Am J Pathol, 2001. **159**(2): p. 431-7.
51. Ruggeri, B.A., et al., *Amplification and overexpression of the AKT2 oncogene in a subset of human pancreatic ductal adenocarcinomas.* Mol Carcinog, 1998. **21**(2): p. 81-6.
52. Altomare, D.A. and J.R. Testa, *Perturbations of the AKT signaling pathway in human cancer.* Oncogene, 2005. **24**(50): p. 7455-64.
53. Bellacosa, A., et al., *Molecular alterations of the AKT2 oncogene in ovarian and breast carcinomas.* Int J Cancer, 1995. **64**(4): p. 280-5.

54. Cheng, J.Q., et al., *Amplification of AKT2 in human pancreatic cells and inhibition of AKT2 expression and tumorigenicity by antisense RNA.* Proc Natl Acad Sci U S A, 1996. **93**(8): p. 3636-41.

55. Yang, W.L., et al., *The E3 ligase TRAF6 regulates Akt ubiquitination and activation.* Science, 2009. **325**(5944): p. 1134-8.

56. Carpten, J.D., et al., *A transforming mutation in the pleckstrin homology domain of AKT1 in cancer.* Nature, 2007. **448**(7152): p. 439-44.

57. Cen, L., et al., *PDK-1/AKT pathway as a novel therapeutic target in rhabdomyosarcoma cells using OSU-03012 compound.* Br J Cancer, 2007. **97**(6): p. 785-91.

58. Flynn, P., et al., *Inhibition of PDK-1 activity causes a reduction in cell proliferation and survival.* Curr Biol, 2000. **10**(22): p. 1439-42.

59. Xie, Z., et al., *Transformation of mammary epithelial cells by 3-phosphoinositide- dependent protein kinase-1 activates beta-catenin and c-Myc, and down-regulates caveolin-1.* Cancer Res, 2003. **63**(17): p. 5370-5.

60. Finlay, D.K., et al., *Phosphoinositide-dependent kinase 1 controls migration and malignant transformation but not cell growth and proliferation in PTEN-null lymphocytes.* J Exp Med, 2009. **206**(11): p. 2441-54.

61. Bayascas, J.R., et al., *Hypomorphic mutation of PDK1 suppresses tumorigenesis in PTEN(+/-) mice.* Curr Biol, 2005. **15**(20): p. 1839-46.

62. Brugge, J., M.C. Hung, and G.B. Mills, *A new mutational AKTivation in the PI3K pathway.* Cancer Cell, 2007. **12**(2): p. 104-7.

63. Vasudevan, K.M., et al., *AKT-independent signaling downstream of oncogenic PIK3CA mutations in human cancer.* Cancer Cell, 2009. **16**(1): p. 21-32.

64. Berg, J.M., J.L. Tymoczko, and L. Stryer, *Biochemistry*. 5th ed. 2002, New York: W.H. Freeman. 1 v. (various pagings).

65. Chen, Z.J.J. and L.J.J. Sun, *Nonproteolytic Functions of Ubiquitin in Cell Signaling.* Molecular Cell, 2009. **33**(3): p. 275-286.

66. Kim, H.T., et al., *Certain pairs of ubiquitin-conjugating enzymes (E2s) and ubiquitin-protein ligases (E3s) synthesize nondegradable forked ubiquitin chains containing all possible isopeptide linkages.* J Biol Chem, 2007. **282**(24): p. 17375-86.

67. David, Y., et al., *The E2 ubiquitin-conjugating enzymes direct polyubiquitination to preferred lysines.* J Biol Chem, 2010. **285**(12): p. 8595-604.

