

Electronic Thesis and Dissertation Repository

5-29-2019 11:00 AM

Differentially activating the oncogenic kinase Akt1

Nileeka Balasuriya

The University of Western Ontario

Supervisor

O'Donoghue, Patrick.

The University of Western Ontario

Li, Shawn S.-C.

The University of Western Ontario

Junop, Murray.

The University of Western Ontario

Graduate Program in Biochemistry

A thesis submitted in partial fulfillment of the requirements for the degree in Doctor of Philosophy

© Nileeka Balasuriya 2019

Follow this and additional works at: <https://ir.lib.uwo.ca/etd>



Part of the [Biochemistry Commons](#)

Recommended Citation

Balasuriya, Nileeka, "Differentially activating the oncogenic kinase Akt1" (2019). *Electronic Thesis and Dissertation Repository*. 6222.

<https://ir.lib.uwo.ca/etd/6222>

This Dissertation/Thesis is brought to you for free and open access by Scholarship@Western. It has been accepted for inclusion in Electronic Thesis and Dissertation Repository by an authorized administrator of Scholarship@Western. For more information, please contact wlsadmin@uwo.ca.

Abstract

The proto-oncogene Akt/protein kinase B plays a pivotal role in cell growth and survival. Phosphorylation of Akt at Thr308 and Ser473 activates the kinase following growth factor stimulation. Delineating specific role of each activation site in Akt1 on kinase activation, inhibition and substrate selection remain elusive.

We designed a unique set of tools, relying on genetic code expansion with phosphoserine and in vivo enzymatic phosphorylation, to produce differentially phosphorylated Akt1 variants. We found that having both sites phosphorylated increased the apparent catalytic rate of the enzyme by 1500-fold relative to the unphosphorylated enzyme. This increment was mainly due to the phosphorylation of Thr308 but not Ser473 which was confirmed via live cell imaging. We further found that the traditional use of phosphomimetics was unable to mimic the effect of p-Thr308 in the test tube and in cells.

Akt1 activity is also regulated via interactions between the kinase domain and the N-terminal auto-inhibitory pleckstrin homology (PH) domain. Using the same strategy, we produced Akt1 variants containing programmed phosphorylation to probe the interplay between Akt1 phosphorylation status and the auto-inhibitory function of the PH domain. Deletion of the PH domain increased the enzyme activity for all three Akt1 phospho-variants. For the doubly phosphorylated enzyme, deletion of the PH domain relieved auto-inhibition by 295-fold. The robustly active PH domain deleted enzyme variants were used in enzyme inhibition and substrate selectivity studies. We found that phosphorylation at Ser473 provided resistance to chemical inhibition by the Akt inhibitor Akti-1/2.

Finally, we used both defined and randomized peptide libraries to map the substrate selectivity of singly and doubly phosphorylated Akt1 variants. The data revealed that each phospho-form of Akt1 has distinct substrate requirements. Surprisingly, phosphorylation of Ser473 in the context of a pAkt1^{T308} enzyme led to increased activity on some, but not all Akt1 substrates. We also verified a new Akt1 target as the terminal nucleotidyltransferase (germline development 2) Gld2.

In conclusion, the site-specifically phosphorylated Akt1 variants that we produced enabled in characterizing phosphorylation-dependent activity, inhibition and substrate selectivity of the oncogenic kinase Akt1. Since phosphorylation status of Akt1 is used as a cancer biomarker, these variants can act as indispensable tools in further characterizing downstream oncogenic pathways and screening potential drug candidates.

Key words: Akt, Akt1/2 inhibitor, genetic code expansion, kinase, peptide libraries, PH domain, phosphorylation

Co-Authorship Statement

Chapter 2: This chapter contains the pre-print version of published manuscript Balasuriya, N.; Kunkel, M.T.; Liu, X.; Biggar, K.K.; Li, S.S.; Newton, A.C.; O'Donoghue, P. Genetic code expansion and live cell imaging reveal that Thr308 phosphorylation is irreplaceable and sufficient for akt1 activity. *J Biol Chem* **2018**. 293, 10744–10756. M.T. Kunkel conducted the FRET based cell assays. X. Liu and K.K. Biggar performed the mass spec analysis. All other experiments were conducted by N. Balasuriya. Manuscript was written by N. Balasuriya, M.T. Kunkel, A.C. Newton and P. O'Donoghue.

Chapter 3: This chapter contains the preprint version of the published manuscript Balasuriya, N., McKenna, M., Liu, X., Li, S. S. C., and O'Donoghue, P. (2018) Phosphorylation-Dependent Inhibition of Akt1. *Genes (Basel)* **9**. X. Liu conducted the mass spec analysis. Rest of the experiments were conducted by N. Balasuriya. Manuscript was written by N. Balasuriya, M. McKenna and P. O'Donoghue.

Chapter 4: This chapter contains my unpublished data for a manuscript in preparation: Balasuriya, N., Liu, H., Biggar, K., McKenna, M., Li, S-C. S., O'Donoghue, P. Phosphorylation-dependent substrate selectivity of Akt1. In prep, 2019. The chapter also include data I produced for a published manuscript: Chung, C., Balasuriya, N., Manni, E., Liu, X., Li, S.-C., O'Donoghue, P., and Heinemann, I. U. (2019) Gld2 activity is regulated by phosphorylation in the terminal domain. *RNA Biol*, DOI:10.1080/15476286.15472019.11608754.

Dedication

Dedicated to my beloved parents

Sujatha Herath and Senarath Balasuriya

*&
family*

Acknowledgements

First and foremost, I would like to extend my sincere thanks to my supervisor Dr. Patrick O'Donoghue for giving me a chance to pursue my PhD under your supervision. Your constant support, guidance and encouragement were much appreciated throughout the years as a graduate student. You are a truly inspiring scientist and a great mentor.

Many thanks to my supervisory committee Dr. Shawn Li, Dr. David Litchfield and Dr. Murray Junop for your thoughtful discussions and feedbacks throughout the project. Also, I would like to thank Dr. Ilka Heinemann for her kind support and advice.

A big thank you to all the past and present lab members including Susanna George, David Wright, Jeremy Lant, McShane McKenna, Christina Chung and Lauren Seidl from both O'Donoghue and Heinemann labs. You all made my PhD journey a memorable one. Many thanks to our (past) lab technician Yumin Bi for your kind helping hand when-ever needed. My sincere thanks go to Dr. Lynn Weir, Barb Green and Kyle Pollard for your kind assistance throughout the journey.

Specially, I would like to thank Dr. Laszlo Gyenis, Dr. Kyle Biggar, Dr. Xuguang Liu, Dr. Huadong Liu, Victoria Clarke and Kristina Jurcic for helping me with various research aspects of my project.

Also I would like to thank all my Sri Lankan friends who had been offering constant support since we moved to London.

Above all its my parents love and affection who made me walk this far. Much love to my loving sister Rushanti for being my listening ear. Last but not least, my love and thanks goes to my ever-loving husband Nadun Karunatileke for his inspiration and tremendous support towards completing my PhD.

Table of contents

Abstract.....	ii
Co-Authorship statement.....	iv
Dedication.....	v
Acknowledgements.....	vi
Table of contents.....	vii
List of tables.....	xii
List of figures.....	xv
List of abbreviations.....	xvi
Chapter 1.....	1
1 Introduction.....	1
1.1 Akt/Protein kinase B.....	1
1.2 Akt structure.....	2
1.3 Regulation of Akt activity.....	4
1.3.1 Phosphorylation dependent Akt activation.....	4
1.3.2 Phosphatase dependent deactivation of Akt1.....	8
1.4 Additional post translational modifications regulate Akt1....	10
1.5 Akt activity drives cell growth and survival.....	11
1.6 Hyperactivation of Akt in cancer.....	14
1.7 Investigating Akt phosphorylation in vitro and in cell.....	16
1.8 Scope of the thesis.....	18
1.9 References.....	21
Chapter 2.....	29
2 Genetic code expansion and live cell imaging reveal that Thr308 phosphorylation is irreplaceable and sufficient for Akt1 activity.....	29

2.1 Abstract.....	29
2.2 Introduction.....	30
2.3 Materials and methods.....	35
2.3.1 Bacterial strains and plasmids.....	35
2.3.2 Protein production and purification.....	36
2.3.3 <i>In vitro</i> Akt1 kinase assay.....	37
2.3.4 Cell culture media and conditions.....	38
2.3.5 Cell imaging.....	38
2.3.6 Western blotting.....	39
2.4 Results.....	40
2.4.1 Activation of full-length pAkt1 variants by genetic code expansion.....	40
2.4.2 Phosphorylation of Thr308 is necessary and sufficient for Akt1 activation.....	44
2.4.3 Enzymatic activity of phosphomimetic Akt1 variants.....	46
2.4.4 Akt1 mutant activity in live cells.....	48
2.4.5 Mutant Akt1 kinases are phosphorylated following EGF stimulation.....	50
2.4.6 Impact of Ser substitution at Thr308 in cells.....	53
2.5 Discussion.....	54
2.5.1 Generation of active Akt1.....	54
2.5.2 Role of phosphorylation in activating Akt1.....	55
2.5.3 Acidic residues do not function as phosphomimetics in Akt.....	59
2.5.4 Akt1 activation threshold in cells.....	62
2.6 Conclusions.....	64

2.7 References.....	66
2.8 Supporting information.....	74
2.8.1 Supporting experimental procedures.....	74
2.8.2 Supporting references.....	78
2.8.3 Supporting figures.....	79
Chapter 3.....	88
3 Phosphorylation-dependent chemical and auto-inhibition of Akt1.....	88
3.1 Abstract.....	88
3.2 Introduction.....	89
3.3 Materials and methods.....	92
3.3.1 Bacterial strains and plasmids.....	92
3.3.2 Protein and phosphoprotein production.....	93
3.3.3 Parallel-reaction monitoring mass spectrometry (PRM-MS) of ppAkt1.....	94
3.3.4 MALDI-TOF/TOF mass spec analysis.....	95
3.3.5 Akt1 kinase activity assay.....	95
3.3.6 Kinase inhibition assay.....	96
3.4 Results.....	97
3.4.1 Production of recombinant Akt1 variants.....	97
3.4.2 Recombinant Akt1 produced in <i>E. coli</i> versus sf9 cells...	98
3.4.3 Impact of PH domain deletion on differentially phosphorylated Akt1.....	101
3.4.4 Chemical inhibition of phosphorylated Akt1 variants....	106
3.5 Discussion.....	109
3.5.1 Phosphorylation-dependent auto inhibition of Akt1.....	110
3.5.2 Phosphorylation-dependent chemical inhibition of Akt1	112
3.5.3 Synthetic biology approach to generate active Akt1.....	114

3.6	References.....	115
3.7	Supporting information.....	120
3.7.1	Supporting experimental procedures.....	120
3.7.2	Supporting references.....	121
3.7.3	Supporting tables.....	121
3.7.4	Supporting figures.....	123
Chapter 4	129
4	Phosphorylation dependent substrate selectivity of Akt1	129
4.1	Abstract.....	129
4.2	Introduction.....	130
4.3	Materials and methods.....	132
4.3.1	Bacterial strains and plasmids.....	132
4.3.2	Protein and phosphoprotein production.....	132
4.3.3	Library of known Akt1 substrate peptides.....	133
4.3.4	Alanine scans.....	133
4.3.5	OPAL Akt1 activity screen.....	133
4.3.6	Sequence Logos and database searching.....	134
4.3.7	NIH3T3 cell culture.....	135
4.3.8	Kinase assay for peptide libraries.....	136
4.3.9	Kinase assay for NIH3T3 cell extracts.....	136
4.3.10	Kinase assay with GLD2 protein substrate	137
4.4	Results.....	137
4.4.1	Akt1 phosphorylation status alters selectivity among known substrates	137
4.4.2	Phosphorylation dependent changes in the Akt1 target motif	144
4.4.3	Alanine scanning of Akt1 target peptides.....	145

4.4.4 Determination of high resolution Akt1 target specificity...	146
4.4.5 Akt1 variants differentially phosphorylate NIH3T3 cellular proteins.....	152
4.4.6 Validation of a novel Akt1 substrate.....	153
4.5 Discussion.....	155
4.5.1 Phosphorylation dependent substrate specificity of Akt1..	156
4.5.2 Phosphorylation dependent changes in the Akt1 substrate motif.....	159
4.5.3 Predictions and validation of potential substrates derived from OPAL screen.....	160
4.6 Conclusion.....	161
4.7 References.....	162
4.8 Supporting information.....	166
Chapter 5.....	175
5 Summary and future perspectives.....	175
5.1 Summary.....	175
5.2 Future directions.....	178
5.2.1 Validation of predicted substrates.....	178
5.2.2 Targeting Akt1 in cancer treatment.....	179
5.3 Conclusion.....	180
5.4 References.....	181
Curriculum vitae.....	182

List of figures

Figure 1.1 Domain structure and phosphorylation sites of Akt isozymes....	4
Figure 1.2 Akt1 activation via phosphorylating sites Thr308 and Ser473.....	6
Figure 1.3 Structure of human Akt1.....	12
Figure 2.1 A novel route to doubly phosphorylated and active Akt1.....	33
Figure 2.2 Activity of singly phosphorylated Akt1 variants with regulatory site mutations.....	45
Figure 2.3 Activity of T308S and phosphomimetic Akt1 variants.....	46
Figure 2.4 Cellular activity of Akt1 variants.....	49
Figure 2.5 Reduced, but active, signaling from Akt T308S.....	52
Figure 2.6 Structure of human Akt in complex with GSK-3 β substrate peptide.....	62
Figure S2.1 Production of purified full-length Akt1 and pAkt1 variants.....	79
Figure S2.2 Physical characterization of ppAkt1 ^{T308, S473}	80
Figure S2.3 Mass spectra confirming quantitative, genetically encoded pS473 in Akt1.....	81
Figure S2.4 Physical characterization of pAkt1 ^{T308}	82
Figure S2.5 Activity of pAkt1 ^{T308} variants at reduced enzyme concentrations	83
Figure S2.6 Autoradiographs of γ -[³² P]-ATP kinase assays with pAkt1 variants	84
Figure S2.7 Production of pAkt1 ^{S308}	85
Figure S2.8 Cellular activity of Akt1 variants with glutamate substitutions....	86
Figure S2.9 Cellular activity of phospho-ablated Akt1 variant S473A.....	87
Figure 3.1 Simplified schematic of Akt1 activation via phosphorylation of sites T308 and S473.....	90
Figure 3.2 Production of Akt1 variants with programmed phosphorylation.....	91

Figure 3.3 Activity of full length ppAkt1 and commercially available active Akt1.....	101
Figure 3.4 Enzyme activity of Δ PH-Akt1 variants.....	103
Figure 3.5 Impact of PH domain on releasing the activity of differentially phosphorylated Akt1.....	105
Figure 3.6 Structure of Akt1 in active and inhibitor bound forms.....	107
Figure 3.7 Chemical inhibition of full length phosphorylated Akt1 variants....	108
Figure S3.1 Mass spectra confirming genetically encoded phosphoserine in Δ PH-ppAkt1.....	123
Figure S3.2 Initial velocity measurements for highly active Δ PH Akt1 variants.....	124
Figure S3.3 Inhibition of the full length Akt1 variants incubated with Akti-1/2 inhibitor VIII.....	125
Figure S3.4 Autoradiographs of Akt1 inhibitor assays with ppAkt1 ^{T308, S473}	126
Figure S3.5 Autoradiographs of Akt1 inhibitor assays with pAkt1 ^{T308}	127
Figure S3.6 Autoradiographs of Akt1 inhibitor assays with pAkt1 ^{S473}	128
Figure 4.1 Phosphorylation of known substrate peptides by pAkt1 variants....	140
Figure 4.2 String diagrams showing the interactions among highly active peptide substrates of Akt1 variants.....	142
Figure 4.3 Comparison of substrate preferences of ppAkt1 ^{T308,S473} with ppAkt1 ^{T308}	143
Figure 4.4 Sequence logos representing the amino acid preferences adjacent to the Akt1 target motif.....	145
Figure 4.5 Alanine scan of each residue in the GSK-3 β derived peptide.....	147
Figure 4.6 Oriented peptide array library screens (OPAL) activity assays.....	149
Figure 4.7 Sequence logos generated from the OPAL data.....	150

Figure 4.8 Interactions among highly active known and novel substrates of Akt1 variants derived from OPAL screen.....	151
Figure 4.9 NIH 3T3 cell lysates were incubated with the Akt1 indicated variants and γ -[³² P]-ATP.....	153
Figure 4.10 Kinase activity assays of Akt1 and CK2- α with GLD2 protein substrate.....	154
Figure 4.11 Akt1 and PKA phosphorylate Gld2 at S116.....	155
Figure S4.1 Production of Δ PH Akt1 variants.....	170
Figure S4.2 Autoradiographs of OPAL assay conducted for pAkt1 ^{S473}	171
Figure S4.3. Autoradiographs of OPAL assay conducted for pAkt1 ^{T473}	172
Figure S4.4 Autoradiographs of OPAL assay conducted for pAkt1 ^{T308, S473}	173
Figure S4.5 Kinase activity assays of Akt1 and CK2- α with GLD2 and γ -[³² P]-ATP as substrates.....	174

List of tables

Table 2.1 Specific activity of Akt1 variants.....	43
Table 2.2 Akt enzyme activity comparison in vitro and in COS7 cells....	58
Table 3.1 Protein yields for Akt1 variants.....	98
Table 3.2 Activity of Akt1 variants.....	99
Table 3.3 Relative activity of full length versus Δ PH Akt1 variants.....	102
Table 3.4 Akt1/2 inhibitor half maximal inhibitory concentration (IC50) for pAkt1 variants.....	107
Table S3.1 Plasmids used in this study.....	121
Table S4.1 OPAL predictions of known and unknown Akt1 substrates...	166

List of Abbreviations

ATP' = ANP-PNP	non-hydrolyzable ATP analog
CDK2	cyclin dependent kinase 2
CFP	cyan fluorescent protein
CLK2	CDC like kinase 2
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
EF5ep	elongation factor mutant
EGF	epidermal growth factor
EGTA	ethylene glycol-bis(β -aminoethyl ether)-N,N,N',N'-tetraacetic acid
FPLC	fast protein liquid chromatography
FRET	förster resonance energy transfer
Gab2	Grb2 associated binding protein
GSK-3 β	glycogen synthase kinase
HBBS	Hank's Balanced Salt Solution
IAA	iodoacetamide
ILK	integrin-linked kinase
IPTG	isopropyl β -D-1-thiogalactopyranoside
k_{app}	apparent reaction constant
MCS	multi-cloning site
MEK1	human mitogen-activated ERK activating kinase 1
MOPS	3-(N-morpholino)propanesulfonic acid
MRM-MS/MS	multiple reaction monitoring tandem mass spectrometry

mTORC2	mechanistic target of rapamycin complex
OPAL	oriented peptide array library
PDK1	phosphoinositide dependent kinase 1
PH	pleckstrin homology
PHLPP1	PH domain leucine-rich repeat protein phosphatase 1
PHLPP2	PH domain leucine-rich repeat protein phosphatase 2
PI3K	phosphoinositide 3-kinase
PIP2	phosphatidylinositol-4, 5-bisphosphate
PIP3	phosphatidylinositol-4, 5-triphosphate
PKA	protein kinase A
PKB	protein kinase B
PMSF	phenylmethylsulfonyl fluoride
PP2A	phosphatase 2A
PRM-MS/MS	Parallel Reaction Monitoring Tandem Mass Spectrometry
pSer	phosphoserine
PSSMs	position specific scoring matrices
RAC	related to A and C
S6K	S6 kinase
SDS	sodium dodecyl sulphate
SepRS	phosphoseryl-tRNA synthetase
TCEP	tris(2-carboxyethyl)phosphine
TSC2	tuberous sclerosis factor 2
WT	wild type
Δ PH-Akt1	Akt1 lacking the PH domain

Chapter 1

1 Introduction

1.1 Protein kinase B (Akt)

Murine oncogene v-AKT was initially identified from an Akt8 transforming retrovirus in spontaneous thymomas of high leukemic mice (1). Later three groups independently characterized the cellular homolog of v-AKT. These initial studies referred to the same kinase by different names: c-Akt (2), related to A and C (RAC) kinase (3) or protein kinase B (PKB) since it showed close similarity to protein kinase A and C (4). Akt was found to be a protein kinase that phosphorylated multiple protein substrates involved in cell survival signaling (5).

There are three isozymes of Akt found in mammalian cells: Akt1/PKB α , Akt2/PKB β , Akt3/PKB γ (Figure 1.1). Isozymes 1 and 2 are ubiquitously expressed in all tissues while isozyme 3 is predominantly found in brain and testis (6). Recent studies conducted on Akt isozyme specific knock-out mice revealed that the isozymes contribute to functional diversity of Akt. Akt1 null mice suffered from growth retardation and increased apoptosis (7). When Akt2 was knocked out, mice suffered from type 2 diabetes related complications (8). Akt3 null mice had impaired brain development (9). The cellular mechanism related to differential activity of these isozymes is still not well established.

1.2 Akt structure

All three isozymes of Akt share ~80% sequence identity and extensive structural similarity. Akt is composed of three functional domains: 1) the N-terminal pleckstrin homology domain (PH domain); 2) the kinase or catalytic domain; and 3) the small C-terminal regulatory domain referred to as the hydrophobic motif (HM) (Figure 1.1). Akt1 is known to be activated by phosphorylation at two key sites (5), Thr308 in the catalytic domain and Ser473 in the hydrophobic motif.

The PH domain was named after the pleckstrin protein, which is a major substrate of protein kinase C in platelets, lymphocytes, macrophages and monocytes (10). All three Akt isozymes possess an N-terminal PH domain. In its inactive conformation, the PH domain is settled in between the N- and C-terminal lobes of the kinase domain, which inhibits Akt activation by upstream kinase phosphorylation (11,12). Solution phase hydrogen/deuterium (H/D) exchange coupled with mass spectrometry analysis revealed that four β sheets (β 1, β 2, β 5, β 6) of the PH domain bear positively charged residues that are readily available for solvent accessibility in the inactive state and, which also makes it easier to bind with the negatively charged lipid membrane. This indicates that the conformation of the PH domain facilitates membrane binding (13) which is important for Akt1 activity in cells (see Section 1.3). Binding of the PH domain with phosphatidylinositols in the plasma membrane and endomembranes releases the auto-inhibitory effect of the PH domain. Akt1 lacking a PH domain shows higher activity compared to the full-length enzyme (14,15). A mutational screen of the PH/kinase domain interface showed that Akt can be activated by disruption of this interface interactions in cells. Some of these mutations

(Leu52Arg of PH domain, Asp323His of kinase domain) were demonstrated in human cancers. Both mutations cause cellular transformation and are oncogenic in vivo (16).

Structurally, the kinase domain of Akt is bilobal. The N terminal lobe is comprised of 5 β sheets and a partially ordered α C helix. There is a deep cleft in between the two lobes (17).

In the inactive state, certain regions of the Akt1 active site exist in a disordered state. The disordered region of the N-terminal lobe transforms into an ordered state following Thr308 phosphorylation (17,18). Hydrogen bond formation between the phosphate group and Arg273 and Lys297 are facilitated when Thr308 is phosphorylated. The hydrophobic interaction of the γ -methyl group of Thr308 with Cys310 is essential to establishing this hydrogen-bonding network.

The C-terminal hydrophobic motif includes the Ser473 phosphorylation site. Many studies suggest that full activation of the kinase requires phosphorylation at both Thr308 and Ser473 (5). Once phosphorylated at Ser473 the hydrophobic motif interacts with the kinase domain and facilitates disordered to ordered transition of the α C helix of the kinase domain (18).

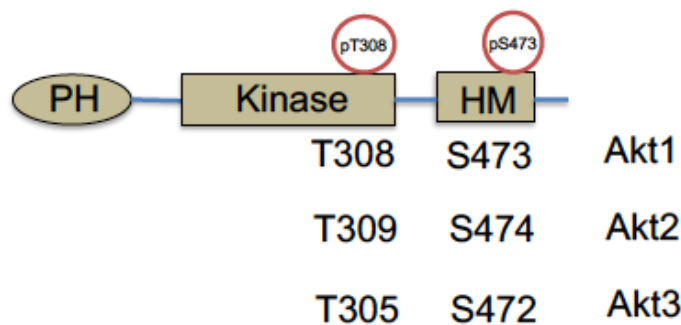


Figure 1.1 Domain structure and regulatory phosphorylation sites in Akt isozymes. The N-terminal pleckstrin homology (PH) domain, kinase domain with the activation site Thr308 and hydrophobic motif (HM) with the activation site Ser473 are shown. The homologous phosphorylation sites in Akt2 and Akt3 are noted.

1.3 Regulation of Akt1 activity

1.3.1 Phosphorylation-dependent activation of Akt

Akt1 is a major kinase in the phosphoinositide 3-kinase (PI3K)/Akt1 signaling pathway, which controls cell growth and survival (5). The impact of active Akt on cell survival occurs mainly via inhibiting the activity of its downstream targets as a result of phosphorylation. The activation of the Akt enzyme occurs in response to growth factors (GF), insulin, or cytokine mediated stimulation (20,21). GFs bind to receptor tyrosine kinases (RTK) in the plasma membrane. This leads to PI3K activation via three independent pathways: i) Once GFs bind to RTKs, the receptor kinases dimerize and auto-phosphorylate tyrosine residues. Upon phosphorylation, the p85 subunit of PI3K will bind to

RTKs and this will lead to the activation of the p110 catalytic subunit of PI3K (22).
ii) The next pathway of PI3K activation occurs through the effector protein growth factor receptor bound protein (GRB2). GRB2 is phosphorylated by activated RTKs (23). GRB2 also binds to the p85 subunit of PI3K, contributing to PI3K activation. iii) The third mode of activation is via RAS. RAS directly activates the p110 catalytic subunit of PI3K independent of p85 (24). PI3K then phosphorylates its immediate downstream target, a lipid second messenger in the plasma membrane, phosphatidylinositol-4, 5-bisphosphate (PIP₂), converting PIP₂ into phosphatidylinositol-4, 5-triphosphate (PIP₃). PH domain containing proteins like Akt1 and PDK1 bind to PIP₃ with high affinity (25) (Figure 1.2).

In Akt1, upon PH domain binding to PIP₃, the auto-inhibitory effect of PH domain is released, exposing the activation sites for phosphorylation (5) by the up-stream kinases, mechanistic target of rapamycin complex 2 (mTORC2) (26) and PDK1 (27). mTORC2 is the Ser473 kinase and PDK1 phosphorylates Thr308 in Akt1.

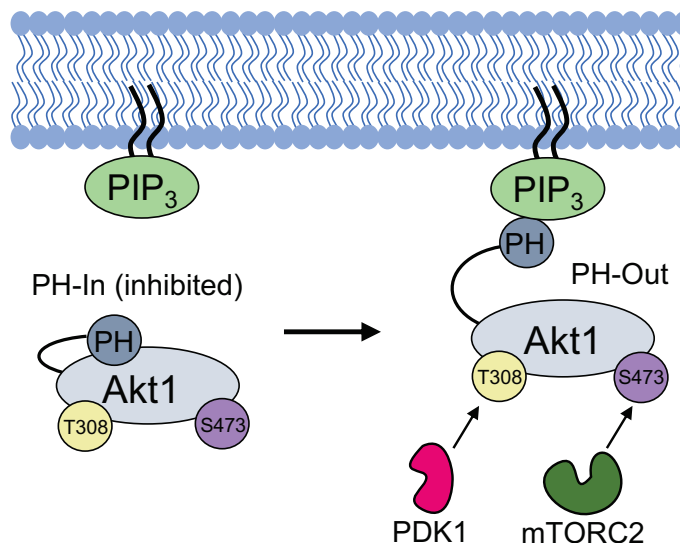


Figure 1.2: Akt1 activation via phosphorylation at sites Thr308 and Ser473. The upstream kinase for the Thr308 phosphorylation is phosphoinositide dependent kinase 1 (PDK1). Complete activation of Akt1 requires C terminal Ser473 phosphorylation. mTORC2 phosphorylates Ser473.

PDK1 is an AGC family of kinase related to Akt. Phosphorylation at Ser241 is essential for PDK1 activation. This is achieved through auto-phosphorylation and this leads to constitutively active enzyme in cell (28). The level of PDK1 phosphorylation does not change upon stimulation of the cells with insulin-like growth factors (29). PDK1 bears a PH domain which facilitates its binding to PIP2 and PIP3. Interestingly, PDK1 also activates kinases that lack PH domains, e.g., p70 ribosomal S6 kinase, p90 ribosomal S6 kinase. Instead, these kinases carry PDK1 docking sites in their C-terminal hydrophobic motifs (28).

mTORC2 is active as an effector protein upon growth factor and insulin mediated PI3K activation. The Sin1 subunit of mTORC2 includes a PH domain, which facilitates binding with PIP3 and co-localization with Akt (30). As with

Akt, upon PIP3 binding the auto-inhibition of Sin1 is released leading to the activation of mTORC2. mTORC2 is also responsible for phosphorylating the turn motif at Thr450 which leads to stabilization of newly synthesized Akt (31).

Since Cohen's group discovered PDK1 for the first time in 1997 (32), no other kinase has been reported-as a potential phosphorylating agent for Thr308 (27,32). PDK1 was originally extracted from rabbit skeletal muscle. Once Akt was incubated with this novel kinase PDK1, the level of phosphorylation and enzyme activity of Akt is increased. Since PDK1 activity was dependent on PIP3 concentration in activity assays, they named this novel kinase as phosphoinositide dependent kinase or PDK1 (32). The kinase for Ser473 was debated during earlier studies. Integrin-linked kinase (ILK) (33), mechanistic target of rapamycin complex (mTORC2) (26) and autophosphorylation by Akt itself (34) are among the possibilities for Ser473 phosphorylation. mTORC2 activation is now considered as the major route to Ser473 phosphorylation because the rictor-mTOR complex directly phosphorylates full length Akt1 *in vitro*, in *Drosophila* and human cells (26). Knocking down rictor or mTOR decreases Akt1 phosphorylation significantly at Ser473 in several cell lines including HEK293T, PC3 and HeLa (26).

Once phosphorylated, Akt1 in its active confirmation is released to the cytosol and nucleus to phosphorylate its substrates. According to live cell imaging studies using a Förster resonance energy transfer (FRET) based reporter system developed for Akt1 (B kinase reporter, BKAR), active Akt1 was present in the cytosol up to 10 min after growth factor stimulation (35). Akt1-dependent phosphorylation of perhaps as many as 100 substrates is known to occur in the cytosol (36) and nucleus (37). Fork head box O (FOXO) transcription factors are a group of well-known Akt substrates and FOXOs can be phosphorylated by Akt1

either in the cytosol or in the nucleus (38,39). Akt phosphorylation can direct trafficking of β -catenin in different cellular locations to the cytosol and nucleus (40). A recent study concluded that Akt1 is de-phosphorylated on a time scale of 10 minutes following its dissociation from the membrane (41). Although this study indicated that Akt1 activity may be restricted to the plasma membrane (41), multiple independent reports suggest Akt1-dependent substrate phosphorylation occurs on the time scale of seconds to minutes in different sub-cellular compartments (5,35,36).

1.3.2 Phosphatase-dependent deactivation of Akt1

Akt1 is deactivated following dephosphorylation of the lipid second messenger PIP3 by a lipid phosphatase, i.e., phosphatase and tensin homolog (PTEN) (42). *PTEN* was initially identified as a tumor suppressor gene. Its presence at the human chromosome 10q23.3 makes it highly susceptible to genetic alteration in human cancers (43). PTEN de-phosphorylates multiple substrates including tyrosines in proteins and lipids second messengers, e.g., PIP3. Further PTEN acts as a scaffolding protein in both the nucleus and cytoplasm. Loss of function of PTEN leads to high plasma concentrations of PIP3 and constitutively active Akt isozymes (44,45). Other than Akt mediated cellular functions, PTEN regulates the activation of c-Jun-N-terminal kinase (JNK) pathway, which regulates cell proliferation, embryonic development and apoptosis (46).

Specific phosphatases are known to dephosphorylate the regulatory sites in Akt1. Thr308 is dephosphorylated by protein phosphatase 2A (PP2A) (47,48). PP2A activity is highly regulated by a multiple regulatory subunits, which

determine the substrate specificity of this phosphatase. The catalytic subunit and the regulatory subunit A comprise the core structure of the phosphatase. The third regulatory subunit B varies depending on the functionality (47). The regulatory subunit B56 of PP2A recruits PP2A to Akt1 and de-phosphorylates Thr308 specifically (48). Other than cell cycle regulation and apoptosis, PP2A activity in maintaining cell adhesion and cytoskeleton maintenance are also established (49). PP2A is a tumour suppressor protein. Loss of activity of PP2A is associated with the onset of lung carcinoma and melanoma (50).

Phosphatases acting on Ser473 are the PH domain leucine-rich repeat protein phosphatases, PHLPP1 and PHLPP2. Other AGC kinases dephosphorylated by PHLPP family members include p70S6 kinase (S6K) and protein kinase C (PKC). PHLPP is also capable of suppressing histone phosphorylation to down regulate gene expression of RTKs (51).

A knockdown study revealed that p27 phosphorylation by Akt3 is regulated by PHLPP2. In breast cancer cells, knockdown of PHLPP2 increased p27 phosphorylation and knockdown of Akt3 decreased p27 phosphorylation. Knocking down of either isoform of PHLPP decreased the phosphorylation of Akt1 substrates, such as FOXO1 (52). This study concluded that PHLPP isoforms de-phosphorylate Akt in an isozyme dependent manner: PHLPP1 de-phosphorylates pSer473 of Akt isozymes 2 and 3 and PHLPP2 dephosphorylates pSer473 in Akt1 and Akt3 (52).

1.4 Additional post-translational modifications regulate Akt1

Apart from the two activation sites Thr308 and Ser473, independent studies have identified 25 additional phosphorylation sites on Akt1 (Figure 1.3). Thr450, which is found in the turn-motif is constitutively phosphorylated by mTORC2 (53). Phosphorylation of Thr450 enhances proper folding and stability of Akt1 by preventing ubiquitin-dependent degradation of the kinase (54). Ser124 was also identified as constitutively phosphorylated in different cell lines including HEK293 cells, (55) BT74 cell lysates (56) and HTC116 (57,58). There are 23 other phospho-sites identified in proteomic studies, yet for most sites the function is not known. Some phosphorylation sites are located in known substrate motifs for kinases upstream of Akt. For example, Ser129 in Akt1 is phosphorylated by casein kinase 2 (CK2), which increases Akt catalytic activity and promotes cell survival (59).

Other types of post-translational modifications are also involved in Akt regulation such as acetylation, ubiquitination, sumoylation and glycosylation (60). Acetylation of Akt is associated with reduced levels of phosphorylation and Akt activity. The acetyltransferase p300 is involved in Akt acetylation while sirtuin 1 (SIRT1) deacetylates Akt, promoting localization with PIP3 (61). Ubiquitination at Lys48 degrades Akt and abolishes Akt signaling (62). It is thought that sumoylation is an important modification for Akt activation (60), yet the exact mechanism of action is not known; the enzyme can be highly activated in the absence of SUMO (14). Finally, glycosylation of Thr308 inhibits Akt activation (63).

1.5 Akt activity drives cell growth and survival programs

Akt blocks pro-apoptotic proteins to promote cell survival. Key substrates regulated by Akt1-dependent phosphorylation include B cell lymphoma-2 (Bcl-2) homology domain 3 protein (BAD), the FOXO family of transcription factors, as well as p53 and the p53 regulating E3 ubiquitin ligase MDM2 (5).

Akt1-dependent phosphorylation impacts target proteins through multiple mechanisms. For example, Akt1 phosphorylates BAD at Ser136. Once phosphorylated, phospho-BAD binds to the regulatory protein 14-3-3 which prevents heterodimerization with BCL-2 and BCL-xl. Heterodimerization of BAD initiates cell death (64).

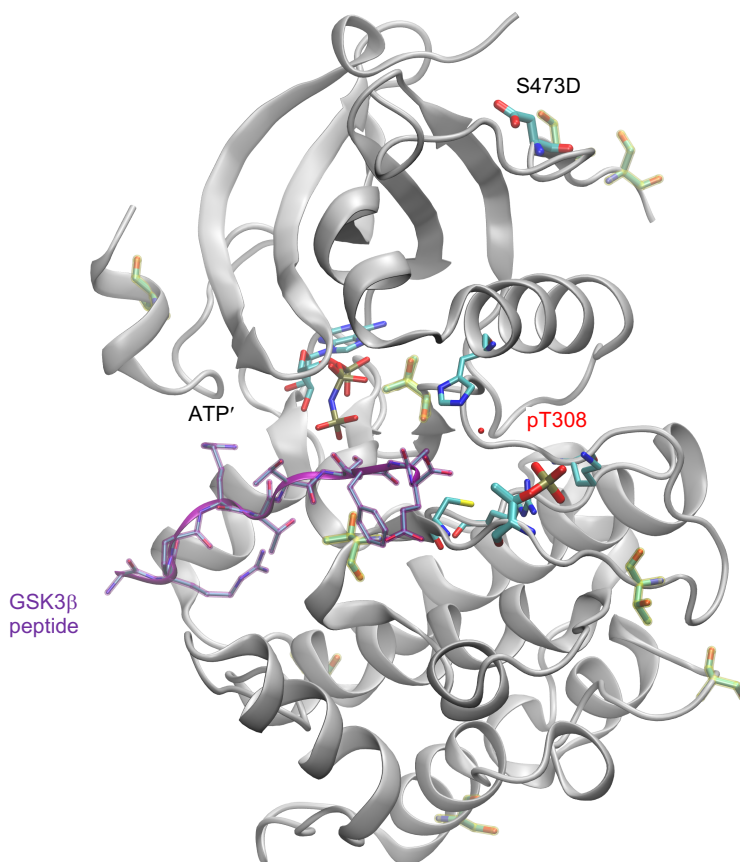


Figure 1.3. Structure of human Akt1. The two activation sites, Thr308 and Ser473; several additional phosphorylation sites (yellow highlight), GSK-3 β peptide in complex with the kinase domain (purple) of Akt1 and a non-hydrolyzable ATP analog (ATP' = AMP-PNP) are shown. Structural data were obtained from PDB codes: 3CQU (65), 1O6K (18).

GSK-3 isoforms were the first identified substrates of Akt1 (66). GSK-3 was initially identified as a key regulator in glycogen synthesis (67). Later it was discovered that GSK-3 regulates a plethora of cellular substrates involved in metabolism, signaling and structural functions. Akt targets include glycogen

synthase, β -catenin, cyclic-AMP response element binding (CREB) protein, insulin receptor substrate 1 (IRS1) and many others (68).

The prototypical Akt substrate, GSK-3 is a key mediator of both insulin signaling and Wnt signaling pathways (6,69). Akt1 inactivates GSK-3 by phosphorylating the N-terminal residues Ser21 in GSK3 α and Ser9 in GSK3 β , respectively (66). Inhibition of GSK3 activates glycogen synthase and converts glucose into glycogen. Inhibition of GSK-3 further promotes protein synthesis by activating the elongation factor 2B (eEF2B) (70).

A similar regulatory mechanism is associated with Akt1-dependent phosphorylation of the FOXO subfamily of fork head transcription factors, which includes FOXO1, FOXO3 and FOXO4. These transcription factors reside in the nucleus in growth factor deprived cells. The function of FOXO transcription factors varies depending on the cell type. In general, these factors promote expression of genes associated with cell differentiation, apoptosis, proliferation, and DNA damage (71). Once the cells are stimulated with growth factors and Akt is activated, Akt phosphorylates FOXO3 at phosphorylation sites Thr32, Ser253, and Ser315 (38). Phosphorylated FOXO3 is bound to 14-3-3, which displaces these transcription factors from their target apoptotic genes and facilitates their shuttling to the cytosol (38,72). Akt also acts on other FOXO members by a similar mechanism (reviewed in (73)).

Another target of Akt1 is the E3 ubiquitin ligase, MDM2, that negatively regulates the activity of the tumor suppressor protein and transcription factor p53 (74). MDM2 polyubiquitinates p53 and directs p53 for proteasomal degradation by enabling the association of p53 with p300, which is an E4 ubiquitin ligase. Activation of p53 by cellular responses including DNA damage,

promote cell cycle arrest and apoptosis (75,76). Akt promotes cell survival in part by counteracting p53-dependent apoptosis. Akt phosphorylates MDM2 at two sites, Ser166 and Ser186. Phosphorylated MDM2 translocate to the nucleus where it triggers p53 ubiquitination and degradation (5).

The mTORC1 protein complex acts as a central regulatory body in protein synthesis and cell growth. Akt phosphorylates tuberous sclerosis factor 2 (TSC2), a negative regulator of mTORC1 (77). Furthermore, Akt phosphorylates the PRAS40 subunit of mTORC1. PRAS40 is also a negative regulator of mTORC1 and Akt1 phosphorylation stabilizes mTORC1 activity by relieving the inhibitory effect of the PRAS40 domain (78). Through inactivation mechanisms of TSC2 and PRAS40 by Akt, mTORC1 activity is stabilized to promote protein synthesis and cell growth (77,78).

1.6 Hyper-activation of Akt in cancer

The significance of Akt activation in cancer has been a widely studied due to the prominent role of Akt1 in stimulating cell growth and survival pathways (79,80). Hyper-phosphorylated and hyper-active Akt1 is known to facilitate cancer cell survival creating a chemo-resistant environment (81). Due to these reasons Akt1 hyper-activation is considered as a hallmark of cancers, including breast (56), lung (82,83), and head and neck (84). Certain somatic mutations also cause Akt mediated carcinogenesis. Glu17Lys mutation at the PH domain promotes Akt1 binding to plasma membrane, which constitutively activates Akt1 (16). The other Akt isozymes are also hyperactive in certain cancers (1,79,85).

The phosphorylation status of Akt1 at positions Ser473 and Thr308 are used as diagnostic markers for multiple cancers (82,83,86). Higher levels of Thr308 and Ser473 phosphorylation is correlated with poor clinical outcome with patients with head and neck squamous cell carcinoma (84,87). Ser473 phosphorylation is the most commonly used biomarker for hyper-active Akt1 (82,88). Thr308 phosphorylation is also as a prognostic biomarker in non-small cell lung cancer (83) and acute myeloid leukemia (86).

Different cancer types are associated with isozyme specific Akt activity. Akt1 promotes cell proliferation in breast cancers (89), Akt2 highly active in hepatocellular carcinoma (90) and colorectal cancers (85) and Akt3 is hyper-active in estrogen receptor negative breast tumors and melanoma (80).

A somatic mutation; Glu17Lys in Akt1 was identified in breast cancers (91). This mutation is present in the PH domain and helps in constitutive membrane localization of Akt1 (91). Yet, there is no evidence of a similar PH domain mutation in Akt2 (80). A later study revealed the presence of Glu17Lys in Akt3 in two melanomas of one patient (92).

Since more than 50% of human tumors are characterized by hyper-active Akt, numerous studies have been conducted to develop Akt specific anticancer drugs (6). After successful in vitro, cell and animal model testing, small molecule inhibitors for Akt have been introduced to clinical trials. However, most of them failed in clinical trials. Inefficiencies during patient stratification, activation of the negative feed-back loops while Akt is inhibited (93), function of other PI3K effectors such as serum and glucocorticoid-induced protein kinase isoforms that activate downstream targets of PI3K/Akt pathway (94) are among the challenges for future work and in the development of clinically successful Akt inhibitors.

1.7 Investigating Akt phosphorylation in vitro and in cells

Amino acid substitutions are often used as a genetic tool to mimic the function of protein modifications. In studies of protein kinases there are multiple challenges that exist to producing proteins with site-specific phosphorylation(s). It is challenging to characterize the functionality of an individual phosphorylation site since a functional protein may require more than one phosphorylation for activity. For >90% of human phosphorylation sites, the upstream kinases for the site is not known (95). Even when upstream kinases are known, they often must be activated themselves by phosphorylation and many kinases phosphorylate at multiple sites in one protein, thus, hampering our ability to generate protein with programmed phosphorylation.

As a resolution to this problem, acidic amino acids like glutamate (Glu) or aspartate (Asp) are used to mimic phosphorylation. Though a phosphate adds two negative charges to the modified protein, the closest resemblance for this to be selected from the canonical amino acid pool are the acidic residues Glu or Asp. Ala mutations are used to mimic non-phosphorylation. Although mimics may be interesting mutants, they are unable to mimic true phosphorylation sometimes.

Thr308Asp mutant of Akt1 expressed and purified as an immunoprecipitate in serum starved COS-1 cell line was 5-fold more active than the wild type Akt (55). When MAPKAP-kinase 2 activated this mutant in vitro, it was 5-fold more active relative to the activated wild type Akt1 in an in vitro kinase assay. The authors conclude that the Asp308 is a suitable mutation for

mimicking phosphorylation (55). However, when the same construct was used to express Thr308Asp mutant in HEK293 cells, the enzyme was totally inactive according to an independent group of investigators (15). Further studies revealed that Asp308 mutant did not show transforming ability in contrast to the truly phosphorylated Akt in chicken embryonic fibroblasts (96). Akt studies often use these mimics to characterize Akt functionality (41,58), however, the preponderance of direct evidence suggests that in Akt1, acidic amino acids fail to mimic phosphorylation (96).

To overcome the drawbacks listed above, novel synthetic biology approaches enable expansion of the genetic code with phosphoserine (97). Genetic code expansion refers to the ability to reassign codons, normally a stop codon, to include additional or unnatural amino acids beyond the canonical 20. These systems often require a tRNA and aminoacyl-tRNA synthetase pair that is orthogonal to the host expression system (98,99), which means the tRNA and synthetase enzyme pair should not cross react with the host tRNAs and tRNA synthetase enzymes. In these systems the unnatural or modified amino acids are incorporated into proteins during translation. Genetic code expansion is used to synthesize phosphoproteins in *Escherichia coli* with site-specifically introduced phosphoserine residues. George et al. (2016) have site specifically incorporated phosphoserine into ubiquitin (100). An active form of human mitogen-activated ERK activating kinase 1 (MEK1) was the first kinase to be produced by site-specific incorporation of phosphoserine (97). Recent studies have reported site-specific incorporation of phosphotyrosine and phosphothreonine following related methodologies (101,102). Incorporation of phosphotyrosine has confirmed by electron spray ionization mass spec (ESI-MS) analysis using *Xenopus* calmodulin and green fluorescent protein (GFP) (101). The yields of calmodulin

and GFP were 1.0 mg/L and 1.5 mg/L, respectively. Incorporation of phosphotyrosine produced a mixture of phosphorylated and non-phosphorylated tyrosines. Removal of a phosphatase gene (*ycdX*) enabled production of a homogeneous mixture of phosphothreonine-incorporated ubiquitin (102).

Co-expression of upstream kinases to phosphorylate its target substrates is another feasible method used to characterize the functionality of phosphorylation sites. This method has already being used to phosphorylate Thr308 of Akt1 by co-expressing upstream kinase PDK1 in *E. coli* (15).

Another method is the in vitro phosphorylation of target substrates. In this method the upstream kinases along with ATP are incubated to facilitate phosphorylation. However, in kinases like Akt there exist special cellular requirements like the presence of lipid second messengers and phosphorylation of multiple upstream kinases to facilitate phosphorylation of Akt1, limiting the yield of phospho-protein and the usefulness of this approach for Akt1 (103).