68. Xu, P., et al., *Quantitative proteomics reveals the function of unconventional ubiquitin chains in proteasomal degradation.* Cell, 2009. **137**(1): p. 133-45.
69. Ikeda, F. and I. Dikic, *Atypical ubiquitin chains: new molecular signals. 'Protein Modifications: Beyond the Usual Suspects' review series.* EMBO Rep, 2008. **9**(6): p. 536-42.
70. Hershko, A. and A. Ciechanover, *The ubiquitin system.* Annu Rev Biochem, 1998. **67**: p. 425-79.
71. Johnson, E.S., et al., *A proteolytic pathway that recognizes ubiquitin as a degradation signal.* J Biol Chem, 1995. **270**(29): p. 17442-56.
72. Jin, L., et al., *Mechanism of ubiquitin-chain formation by the human anaphase-promoting complex.* Cell, 2008. **133**(4): p. 653-65.
73. Shi, C.S. and J.H. Kehrl, *Tumor necrosis factor (TNF)-induced germinal center kinase-related (GCKR) and stress-activated protein kinase (SAPK) activation depends upon the E2/E3 complex Ubc13-Uev1A/TNF receptor-associated factor 2 (TRAF2).* J Biol Chem, 2003. **278**(17): p. 15429-34.
74. Hatakeyama, S., et al., *U box proteins as a new family of ubiquitin-protein ligases.* J Biol Chem, 2001. **276**(35): p. 33111-20.
75. Nishikawa, H., et al., *Mass spectrometric and mutational analyses reveal Lys-6-linked polyubiquitin chains catalyzed by BRCA1-BARD1 ubiquitin ligase.* J Biol Chem, 2004. **279**(6): p. 3916-24.
76. Haglund, K., et al., *Multiple monoubiquitination of RTKs is sufficient for their endocytosis and degradation.* Nat Cell Biol, 2003. **5**(5): p. 461-6.
77. Hoeller, D., et al., *Regulation of ubiquitin-binding proteins by monoubiquitination.* Nat Cell Biol, 2006. **8**(2): p. 163-9.
78. Sundvall, M., et al., *Isoform-specific monoubiquitination, endocytosis, and degradation of alternatively spliced ErbB4 isoforms.* Proc Natl Acad Sci U S A, 2008. **105**(11): p. 4162-7.
79. Gupta-Rossi, N., et al., *Monoubiquitination and endocytosis direct gamma-secretase cleavage of activated Notch receptor.* J Cell Biol, 2004. **166**(1): p. 73-83.
80. Hicke, L., *Protein regulation by monoubiquitin.* Nat Rev Mol Cell Biol, 2001. **2**(3): p. 195-201.
81. Hicke, L., H.L. Schubert, and C.P. Hill, *Ubiquitin-binding domains.* Nat Rev Mol Cell Biol, 2005. **6**(8): p. 610-21.
82. Nakanishi, S., et al., *Histone H2BK123 monoubiquitination is the critical determinant for H3K4 and H3K79 trimethylation by COMPASS and Dot1.* J Cell Biol, 2009. **186**(3): p. 371-7.

83. Wang, B., H. Suzuki, and M. Kato, *Roles of mono-ubiquitinated Smad4 in the formation of Smad transcriptional complexes.* Biochem Biophys Res Commun, 2008. **376**(2): p. 288-92.

84. Li, M., et al., *Mono- versus polyubiquitination: differential control of p53 fate by Mdm2.* Science, 2003. **302**(5652): p. 1972-5.

85. Trotman, L.C., et al., *Ubiquitination regulates PTEN nuclear import and tumor suppression.* Cell, 2007. **128**(1): p. 141-56.

86. Hussain, S., Y. Zhang, and P.J. Galardy, *DUBs and cancer: the role of deubiquitinating enzymes as oncogenes, non-oncogenes and tumor suppressors.* Cell Cycle, 2009. **8**(11): p. 1688-97.

87. Nijman, S.M., et al., *A genomic and functional inventory of deubiquitinating enzymes.* Cell, 2005. **123**(5): p. 773-86.

88. Singhal, S., M.C. Taylor, and R.T. Baker, *Deubiquitylating enzymes and disease.* BMC Biochem, 2008. **9 Suppl 1**: p. S3.

89. Papa, F.R. and M. Hochstrasser, *The yeast DOA4 gene encodes a deubiquitinating enzyme related to a product of the human tre-2 oncogene.* Nature, 1993. **366**(6453): p. 313-9.

90. Schwickart, M., et al., *Deubiquitinase USP9X stabilizes MCL1 and promotes tumour cell survival.* Nature. **463**(7277): p. 103-7.

91. Nijman, S.M., et al., *The deubiquitinating enzyme USP1 regulates the Fanconi anemia pathway.* Mol Cell, 2005. **17**(3): p. 331-9.

92. Wang, X., P.R. Andreassen, and A.D. D'Andrea, *Functional interaction of monoubiquitinated FANCD2 and BRCA2/FANCD1 in chromatin.* Mol Cell Biol, 2004. **24**(13): p. 5850-62.

93. Bignell, G.R., et al., *Identification of the familial cylindromatosis tumour-suppressor gene.* Nat Genet, 2000. **25**(2): p. 160-5.

94. Biggs, P.J., et al., *Familial cylindromatosis (turban tumour syndrome) gene localised to chromosome 16q12-q13: evidence for its role as a tumour suppressor gene.* Nat Genet, 1995. **11**(4): p. 441-3.

95. Brummelkamp, T.R., et al., *Loss of the cylindromatosis tumour suppressor inhibits apoptosis by activating NF-kappaB.* Nature, 2003. **424**(6950): p. 797-801.

96. Wertz, I.E., et al., *De-ubiquitination and ubiquitin ligase domains of A20 downregulate NF-kappaB signalling.* Nature, 2004. **430**(7000): p. 694-9.

97. Li, M., et al., *Deubiquitination of p53 by HAUSP is an important pathway for p53 stabilization.* Nature, 2002. **416**(6881): p. 648-53.

98. Eom, Y.W., et al., *Two distinct modes of cell death induced by doxorubicin: apoptosis and cell death through mitotic catastrophe accompanied by senescence-like phenotype.* Oncogene, 2005. **24**(30): p. 4765-77.

99. Airley, R., *Cancer chemotherapy*. 2009, Chichester, UK ; Hoboken, NJ: Wiley-Blackwell. x, 341 p., 8 p. of plates.

100. van't Veer, L.J. and R. Bernards, *Enabling personalized cancer medicine through analysis of gene-expression patterns.* Nature, 2008. **452**(7187): p. 564-70.

101. Jackson, E.L., et al., *Analysis of lung tumor initiation and progression using conditional expression of oncogenic K-ras.* Genes Dev, 2001. **15**(24): p. 3243-8.

102. Verma, U.N., et al., *Small interfering RNAs directed against beta-catenin inhibit the in vitro and in vivo growth of colon cancer cells.* Clin Cancer Res, 2003. **9**(4): p. 1291-300.

103. Piccart-Gebhart, M.J., et al., *Trastuzumab after adjuvant chemotherapy in HER2-positive breast cancer.* N Engl J Med, 2005. **353**(16): p. 1659-72.

104. Slamon, D.J., et al., *Use of chemotherapy plus a monoclonal antibody against HER2 for metastatic breast cancer that overexpresses HER2.* N Engl J Med, 2001. **344**(11): p. 783-92.

105. Weinstein, I.B. and A.K. Joe, *Mechanisms of disease: Oncogene addiction--a rationale for molecular targeting in cancer therapy.* Nat Clin Pract Oncol, 2006. **3**(8): p. 448-57.

106. Hahn, C.K., et al., *Proteomic and genetic approaches identify Syk as an AML target.* Cancer Cell, 2009. **16**(4): p. 281-94.

107. Weinstein, I.B. and A. Joe, *Oncogene addiction.* Cancer Res, 2008. **68**(9): p. 3077-80; discussion 3080.

108. Jain, R.K., *Transport of molecules in the tumor interstitium: a review.* Cancer Res, 1987. **47**(12): p. 3039-51.

109. Almasri, N.M. and M. Al Hamad, *Immunohistochemical evaluation of human epidermal growth factor receptor 2 and estrogen and progesterone receptors in breast carcinoma in Jordan.* Breast Cancer Res, 2005. **7**(5): p. R598-604.

110. Lapenna, S. and A. Giordano, *Cell cycle kinases as therapeutic targets for cancer.* Nat Rev Drug Discov, 2009. **8**(7): p. 547-66.

111. Steinberg, M., *Dasatinib: a tyrosine kinase inhibitor for the treatment of chronic myelogenous leukemia and philadelphia chromosome-positive acute lymphoblastic leukemia.* Clin Ther, 2007. **29**(11): p. 2289-308.

112. Rouleau, M., et al., *PARP inhibition: PARP1 and beyond.* Nat Rev Cancer.

113. Knight, Z.A. and K.M. Shokat, *Chemically targeting the PI3K family.* Biochem Soc Trans, 2007. **35**(Pt 2): p. 245-9.

114. Zhao, J.J., et al., *The p110alpha isoform of PI3K is essential for proper growth factor signaling and oncogenic transformation.* Proc Natl Acad Sci U S A, 2006. **103**(44): p. 16296-300.

115. Puri, K.D., et al., *Mechanisms and implications of phosphoinositide 3-kinase delta in promoting neutrophil trafficking into inflamed tissue.* Blood, 2004. **103**(9): p. 3448-56.

116. Jackson, S.P., et al., *PI 3-kinase p110beta: a new target for antithrombotic therapy.* Nat Med, 2005. **11**(5): p. 507-14.

117. Sasaki, T., et al., *Function of PI3Kgamma in thymocyte development, T cell activation, and neutrophil migration.* Science, 2000. **287**(5455): p. 1040-6.

118. Fan, Q.W., et al., *A dual PI3 kinase/mTOR inhibitor reveals emergent efficacy in glioma.* Cancer Cell, 2006. **9**(5): p. 341-9.