1.8 Scope of the thesis

Since Akt1 is a proto-oncogene, its activation mechanisms and therapeutic interventions have been studied extensively (5). However, apart from the known contribution to activation, the specific role of each activation site, i.e., Thr308 and Ser473, on Akt1 activity are not known. Because the phosphorylation of each activation site is used as a biomarker for different types of cancers, the relevance of Akt1 phosphorylation status to disease is well documented (82-84). The inability to produce site specifically phosphorylated Akt variants have created a

barrier in unraveling the undiscovered and unique functionality of the two activation sites.

As discussed in Section 1.7, since the methods used in recent studies (i.e. phosphomimetics, Ala mutations, partially active Akt produced in eukaryotic cells) are not the best suited candidates for this purpose, I developed a facile strategy to produce site-specifically and quantitatively phosphorylated Akt1 variants in *E. coli*. I used a combined approach of using genetic code expansion strategy with upstream kinase expression to produce doubly phosphorylated Akt1. Expression of these systems individually enabled production of singly phosphorylated Akt1 variants.

In chapter 2, I quantified how each phosphorylation site contributes to the overall activation of Akt1. We have used *in vitro* assays, mass spectrometry and, in collaboration with Alexandra Newton (UCSD) live cell imaging techniques to discover the unique role of each phosphorylation site on the activation of Akt1 *in vitro* and in mammalian cells (19).

Chapter 3 focuses on the impact of each phosphorylation site on the auto- and chemical inhibition of Akt1. Deletion of PH domain to release the auto-inhibitory effect increased the enzyme activity for all three phosphorylated Akt1 variants. Phosphorylation of Ser473 showed resistance to chemical inhibition by the clinically relevant inhibitor Akti1/2.

The exact mechanism of Akt's substrate selection is still under investigation. In chapter 4, I determined how the phosphorylation status of Akt1 modulates substrate selectivity. I used peptides derived from all known substrates of Akt1, alanine mutagenic scans, and oriented peptide array library screens to systematically reveal the ability of Akt1 phosphorylation status to

globally regulate substrate selectivity. The data revealed that each phospho-form of Akt1 has distinct substrate requirements and Ser473 plays a key role in modulating global substrate selectivity.

Using genetic code expansion and upstream kinase phosphorylation, I was able to produce site specifically phosphorylated Akt1 variants which act as indispensable tools in characterizing the functionality of each activation site of Akt1. This is the first time that genetic code expansion has been used to produce Akt1 carrying single phosphorylation at Ser473. Further we have shown that upstream kinase expression is a compatible mode of phosphorylation with genetic code expansion. This novel methodological advancement could be employed with other cellular proteins, especially with kinases, to generate site specifically phosphorylated protein variants that will help to overcome the existing limitations on characterizing the unique functionality of specific phosphorylation sites.

1.9 References

1. Staal, S. P. (1987) 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* **84**, 5034-5037
2. Bellacosa, A., Testa, J. R., Staal, S. P., and Tsichlis, P. N. (1991) A retroviral oncogene, akt, encoding a serine-threonine kinase containing an SH2-like region. *Science* **254**, 274-277
3. Jones, P. F., Jakubowicz, T., Pitossi, F. J., Maurer, F., and Hemmings, B. A. (1991) Molecular cloning and identification of a serine/threonine protein kinase of the second-messenger subfamily. *Proc Natl Acad Sci U S A* **88**, 4171-4175
4. Coffey, P. J., and Woodgett, J. R. (1991) Molecular cloning and characterisation of a novel putative protein-serine kinase related to the cAMP-dependent and protein kinase C families. *Eur J Biochem* **201**, 475-481
5. Manning, B. D., and Cantley, L. C. (2007) AKT/PKB signaling: navigating downstream. *Cell* **129**, 1261-1274
6. Manning, B. D., and Toker, A. (2017) AKT/PKB Signaling: Navigating the Network. *Cell* **169**, 381-405
7. Chen, W. S., Xu, P. Z., Gottlob, K., Chen, M. L., Sokol, K., Shiyanova, T., Roninson, I., Weng, W., Suzuki, R., Tobe, K., Kadowaki, T., and Hay, N. (2001) Growth retardation and increased apoptosis in mice with homozygous disruption of the Akt1 gene. *Genes Dev* **15**, 2203-2208
8. Garofalo, R. S., Orena, S. J., Rafidi, K., Torchia, A. J., Stock, J. L., Hildebrandt, A. L., Coskran, T., Black, S. C., Brees, D. J., Wicks, J. R., McNeish, J. D., and Coleman, K. G. (2003) Severe diabetes, age-dependent loss of adipose tissue, and mild growth deficiency in mice lacking Akt2/PKB beta. *J Clin Invest* **112**, 197-208
9. Tschopp, O., Yang, Z. Z., Brodbeck, D., Dummler, B. A., Hemmings-Mieszczak, M., Watanabe, T., Michaelis, T., Frahm, J., and Hemmings, B. A. (2005) Essential role of protein kinase B gamma (PKB gamma/Akt3) in postnatal brain development but not in glucose homeostasis. *Development* **132**, 2943-2954
10. Lian, L., Wang, Y., Flick, M., Choi, J., Scott, E. W., Degen, J., Lemmon, M. A., and Abrams, C. S. (2009) Loss of pleckstrin defines a novel pathway for PKC-mediated exocytosis. *Blood* **113**, 3577-3584
11. Calleja, V., Alcor, D., Laguerre, M., Park, J., Vojnovic, B., Hemmings, B. A., Downward, J., Parker, P. J., and Larijani, B. (2007) Intramolecular and intermolecular interactions of protein kinase B define its activation in vivo. *PLoS Biol* **5**, e95
12. Wu, W. I., Voegtli, W. C., Sturgis, H. L., Dizon, F. P., Vigers, G. P., and Brandhuber, B. J. (2010) Crystal structure of human AKT1 with an allosteric inhibitor reveals a new mode of kinase inhibition. *PLoS One* **5**, e12913
13. Guo, M., Huang, B. X., and Kim, H. Y. (2009) Conformational changes in Akt1 activation probed by amide hydrogen/deuterium exchange and nano-

- electrospray ionization mass spectrometry. *Rapid Commun Mass Spectrom* **23**, 1885-1891
14. Balasuriya, N., McKenna, M., Liu, X., Li, S. S. C., and O'Donoghue, P. (2018) Phosphorylation-Dependent Inhibition of Akt1. *Genes (Basel)* **9**
 15. Klein, S., Geiger, T., Linchevski, I., Lebendiker, M., Itkin, A., Assayag, K., and Levitzki, A. (2005) Expression and purification of active PKB kinase from *Escherichia coli*. *Protein Expr Purif* **41**, 162-169
 16. Parikh, C., Janakiraman, V., Wu, W. I., Foo, C. K., Kljavin, N. M., Chaudhuri, S., Stawiski, E., Lee, B., Lin, J., Li, H., Lorenzo, M. N., Yuan, W., Guillory, J., Jackson, M., Rondon, J., Franke, Y., Bowman, K. K., Sagolla, M., Stinson, J., Wu, T. D., Wu, J., Stokoe, D., Stern, H. M., Brandhuber, B. J., Lin, K., Skelton, N. J., and Seshagiri, S. (2012) Disruption of PH-kinase domain interactions leads to oncogenic activation of AKT in human cancers. *Proc Natl Acad Sci U S A* **109**, 19368-19373
 17. Huang, X., Begley, M., Morgenstern, K. A., Gu, Y., Rose, P., Zhao, H., and Zhu, X. (2003) Crystal structure of an inactive Akt2 kinase domain. *Structure* **11**, 21-30
 18. Yang, J., Cron, P., Good, V. M., Thompson, V., Hemmings, B. A., and Barford, D. (2002) Crystal structure of an activated Akt/protein kinase B ternary complex with GSK3-peptide and AMP-PNP. *Nat Struct Biol* **9**, 940-944
 19. Balasuriya, N., Kunkel, M. T., Liu, X., Biggar, K. K., Li, S. S., Newton, A. C., and O'Donoghue, P. (2018) Genetic code expansion and live cell imaging reveal that Thr308 phosphorylation is irreplaceable and sufficient for Akt1 activity. *J Biol Chem* **293**, 10744-10756
 20. Castellano, E., and Downward, J. (2011) RAS Interaction with PI3K: More Than Just Another Effector Pathway. *Genes Cancer* **2**, 261-274
 21. Liao, Y., and Hung, M. C. (2010) Physiological regulation of Akt activity and stability. *Am J Transl Res* **2**, 19-42
 22. Domchek, S. M., Auger, K. R., Chatterjee, S., Burke, T. R., Jr., and Shoelson, S. E. (1992) Inhibition of SH2 domain/phosphoprotein association by a nonhydrolyzable phosphonopeptide. *Biochemistry* **31**, 9865-9870
 23. Pawson, T. (2004) Specificity in signal transduction: from phosphotyrosine-SH2 domain interactions to complex cellular systems. *Cell* **116**, 191-203
 24. Castellano, E., and Downward, J. (2010) Role of RAS in the regulation of PI 3-kinase. *Curr Top Microbiol Immunol* **346**, 143-169
 25. Musacchio, A., Gibson, T., Rice, P., Thompson, J., and Saraste, M. (1993) The PH domain: a common piece in the structural patchwork of signalling proteins. *Trends in biochemical sciences* **18**, 343-348
 26. Sarbassov, D. D., Guertin, D. A., Ali, S. M., and Sabatini, D. M. (2005) Phosphorylation and regulation of Akt/PKB by the rictor-mTOR complex. *Science* **307**, 1098-1101
 27. Alessi, D. R., and Cohen, P. (1998) Mechanism of activation and function of protein kinase B. *Curr Opin Genet Dev* **8**, 55-62

28. Komander, D., Kular, G., Deak, M., Alessi, D. R., and van Aalten, D. M. (2005) Role of T-loop phosphorylation in PDK1 activation, stability, and substrate binding. *J Biol Chem* **280**, 18797-18802
29. Casamayor, A., Morrice, N. A., and Alessi, D. R. (1999) 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* **342 (Pt 2)**, 287-292
30. Saxton, R. A., and Sabatini, D. M. (2017) mTOR Signaling in Growth, Metabolism, and Disease. *Cell* **169**, 361-371
31. Liu, P., Gan, W., Chin, Y. R., Ogura, K., Guo, J., Zhang, J., Wang, B., Blenis, J., Cantley, L. C., Toker, A., Su, B., and Wei, W. (2015) PtdIns(3,4,5)P3-Dependent Activation of the mTORC2 Kinase Complex. *Cancer Discov* **5**, 1194-1209
32. Alessi, D. R., James, S. R., Downes, C. P., Holmes, A. B., Gaffney, P. R., Reese, C. B., and Cohen, P. (1997) Characterization of a 3-phosphoinositide-dependent protein kinase which phosphorylates and activates protein kinase Balpha. *Curr Biol* **7**, 261-269
33. Persad, S., Attwell, S., Gray, V., Mawji, N., Deng, J. T., Leung, D., Yan, J., Sanghera, J., Walsh, M. P., and Dedhar, S. (2001) Regulation of protein kinase B/Akt-serine 473 phosphorylation by integrin-linked kinase: critical roles for kinase activity and amino acids arginine 211 and serine 343. *J Biol Chem* **276**, 27462-27469
34. Toker, A., and Newton, A. C. (2000) Akt/protein kinase B is regulated by autophosphorylation at the hypothetical PDK-2 site. *J Biol Chem* **275**, 8271-8274
35. Kunkel, M. T., Ni, Q., Tsiens, R. Y., Zhang, J., and Newton, A. C. (2005) Spatio-temporal dynamics of protein kinase B/Akt signaling revealed by a genetically encoded fluorescent reporter. *J Biol Chem* **280**, 5581-5587
36. Rosner, M., Hanneder, M., Freilinger, A., and Hengstschlager, M. (2007) Nuclear/cytoplasmic localization of Akt activity in the cell cycle. *Amino Acids* **32**, 341-345
37. Martelli, A. M., Tabellini, G., Bressanin, D., Ognibene, A., Goto, K., Cocco, L., and Evangelisti, C. (2012) The emerging multiple roles of nuclear Akt. *Biochim Biophys Acta* **1823**, 2168-2178
38. Tzivion, G., Dobson, M., and Ramakrishnan, G. (2011) FoxO transcription factors; Regulation by AKT and 14-3-3 proteins. *Biochim Biophys Acta* **1813**, 1938-1945
39. Uranga, R. M., Katz, S., and Salvador, G. A. (2013) Enhanced phosphatidylinositol 3-kinase (PI3K)/Akt signaling has pleiotropic targets in hippocampal neurons exposed to iron-induced oxidative stress. *J Biol Chem* **288**, 19773-19784
40. Fang, D., Hawke, D., Zheng, Y., Xia, Y., Meisenhelder, J., Nika, H., Mills, G. B., Kobayashi, R., Hunter, T., and Lu, Z. (2007) Phosphorylation of beta-catenin by AKT promotes beta-catenin transcriptional activity. *J Biol Chem* **282**, 11221-11229

41. Ebner, M., Lucic, I., Leonard, T. A., and Yudushkin, I. (2017) PI(3,4,5)P3 Engagement Restricts Akt Activity to Cellular Membranes. *Mol Cell* **65**, 416-431 e416
42. Chalhoub, N., and Baker, S. J. (2009) PTEN and the PI3-kinase pathway in cancer. *Annu Rev Pathol* **4**, 127-150
43. Steck, P. A., Pershouse, M. A., Jasser, S. A., Yung, W. K., Lin, H., Ligon, A. H., Langford, L. A., Baumgard, M. L., Hattier, T., Davis, T., Frye, C., Hu, R., Swedlund, B., Teng, D. H., and Tavtigian, S. V. (1997) Identification of a candidate tumour suppressor gene, MMAC1, at chromosome 10q23.3 that is mutated in multiple advanced cancers. *Nat Genet* **15**, 356-362
44. Lee, Y. R., Chen, M., and Pandolfi, P. P. (2018) The functions and regulation of the PTEN tumour suppressor: new modes and prospects. *Nat Rev Mol Cell Biol* **19**, 547-562
45. Song, M. S., Salmena, L., and Pandolfi, P. P. (2012) The functions and regulation of the PTEN tumour suppressor. *Nat Rev Mol Cell Biol* **13**, 283-296
46. Vivanco, I., Palaskas, N., Tran, C., Finn, S. P., Getz, G., Kennedy, N. J., Jiao, J., Rose, J., Xie, W., Loda, M., Golub, T., Mellinghoff, I. K., Davis, R. J., Wu, H., and Sawyers, C. L. (2007) Identification of the JNK signaling pathway as a functional target of the tumor suppressor PTEN. *Cancer Cell* **11**, 555-569
47. Janssens, V., and Goris, J. (2001) Protein phosphatase 2A: a highly regulated family of serine/threonine phosphatases implicated in cell growth and signalling. *Biochem J* **353**, 417-439
48. Kuo, Y. C., Huang, K. Y., Yang, C. H., Yang, Y. S., Lee, W. Y., and Chiang, C. W. (2008) Regulation of phosphorylation of Thr-308 of Akt, cell proliferation, and survival by the B55alpha regulatory subunit targeting of the protein phosphatase 2A holoenzyme to Akt. *J Biol Chem* **283**, 1882-1892
49. Young, M. R., Liu, S. W., and Meisinger, J. (2003) Protein phosphatase-2A restricts migration of Lewis lung carcinoma cells by modulating the phosphorylation of focal adhesion proteins. *Int J Cancer* **103**, 38-44
50. Calin, G. A., di Iasio, M. G., Caprini, E., Vorechovsky, I., Natali, P. G., Sozzi, G., Croce, C. M., Barbanti-Brodano, G., Russo, G., and Negrini, M. (2000) Low frequency of alterations of the alpha (PPP2R1A) and beta (PPP2R1B) isoforms of the subunit A of the serine-threonine phosphatase 2A in human neoplasms. *Oncogene* **19**, 1191-1195
51. Grzechnik, A. T., and Newton, A. C. (2016) PHLPPing through history: a decade in the life of PHLPP phosphatases. *Biochem Soc Trans* **44**, 1675-1682
52. Brognard, J., Sierrecki, E., Gao, T., and Newton, A. C. (2007) PHLPP and a second isoform, PHLPP2, differentially attenuate the amplitude of Akt signaling by regulating distinct Akt isoforms. *Mol Cell* **25**, 917-931
53. Ikenoue, T., Inoki, K., Yang, Q., Zhou, X., and Guan, K. L. (2008) Essential function of TORC2 in PKC and Akt turn motif phosphorylation, maturation and signalling. *EMBO J* **27**, 1919-1931

54. Facchinetti, V., Ouyang, W., Wei, H., Soto, N., Lazorchak, A., Gould, C., Lowry, C., Newton, A. C., Mao, Y., Miao, R. Q., Sessa, W. C., Qin, J., Zhang, P., Su, B., and Jacinto, E. (2008) The mammalian target of rapamycin complex 2 controls folding and stability of Akt and protein kinase C. *EMBO J* **27**, 1932-1943
55. Alessi, D. R., Andjelkovic, M., Caudwell, B., Cron, P., Morrice, N., Cohen, P., and Hemmings, B. A. (1996) Mechanism of activation of protein kinase B by insulin and IGF-1. *EMBO J* **15**, 6541-6551
56. Iacovides, D. C., Johnson, A. B., Wang, N., Boddapati, S., Korkola, J., and Gray, J. W. (2013) Identification and quantification of AKT isoforms and phosphoforms in breast cancer using a novel nanofluidic immunoassay. *Mol Cell Proteomics* **12**, 3210-3220
57. Guo, H., Gao, M., Lu, Y., Liang, J., Lorenzi, P. L., Bai, S., Hawke, D. H., Li, J., Dogruluk, T., Scott, K. L., Jonasch, E., Mills, G. B., and Ding, Z. (2014) Coordinate phosphorylation of multiple residues on single AKT1 and AKT2 molecules. *Oncogene* **33**, 3463-3472
58. Bellacosa, A., Chan, T. O., Ahmed, N. N., Datta, K., Malstrom, S., Stokoe, D., McCormick, F., Feng, J., and Tsichlis, P. (1998) Akt activation by growth factors is a multiple-step process: the role of the PH domain. *Oncogene* **17**, 313-325
59. Di Maira, G., Salvi, M., Arrigoni, G., Marin, O., Sarno, S., Brustolon, F., Pinna, L. A., and Ruzzene, M. (2005) Protein kinase CK2 phosphorylates and upregulates Akt/PKB. *Cell Death Differ* **12**, 668-677
60. Chan, C. H., Jo, U., Kohrman, A., Rezaeian, A. H., Chou, P. C., Logothetis, C., and Lin, H. K. (2014) Posttranslational regulation of Akt in human cancer. *Cell Biosci* **4**, 59
61. Sundaresan, N. R., Pillai, V. B., Wolfgeher, D., Samant, S., Vasudevan, P., Parekh, V., Raghuraman, H., Cunningham, J. M., Gupta, M., and Gupta, M. P. (2011) The deacetylase SIRT1 promotes membrane localization and activation of Akt and PDK1 during tumorigenesis and cardiac hypertrophy. *Sci Signal* **4**, ra46
62. Xiang, T., Ohashi, A., Huang, Y., Pandita, T. K., Ludwig, T., Powell, S. N., and Yang, Q. (2008) Negative Regulation of AKT Activation by BRCA1. *Cancer Res* **68**, 10040-10044
63. Wang, S., Huang, X., Sun, D., Xin, X., Pan, Q., Peng, S., Liang, Z., Luo, C., Yang, Y., Jiang, H., Huang, M., Chai, W., Ding, J., and Geng, M. (2012) Extensive crosstalk between O-GlcNAcylation and phosphorylation regulates Akt signaling. *PLoS One* **7**, e37427
64. Datta, S. R., Dudek, H., Tao, X., Masters, S., Fu, H., Gotoh, Y., and Greenberg, M. E. (1997) Akt phosphorylation of BAD couples survival signals to the cell-intrinsic death machinery. *Cell* **91**, 231-241
65. Lippa, B., Pan, G., Corbett, M., Li, C., Kauffman, G. S., Pandit, J., Robinson, S., Wei, L., Kozina, E., Marr, E. S., Borzillo, G., Knauth, E., Barbacci-Tobin, E. G., Vincent, P., Troutman, M., Baker, D., Rajamohan, F., Kakar, S., Clark, T., and Morris, J. (2008) Synthesis and structure based optimization of novel Akt inhibitors. *Bioorganic & medicinal chemistry letters* **18**, 3359-3363

66. Cross, D. A., Alessi, D. R., Cohen, P., Andjelkovich, M., and Hemmings, B. A. (1995) Inhibition of glycogen synthase kinase-3 by insulin mediated by protein kinase B. *Nature* **378**, 785-789
67. Woodgett, J. R., and Cohen, P. (1984) Multisite phosphorylation of glycogen synthase. Molecular basis for the substrate specificity of glycogen synthase kinase-3 and casein kinase-II (glycogen synthase kinase-5). *Biochim Biophys Acta* **788**, 339-347
68. Lee, J., and Kim, M. S. (2007) The role of GSK3 in glucose homeostasis and the development of insulin resistance. *Diabetes Res Clin Pract* **77 Suppl 1**, S49-57
69. Beurel, E., Grieco, S. F., and Jope, R. S. (2015) Glycogen synthase kinase-3 (GSK3): regulation, actions, and diseases. *Pharmacol Ther* **148**, 114-131
70. Welsh, G. I., and Proud, C. G. (1993) Glycogen synthase kinase-3 is rapidly inactivated in response to insulin and phosphorylates eukaryotic initiation factor eIF-2B. *Biochem J* **294 (Pt 3)**, 625-629
71. Farhan, M., Wang, H., Gaur, U., Little, P. J., Xu, J., and Zheng, W. (2017) FOXO Signaling Pathways as Therapeutic Targets in Cancer. *Int J Biol Sci* **13**, 815-827
72. Brunet, A., Park, J., Tran, H., Hu, L. S., Hemmings, B. A., and Greenberg, M. E. (2001) Protein kinase SGK mediates survival signals by phosphorylating the forkhead transcription factor FKHRL1 (FOXO3a). *Mol Cell Biol* **21**, 952-965
73. Burgering, B. M., and Medema, R. H. (2003) Decisions on life and death: FOXO Forkhead transcription factors are in command when PKB/Akt is off duty. *Journal of leukocyte biology* **73**, 689-701
74. Mayo, L. D., and Donner, D. B. (2001) A phosphatidylinositol 3-kinase/Akt pathway promotes translocation of Mdm2 from the cytoplasm to the nucleus. *Proc Natl Acad Sci U S A* **98**, 11598-11603
75. Chen, J. (2016) The Cell-Cycle Arrest and Apoptotic Functions of p53 in Tumor Initiation and Progression. *Cold Spring Harb Perspect Med* **6**, a026104
76. Shi, D., and Gu, W. (2012) Dual Roles of MDM2 in the Regulation of p53: Ubiquitination Dependent and Ubiquitination Independent Mechanisms of MDM2 Repression of p53 Activity. *Genes Cancer* **3**, 240-248
77. Huang, J., and Manning, B. D. (2009) A complex interplay between Akt, TSC2 and the two mTOR complexes. *Biochem Soc Trans* **37**, 217-222
78. Wiza, C., Nascimento, E. B., and Ouwens, D. M. (2012) Role of PRAS40 in Akt and mTOR signaling in health and disease. *Am J Physiol Endocrinol Metab* **302**, E1453-1460
79. Altomare, D. A., and Testa, J. R. (2005) Perturbations of the AKT signaling pathway in human cancer. *Oncogene* **24**, 7455-7464
80. Gonzalez, E., and McGraw, T. E. (2009) The Akt kinases: isoform specificity in metabolism and cancer. *Cell Cycle* **8**, 2502-2508
81. Bellacosa, A., Kumar, C. C., Di Cristofano, A., and Testa, J. R. (2005) Activation of AKT kinases in cancer: implications for therapeutic targeting. *Adv Cancer Res* **94**, 29-86

82. Tsao, A. S., McDonnell, T., Lam, S., Putnam, J. B., Bekele, N., Hong, W. K., and Kurie, J. M. (2003) Increased phospho-AKT (Ser(473)) expression in bronchial dysplasia: implications for lung cancer prevention studies. *Cancer Epidemiol Biomarkers Prev* **12**, 660-664
83. Vincent, E. E., Elder, D. J., Thomas, E. C., Phillips, L., Morgan, C., Pawade, J., Sohail, M., May, M. T., Hetzel, M. R., and Tavares, J. M. (2011) Akt phosphorylation on Thr308 but not on Ser473 correlates with Akt protein kinase activity in human non-small cell lung cancer. *Br J Cancer* **104**, 1755-1761
84. Freudlsperger, C., Horn, D., Weissfuss, S., Weichert, W., Weber, K. J., Saure, D., Sharma, S., Dyckhoff, G., Grabe, N., Plinkert, P., Hoffmann, J., Freier, K., and Hess, J. (2015) Phosphorylation of AKT(Ser473) serves as an independent prognostic marker for radiosensitivity in advanced head and neck squamous cell carcinoma. *Int J Cancer* **136**, 2775-2785
85. Cheng, J. Q., Ruggeri, B., Klein, W. M., Sonoda, G., Altomare, D. A., Watson, D. K., and Testa, J. R. (1996) Amplification of AKT2 in human pancreatic cells and inhibition of AKT2 expression and tumorigenicity by antisense RNA. *Proc Natl Acad Sci U S A* **93**, 3636-3641
86. Gallay, N., Dos Santos, C., Cuzin, L., Bousquet, M., Simmonet Gouy, V., Chaussade, C., Attal, M., Payrastre, B., Demur, C., and Recher, C. (2009) The level of AKT phosphorylation on threonine 308 but not on serine 473 is associated with high-risk cytogenetics and predicts poor overall survival in acute myeloid leukaemia. *Leukemia* **23**, 1029-1038
87. Massihnia, D., Avan, A., Funel, N., Maftouh, M., van Krieken, A., Granchi, C., Raktoe, R., Boggi, U., Aicher, B., Minutolo, F., Russo, A., Leon, L. G., Peters, G. J., and Giovannetti, E. (2017) Phospho-Akt overexpression is prognostic and can be used to tailor the synergistic interaction of Akt inhibitors with gemcitabine in pancreatic cancer. *Journal of hematology & oncology* **10**, 9
88. Slipicevic, A., Holm, R., Nguyen, M. T., Bohler, P. J., Davidson, B., and Florenes, V. A. (2005) Expression of activated Akt and PTEN in malignant melanomas: relationship with clinical outcome. *Am J Clin Pathol* **124**, 528-536
89. Riggio, M., Perrone, M. C., Polo, M. L., Rodriguez, M. J., May, M., Abba, M., Lanari, C., and Novaro, V. (2017) AKT1 and AKT2 isoforms play distinct roles during breast cancer progression through the regulation of specific downstream proteins. *Scientific reports* **7**, 44244
90. Xu, X., Sakon, M., Nagano, H., Hiraoka, N., Yamamoto, H., Hayashi, N., Dono, K., Nakamori, S., Umeshita, K., Ito, Y., Matsuura, N., and Monden, M. (2004) Akt2 expression correlates with prognosis of human hepatocellular carcinoma. *Oncology reports* **11**, 25-32
91. Carpten, J. D., Faber, A. L., Horn, C., Donoho, G. P., Briggs, S. L., Robbins, C. M., Hostetter, G., Boguslawski, S., Moses, T. Y., Savage, S., Uhlik, M., Lin, A., Du, J., Qian, Y. W., Zeckner, D. J., Tucker-Kellogg, G., Touchman, J., Patel, K., Mousses, S., Bittner, M., Schevitz, R., Lai, M. H., Blanchard, K. L., and Thomas, J. E. (2007)

- A transforming mutation in the pleckstrin homology domain of AKT1 in cancer. *Nature* **448**, 439-444
92. Davies, M. A., Stemke-Hale, K., Tellez, C., Calderone, T. L., Deng, W., Prieto, V. G., Lazar, A. J., Gershenwald, J. E., and Mills, G. B. (2008) A novel AKT3 mutation in melanoma tumours and cell lines. *Br J Cancer* **99**, 1265-1268
 93. Chandarlapaty, S., Sawai, A., Scaltriti, M., Rodrik-Outmezguine, V., Grbovic-Huezo, O., Serra, V., Majumder, P. K., Baselga, J., and Rosen, N. (2011) AKT inhibition relieves feedback suppression of receptor tyrosine kinase expression and activity. *Cancer Cell* **19**, 58-71
 94. Vasudevan, K. M., Barbie, D. A., Davies, M. A., Rabinovsky, R., McNear, C. J., Kim, J. J., Hennessy, B. T., Tseng, H., Pochanard, P., Kim, S. Y., Dunn, I. F., Schinzel, A. C., Sandy, P., Hoersch, S., Sheng, Q., Gupta, P. B., Boehm, J. S., Reiling, J. H., Silver, S., Lu, Y., Stemke-Hale, K., Dutta, B., Joy, C., Sahin, A. A., Gonzalez-Angulo, A. M., Lluch, A., Rameh, L. E., Jacks, T., Root, D. E., Lander, E. S., Mills, G. B., Hahn, W. C., Sellers, W. R., and Garraway, L. A. (2009) AKT-independent signaling downstream of oncogenic PIK3CA mutations in human cancer. *Cancer Cell* **16**, 21-32
 95. Hornbeck, P. V., Kornhauser, J. M., Tkachev, S., Zhang, B., Skrzypek, E., Murray, B., Latham, V., and Sullivan, M. (2012) PhosphoSitePlus: a comprehensive resource for investigating the structure and function of experimentally determined post-translational modifications in man and mouse. *Nucleic acids research* **40**, D261-270
 96. Hart, J. R., and Vogt, P. K. (2011) Phosphorylation of AKT: a mutational analysis. *Oncotarget* **2**, 467-476
 97. Park, H. S., Hohn, M. J., Umehara, T., Guo, L. T., Osborne, E. M., Benner, J., Noren, C. J., Rinehart, J., and Soll, D. (2011) Expanding the genetic code of *Escherichia coli* with phosphoserine. *Science* **333**, 1151-1154
 98. O'Donoghue, P., Ling, J., Wang, Y. S., and Soll, D. (2013) Upgrading protein synthesis for synthetic biology. *Nat Chem Biol* **9**, 594-598
 99. Wang, L., Xie, J., and Schultz, P. G. (2006) Expanding the genetic code. *Annu Rev Biophys Biomol Struct* **35**, 225-249
 100. George, S., Aguirre, J. D., Spratt, D. E., Bi, Y., Jeffery, M., Shaw, G. S., and O'Donoghue, P. (2016) Generation of phospho-ubiquitin variants by orthogonal translation reveals codon skipping. *FEBS Lett* **590**, 1530-1542
 101. Hoppmann, C., Wong, A., Yang, B., Li, S., Hunter, T., Shokat, K. M., and Wang, L. (2017) Site-specific incorporation of phosphotyrosine using an expanded genetic code. *Nat Chem Biol* **13**, 842-844
 102. Zhang, M. S., Brunner, S. F., Huguenin-Dezot, N., Liang, A. D., Schmied, W. H., Rogerson, D. T., and Chin, J. W. (2017) Biosynthesis and genetic encoding of phosphothreonine through parallel selection and deep sequencing. *Nat Methods* **14**, 729-736

103. Zhang, X., Zhang, S., Yamane, H., Wahl, R., Ali, A., Lofgren, J. A., and Kendall, R. L. (2006) Kinetic mechanism of AKT/PKB enzyme family. *J Biol Chem* **281**, 13949-13956

Chapter 2

2 Genetic code expansion and live cell imaging reveal that Thr308 phosphorylation is irreplaceable and sufficient for Akt1 activity

2.1 Abstract

The proto-oncogene Akt/protein kinase B (PKB) is a pivotal signal transducer for growth and survival. Growth factor stimulation leads to Akt phosphorylation at two regulatory sites (Thr308, Ser473), acutely activating Akt signaling. Delineating the exact role of each regulatory site is, however, technically challenging and has remained elusive. Here, we used genetic code expansion to produce site-specifically phosphorylated Akt1 in order to dissect the contribution of each regulatory site to Akt1 activity. We achieved recombinant production of full length Akt1 containing site-specific pThr and pSer residues for the first time. Our analysis of Akt1 site-specifically phosphorylated at either or both sites revealed that phosphorylation at both sites increases the apparent catalytic rate 1500-fold relative to un-phosphorylated Akt1, an increase attributable primarily to phosphorylation at Thr308. Live imaging of COS7 cells confirmed that phosphorylation of Thr308, but not Ser473, is required for cellular activation

of Akt. We found in vitro and in the cell that pThr308 function cannot be mimicked with acidic residues nor could un-phosphorylated Thr308 be mimicked by an Ala mutation. An Akt1 variant with pSer308 achieved only partial enzymatic and cellular signaling activity, revealing a critical interaction between the γ -methyl group of pThr308 and Cys310 in the Akt1 active site. Thus, pThr308 is necessary and sufficient to stimulate Akt signaling in cells and the common use of phosphomimetics is not appropriate for studying the biology of Akt signaling. Our data also indicate that pThr308 should be regarded as the primary diagnostic marker of Akt activity.

2.2 Introduction

The proto-oncogene Akt/protein kinase B (PKB) is a central transducer of growth and survival signaling (1). There are three isozymes of Akt in mammals. Akt1, Akt2, and Akt3 include kinase domains with extensive homology to those of protein kinases A, G and C, defining them as members of the AGC family of Ser/Thr protein kinases (2). Akt transduces signals in the phosphoinositide 3-kinase (PI3K) signaling cascade, which is one of the most commonly deregulated pathways in human cancer (3,4). Thus, enormous efforts are directed at understanding the mechanisms of activation of Akt and how to target these enzymes therapeutically (5).

Akt activity in the cell is acutely controlled by growth factor-dependent phosphorylation mechanisms (6). Following activation by agonist-bound receptor tyrosine kinases at the plasma membrane, PI3K phosphorylates phosphatidylinositol-4, 5-bisphosphate (PIP₂) to generate the lipid second

messenger phosphatidylinositol-3,4,5-triphosphate (PIP₃) (7). This second messenger engages Akt at the plasma membrane by binding its autoinhibitory pleckstrin homology (PH) domain, resulting in a conformational change that exposes the kinase domain for phosphorylation (8). The phosphoinositide dependent kinase 1 (PDK-1) phosphorylates a conserved Thr on the activation loop (Thr308 in Akt1), leading to partial activation of Akt (9). Full activation of Akt1 results from a second phosphorylation event in the C-terminal tail, a regulatory region referred to as the 'hydrophobic motif' (Ser473 in Akt1) that was originally identified in protein kinase C and S6 kinase (10-12). Phosphorylation at Ser473 in Akt1 depends on the mammalian target of rapamycin complex 2 (mTORC2) (13). In addition, Akt is co-translationally and constitutively phosphorylated at another C-terminal site termed the 'turn motif' (Thr450 in Akt1) by mTORC2, a modification that regulates the stability of the enzyme (14). Given that Akt is activated by phosphorylation of Thr308 and Ser473, phospho-specific antibodies to these sites are widely used as diagnostic markers (15-17). To a striking degree, many clinical (18-21) and biochemical (22) studies rely solely on Ser473 phosphorylation as a proxy for Akt activity.

The activation of Akt by phosphorylation is well established (8), yet the contribution of each specific phosphorylation site towards the maximal Akt activity and pathogenesis is less well defined. This knowledge gap resulted from the inability previously to prepare Akt variants in site-specifically phosphorylated forms. Earlier work established production of partially active and truncated Akt1 in *E. coli*. The attempt was unsuccessful in producing a sufficient amount of full length Akt1 to determine activity (23). Instead this study relied on a construct lacking the PH domain with Ser473 substituted by glutamate, and the authors were unable to show how the phosphomimetic

compared to pSer at position 473 (23). A protocol for active Akt1 production in insect (Sf9) cells has been established (24). The ability to generate ppAkt1 required a complex and low yield in vitro procedure to phosphorylate Akt with 2 additional purified upstream kinases in the presence of lipid vesicles (24). Protein production in Sf9 cells fails to produce Akt1 with site-specific or programmed phosphorylation. The resulting protein is a mixture of singly and doubly phosphorylated species (25) and includes phosphorylation at Thr450 in addition to potentially other modifications, so the ability to isolate the activity of each regulatory site individually and in precise combinations has remained elusive. As demonstrated below, we have developed a facile and efficient approach that is a novel combination of in vivo enzymatic phosphorylation with genetic code expansion to produce pAkt1 and ppAkt1 variants with specifically programmed phosphorylation (Figure 2.1).

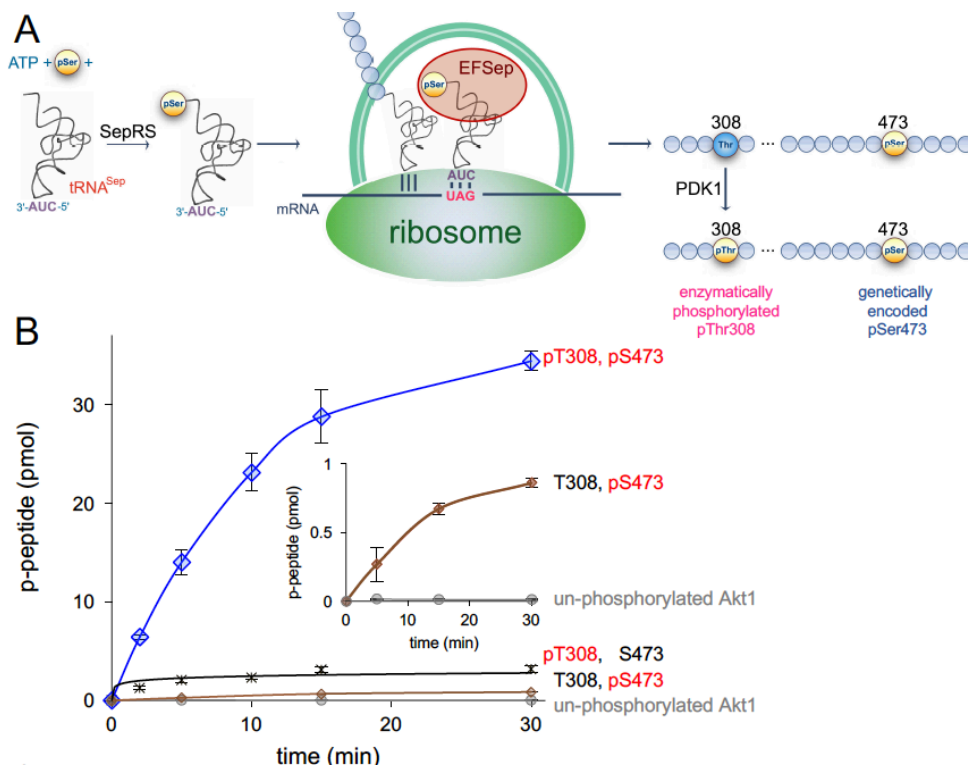


Figure 2.1 A novel route to doubly phosphorylated and active Akt1. (A) Schematic representation of recombinant Akt1 biosynthesis with pSer473 genetically encoded in response to the UAG codon and pThr308 enzymatically phosphorylated *in vivo* in *E. coli*. Genetically encoded pSer incorporation requires phosphoseryl-tRNA synthetase (SepRS), a UAG-decoding tRNA^{Sep}, and the elongation factor mutant (EFSep). (B) Enzyme activity of differentially phosphorylated Akt1 variants with a GSK-3 β substrate peptide. Akt1 quantitatively phosphorylated at both 308 and 473 (ppAkt^{S473, T308}, blue diamonds) showed maximal activity compared to the un-phosphorylated Akt1 (gray circles), and singly phosphorylated Akt1 variants: pAkt^{T308} (black cross) and pAkt^{S473} (brown diamonds). The reported values represent the mean of triplicate experiments with error bars indicating 1 standard deviation. Lower activity variants show above background kinase activity (inset).

Because of the previous technological barriers to producing specifically phosphorylated kinase variants, a significant literature continues to accumulate for Akt (23,26-32) and other kinases (33), in which 'phosphomimetic' substitutions are used as a genetic tool to interrogate the biological consequences of phosphate on a site of interest (33). The rationale behind these experiments is that the acidic residues, Asp and Glu, are negatively charged like phosphate. Yet the carboxylate of an acidic amino acid has considerably less electronegativity than a phosphate and a significantly smaller hydration sphere and volume (34,35). A complementary approach involves nullifying the effect of a particular phosphorylation site by introducing a non-phosphorylatable Ala mutation (26); however, an Ala is considerably smaller and less polar than a Ser or Thr, and Ala lacks the capacity to form hydrogen bonds. In light of these observations, the widespread use of Ala and phosphomimetic mutants in signaling studies begs the question of how appropriate these mutations are to interrogate the cellular function of Akt or other phosphorylated proteins.

Here we provide, for the first time, a quantitative analysis of the contribution of phosphate at positions 308 and 473 in the catalytic activity of Akt1 and, additionally, examine the effects of substitutions of acidic residues or Ala at these positions. To do this, we developed an optimal strategy to produce recombinant full-length human Akt1 in *Escherichia coli* with genetically encoded phosphoserine (pSer) introduced at specific phosphorylation sites. We measured the activity of singly and doubly phosphorylated Akt1 variants with phosphate at positions 308 (pAkt1^{S308}, pAkt1^{T308}) and/or 473 (pAkt1^{S473}) in comparison to inactive and un-phosphorylated Akt1 as well as Akt1 variants with phosphomimetic Glu or Asp mutations. With a Förster resonance energy transfer (FRET)-based Akt activity sensor (BKAR) (36), we conducted complementary

experiments of specific Akt1 variants in live cells. Our data reveal that, in comparison to un-phosphorylated enzyme, phosphorylation of Thr308 alone increases the apparent catalytic rate by nearly 400-fold, which is sufficient to observe maximal signaling in cells. Phosphorylation of only Ser473 boosts Akt1 activity by ~80-fold over un-phosphorylated enzyme, however, our data suggest that phosphorylation at Ser473 alone may not be sufficient to elicit Akt1 signaling in cells. Thus, phosphorylation of Thr308 is necessary and sufficient for the activity of Akt. Importantly, our cell based observations and biochemical data confirm that Ala does not mimic a non-phosphorylated Thr308 and further that acidic residues fail to activate Akt1. Phosphomimetic substitutions, therefore, do not mimic phosphorylation in Akt1 at either of its two key regulatory phosphorylation sites.

2.3 Materials and methods

2.3.1 Bacterial strains and plasmids. The full length human *AKT1* gene was cloned into a pUC18-derived vector (pDS1(38)) and pCDFDuet1 vector (see SI methods). Ser473 and Thr308 sites were mutated by site-directed mutagenesis in *E. coli* DH5 α to amber (TAG), Asp (GAC), or Glu (GAG) codons according to previously described methods (65). All clones were verified by DNA sequencing (London Genomics Research Center, Robarts Research Institute, London; Genewiz Inc. NJ, USA). Phosphoproteins were produced by genetically encoding pSer at UAG codons with the 2nd generation pSer incorporation system (pDS-pSer2, kanamycin resistant) (38,66). Recombinant Akt1 and pAkt1 variants were

over expressed in *E. coli* BL21(DE3) (Invitrogen, California, USA); exogenous pSer (5 mM) was added to the expression media for production of pAkt1 variants (see SI methods for a detailed protocol). For the imaging studies, the mouse *AKT1* gene (a gift from A. Toker; 98.3% amino acid identity to human Akt1) was subcloned into pcDNA3 (Invitrogen) containing mCherry at its amino-terminus. Mutations were generated by QuikChange mutagenesis (Agilent Genomics) and confirmed by Sanger sequencing (Eton Bioscience). Generation of BKAR was previously described (36).

2.3.2 Protein production and purification. For phosphoprotein production, pDS1-Akt1 expression plasmid variants with TAG codons at the indicated phospho-site were co-transformed with pDS-pSer2 into *E. coli* BL21(DE3). For unphosphorylated and phosphomimetic Akt1 variants, the appropriate pDS1-Akt1 plasmid was transformed alone into BL21 (DE3). Cells were grown and pelleted at $5000 \times g$ as described in SI methods. The cell pellets were re-suspended in lysis buffer (20 mM HEPES, 150 mM NaCl, 3 mM β -mercaptoethanol, 3mM Dithiothreitol (DTT), 10 mM Imidazole) at 10 ml per gram of cells. Lysis buffer for phosphoproteins contained phosphatase inhibitors (1 mM Na_3VO_4 and 5 mM NaF). One tablet of ethylenediaminetetraacetic acid (EDTA)-free mini protease inhibitor cocktail (Roche, Mississauga, ON, Canada) and 1 mM phenylmethylsulfonyl fluoride (PMSF) were added to the cell suspension (typically 200 ml). Cells were treated with lysozyme (1 mg/ml) for 20 min, shaking at 4°C and lysed using French Pressure Cell Press (American Instrument Co. Inc. Maryland, USA) at 1000 psi. Cell lysates were centrifuged at $38,000 \times g$ for 1 h at 4°C. The supernatant was filtered through a 1.2 μm filter, mixed with Ni-NTA affinity beads (Thermo Scientific) and pre-equilibrated with

lysis buffer for 1 h. Finally, proteins bound to beads were purified under gravity flow. Elution fractions were further purified using Superdex200 gel filtration column attached to an AKTA Pure L1 fast protein liquid chromatography (FPLC) system (GE Healthcare, Little Chalfont, UK) (see SI Methods). Protein yields ranged from 20-100 $\mu\text{g/l}$ *E. coli* culture.

2.3.3 *In vitro* Akt1 kinase assay. For the kinase activity assay, we established a set of common conditions to assay a series of Akt1 variants of widely varying activity. To achieve this, we worked in a regime of sub-saturating ATP, thus, our apparent reaction constants (k_{app}) serve well to compare the Akt1 variants to one another, but these rates are significantly lower than k_{cat} . Our data represent single turnover kinetics. Akt1 activity was determined using 200 μM substrate peptide CKRPRAASFAE (SignalChem, Vancouver, BC, Canada) derived from the natural Akt1 substrate, glycogen synthase kinase (GSK-3 β). The reported K_{M} of Akt1 for a similar substrate peptide is 18 μM (67), so the peptide concentration is in excess. Assays were performed in 3-(N-morpholino)propanesulfonic acid (MOPS, 25 mM, pH 7.0), β -glycerolphosphate (12.5 mM), MgCl_2 (25 mM), ethylene glycol-bis(β -aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA, 5 mM, pH 8.0), EDTA (2 mM), ATP (20 μM) and 0.4 μCi (33 nM) γ -[^{32}P]-ATP in a 30 μl reaction volume, at 37°C in 30 min time courses. Unless otherwise indicated, reactions were initiated by the addition of 18 pmol of the indicated Akt1 variant (to yield a concentration of 600 nM) and quenched by spotting 5 μl from each reaction on P81 paper at specified time points (68). Following washes with 1% phosphoric acid (3 \times 10 min) and 95% ethanol (1 \times 5 min), P81 paper was air-dried and exposed to a phosphor screen. The ^{32}P -peptide products were imaged and

quantitated using a Storm 860 Molecular Imager and ImageQuant TL software (Molecular Dynamics, Caesarea, Israel).

2.3.4 Cell culture media and conditions. COS-7 cells were maintained in DMEM (Cellgro) containing 10% fetal bovine serum and 1% penicillin/streptomycin at 37°C in 5% CO₂. For cell imaging experiments, cells were plated onto sterilized glass coverslips in 35-mm dishes prior to transfection with 1 µg pcDNA3-BKAR with or without 1 µg pcDNA3-mCherry-Akt using Lipofectamine 3000 (Invitrogen). For experiments in which low expression levels of Cherry-Akt were desired, cells were transfected with 1 µg pcDNA3-BKAR and 0.05 µg pcDNA3-mCherry-Akt. Cells were imaged within 24 hours following transfection. For Western blot experiments, in which average overexpressed Akt levels were desired to be at a similar level to endogenous Akt, COS-7 cells were transfected with 0.05 µg pcDNA3-mCherry-Akt using Lipofectamine 3000 (Invitrogen) in 6-well dishes.