119. Garcia-Echeverria, C. and W.R. Sellers, *Drug discovery approaches targeting the PI3K/Akt pathway in cancer.* Oncogene, 2008. **27**(41): p. 5511-26.

120. Guertin, D.A. and D.M. Sabatini, *Defining the role of mTOR in cancer.* Cancer Cell, 2007. **12**(1): p. 9-22.

121. Faivre, S., G. Kroemer, and E. Raymond, *Current development of mTOR inhibitors as anticancer agents.* Nat Rev Drug Discov, 2006. **5**(8): p. 671-88.

122. Hilgard, P., et al., *D-21266, a new heterocyclic alkylphospholipid with antitumour activity.* Eur J Cancer, 1997. **33**(3): p. 442-6.

123. Luo, Y., et al., *Pseudosubstrate peptides inhibit Akt and induce cell growth inhibition.* Biochemistry, 2004. **43**(5): p. 1254-63.

124. Cheng, J.Q., et al., *The Akt/PKB pathway: molecular target for cancer drug discovery.* Oncogene, 2005. **24**(50): p. 7482-92.

125. Peifer, C. and D.R. Alessi, *New anti-cancer role for PDK1 inhibitors: preventing resistance to tamoxifen.* Biochem J, 2009. **417**(1): p. e5-7.

126. Iorns, E., C.J. Lord, and A. Ashworth, *Parallel RNAi and compound screens identify the PDK1 pathway as a target for tamoxifen sensitization.* Biochem J, 2009. **417**(1): p. 361-70.

127. Liang, K., et al., *Differential roles of phosphoinositide-dependent protein kinase-1 and akt1 expression and phosphorylation in breast cancer cell resistance to Paclitaxel, Doxorubicin, and gemcitabine.* Mol Pharmacol, 2006. **70**(3): p. 1045-52.

128. Barnett, S.F., et al., *Identification and characterization of pleckstrin-homology-domain-dependent and isoenzyme-specific Akt inhibitors.* Biochem J, 2005. **385**(Pt 2): p. 399-408.

129. Green, C.J., et al., *Use of Akt inhibitor and a drug-resistant mutant validates a critical role for protein kinase B/Akt in the insulin-dependent regulation of glucose and system A amino acid uptake.* J Biol Chem, 2008. **283**(41): p. 27653-67.

130. Xu, Z., et al., *Development of High-Throughput TR-FRET and AlphaScreen Assays for Identification of Potent Inhibitors of PDK1.* J Biomol Screen, 2009.

131. Komander, D., et al., *Structural basis for UCN-01 (7-hydroxystaurosporine) specificity and PDK1 (3-phosphoinositide-dependent protein kinase-1) inhibition.* Biochem J, 2003. **375**(Pt 2): p. 255-62.

132. Arico, S., et al., *Celecoxib induces apoptosis by inhibiting 3-phosphoinositide-dependent protein kinase-1 activity in the human colon cancer HT-29 cell line.* J Biol Chem, 2002. **277**(31): p. 27613-21.

133. Zhu, J., et al., *From the cyclooxygenase-2 inhibitor celecoxib to a novel class of 3-phosphoinositide-dependent protein kinase-1 inhibitors.* Cancer Res, 2004. **64**(12): p. 4309-18.

134. Meuillet, E.J., et al., *Molecular pharmacology and antitumor activity of PHT-427, a novel Akt/phosphatidylinositide-dependent protein kinase 1 pleckstrin homology domain inhibitor.* Mol Cancer Ther, 2010. **9**(3): p. 706-17.

135. Yang, Y., et al., *Targeting the ubiquitin-proteasome system for cancer therapy.* Cancer Sci, 2009. **100**(1): p. 24-8.

136. Hoeller, D. and I. Dikic, *Targeting the ubiquitin system in cancer therapy.* Nature, 2009. **458**(7237): p. 438-44.

137. Miller, C.P., et al., *NPI-0052, a novel proteasome inhibitor, induces caspase-8 and ROS-dependent apoptosis alone and in combination with HDAC inhibitors in leukemia cells.* Blood, 2007. **110**(1): p. 267-77.

138. Bornstein, G., et al., *Role of the SCFSkp2 ubiquitin ligase in the degradation of p21Cip1 in S phase.* J Biol Chem, 2003. **278**(28): p. 25752-7.

139. Carrano, A.C., et al., *SKP2 is required for ubiquitin-mediated degradation of the CDK inhibitor p27.* Nat Cell Biol, 1999. **1**(4): p. 193-9.