2.3.5 Cell imaging. Transfected COS-7 cells were serum-starved at least 4 hours (as long as overnight) prior to imaging. Cells were washed one time in Hank's Balanced Salt Solution (HBSS, Cellgro) supplemented with 1 mM CaCl₂ and imaged in this HBSS in the dark at room temperature. Data were collected on a Zeiss Axiovert microscope (Carl Zeiss Microimaging, Inc) using a MicroMax digital camera (Roper-Princeton Instruments) controlled by MetaFluor software (Universal Imaging, Corp.). Optical filters were obtained from Chroma Technologies and Semrock. Data were collected through a 10% neutral density filter. Cyan fluorescent protein (CFP) and FRET images were obtained every 15

seconds through a 420/20 nm excitation filter, a 450 nm dichroic mirror and a 475/40 nm or 535/25 emission filter for CFP and FRET, respectively. Cherry images were acquired through a 560/25 excitation filter, a 593 nm dichroic mirror and a 629/53 emission filter. Excitation and emission filters were switched in filter wheels (Lambda 10-2, Sutter). Integration times were 200 ms for CFP and FRET and 150 ms for mCherry. Cells were stimulated with 50 ng/ml EGF (PeproTech, Inc.) and then treated with 20 μ M GDC 0068 (Selleckchem.com) at the indicated times. Data were normalized to the first 3 minutes of the experiment for each cell. Error bars represent 1 standard error of the mean.

2.3.6 Western Blotting. Within 24 hours after COS-7 cell transfection, cells were serum-starved for at least 4 hours. Cells were rinsed once in HBSS/1 mM Ca^{2+} and then left untreated or stimulated with 50 ng/ml EGF for 10 minutes at room temperature. Cells were lysed in 50 mM Na_2HPO_4 , 1 mM $\text{Na}_4\text{P}_2\text{O}_7$, 20 mM NaF, 2 mM EDTA, 2 mM EGTA, 1% Triton X-100 (supplemented with 1 mM DTT, 200 μ M benzamidine, 40 μ g/ml leupeptin, 300 μ M phenylmethylsulfonyl fluoride, and 1 μ M microcystin) and cleared by a high-speed centrifugation for 2.5 min. The cleared lysates were analyzed by Western blotting to determine the relative amounts of Akt-Thr308 (Phospho-Akt (Thr308), Cell Signaling #9275) or Akt-Ser473 (Phospho-Akt (Ser473)(D9E) XP, Cell Signaling #4060) phosphorylation. Total Akt levels were assessed with an antibody against all Akt isozymes (Anti-AKT1/2/3, Abcam #126811). Western blots were developed using chemiluminescence.

2.4 Results

2.4.1 Activation of full-length pAkt1 variants by genetic code expansion. We used *in vitro* radioactive kinase assays with γ -[³²P]-ATP to test the activity of full length Akt1 variants that were made with site-specifically incorporated pSer residues (Figure S2.1, S2.2, S2.33). The assays quantify the ability of each Akt1 variant to phosphorylate an Akt1 substrate peptide (CKRPRAASFAE) that is derived from the natural Akt1 target glycogen synthase kinase 3 β (GSK-3 β). Using genetic code expansion (37,38), we reassigned the UAG stop codon to genetically encode pSer in recombinant Akt1 proteins produced in *E. coli*. For example, we expressed pAkt1^{S473} with a genetically-encoded pSer residue inserted in response to a UAG codon at position 473 in the Akt1 construct. We used Multiple Reaction Monitoring Tandem Mass Spectrometry (MRM-MS/MS) to unambiguously identify pSer at position 473 (Figure S2.3). Genetically-encoded pAkt1^{S473} showed a clear signal for phosphorylation at position 473 with no evidence of de-phosphorylation (Figure S2.3A,B). In wild type or un-phosphorylated Akt1, Ser473 is readily detected with no evidence of pSer473 (Figure S2.3C,D).

For pThr incorporation at position 308, we relied on enzymatic phosphorylation by the upstream kinase PDK1 (23). We produced phosphorylated pAkt1^{T308} as well as the doubly-phosphorylated ppAkt1^{T308,S473} by co-expressing PDK1 in *E. coli* with either the wild-type Akt1 or pAkt1^{S473} constructs, respectively (Figure 2.1A). We confirmed phosphorylation at Thr308 (Figure S2.4) by Parallel Reaction Monitoring Tandem Mass Spectrometry (PRM-MS/MS). The MS/MS data identified an insignificant level of Thr308 in the pAkt1^{T308} sample, indicating essentially quantitative phosphorylation of Thr308

by PDK1 in *E. coli*. Finally, we confirmed site-specific phosphorylation of the ppAkt1^{T308,S473} variant using PRM-MS/MS (Figure S2.2). In the MS/MS analysis, we achieved up to 90% coverage of the full length Akt1 (Figure S2.2D), and we were unable to identify significant peptides with modifications other than the anticipated and programmed phosphorylations. The data indicate that both genetic code expansion and PDK1 phosphorylation are highly site-specific, and that endogenous modification or de-phosphorylation by *E. coli* enzymes is absent or minimal.

We first measured the activity of Akt phosphorylated at both positions (ppAkt1^{T308/S473}). Doubly phosphorylated Akt1 (Figure S2.4) had an apparent reaction rate of 44 ± 6 fmol/min/pmol of enzyme (Figure 2.1, Table 2.1, Figure S2.5, S2.6). We performed these kinase assays in conditions of sub-saturating ATP. We chose these conditions so that Akt1 protein variants with a wide range of activities could be assayed using the same experimental conditions. In order to compare the relative activities of Akt1 and pAkt1 variants, we determined an apparent catalytic rate ($k_{app} = v_o/[Akt1]$) based on the initial velocity (v_o) observed in the kinase assays. Under sub-saturating ATP, our reaction velocities are far below V_{max} for Akt and related kinases (24,39); k_{app} is not intended to estimate k_{cat} . Interestingly, the bacterially expressed ppAkt1 construct consistently migrated slightly faster than the other pAkt1 variants and Akt1 mutants on sodium dodecyl sulphate (SDS)-PAGE (Figure S2.1, Figure S2.2A). We determined that this was not a result of premature termination of the translation product because PRM-MS/MS unambiguously identified intact full length and doubly phosphorylated ppAkt^{T308, S473} (Figure S2.2B-D). We found no evidence of truncation or stopping at the UAG473 codon, or de-phosphorylation according to mass spectrometry. Phosphorylation at Thr450 is known to specifically decrease

the electrophoretic mobility of Akt1 expressed in mammalian cells (40). Our bacterially expressed pAkt1 variants show no evidence of phosphorylation at Thr450. These observations together suggest that phosphorylation status impacts the mobility of Akt1 on SDS-PAGE.

Akt1 variants that contain pThr308 were so highly active under the conditions of our assays that we reduced the Akt1 concentration 10-fold in order to establish a linear range of activity to determine v_0 (Figure S2.5). Un-phosphorylated Akt1 was essentially inactive, showing a low basal activity of 0.03 ± 0.01 fmol/min/pmol of enzyme (Table 2.1). Thus, in comparison to un-phosphorylated enzyme, phosphorylation at both activating sites increased the catalytic rate of Akt1 by 1500-fold. We note this is the first time that the activity of Akt1 has been definitively measured and reported for homogenously pure variants with site-specifically programmed phosphorylation at both key regulatory sites and without phosphate occupancy at other sites (e.g., pThr450).

Table 2.1 Specific activity of Akt1 variants

Akt1 variant	Akt1 amount (pmol)	Initial velocity (v_0) (fmol/min)	Apparent catalytic rate, k_{app} (fmol/min/pmol Akt1)	Activation (fold increase)
Akt1 (unphosphorylated)	18	0.6 ± 0.2	0.03 ± 0.01	1
pAkt1				
pS473	18	46 ± 5	2.5 ± 0.3	83
pS308	6	2.9 ± 0.2	0.48 ± 0.03	16
pT308*	1.8	22 ± 4	12 ± 2	400
ppAkt1 (pT308, pS473)*	1.8	79 ± 11	44 ± 6	1467
Phosphomimetics				
E473	18	1.5 ± 0.1	0.083 ± 0.006	2.7
E308	18	1.0 ± 0.1	0.056 ± 0.006	1.9
D473	18	0.40 ± 0.03	0.022 ± 0.002	0.7
D308	18	2.6 ± 0.1	0.14 ± 0.01	5
pAkt1 ^{S473} mutants				
D308	18	83 ± 1	4.6 ± 0.1	153
A308	18	5.4 ± 0.3	0.30 ± 0.02	10
pAkt1 ^{T308} mutants				
D473	18	188 ± 11	10.4 ± 0.6	348
E473	18	120 ± 16	6.6 ± 0.8	222
A473	18	74 ± 3	4.1 ± 0.2	137

Initial velocities (v_0) and apparent catalytic rates ($v_0/[Akt1]$) of each enzyme variant were calculated using linear regression analysis of the activity plots.

*In order to measure the initial velocity accurately, the most active variants were assayed at a 10-fold reduced enzyme concentration (Figure S2.5).

2.4.2 Phosphorylation of Thr308 is necessary and sufficient for Akt1 activation.

We next examined the contribution of phosphate at each site individually to the maximal activity of Akt1. Based on measurements of the initial velocity, the activity of mono-phosphorylated pAkt1^{T308} corresponded to ~27% that of the doubly-phosphorylated species (Figure 2.1, Table 2.1). Thus, phosphorylation at the PDK-1 site alone results in an over 400-fold increase in activity compared to the un-phosphorylated enzyme. These data establish that phosphorylation at Thr308 alone is sufficient for robust activation of Akt. Purified, mono-phosphorylated pAkt1^{S473} was considerably less active than protein with phosphate at Thr308 (Figure 2.1). pAkt1^{S473} had activity that corresponded to about 5% of the activity of the doubly-phosphorylated species (Table 2.1). Although significantly reduced relative to that of the doubly-phosphorylated species, activity from pAkt1^{S473} still represents an 80-fold increase over the un-phosphorylated Akt1 enzyme. Thus, genetic code expansion with pSer enables production of active Akt1 without the need to purify and activate upstream Akt1 kinases (24). In the absence of substrate peptide, we were able to detect low, but above background, phosphorylation that may be attributed to auto-phosphorylation by pAkt1^{S473} (Figure 2.2).

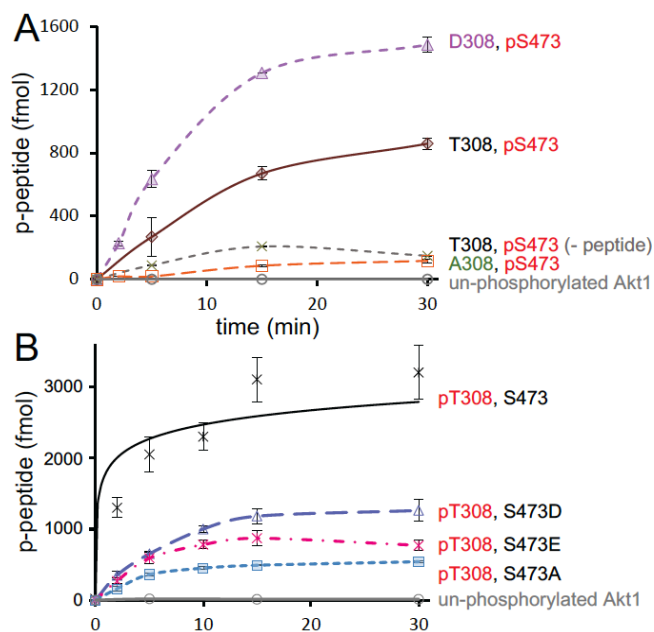


Figure 2.2 Activity of singly phosphorylated Akt1 variants with regulatory site mutations. (A) Akt1 enzyme activity is shown for variants with S473 phosphorylated and T308 un-phosphorylated (brown diamond), or mutated: T308D (purple triangles), T308A (orange squares). Controls include unphosphorylated Akt1 (gray circles), and pAkt1^{S473} in the absence of substrate peptide (-peptide, green crosses). (B) In comparison to pAkt1^{T308} activity (black crosses) and in the context of Akt1 phosphorylated at T308, mutations S473D (blue triangles), S473E (magenta crosses) or S473A (cyan squares) resulted in marginally reduced activity. All reported values represent the mean of triplicate experiments with error bars indicating 1 standard deviation.

Genetic code expansion was also used to produce pAkt1^{S308}. The Akt enzyme containing pSer308 had above background but low activity (Figure 3A). Western blot analysis revealed a band co-migrating with full-length Akt but also a species with an apparent MW of ~35 kDa (Figure S2.7). This could result either from

degradation or premature truncation from stopping at UAG308, which would produce a protein of 37.6 kDa. We adjusted our estimated rate for this enzyme by accounting for the fraction of full-length pAkt1^{S308} in our preparation. Although it is possible we underestimated the activity Akt1 with pS308 (Figure 2.3A), the data show that pSer cannot substitute for pThr308 to produce optimally active Akt1.

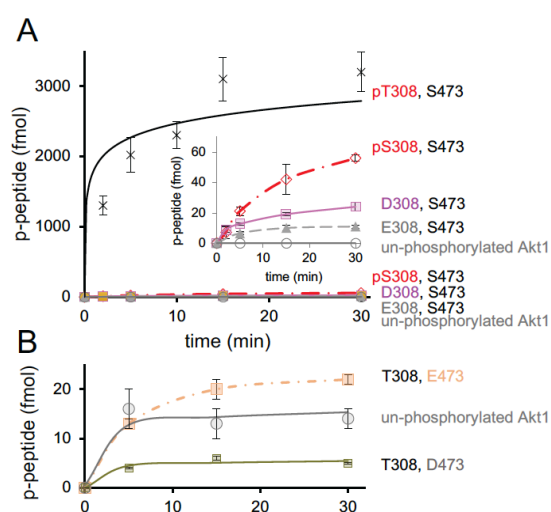


Figure 2.3 Activity of T308S and phosphomimetic Akt1 variants. (A) In comparison to pAkt1^{T308} activity (black crosses), mutations of T308 to either D (pink squares), E (green triangles), or pS (red diamonds) resulted in low but above background kinase activity (inset). (B) Kinase activity was also measured in the context of an unphosphorylated Akt1 (gray circles) with phosphomimetic mutations S473D (green squares) and S473E (peach squares). All reported values represent the mean of triplicate experiments with error bars indicating 1 standard deviation.

2.4.3 Enzymatic activity of phosphomimetic Akt1 variants. We next tested the activity of purified phosphomimetic Akt1 mutants (Figure S2.6). In these

experiments, we programmed either Ser473 phosphorylation (Figure 2.2A) or un-phosphorylated Ser473 (Figure 2.3A) and examined the effect of residue substitutions at position 308. Replacement of Thr308 with Asp increased the activity of pAkt1^{S473} by a modest 2-fold (from 2.5 to 4.6 fmol/min/pmol Akt), resulting in enzyme that displayed only 10% of the activity of doubly-phosphorylated enzyme (Figure 2.2A, S2.6, Table 2.1). Surprisingly, replacing the Thr308 with an Ala eliminated the activity of the mono-phosphorylated pAkt1^{S473} (Figure 2.2A), demonstrating that Ala308 is not an appropriate substitute for un-phosphorylated Thr.

In the context of Akt1 that was not phosphorylated at Ser473 (Figure 2.3B), Akt1^{D308} demonstrated 5-fold higher enzyme activity in comparison to un-phosphorylated Akt1, but this was still ~100-times lower than that of pAkt1^{T308} (Figure 2.3A, S2.6, Table 2.1). Akt1^{E308} was >200-fold less active than pAkt1^{T308} (Figure 2.3A, S2.6). Interestingly, production of pAkt1^{S308} by our genetic code expansion approach resulted in an enzyme with only 3-fold more activity than Akt1^{D308} (Figure 2.3A), suggesting that Asp308 may, to some extent, mimic pSer308, but not the natural pThr308 residue in Akt1.

Phosphomimetic substitution at Ser473 also failed to activate Akt1. We tested Ser473 mutations in the context of un-phosphorylated Akt1 (Figure 2.3B, S2.6) and in Akt1 variants phosphorylated at Thr308 (Figure 2.2B). For un-phosphorylated Akt1, we examined the effect of Asp and Glu substitutions at position 473 (Figure S2.6). Substitution with Asp at position 473 lead to essentially inactive enzyme (Figure 2.3B, S2.6, Table 2.1). Replacement of Ser473 with Glu resulted in an insignificant increase above the activity of un-phosphorylated Akt1 enzyme (Figure 2.2C, S2.6, Table 2.1). In the context of pAkt1^{T308} (Figure 2.2B), phosphomimetic mutations (D and E) failed to stimulate

activity above pAkt1^{T308} with S473. Indeed, all S473 variants reduced pAkt1^{T308} activity with S473A showing the most significant reduction (Tables 2.1, 2.2).

2.4.4 Akt1 mutant activity in live cells. Having assessed the Akt mutants in vitro, we examined the activity of Akt1 phosphorylation site mutants in live cells. To this end, we utilized our quantitative FRET-based kinase activity reporter, BKAR, and examined Akt signaling (36). Co-expression of kinase constructs with FRET-based activity sensors affords a sensitive assay for examining agonist-evoked signalling in a cellular context (41). As the response to endogenous Akt activity is so low, it provides an ideal system to evaluate signaling by introduced Akt variants as a significant response can be observed from the overexpressed kinase.

Serum-starved COS-7 cells expressing BKAR and mCherry-tagged Akt variants were stimulated with epidermal growth factor (EGF) followed by Akt inhibition with GDC 0068. In real time, we monitored resulting changes in the BKAR FRET ratio (CFP/FRET) that are reflective of cellular Akt activity (Figure 2.4A). For these experiments, cells selected for analysis had comparable levels of Akt1 expression as assessed by quantifying mCherry levels; thus, in this system, one can clearly compare the relative signaling competence between wild type and mutant Akt. We have documented previously that the mCherry tag does not significantly impact Akt1 activity (41).

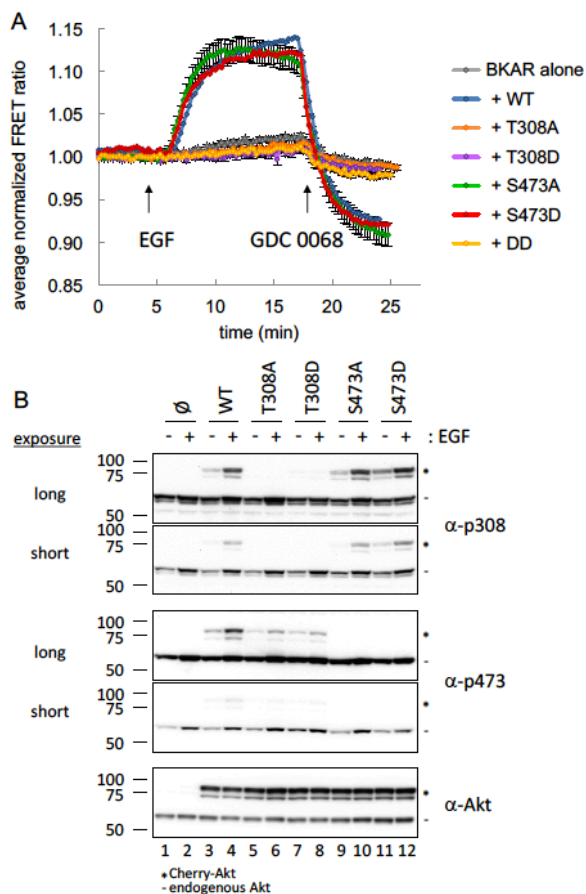


Figure 2.4 Cellular activity of Akt1 variants. (A) Serum-starved COS-7 cells expressing BKAR alone (gray) or BKAR with Cherry-tagged Akt WT (blue), T308A (orange), T308D (purple), S473A (green), S473D (red), or T308D, S473D (DD, yellow) were imaged, stimulated with EGF, and then treated with the Akt inhibitor GDC 0068. Multiple cells were included from at least two independent experiments for analysis. FRET ratios from each cell were normalized and their average plotted over time. Error bars represent SEM. (B) Following a 10-minute treatment with EGF, lysates from serum-starved COS-7 cells expressing the indicated Cherry-tagged Akt were analyzed by Western blotting for Akt activation using phospho-specific antibodies toward the activation loop (α -p308) and hydrophobic motif (α -p473). The asterisk (*) marks migration of Cherry-Akt and the hyphen (-) marks endogenous Akt. (Experiment by Maya Kunkel)

Overexpression of wild type (WT) Akt1 resulted in a significant BKAR response to EGF treatment compared to the response observed from endogenous Akt (Figure 2.4A, blue vs. grey trace). Interestingly, despite the level of overexpressed Akt in these experiments being in vast excess compared to endogenous Akt, substitution of either the non-phosphorylatable Ala or the 'phosphomimetic' Asp or Glu at the Thr308 site yielded an Akt1 enzyme that displayed no signaling in the cell (Figure 2.4A, orange and purple traces, Figure S2.8). These data establish the requirement of pThr308 for detectable Akt1 activation in cells and show that T308D is not a constitutively active Akt1 variant.

In contrast to the strict requirement for phosphorylation at Thr308, either Ala, Asp or Glu substitution at the Ser473 site were tolerated without detrimental effects on cellular Akt activity as assessed using BKAR (Figure 2.4A, green and red trace, Figure S2.8). Importantly, a double mutation of both 308 and 473 to Asp resulted in an inactive enzyme. Thus, the activity of Akt mono-phosphorylated at Thr308 is necessary and sufficient for maximal signaling in the context of cells.

2.4.5 Mutant Akt1 kinases are phosphorylated following EGF stimulation. We next examined the phosphorylation state of Thr308 and Ser473 of the mutant Akt kinases in our cell-based assay (Figure 2.4B). In experiments in Figure 2.5, we reduced the expression of the tagged Akt variants to be equivalent to the endogenous Akt expression level as determined by Western blot. EGF stimulation (all even numbered lanes) induced phosphorylation of both endogenous and wild-type Akt1 (lane 4) as assessed by Western blot (Figure 2.5). Importantly, phosphorylation of Thr308 in the Ser473 mutants (lanes 10 and 12)

was at the same level as the WT Akt1 (lane 4) demonstrating that PDK1 phosphorylation downstream of PI3K signaling was intact. Analysis of Ser473 phosphorylation revealed that, while this site was phosphorylated downstream of PI3K signaling, the level of phosphorylation at Ser473 was reduced in the T308A and T308D mutants (lanes 6 and 8) compared to WT Akt1 (lane 4). This suggests that lack of phosphate on Thr308 impairs phosphorylation of Ser473. Even though the level of Ser473 phosphorylation was reduced in these mutants, the level of total overexpressed Akt protein was saturating in Figure 2.4A. Given the excessive amount of Akt overexpressed in the experiments in Figure 2.4A, this lack of activity observed using BKAR indicates that T308A, T308D and T308E (Figure S2.8) are clearly not able to signal, and any catalytic activity observed in these variants is below the threshold to result in detectable cellular activity.

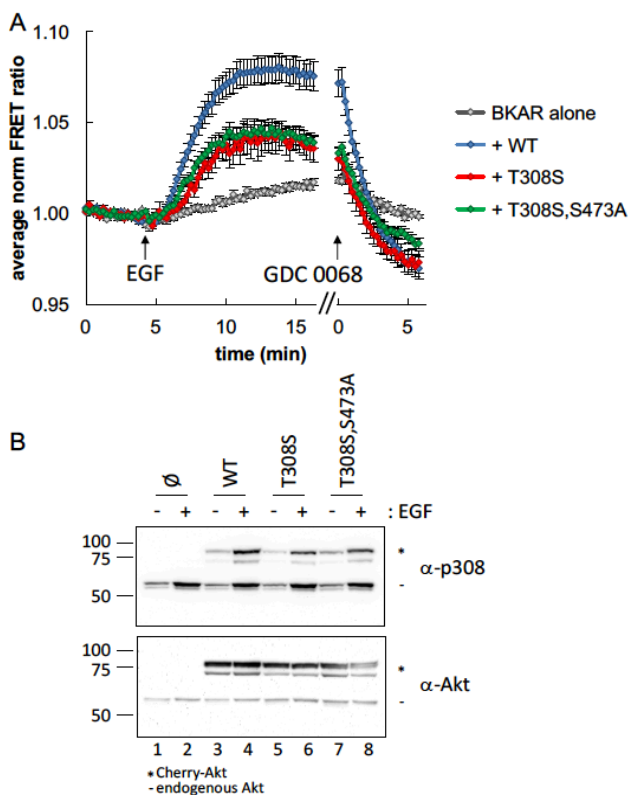


Figure 2.5 Reduced, but active, signaling from Akt T308S. (A) Serum-starved COS-7 cells expressing BKAR with or without minimally detectable levels of Cherry-tagged Akt were imaged during stimulation with EGF followed by treatment with the Akt inhibitor GDC 0068. Normalized average FRET ratios for WT (blue), T308S (red) and T308S, S473A (green) in comparison to BKAR alone (gray) are shown. Data were analyzed from cells expressing equal low levels of Cherry-Akt. Multiple cells were included from at least two independent experiments. FRET ratios from each cell were normalized and their average plotted over time. Error bars represent SEM. (B) Following a 10-minute treatment with EGF, lysates from serum-starved COS-7 cells expressing the indicated Cherry-tagged Akt were analyzed by Western blotting for Akt activation using phospho-specific antibodies toward the activation loop (α -p308) and hydrophobic motif (α -p473). The asterisk (*) marks migration of Cherry-Akt and the hyphen (-) marks endogenous Akt. (Experiment by Maya Kunkel)

2.4.6 Impact of Ser substitution at Thr308 in cells. As the recombinant Akt1^{D308} had similar activity as the pAkt1^{S308} protein *in vitro*, we were interested to examine the impact of Ser at 308 in the overexpressed mCherry-Akt system using BKAR. Overexpression of mCherry-Akt-T308S resulted in a similar BKAR response to that of WT Akt1 (data not shown). As this imaging system is monitoring an excess of the overexpressed mutant Akt, we attempted to express reduced levels of the kinase in order to discern whether there was a difference in cellular activity between WT Akt1 and Akt1 with Ser at position 308. Indeed, titrating down the expression levels and selecting for the lowest expressing cells based on mCherry intensity, we observed a reduction in Akt1 signaling output from the Ser308 enzyme compared to the WT enzyme with Thr308 (Figure 2.5A, blue and red traces). In the impaired kinase (Akt1^{S308}), we then substituted the 473 site with Ala (Ser308, Ala473), however, even in the context of an impaired Akt kinase, mutation of the 473 site did not influence Akt signaling (Figure 2.5A, green trace). Under the same conditions noted here to analyze minimal levels of overexpressed kinase, we were unable to detect a reduced level in signaling from the S473A mutant compared to WT Akt1. This result is in agreement with our observations that mutation of Ser473 to Ala in the context of Akt1 phosphorylated at residue 308 either on Thr (Figure 2.4, green trace) or Ser (Figure 2.5, green trace) does not impact signaling in cells. Furthermore, the data indicate that the ability of pAkt1^{T308} S473A to signal is greater than that of Akt1 T308S.

Western blot analysis confirmed that phosphorylation of the 308 site (even with a Ser substitution) was intact following EGF stimulation (Figure 5B). Our data in live cells indicate that phosphate, specifically, not an acidic residue, at position 308 is the most critical component to induce Akt activity. In the cell, as

we found *in vitro*, phosphomimetic substitutions of Asp or Glu at the 308 site are unable to propagate Akt signaling, and, in agreement with our enzymatic data, substitution of Thr308 to Ser leads to reduced Akt activity and signaling.

2.5 Discussion

Using genetic code expansion and enzymatic phosphorylation, we produced fully active Akt1 directly from *E. coli* for the first time. We validated our biochemical findings in cells using a genetically-encoded reporter for Akt signaling. This allowed us to 1) measure the specific activity of Akt1 site-specifically and exclusively phosphorylated at either or both regulatory sites, Thr308 and Ser473, 2) systematically examine the role of phosphate at each site in modulating the intrinsic catalytic activity of the pure enzyme, and 3) examine the effectiveness of amino acid substitutions to mimic phosphorylated or unphosphorylated residues in cells.

2.5.1 Generation of active Akt1. We demonstrated the compatibility of enzymatic phosphorylation with genetically-encoded phosphoserine incorporation by producing site-specific and doubly-phosphorylated Akt1 (Figure 2.1A). This was achieved by co-expressing the upstream kinase PDK1, to induce Thr308 phosphorylation, and simultaneously genetically encoded pSer473 in Akt1. To our knowledge, this is the first demonstration of protein production in *E. coli* with programmed pSer and pThr residues in the same protein. Consistent with numerous studies (24), our *in vitro* kinase assays revealed robust activity of ppAkt1 activity in the absence of PIP₃, suggesting PIP₃ is not required

for Akt1 activity *per se*. This contradicts a recent study proposing that PIP₃ binding may be essential for both Akt1 activation and activity in cells (42). As noted above, producing site-specifically and doubly phosphorylated, and thus fully-activated Akt1 has previously not been achieved via recombinant expression *in vivo* in *E. coli* given the complicated nature of Akt activation by multiple kinases, chaperones, and lipid second messengers (24). Our experiments demonstrate a novel approach, taking advantage of genetic code expansion and enzymatic phosphorylation, that overcomes the existing limitations in both methods to produce fully-active human kinases.

2.5.2 Role of phosphorylation in activating Akt1. The kinetic mechanisms of Akt activation by phosphorylation are less well characterized relative to other kinases such as PKA (43) and PKC (44), stemming in part from the difficulty in obtaining Akt in specifically phosphorylated forms. Most studies relied on immunoprecipitating Akt from unstimulated and growth factor-stimulated cells. Akt1 activity is then determined using a kinase activity assay in the immunoprecipitates. Such studies typically report a 10–50-fold stimulation of the activity of Akt immunoprecipitated from growth factor-treated compared to unstimulated cells, e.g., (45-49). Alessi and co-workers showed that Akt1 had 45-fold higher activity when immunoprecipitated from cells treated with IGF-1 compared to untreated cells (6). In the context of an Akt1 mutant with Ala present at position 473, the increase was only 5-fold, and if Ala was present at position 308, there was essentially no stimulated activity. Similar results were reported by Hemmings and coworkers for Akt3 (50). These data are qualitatively similar to the results from our study, yet our quantitative analysis defines Thr308 as the critical regulator of Akt activity and its phosphorylation alone increases

activity to approximately one-third that of the doubly-phosphorylated enzyme. Our unique ability to produce recombinant fully-phosphorylated Akt1 from bacteria has revealed that specific phosphorylation at both regulatory sites increases the intrinsic catalytic activity by over three orders of magnitude, defining the stringency with which Akt signaling is 'silent' in the absence of agonist stimulation and the resulting phosphorylation of Akt.

Several lines of evidence support our finding that phosphorylation of Thr308, but not Ser473, is necessary and sufficient for the activation of the intrinsic catalytic activity of Akt. First, Vogt and colleagues concluded that phosphorylation of this site, but not Ser473, was sufficient for the oncogenic potential of Akt (26). Specifically, they showed that mutating Thr308 prevented the phosphorylation of Akt substrates and transformation of chicken embryonic fibroblasts. Secondly, Jacinto and coworkers showed that in cells lacking mTORC2, Akt was not phosphorylated on Ser473, yet retained the ability to phosphorylate a subset of Akt substrates (14). Based on this and other reports from the literature, phosphorylation of Ser473 was suggested to determine substrate specificity of Akt. Supporting a role of Ser473 in the cellular function of Akt, loss of phosphorylation of this site by overexpressing the PH domain Leucine-rich repeat Protein Phosphatase (PHLPP) (which dephosphorylates Ser473) results in increased apoptosis (51) and, conversely, enhancing phosphorylation at Ser473 by depletion of PHLPP results in suppression of tumors in a xenograft model. Although these effects on apoptosis may arise from non-Akt functions of PHLPP (52), they support a role of Ser473 phosphorylation in Akt biology. Phosphorylation at Ser473 may function to tune rather than activate Akt signaling. However, our results establish that the phosphorylation state of Thr308 is a superior read-out for the activation state of Akt. Whereas

phosphorylation at Thr308 alone is able to activate Akt1, phosphorylation of Ser473 alone leads to relatively weak activity *in vitro*, and no detectable activity in the cell. Neither the Asp308 nor Glu308 Akt1 mutants had EGF-stimulated activity in cells. Although the pAkt1^{S473} Thr308Asp mutant had 10% of the activity of the doubly-phosphorylated Akt1 *in vitro* (Table 2.2), we observed no cellular Akt1 activity and impaired Ser473 phosphorylation in Akt1 variants with Asp, Glu or Ala mutations at position 308. Even if these variants are active in the cell, the low intrinsic activity of the Asp308 mutant, for example, may be too low to overcome the opposing de-phosphorylation reaction catalyzed by cellular phosphatases acting on Akt substrates, including the BKAR reporter. Akt1 Thr308Asp mutants can no longer be regarded as constitutively active Akt1 variants (30-32).

The phosphorylation status of Akt1 is indeed clinically relevant. Whereas pSer473 is the most commonly used biomarker (53,54), pThr308 was identified as a better prognostic biomarker in human non-small cell lung cancer (55) and acute myeloid leukemia (56). Our study underscores the relevance of examining the phosphorylation state of Thr308 as a general marker for the activation state of Akt in basic research and clinical settings.

Table 2.2 Akt enzyme activity comparison in vitro and in COS7 cells

Akt1 sites		Akt1 activity	
308	473	in vitro (%)	in cell
pThr	pSer	100	+
Thr	Ser	<1	N.D.
pThr	Ser	27	N.D.
pThr	Asp	24	+
pThr	Glu	15	+
pThr	Ala	9	+
Thr	pSer	5	N.D.
Asp	pSer	10	0
Glu	pSer	N.D.	0
Ala	pSer	<0.001	0
pSer	pSer	N.D.	+*
pSer	Ser	1	N.D.
pSer	Ala	N.D.	+*
Thr	Asp	<1	N.D.
Thr	Glu	<1	N.D.
Asp	Ser	<1	N.D.
Glu	Ser	<1	N.D.

Enzyme activity determined biochemically is given as a percentage of the maximally active ppAkt1. Agonist-evoked enzyme activity in cells is denoted by a plus (+) sign. No detectible activity is denoted by zero (0). N.D. Not determined.

*Observed a reduced response compared to WT when the Akt1 mutant indicated was expressed at a reduced level, similar to the level of endogenous Akt (Figure 3B).

2.5.3 Acidic residues do not function as phosphomimetics in Akt1. Koshland and colleagues first introduced the use of acidic amino acids to mimic phosphate, showing that an Asp mimicked the functional effect of phosphate to inactivate isocitrate dehydrogenase (57). There are other examples where the negative charge of the amino acid effectively mimics that of phosphate and, indeed, nature has used the trick in reverse: it is estimated that 5% of pSer sites may have evolved from an ancestor with Glu or Asp at the homologous position (34). However, acidic residues are not necessarily 'phosphomimetics': their negative charge, hydration sphere, and size are considerably different from a phosphorylated Ser or Thr residue. Acidic amino acids have a net negative charge of 1 compared to 2 for phosphate at neutral pH, the hydration sphere is 4 waters compared to 14 for phosphate, and their volume is considerably smaller than the phospho-amino acids they replace.

Because kinases are often activated by phosphorylation, Glu and Asp mutations are routinely used to mimic the function of phosphorylated serine or threonine residues in many kinases (33), including Akt1 (26,29). Our current study reveals that acidic residues do not mimic phosphorylation in Akt1. This is an important finding given the widespread and continued use of phosphomimetics in interrogating Akt signaling, and kinase signaling generally, in cells. The phosphomimetic variants that were active in our assays are 30 to 210-fold less active than their monophosphorylated counterparts. Based on our data in live cells, Thr308 phosphomimetic variants fail to activate Akt signaling (Figure 2.4A). Although, Akt1 Thr308Asp was the most active of the phosphomimetic mutants *in vitro*, this level of activity is insufficient to produce a cellular response. This result is consistent with a study from Vogt and colleagues who reported that Thr308Asp was an 'unsuitable' substitution for pThr308

(26,29). Their data showed that Akt Thr308Asp did not cause the phosphorylation of cellular Akt substrates such as GSK-3 β nor did it induce oncogenic transformation, functions that were robustly mediated by wild-type Akt. Nonetheless, phosphomimetics constructs of Akt pervade the literature on Akt signaling mechanisms. Because phosphomimetics do not result in physiologically relevant activation of the kinase activity of Akt, it is possible that any biological effects of such mutations may arise from scaffolding functions or non-catalytic mechanisms, as increasingly described for protein kinase family members (58).

An interesting finding from our study is that pSer is not well tolerated at position 308. According to the *in vitro* kinase assays, pAkt1^{S308} is more active than phosphomimetic 308 mutants, yet pAkt1^{T308} is ~25-fold more active than pAkt1^{S308}. Furthermore, when phosphorylated post-translationally in the cell, pAkt1^{S308} also showed reduced activity compared to that of pAkt1^{T308}. To the best of our understanding, there is no literature-based evidence on the effect of pS308 on the activation of Akt1 *in vivo* or *in vitro*. Our results indicate that the γ -methyl group of Thr308 optimally positions the phosphate to create the ordered hydrogen-bonding network between the phosphate of pThr308, His194 and Arg273, which facilitates the binding to ATP and substrate at the Akt1 active site (26). Indeed, this observation is supported by crystallographic structures of Akt in partially active form and in complex with substrate peptide and an ATP analog (59,60). The structure shows pThr308 is involved in salt bridge interactions with Arg273 and Lys297, and a water-mediated hydrogen bond with His194 is also evident (Figure 2.6). In the structure, steric complementarity can be seen between the γ -methyl of pThr308 and Cys310 (Figure 2.6B). In agreement with our cell-based and biochemical data, the structure suggests the interaction between the γ -

methyl of pThr308 and Cys310 optimally orients the phosphate for interaction with its positively charged counterparts in the salt bridges. We hypothesize that pS308, which lacks this methyl group, will rotate more freely in the active site, perhaps shifting the enzyme in and out of its active conformation.

Our study also revealed that Ala is not a mimic of un-phosphorylated Thr308. Ala at position 308 resulted in a 10-fold reduction in the basal activity of Akt1 mono-phosphorylated at Ser473. Taylor and colleagues also found that Ala at the activation loop of protein kinase A (PKA) results in a more severe kinetic defect compared to having an un-phosphorylated Thr at the activation loop (61,62). The detrimental effect of Ala may arise from a loss of hydrogen bonding ability. The Ala mutation favors a helical conformation of the activation loop that may also drive structural ensembles away from the active conformation or it may favor an inhibited conformation due to interactions with other domains or proteins (A. Kornev and S. Taylor, personal communication).

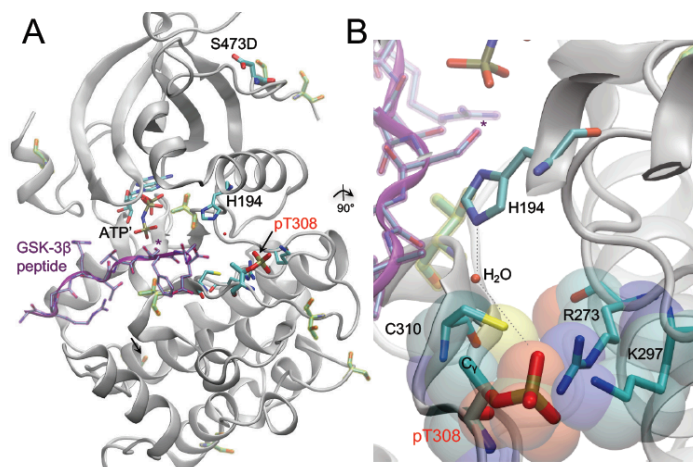


Figure 2.6 Structure of human Akt in complex with GSK-3 β substrate peptide.

(A) Structure of the active human pAkt^{T308} (S473D) kinase domain (gray, cartoon) shown in complex with GSK-3 β peptide (purple) and a non-hydrolyzable ATP analog (ATP' = AMP-PNP). The target of Akt1 phosphorylation on the GSK-3 β substrate peptide is indicated (*). Key residues are labeled and in addition to the regulatory phosphorylation sites (pThr308, and phosphomimetic mutation S473D), other Ser/Thr phosphorylation sites are highlighted (yellow). (B) A 90° rotated and close up view of the Akt1 active site, focused on the position of pThr308. The phosphate at position 308 makes extensive salt bridge interactions with Arg273 and Lys297; pThr308 also participates in a water mediated hydrogen bond network with His194. The C γ methyl group on pThr308 forms a hydrophobic interaction with Cys310 as indicated in the van der Waals surface representation (transparent). The figure includes structural data from PDB codes 3CQU (59) and 1O6K (60).

2.5.4 Akt1 activation threshold in cells. Live cell imaging experiments revealed that all forms of Akt1 that had phosphorylation of Thr at position 308 had maximal signaling output following EGF stimulation of cells, regardless of the

amino acid at position 473 (Table 2.2). In contrast, no activity was observed if the residue at position 308 was not phosphorylated, regardless of the amino acid at position 473 (Table 2.2). Based on *in vitro* data, we can estimate a threshold to observe Akt activity in cells: our *in vitro* studies reveal that phosphorylation of Ser473 alone stimulated Akt1 activity 80-fold over the un-phosphorylated enzyme, yet this level of Akt1 activity is apparently below the threshold required to elicit Akt1 signaling in the cell. *In vitro*, the activity of mono-phosphorylated Akt1 at Thr308 was reduced by less than 3-fold compared to the maximally-active and doubly-phosphorylated enzyme, and this level of activity is sufficient for maximal signaling output in cells. In addition, pAkt1^{T308} S473A was 10-fold less active than the doubly phosphorylated enzyme, yet this enzyme was sufficient for robust Akt1 signaling in the cell. Taken together, our data suggest that Akt1 variants whose intrinsic catalytic activity is greater than ~10% that of doubly phosphorylated enzyme are robustly active in cells. This novel finding further suggests that in cells only a fraction of Akt needs to be activated by phosphorylation for full Akt-dependent signaling.

Although previous reports have identified a 10-fold activity increase associated with Ser473 phosphorylation in immuno-precipitates, the stoichiometry of phosphorylation was unknown (6). Furthermore, the phosphorylation state of Akt1 may differentially impact substrates based on the local concentration or abundance of the substrate. For example, if Akt1 is poised on a scaffold and co-localized with substrate, a lack of Ser473 phosphorylation may be irrelevant as the local concentration of substrate is high. For phosphorylation of an untethered or low abundant substrate, that 3-fold difference in activity may reduce or eliminate Akt1 signaling in a substrate-specific manner. Indeed, previous work suggested that phosphorylation at Ser473

may increase the activity of Akt1 for particular substrates, e.g., FoxO1/3a (14), yet significant future efforts are required to define the impact of Akt1 phosphorylation status on substrate selectivity.

Mutation and phosphorylation state can impact Akt1 activity directly, but also indirectly by altering the accessibility of phosphatases. For example, previous studies showed that Ala substitution in PKC at the hydrophobic motif increased phosphatase sensitivity of PKC at additional sites (63). Our finding that Akt1 mutants with very low activity did not signal in cells suggests that localized activation needs to exceed a threshold to outcompete phosphatases in the cellular environment. Thus, the dynamic range of Akt signaling in a cell is such that sufficient Akt needs to be activated to overcome phosphatase suppression, but additional changes in phosphorylation site occupancy may or may not be needed for maximal signaling, particularly if localized to protein scaffolds and microdomains (64).

2.6 Conclusion

We established a robust protocol to generate and characterize fully active and differentially active Akt1 variants. These Akt1 variants will next be employed as a unique set of tools to investigate Akt1 substrate specificity and to screen for potential drug candidates against the most potent and active forms of the oncogenic kinase. The ability to produce optimally active Akt1 will have broad implications for drug discovery efforts, as we hypothesize that drug screening against a fully active Akt1 enzyme will more likely produce a potent and selective inhibitor.

We presented a systematic study of the impact of phosphorylation and phosphomimetic substitutions on the activity of Akt1 in the test tube and in living cells. Our data suggest that phosphomimetic substitutions should be tested for their ability to mimic phosphorylation, and Ala substitutions should also be assessed for their ability to act as non-phosphorylatable counterparts. We found these common assumptions simply do not hold for Akt1 and complementary experiments *in vitro* and in the cell were invaluable in reaching this conclusion. Viewing the cell-based assays in isolation of the kinase activity data may have led to the erroneous conclusion that phosphomimetic substitution at 473 supports full Akt1 activity. Rather the situation in the cell is more complex. Phosphorylation at 308 alone is sufficient to observe maximal Akt1 signaling, and mutation of 473 to Ala, Asp or Glu had no apparent impact on Akt1 activity in the cell. In the test tube, we determined that phosphomimetics fall far short of activating Akt1 to the same extent as true phosphorylation at both regulatory sites. Indeed and counter-intuitively, we found that Thr308Asp is a good mimic of a *lack* of phosphorylation at 308, while Thr308Ala is a kinase-dead enzyme that is not active *in vitro* despite phosphorylation at 473. In the cell, only true phosphorylation at 308 led to observable activity, suggesting that mimics of pThr are particularly inappropriate at this site. Interestingly, pSer308 shows reduced activity, which was still sufficient to observed Akt1 signaling in the cell. The data demonstrate conclusively that pSer is a far better mimic of pThr than Asp or Glu phosphomimetic substitutions.

The foregoing experiments reveal that phosphorylated Thr308 is necessary, sufficient, and irreplaceable for activation of Akt *in vitro* and in cells. We showed that no other residue is tolerated at that site, including acidic amino acids, which are frequently used as 'phosphomimetics'. Our findings also question the

generally assumed validity of using Ala mutation to mimic a non-phosphorylated form of residues like Ser and Thr. In total, our results beg caution in using amino acid substitutions to examine the role of phosphorylation in protein function and in the biology of cell signaling.

Acknowledgements

We are grateful to Ilka Heinemann and David Litchfield for critical discussions and suggestions on the manuscript and to Alexandr Kornev and Susan Taylor for helpful discussions.