140. Vassilev, L.T., *MDM2 inhibitors for cancer therapy.* Trends Mol Med, 2007. **13**(1): p. 23-31.

141. VanderBorght, A., et al., *Effect of an hdm-2 antagonist peptide inhibitor on cell cycle progression in p53-deficient H1299 human lung carcinoma cells.* Oncogene, 2006. **25**(50): p. 6672-7.

142. Hjerpe, R. and M.S. Rodriguez, *Alternative UPS drug targets upstream the 26S proteasome.* Int J Biochem Cell Biol, 2008. **40**(6-7): p. 1126-40.

143. Maraganore, D.M., et al., *Case-control study of the ubiquitin carboxy-terminal hydrolase L1 gene in Parkinson's disease.* Neurology, 1999. **53**(8): p. 1858-60.

144. Liu, Y., et al., *The UCH-L1 gene encodes two opposing enzymatic activities that affect alpha-synuclein degradation and Parkinson's disease susceptibility.* Cell, 2002. **111**(2): p. 209-18.

145. Caballero, O.L., et al., *Interaction and colocalization of PGP9.5 with JAB1 and p27(Kip1).* Oncogene, 2002. **21**(19): p. 3003-10.

146. Love, K.R., et al., *Mechanisms, biology and inhibitors of deubiquitinating enzymes.* Nat Chem Biol, 2007. **3**(11): p. 697-705.

147. Liu, Y., et al., *Discovery of inhibitors that elucidate the role of UCH-L1 activity in the H1299 lung cancer cell line.* Chem Biol, 2003. **10**(9): p. 837-46.

148. Rigaut, G., et al., *A generic protein purification method for protein complex characterization and proteome exploration.* Nat Biotechnol, 1999. **17**(10): p. 1030-2.

149. Glatter, T., et al., *An integrated workflow for charting the human interaction proteome: insights into the PP2A system.* Mol Syst Biol, 2009. **5**: p. 237.

150. Vasilescu, J. and D. Figeys, *Mapping protein-protein interactions by mass spectrometry.* Curr Opin Biotechnol, 2006. **17**(4): p. 394-9.

151. Bayascas, J.R., et al., *Mutation of the PDK1 PH domain inhibits protein kinase B/Akt, leading to small size and insulin resistance.* Mol Cell Biol, 2008. **28**(10): p. 3258-72.

152. Larsen, M.R. and P. Roepstorff, *Mass spectrometric identification of proteins and characterization of their post-translational modifications in proteome analysis.* Fresenius J Anal Chem, 2000. **366**(6-7): p. 677-90.

153. Garza, S. and M. Moini, *Analysis of complex protein mixtures with improved sequence coverage using (CE-MS/MS)n.* Anal Chem, 2006. **78**(20): p. 7309-16.

154. Xirodimas, D.P., et al., *Mdm2-mediated NEDD8 conjugation of p53 inhibits its transcriptional activity.* Cell, 2004. **118**(1): p. 83-97.

155. Nielsen, M.L., et al., *Iodoacetamide-induced artifact mimics ubiquitination in mass spectrometry.* Nat Methods, 2008. **5**(6): p. 459-60.

156. Hannich, J.T., et al., *Defining the SUMO-modified proteome by multiple approaches in Saccharomyces cerevisiae.* J Biol Chem, 2005. **280**(6): p. 4102-10.

157. Jeram, S.M., et al., *Using mass spectrometry to identify ubiquitin and ubiquitin-like protein conjugation sites.* Proteomics, 2009. **9**(4): p. 922-34.

158. Mayor, T., et al., *Quantitative profiling of ubiquitylated proteins reveals proteasome substrates and the substrate repertoire influenced by the Rpn10 receptor pathway.* Mol Cell Proteomics, 2007. **6**(11): p. 1885-95.

159. Bylebyl, G.R., I. Belichenko, and E.S. Johnson, *The SUMO isopeptidase Ulp2 prevents accumulation of SUMO chains in yeast.* J Biol Chem, 2003. **278**(45): p. 44113-20.

160. Hodgins, R.R., K.S. Ellison, and M.J. Ellison, *Expression of a ubiquitin derivative that conjugates to protein irreversibly produces phenotypes consistent with a ubiquitin deficiency.* J Biol Chem, 1992. **267**(13): p. 8807-12.

161. Carlson, C.B., R.A. Horton, and K.W. Vogel, *A toolbox approach to high-throughput TR-FRET-based SUMOylation and DeSUMOylation assays.* Assay Drug Dev Technol, 2009. **7**(4): p. 348-55.

162. Berggard, T., S. Linse, and P. James, *Methods for the detection and analysis of protein-protein interactions.* Proteomics, 2007. **7**(16): p. 2833-42.