2.7 References

1. Manning, B. D., and Cantley, L. C. (2007) AKT/PKB signaling: navigating downstream. *Cell* **129**, 1261-1274
2. Hanada, M., Feng, J., and Hemmings, B. A. (2004) Structure, regulation and function of PKB/AKT--a major therapeutic target. *Biochim Biophys Acta* **1697**, 3-16
3. Altomare, D. A., and Testa, J. R. (2005) Perturbations of the AKT signaling pathway in human cancer. *Oncogene* **24**, 7455-7464
4. Dai, D. L., Martinka, M., and Li, G. (2005) Prognostic significance of activated Akt expression in melanoma: a clinicopathologic study of 292 cases. *J Clin Oncol* **23**, 1473-1482
5. Wong, K. K., Engelman, J. A., and Cantley, L. C. (2010) Targeting the PI3K signaling pathway in cancer. *Curr Opin Genet Dev* **20**, 87-90
6. Alessi, D. R., Caudwell, F. B., Andjelkovic, M., Hemmings, B. A., and Cohen, P. (1996) Molecular basis for the substrate specificity of protein kinase B; comparison with MAPKAP kinase-1 and p70 S6 kinase. *FEBS Lett* **399**, 333-338
7. Hassan, B., Akcakanat, A., Holder, A. M., and Meric-Bernstam, F. (2013) Targeting the PI3-kinase/Akt/mTOR signaling pathway. *Surg Oncol Clin N Am* **22**, 641-664

8. Martini, M., De Santis, M. C., Braccini, L., Gulluni, F., and Hirsch, E. (2014) PI3K/AKT signaling pathway and cancer: an updated review. *Ann Med* **46**, 372-383
9. Alessi, D. R., James, S. R., Downes, C. P., Holmes, A. B., Gaffney, P. R., Reese, C. B., and Cohen, P. (1997) Characterization of a 3-phosphoinositide-dependent protein kinase which phosphorylates and activates protein kinase Balpha. *Curr. Biol.* **7**, 261-269
10. Keranen, L. M., Dutil, E. M., and Newton, A. C. (1995) Protein kinase C is regulated in vivo by three functionally distinct phosphorylations. *Curr Biol* **5**, 1394-1403
11. Tsutakawa, S. E., Medzihradzky, K. F., Flint, A. J., Burlingame, A. L., and Koshland, D. E., Jr. (1995) Determination of in vivo phosphorylation sites in protein kinase C. *J Biol Chem* **270**, 26807-26812
12. Pearson, R. B., Dennis, P. B., Han, J.-W., Williamson, N. A., Kozma, S. C., Wettenhall, R. E. H., and Thomas, G. (1995) The principal target of rapamycin-induced p70s6k inactivation is a novel phosphorylation site within a conserved hydrophobic domain. *EMBO J.* **14**, 5279-5287
13. Sarbassov, D. D., Guertin, D. A., Ali, S. M., and Sabatini, D. M. (2005) Phosphorylation and regulation of Akt/PKB by the rictor-mTOR complex. *Science* **307**, 1098-1101
14. Jacinto, E., Facchinetti, V., Liu, D., Soto, N., Wei, S., Jung, S. Y., Huang, Q., Qin, J., and Su, B. (2006) SIN1/MIP1 maintains rictor-mTOR complex integrity and regulates Akt phosphorylation and substrate specificity. *Cell* **127**, 125-137
15. Iacovides, D. C., Johnson, A. B., Wang, N., Boddapati, S., Korkola, J., and Gray, J. W. (2013) Identification and quantification of AKT isoforms and phosphoforms in breast cancer using a novel nanofluidic immunoassay. *Mol Cell Proteomics* **12**, 3210-3220
16. LoRusso, P. M. (2016) Inhibition of the PI3K/AKT/mTOR Pathway in Solid Tumors. *J Clin Oncol*
17. Massihnia, D., Avan, A., Funel, N., Maftouh, M., van Krieken, A., Granchi, C., Raktoe, R., Boggi, U., Aicher, B., Minutolo, F., Russo, A., Leon, L. G., Peters, G. J., and Giovannetti, E. (2017) Phospho-Akt overexpression is prognostic and can be used to tailor the synergistic interaction of Akt inhibitors with gemcitabine in pancreatic cancer. *J Hematol Oncol* **10**, 9
18. Liao, Y., Yuan, S., Chen, X., Zhu, P., Li, J., Qin, L., and Liao, W. (2017) Up-regulation of BRCA1-associated RING Domain 1 Promotes Hepatocellular Carcinoma Progression by Targeting Akt Signaling. *Sci Rep* **7**, 7649

19. Xiao, J., Yu, W., Hu, K., Li, M., Chen, J., and Li, Z. (2017) miR-92a promotes tumor growth of osteosarcoma by targeting PTEN/AKT signaling pathway. *Oncol Rep* **37**, 2513-2521
20. Parker, L., Levinger, I., Mousa, A., Howlett, K., and de Courten, B. (2016) Plasma 25-Hydroxyvitamin D Is Related to Protein Signaling Involved in Glucose Homeostasis in a Tissue-Specific Manner. *Nutrients* **8**
21. Tang, H., Wu, Y., Liu, M., Qin, Y., Wang, H., Wang, L., Li, S., Zhu, H., He, Z., Luo, J., Wang, H., Wang, Q., and Luo, S. (2016) SEMA3B improves the survival of patients with esophageal squamous cell carcinoma by upregulating p53 and p21. *Oncol Rep* **36**, 900-908
22. Yang, Y., Huang, Y., Wang, Z., Wang, H. T., Duan, B., Ye, D., Wang, C., Jing, R., Leng, Y., Xi, J., Chen, W., Wang, G., Jia, W., Zhu, S., and Kang, J. (2016) HDAC10 promotes lung cancer proliferation via AKT phosphorylation. *Oncotarget* **7**, 59388-59401
23. Klein, S., Geiger, T., Linchevski, I., Lebendiker, M., Itkin, A., Assayag, K., and Levitzki, A. (2005) Expression and purification of active PKB kinase from *Escherichia coli*. *Protein Expr Purif* **41**, 162-169
24. Zhang, X., Zhang, S., Yamane, H., Wahl, R., Ali, A., Lofgren, J. A., and Kendall, R. L. (2006) Kinetic mechanism of AKT/PKB enzyme family. *J Biol Chem* **281**, 13949-13956
25. Fabbro, D., Batt, D., Rose, P., Schacher, B., Roberts, T. M., and Ferrari, S. (1999) Homogeneous purification of human recombinant GST-Akt/PKB from Sf9 cells. *Protein Expr Purif* **17**, 83-88
26. Hart, J. R., and Vogt, P. K. (2011) Phosphorylation of AKT: a mutational analysis. *Oncotarget* **2**, 467-476
27. Tobisawa, T., Yano, T., Tanno, M., Miki, T., Kuno, A., Kimura, Y., Ishikawa, S., Kouzu, H., Nishizawa, K., Yoshida, H., and Miura, T. (2017) Insufficient activation of Akt upon reperfusion because of its novel modification by reduced PP2A-B55alpha contributes to enlargement of infarct size by chronic kidney disease. *Basic Res Cardiol* **112**, 31
28. Warsi, J., Fezai, M., Fores, M., Elvira, B., and Lang, F. (2015) Up-Regulation of Voltage Gated K⁺ Channels Kv1.3 and Kv1.5 by Protein Kinase PKB/Akt. *Cell Physiol Biochem* **37**, 2454-2463
29. Liu, P., Begley, M., Michowski, W., Inuzuka, H., Ginzberg, M., Gao, D., Tsou, P., Gan, W., Papa, A., Kim, B. M., Wan, L., Singh, A., Zhai, B., Yuan, M., Wang, Z., Gygi, S. P., Lee, T. H., Lu, K. P., Toker, A., Pandolfi, P. P., Asara, J. M., Kirschner, M. W., Sicinski, P., Cantley, L., and Wei, W. (2014) Cell-cycle-regulated activation of Akt kinase by phosphorylation at its carboxyl terminus. *Nature* **508**, 541-545

30. Zhuo, D. X., Zhang, X. W., Jin, B., Zhang, Z., Xie, B. S., Wu, C. L., Gong, K., and Mao, Z. B. (2013) CSTP1, a novel protein phosphatase, blocks cell cycle, promotes cell apoptosis, and suppresses tumor growth of bladder cancer by directly dephosphorylating Akt at Ser473 site. *PLoS One* **8**, e65679
31. Munoz, C., Almilaji, A., Setiawan, I., Foller, M., and Lang, F. (2013) Up-regulation of the inwardly rectifying K(+) channel Kir2.1 (KCNJ2) by protein kinase B (PKB/Akt) and PIKfyve. *J Membr Biol* **246**, 189-197
32. Berndt, N., Yang, H., Trinczek, B., Betzi, S., Zhang, Z., Wu, B., Lawrence, N. J., Pellecchia, M., Schonbrunn, E., Cheng, J. Q., and Sebti, S. M. (2010) The Akt activation inhibitor TCN-P inhibits Akt phosphorylation by binding to the PH domain of Akt and blocking its recruitment to the plasma membrane. *Cell Death Differ* **17**, 1795-1804
33. Dissmeyer, N., and Schnittger, A. (2011) Use of phospho-site substitutions to analyze the biological relevance of phosphorylation events in regulatory networks. *Methods Mol Biol* **779**, 93-138
34. Pearlman, S. M., Serber, Z., and Ferrell, J. E., Jr. (2011) A mechanism for the evolution of phosphorylation sites. *Cell* **147**, 934-946
35. Hunter, T. (2012) Why nature chose phosphate to modify proteins. *Philos Trans R Soc Lond B Biol Sci* **367**, 2513-2516
36. Kunkel, M. T., Ni, Q., Tsien, R. Y., Zhang, J., and Newton, A. C. (2005) Spatio-temporal dynamics of protein kinase B/Akt signaling revealed by a genetically encoded fluorescent reporter. *J Biol Chem* **280**, 5581-5587
37. Park, H. S., Hohn, M. J., Umehara, T., Guo, L. T., Osborne, E. M., Benner, J., Noren, C. J., Rinehart, J., and Söll, D. (2011) Expanding the genetic code of *Escherichia coli* with phosphoserine. *Science* **333**, 1151-1154
38. George, S., Aguirre, J. D., Spratt, D. E., Bi, Y., Jeffery, M., Shaw, G. S., and O'Donoghue, P. (2016) Generation of phospho-ubiquitin variants by orthogonal translation reveals codon skipping. *FEBS Lett* **590**, 1530-1542
39. Adams, J. A., and Taylor, S. S. (1992) Energetic limits of phosphotransfer in the catalytic subunit of cAMP-dependent protein kinase as measured by viscosity experiments. *Biochemistry* **31**, 8516-8522
40. Toker, A., and Newton, A. C. (2000) Akt/protein kinase B is regulated by autophosphorylation at the hypothetical PDK-2 site. *J Biol Chem* **275**, 8271-8274
41. Kunkel, M. T., and Newton, A., C. (2009) Spatiotemporal Dynamics of Kinase Signaling Visualized by Targeted Reporters. *Curr. Protoc. Chem Biol.* **1**, 17-28
42. Ebner, M., Lucic, I., Leonard, T. A., and Yudushkin, I. (2017) PI(3,4,5)P3 Engagement Restricts Akt Activity to Cellular Membranes. *Mol Cell* **65**, 416-431 e416

43. Walsh, D. A., Perkins, J. P., and Krebs, E. G. (1968) An adenosine 3',5'-monophosphate-dependant protein kinase from rabbit skeletal muscle. *J Biol Chem* **243**, 3763-3765
44. Antal, C. E., and Newton, A. C. (2014) Tuning the signalling output of protein kinase C. *Biochemical Society transactions* **42**, 1477-1483
45. Andjelkovic, M., Alessi, D. R., Meier, R., Fernandez, A., Lamb, N. J., Frech, M., Cron, P., Cohen, P., Lucocq, J. M., and Hemmings, B. A. (1997) Role of translocation in the activation and function of protein kinase B. *J Biol Chem* **272**, 31515-31524
46. Kohn, A. D., Takeuchi, F., and Roth, R. A. (1996) Akt, a pleckstrin homology domain containing kinase, is activated primarily by phosphorylation. *J Biol Chem* **271**, 21920-21926
47. Andjelkovic, M., Maira, S.-M., Cron, P., Parker, P. J., and Hemmings, B. A. (1999) Domain swapping used to investigate the mechanism of protein kinase B regulation by 3-phosphoinositide-dependent protein kinase 1 and Ser473 kinase. *Mol. Cell Biol.* **19**, 5061-5072
48. Scheid, M. P., Marignani, P. A., and Woodgett, J. R. (2002) Multiple phosphoinositide 3-kinase-dependent steps in activation of protein kinase B. *Mol Cell Biol* **22**, 6247-6260
49. Hauge, C., Antal, T. L., Hirschberg, D., Doehn, U., Thorup, K., Idrissova, L., Hansen, K., Jensen, O. N., Jorgensen, T. J., Biondi, R. M., and Frodin, M. (2007) Mechanism for activation of the growth factor-activated AGC kinases by turn motif phosphorylation. *Embo J* **26**, 2251-2261
50. Brodbeck, D., Cron, P., and Hemmings, B. A. (1999) A human protein kinase Bgamma with regulatory phosphorylation sites in the activation loop and in the C-terminal hydrophobic domain. *J Biol Chem* **274**, 9133-9136
51. Gao, T., Furnari, F., and Newton, A. C. (2005) PHLPP: a phosphatase that directly dephosphorylates Akt, promotes apoptosis, and suppresses tumor growth. *Mol Cell* **18**, 13-24
52. Newton, A. C., and Trotman, L. C. (2014) Turning off AKT: PHLPP as a drug target. *Annual review of pharmacology and toxicology* **54**, 537-558
53. Slipicevic, A., Holm, R., Nguyen, M. T., Bohler, P. J., Davidson, B., and Florenes, V. A. (2005) Expression of activated Akt and PTEN in malignant melanomas: relationship with clinical outcome. *Am J Clin Pathol* **124**, 528-536
54. Tsao, A. S., McDonnell, T., Lam, S., Putnam, J. B., Bekele, N., Hong, W. K., and Kurie, J. M. (2003) Increased phospho-AKT (Ser(473)) expression in bronchial dysplasia: implications for lung cancer prevention studies. *Cancer Epidemiol Biomarkers Prev* **12**, 660-664
55. Vincent, E. E., Elder, D. J., Thomas, E. C., Phillips, L., Morgan, C., Pawade, J., Sohail, M., May, M. T., Hetzel, M. R., and Tavare, J. M. (2011) Akt

- phosphorylation on Thr308 but not on Ser473 correlates with Akt protein kinase activity in human non-small cell lung cancer. *Br J Cancer* **104**, 1755-1761
56. Gallay, N., Dos Santos, C., Cuzin, L., Bousquet, M., Simmonet Gouy, V., Chaussade, C., Attal, M., Payrastre, B., Demur, C., and Recher, C. (2009) The level of AKT phosphorylation on threonine 308 but not on serine 473 is associated with high-risk cytogenetics and predicts poor overall survival in acute myeloid leukaemia. *Leukemia* **23**, 1029-1038
 57. Thorsness, P. E., and Koshland, D. E. (1987) Inactivation of isocitrate dehydrogenase by phosphorylation is mediated by the negative charge of the phosphate. *J. Biol. Chem.* **262**, 10422-10425
 58. Hu, J., Ahuja, L. G., Meharena, H. S., Kannan, N., Kornev, A. P., Taylor, S. S., and Shaw, A. S. (2015) Kinase regulation by hydrophobic spine assembly in cancer. *Mol Cell Biol* **35**, 264-276
 59. Lippa, B., Pan, G., Corbett, M., Li, C., Kauffman, G. S., Pandit, J., Robinson, S., Wei, L., Kozina, E., Marr, E. S., Borzillo, G., Knauth, E., Barbacci-Tobin, E. G., Vincent, P., Troutman, M., Baker, D., Rajamohan, F., Kakar, S., Clark, T., and Morris, J. (2008) Synthesis and structure based optimization of novel Akt inhibitors. *Bioorg Med Chem Lett* **18**, 3359-3363
 60. Yang, J., Cron, P., Good, V. M., Thompson, V., Hemmings, B. A., and Barford, D. (2002) Crystal structure of an activated Akt/protein kinase B ternary complex with GSK3-peptide and AMP-PNP. *Nat Struct Biol* **9**, 940-944
 61. Steichen, J. M., Kuchinskas, M., Keshwani, M. M., Yang, J., Adams, J. A., and Taylor, S. S. (2012) Structural basis for the regulation of protein kinase A by activation loop phosphorylation. *J Biol Chem* **287**, 14672-14680
 62. Adams, J. A., McGlone, M. L., Gibson, R., and Taylor, S. S. (1995) Phosphorylation modulates catalytic function and regulation in the cAMP-dependent protein kinase. *Biochemistry* **34**, 2447-2454
 63. Bornancin, F., and Parker, P. J. (1997) Phosphorylation of protein kinase C-alpha on serine 657 controls the accumulation of active enzyme and contributes to its phosphatase-resistant state [published erratum appears in *J Biol Chem* 1997 May 16;272(20):13458]. *J Biol Chem* **272**, 3544-3549
 64. Antal, C. E., and Newton, A. C. (2013) Spatiotemporal dynamics of phosphorylation in lipid second messenger signaling. *Mol Cell Proteomics* **12**, 3498-3508
 65. Edelheit, O., Hanukoglu, A., and Hanukoglu, I. (2009) Simple and efficient site-directed mutagenesis using two single-primer reactions in parallel to generate mutants for protein structure-function studies. *BMC Biotechnol* **9**, 61

66. Lee, S., Oh, S., Yang, A., Kim, J., Soll, D., Lee, D., and Park, H. S. (2013) A facile strategy for selective incorporation of phosphoserine into histones. *Angew Chem Int Ed Engl* **52**, 5771-5775
67. Reuveni, H., Livnah, N., Geiger, T., Klein, S., Ohne, O., Cohen, I., Benhar, M., Gellerman, G., and Levitzki, A. (2002) Toward a PKB inhibitor: modification of a selective PKA inhibitor by rational design. *Biochemistry* **41**, 10304-10314
68. Turowec, J. P., Duncan, J. S., French, A. C., Gyenis, L., St Denis, N. A., Vilks, G., and Litchfield, D. W. (2010) Protein kinase CK2 is a constitutively active enzyme that promotes cell survival: strategies to identify CK2 substrates and manipulate its activity in mammalian cells. *Methods Enzymol* **484**, 471-493
69. Manning, B. D., and Toker, A. (2017) AKT/PKB Signaling: Navigating the Network. *Cell* **169**, 381-405
70. Agarwal, E., Brattain, M. G., and Chowdhury, S. (2013) Cell survival and metastasis regulation by Akt signaling in colorectal cancer. *Cell Signal* **25**, 1711-1719
71. Spencer, A., Yoon, S. S., Harrison, S. J., Morris, S. R., Smith, D. A., Brigandi, R. A., Gauvin, J., Kumar, R., Opalinska, J. B., and Chen, C. (2014) The novel AKT inhibitor afuresertib shows favorable safety, pharmacokinetics, and clinical activity in multiple myeloma. *Blood* **124**, 2190-2195
72. Antonelli, M., Massimino, M., Morra, I., Garre, M. L., Gardiman, M. P., Buttarelli, F. R., Arcella, A., and Giangaspero, F. (2012) Expression of pERK and pAKT in pediatric high grade astrocytomas: correlation with YKL40 and prognostic significance. *Neuropathology* **32**, 133-138
73. Suzuki, Y., Shirai, K., Oka, K., Mobaraki, A., Yoshida, Y., Noda, S. E., Okamoto, M., Suzuki, Y., Itoh, J., Itoh, H., Ishiuchi, S., and Nakano, T. (2010) Higher pAkt expression predicts a significant worse prognosis in glioblastomas. *J Radiat Res* **51**, 343-348
74. Blachly, J. S., and Baiocchi, R. A. (2014) Targeting PI3-kinase (PI3K), AKT and mTOR axis in lymphoma. *Br J Haematol* **167**, 19-32
75. Westin, J. R. (2014) Status of PI3K/Akt/mTOR pathway inhibitors in lymphoma. *Clin Lymphoma Myeloma Leuk* **14**, 335-342
76. Balasuriya, N., Kunkel, M. T., Liu, X., Biggar, K. K., Li, S. S., Newton, A. C., and O'Donoghue, P. (2018) Genetic code expansion and live cell imaging reveal that Thr308 phosphorylation is irreplaceable and sufficient for Akt1 activity. *J Biol Chem*
77. Parikh, C., Janakiraman, V., Wu, W. I., Foo, C. K., Kljavin, N. M., Chaudhuri, S., Stawiski, E., Lee, B., Lin, J., Li, H., Lorenzo, M. N., Yuan, W., Guillory, J., Jackson, M., Rondon, J., Franke, Y., Bowman, K. K., Sagolla, M., Stinson, J., Wu, T. D., Wu, J., Stokoe, D., Stern, H. M., Brandhuber, B. J., Lin, K., Skelton, N. J., and Seshagiri, S. (2012) Disruption of PH-kinase domain interactions

- leads to oncogenic activation of AKT in human cancers. *Proc Natl Acad Sci U S A* **109**, 19368-19373
78. Gijzen, M., King, P., Perera, T., Parker, P. J., Harris, A. L., Larijani, B., and Kong, A. (2010) HER2 phosphorylation is maintained by a PKB negative feedback loop in response to anti-HER2 herceptin in breast cancer. *PLoS Biol* **8**, e1000563
 79. George, S., Wang, S. M., Bi, Y., Treidlinger, M., Barber, K. R., Shaw, G. S., and O'Donoghue, P. (2017) Ubiquitin phosphorylated at Ser57 hyperactivates parkin. *Biochim Biophys Acta* **1861**, 3038-3046
 80. Aerni, H. R., Shifman, M. A., Rogulina, S., O'Donoghue, P., and Rinehart, J. (2015) Revealing the amino acid composition of proteins within an expanded genetic code. *Nucleic acids research* **43**, e8
 81. Calleja, V., Alcor, D., Laguerre, M., Park, J., Vojnovic, B., Hemmings, B. A., Downward, J., Parker, P. J., and Larijani, B. (2007) Intramolecular and intermolecular interactions of protein kinase B define its activation in vivo. *PLoS Biol* **5**, e95
 82. Wu, W. I., Voegtli, W. C., Sturgis, H. L., Dizon, F. P., Vigers, G. P., and Brandhuber, B. J. (2010) Crystal structure of human AKT1 with an allosteric inhibitor reveals a new mode of kinase inhibition. *PLoS One* **5**, e12913

2.8 Supporting information:

2.8.1 Supporting experimental procedures

Bacterial strains and plasmids

The human *akt1* gene was purchased from Harvard PlasmidID repository service (Boston, MA, USA) and subcloned (*NdeI/BamHI*) into an IPTG (isopropyl β -D-1-thiogalactopyranoside) inducible T7 promoter driven expression vector (pDS1) (38). In addition, the *akt1* gene was subcloned (*NcoI/NotI*) into T7lac promoter preceded pCDFDuet-1 vector. *PDPK1* gene, which was also purchased from Harvard PlasmidID repository service, was subcloned (*KpnI/NdeI*) into the second cloning site of pCDFDuet-1. The codons for Ser and Thr residues in *akt1* at positions Ser473 and Thr308, were mutated to amber (TAG), Asp (GAC) and Glu (GAG) codons using site-directed mutagenesis according to previously described methods(65). Successful cloning was verified by DNA sequencing (London Regional Genomics Centre, London, ON, Canada and Genewiz, Cambridge, MA, USA).

Protein and phosphoprotein production. Recombinant proteins were expressed in BL21(DE3) (Invitrogen). Phosphoproteins were produced using 2nd generation mutants of the pSer incorporation system (SepRS9, EFSep21) (66). The system is encoded on the pDS-pSer2 plasmid (described previously (38)), which contains 5 copies of tRNA^{Sep}, SepRS9 and EFSep21. pDS1 and pDS-pSer2 plasmids for phosphoprotein expression were co-transformed into *E. coli* BL21(DE3) and plated on LB-agar plates with 25 μ g/ml kanamycin and 100 μ g/ml ampicillin. A single

colony was used to inoculate 70 ml of LB (with ampicillin 100 $\mu\text{g/ml}$, kanamycin 25 $\mu\text{g/ml}$), which was grown, shaking, overnight at 37°C. From this starter culture, a 10 ml inoculum was added to 1 l of LB medium with antibiotics (as above) and *O*-phospho-L-serine (pSer, 2.5 mM final concentration, Sigma Aldrich). These cultures were grown at 37°C until $\text{OD}_{600} = 0.6$ at which point 2.5 mM of additional pSer was added to the culture. Protein expression was induced by adding 300 μM of IPTG at $\text{OD}_{600} = 0.8$. Cultures were further incubated at 16°C for 18 h. The same protocol was used in producing pAkt1 variants from pCDFDuet-1 construct, except that here streptomycin (50 $\mu\text{g/ml}$) was used as the antibiotic to maintain pCDF. In expressing phosphomimetic proteins, ampicillin at a concentration of 100 $\mu\text{g/ml}$ was used and no pSer was added to the cultures.

Affinity column chromatography. The His-tagged proteins were purified using Ni affinity column chromatography. 0.5 ml of Ni-NTA resin (Thermo-Fisher Scientific) was used for 1 l of *E. coli* culture. The cell lysates (see Methods) were loaded into the column and washed extensively with wash buffer A (20 mM Hepes, 150 mM NaCl, 3 mM β -mercaptoethanol, 3mM Dithiothreitol, 1 mM Na_3VO_4 and 5 mM NaF, 15 mM Imidazole) followed by wash buffer B (20 mM Hepes, 150 mM NaCl, 3 mM β -mercaptoethanol, 3mM DTT, 1 mM Na_3VO_4 and 5 mM NaF, 20 mM Imidazole). Protein was eluted using an elution buffer (20 mM Hepes, 150 mM NaCl, 3 mM β -mercaptoethanol, 3mM dithiothreitol, 1 mM Na_3VO_4 and 5 mM NaF) with 75 mM Imidazole. The same protocol was used for Akt1 variants with phosphomimetic mutations except: 1) phosphatase inhibitors were excluded from the buffers, 2) the imidazole concentrations of the wash buffers were 20 - 50 mM, and 3) the imidazole concentration of the elution buffer was 200 mM. Fractions were run on 10% SDS (sodium dodecyl sulfate) poly-

acrylamide gels. Akt1 variants were further purified by size exclusion chromatography.

Size exclusion chromatography. Purification was performed in AKTA Pure L1 FPLC system (GE Healthcare, Little Chalfont, UK) using Superdex200 (GE Healthcare Uppsala, Sweden) gel filtration column equilibrated with buffer (25 mM HEPES pH 7.0, 100 mM NaCl, 0.25 mM Tris(2-carboxyethyl)phosphine (TCEP)). The flow rate was maintained at 0.1 ml/min and 1 ml fractions were collected. Fractions were run on 10% SDS-PAGE and pure fractions were pooled and concentrated using Vivaspin 6 (6 ml, 10 kDa, GE Health Care, Buckinghamshire, UK) or Amicon ultra (0.5 ml, 3 kDa, Merk Millipore Ltd., Cork, Ireland) concentration units. Protein concentrations were determined using Bradford assay and samples were stored in aliquots at -80°C in storage buffer (20 mM HEPES pH 7.0, 150 mM NaCl, 3 mM β -mercaptoethanol, 3mM DTT) containing 50% glycerol until further analysis.

Western blotting. The purified proteins were denatured using standard SDS sample buffer and run on a 10% SDS gel. The gel was blotted onto a nitrocellulose membrane using Turbo-Blot Turbo transfer system (Bio-Rad). The membrane was immunoblotted with mouse anti-histidine primary antibody (GE Health Care Life Sciences) followed by anti-mouse horseradish peroxidase linked secondary antibody (GE Health Care Life Sciences). Bands were visualized by chemiluminescence and detected with Gel Doc XR plus (Bio-Rad).

Multiple Reaction Monitoring (MRM) of pAkt1^{S473}. Following protein purification, phosphorylated Akt protein was re-suspended in trypsin digestion buffer (100 mM Tris pH 8.5, 1 mM CaCl₂) and digested at a ratio of 20:1 w/w with sequencing grade trypsin (Roche Diagnostics) overnight at 37°C. The digest was then analyzed by positive electrospray ionization LC-MS/MS on a triple quadrupole mass spectrometer (4000 QTRAP AB Sciex, Concord, ON, Canada) with Q3 used as a linear ion trap. A NanoAcquity UPLC system (Waters, Milford, MA, USA) equipped with a C18 analytical column (1.7 μm, 75 μm × 200 mm) was used to separate the peptides at the flow rate of 300 μl/min and operating pressure of 8000 psi. Peptides were eluted using a 62 min gradient from 95% solvent A (H₂O, 0.1% formic acid) and 5% B (acetonitrile, 0.1% formic acid) to 50% B in 41 min, 6 min at 90% B, and back to 5% for 10 min. Eluted peptides were directly electrosprayed (Nanosource, ESI voltage +2000V) into the mass spectrometer. The instrument was set to monitor 49 transitions in each sample with a dwelling time of 100 msec/transition. The in silico protease digest patterns and the corresponding MRM-MS transitions were compiled with Skyline software. Transitions that were larger than the precursor ion were selected on the basis of the Skyline predictions and the specific b/y ions that allow unambiguous identification of the selected phosphorylated Akt serine residues (Ser473) were included. Akt peptides were monitored for serine phosphorylation, non-phosphorylated serine, as well as a serine deletion or mistranslation with glutamine.

Parallel-Reaction Monitoring (PRM) of pAkt1^{T308}. Following protein purification, the Akt protein was precipitated in ice-cold acetone/ethanol/acetic acid (50/50/0.1, vol/vol/vol). The protein precipitate was re-suspended in 8 M Urea, then reduced

in 5mM dithiothreitol (DTT) at 37°C for 1h and alkylated in 14 mM iodoacetamide (IAA) in darkness at room temperature for 1h. Unreacted IAA was neutralized by adding 5 mM DTT in the suspension. The final protein concentration was determined by Bradford assay. Glu-C digestion was performed at 37°C overnight with a protein:Glu-C ratio of 20:1 w/w. The digest was desalted in C18 column (Phenomenex) according to the manufacture's protocol and re-suspended in MS-grade water. A Q Exactive Hybrid Quadrupole Orbitrap MS (Thermo Fisher Scientific) was used to analyze the peptides. Data were analyzed using Skyline software.

2.8.2 Supporting References

1. George, S., Aguirre, J. D., Spratt, D. E., Bi, Y., Jeffery, M., Shaw, G. S., and O'Donoghue, P. (2016) Generation of phospho-ubiquitin variants by orthogonal translation reveals codon skipping. *FEBS Lett* **590**, 1530-1542
2. Edelheit, O., Hanukoglu, A., and Hanukoglu, I. (2009) Simple and efficient site-directed mutagenesis using two single-primer reactions in parallel to generate mutants for protein structure-function studies. *BMC Biotechnol* **9**, 61
3. Lee, S., Oh, S., Yang, A., Kim, J., Soll, D., Lee, D., and Park, H. S. (2013) A facile strategy for selective incorporation of phosphoserine into histones. *Angew Chem Int Ed Engl* **52**, 5771-5775

2.8.3 Supporting Figures

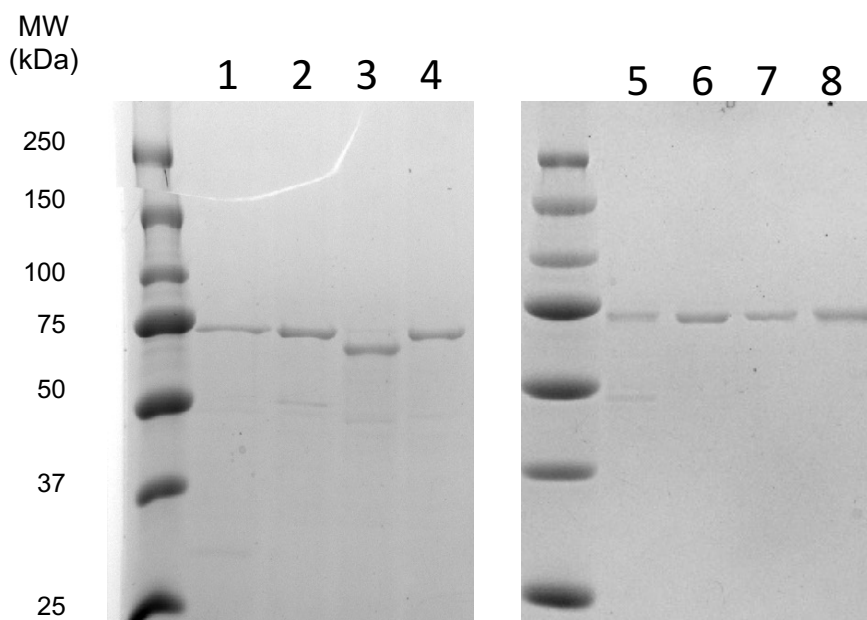


Figure S2.1 Production of purified full-length Akt1 and pAkt1 variants. Following affinity chromatography, purified fractions from size exclusion chromatography are visualized on SDS-PAGE. The Coomassie stained gel shows successful purification of full-length Akt1 variants: un-phosphorylated Akt (lane 1), pAkt^{T308} (lane 2), ppAkt^{T308, S473} (lane 3), Akt^{T308D} (lane 4), pAkt^{S473} (lane 5), pAkt^{S473} T308A (lane 6), pAkt^{S473} T308D (lane 7), Akt^{S473D} (lane 8). The ppAkt1 variant, likely due to its double negative charge, consistently runs slightly faster than anticipated. We found this to be reproducible from 3 independent preparations. MS/MS analysis confirmed the full-length ppAkt1 protein with the expected protein sequence (Figure S2.2).

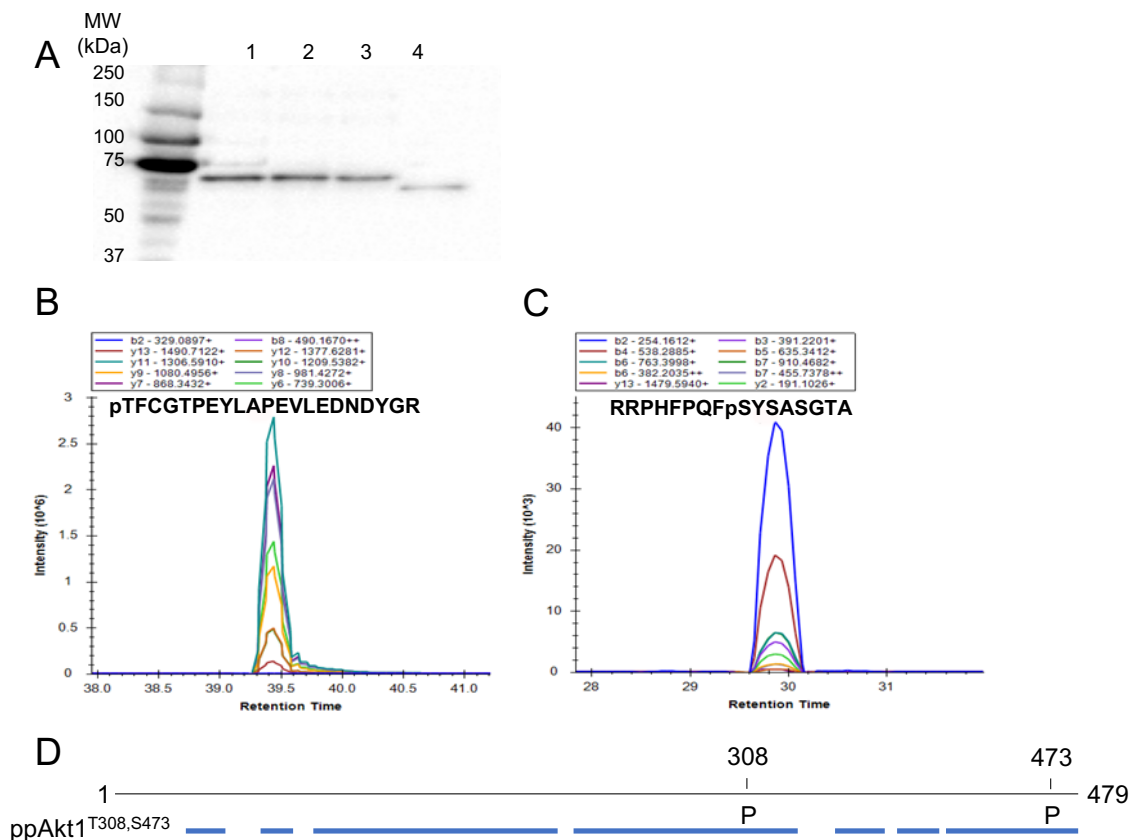


Figure S2.2 Physical characterization of ppAkt1^{T308, S473}. (A) Western blots of His₆-tagged un-phosphorylated Akt1 (lane 1), pAkt1^{T308} (lane 2), pAkt1^{S473} (lane 3), and ppAkt1^{T308, S473} (lane 4) with anti-his antibody. Mass spectra confirming enzymatically phosphorylated ppAkt1^{T308, S473}. PRM-MS/MS was used to identify peptides with pT308 (B) and pS473 (C) in tryptic or GluC-digested samples of ppAkt1^{T308, S473}, we were unable to identify peaks corresponding to de-phosphorylation or truncation of the ppAkt1. (D) Coverage map shows the locations in the protein sequence where one or more significant tryptic or GluC peptides were identified (blue line) by PRM-MS/MS in the ppAkt1^{T308, S473} sample. Identified phosphorylation sites are indicated (P).

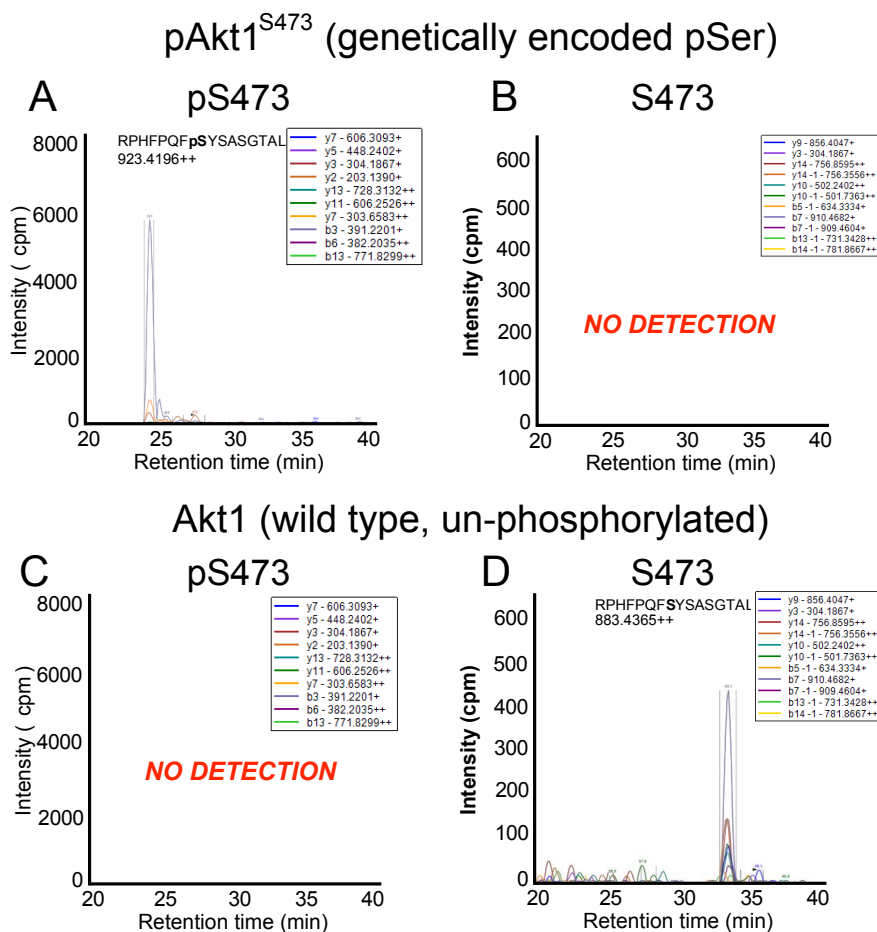


Figure S2.3 Mass spectra confirming quantitative, genetically encoded pS473 in Akt1. MRM-MS/MS was used to identify pS473 (A) but not Ser473 in a tryptic digested sample of pure pAkt1^{S473} (A, B). MRM-MS/MS also identified a peptide containing S473 (D) but not pS473 (C) in samples of purified and un-phosphorylated Akt1 (C, D).

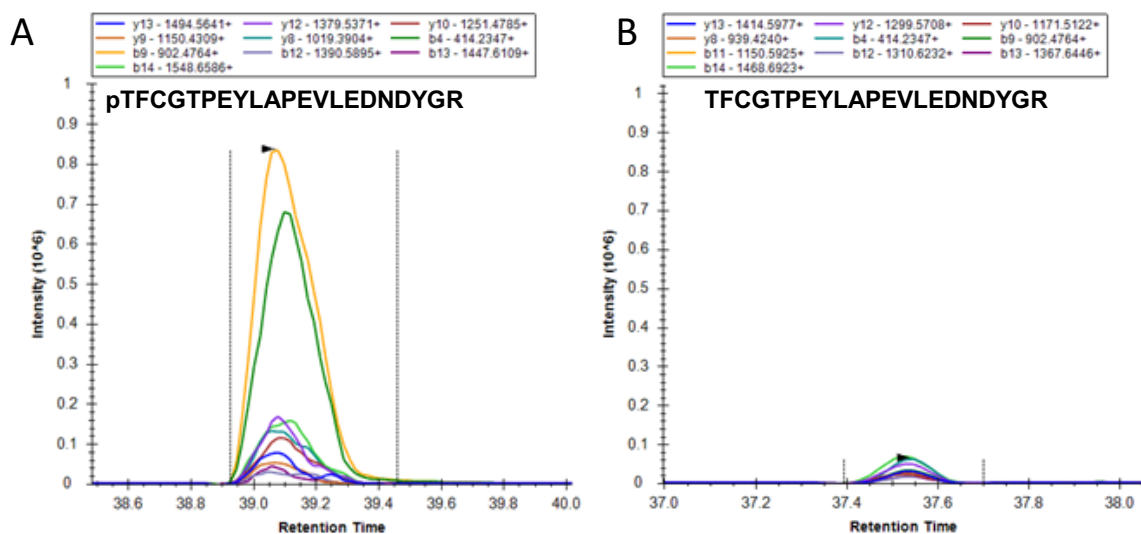


Figure S2.4 Physical characterization of pAkt1^{T308}. Mass spectra (A) confirming enzymatically phosphorylated pAkt1^{T308}. PRM-MS/MS was used to identify peptides in tryptic digested samples of pAkt1^{T308}. Abundant occurrence of peptide containing pThr308 (A) and relatively very low levels of unphosphorylated form of peptide containing T308 (B) were identified.

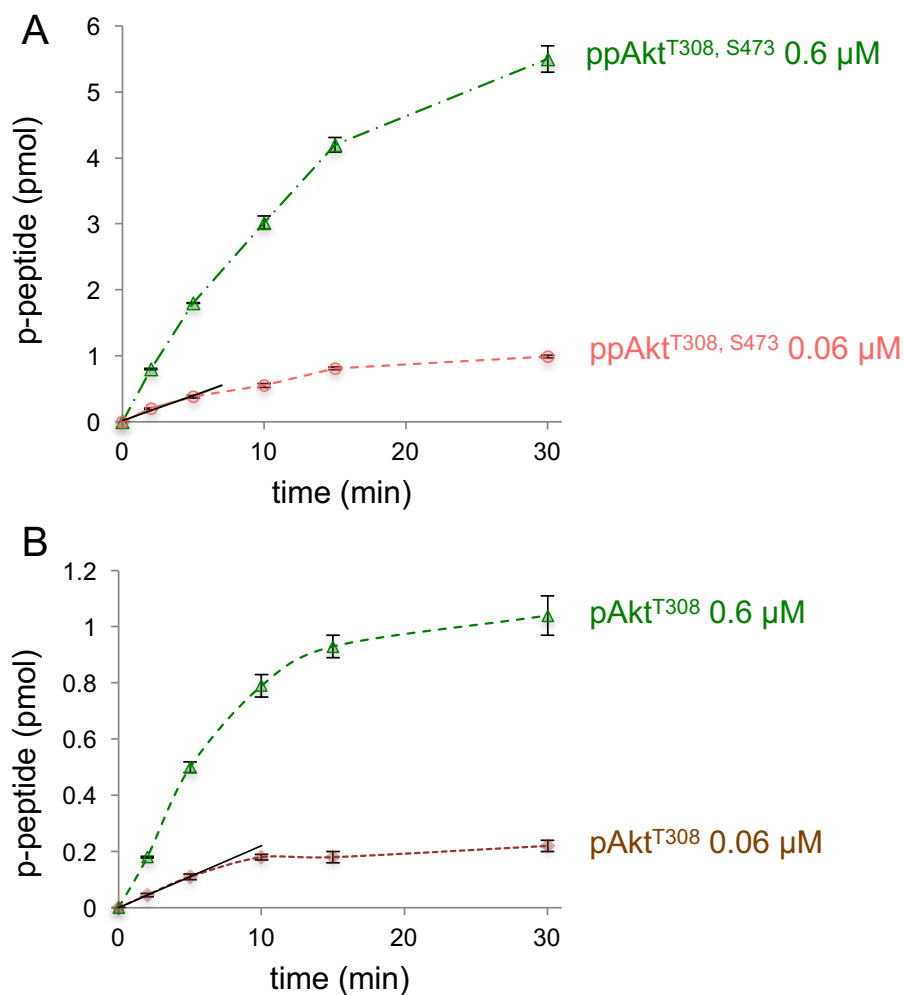


Figure S2.5 Activity of pAkt1^{T308} variants at reduced enzyme concentrations. A) ppAkt1 and B) pAkt1^{T308} enzyme concentrations were reduced by 10-fold in order to provide a sufficient linear phase to measure the reaction velocity accurately.

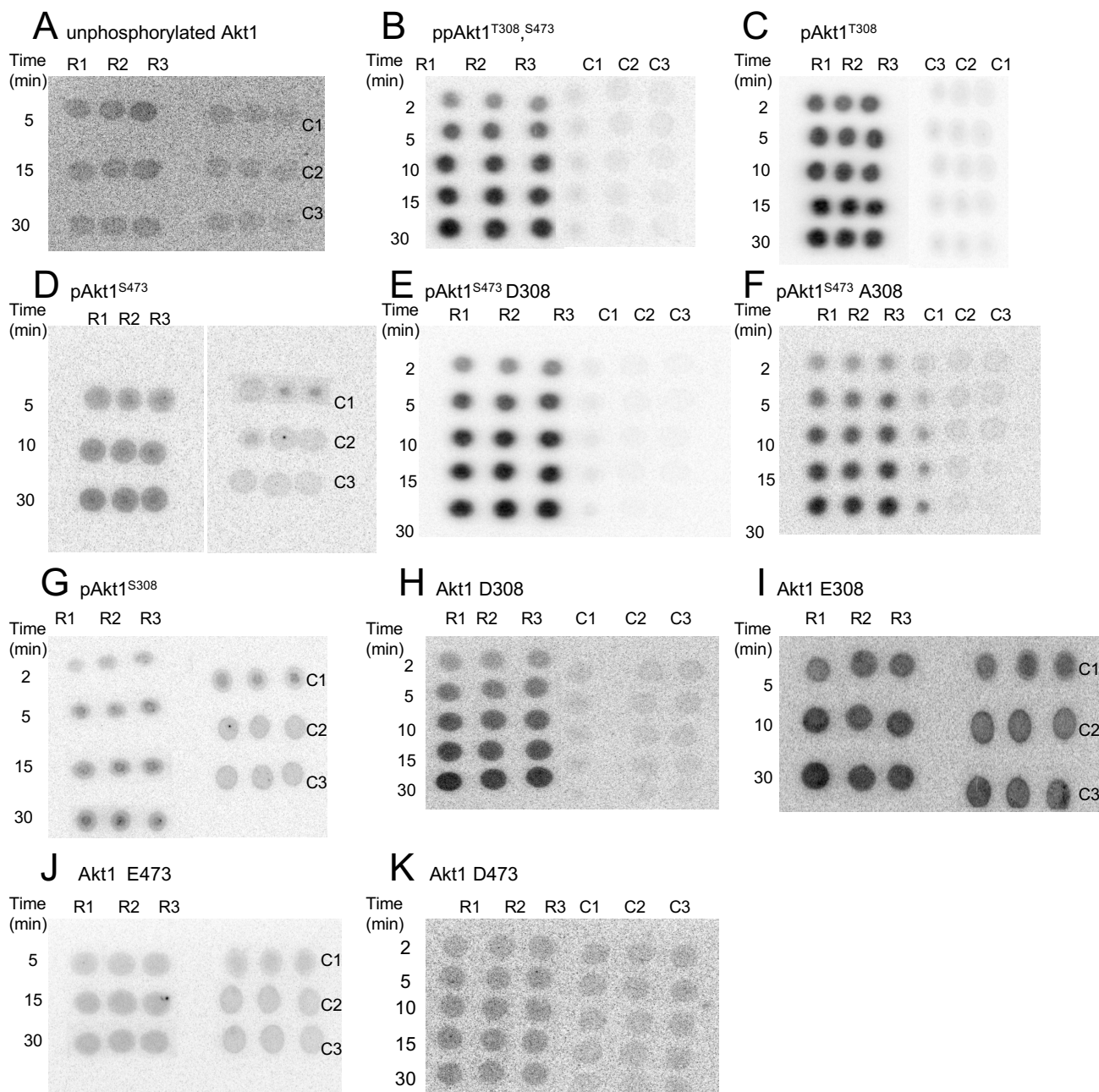


Figure S2.6 Autoradiographs of γ -[³²P]-ATP kinase assays with pAkt1 variants.