163. Guerrero, C., et al., *An integrated mass spectrometry-based proteomic approach: quantitative analysis of tandem affinity-purified in vivo cross-linked protein complexes (QTAX) to decipher the 26 S proteasome-interacting network.* Mol Cell Proteomics, 2006. **5**(2): p. 366-78.

164. Trompouki, E., et al., *CYLD is a deubiquitinating enzyme that negatively regulates NF-kappaB activation by TNFR family members.* Nature, 2003. **424**(6950): p. 793-6.

165. Sun, S.C., *Deubiquitylation and regulation of the immune response.* Nat Rev Immunol, 2008. **8**(7): p. 501-11.

166. Kulka, R.G., et al., *A Chinese hamster cell cycle mutant arrested at G2 phase has a temperature-sensitive ubiquitin-activating enzyme, E1.* J Biol Chem, 1988. **263**(30): p. 15726-31.

167. Domon, B. and R. Aebersold, *Mass spectrometry and protein analysis.* Science, 2006. **312**(5771): p. 212-7.

168. O'Reilly, K.E., et al., *mTOR inhibition induces upstream receptor tyrosine kinase signaling and activates Akt.* Cancer Res, 2006. **66**(3): p. 1500-8.

169. Baryawno, N., et al., *Small-molecule inhibitors of phosphatidylinositol 3-kinase/Akt signaling inhibit Wnt/beta-catenin pathway cross-talk and suppress medulloblastoma growth.* Cancer Res. **70**(1): p. 266-76.

170. Kocher, T. and G. Superti-Furga, *Mass spectrometry-based functional proteomics: from molecular machines to protein networks.* Nat Methods, 2007. **4**(10): p. 807-15.

171. Huang, F., et al., *Differential regulation of EGF receptor internalization and degradation by multiubiquitination within the kinase domain.* Mol Cell, 2006. **21**(6): p. 737-48.

172. Cottrell, J.S., *Protein identification by peptide mass fingerprinting.* Pept Res, 1994. **7**(3): p. 115-24.

173. Kirkpatrick, D.S., C. Denison, and S.P. Gygi, *Weighing in on ubiquitin: the expanding role of mass-spectrometry-based proteomics.* Nat Cell Biol, 2005. **7**(8): p. 750-7.

174. Dass, C., *Fundamentals of contemporary mass spectrometry*. Wiley-Interscience series on mass spectrometry. 2007, Hoboken, N.J.: Wiley-Interscience. xx, 585 p.

175. Ardrey, R.E., *Liquid chromatography-mass spectrometry : an introduction*. Analytical techniques in the sciences. 2003, New York: J. Wiley. xviii, 276 p.

176. Kiger, A.A., et al., *A functional genomic analysis of cell morphology using RNA interference.* J Biol, 2003. **2**(4): p. 27.

177. Lee, S.S., et al., *A systematic RNAi screen identifies a critical role for mitochondria in C. elegans longevity.* Nat Genet, 2003. **33**(1): p. 40-8.

178. Berns, K., et al., *A large-scale RNAi screen in human cells identifies new components of the p53 pathway.* Nature, 2004. **428**(6981): p. 431-7.

179. Graham, F.L., et al., *Characteristics of a human cell line transformed by DNA from human adenovirus type 5.* J Gen Virol, 1977. **36**(1): p. 59-74.

180. Schmidt, T.G. and A. Skerra, *The Strep-tag system for one-step purification and high-affinity detection or capturing of proteins.* Nat Protoc, 2007. **2**(6): p. 1528-35.

181. Junttila, M.R., et al., *Single-step Strep-tag purification for the isolation and identification of protein complexes from mammalian cells.* Proteomics, 2005. **5**(5): p. 1199-203.

7 Appendix

7.1 Abbreviations used

HA	Haemaglutinin (tag)
His	Histidine (tag)
AP	Affinity purification
AGC	cAMP-dependent, cGMP-dependent, protein kinase C kinase family
AML	Acute myeloid Leukaemia
DUB	Deubiquitinating enzyme
ESI	Electron spray ionisation
FCS	Foetal Calf Serum
GPCR	G-Protein coupled receptor
IP	Immunoprecipitation
LC	Liquid chromatography
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
PAGE	Polyacrylamide gel electrophoresis
PI	Phosphatidylinositol
PIP	phosphatidylinositol‑phosphate
PIP_2	Phosphatidylinositol-bisphosphate
PIP_3	Phosphatidylinositol-triphosphate
PM	Plasma membrane
RTK	Receptor Tyrosine kinase
Ub	Ubiquitin
Ubl	Ubiquitin-like modifier
WCL	Whole cell lysate

7.2 Table of Figures

Figure 1 – Generation of PIP_3 by PI3K and dephosphorylation to PIP_2 by PTEN 8
Figure 2 – The PI3K/AKT Pathway and downstream targets. 9
Figure 3 – Ubiquitin conjugation steps. 19
Figure 4 – The five DUB Families. 22
Figure 5 – Target nodes of the PI3K pathway with examples of drugs in use or in clinical development. 27
Figure 6 – Small molecule inhibitors of PDK1. Adapted from Garcia-Echeverria et al. 2008 [119] 30
Figure 7 – PDK1 is mono-ubiquitinated. 34
Figure 8 – Inter species sequence alignment of PDK1. 35
Figure 9 – Mapping the mono-ubiquitinated lysine residue. 36
Figure 10 – Affinity purification (AP) approaches to isolate PDK1-Ubiquitin for MS/MS. 40
Figure 11 – Affinity purification of ubiquitinated PDK1 and silver staining. 41
Figure 12 – MASCOT readout from MS/MS analysis of A05 and A06 trypsin digested bands. 45
Figure 13 – Loss of function screen for a DUB acting on PDK1. 47
Figure 14 – Functional characterization of PDK1 mono-ubiquitination. 50

7.3 Zusammenfassung

Durch die Aktivität der zentralen Effektorkinase AKT (PKB) spielt der PI3K/AKT Singalweg eine wichtige Rolle bei der Transduktion von anti-apoptotischen Signalen in Zellteilung und Zellproliferation. Weiters besteht ein Zusammenhang zwischen der Deregulation des PI3K/AKT Signalwegs und dem Auftreten zahlreicher maligner Erkrankungen wie Brust- und Prostatakrebs und der Entstehung von Glioblastomen. Dies macht die Komponenten dieses Signaltransduktionsweges zu wichtigen therapeutischen Zielen. Die Aktivierung von AKT ist abhängig von der Kinase phosphoinositide-dependent kinase 1 (PDK1), welche AKT unter Mitwirkung von PIP_3 aktiviert, und so als essentieller Hauptaktivator für AKT fungiert. PDK1 aktiviert auch weitere downstream Proteine der AGC Familie, welche eine wichtige Rolle für Metabolismus und Translation spielen. Es ist etabliert, dass PDK1 konstitutiv aktiv ist und Wachstumsfaktor- und Insulinsignaltransduktion indirekt die Fähigkeit von PDK1 beinflussen Substrate zu binden. Die Regulation von PDK1 ist jedoch nicht zur Gänze aufgeklärt. Weiters hat PDK1 im Laufe der letzten Jahre als therapeutisches Zielprotein an Popularität gewonnen und mehrere Inhibitoren befinden sich in klinischer Testphase. Erstmals wird mit der vorliegenden Arbeit gezeigt, dass PDK1 monoubiquitiniert ist. Zudem sollen die Position, die Rolle und die molekularen Antagonisten dieser posttranslationalen Modifikation charakterisiert werden. Ubiquitin ist bekannt für seine Rolle in proteosomaler Degradation, jedoch können verschiedene Typen von konjugierten Ubiquitinepitopen auch Lokalisation, Aktivität und Konformation von ubiqutinierten Proteinen beeinflussen. Die Chrakaterisierung der PDK1 Monoubiquitinierung verspricht neue Einsicht in die Regulationsmechanismen von PDK1 in normalen und transformierten Zellen zu gewinnen. Weiters wird das Ubiquitinsystem zunehmend als therapeutisches Ziel betrachtet, da es auf enzymatischen Reaktionen basiert, die potentiell durch niedermolekulare Substanzen inhibiert werden können. Dies eröffnet die Möglichkeit Monoubiquitinierung als alternativen Winkel auszunutzen um den PI3K/AKT Signalweg über PDK1 zu inhibieren. Dieses Projekt versucht die Position der ubiqutinierten Aminosäure in PDK1 durch Mutagenese, Truncations, und Massenspektrometrie zu identifizieren. Obzwar die genaue Position des ubiquitinierten Aminosäurerests nicht definiert werden konnte, konnte die Monoubiquitinierungsstelle der Kinasendomäne zugeordnet werden. Zudem wurde versucht durch Experimente zu intrazellulärer Lokalisation, katalytischer Aktivität und PIP_3 Bindung, die Funktion der Monoubiquitinierung zu ergründen. Den hier beschriebenen Resultaten zufolge ist auszuschließen, dass PDK1 Monoubiquitinierung PIP_3 Bindung beeinflusst und dass diese keine

Vorraussetzung für die Monoubiquitinierung von PDK1 ist. Die Position von PDK1 und seine Fähigkeit AKT zu phosphorylieren waren auch nicht von Monoubiquitinierung abhängig. Letztlich haben wir einen „Loss of function“ RNA-Interferenzscreen durchgeführt um eine PDK1 spezifische Deubiquitinase (DUB) zu identifizieren. Es wurde allerdings keine DUB gefunden. Obwohl die Charakterisierung der Monoubiquitinierung weitere Arbeit verlangen wird, haben wir die erste post-translationale Modifikation von PDK1 durch Ubiquitin beschrieben.