Time courses are shown for reactions catalyzed by (A) unmodified Akt1, (B) ppAkt1^{T308, S473}, (C) pAkt1^{T308} (D) pAkt1^{S473} (E) pAkt1^{S473} D308 (F) pAkt1^{S473} A308 (G) pAkt1^{S308} (H) Akt1^{D308} (I) Akt1^{E308} (J) Akt1^{E473} (K) Akt1^{D473}. Assays were performed in triplicate (as indicated by R1-R3). Controls include: C1 without substrate peptide, C2 without enzyme, C3 only kinase assay buffer.

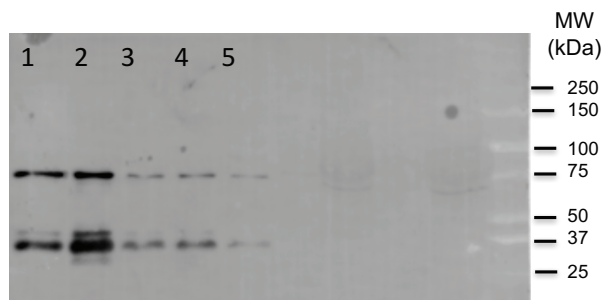


Figure S2.7 Production of pAkt1^{S308}. Fractions from affinity chromatography are visualized on SDS-PAGE by anti-His immunoblot. Elution gradient of pAkt1^{S308} (lanes 1-5) with increasing imidazole concentrations.

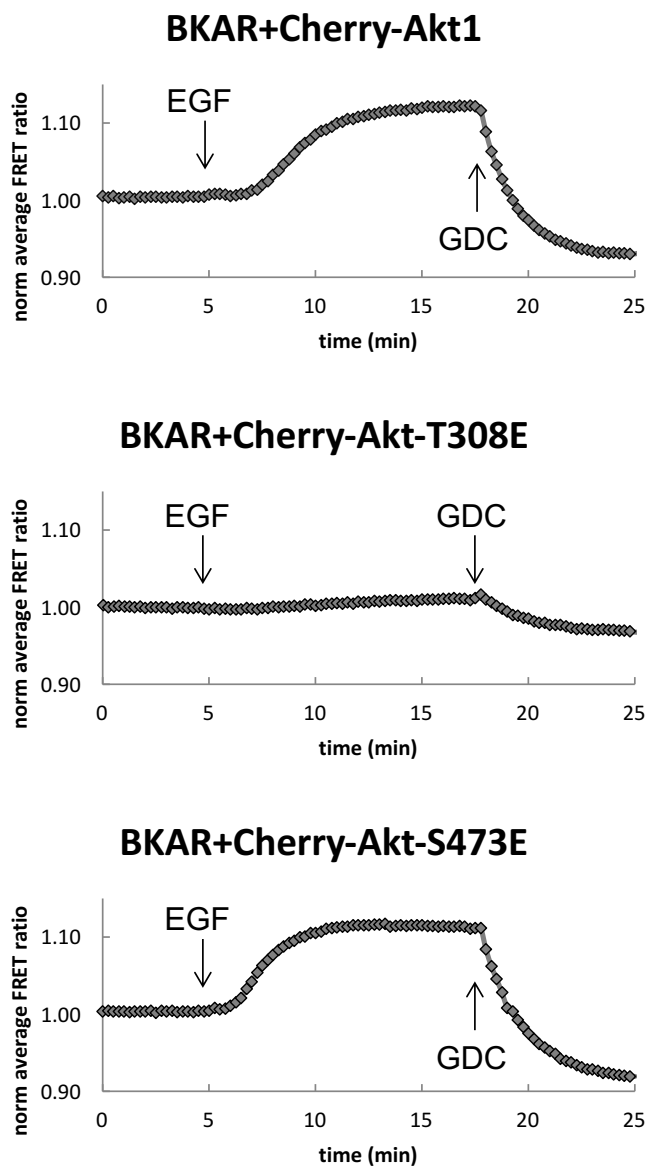


Figure S2.8 Cellular activity of Akt1 variants with glutamate substitutions. COS-7 cells co-expressing BKAR and equal levels of the indicated Cherry-tagged Akt were imaged and the CFP/FRET ratio examined following EGF stimulation and Akt inhibition with GDC 0068 (GDC). Mutation of Thr308 to Glu resulted in an inactive kinase (middle plot), whereas mutation of Ser473 to Glu did not impair its activity (bottom plot). Data were normalized to the first 4 minutes and plotted. Graphs are representative of three independent experiments. (Experiment by Maya Kunkel)

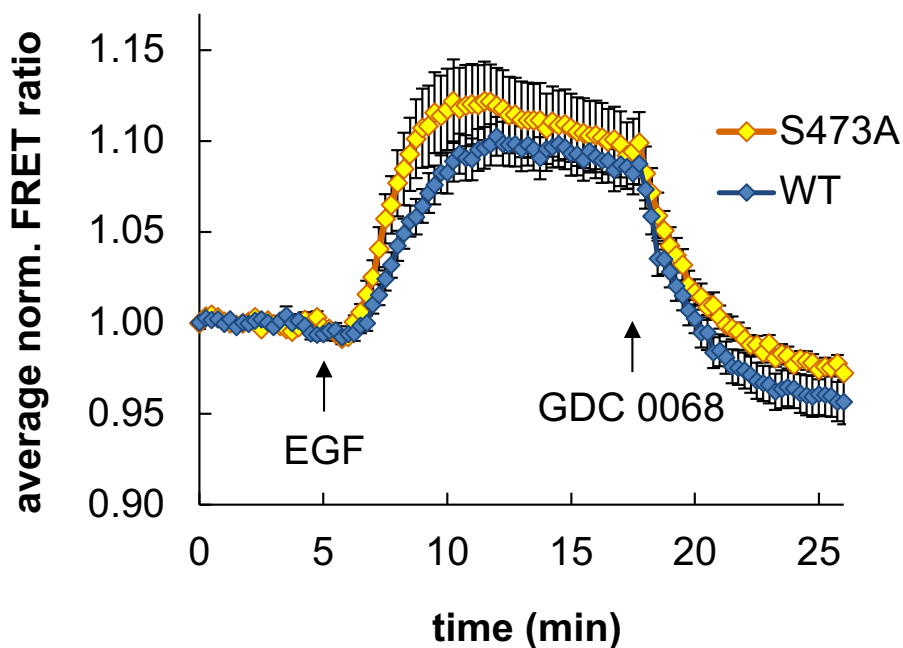


Figure S2.9 Cellular activity of phospho-ablated Akt1 variant S473A. Serum-starved COS7 cells expressing BKAR with minimally detectable levels of Cherry-tagged Akt were imaged during stimulation with EGF followed by treatment with the Akt inhibitor GDC 0068. The average FRET ratios for WT (blue) and S473A (yellow) are shown. Data were analyzed from cells expressing equal low levels of Cherry-Akt (n=12 for WT and n=8 for S473A) from three independent experiments. FRET ratios from each cell were normalized and their average plotted over time. Error bars represent SEM. (Experiment by Maya Kunkel)

Chapter 3

3 Phosphorylation-dependent chemical and auto-inhibition of Akt1

3.1 Abstract

Akt1 is a proto-oncogene that is over active in most cancers. Akt1 activation requires phosphorylation at Thr308; phosphorylation at Ser473 further enhances catalytic activity. Akt1 activity is also regulated via interactions between the kinase domain and the N-terminal auto-inhibitory pleckstrin homology (PH) domain. As it was previously difficult to produce Akt1 in site-specifically phosphorylated forms, the contribution of each activating phosphorylation site to auto-inhibition was unknown. Using a combination of genetic code expansion and *in vivo* enzymatic phosphorylation, we produced Akt1 variants containing programmed phosphorylation to probe the interplay between Akt1 phosphorylation status and the auto-inhibitory function of the PH domain. Deletion of the PH domain increased the enzyme activity for all three phosphorylated Akt1 variants. For the doubly phosphorylated enzyme, deletion of the PH domain relieved auto-inhibition by 295-fold. We next found that phosphorylation at Ser473 provided resistance to chemical inhibition by Akti-1/2 inhibitor VIII. The Akti-1/2 inhibitor was most effective against pAkt1^{T308} and showed 4-fold decreased potency with Akt1 variants phosphorylated at Ser473.

The data highlight the need to design more potent Akt1 inhibitors that are effective against the doubly phosphorylated and most pathogenic form of Akt1.

3.2 Introduction

Protein kinase B (Akt) is a human serine-threonine kinase and a member of the AGC family of protein kinases (1,2). The pathway regulated by Akt is the most commonly activated signaling pathway in human cancers (3). Given that more than 50% of human tumors contain hyper-activated Akt (2), effective inhibition of active Akt has the potential to treat several distinct cancers. There are three Akt genes in humans, encoding the isozymes Akt1, Akt2, and Akt3. The Akt1 isozyme has well established roles in many human cancers. Over-active Akt1 is a hallmark of diverse human malignancies (3,4) and linked to reduced survival outcomes (5,6). Indeed, Akt1 is a leading drug target in cancer (7,8). Over 300 clinical trials are completed or underway that involve targeting the Akt1 signaling pathway.

Akt1 is a key regulator of the phosphoinositide 3-kinase (PI3K)/Akt1 signaling cascade that controls cell growth and survival (1). In human cells, the activation of Akt1 occurs in response to growth factor stimulation (Figure 3.1). Following activation by a receptor tyrosine kinase at the plasma membrane, phosphoinositide-3-phosphate kinase (PI3K) phosphorylates its immediate downstream target, a lipid second messenger called phosphatidylinositol-4, 5-bisphosphate (PIP₂), converting PIP₂ into phosphatidylinositol-3, 4, 5-triphosphate (PIP₃) (2). Membrane anchored PIP₃ is a binding site for pleckstrin homology (PH) domain-containing proteins such as Akt1 and one of the

upstream kinases that activates Akt1, phosphoinositide dependent kinase 1 (PDK1) (9). Co-localization of Akt1 with PDK1 leads to partial activation of Akt1 by PDK1-mediated phosphorylation of Thr308 in the kinase domain of Akt1. Full activation of Akt1 results from a second phosphorylation event at Ser473 in the regulatory domain of Akt1 by mechanistic target of rapamycin complex 2 (mTORC2) (Figure 3.1).

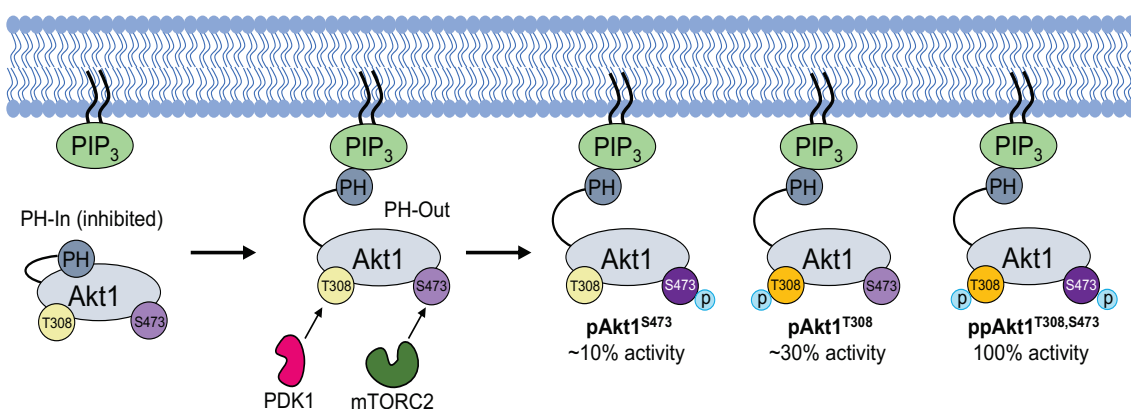


Figure 3.1 Simplified schematic of Akt1 activation via phosphorylation of sites Thr308 and Ser473. The transition from Akt1's inactive state (PH-in) to its fully active state (ppAkt1^{T308/S473}) requires the release of PH domain mediated auto-inhibition. This release occurs when Akt1's PH domain interacts with PIP₃ (PH-out). In the PH-out conformation, Akt1 is more susceptible to phosphorylation at Thr308 and Ser473 by PDK1 and mTORC2 respectively. Upon release from PIP₃, Akt1 distributes rapidly in the cytosol and translocates to the nucleus to phosphorylate >100 cellular proteins(2,10).

Leveraging our ability to produce Akt1 protein in specifically phosphorylated forms (Figure 3.2), we recently quantified the precise contribution of pThr308 and pSer473 to Akt1 activity in vitro and in mammalian

cells (11). In studies with purified full length Akt1, we found that pAkt1^{T308} achieves 30% of the activity of the doubly phosphorylated kinase. We also observed in COS-7 cells that pSer473 is dispensable for Akt1 signaling and phosphorylation of Thr308 alone was sufficient for maximal Akt1 signal propagation. These results indicated Thr308 phosphorylation status is a superior biomarker for Akt1 activity (11) and called into question the frequent use of Ser473 phosphorylation as a diagnostic marker for Akt1 activity in cancer patients (12-15).

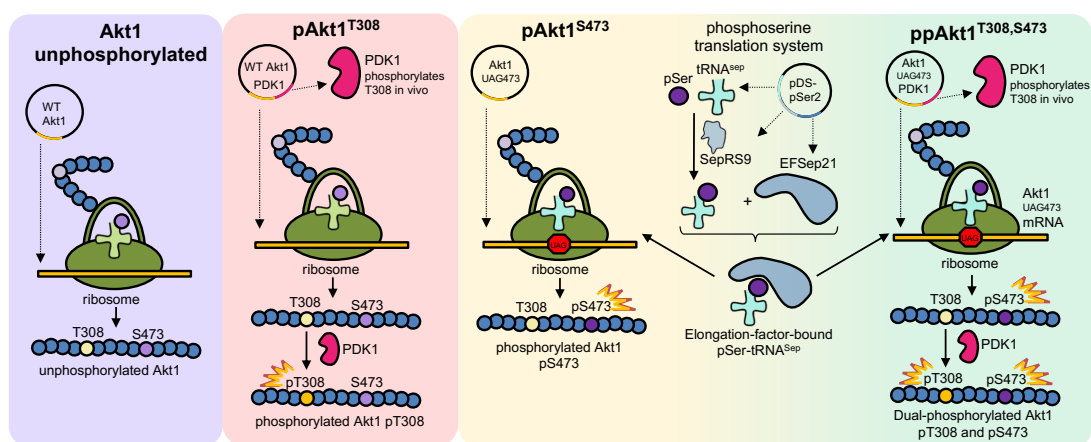


Figure 3.2 Production of Akt1 variants with programmed phosphorylation. To produce pAkt1^{T308}, PDK1 (Akt1's natural upstream kinase) was co-expressed along with Akt1. To produce pAkt1^{S473}, the phosphoserine orthogonal translation system was used to genetically incorporate phosphoserine at position 473 in response to an amber (UAG) codon. The ppAkt1^{T308,S473} variant was produced by combining both methods.

The N-terminal PH domain is auto-inhibitory to Akt1 activity. Due to previous roadblocks in preparing Akt1 with programmed phosphorylation(s), there are no reports that measure the contribution of a phosphate at each key regulatory site to the auto-inhibition of Akt1 by the PH domain. In the current

model, Akt1 exists in an auto-inhibited (PH-in) and activated conformation (PH-out). In its auto-inhibited conformation (PH-in), the PH domain binds between the N- and C-terminal lobes of the un-phosphorylated Akt1 kinase domain. During growth factor-mediated activation of Akt1, the PH domain forms a new interaction with PIP3 causing it to move outward from the now more accessible kinase domain. This PH-out conformation is readily activated via phosphorylation at Thr308 by PDK1 and Ser473 by mTORC2. Disruption of the PH and kinase domain interaction was identified as a plausible cause of increased Akt1 phosphorylation and subsequent activity in cancer (16).

Here we quantified the ability of the PH domain to auto-inhibit Akt1 variants phosphorylated at either or both key regulatory sites Thr308 and Ser473. We produced novel recombinant Akt1 variants lacking the PH domain (Δ PH-Akt1) that also contained programmed phosphorylation at each site separately or with both sites phosphorylated. In the context of full length phosphorylated Akt1 variants, we quantified the contribution of both regulatory phosphorylation sites to chemical inhibition with the PH domain dependent allosteric inhibitor Akti-1/2.

3.3 Materials and Methods

3.3.1 Bacterial strains and plasmids. We designed a codon optimized PH domain deficient (Δ PH) human *akt1* gene (residues 109-480), which was synthesized by ATUM (Newark, California, USA). The Δ PH-*akt1* gene was sub-cloned (*NcoI/NotI*) into an isopropyl β -D-1-thiogalactopyranoside (IPTG) inducible T7lac promoter driven pCDF-Duet1 vector with CloDF13-derived CDF replicon and

streptomycin/spectinomycin resistance (pCDF-Duet1- Δ PHAkt1). The *PDPK1* gene was purchased from Harvard PlasmidID repository service (plasmid ID: HsCD00001584, Boston, MA, USA) and sub-cloned (*KpnI/NdeI*) into the second multi-cloning site (MCS) of pCDF-Duet-1. Full length pAkt1 variants were produced from pCDF-Duet1 plasmids as described previously (11). The genetic code expansion system for phosphoserine (pSer) is encoded on the pDS-pSer2 plasmid (11,17,18), which contains 5 copies of tRNA^{Sep} (19), phosphoseryl-tRNA synthetase (SepRS9), and elongation factor Tu mutant (EFSep21) (20). Incorporation of pSer also required site-directed mutagenesis of the Ser473 codon to TAG in the Δ PH-*akt1* constructs. Successful cloning was verified by DNA sequencing at the London Regional Genomics Centre (London, ON, Canada) and Genewiz (Cambridge, MA, USA).

3.3.2 Protein and phosphoprotein production. Recombinant Akt1 protein variants were expressed in BL21(DE3) (Invitrogen) (Figure 3.2). The pDS-pSer2 plasmid (18) was used as before (11) to incorporate pSer in response to a UAG codon at position 473 in Δ PHAkt1 and full length Akt1 variants. To produce both full length and PH domain deficient Akt1 variants containing pSer473 (Table S3.1), the pCDFDuet-1 Akt1 bearing plasmid was co-transformed with pDS-pSer2 into *E. coli* BL21(DE3) and plated on LB agar plates with 25 μ g/ml kanamycin and 50 μ g/ml streptomycin. To produce pAkt1^{T308} variants, a pCDF-Duet1 plasmid containing both the Akt1 variant (multiple cloning site 2 (MSC 2)) and PDK1 (multiple cloning site 1 (MSC 1)) was transformed into *E. coli* BL21(DE3) and plated on LB agar plates with 50 μ g/ml streptomycin. To produce ppAkt1^{T308,S473} variants, a pCDF-Duet1 plasmid containing both the Akt1 variant (MSC 2) with a TAG codon at position 473 and PDK1 (MSC 1) (11) was co-transformed with

pDS-pSer2 into *E. coli* BL21(DE3) and plated on LB-agar plates with 25 µg/ml kanamycin and 50 µg/ml streptomycin.

In all cases, a single colony was used to inoculate 70 ml of LB (with streptomycin 50 µg/ml and, if needed, kanamycin 25 µg/ml), which was grown, shaking, overnight at 37 °C. From this starter culture, a 10 ml inoculum was added to 1 L of LB with antibiotics (as above) and, for pSer473 containing variants only, *O*-phospho-L-serine (Sigma Aldrich, Oakville, Ontario) was added to a final concentration of 2.5 mM. The cultures were grown at 37 °C until OD₆₀₀ = 0.6 at which point, for pSer473 containing variants only, 2.5 mM of additional pSer was added to the culture. Protein expression was induced by adding 300 µM of IPTG at OD₆₀₀ = 0.8. Cultures were then incubated at 16°C for 18 h. Cells were grown and pelleted at 5000 × g and stored at -80°C until further analysis. Akt1 protein variants were purified from the cell pellets using Ni-Nitrilotriacetic acid affinity column chromatography (see Supplementary Methods).

3.3.3 Parallel-Reaction Monitoring Mass Spectrometry (PRM-MS) of ppAkt1.

The ppAkt1 protein produced as noted above and the commercially available active Akt1 (Abcam, lot 1) were precipitated in ice-cold acetone/ethanol/acetic acid (50/50/0.1, vol/vol/vol). The protein precipitate was re-suspended in 8 M urea, then reduced in 5 mM dithiothreitol (DTT) at 37°C for 1 h and alkylated in 14 mM iodoacetamide (IAA) in darkness at room temperature for 1 h. Unreacted IAA was neutralized by adding 5 mM DTT. The final protein concentration was determined by Bradford assay. Glu-C digestion was performed at 37 °C overnight with a Glu-C to-protein ratio of 1/20 (w/w). The digest was desalted in C18 column (Phenomenex) according to the manufacture's protocol and re-

suspended in MS-grade water. A Q Exactive Hybrid Quadrupole Orbitrap MS (Thermo Fisher Scientific) was used to analyze the peptides. Data were analyzed using Skyline software.

3.3.4 MALDI-TOF/TOF Mass Spec Analysis. In-gel digestion was performed using a MassPREP automated digester station (PerkinElmer). Gel pieces were de-stained using 50mM ammonium bicarbonate and 50% acetonitrile, which was followed by protein reduction using 10 mM dithiotreitol (DTT), alkylation using 55 mM IAA, and tryptic digestion in 50 mM ammonium bicarbonate, pH 8. Peptides were extracted using a solution of 1% formic acid and 2% acetonitrile and lyophilized. Prior to mass spectrometric analysis, dried peptide samples were re-dissolved in a 10% acetonitrile and 0.1 % trifluoroacetic acid solution. MALDI matrix, α -cyano-4-hydroxycinnamic acid, was prepared as 5 mg/ml in 6 mM ammonium phosphate monobasic, 50% acetonitrile, 0.1 % trifluoroacetic acid and mixed with the sample at 1:1 ratio (v/v). Mass Spectrometry data (Figure S3.1) were obtained using an AB Sciex 5800 MALDI TOF/TOF System (Framingham, MA, USA). Data acquisition and data processing were done using a TOF TOF Series Explorer and Data Explorer (both from AB Sciex), respectively. The instrument is equipped with a 349 nm Nd:YLF OptiBeam On-Axis laser. The laser pulse rate is 400 Hz. Reflectron positive mode was used. Reflectron mode was externally calibrated at 50 ppm mass tolerance and internally at 10 ppm. Each mass spectrum was collected as a sum of 500 shots.

3.3.5 Akt1 kinase activity assay. The activity of each Akt1 variant was characterized by performing kinase assays in the presence of 200 μ M substrate

peptide CKRPRAASFAE (SignalChem, Vancouver, BC, Canada) derived from the natural Akt1 substrate, glycogen synthase kinase (GSK-3 β). Assays were performed in 3-(N-morpholino)propanesulfonic acid (MOPS, 25 mM, pH 7.0), β -glycerolphosphate (12.5 mM), MgCl₂ (25 mM), ethylene glycol-bis(β -aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA, 5 mM, pH 8.0), EDTA (2 mM), ATP (0.02 mM) and 0.4 μ Ci (0.033 μ M) γ -[³²P]-ATP in a 30 μ l reaction volume. Reactions were incubated at 37 °C and time points were taken over 30 min time courses. As previously (11), reactions were initiated by the addition of 18 pmol of the indicated Akt1 variant to yield a final enzyme concentration of 600 nM and quenched by spotting on P81 paper (21). For highly active Akt1 variants, the level of Akt1 was titrated to identify a linear range to accurately determine initial velocity (v_0) (Figure S3.2). For this reason, Akt1 activity is compared based on $k_{app} = v_0/[Akt1]$. Samples from each reaction (5 μ l) were spotted on P81 paper at specified time points. Following washes with 1% phosphoric acid (3 \times 10 min) and 95% ethanol (1 \times 5 min), the P81 paper was air-dried. Incorporation of ³²P into the substrate peptide was detected by exposing the P81 paper to a phosphor-imaging screen. The ³²P-peptide products were imaged and quantitated using a Storm 860 Molecular Imager and ImageQuant TL software (GE Healthcare).

3.3.6 Kinase inhibition assay. A concentration gradient (0.001, 0.05, 0.5, 1, 5, 10 μ M) of Akti-1/2 inhibitor VIII (Sellekchem, Houston, TX, USA) (22) was selected to determine the concentration dependence of Akt1 inhibition. Kinase inhibition assays were performed exactly as described above (section 3.3.5) and with the addition of dimethyl sulfoxide at 10% (v/v) (control) or with the indicated concentration of Akti-1/2 inhibitor. For each condition, initial velocity was determined over a time course of 10 minutes with time points at 0, 2, 5 and 10

min (Figure S3.3). In the inhibition assays, following established protocols (16), the inhibitor was pre-incubated with the enzyme for 5 min at 37°C before addition of ATP and Akt1 substrate peptide to start the reaction. The time courses were used to determine the initial velocities of each reaction. The fraction of enzyme inhibition was measured based on initial velocities of uninhibited and inhibited reactions. From these data, half maximal inhibitory concentration (IC_{50}) values were calculated using Sigma Plot (Systat Software, Inc, San Jose, CA).

3.4 Results

3.4.1 Production of recombinant Akt1 variants. We recently established a method to produce full length Akt1 variants with programmed phosphorylations (11). Here we applied this method to produce site-specifically phosphorylated Akt1 variants lacking the auto-inhibitory PH domain (Δ PH-Akt1). The approach combines *in vivo* enzymatic phosphorylation with genetic code expansion to produce Akt1 variants containing either or both pThr308 and pSer473 (Figure 3.2).

Using genetic code expansion (11,18), we incorporated pSer in response to a UAG stop codon at position 473 in the relevant Akt1 constructs. Thr308 was site-specifically phosphorylated by the co-expression of the upstream kinase PDK1 in *E. coli*. We (11) and others (23) have demonstrated that PDK1 is strictly specific in phosphorylating only the 308 site in Akt1. We used these methods in isolation or in combination to produce the three physiologically relevant Akt1 variants pAkt1^{S473}, pAkt1^{T308}, and ppAkt1^{T308,S473} (Figure 3.2). We observed that the Δ PH-Akt1 variants were all more soluble and were produced at 1.5 to 7-fold greater yield per liter of *E. coli* culture than the corresponding full length Akt1

variants (Table 3.1). As previously (11), we confirmed phosphorylation of the Thr308 site (Figure 3.3C) and the Ser473 site (Figure S3.1) by mass spectrometry.

Table 3.1 Protein yields for Akt1 variants.

Akt1 variant	Protein Yields ($\mu\text{g/L}$ <i>E. coli</i> culture)	
	full length	$\Delta\text{PH Akt1}$
Akt1 (unphosphorylated)	46	330
pAkt1 ^{S473}	100	150
pAkt1 ^{T308}	37	235
ppAkt1 ^{T308,S473}	45	224

3.4.2 Recombinant Akt1 produced in *E. coli* versus Sf9 cells. Earlier work established production of partially active and truncated Akt1 in *E. coli*, which was unsuccessful in producing a sufficient amount of full length Akt1 to determine activity (24). Although we recently overcame these difficulties and developed a robust protocol to produce full length Akt1 from *E. coli* with programmed phosphorylation, studies in the intervening period relied on insect Sf9 cell culture to produce recombinant Akt1 (25). The ability to generate ppAkt1 from insect cells, however, requires a complex and low yield in vitro procedure to phosphorylate Akt with 2 additional purified upstream kinases in the presence of lipid vesicles (25). Protein production in Sf9 cells fails to produce Akt1 with site-specific or programmed phosphorylation. The resulting protein is a mixture of singly and doubly phosphorylated species (26).

In order to benchmark the activity of the recombinant phosphorylated Akt1 protein we produce in *E. coli*, we compared the activity of full length ppAkt1 to active Akt1 purchased from Abcam. The commercially available full-

length Akt1 is made by a protocol similar to that established previously (25) in which Akt1 protein production in Sf9 cells followed by in vitro phosphorylation of the purified Akt1 with purified PDK1. In testing an initial lot of active Akt1 (lot 1), we found that the commercially available Akt1 enzyme was catalytically deficient by a factor of 8-fold (Table 3.2) compared to the full length ppAkt1 we produced (Figure 3.3A).

Table 3.2 Activity of Akt1 variants

Akt1 variant	Akt1 amount (pmol)	Initial velocity v_0 (fmol/min)	Apparent catalytic rate k_{app} (fmol/min/pmol Akt1)	Activation (fold increase)	Reference
Akt1 (un-phosphorylated)	18	0.6 ± 0.2	0.03 ± 0.01	1.0 ± 0.3	[10]
pAkt1 ^{S473}	18	46 ± 5	2.6 ± 0.3	85 ± 9	[10]
pAkt1 ^{T308}	1.8	22 ± 4	12 ± 2	400 ± 70	[10]
ppAkt1 ^{T308,S473}	1.8	79 ± 11	44 ± 6	1500 ± 200	[10]
Δ PHAkt1 (un-phosphorylated)	18	1.4 ± 0.1	0.079 ± 0.006	2.7 ± 0.2	this study
	18	210 ± 20	12 ± 1	390 ± 40	this study
Δ PH pAkt1 ^{S473}	0.18	370 ± 100	2100 ± 600	6900 ± 1800	this study
Δ PH pAkt1 ^{T308}	0.18	2300 ± 600	$(13 \pm 3) \times 10^3$	$(4 \pm 1) \times 10^5$	this study
Δ PH ppAkt1 ^{T308,S473}					
Commercial Akt1 (abcam)	18	100 ± 20	6 ± 1	200 ± 30	this study
Lot 1					
Lot 2	18	2200 ± 700	120 ± 40	4000 ± 1000	this study

PRM-MS/MS was used to determine the identity of the residue at position 308 and the level of phosphorylation in ppAkt1 we produced and in the purchased protein. Mass spectrometry revealed that the low activity of lot 1 enzyme was attributable to the low level of phosphorylation at position 308 (peak intensity 1.3×10^1) and a relatively high level of un-phosphorylated Thr at position 308 (Figure 3.3B). In contrast, ppAkt1^{T308,S473} that we produced showed a high level (peak intensity 1.6×10^6) of Thr308 phosphorylation and un-phosphorylated Thr308 was not detected (Figure 3.3C). We then purchased a second lot of Akt1 enzyme (lot 2), and this enzyme displayed significantly more but highly variable activity compared to lot 1 (Table 3.2). Presumably, the second lot was quantitatively phosphorylated during production. In contrast to the method we developed for pAkt1 production in *E. coli* (Figure 3.2), the protocol to generate active Akt1 relying on Sf9 cells and subsequent in vitro phosphorylation appears to lead to a variable level of phosphorylation and activity in the resulting Akt1 preparations.

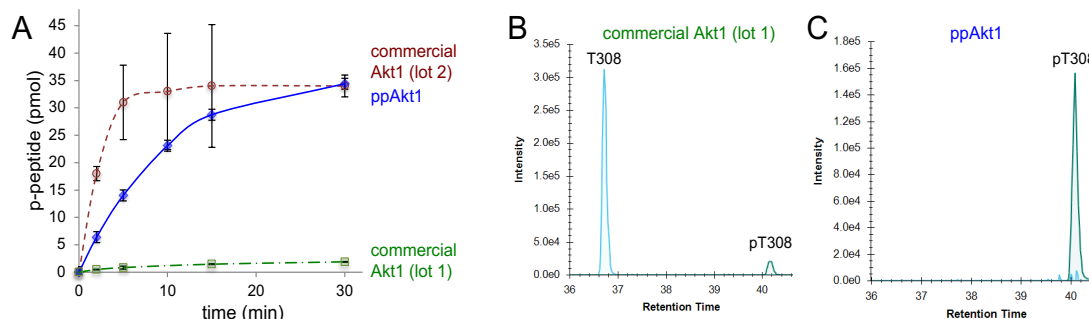


Figure 3.3 Activity of full length ppAkt1 and commercially available active Akt1. (A) Kinase activity assays over a 30-minute time course show substantially reduced activity of commercial Akt1 (lot 1, green squares) compared to full length ppAkt1 (blue diamonds) produced in *E. coli*. Commercial Akt1 lot 2 (red circles) showed highly variable but similar activity to full length ppAkt1. (B) Tryptic peptides from commercial Akt1 and ppAkt1 were analyzed by PRM-MS analysis. The purchased ‘active’ Akt1 (lot 1) showed a low intensity peak for phosphorylation at Thr308 (green peak at a retention time of ~40 min) and high intensity peak for non-phosphorylated Thr308 (cyan peak, retention time of ~37 min). (C) PRM-MS analysis of ppAkt1^{T308,S473} showed a high intensity peak for phosphorylation at Thr308 (green peak at a retention time of 40) and the non-phosphorylated Thr308 was undetectable.

3.4.3 Impact of PH domain deletion on differentially phosphorylated Akt1. In order to determine the impact of the PH domain on each phospho-form of Akt1, we next assayed the activity of specifically phosphorylated Δ PH-Akt1 variants using γ -[³²P]-ATP and a substrate peptide for Akt1 (CKRPRAASFAE) that was derived from GSK-3 β , a well-established Akt1 substrate (11,27). In kinase assay conditions that we previously optimized (11) to measure a wide range of Akt1 activity, we determined the apparent catalytic rate of each Δ PH-Akt1 variant (un-

phosphorylated, pAkt1^{S473}, pAkt1^{T308}, ppAkt1^{T308,S473}). We then compared the apparent catalytic rates to the measurements we had made previously with full-length Akt1 and pAkt1 variants to ascertain the relative impact of each phosphorylation state on the auto-inhibition by the PH domain.

As anticipated, all Akt1 variants lacking the PH domain were significantly more active than their full-length counterparts (Table 3.1). Doubly phosphorylated Δ PH-Akt1 (ppAkt1^{T308,S473}) showed the highest activity among all three variants (Figure 3.3). The un-phosphorylated Δ PH-Akt1 showed a basal level of activity that was significantly higher (2.7 ± 0.3 -fold) than the background activity we recorded for full length un-phosphorylated Akt1 (Table 3.3). Although both un-phosphorylated Akt1s would not be of sufficient activity to induce Akt1-dependent signaling in cells (2,11), it is interesting to note that the auto-inhibitory effect of the PH domain is indeed measurable in the increased minimal activity of un-phosphorylated Δ PH-Akt1 (Figure 3.4B).

Table 3.3 Relative activity of full length versus Δ PH Akt1 variants

Akt1 variant	full length Akt1	ΔPH Akt1
Akt1 (un-phosphorylated)	1.0 \pm 0.3	2.7 \pm 0.2
pAkt1 ^{S473}	1.0 \pm 0.1	4.6 \pm 0.4
pAkt1 ^{T308}	1.0 \pm 0.2	175 \pm 50
ppAkt1 ^{T308,S473}	1.0 \pm 0.1	295 \pm 68

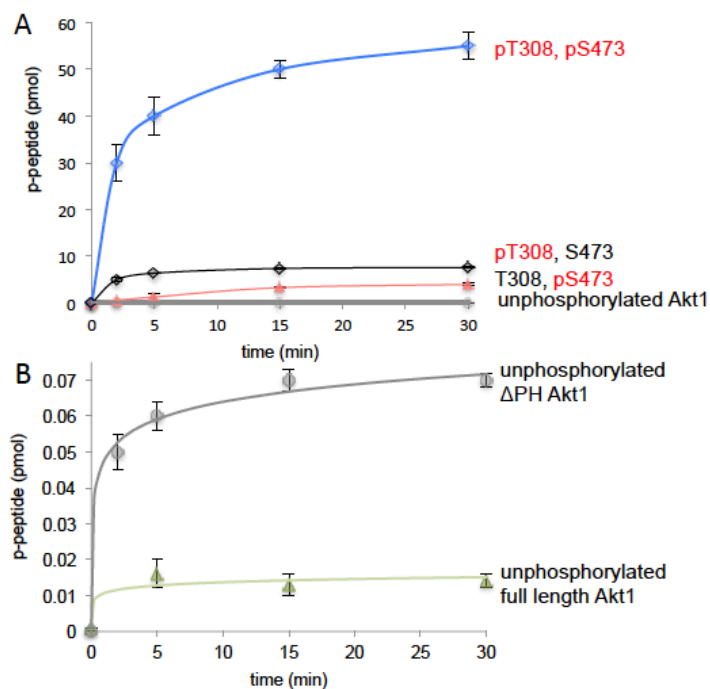


Figure 3.4 Enzyme activity of Δ PH-Akt1 variants. (A) The activity of differentially phosphorylated Δ PH Akt1 variants with the GSK-3 β substrate peptide was measured over a 30-minute time course. Akt1 phosphorylated at both 308 and 473 (Δ PH ppAkt^{S473, T308}, blue diamonds) showed maximal activity compared to the un-phosphorylated Δ PH-Akt1 (gray circles), and singly phosphorylated Akt1 variants: Δ PH pAkt1^{T308} (black cross) and Δ PH pAkt1^{S473} (pink diamonds). (B) The basal activity of un-phosphorylated Δ PH Akt1 (gray circles) was compared to full length

Compared to un-phosphorylated Δ PH-Akt1, the most active doubly phosphorylated Akt1 variant (Δ PH- ppAkt^{T308,S473}) showed 150,000-fold increase in relative catalytic rate (Table 3.2, Figure 3.4). Since rapid enzyme kinetics were observed with 18 pmol of enzyme with both these variants containing Thr308 phosphorylation, the enzyme concentrations were subsequently reduced by 10-

fold and 100-fold to obtain a highly accurate initial velocity with which to determine the apparent rate ($k_{app} = v_o/[enzyme]$) (Figure S3.2).

Interestingly, dual phosphorylation of ΔPH -Akt1 leads to 100-fold greater increases in activity than that observed upon dual phosphorylation of the full-length enzyme, which is 1500-fold more active than the un-phosphorylated full-length enzyme. The data indicate the PH domain significantly dampens the catalytic activity endowed by phosphorylation at Thr308 and Ser473 (Figure 3.5). The ΔPH -Akt1 variant with a single phosphorylation at Thr308 site was robustly active, 2500-fold above the unphosphorylated ΔPH -Akt1, yet 60-fold reduced activity compared to the doubly phosphorylated enzyme ΔPH -ppAkt^{T308/S473} (Table 3.2). Phosphorylation at the C-terminal site Ser473 activated the enzyme 140-fold above the un-phosphorylated control, but to a lesser extent (18-fold) than the ΔPH -pAkt1^{T308}. We previously observed a quantitatively similar pattern of activity with the full length Akt1 variants (Table 3.2, (11)).

We found that the PH domain exerts an auto-inhibitory effect, the strength of which depends on the phosphorylation status of the Akt1 enzyme. We compared the apparent catalytic rates (k_{app}) (Figure 3.5A) and normalized relative catalytic rates (Figure 3.5B) between the full length (11) and PH domain deficient Akt1 variants. In the context of a single phosphorylation at Ser473, the PH domain is associated with a ~5-fold reduction in activity (Table 3.3, Figure 3.5). In the singly phosphorylated pAkt1^{T308} enzyme the auto-inhibition is far stronger (175-fold). The two phosphorylations together led to a super-additive inhibitory effect (295-fold) in the doubly phosphorylated enzyme (Figure 3.5).

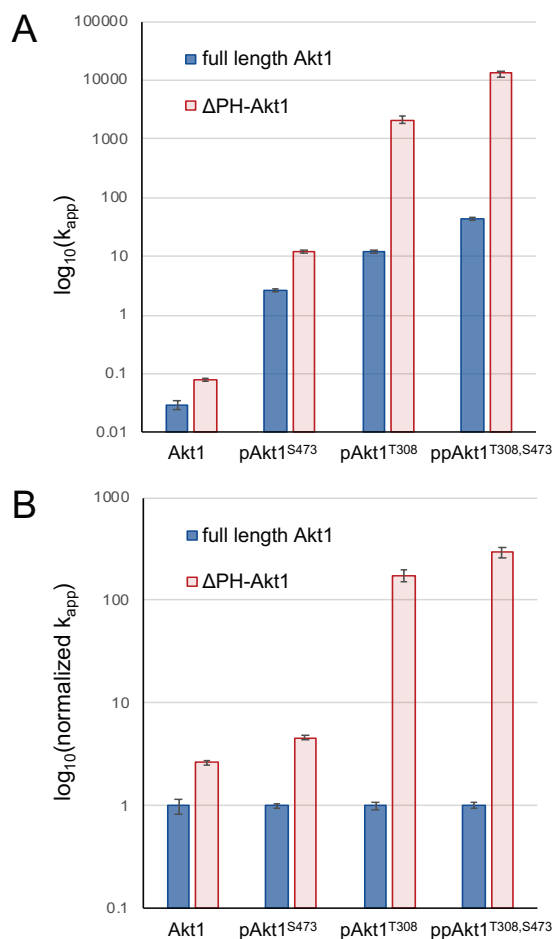


Figure 3.5 Impact of the PH domain on the activity of differentially phosphorylated Akt1. (A) Apparent catalytic rates (k_{app}) and (B) normalized k_{app} values of full-length Akt1 variants (blue) and Δ PH Akt1 variants (red) are shown. Error bars represent 1 standard deviation of triplicate measurements.

We found that the PH domain exerts an auto-inhibitory effect, the strength of which depends on the phosphorylation status of the Akt1 enzyme. We compared the apparent catalytic rates (k_{app}) (Figure 3.5A) and normalized relative catalytic rates (Figure 3.5B) between the full length (11) and PH domain deficient Akt1 variants. In the context of a single phosphorylation at Ser473, the PH domain is

associated with a ~5-fold reduction in activity (Table 3.3, Figure 3.5). In the singly phosphorylated pAkt1^{T308} enzyme the auto-inhibition is far stronger (175-fold). The two phosphorylations together led to a super-additive inhibitory effect (295-fold) in the doubly phosphorylated enzyme (Figure 3.5).

3.4.4 Chemical inhibition of phosphorylated Akt1 variants. Given the differential impact of Akt1 phosphorylation on auto-inhibition, we next identified phosphorylation dependence in the interaction between Akt1 and a clinically relevant drug scaffold, Akti-1/2 inhibitor VIII. Several classes of chemical inhibitors were developed to repress aberrant Akt1 activity in cancer cells (28). Early Akt1 inhibitors focused on ATP competitive compounds (29), yet these molecules suffered from significant cross reactivity with other AGC family kinases (30). For this reason, subsequent efforts focused on allosteric Akt1 inhibitors. Akti-1/2 is an allosteric inhibitor that binds Akt1 in a cleft between the kinase and PH domains, locking the enzyme in a non-catalytic conformation (Figure 3.6). Biochemical data with different Akt1 preparations have provided estimates of IC₅₀ of Akt1 for the Akti-1/2 inhibitor ranging from 58 nM to 2.7 μM (22,31,32) (Table 3.4). We suspected that this range of values result from preparations of Akt1 with various levels of phosphorylation with less active Akt1 preparations leading to an underestimate of IC₅₀. In addition, previous studies were unable to isolate the contribution of each regulatory phosphorylation site to inhibition by Akti-1/2.

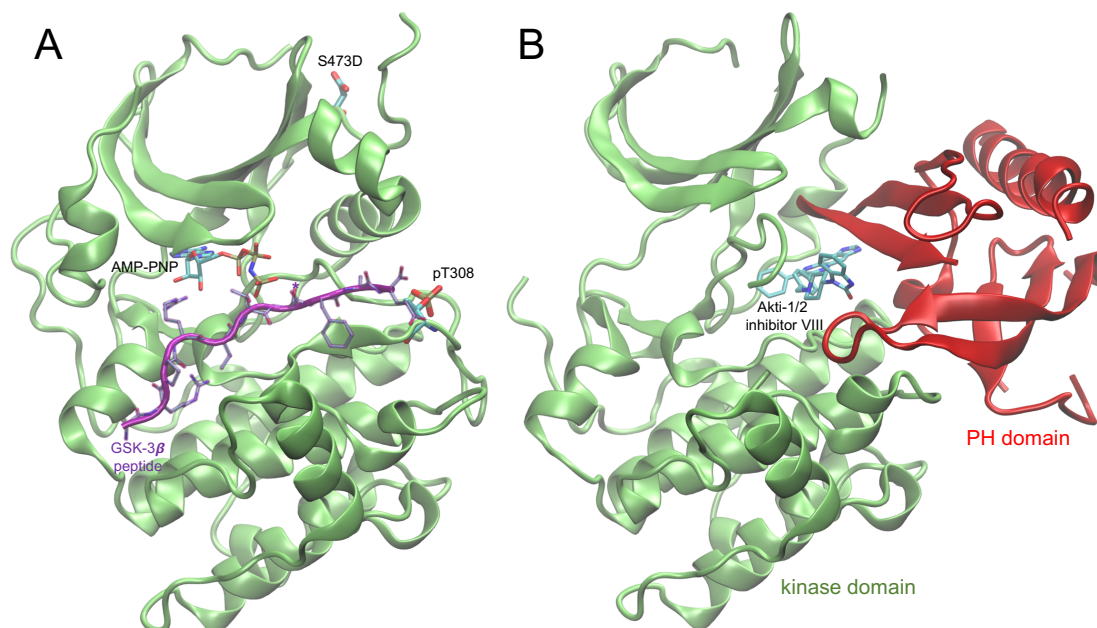


Figure 3.6 Structure of Akt1 in active and inhibitor bound forms. (A) The structure of Δ PH-pAkt1^{Thr308} Ser473Asp (PDB 1O6K (33)) is shown in complex with ATP analog (AMP-PNP) and substrate peptide (purple). (B) Structure of the full length Akt1 (un-phosphorylated) is shown in complex with the Akti-1/2 inhibitor VIII (PDB 1O96 (34)) binding in the cleft between the kinase domain (green) and the N-terminal PH domain (red).

Table 3.4 Akti-1/2 inhibitor half maximal inhibitory concentration (IC₅₀) for pAkt1 variants

Akt1 variant	inhibitor type	IC ₅₀ (μ M)	expression system	reference
ppAkt1 ^{T308,S473}	Akti-1/2 inhibitor VIII	1.2 \pm 0.3	<i>E. coli</i>	this study
pAkt1 ^{T308}	Akti-1/2 inhibitor VIII	0.3 \pm 0.1	<i>E. coli</i>	this study
pAkt1 ^{S473}	Akti-1/2 inhibitor VIII	1.3 \pm 0.1	<i>E. coli</i>	this study
active	Akti-1/2 inhibitor VIII	0.058	<i>Drosophila</i> S2 cells	[22]
active	Akti-1/2 inhibitor VIII	0.1	HEK 293 cells	[31]
active	Akti-1/2 inhibitor	2.7	<i>Drosophila</i> S2 cells	[32]
active	Akti-1 inhibitor	4.6	<i>Drosophila</i> S2 cells	[32]

We conducted Akt1 inhibition assays using Akti-1/2 concentrations varying from 0.01 to 10 μM . All three full-length phosphorylated Akt1 variants showed concentration responsive enzyme inhibition (Figures 3.7, S3.3-S3.6). Akti-1/2 was most potent ($\text{IC}_{50} = 300 \text{ nM}$) when only the Thr308 site was phosphorylated (Table 3.4). Interestingly, at the lower concentrations of Akti-1/2 (0.001, 0.05, 0.5 μM) the compound was not effective in inhibiting the activity of pAkt1^{S473}. Akt1 phosphorylated at the C-terminal Ser473 site was overall more resistant to inhibitor VIII inhibition resulting an IC_{50} value of 1.3 μM . In a surprising finding, the doubly phosphorylated enzyme had an indistinguishable IC_{50} from the pAkt1^{S473} enzyme. Together the data indicate that Ser473 phosphorylation provides a 4-fold increase in resistance to the Akti-1/2 inhibitor even in the presence of phosphorylation at Thr308. This is the first report that demonstrating phosphorylation dependence in the interaction between Akt1 and an inhibitor.

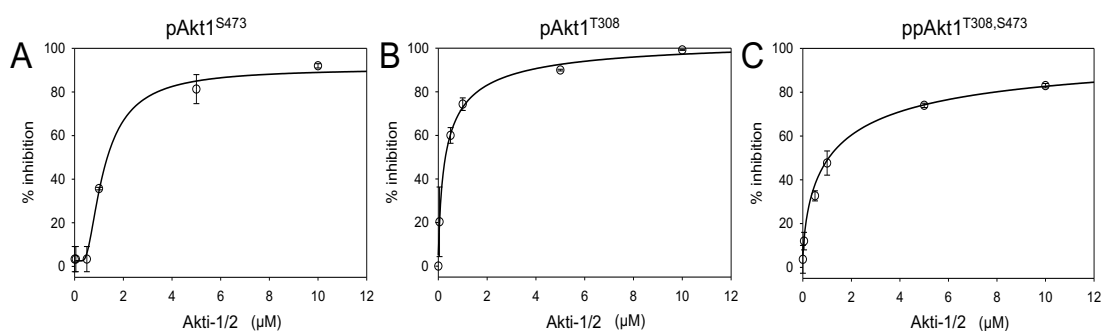


Figure 3.7 Chemical inhibition of full length phosphorylated Akt1 variants. (A) Inhibition of (A) pAkt1^{S473}, (B) pAkt1^{T308}, and (C) ppAkt1^{T308,S473} with varying concentrations (0.001, 0.05, 0.5, 1, 5, 10 μM) of Akti-1/2 inhibitor VIII. The resulting IC_{50} values are in Table 3.4.

3.5 Discussion

Akt1 is a prime target for therapeutic intervention due to its involvement in regulating a multitude of cellular pathways (35,36). Aberrant Akt function has been linked to cancers and a variety of human diseases related to metabolic regulation, immune function, and neurological development (2). The broad range of cellular processes governed by Akt, therefore, presents an opportunity for single-target therapeutic intervention of a variety of conditions. To capitalize on this opportunity, compounds that selectively attenuate Akt1 activity rather than global kinase inactivation are required. This need is reflected in clinical trials which show that non-specific Akt inhibition in cancer therapy, due to inadvertent off-targeting of closely related kinases or multiple Akt isozymes, can result in detrimental side-effects including liver damage and metabolic disorders (37-40). Accordingly, the development of small molecule inhibitors for Akt has reflected this sentiment towards engineering greater target specificity (28). From the earliest ATP-competitive inhibitors that unintentionally inhibited related AGC kinases, to more recent allosteric inhibitors that preferentially target Akt or even a subset of Akt isozymes, the continued pursuit for Akt1 inhibitor specificity requires an expanded understanding of the molecular basis of Akt1 activation and inhibition.

Previous work was unable to uncover the role of each regulatory site (Thr308 and Ser473) in the inhibition kinetics of Akt1. Given this critical knowledge gap, we investigated the role of Akt1 phosphorylation status on the auto-inhibitory effect of the PH-domain and on allosteric inhibition by Akti-1/2.

3.5.1 Phosphorylation-Dependent Auto-Inhibition of Akt1. The N-terminal PH domain is shared amongst all three Akt isozymes and its direct function is to mediate protein-lipid interactions. It is well documented that the PH domain binding to a lipid second messenger PIP3 in cell membranes releases the inhibitory effect of the PH domain (2). Here, we produced differentially phosphorylated Akt1 variants with and without the PH domain in *E. coli* using genetic code expansion to incorporate pSer473 and the upstream Thr308 kinase PDK1 was co-expressed in *E. coli*, leading to quantitative phosphorylation of the recombinant Akt1 protein. Compared to un-phosphorylated Akt1, all phosphorylated variants showed increased enzyme activity.

Previously, live cell imaging studies revealed that once phosphorylated, Akt1 favors a 'PH-out' confirmation that increases Akt1 activity in the downstream phosphorylation of substrate proteins both in the cytoplasm and nucleus. In addition, binding of Akt1 to PIP3 in the plasma membrane further releases the auto-inhibitory effect of the PH domain (41,42). Although phosphorylation of the kinase domain at Thr308 is known to reduce the PH domain's affinity towards the kinase domain (41), the contribution of Thr308 and Ser473 phosphorylation to auto-inhibition by the PH domain was unknown.

We quantitated the release of PH domain mediated inhibition corresponding to each phosphorylation site and both sites in combination. Our data revealed that the magnitude of the auto-inhibitory effect of the PH domain was sensitively dependent on the phosphorylation status of Akt1. In comparison to the activity of full length Akt1 phospho-variants, deletion of the PH domain significantly increased activity. In comparison to singly phosphorylated Akt1 variants, the auto-inhibitory effect of the PH domain was strongest (295-fold) in the most active and doubly phosphorylated Akt1 variant. Interestingly, the doubly

phosphorylated Akt1 displayed an increase in activity upon PH domain deletion that was greater than the sum of the effects observed for the singly phosphorylated Akt1 variants. The data suggest a novel finding that the PH domain dampens the activity of Akt1 variants in a phosphorylation-dependent manner.

The combination of phosphorylation and PH domain release regulate the kinase activity of Akt1 over a 400,000-fold range. The data indicate the degree to which the un-phosphorylated PH-in conformation of Akt1 is suppressed in comparison to the PH-out doubly phosphorylated version of the enzyme that exists at the plasma membrane in living cells. Recent *in vitro* biochemical work suggested that association with PIP3 containing lipid vesicles stimulated the activity of a full length Akt1 protein by 7-fold (42). The authors suggested that this may be an underestimate due to low levels of phosphorylation (10% Thr308, 1% Ser473) in their Akt1 prepared from Sf9 cells. Our data suggest release of the PH domain in the context of doubly phosphorylated Akt1 would lead to maximal activity increase of 300-fold; a PH domain dependent reduction of less than this value would indicate there may be significant interaction between the Akt1 kinase and PH domains even in the PIP3 bound state at the plasma membrane.

Indeed a recent debate in the literature (43) is underway to explain how Akt1 activity can be maintained far from the membrane. Recent findings suggest that Akt1 is de-phosphorylated on a time scale of ~10 minutes following dissociation from PIP3 on the plasma membrane (44). Multiple nuclear substrates of Akt1 are known to be phosphorylated much more rapidly, on a time scale of seconds (45). Although the authors suggested that all or perhaps most of the active Akt1 is membrane-associated (44), this finding disputes several independent studies identifying many cytosolic and nuclear targets of Akt1-dependent

phosphorylation. Multiple studies clearly identified both phospho-Akt1, e.g., (45-47), and directly measured Akt1 activity in the nucleus (10). Using a fluorescent reporter that quantifies Akt1 activity in live cells in real time, Kunkel and Newton observed Akt1 activity distributed in both the cytoplasm and nucleus on a time scale of seconds to minutes following growth factor stimulation. Their data further show similar levels of Akt1 activity in the nucleus and cytoplasm, with a delay in the peak activity in the nucleus of ~2 minutes. Additional experiments confirmed this lag is related to the time it takes for active Akt1 to translocate from the plasma membrane to the nucleus (10). In light of our data and these findings, the membrane associated Akt1 is likely significantly more active than doubly phosphorylated Akt1 found in the nucleus and cytosol. Phosphorylated Akt1, nevertheless, does persist away from the membrane, retaining significant activity in comparison to the un-phosphorylated Akt1.

3.5.2 Phosphorylation-Dependent Chemical Inhibition of Akt1. Post-translational-modifications, of which phosphorylation is the most common, are a ubiquitous mechanism that cells use to tune the activity of individual proteins and increase the functional diversity of the proteome. Phosphorylation can have a significant impact on the activity of proteins, kinases are a prime example, and phosphorylation status of a protein target can accordingly impact the ability of small molecules to inhibit enzyme activity. In the experiments presented here, we determined precisely how the phosphorylation-status of Akt1 influences the repression of kinase activity by chemical inhibition. We investigated the Akt-specific allosteric inhibitor Akti-1/2. Based on the results of our assays, the phosphorylation status of Akt1 can indeed influence the degree to which Akti-1/2 is able to act on Akt1. Akti-1/2 was most effective at inhibiting pAkt1^{T308}, whereas

phosphorylation at position Ser473 in either pAkt^{S473} or ppAkt^{T308/S473}, reduced the relative effectiveness of the inhibitor by a factor of 4.

Ser473 phosphorylation appears to act as a built-in mechanism of drug resistance for Akt1. It is well established that hyper-phosphorylation of Akt1 at both regulatory sites is linked to poor prognoses and therapeutic resistance (3-6,48). The accepted model for Akti-1/2 activity suggests that the compound locks Akt1 into the auto-inhibited or PH-in confirmation and prevents Akt1 from binding PIP3 in the plasma membrane, inhibiting phosphorylation at Thr308 and Ser473 (49). Our data also suggest that although limited by Ser473 phosphorylation, the Akti-1/2 has significant activity in inhibiting the pre-phosphorylated Akt1 variants that the inhibitor initially encounters in the cell. Studies in cell culture and mouse models show that the short-term impact of Akti-1/2 treatment is that pThr308 and pSer473 levels are depleted within hours. A related allosteric Akt inhibitor, and the most clinically promising (50) compound, MK-2206 significantly reduces Ser473 phosphorylation after 10 hours in mouse models with patient derived xenografts of endometrial tumors (51).

Surprisingly, breast cancer cells (BT474) treated with Akti-1/2 (1 μ M) showed a rebound effect in Akt1 phosphorylation status following Akti-1/2 treatment. These cells have amplified *HER2* genes and constitutively activate the PI3K/Akt signaling cascade. In short term experiments (minutes to hours), Akt1 phosphorylation reduces in response to inhibitor treatment, but at longer times (2-3 days) Akt1 phosphorylation status returns to stimulated levels in these cancer cells. In this longer time frame, phosphorylation of downstream Akt1 target, S6K, was not restored, but PRAS40 phosphorylation was partially restored concomitant with increased Akt1 phospho-status. The study provided evidence that Akti-1/2 ultimately induces the expression and activation of receptor

tyrosine kinases EGFR, HER3, and HER4, which in turn re-activate Akt1 (52). Interestingly, in mouse models a similar rebound in Ser473 phosphorylation following treatment with MK-2206 was observed on time courses of 5 to 20 days (53). In these cases, Ser473 phosphorylation may provide the tumor cell with a means to reduce the effectiveness of Akt1 inhibitor. The data suggest the need to develop an inhibitor that is more effective towards the most active and doubly phosphorylated form of Akt1.

3.5.3 Synthetic Biology Approach to Generate Active Akt1. In our current study, and previous work (11,17-19), we demonstrated that genetic code expansion with pSer in *E. coli* provides a simple route to produce designer phosphoproteins. Here, we have produced differentially phosphorylated Akt1 variants with and without the PH domain in *E. coli* using genetic code expansion and enzymatic phosphorylation in *E. coli*. We found that the variants lacking the PH domain were produced at significantly higher yield compared to the full length Akt1 protein. The ability to produce recombinant Akt1 protein with programmed phosphorylation(s) was essential for our investigations in the role of each phospho-site in Akt1 inhibition.

In comparison with our previous work (11), we found the system we developed to produce Akt1 with either or both regulatory phosphorylation sites (Figure 3.2) leads to a consistently active Akt1 variant with indistinguishable batch to batch variability. This is in contrast to the commercially available Akt1 produced in using Sf9 cell system followed by in vitro PDK1 phosphorylation of Thr308 (25). We observed a vast batch-to-batch variability of enzyme activity in the commercially available Akt1, which warrants cautious attention in its experimental use. Previous work also established that Akt1 produced in Sf9 cells

leads to a mixture of singly and doubly phosphorylated Akt1 variants (26) at times leading to a low stoichiometry of phosphorylation (42) (Figure 3.3B). Our approach consistently results in site-specifically phosphorylated and active Akt1 variants that provide a reliable and consistent source of protein that for biochemical studies and applications in inhibitor screening.

Acknowledgements: We are grateful to Ilka Heinemann and Shawn Li for critical discussions and suggestions.

3.6 References

1. Manning, B. D., and Cantley, L. C. (2007) AKT/PKB signaling: navigating downstream. *Cell* **129**, 1261-1274
2. Manning, B. D., and Toker, A. (2017) AKT/PKB Signaling: Navigating the Network. *Cell* **169**, 381-405
3. Agarwal, E., Brattain, M. G., and Chowdhury, S. (2013) Cell survival and metastasis regulation by Akt signaling in colorectal cancer. *Cell Signal* **25**, 1711-1719
4. Spencer, A., Yoon, S. S., Harrison, S. J., Morris, S. R., Smith, D. A., Brigandi, R. A., Gauvin, J., Kumar, R., Opalinska, J. B., and Chen, C. (2014) The novel AKT inhibitor afuresertib shows favorable safety, pharmacokinetics, and clinical activity in multiple myeloma. *Blood* **124**, 2190-2195
5. Antonelli, M., Massimino, M., Morra, I., Garre, M. L., Gardiman, M. P., Buttarelli, F. R., Arcella, A., and Giangaspero, F. (2012) Expression of pERK and pAKT in pediatric high grade astrocytomas: correlation with YKL40 and prognostic significance. *Neuropathology* **32**, 133-138
6. Suzuki, Y., Shirai, K., Oka, K., Mobaraki, A., Yoshida, Y., Noda, S. E., Okamoto, M., Suzuki, Y., Itoh, J., Itoh, H., Ishiuchi, S., and Nakano, T. (2010) Higher pAkt expression predicts a significant worse prognosis in glioblastomas. *J Radiat Res* **51**, 343-348
7. Blachly, J. S., and Baiocchi, R. A. (2014) Targeting PI3-kinase (PI3K), AKT and mTOR axis in lymphoma. *Br J Haematol* **167**, 19-32

8. Westin, J. R. (2014) Status of PI3K/Akt/mTOR pathway inhibitors in lymphoma. *Clin Lymphoma Myeloma Leuk* **14**, 335-342
9. Altomare, D. A., and Testa, J. R. (2005) Perturbations of the AKT signaling pathway in human cancer. *Oncogene* **24**, 7455-7464
10. Kunkel, M. T., Ni, Q., Tsien, R. Y., Zhang, J., and Newton, A. C. (2005) Spatio-temporal dynamics of protein kinase B/Akt signaling revealed by a genetically encoded fluorescent reporter. *J Biol Chem* **280**, 5581-5587
11. Balasuriya, N., Kunkel, M. T., Liu, X., Biggar, K. K., Li, S. S., Newton, A. C., and O'Donoghue, P. (2018) Genetic code expansion and live cell imaging reveal that Thr308 phosphorylation is irreplaceable and sufficient for Akt1 activity. *J Biol Chem* **293**, 10744-10756
12. Liao, Y., Yuan, S., Chen, X., Zhu, P., Li, J., Qin, L., and Liao, W. (2017) Up-regulation of BRCA1-associated RING Domain 1 Promotes Hepatocellular Carcinoma Progression by Targeting Akt Signaling. *Sci Rep* **7**, 7649
13. Parker, L., Levinger, I., Mousa, A., Howlett, K., and de Courten, B. (2016) Plasma 25-Hydroxyvitamin D Is Related to Protein Signaling Involved in Glucose Homeostasis in a Tissue-Specific Manner. *Nutrients* **8**
14. Tang, H., Wu, Y., Liu, M., Qin, Y., Wang, H., Wang, L., Li, S., Zhu, H., He, Z., Luo, J., Wang, H., Wang, Q., and Luo, S. (2016) SEMA3B improves the survival of patients with esophageal squamous cell carcinoma by upregulating p53 and p21. *Oncol Rep* **36**, 900-908
15. Xiao, J., Yu, W., Hu, K., Li, M., Chen, J., and Li, Z. (2017) miR-92a promotes tumor growth of osteosarcoma by targeting PTEN/AKT signaling pathway. *Oncol Rep* **37**, 2513-2521
16. Parikh, C., Janakiraman, V., Wu, W. I., Foo, C. K., Kljavin, N. M., Chaudhuri, S., Stawiski, E., Lee, B., Lin, J., Li, H., Lorenzo, M. N., Yuan, W., Guillory, J., Jackson, M., Rondon, J., Franke, Y., Bowman, K. K., Sagolla, M., Stinson, J., Wu, T. D., Wu, J., Stokoe, D., Stern, H. M., Brandhuber, B. J., Lin, K., Skelton, N. J., and Seshagiri, S. (2012) Disruption of PH-kinase domain interactions leads to oncogenic activation of AKT in human cancers. *Proc Natl Acad Sci U S A* **109**, 19368-19373
17. George, S., Wang, S. M., Bi, Y., Treidlinger, M., Barber, K. R., Shaw, G. S., and O'Donoghue, P. (2017) Ubiquitin phosphorylated at Ser57 hyper-activates parkin. *Biochim Biophys Acta* **1861**, 3038-3046
18. George, S., Aguirre, J. D., Spratt, D. E., Bi, Y., Jeffery, M., Shaw, G. S., and O'Donoghue, P. (2016) Generation of phospho-ubiquitin variants by orthogonal translation reveals codon skipping. *FEBS Lett* **590**, 1530-1542
19. Aerni, H. R., Shifman, M. A., Rogulina, S., O'Donoghue, P., and Rinehart, J. (2015) Revealing the amino acid composition of proteins within an expanded genetic code. *Nucleic acids research* **43**, e8

20. Lee, S., Oh, S., Yang, A., Kim, J., Soll, D., Lee, D., and Park, H. S. (2013) A facile strategy for selective incorporation of phosphoserine into histones. *Angew Chem Int Ed Engl* **52**, 5771-5775
21. Turowec, J. P., Duncan, J. S., French, A. C., Gyenis, L., St Denis, N. A., Vilk, G., and Litchfield, D. W. (2010) Protein kinase CK2 is a constitutively active enzyme that promotes cell survival: strategies to identify CK2 substrates and manipulate its activity in mammalian cells. *Methods Enzymol* **484**, 471-493
22. Lindsley, C. W., Zhao, Z., Leister, W. H., Robinson, R. G., Barnett, S. F., Defeo-Jones, D., Jones, R. E., Hartman, G. D., Huff, J. R., Huber, H. E., and Duggan, M. E. (2005) Allosteric Akt (PKB) inhibitors: discovery and SAR of isozyme selective inhibitors. *Bioorg Med Chem Lett* **15**, 761-764
23. Alessi, D. R., James, S. R., Downes, C. P., Holmes, A. B., Gaffney, P. R., Reese, C. B., and Cohen, P. (1997) Characterization of a 3-phosphoinositide-dependent protein kinase which phosphorylates and activates protein kinase B α . *Curr Biol* **7**, 261-269
24. Klein, S., Geiger, T., Linchevski, I., Lebendiker, M., Itkin, A., Assayag, K., and Levitzki, A. (2005) Expression and purification of active PKB kinase from *Escherichia coli*. *Protein Expr Purif* **41**, 162-169
25. Zhang, X., Zhang, S., Yamane, H., Wahl, R., Ali, A., Lofgren, J. A., and Kendall, R. L. (2006) Kinetic mechanism of AKT/PKB enzyme family. *J Biol Chem* **281**, 13949-13956
26. Fabbro, D., Batt, D., Rose, P., Schacher, B., Roberts, T. M., and Ferrari, S. (1999) Homogeneous purification of human recombinant GST-Akt/PKB from Sf9 cells. *Protein Expr Purif* **17**, 83-88
27. Alessi, D. R., Caudwell, F. B., Andjelkovic, M., Hemmings, B. A., and Cohen, P. (1996) Molecular basis for the substrate specificity of protein kinase B; comparison with MAPKAP kinase-1 and p70 S6 kinase. *FEBS Lett* **399**, 333-338
28. Nitulescu, G. M., Margina, D., Juzenas, P., Peng, Q., Olaru, O. T., Saloustros, E., Fenga, C., Spandidos, D., Libra, M., and Tsatsakis, A. M. (2016) Akt inhibitors in cancer treatment: The long journey from drug discovery to clinical use (Review). *Int J Oncol* **48**, 869-885
29. Reuveni, H., Livnah, N., Geiger, T., Klein, S., Ohne, O., Cohen, I., Benhar, M., Gellerman, G., and Levitzki, A. (2002) Toward a PKB inhibitor: modification of a selective PKA inhibitor by rational design. *Biochemistry* **41**, 10304-10314
30. Yap, T. A., Walton, M. I., Grimshaw, K. M., Te Poele, R. H., Eve, P. D., Valenti, M. R., de Haven Brandon, A. K., Martins, V., Zetterlund, A., Heaton, S. P., Heinzmann, K., Jones, P. S., Feltell, R. E., Reule, M.,

- Woodhead, S. J., Davies, T. G., Lyons, J. F., Raynaud, F. I., Eccles, S. A., Workman, P., Thompson, N. T., and Garrett, M. D. (2012) AT13148 is a novel, oral multi-AGC kinase inhibitor with potent pharmacodynamic and antitumor activity. *Clin Cancer Res* **18**, 3912-3923
31. Green, C. J., Goransson, O., Kular, G. S., Leslie, N. R., Gray, A., Alessi, D. R., Sakamoto, K., and Hundal, H. S. (2008) 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* **283**, 27653-27667
 32. Barnett, S. F., Defeo-Jones, D., Fu, S., Hancock, P. J., Haskell, K. M., Jones, R. E., Kahana, J. A., Kral, A. M., Leander, K., Lee, L. L., Malinowski, J., McAvoy, E. M., Nahas, D. D., Robinson, R. G., and Huber, H. E. (2005) Identification and characterization of pleckstrin-homology-domain-dependent and isoenzyme-specific Akt inhibitors. *Biochem J* **385**, 399-408
 33. Yang, J., Cron, P., Good, V. M., Thompson, V., Hemmings, B. A., and Barford, D. (2002) Crystal structure of an activated Akt/protein kinase B ternary complex with GSK3-peptide and AMP-PNP. *Nat Struct Biol* **9**, 940-944
 34. Wu, W. I., Voegtli, W. C., Sturgis, H. L., Dizon, F. P., Vigers, G. P., and Brandhuber, B. J. (2010) Crystal structure of human AKT1 with an allosteric inhibitor reveals a new mode of kinase inhibition. *PLoS One* **5**, e12913
 35. Massihnia, D., Avan, A., Funel, N., Maftouh, M., van Krieken, A., Granchi, C., Raktoe, R., Boggi, U., Aicher, B., Minutolo, F., Russo, A., Leon, L. G., Peters, G. J., and Giovannetti, E. (2017) Phospho-Akt overexpression is prognostic and can be used to tailor the synergistic interaction of Akt inhibitors with gemcitabine in pancreatic cancer. *J Hematol Oncol* **10**, 9
 36. Slipicevic, A., Holm, R., Nguyen, M. T., Bohler, P. J., Davidson, B., and Florenes, V. A. (2005) Expression of activated Akt and PTEN in malignant melanomas: relationship with clinical outcome. *Am J Clin Pathol* **124**, 528-536
 37. Wang, Q., Chen, X., and Hay, N. (2017) Akt as a target for cancer therapy: more is not always better (lessons from studies in mice). *Br J Cancer* **117**, 159-163
 38. Lu, M., Wan, M., Leavens, K. F., Chu, Q., Monks, B. R., Fernandez, S., Ahima, R. S., Ueki, K., Kahn, C. R., and Birnbaum, M. J. (2012) Insulin regulates liver metabolism in vivo in the absence of hepatic Akt and Foxo1. *Nat Med* **18**, 388-395
 39. Crouthamel, M. C., Kahana, J. A., Korenchuk, S., Zhang, S. Y., Sundaresan, G., Eberwein, D. J., Brown, K. K., and Kumar, R. (2009) Mechanism and

- management of AKT inhibitor-induced hyperglycemia. *Clin Cancer Res* **15**, 217-225
40. Cho, H., Mu, J., Kim, J. K., Thorvaldsen, J. L., Chu, Q., Crenshaw, E. B., 3rd, Kaestner, K. H., Bartolomei, M. S., Shulman, G. I., and Birnbaum, M. J. (2001) Insulin resistance and a diabetes mellitus-like syndrome in mice lacking the protein kinase Akt2 (PKB beta). *Science* **292**, 1728-1731
 41. Calleja, V., Alcor, D., Laguerre, M., Park, J., Vojnovic, B., Hemmings, B. A., Downward, J., Parker, P. J., and Larijani, B. (2007) Intramolecular and intermolecular interactions of protein kinase B define its activation in vivo. *PLoS Biol* **5**, e95
 42. Lucic, I., Rathinaswamy, M. K., Truebestein, L., Hamelin, D. J., Burke, J. E., and Leonard, T. A. (2018) Conformational sampling of membranes by Akt controls its activation and inactivation. *Proc Natl Acad Sci U S A* **115**, E3940-E3949
 43. Agarwal, A. K. (2018) How to explain the AKT phosphorylation of downstream targets in the wake of recent findings. *Proc Natl Acad Sci U S A* **115**, E6099-E6100
 44. Ebner, M., Lucic, I., Leonard, T. A., and Yudushkin, I. (2017) PI(3,4,5)P3 Engagement Restricts Akt Activity to Cellular Membranes. *Mol Cell* **65**, 416-431 e416
 45. Humphrey, S. J., Azimifar, S. B., and Mann, M. (2015) High-throughput phosphoproteomics reveals in vivo insulin signaling dynamics. *Nat Biotechnol* **33**, 990-995
 46. Zhu, L., Hu, C., Li, J., Xue, P., He, X., Ge, C., Qin, W., Yao, G., and Gu, J. (2007) Real-time imaging nuclear translocation of Akt1 in HCC cells. *Biochem Biophys Res Commun* **356**, 1038-1043
 47. Hixon, M. L., and Boekelheide, K. (2003) Expression and localization of total Akt1 and phosphorylated Akt1 in the rat seminiferous epithelium. *J Androl* **24**, 891-898
 48. Freudlsperger, C., Horn, D., Weissfuss, S., Weichert, W., Weber, K. J., Saure, D., Sharma, S., Dyckhoff, G., Grabe, N., Plinkert, P., Hoffmann, J., Freier, K., and Hess, J. (2015) Phosphorylation of AKT(Ser473) serves as an independent prognostic marker for radiosensitivity in advanced head and neck squamous cell carcinoma. *Int J Cancer* **136**, 2775-2785
 49. Okuzumi, T., Fiedler, D., Zhang, C., Gray, D. C., Aizenstein, B., Hoffman, R., and Shokat, K. M. (2009) Inhibitor hijacking of Akt activation. *Nat Chem Biol* **5**, 484-493
 50. Wisinski, K. B., Tevaarwerk, A. J., Burkard, M. E., Rampurwala, M., Eickhoff, J., Bell, M. C., Kolesar, J. M., Flynn, C., and Liu, G. (2016) Phase I Study of an AKT Inhibitor (MK-2206) Combined with Lapatinib in Adult

- Solid Tumors Followed by Dose Expansion in Advanced HER2+ Breast Cancer. *Clin Cancer Res* **22**, 2659-2667
51. Winder, A., Unno, K., Yu, Y., Lurain, J., and Kim, J. J. (2017) The allosteric AKT inhibitor, MK2206, decreases tumor growth and invasion in patient derived xenografts of endometrial cancer. *Cancer Biol Ther* **18**, 958-964
 52. Chandarlapaty, S., Sawai, A., Scaltriti, M., Rodrik-Outmezguine, V., Grbovic-Huezo, O., Serra, V., Majumder, P. K., Baselga, J., and Rosen, N. (2011) AKT inhibition relieves feedback suppression of receptor tyrosine kinase expression and activity. *Cancer Cell* **19**, 58-71
 53. Lin, A., Piao, H. L., Zhuang, L., Sarbassov dos, D., Ma, L., and Gan, B. (2014) FoxO transcription factors promote AKT Ser473 phosphorylation and renal tumor growth in response to pharmacologic inhibition of the PI3K-AKT pathway. *Cancer Res* **74**, 1682-1693

3.7 Supporting information:

3.7.1 Supporting experimental procedures

Affinity column chromatography. The His-tagged proteins were purified using Ni affinity column chromatography. 0.5 ml of Ni-NTA resin (Thermo-Fisher Scientific) was used for 1 l of *E. coli* culture. The cell lysates (see Methods) were loaded into the column (2 ml column volume) and washed with 20 column volumes of wash buffer A (20 mM Hepes, 150 mM NaCl, 3 mM β -mercaptoethanol, 3mM Dithiothreitol, 1 mM Na_3VO_4 and 5 mM NaF, 15 mM imidazole) followed by 10 column volumes of wash buffer B (20 mM Hepes, 150 mM NaCl, 3 mM β -mercaptoethanol, 3mM DTT, 1 mM Na_3VO_4 and 5 mM NaF, 20 mM imidazole). Protein was eluted using an elution buffer (20 mM Hepes, 150 mM NaCl, 3 mM β -mercaptoethanol, 3mM dithiothreitol, 1 mM Na_3VO_4 and 5 mM NaF) with 75 mM Imidazole. The same protocol was used for Akt1 variants with phosphomimetic mutations except: 1) phosphatase inhibitors were excluded

from the buffers, 2) the imidazole concentrations of the wash buffers were 20 - 50 mM, and 3) the imidazole concentration of the elution buffer was 200 mM. Fractions were run on 10% SDS (sodium dodecyl sulfate) poly-acrylamide gels. As detailed previously [1], Akt1 variants were further purified by size exclusion chromatography.

3.7.2 Supporting References

1. Balasuriya, N.; Kunkel, M.T.; Liu, X.; Biggar, K.K.; Li, S.S.; Newton, A.C.; O'Donoghue, P. (2018) Genetic code expansion and live cell imaging reveal that thr308 phosphorylation is irreplaceable and sufficient for akt1 activity. *J Biol Chem*, **293**, 10744-10756
2. George, S.; Aguirre, J.D.; Spratt, D.E.; Bi, Y.; Jeffery, M.; Shaw, G.S.; O'Donoghue, P. (2016) Generation of phospho-ubiquitin variants by orthogonal translation reveals codon skipping. *FEBS Lett*, **590**, 1530-1542

3.7.3 Supporting Tables

Supplementary Table S3.1 Plasmids used in this study

Plasmid ID	Plasmid backbone	Selectable marker	Inserted genes	Genetic modifications	Protein product
(1) WT Akt1	pDS0 (pUC) [2]	Amp	<i>AKT1</i>	None.	Akt1, unphosphorylated. (full-length, inactive) [1]
(2) WT Akt1-PDK1	pCDF-Duet-1	Strep	<i>AKT1</i> <i>PDK1</i>	None.	Akt1, phosphorylated at position T308. (full-length, active) [1]
(3) Akt1 UAG473	pCDF-Duet-1	Strep	<i>AKT1</i>	UAG mutation at position 473 in <i>AKT1</i> .	Akt1, phosphorylated at position S473. (full-length, slightly active) [1]
(4) Akt1-UAG473-PDK1	pCDF-Duet-1	Strep	<i>AKT1</i> <i>PDK1</i>	UAG mutation at position 473 in <i>AKT1</i> .	Akt1, phosphorylated at positions T308 and S472. (full-length, highly active) [1]
(5) pSer-OTS	pDS-pSer2	Kan	tRNA ^{ser} (5x) SepRS9 EFSep21	This system is comprised of the 2 nd generation mutants of the pSer incorporation system.	pSer orthogonal translation system, capable of decoding UAG codons and incorporating pSer into nascent peptides [2]
(6) ΔPH-WT Akt1	pDS0 (pUC) [2]	Amp	<i>AKT1</i>	Deletion of the PH domain from <i>AKT1</i> (Δ residues 2-109).	ΔPH-Akt1, unphosphorylated. (truncated, inactive)
(7) ΔPH-WT Akt1-PDK1	pCDF-Duet-1	Strep	<i>AKT1</i> <i>PDK1</i>	Deletion of the PH domain from <i>AKT1</i> (Δ residues 2-109).	ΔPH-Akt1, phosphorylated at position T308. (truncated, active)
(8) ΔPH-Akt1-UAG473	pCDF-Duet-1	Strep	<i>AKT1</i>	Deletion of the PH domain from <i>AKT1</i> (Δ residues 2-109). UAG mutation at position 473 in <i>AKT1</i>	ΔPH-Akt1, phosphorylated at position S473. (truncated, slightly active)
(9) ΔPH-Akt1 UAG473-PDK1	pCDF-Duet-1	Strep	<i>AKT1</i> <i>PDK1</i>	Deletion of the PH domain from <i>AKT1</i> (Δ residues 2-109). UAG mutation at position 473 in <i>AKT1</i> .	ΔPH-Akt1, phosphorylated at positions T308 and S472. (truncated, highly active)

3.7.4 Supporting Figures

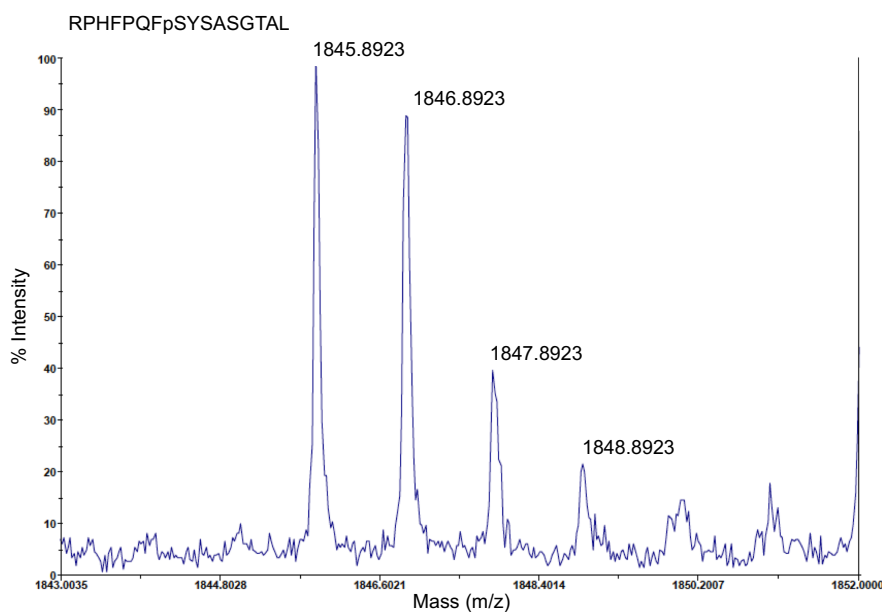


Figure S3.1 Mass spectra confirming genetically encoded phosphoserine in Δ PH-ppAkt1. Tryptic digested peptides of Δ PH-ppAkt1^{T308,S473} were subjected to MALDI-TOF mass spectrometry. The C-terminal peptide of Δ PH-Akt1 carrying a single phosphorylation was readily detected. The isotopic masses of the peptide carrying the most abundant peak of ¹²C isotope (observed m/z = 1845.8923; expected m/z = 1845.8378) and the 3 consecutive peaks with ¹³C isotopes (m/z = 1846.8923, 1847.8923, 1848.8923, respectively) are shown.

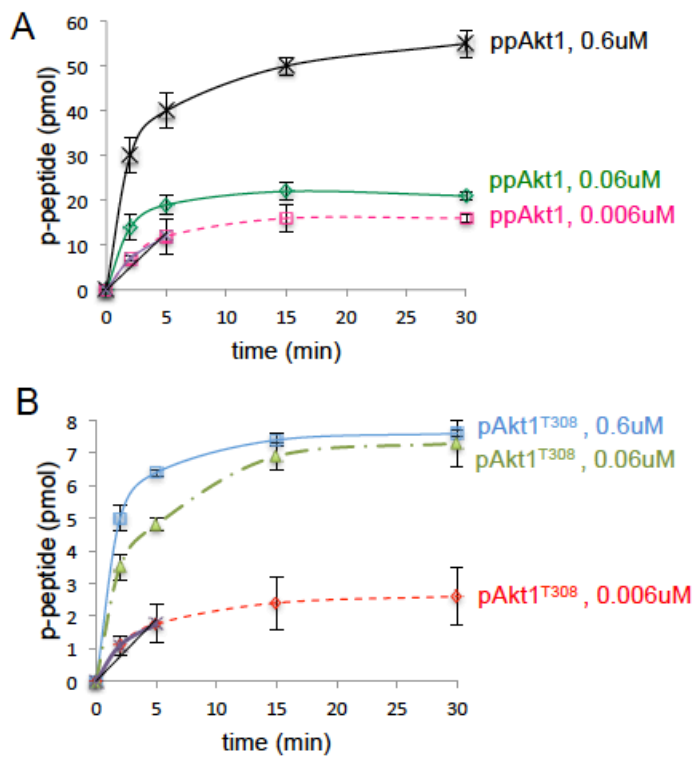


Figure S3.2 Initial velocity measurements for highly active Δ PH Akt1 variants. (A) Δ PH-ppAkt1^{T308, S473} and (B) Δ PH-pAkt1^{T308} were diluted by 10-fold and 100-fold, respectively, to obtain a linear range of increasing enzyme activity. Initial velocities were calculated using the data points at the linear range of 100-fold diluted enzyme.

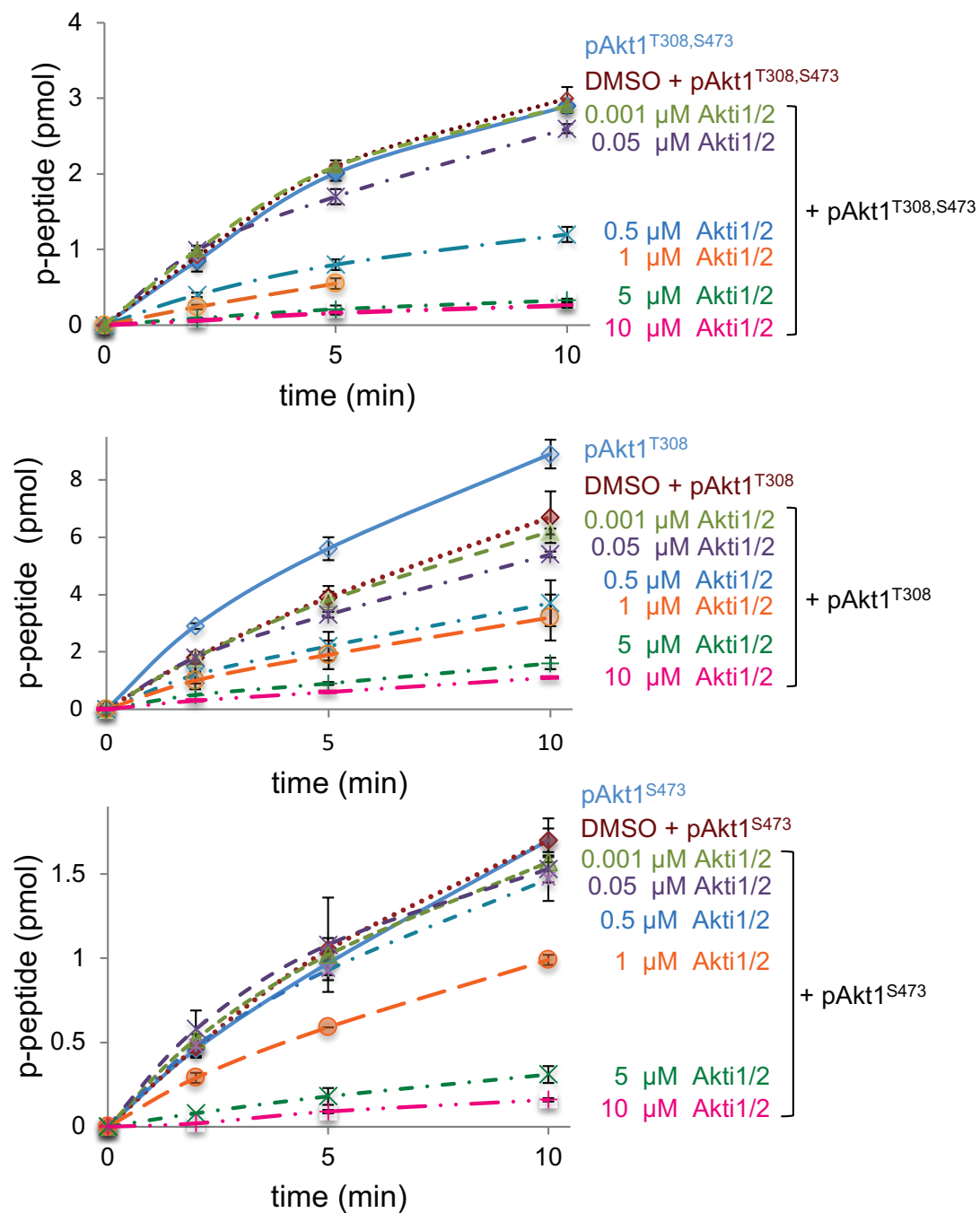


Figure S3.3 Inhibition of the full length Akt1 variants incubated with Akti-1/2 inhibitor VIII. (A) pAkt1^{T308, S473}, (B) pAkt1^{T308}, (3) pAkt1^{S473} variants were incubated with increasing concentrations (0.001 uM to 10 uM) of Akti1/2. Error bars indicate 1 standard deviation based on triplicate experiments.

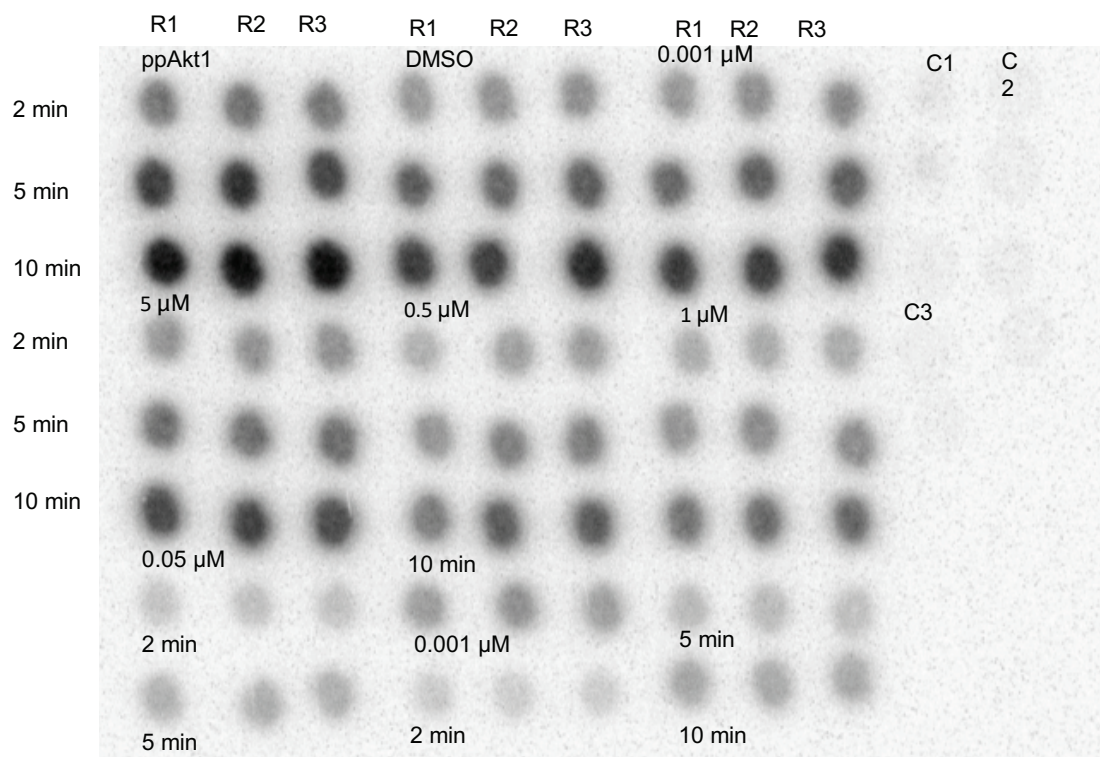


Figure S3.4 Autoradiographs of Akt1 inhibitor assays with ppAkt1^{T308, S473}. Time courses are shown for the reaction catalyzed by ppAkt1^{T308, S473} with the indicated concentrations of inhibitor Akti1/2. Assays were performed in triplicate (as indicated by R1-R3). Controls include: C1 without substrate peptide, C2 without enzyme, C3 only kinase assay buffer.

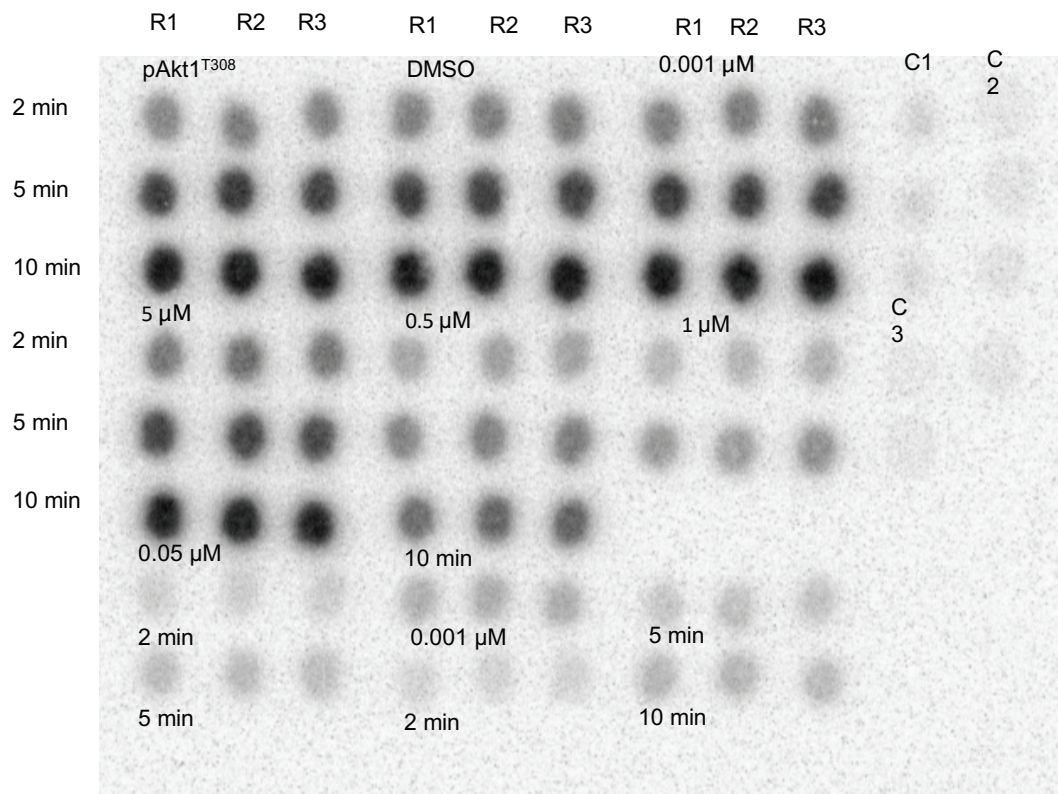


Figure S3.5 Autoradiographs of Akt1 inhibitor assays with pAkt1^{T308}. Time courses are shown for the reaction catalyzed by pAkt1^{T308} with the indicated concentrations of inhibitor Akti1/2. Assays were performed in triplicate (as indicated by R1-R3). Controls include: C1 without substrate peptide, C2 without enzyme, C3 only kinase assay buffer.