7.4 Abstract

The PI3K/AKT signaling pathway mediates anti-apoptotic signals and drives cell growth and proliferation via the activity of its central effector kinase AKT (PKB). Deregulation of the PI3K/AKT pathway has been linked to a large number of human malignancies such as glioblastoma, breast and prostate cancer, which makes its components interesting targets for therapy. Activation of AKT depends on the action of phosphoinositide-dependent kinase 1 (PDK1), which phosphorylates and activates AKT in a PIP_3 dependent manner, and thus acts as an essential master activator of the pathway. PDK1 also activates other downstream targets of the AGC kinase family that drive metabolism and translation. It is established that PDK1 is constitutively active and that insulin and growth factor signaling indirectly affect PDK1's ability to bind its substrates, yet its regulation is incompletely understood. Furthermore PDK1 has gained popularity as a therapeutic target over the last years, and several inhibitors have been identified which are currently being tested in clinical trials. Here we show for the first time that PDK1 is mono-ubiquitinated and have attempted to elucidate the site, role and molecular antagonists of this post translational modification. Ubiquitination is known for its role in proteasomal degradation, but depending on the type of conjugated ubiquitin epitope, can also influence localization, activity and conformation of the ubiquitinated protein. Identifying the role of PDK1 mono-ubiquitination may give novel mechanistic insight into PDK1 regulation in normal and transformed cells. Additionally, the ubiquitin system is increasingly considered as a therapeutic target because it involves enzymatic reactions which can potentially be inhibited with low molecular compounds. This raises the possibility that mono-ubiquitination may be exploited as an alternative angle to target the PI3K/AKT pathway via PDK1 in cancer. In this project we have attempted to map the ubiquitinated lysine residue by mutagenesis, truncation experiments and mass spectrometry. We were able to map the ubiquitination site to the kinase domain, but finding the specific ubiquitinated residue will require further efforts. We have also attempted to identify the function of this post-translational modification by investigating localization, catalytic activity and PIP_3 binding. We were able to exclude that PDK1 mono-ubiquitination affects PIP_3 binding and that it is dependent on it. Our data also suggests that localization and PDK1's ability to phosphorylate AKT are not affected by mono-ubiquitin. Finally an RNAi loss of function screen was performed to identify a de-ubiquitinase (DUB), but no DUB was found. Although our efforts to characterize PDK1 mono-ubiquitination will require further work, we have described the first post-translational modification of PDK1 by ubiquitin.

7.5 Curriculum Vitae

NAME

Thomas List (list.thomas@gmail.com)

DATE OF BIRTH

October 22nd 1985, Vienna, Austria.

EDUCATION

2004- 2010	BSc and MSc Molecular Microbiology and Immunology at the University of Vienna
2007-2008	Studies at the University of Leicester, UK. Department of Infection and Immunity.
2003-2004	Military service.
2003	French and Austrian A-levels ("Baccalauréat" and "Matura")
1991-2003	Lycée Français in Jakarta (Indonesia), Tokyo (Japan), La Marsa (Tunisia), Brussels (Belgium) and Vienna (Austria).

CURRENT EXPERIENCE

June 2010-November 2010	Research assistant in at Sebastian Nijman's lab at CeMM
March 2009 – 2010	MSc project at Dr. Sebastian Nijman's lab on "Mono-ubiquitination of Phosphoinositide-dependent-kinase 1 (PDK1)" at CeMM
January-March 2009	Internship at CeMM (Center for Molecular Medicine, Austrian Academy of Sciences)
July-August 2008	Internship at Bayer CropScience in Monheim am Rhein, Germany
2008	Research Project at the Department of Infection and Immunity, University of Leicester, UK.
July-August 2007	Internship at Bayer CropScience in Monheim am Rhein, Germany
July 2005	Internship at Vienna Biocenter, Department Genetics and Microbiology
July 2004	Internship at the Erste Bank in Vienna. Section "Futures and Options"