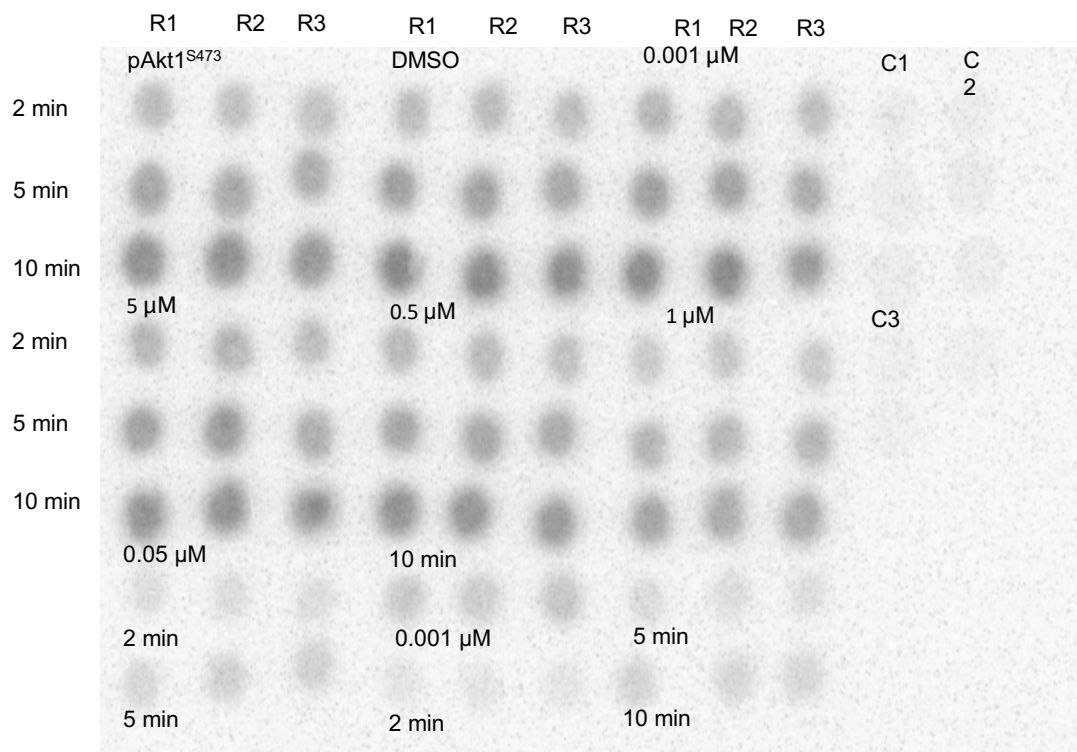


Figure S3.6 Autoradiographs of Akt1 inhibitor assays with pAkt1^{S473}. Time courses are shown for the reaction catalyzed by pAkt1^{S473} with the indicated concentrations of inhibitor Akti1/2. Assays were performed in triplicate (as indicated by R1-R3). Controls include: C1 without substrate peptide, C2 without enzyme, C3 only kinase assay buffer.

Chapter 4

4 Phosphorylation-dependent substrate selectivity of Akt1

4.1 Abstract

Protein kinase B (Akt1) is a central node in a signaling pathway that regulates cell survival. The diverse pathways regulated by Akt1 are communicated in the cell via the phosphorylation of perhaps as many as ~100 different cellular substrates. Akt1 is itself activated by phosphorylation at Thr308 and Ser473. We recently developed a method to produce Akt1 with programmed phosphorylation at either or both key regulatory sites. This breakthrough previously enabled resolution of the contribution of each phospho-site to the activation of Akt1. Here we used both defined and randomized peptide libraries to map the substrate selectivity of singly and doubly phosphorylated Akt1 variants. To globally quantitate Akt1 substrate preferences we synthesized two Akt1 substrate peptide libraries: one based on 84 “known” substrates and a larger oriented peptide array library (OPAL) of 10^{11} peptides. The data revealed that each phospho-form of Akt1 has distinct substrate requirements. Surprisingly, phosphorylation of Ser473 in the context of a pAkt1^{T308} enzyme led to increased activity on some, but not all Akt1 substrates. This is the first report that Ser473 phosphorylation does not simply increase Akt1 kinase activity, rather we found that phosphorylation at Ser473 modulates global substrate preferences by selectively increasing, decreasing or not altering activity compared to the kinase singly phosphorylated at Thr308. We also verified a new Akt1 target as the terminal nucleotidyltransferase (germline development 2) Gld2.

Our data show that Akt1 site-specifically phosphorylates Gld2 at Ser116. Together these data suggest new and expanded roles for Akt1 signaling in regulating cellular processes.

4.2 Introduction

Protein kinase B (PKB, Akt1) is a central node in the Phosphoinositide 3-kinase (PI3K)/Akt signaling pathway that regulates cell survival. There are three isozymes of Akt (Akt1, Akt2, Akt3) identified in mammalian cells (1) with both distinct and overlapping roles. Akt1 is an oncogenic kinase that is overactive and hyper-phosphorylated in most cancers and a prime target in cancer therapy (2). Although direct Akt1 inhibitors have not been generally successful as monotherapies (3), Akt1 inhibitors show promise in combination with, e.g., mitogen-activated protein kinase inhibitors (4). Activated Akt1 phosphorylates downstream targets that regulate many cellular processes including cell survival and apoptosis (5). The diverse cellular functions of Akt1 are mediated by its ability to phosphorylate perhaps as many as hundreds of cellular substrates, although the precise number is not known. Based on a large number of reports from the literature, a catalog of ~200 known Akt substrates have been reported (6).

At present it is unclear if all of these substrates are truly phosphorylated by Akt1 or to what extent these substrates are differentially phosphorylated by active Akt1. Because the downstream phosphorylation profile of active Akt1 determines which cellular programs are turned on or off by the enzyme, the ability to identify novel Akt1 substrates will have significant impact on target selection and drug discovery in Akt1 mediated chronic diseases such as cancer (1) and diabetes (7).

We recently developed an approach to produce Akt1 variants with site-specific phosphorylation at either or both key regulatory sites (8,9). Here we show the method provides an indispensable tool for identifying the role of Akt1 phosphorylation status in substrate selectivity. The substrate selectivity associated with specific Akt1 phospho-forms is challenging to resolve in the complex environment of the cells (10). The phosphorylation status of Akt1 substrates depend on many factors including the expression level and cellular localization of Akt1 substrates as well as the off-rate of phosphate on these substrates as a result of phosphatase activity (11). However, there remains a lack of understanding regarding how the two key activating phosphorylation sites on Akt1 lead to differences in substrate selection and downstream substrate phosphorylation (10).

The minimal Akt substrate recognition motif is R₋₅ X₋₄ R₋₃ X₋₂ X₋₁ S/T₀ φ₊₁. X represents any amino acid except Thr, Cys; φ represents a bulky hydrophobic residue (Phe, Tyr etc.) and the phospho-accepting site is a Ser or Thr residue (12). This motif was further validated through oriented peptide array library (OPAL) screens where the -5 position was Arg and specific amino acids were designated to -1, -2, +1 and +2 positions (13). The Akt target consensus was initially determined using the amino acid sequences surrounding the phosphorylation site of the first reported Akt substrate: glycogen synthase kinase 3 (GSK-3) (12). Arg residues at position -3 and -5 were found to be specific to Akt whereas other AGC family kinases such as the S6 kinase (S6K1), prefer Lys at -3 and -5 positions (10). Peptide library screening is a gold standard method that has been widely used to determine specific amino acid preferences of the kinase substrate recognition motif. Degenerate peptide libraries or OPAL screens provide a systematic approach to identify the important residues surrounding the phosphorylation site on the substrate (13,14).

Here we synthesized designed and randomized (OPAL) peptide libraries to define the substrate requirements for differentially phosphorylated Akt1 variants. We discovered that the phosphorylation status of Akt1 has a significant impact on substrate selectivity and on the specific residue preferences at each location in the consensus motif. Akt1 kinase activity data over the OPAL library was used to generate a score matrix that we used to rank putative novel Akt1 targets in the human proteome. We validated one of these predicted Akt1 targets, the terminal nucleotidyl transferase Gld2 (15).

4.3 Materials and Methods

4.3.1 Bacterial strains and plasmids. A codon optimized human *AKT1* gene lacking the PH domain was synthesized at ATUM (Newark, California, USA) and sub-cloned (*NcoI/NotI*) into an isopropyl β -D-1-thiogalactopyranoside (IPTG) inducible *T7lac* promoter driven pCDF-Duet vector with CloDF13-derived CDF replicon and streptomycin/spectinomycin resistance (pCDF-Duet1- Δ PHAkt1). The human *PDPK1* gene, which was acquired from the Harvard PlasmidID repository service (HsCD00001584, Boston, MA, USA), was sub-cloned (*KpnI/NdeI*) into the second multiple cloning site of pCDFDuet-1. The codon for the residue in *AKT1* at position Ser473 was mutated to the amber (TAG) codon in the synthetic gene from ATUM. Successful cloning was verified by DNA sequencing (London Regional Genomics Centre, London, ON, Canada and Genewiz, Cambridge, MA, USA).

4.3.2 Protein and phosphoprotein production. Un-phosphorylated and phosphorylated forms of Akt1 (pAkt1^{S473}, pAkt1^{T308}, ppAkt1^{T308,S473}) were produced

according to previously described methods (8,16) (See also the methods and SI sections Chapters 2 and 3).

4.3.3 Library of known Akt1 substrate peptides. Based on reports from the literature collated at the Phosphosite database (6), we selected 84 different peptides to generate a library of 'known' Akt1 substrates. The substrate motifs derived from these Akt1 substrates were synthesized in the following form: biotin-AX₆-X₁S₀/T₀X₁-X₆YRR. The phosphorylation site (Ser or Thr) is located at the 0 position. Each individual in-solution peptide was synthesized according to previously reported methods (17).

4.3.4 Alanine scanning. In my previous studies of Akt1 (8,12) (Chapters 2-3), the GSK-3 β substrate peptide was used to assay a wide variety of phosphorylated Akt1 variants. To identify critical residues for the activity of each Akt1 phospho-variant, a series of 12 GSK-3 β derived peptides were synthesized with one of the positions substituted to alanine. The in-solution peptides were synthesized according to previously reported methods (17).

4.3.5 OPAL Akt1 activity screen. An oriented peptide library was designed as a series of soluble library pools according to previously reported methods (14). The known consensus sequence of Akt1 phosphorylation, which is biotin-AGGX₆X₅X₄R₃X₂X₁S₀X₁X₂X₃, was used as the design for library synthesis. X represents any amino acid other than Ser, Thr or Cys. The phosphorylatable residues (Ser, Thr) were excluded from the screen to avoid false positive signals when probing position S₀. Each of the 17 other canonical amino acids are tested in X positions.

The -3 and 0 positions were fixed to Arg and Ser, respectively, where 0 position is the phosphorylation site and R-3 is essential for substrate phosphorylation by Akt1 (12). In each of 128 distinct sub-library pools, one of the X positions was fixed to one specific amino acid and every other X position was randomized. The total library contains 10^{11} peptides.

4.3.6 Sequence Logos and database searching. Sequence logos of substrates corresponding to each Akt1 variant were generated using WebLogo version 2.8.2 (18) or Seq2Logo 2.0 (19) as indicated. Using the data in Figure 4.1, we generated an alignment of known peptides in which the population of each peptide was linearly related to its activity level. The resulting peptide alignment was, thus, most populated by the highest active peptides and least populated by the low active peptides. This allow us to convert the activity data over known peptides using WebLogo (Figure 4.3).

Using the OPAL data we generated position-specific scoring matrices (PSSMs) according to a similar method used in Scansite (20). For each data set, a selectivity matrix was calculated by dividing all activity values by the average value for the complete array, *i.e.*, a selectivity factor. A scoring matrix was computed as the natural log of the selectivity matrix. Finally, the raw scoring matrices were scaled by a factor of 10 multiplied by the selectivity factor for each data set. The resulting PSSMs based on each OPAL data set were used to produce sequence logos with the program Seg2Logo 2.0 (19). The PSSM-Logo was computed using default values.

As an alternate approach to generate a PSSM, we also converted the OPAL activity data to a set of Z-score matrices as before (21). The preference rank of each

substrate was statistically determined and assigned a z score. The formula is given below.

$$Z_i \text{ score} = \frac{X_i - X'}{S}$$

X_i = activity value of i^{th} peptide sub-library, X' = mean activity over entire peptide library, S =standard deviation of activity over entire peptide library.

The known human phospho-proteome was scored based on the amino acid preferences at each position in the z-score PSSM. The sum z-scores at each position in the target peptide was used to rank all peptides covering the human proteome.

4.3.7 NIH 3T3 cell culture. NIH 3T3 cells were purchased from American type culture collection (CRL-1658), Manassas, VA, USA. Cells were grown at 37°C with 5% CO₂ in Dulbecco's Modified Eagles Medium (DMEM) supplemented with 4 mM L-glutamine, 4.5 g/L glucose, 1 mM sodium pyruvate, 1.5 g/L sodium bicarbonate (Wisenet, 319-007-CL), 1% pen-strep solution (Fisher Scientific, 15140122) and bovine calf serum (ATCC, 30-2030). Cells were grown until 80% confluence and passaged. Cells grown to 3 passages were used for the kinase assays. Cells were harvested with 0.25% (v/v) trypsin-ethylenediaminetetraacetic acid (EDTA) and incubated with cell lysis buffer (50 mM Tris-HCl, 1% Triton-100, 150 mM NaCl, 0.1% sodium dodecyl sulfate (SDS) pH 7.5) for 5 min on ice. 200 µL of lysis buffer was added per well of a 6-well plate. Cell lysate was centrifuged at 38000 × g at 4 °C for 10 min. Supernatants were transferred to sterile micro-centrifuge tubes and lysate protein concentrations were determined using a

Bradford assay according to manufacturer's instructions (Bio-Rad Laboratories, CA, USA).

4.3.8 Kinase assay for peptide libraries. Kinase assays with peptides or peptide sub-libraries in dimethyl sulfoxide (DMSO) solution were conducted using radiolabeled γ -[³²P]-ATP according to previously reported methods (8) with slight modifications as following: Akt1 activity was determined using 400 μ M substrate peptide for the known substrate library. The substrate concentration for the OPAL library was 1 mM. 0.3 ng of kinase was incorporated per reaction. Each assay was conducted in triplicate.

4.3.9 Kinase assay with NIH 3T3 cell extracts. The NIH 3T3 cell extracts in lysis buffer were dialyzed into ATP free kinase assay buffer (25 mM 3-(N-morpholino)propanesulfonic acid (MOPS) pH 7.0, 12.5 mM β -glyceraldehydephosphate, 25 mM MgCl₂, 5 mM ethylene glycol-bis(β -aminoethyl ether-N,N,N',N'-tetraacetic acid (EGTA), 2 mM EDTA) using an Amicon ultral-0.5 ml centrifugal device. Cellular proteins (18 μ g) from the lysates were incubated with 0.1 μ g of the indicated Akt1 enzyme variant in 30 μ L kinase assay buffer containing 0.2 mM unlabeled ATP and 33 nM γ -[³²P]-ATP. 10 μ L from each reaction were taken at 20 min and 80 min time points and boiled for 10 min in 3X SDS loading dye (1 M Tris-HCl pH 6.8, 0.38 g/mL sucrose, 0.1 % bromophenol blue, 20% SDS, 3% (V/V) β -mercaptoethanol) and boiled for 10 min. Samples were rissolved on a 10% polyacrylamide SDS gel, wrapped in plastic wrap, and placed on a Whatman paper, and wrapped in Ceran wrap. The gel was exposed to a

phosphorimaging screen overnight at -80 °C and visualized with a phosphorimager (Storm 860 Molecular Imager).

4.3.10 Kinase assay with Gld2 protein substrate. Gld2 was purified as described previously (15). 30 μ L reaction containing 1.5 μ g Gld2 in kinase buffer (20 mM MOPS [pH 7.0], 25 mM β -glyceraldehydephosphate, 25 mM $MgCl_2$, 5 mM EGTA [pH 8.0], 1 mM Na_2VO_4 , 0.1 mM ATP, 13.2 nM γ - $[^{32}P]$ -ATP) and Akt1 (33 nM) or CK2- α (25 nM) kinases were incubated at 37°C shaking for 15 minutes. Samples of 5 μ l were taken after 10 minutes and the reaction was stopped by spotting on P81 paper. The P81 paper was washed with 1% phosphoric acid, air dried, exposed to a phosphor screen over night and visualized with the Storm 860 Molecular Imager. Purified CK2- α kinase was used as kinase control and was a generous gift from Dr. David Litchfield.

4.4 Results

4.4.1 Akt1 phosphorylation status alters selectivity among known substrates.

Phosphoinositide dependent kinase (PDK1) site specifically phosphorylates Akt1 at Thr308, while the upstream kinase of S473 is unknown. Using a combination of genetic code expansion to encode phosphoserine at S473 and co-expression of PDK1, we were able to produce active full length and PH domain truncated Akt1 variants in *E. coli* with either or both Thr308 and Ser473 sites phosphorylated (Figure S4.1, (8,9)). In previous work, we discovered that the traditional phosphomimetics are incapable of replacing or approximating the functionality of

phosphorylated Ser and Thr at the activation sites of Akt1 and that alanine substitutions at these sites also disable the enzyme (8). The significance of our approach is the ability to generate pAkt1 and ppAkt1 variants without the need for amino acid substitutions with phosphomimetics.

Our previous studies on pAkt1 and ppAkt1 variants focused on their differential activity with a single substrate peptide derived from GSK-3 β (8). Here we quantified the substrate preference for phosphorylated Akt1 variants using a synthetic peptide library of known Akt1 substrates. We found that the deletion of the PH domain abolishes its auto-inhibitory activity and leads to more soluble enzyme (9) appropriate for larger scale screening studies presented here.

Currently “known” Akt1 substrates have been identified and in some cases validated using multiple different experimental approaches, including *in vitro* biochemical (22) and cell-based activity assays (23), proteomic analysis (24), as well as immunoblotting (25) in cells (26) and animals (27).

Our study allowed the evaluation of these distinctively reported substrates using one systematic platform to quantify Akt1 kinase activity. The doubly phosphorylated Akt1 was capable of phosphorylating most, but not all, of the substrate peptides in the library. The ppAkt1 enzyme showed significantly above background activity with 95% of the peptides tested (Figure 4.1A). In our activity assays, the initial reaction velocity varied over an 80-fold range; ppAkt1 produced between 0.1-8.0 pmol/min of phosphorylated peptide.

Overall the level of phosphorylation observed was markedly lower with pAkt1^{S473} compared to pAkt1^{T308} and the ppAkt1^{T308, S473} (Figure 4.1). Compared to the singly phosphorylated variants (Figure 4.1BC), ppAkt1^{T308, S473} usually displayed more activity and was active over the widest range of substrate peptides.

Indeed, we observed a clear trend in activity over the library with pAkt1^{S473} showing the most restricted substrate range (82% of the library) compared to the variants phosphorylated at Thr308 (5% of the library) and ppAkt1 (8 % of the library).

In comparing singly and doubly phosphorylated Akt1, it is clear that the three variants have distinct preferences for particular peptides. The highest active substrates for pAkt1^{S473} variant were completely orthogonal to or distinct from the most active substrates with pAkt1^{T308} or ppAkt1^{T308, S473} (Figure 4.2).

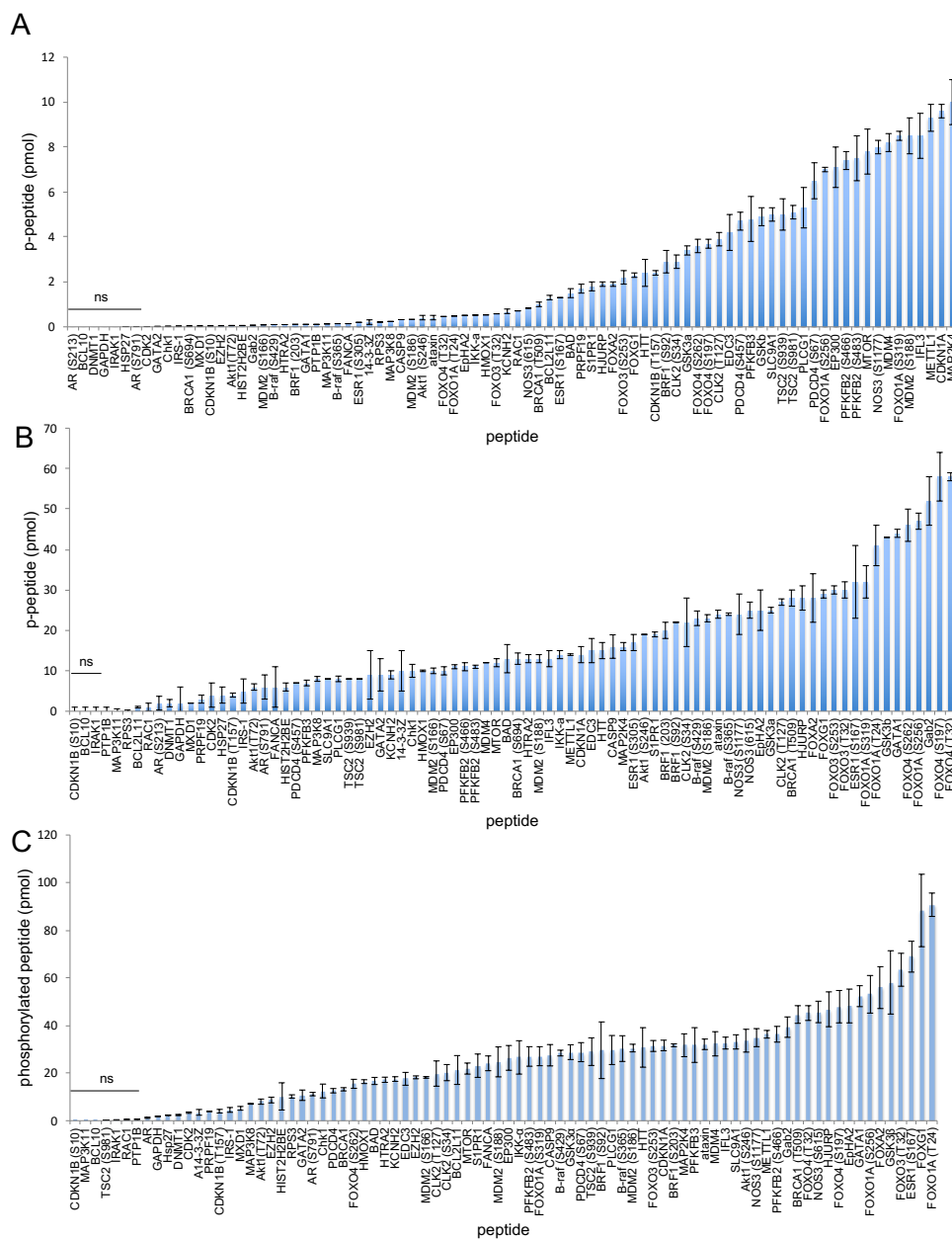


Figure 4.1 Phosphorylation of known substrate peptides by Akt1 variants. The activity (pmol of phosphorylated peptide following a 10 minute reaction) of (A) pAkt1^{S473} (B) pAkt1^{T308} (C) and ppAkt1^{T308,S473} variants with each of the 84 different “known” Akt1 substrate derived peptides. Gene names for each substrate are provided on the horizontal axis. For substrates with multiple phosphorylation sites, separate peptides were synthesized and are indicated by the phosphorylation site residue number in parentheses. All experiments were performed in triplicate; error bars represent ± 1 SD. A minority of the peptides did not support activity that was significantly above background (ns).

The pAkt1^{S473} variant was most active with substrates (Figure 4.2A) that were completely distinct from those observed for pAkt1^{T308} variant. To visualize these distinct substrate preferences, we generated a Search Tool for Retrieval of Interacting Genes/Protein (STRING) diagram (28), including nodes representing all 84 substrates in the library. Overlaid on the STRING diagram we highlighted the most (>50% activity) and least (< 10% activity) active substrates associated with each of the Akt1 phospho-forms (Figure 4.2).

The Fork head box O (FOXO) family of transcription factors are well-established substrates of Akt1 (25,29). The FOXO family transcription factors were among the most active substrates with Akt1 phosphorylated only at Thr308. Indeed, we found that the FOXO target peptides were among the highest activity for both Akt1 variants phosphorylated at Thr308, pAkt1^{T308} and ppAkt1^{T308,S473} (Figure 4.2B, 4.2C). We also observed that while FOXO1 was among the highest active peptide substrates with pAkt1^{S473}, the enzyme singly phosphorylated at S473 displayed low activity with peptides derived from other FOXO family members.

Certain substrate peptides, *e.g.*, including FOXOs and TSC2, carried more than one phosphorylation site, which we represented with distinct synthetic peptides. Each Akt1 phospho-variant showed different preferences for the multiple sites in these substrates. For example, with pAkt1^{S473}, Ser256 and Ser319 of FOXO1 showed relatively high activity, but this enzyme showed low activity with a peptide derived from FOXO1 Thr24. In contrast, for both pAkt1^{T308} and ppAkt1^{T308,S473} FOXO1 Ser319 was the least preferred compared to Ser256 and Thr24.

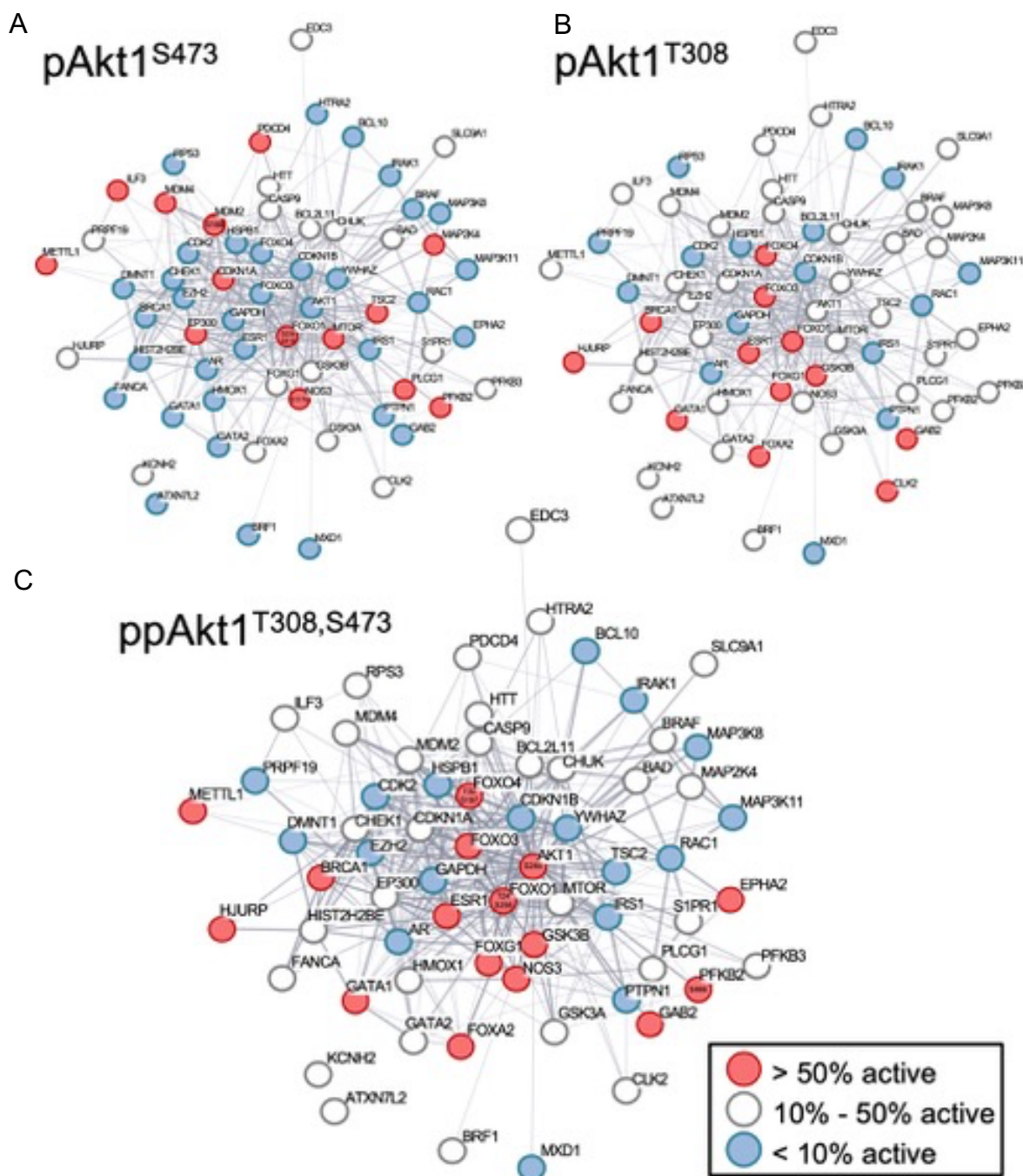


Figure 4.2 String diagrams showing the interactions among highly active peptide substrates of Akt1 variants. (A) Highly active phosphorylated substrates of pAkt1^{S473} shared a unique profile, which was not shared by the other two variants (B) pAkt1^{T308} variant phosphorylated substrates showed a similar profile to the doubly phosphorylated variant. (C) Highly active phosphorylated substrates of ppAkt1^{T308,S473} included most of the FOXO transcription factors.

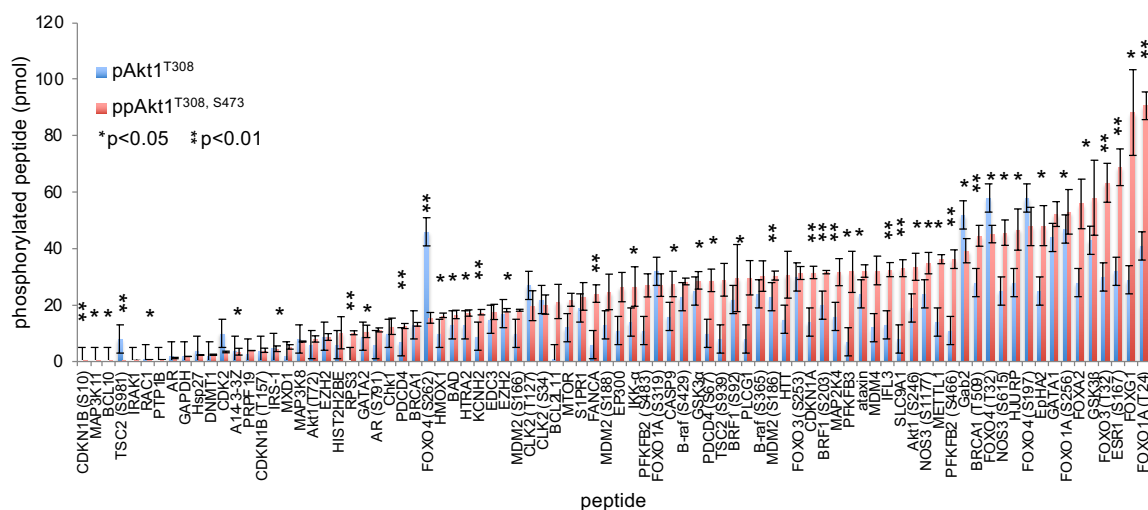


Figure 4.3 Comparison of substrate preferences of ppAkt1^{T308,S473} with pAkt1^{T308}.

Red bars represent the substrate peptides arranged in increasing order of ppAkt1^{T308,S473} activity. The corresponding pAkt1^{T308} phosphorylated substrates are shown in blue. Gene names of each substrates where the peptides synthesis is based on are given in X axis. Phosphorylation sites are given within brackets for peptides carrying more than one phosphorylation site. Error bars represent ± 1 SD.

To better understand the impact of Ser473 phosphorylation on the activity of the doubly phosphorylated Akt1, we plotted kinase activity data of pAkt1^{T308} and ppAkt1^{T308,S473} together (Figure 4.3). We analyzed these comparative data for statistically significant differences. Interestingly, we observed that additional phosphorylation at Ser473 significantly increased Akt1 activity for many (90%) but not all Akt1 substrates.

In the context of Akt1 phosphorylated at Thr308, we found that addition of phosphorylation at Ser473 actually decreased the activity of several competent Akt1 substrates. Phosphorylation at Ser473 reduces activity for all four peptides derived from distinct sites in the FOXO4 transcription factor, two distinct sites in

FOXO1 and FOXO3 as well as sites in cyclin dependent kinase 2 (CDK2), Grb2 associated binding protein (Gab2), CDC like kinase 2 (CLK2), and TSC complex subunit 2 (TSC2). Together the data show that both regulatory phosphorylation sites in Akt1 have a significant and unexpectedly complex impact on substrate selectivity over a library of known Akt1 substrates.

4.4.2 Phosphorylation dependent changes in the Akt1 target motif. Based on the activity data in Figure 4.1, we computed sequence logos for each Akt1 phosphoform. The logos (Figure 4.4) represent the key residues of importance in phosphorylation based on the activity level recorded for each substrate peptide (Figure 4.1). The sequence alignments used to generate the logos was populated with each of the 84 peptides such that the number of occurrences of each peptide in the alignment was linearly related to enzyme activity recorded in Figure 4.1.

The importance of conserved consensus sequence residues R₋₅ and R₋₃ for Akt1 substrates (R₋₅-X-R₋₃-X-X-S/T) was observed with all three variants. As reported previously (12), the presence of a large hydrophobic residue following the phosphorylation site (+1 position) was observed among the peptides with all three Akt1 variants. Pro occupied the +2 position at the highest frequency in pAkt1^{T308} and ppAkt1^{T308, S473} variants, and the most active substrates tend to have Pro at +2. The +2 position favors amino acids capable of producing tight turns in protein structures. According to Obata and others Gly, Ser, Asn and Thr were also found to be favorable at this position (13). Peptide substrates showing high activity with pAkt1^{S473} variant were dominated by those peptides with a Ser rather than a Thr at the phosphorylation site. For pAkt1^{T308} and ppAkt1^{T308, S473}, highly active

peptides were approximately equally likely to have Ser or Thr at the phosphorylation site.

4.4.3 Alanine scanning of Akt1 target peptides. To provide additional data regarding essential amino acid residues for kinase activity with each phosphorylated Akt1 variant, we next synthesized peptides containing alanine substitutions at each position in the consensus motif (Figure 4.4). The consensus motif was derived from the activity data on the known substrates (Figure 4.1). For these studies, we chose a peptide derived from GSK-3 β . Among known substrates, GSK-3 β shows significant activity (>50% maximal) with each Akt1 phospho-form (Figure 4.1); peptides derived from GSK-3 β are commonly used to assess Akt activity (8,12).

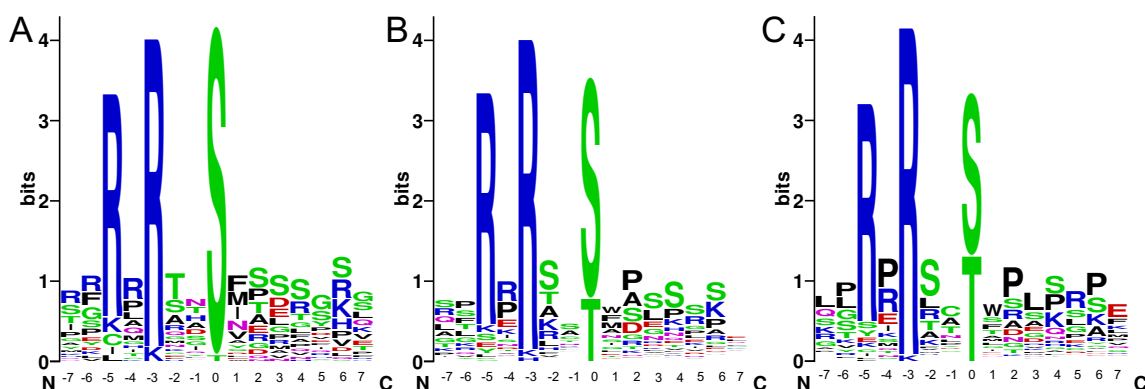


Figure 4.4 Sequence logos representing the amino acid preferences adjacent to the Akt1 target motif. Sequence logos of (A) pAkt1^{S473} phosphorylated (B) pAkt1^{T308} phosphorylated and (C) ppAkt1^{T308, S473} are based on enzyme activity data in Figure 4.1 as described in methods. The phosphorylation site is denoted by the 0 position. Sequence logos were created using the WebLogo server (18).

The pAkt1^{T308} and ppAkt1^{T308,S473} variants showed a similar response to each of the alanine substituted peptides, whereas the pAkt1^{S473} response was distinct (Figure 4.5). For each Akt1 variant, alanine mutation at the phosphorylation site abolished the enzyme activity to a level indistinguishable from background. R-3 was essential for all three variants to be active. Arginine at -5 was important for pAkt1^{S473} activity, where an alanine substitution lead to only 0.4 pmol/min activity, a 9-fold reduction. pAkt1^{T308} did not show a significant ($p < 0.05$) difference with the control at arginine -5, where in contrast, ppAkt1^{T308, S473} showed a 0.8-fold increased activity in comparison to the control peptide. Attesting to the robust activity of the ppAkt1 enzyme according to our previous studies, ppAkt1 displays higher activity for each Ala substitute peptide compared to the singly phosphorylated enzymes.

4.4.4 Determination of high-resolution Akt1 target specificity. Our analysis of known substrates revealed several new facets of Akt1 enzymology, however, these data are biased by our current and perhaps limited knowledge of Akt1 substrate selectivity on 84 previously identified Akt1 substrates. We, therefore, designed a far larger, unbiased oriented library of peptides to test the most preferred amino acid residue at each site of the Akt1 substrate consensus motif. We synthesized 128 different pools of peptide sub-libraries (see Methods) to cover the complete library of 10^{11} peptides. Using each phospho-form of Akt1, we conducted kinase assays with each of the substrate sub-libraries (Figures S4.1, S4.2, S4.3). In each sub-library a single amino acid is held constant for a single position in the consensus, while all other variable positions are allowed to cover the complete sequence space with the exception of Cys, Thr and Ser. The Akt1 activity for singly and doubly phosphorylated Akt1 variants over each peptide sub-library pools are shown in

terms of activity and overlaid as a heat map indicating relative changes in activity (Figure 4.6).

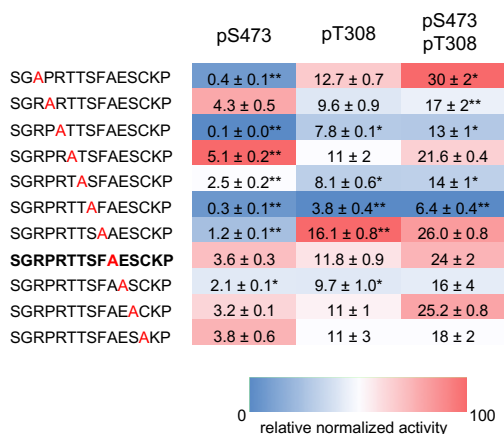


Figure 4.5 Alanine scanning of each residue in the GSK-3 β derived peptide. Corresponding peptide sequences are shown adjacent to the activities for each indicated Akt1 phospho-form. The ‘wild-type’ and unmutated control peptide is bolded. Akt1 activity is presented as absolute activity values (pmol p-peptide/min). Statistically significant changes in comparison to the ‘wild-type’ unmutated peptide control are indicated as $p < 0.05$ (*) or $p < 0.01$ (**).

To visualize the amino acid preferences and anti-determinants we converted the activity data into PSSMs (see Methods). Using Seq2Logo (19) we generated sequence logos representing the OPAL data (Figure 4.7). The results clearly demonstrate that the substrate selectivity of pAkt1^{S473} is both more restricted and totally distinct from the profile we observed for the pAkt1^{T308} and ppAkt1^{T308, S473} enzymes. Both ppAkt1^{T308, S473} and pAkt1^{T308} preferred large hydrophobic residues (Phe, Tyr, Trp) at the +1 position in the target peptide. In contrast, pAkt1^{S473} prefers basic residues (Lys, Arg, His) at the +1 position. Ile at -1 and +2 positions were the least favorable for both pAkt1^{T308} and ppAkt1^{T308,S473} variants. Phe and Pro are least preferred at -1 and +2 positions for pAkt1^{S473}. The least preferred amino acid cluster at position +1 include Pro, Asp, Glu and Gly for

pAkt1^{T308} and ppAkt1^{T308, S473}. Thus the least favoured cluster for pAkt1^{S473} include Asp, Asn, Gln and Pro. Finally, we note that the specific amino acids most preferred at each position is distinct between each Akt1 phospho-form (Figure 4.6, 4.7) in concordance with the unique substrate selective profiles we recorded over the library of known substrates (Figure 4.1).

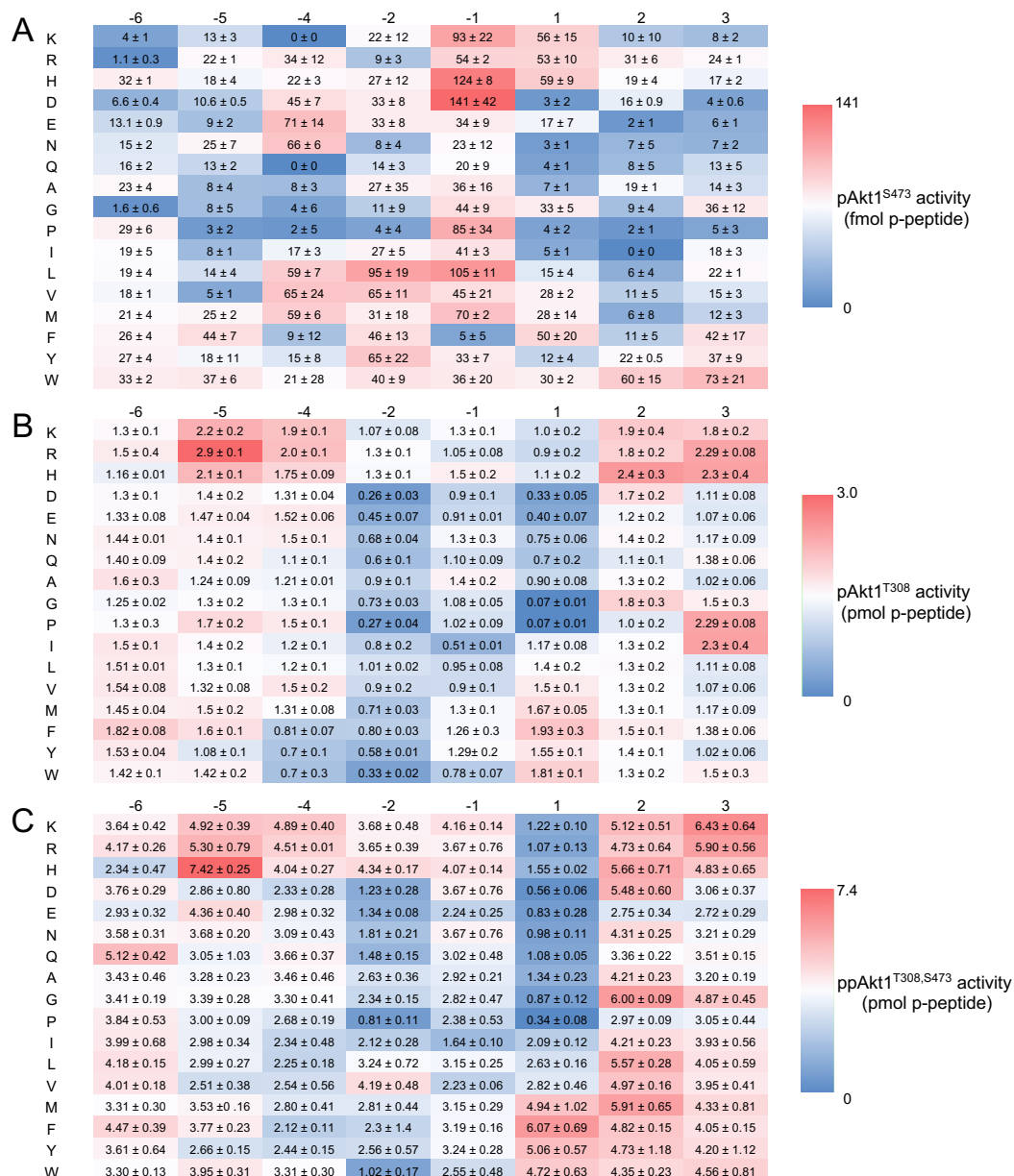


Figure 4.6 Oriented peptide array library (OPAL) activity assays. Each cell in the above matrix represents activity (amount of pmol or fmol phosphorylated peptide/min) with the indicated sub-library in reactions catalyzed by pAkt1^{S473} (A), pAkt1^{T308} (B) or ppAkt1^{T308,S473} (C). The red to blue scale of relative activity corresponds to the preferences of amino acids at each indicated position substrate phosphorylation motif of Akt1. The 0 position is the phosphorylation site which was fixed to a Ser residue and was not shown; -3 position also not shown was fixed to an Arg.

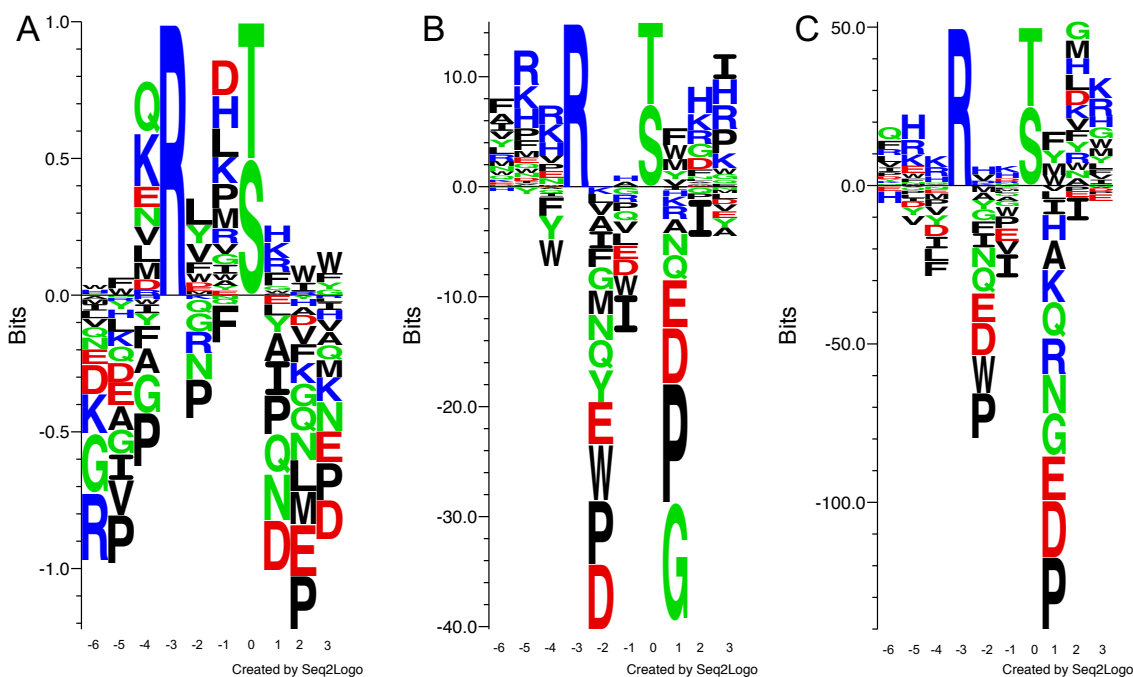


Figure 4.7 Sequence logos generated from the OPAL data. Sequence logos of (A) pAkt1^{S473} (B) pAkt1^{T308} and (C) ppAkt1^{T308,S473} are based on the conversion of activity values from Figure 4.6 into a PSSM. Seq2Logo was used to convert the PSSMs to amino acid preferences as represented by sequence information bits.

Next, we used information from the OPAL experiments to score the known human phospho-proteome to identify new Akt1 substrates. We then converted the Akt1 activity data into PSSMs based on calculating z-scores (see Methods) as before (14). The known human phospho-proteome was scored based on the amino acid preferences at each position in the z-score PSSM (Table S4.1). The scoring of peptides was performed for data based on each phospho-form of Akt1. We then selected the top scoring substrates (normalized sum of z-scores > 3). We included all of these high confidence hits in a STRING diagram to identify potential protein interactions and functional clusters among the known and predicted substrates (Figure 4.8, Table S4.1).

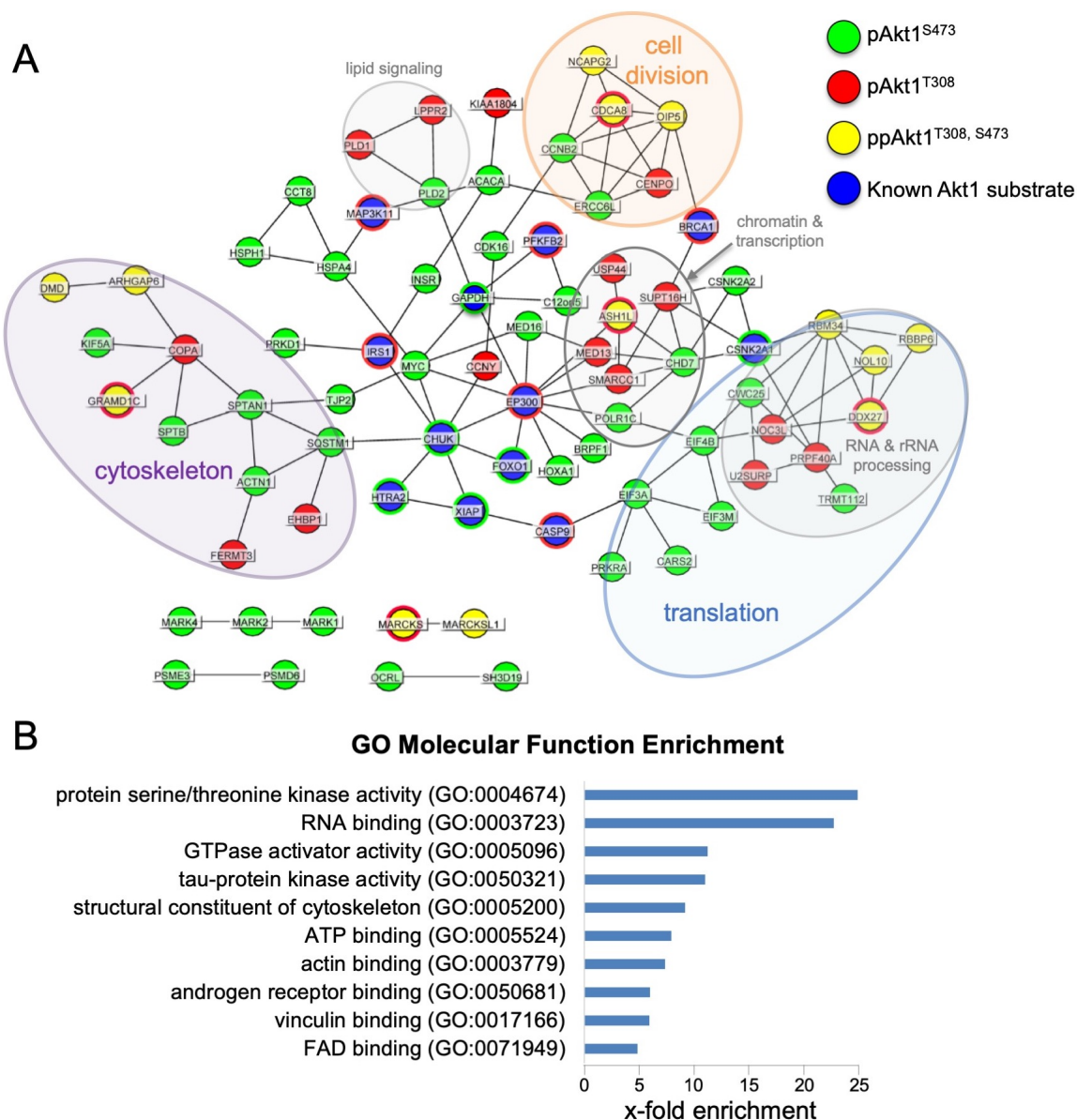


Figure 4.8 Interactions among highly active known and novel substrates of Akt1 variants derived from OPAL screen. (A) String diagram of interactions and (B) GO enrichment analysis conducted using Gene Ontology (30) for the string diagram are shown. Red, yellow and green colors are assigned for novel substrates identified for pAkt1^{T308}, pAkt1^{S473} and ppAkt1^{T308,S473} variants, respectively. Blue color denoted known substrates for ppAkt1^{T308,S473}. Novel clusters of substrates involved in cell division, (orange), translation (blue), cytoskeleton (purple), RNA processing, lipid signaling, chromatin and processing (three gray circles) are encircled.

The search identified both known and unknown Akt1 substrates among the high confidence hits. Interestingly, the prediction suggested the presence of Akt1 substrate in pathways not previously known to be regulated by Akt1. These include clusters of proteins that participate in translation, transcription, cell division, cytoskeletal organization, and lipid signaling (Figure 4.8A). The putative Akt1 substrates are significantly enriched in gene ontology (GO) (30) molecular functions related to protein kinases, RNA binding, and cytoskeletal organization according to GO enrichment analysis (Figure 4.8B). Putative Akt1 substrates include centromeric proteins involved in cellular mitosis. Chromosome associated protein G, boreilin, opa protein 5 and centromere protein O were the identified strong candidates for activity with each phospho-form of Akt1. Another novel cluster of putative Akt1 targets includes nucleolar proteins involved in ribosomal RNA processing. RNA binding motif protein 34, nucleolar protein 10, E3 ubiquitin ligase (RB binding protein 6 ubiquitin ligase) and RNA helicase were among this functional cluster.

4.4.5 Akt1 variants differentially phosphorylate NIH 3T3 cellular proteins. We next measured the overall activity of each Akt1 phospho-form using the soluble cellular proteome derived from mammalian cells. We incubated the Akt1 variants with NIH 3T3 cell extracts to quantify how Akt1 phosphorylation status impacts its kinase activity on natural and full-length protein substrates. The reactions and controls contained γ -[³²P]-ATP. The phosphorylated cell extracts were separated on SDS-PAGE gels and exposed to a phosphor-imaging screen to quantify radio-labeled proteins. Significant activity was observed with the ppAkt1^{T308,S473} and pAkt1^{T308} variants (Figure 4.9). In agreement with the peptide experiments, pAkt1^{S473} demonstrated low activity with cellular extracts. The enzyme with a

single phosphorylation at Thr308 nearly reaches the maximal activity observed with the ppAkt1^{T308, S473} enzyme, validating our previous findings in COS7 cells (Chapter 2, (8)).

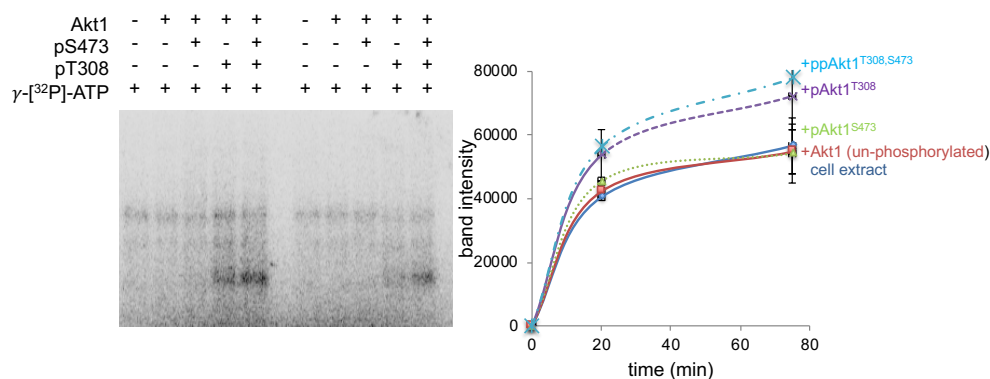


Figure 4.9 NIH 3T3 cell lysates were incubated with the indicated Akt1 phosphorylated variants and γ -[³²P]-ATP. At specified time point, the reactions were quenched and (A) visualized 10% SDS-PAGE gel and exposed to phosphor-imaging screen. (B) The radiolabeled protein intensities were quantified based on duplicate measurements.

4.4.6 Validation of a novel Akt1 substrate. One of the novel potential substrates of Akt1 derived from the OPAL scan was the nucleotidyltransferase Gld2. Gld2 adenylates miRNAs and mRNAs to increase their stability, which impacts gene expression (31). We used doubly phosphorylated Akt1 to test Akt1 activity on recombinantly purified Gld2 *in vitro*. In comparison to CK2- α , which showed no significant activity, Akt1 and protein kinase A (PKA) efficiently phosphorylated Gld2 (Figure 4.10, S4.4). These data were including in our recent publication (15). Mass spectrometry results revealed that ppAkt1 phosphorylated Gld2 specifically at Ser116 (Figure 4.11) (15). We also found that the Gld2 Ser116Ala mutant was not a substrate for Akt1 (15). The data demonstrate that Akt1 site-specifically phosphorylates Gld2 at Ser116. Strikingly, as found by my colleagues,

phosphorylation at this site nearly abolishes Gld2's ability to catalyze nucleotide addition to microRNAs and mRNAs (15). The data suggest Akt1-dependent phosphorylation may be a powerful mechanism to regulate mRNA and miRNA metabolism.

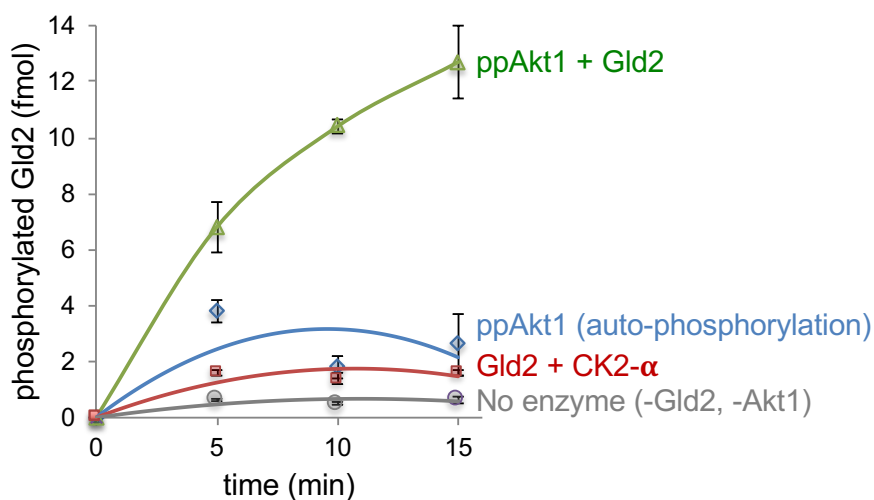


Figure 4.10 Kinase activity assays of Akt1 and CK2- α with Gld2 protein substrate. The enzyme activity was plotted over time. Akt1 but not CK2- α showed significantly increased reaction velocity with Gld2 as a substrate. All reactions were performed in triplicate (see Figure S4.4).

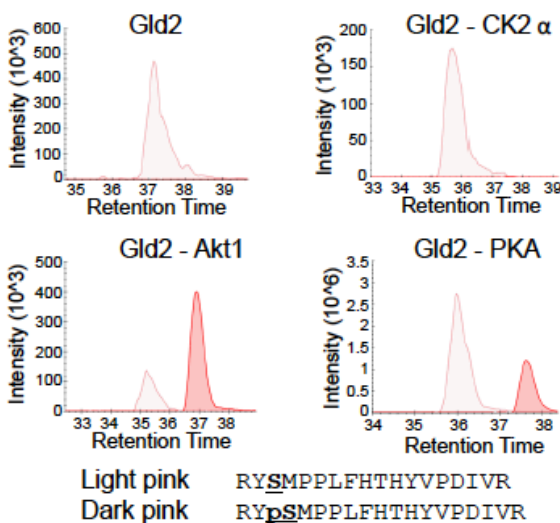


Figure 4.11 Akt1 and PKA phosphorylate Gld2 at S116. Mass spectra of unphosphorylated Gld2 after incubation with CK2- α and phosphorylated after incubation with ppAkt1^{T308,S473} or PKA. Unphosphorylated peptide is indicated by the light pink peak and the phosphorylated peptide by the dark peak. Position 116 is bolded and underlined (15).

4.5 Discussion

We recently developed a new method to produce Akt1 with programmed phosphorylation at either or both key regulatory sites (8). This breakthrough technology enabled resolution of the contribution of each phospho-site to the activation (8) or inhibition (9) of Akt1. Because this approach was only recently developed, there are still very few studies that report on the function or impact of each phosphorylation site on Akt1 substrate specificity (32). Investigations into the substrate specificity of Akt have focused on characterizing the isozyme specific substrate preferences that differ between Akt1, Akt2, and Akt3 (1,33). Indeed, the previous lack of tools to isolate the function of each Akt1 phospho-form inhibited investigations to characterize the impact of each phosphorylation site on substrate specificity. Our approach to produce singly and doubly phosphorylated Akt1

variants using genetic code expansion with or without co-expression of the upstream kinase PDK1 has to overcome this technological barrier (8,9).

4.5.1 Phosphorylation dependent substrate specificity of Akt1. In our previous studies (8), we found that phosphorylation at Thr308 was necessary and sufficient for maximal Akt1 activity in mammalian cells. The addition or ablation of Ser473 had some impact on the activity of the kinase in the test tube but did not influence phosphorylation of an Akt-specific live cell fluorescent reporter (8). These observations lead to our hypothesis that phosphorylation of Ser473 may function to tune rather than activate the Akt1.

Using peptide library approaches, we mapped the substrate preferences for each key phospho-form of Akt1. The data presented here revealed that Ser473 phosphorylation does indeed impact substrate specificity. More generally, we found that the phosphorylation status of Akt1 has a global impact on substrate preference as determined by kinase activity.

A multitude of studies (34,35), including our own (8) have presented evidence indicating that Akt1 activity increases monotonically with $pAkt1^{S473} < pAkt1^{T308} < ppAkt1$. Based on all of our assays, it remains clear that the $pAkt1^{S473}$ enzyme is far less active than the other two. Of the 84 known substrates we tested, this rule only holds completely true for half of the peptides (49%). When comparing $pAkt1^{T308}$ with $ppAkt1^{T308, S473}$, we found that many substrates (48%) show the same level of activity with $pAkt1^{T308}$ and $ppAkt1$, while a minority (3%) are actually more active with the singly phosphorylated $pAkt1^{T308}$ compared with the doubly phosphorylated enzyme.

Our current study further revealed that when only Ser473 is phosphorylated the substrate preference profile is distinct in comparison to Thr308 phosphorylation. This observation was supported by data generated from 'known' substrates as well as the much larger data set generated using the OPAL approach. Several highest active substrates for pAkt^{S473}, including MDM2 (Ser188), MDM4, EP300 and eNOS (S1177), are members of the p53 mediated apoptotic pathway (36) (Figure 4.2A). Phosphorylation of MDM family members and EP300 by Akt1 leads to p53 ubiquitination and degradation and promotes cell survival (36). Akt phosphorylates eNOS, which enhances its activity to produce nitric oxide (NO) (37). Acting as a signaling molecule, NO promotes angiogenesis in tumors (38), however, NO plays a dual role in cancer cell survival (37). Under oxidative stress induced cellular environment, NO promotes accumulation of p53 in the nucleus leading to apoptosis (38). p53 activates phosphatase and tensin homolog (PTEN) which is the phosphatase responsible in dephosphorylating the Akt binding lipid second messenger phosphatidylinositol-4, 5-triphosphate (PIP3) which suppresses Akt activation (39).

The significance of Ser473 phosphorylation in differential substrate phosphorylation had been highlighted anecdotally. Jacinto et al. (2006) found disruption of Ser473 phosphorylation resulted from genetic ablation of *sin1*. Sin1 is a component of the TORC2 complex, which phosphorylates Ser473. In mice or mammalian cells, downstream Akt1 substrate phosphorylation events were subsequently down regulated in a subset of Akt1 substrates, which included FOXO1 and FOXO3 (32). Indeed, although many substrates do not show different activity with ppAkt1^{T308,S473} versus pAkt^{T308}, all of the FOXO peptides except the N-terminal phosphorylation sites T24 and T32 of FOXO1 and FOXO3, respectively showed loss of activity in the absence of pS473 phosphorylation (Figure 4.2, 4.3).

The next example is related to endoplasmic reticulum stress in mammalian cells. Upon ER stress, the ER chaperone protein GRP78 is down regulated and this leads to increased phosphorylation of Akt1 at Ser473 but not Thr308 in JEG3 cells. As a result, increased Akt1-dependent phosphorylation of HDM2 (pSer166), FOXO1 (pSer319) and GSK3- β (pSer9) were observed by Western blotting (40). In our study, FOXO1, FOXO3 and GSK3- β demonstrated higher activity with both ppAkt1^{T308, S473} and pAkt1^{T308} variants in comparison to pAkt1^{S473} (Figure 4.2, Figure 4.3).

Our data clearly indicate that ppAkt1 is normally (but not always) more active and has a broader substrate profile than either of the singly phosphorylated enzymes. In alignment with the essentiality of pThr308 in the activation of Akt1 *in vitro* and in cells (8,35), we found that the two variants phosphorylated at T308 showed greatest similarities in substrate preferences in comparison to the pAkt1^{S473} enzyme. Manning and Cantley (2007) observed that Ser473 was not essential for phosphorylating the non-N-terminal phospho-sites of the FOXO transcription factors, TSC2, or GSK-3 β (10). These observations are in close agreement with and supportive of our findings. The ppAkt1^{T308, S473} enzyme showed high activity with FOXO1 C-terminal phosphorylation sites (S319, S256), TSC2 and GSK-3 β (Figure 4.2, Figure 4.3). For pAkt1^{T308}, all three FOXO1 phospho-sites ranked among the highest active substrates, suggesting that Ser473 is not a critical factor in FOXO1 phosphorylation. In the clinic, phosphorylation of Thr308 was associated with poor cell survival with non-small cell lung cancer (41) and acute myeloid leukemia (42). According to our data here (8), we suggest that phosphorylation of Thr308 alone may be capable of switching-on downstream signaling to promote cell survival by inhibiting the FOXO group of transcription factors. High level of

phosphorylation of Thr308 could contribute to the poor survival rate of lung cancer (38) and leukemic patients (39) with tumors characterized by hyper-active Akt1.

4.5.2 Phosphorylation dependent changes in the Akt1 substrate motif. The molecular basis for kinase substrate selectivity depends on the residues neighboring the phosphorylation site (13). Yet it is important to understand that not all substrates identified for Akt isozymes include the same consensus motif (10). In the context of the cell, even with an optimal substrate according to the consensus motif requirements, phosphorylation by Akt1 will depend on multiple factors, including accessibility, cellular localization, and phosphatase activity (11).

Our data obtained from the OPAL study for ppAkt1^{T308, S473} was in accord with but extended beyond previously reported results (12,13). We fixed the -3 position to an Arg since the essentiality of Arg at -3 is well established (13). According to our results, all Akt1 phospho-forms showed a preference for Arg at position -5. ppAkt1^{T308, S473} and pAkt1^{T308} showed preference for hydrophobic amino acids for position +1. These amino acid preferences are shared by other AGC family kinases including protein kinase C. Obata *et al.* (2000) used degenerative peptide library studies to determine the most relevant substrate motif for active Akt1 (13). Unlike in our study, active Akt1 was derived from SF-9 insect cells and the phosphorylation status of Akt1 enzyme was not precisely defined. The authors reported the amino acid preferences derived from a set of known substrates and a degenerative peptide library. In comparison with these sequence preferences, we observed a strong compatibility with the predicted residues of our OPAL results with positions -5 and +1 with pAkt1^{T308} and ppAkt1^{T308, S473}. The most preferred amino acid at the -5 position was Arg and preferred amino acids at +1 are large hydrophobic residues. Both pAkt1^{T308} and ppAkt1^{T308, S473} enzyme variants showed

preference for Phe at position -6 as observed in the generative library predictions of Obata *et al.* Lys and Arg were predicted to be preferred for position -4 according to our OPAL results. According to their predictions position -4 could be any amino acid. In contrast to the study by Obata *et al.*, we observed no selection for Ser and Thr at +2 with our OPAL data. Thus, Ser and Thr at +2 were the favorable amino acids according to our known library data.

4.5.3 Putative substrates derived from OPAL screening. OPAL data lead to a predicted consensus sequence for all three Akt1 variants (Figure 4.7). These predicted consensus sequences were matched against the human proteome to identify potential novel substrates. Zn finger domain carrying proteins (numbered 211, 251, 484, 668, 773, 792, 835) were abundant among the most likely putative Akt1 substrates. The functionality of Zn fingers includes DNA and/or RNA binding, protein-protein interactions and membrane binding. Indeed, most transcription factors carry Zn finger (43). Since Akt is well known for its phosphorylation-based inactivation of transcription factors like FOXO family proteins, Zn fingers containing transcription factors are interesting potential targets of Akt1.

Validation of predictions based on our biochemical data using cellular or ultimately animal models is warranted in future studies. Our initial investigations towards such validations revealed that the *E. coli* produced Akt1 variants can phosphorylate cellular substrates (Figure 4.9). Next, based on the OPAL predictions we found that Akt1 may be involved in regulating RNA metabolism (Figure 4.8). Gld2, which is a nucleotidyltransferase that regulates RNA stability was one of the predicted targets of ppAkt1^{T308,S473}. Gld2 has been implicated in the

stabilization and maturation of the tumour suppressor microRNAs miR-122 and let-7 (44,45). We found Gld2 was specifically phosphorylated by Akt1 at Ser116, which abolished Gld2 activity in 3'-terminal addition of adenine to miRNA and mRNA substrates (15). As hyperactivity of Akt1 is common in many cancers (46), our results suggest that phosphorylation of Gld2 by Akt1 would be beneficial for cancer cells in order to decrease the levels of miRNAs that act as tumour suppressors such as miR-122 and let-7 (15). Our data, thus, suggest a novel role of Akt1 activity on a miRNA editing enzyme and provides the first link between tumor suppressor miRNA regulation and oncogenic signaling.

4.6 Conclusion

The phosphorylation status of Akt is a commonly used biomarker for human cancer (41). Here we found that Akt1 phosphorylation status has a global impact on substrate selectivity. Fascinatingly, ppAkt1^{T308,S473} showed higher activity with most but not all substrates compared to Akt1 singly phosphorylated at Thr308. Indeed, a select pool of substrates was highly active with pAkt1^{T308}. The pAkt1^{S473} variant had a distinct substrate preference profile compared to Akt1 variants phosphorylated at Thr308. The role of Ser473 phosphorylation appears to involve global tuning of substrate selectivity rather than in truly activating the kinase. Indeed, it remains unknown whether Akt1 with only Ser473 phosphorylated is active in the cell (8,35). Our findings have major implications for the use of Akt1 phosphorylation status as a clinical diagnostic (41,47) or a marker for cancer biology (46).

4.7 References

1. Gonzalez, E., and McGraw, T. E. (2009) The Akt kinases: isoform specificity in metabolism and cancer. *Cell Cycle* **8**, 2502-2508
2. Martini, M., De Santis, M. C., Braccini, L., Gulluni, F., and Hirsch, E. (2014) PI3K/AKT signaling pathway and cancer: an updated review. *Ann Med* **46**, 372-383
3. Ma, C. X., Sanchez, C., Gao, F., Crowder, R., Naughton, M., Pluard, T., Creekmore, A., Guo, Z., Hoog, J., Lockhart, A. C., Doyle, A., Erlichman, C., and Ellis, M. J. (2016) A Phase I Study of the AKT Inhibitor MK-2206 in Combination with Hormonal Therapy in Postmenopausal Women with Estrogen Receptor-Positive Metastatic Breast Cancer. *Clin Cancer Res* **22**, 2650-2658
4. Dumble, M., Crouthamel, M. C., Zhang, S. Y., Schaber, M., Levy, D., Robell, K., Liu, Q., Figueroa, D. J., Minthorn, E. A., Seefeld, M. A., Rouse, M. B., Rabindran, S. K., Heerding, D. A., and Kumar, R. (2014) Discovery of novel AKT inhibitors with enhanced anti-tumor effects in combination with the MEK inhibitor. *PLoS One* **9**, e100880
5. Zhang, X., Tang, N., Hadden, T. J., and Rishi, A. K. (2011) Akt, FoxO and regulation of apoptosis. *Biochim Biophys Acta* **1813**, 1978-1986
6. Hornbeck, P. V., Zhang, B., Murray, B., Kornhauser, J. M., Latham, V., and Skrzypek, E. (2015) PhosphoSitePlus, 2014: mutations, PTMs and recalibrations. *Nucleic Acids Res* **43**, D512-520
7. Hers, I., Vincent, E. E., and Tavaré, J. M. (2011) Akt signalling in health and disease. *Cell Signal* **23**, 1515-1527
8. Balasuriya, N., Kunkel, M. T., Liu, X., Biggar, K. K., Li, S. S., Newton, A. C., and O'Donoghue, P. (2018) Genetic code expansion and live cell imaging reveal that Thr308 phosphorylation is irreplaceable and sufficient for Akt1 activity. *J Biol Chem*
9. Balasuriya, N., McKenna, M., Liu, X., Li, S. S. C., and O'Donoghue, P. (2018) Phosphorylation-Dependent Inhibition of Akt1. *Genes (Basel)* **9**
10. Manning, B. D., and Cantley, L. C. (2007) AKT/PKB signaling: navigating downstream. *Cell* **129**, 1261-1274
11. Manning, B. D., and Toker, A. (2017) AKT/PKB Signaling: Navigating the Network. *Cell* **169**, 381-405
12. Alessi, D. R., Caudwell, F. B., Andjelkovic, M., Hemmings, B. A., and Cohen, P. (1996) Molecular basis for the substrate specificity of protein kinase B; comparison with MAPKAP kinase-1 and p70 S6 kinase. *FEBS Lett* **399**, 333-338
13. Obata, T., Yaffe, M. B., Leparac, G. G., Piro, E. T., Maegawa, H., Kashiwagi, A., Kikkawa, R., and Cantley, L. C. (2000) Peptide and protein library screening defines optimal substrate motifs for AKT/PKB. *J Biol Chem* **275**, 36108-36115
14. Rodriguez, M., Li, S. S., Harper, J. W., and Songyang, Z. (2004) An oriented peptide array library (OPAL) strategy to study protein-protein interactions. *J Biol Chem* **279**, 8802-8807

15. Chung, C., Balasuriya, N., Manni, E., Liu, X., Li, S.-C., O'Donoghue, P., and Heinemann, I. U. (2019) Gld2 activity is regulated by phosphorylation in the N-terminal domain. *RNA Biol*, DOI:10.1080/15476286.15472019.11608754
16. George, S., Aguirre, J. D., Spratt, D. E., Bi, Y., Jeffery, M., Shaw, G. S., and O'Donoghue, P. (2016) Generation of phospho-ubiquitin variants by orthogonal translation reveals codon skipping. *FEBS Lett* **590**, 1530-1542
17. Wei, R., Kaneko, T., Liu, X., Liu, H., Li, L., Voss, C., Liu, E., He, N., and Li, S. S. C. (2017) Interactome mapping uncovers a general role for Numb in protein kinase regulation. *Mol Cell Proteomics*
18. Crooks, G. E., Hon, G., Chandonia, J. M., and Brenner, S. E. (2004) WebLogo: a sequence logo generator. *Genome Res* **14**, 1188-1190
19. Thomsen, M. C., and Nielsen, M. (2012) Seq2Logo: a method for construction and visualization of amino acid binding motifs and sequence profiles including sequence weighting, pseudo counts and two-sided representation of amino acid enrichment and depletion. *Nucleic Acids Res* **40**, W281-287
20. Obenaus, J. C., Cantley, L. C., and Yaffe, M. B. (2003) Scansite 2.0: Proteome-wide prediction of cell signaling interactions using short sequence motifs. *Nucleic Acids Res* **31**, 3635-3641
21. Huang, H., Li, L., Wu, C., Schibli, D., Colwill, K., Ma, S., Li, C., Roy, P., Ho, K., Songyang, Z., Pawson, T., Gao, Y., and Li, S. S. (2008) Defining the specificity space of the human SRC homology 2 domain. *Mol Cell Proteomics* **7**, 768-784
22. Bonni, A., Brunet, A., West, A. E., Datta, S. R., Takasu, M. A., and Greenberg, M. E. (1999) Cell survival promoted by the Ras-MAPK signaling pathway by transcription-dependent and -independent mechanisms. *Science* **286**, 1358-1362
23. Maiti, D., Bhattacharyya, A., and Basu, J. (2001) Lipoarabinomannan from *Mycobacterium tuberculosis* promotes macrophage survival by phosphorylating Bad through a phosphatidylinositol 3-kinase/Akt pathway. *J Biol Chem* **276**, 329-333
24. Michell, B. J., Harris, M. B., Chen, Z. P., Ju, H., Venema, V. J., Blackstone, M. A., Huang, W., Venema, R. C., and Kemp, B. E. (2002) Identification of regulatory sites of phosphorylation of the bovine endothelial nitric-oxide synthase at serine 617 and serine 635. *J Biol Chem* **277**, 42344-42351
25. Huang, H., Regan, K. M., Wang, F., Wang, D., Smith, D. I., van Deursen, J. M., and Tindall, D. J. (2005) Skp2 inhibits FOXO1 in tumor suppression through ubiquitin-mediated degradation. *Proc Natl Acad Sci U S A* **102**, 1649-1654
26. Guan, K. L., Figueroa, C., Brtva, T. R., Zhu, T., Taylor, J., Barber, T. D., and Vojtek, A. B. (2000) Negative regulation of the serine/threonine kinase B-Raf by Akt. *J Biol Chem* **275**, 27354-27359
27. Giamas, G., Hirner, H., Shoshiashvili, L., Grothey, A., Gessert, S., Kuhl, M., Henne-Bruns, D., Vorgias, C. E., and Knippschild, U. (2007) Phosphorylation of CK1delta: identification of Ser370 as the major phosphorylation site targeted by PKA in vitro and in vivo. *Biochem J* **406**, 389-398

28. Szklarczyk, D., Franceschini, A., Wyder, S., Forslund, K., Heller, D., Huerta-Cepas, J., Simonovic, M., Roth, A., Santos, A., Tsafou, K. P., Kuhn, M., Bork, P., Jensen, L. J., and von Mering, C. (2015) STRING v10: protein-protein interaction networks, integrated over the tree of life. *Nucleic Acids Res* **43**, D447-452
29. Rena, G., Guo, S., Cichy, S. C., Unterman, T. G., and Cohen, P. (1999) Phosphorylation of the transcription factor forkhead family member FKHR by protein kinase B. *J Biol Chem* **274**, 17179-17183
30. Mi, H., Huang, X., Muruganujan, A., Tang, H., Mills, C., Kang, D., and Thomas, P. D. (2017) PANTHER version 11: expanded annotation data from Gene Ontology and Reactome pathways, and data analysis tool enhancements. *Nucleic Acids Res* **45**, D183-D189
31. Chung, C. Z., Jo, D. H., and Heinemann, I. U. (2016) Nucleotide specificity of the human terminal nucleotidyltransferase Gld2 (TUT2). *Rna* **22**, 1239-1249
32. Jacinto, E., Facchinetti, V., Liu, D., Soto, N., Wei, S., Jung, S. Y., Huang, Q., Qin, J., and Su, B. (2006) SIN1/MIP1 maintains rictor-mTOR complex integrity and regulates Akt phosphorylation and substrate specificity. *Cell* **127**, 125-137
33. Lee, R. S., House, C. M., Cristiano, B. E., Hannan, R. D., Pearson, R. B., and Hannan, K. M. (2011) Relative Expression Levels Rather Than Specific Activity Plays the Major Role in Determining In Vivo AKT Isoform Substrate Specificity. *Enzyme research* **2011**, 720985
34. Alessi, D. R., Andjelkovic, M., Caudwell, B., Cron, P., Morrice, N., Cohen, P., and Hemmings, B. A. (1996) Mechanism of activation of protein kinase B by insulin and IGF-1. *EMBO J* **15**, 6541-6551
35. Hart, J. R., and Vogt, P. K. (2011) Phosphorylation of AKT: a mutational analysis. *Oncotarget* **2**, 467-476
36. Shi, D., and Gu, W. (2012) Dual Roles of MDM2 in the Regulation of p53: Ubiquitination Dependent and Ubiquitination Independent Mechanisms of MDM2 Repression of p53 Activity. *Genes Cancer* **3**, 240-248
37. Bonavida, B., and Baritaki, S. (2011) Dual role of NO donors in the reversal of tumor cell resistance and EMT: Downregulation of the NF-kappaB/Snail/YY1/RKIP circuitry. *Nitric oxide : biology and chemistry* **24**, 1-7
38. Muntane, J., and la Mata, M. D. (2010) Nitric oxide and cancer. *World J Hepatol* **2**, 337-344
39. Stambolic, V., MacPherson, D., Sas, D., Lin, Y., Snow, B., Jang, Y., Benchimol, S., and Mak, T. W. (2001) Regulation of PTEN transcription by p53. *Mol Cell* **8**, 317-325
40. Yung, H. W., Charnock-Jones, D. S., and Burton, G. J. (2011) Regulation of AKT phosphorylation at Ser473 and Thr308 by endoplasmic reticulum stress modulates substrate specificity in a severity dependent manner. *PLoS One* **6**, e17894
41. Vincent, E. E., Elder, D. J., Thomas, E. C., Phillips, L., Morgan, C., Pawade, J., Sohail, M., May, M. T., Hetzel, M. R., and Tavaré, J. M. (2011) Akt phosphorylation on Thr308 but not on Ser473 correlates with Akt protein kinase activity in human non-small cell lung cancer. *Br J Cancer* **104**, 1755-1761

42. Gallay, N., Dos Santos, C., Cuzin, L., Bousquet, M., Simmonet Gouy, V., Chaussade, C., Attal, M., Payrastre, B., Demur, C., and Recher, C. (2009) The level of AKT phosphorylation on threonine 308 but not on serine 473 is associated with high-risk cytogenetics and predicts poor overall survival in acute myeloid leukaemia. *Leukemia* **23**, 1029-1038
43. Laity, J. H., Lee, B. M., and Wright, P. E. (2001) Zinc finger proteins: new insights into structural and functional diversity. *Curr Opin Struct Biol* **11**, 39-46
44. Katoh, T., Sakaguchi, Y., Miyauchi, K., Suzuki, T., Kashiwabara, S., Baba, T., and Suzuki, T. (2009) Selective stabilization of mammalian microRNAs by 3' adenylation mediated by the cytoplasmic poly(A) polymerase GLD-2. *Genes Dev* **23**, 433-438
45. Rissland, O. S., Mikulasova, A., and Norbury, C. J. (2007) Efficient RNA polyuridylation by noncanonical poly(A) polymerases. *Mol Cell Biol* **27**, 3612-3624
46. Bellacosa, A., Kumar, C. C., Di Cristofano, A., and Testa, J. R. (2005) Activation of AKT kinases in cancer: implications for therapeutic targeting. *Adv Cancer Res* **94**, 29-86
47. Freudlsperger, C., Horn, D., Weissfuss, S., Weichert, W., Weber, K. J., Saure, D., Sharma, S., Dyckhoff, G., Grabe, N., Plinkert, P., Hoffmann, J., Freier, K., and Hess, J. (2015) Phosphorylation of AKT(Ser473) serves as an independent prognostic marker for radiosensitivity in advanced head and neck squamous cell carcinoma. *Int J Cancer* **136**, 2775-2785

4.8 Supporting Information

Table S4.1 OPAL predictions of known and unknown Akt1 substrates

Uniprot_ AC/ID	Protein Name	Site	Presence at phosphosite
Q9UNQ0	ATP-binding cassette sub-family G member 2 (Breast cancer resistance protein) (CDw338) (Mitoxantrone resistance-associated protein) (Placenta-specific ATP-binding cassette transporter) (Urate exporter) (CD antigen CD338)	S50	yes
P12814	Alpha-actinin-1 (Alpha-actinin cytoskeletal isoform) (F-actin cross-linking protein) (Non-muscle alpha-actinin-1)	S763	yes
Q8IVF2	Protein AHNAK2	S4962	yes
Q9BT22	Chitobiosyldiphosphodolichol beta-mannosyltransferase (EC 2.4.1.142) (Asparagine-linked glycosylation protein 1 homolog) (Beta-1,4-mannosyltransferase) (GDP-Man:GlcNAc2-PP-dolichol mannosyltransferase) (GDP-mannose-dolichol diphosphochitobiose mannosyltransferase) (Mannosyltransferase-1) (MT-1) (hMat-1)	S239	yes
Q8IZ07	Ankyrin repeat domain-containing protein 13A (Protein KE03)	S248	yes
Q14678	KN motif and ankyrin repeat domain-containing protein 1 (Ankyrin repeat domain-containing protein 15) (Kidney	S186	yes

	ankyrin repeat-containing protein)		
Q8NB78	Lysine-specific histone demethylase 1B (EC 1.-.-.) (Flavin-containing amine oxidase domain-containing protein 1) (Lysine-specific histone demethylase 2)	S13	yes
O94875	Sorbin and SH3 domain-containing protein 2 (Arg-binding protein 2) (ArgBP2) (Arg/Abl-interacting protein 2) (Sorbin)	S712	yes
Q53QZ3	Rho GTPase-activating protein 15 (ArhGAP15) (Rho-type GTPase-activating protein 15)	S64	yes
O43182	Rho GTPase-activating protein 6 (Rho-type GTPase-activating protein 6) (Rho-type GTPase-activating protein RhoGAPX-1)	S264	yes
Q9BRR9	Rho GTPase-activating protein 9 (Rho-type GTPase-activating protein 9)	S496	yes
Q9NR48	Histone-lysine N-methyltransferase ASH1L (EC 2.1.1.43) (ASH1-like protein) (huASH1) (Absent small and homeotic disks protein 1 homolog) (Lysine N-methyltransferase 2H)	S1226	yes
Q9ULI0	ATPase family AAA domain-containing protein 2B	S1138	yes
O60241	Adhesion G protein-coupled receptor B2 (Brain-specific angiogenesis inhibitor 2)	S1543	yes
Q53HL2	Borealin (Cell division cycle-associated protein 8) (Dasra-B) (hDasra-B) (Pluripotent embryonic stem cell-related gene 3 protein)	S23	yes

Q9HA77	Probable cysteine--tRNA ligase, mitochondrial (EC 6.1.1.16) (Cysteinyl-tRNA synthetase) (CysRS)	S401	yes
P55211	Caspase-9 (CASP-9) (EC 3.4.22.62) (Apoptotic protease Mch-6) (Apoptotic protease-activating factor 3) (APAF-3) (ICE-like apoptotic protease 6) (ICE-LAP6) [Cleaved into: Caspase-9 subunit p35; Caspase-9 subunit p10]	S196	yes
Q8ND76	Cyclin-Y (Cyc-Y) (Cyclin box protein 1) (Cyclin fold protein 1) (cyclin-X)	S83	yes
Q0P6D6	Coiled-coil domain-containing protein 15	S186	yes
Q9NXE8	Pre-mRNA-splicing factor CWC25 homolog (Coiled-coil domain-containing protein 49) (Spliceosome-associated protein homolog CWC25)	S381	yes
Q9Y3X0	Coiled-coil domain-containing protein 9	S349	yes
O95067	G2/mitotic-specific cyclin-B2	S274	yes
Q8N7R7	Cyclin-Y-like protein 1	S105	yes
P50990	T-complex protein 1 subunit theta (TCP-1-theta) (CCT-theta) (Renal carcinoma antigen NY-REN-15)	S317	yes
Q96GN5	Cell division cycle-associated 7-like protein (Protein JPO2) (Transcription factor RAM2)	S62	yes
Q96GN5	Cell division cycle-associated 7-like protein (Protein JPO2) (Transcription factor RAM2)	S321	yes
Q00536	Cyclin-dependent kinase 16 (EC 2.7.11.22) (Cell division protein kinase 16) (PCTAIRE-motif protein kinase 1)	S146	yes

	(Serine/threonine-protein kinase PCTAIRE-1)		
A8MXV6	CMT1A duplicated region transcript 15 protein-like protein	S205	yes
Q9BU64	Centromere protein O (CENP-O) (Interphase centromere complex protein 36)	S72	yes
Q6ZU80	Centrosomal protein of 128 kDa (Cep128)	S187	yes
P68400	Casein kinase II subunit alpha (CK II alpha) (EC 2.7.11.1)	S194	yes
P19784	Casein kinase II subunit alpha' (CK II alpha') (EC 2.7.11.1)	S195	yes
P53621	Coatomer subunit alpha (Alpha-coat protein) (Alpha-COP) (HEP-COP) (HEPCOP) [Cleaved into: Xenin (Xenopsin-related peptide); Proxenin]	S16	yes
P05813	Beta-crystallin A3 [Cleaved into: Beta-crystallin A3, isoform A1, Delta4 form; Beta-crystallin A3, isoform A1, Delta7 form; Beta-crystallin A3, isoform A1, Delta8 form]	S112	yes
Q9Y534	Cold shock domain-containing protein C2 (RNA-binding protein PIPPin)	S32	yes
Q8NEV1	Casein kinase II subunit alpha 3 (CK II alpha 3) (EC 2.7.11.1) (Casein kinase II alpha 1 polypeptide pseudogene)	S194	No
P14416	D(2) dopamine receptor (Dopamine D2 receptor)	S148	yes
O60469	Down syndrome cell adhesion molecule (CHD2)	S860	yes
P11532	Dystrophin	S616	yes
Q96F86	Enhancer of mRNA-decapping protein 3 (LSM16 homolog) (YjeF N-terminal domain-containing	S161	yes

	protein 2) (YjeF_N2) (hYjeF_N2) (YjeF domain-containing protein 1)		
Q8NDI1	EH domain-binding protein 1	S781	yes
Q9H329	Band 4.1-like protein 4B (FERM-containing protein CG1) (Protein EHM2)	S414	yes
Q14152	Eukaryotic translation initiation factor 3 subunit A (eIF3a) (Eukaryotic translation initiation factor 3 subunit 10) (eIF-3-theta) (eIF3 p167) (eIF3 p180) (eIF3 p185)	S888	yes
P23588	Eukaryotic translation initiation factor 4B (eIF-4B)	S230	yes
Q9HCM4	Band 4.1-like protein 5	S372	yes
Q9H2F5	Enhancer of polycomb homolog 1	S535	yes
Q5BKY9	Protein FAM133B	S82	yes
Q7L5A3	Protein FAM214B	S268	yes
Q9NZ56	Formin-2	S624	yes

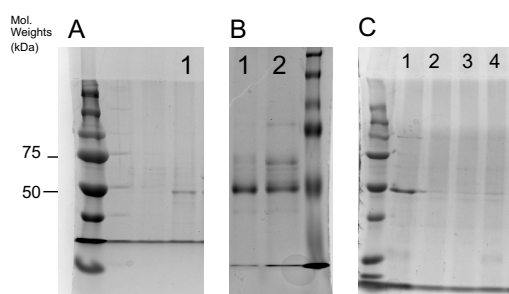


Figure S4.1 Production of Δ PH Akt1 variants. Purified fractions of (A) unphosphorylated Akt1 (lane 1) (B) pAkt^{S473} (lane 1), pAkt^{T308} (lane 2) and (C) ppAkt^{T308, S473} (lanes 1-4) visualized on Coomassie stained SDS-PAGE after affinity and size exclusion chromatography.

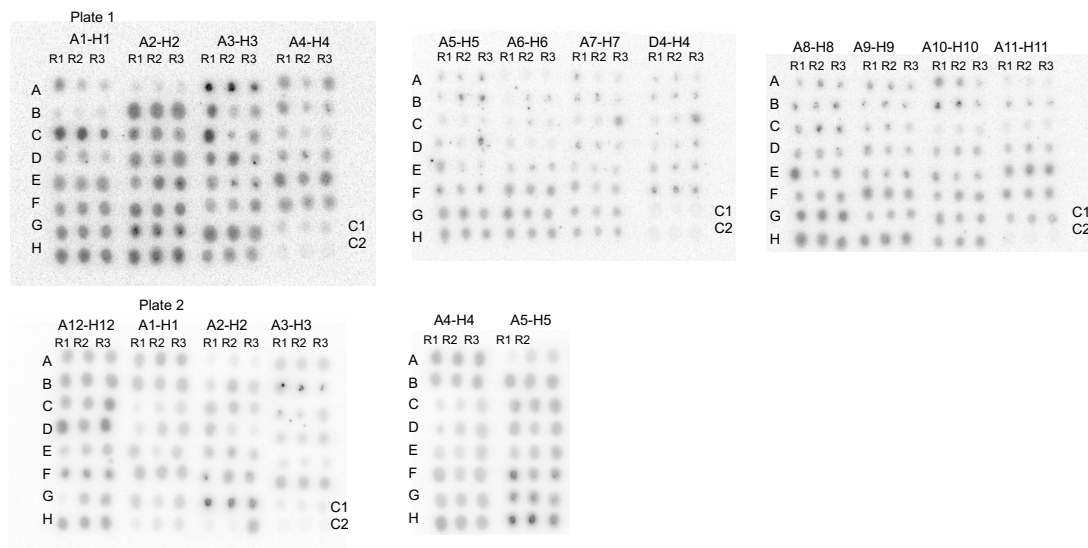


Figure S4.2 Autoradiographs on OPAL assay conducted for pAkt^{S473}. A_x-H_x are sub-library pools of peptides where one amino acid has kept unchanged in the peptide sequence while the rest is randomized with 17 amino acids except serine, threonine and cysteine. Assays were performed in triplicate (as indicated by R1-R3). Controls include: C1 enzyme without substrate peptide, C2 only kinase assay buffer.

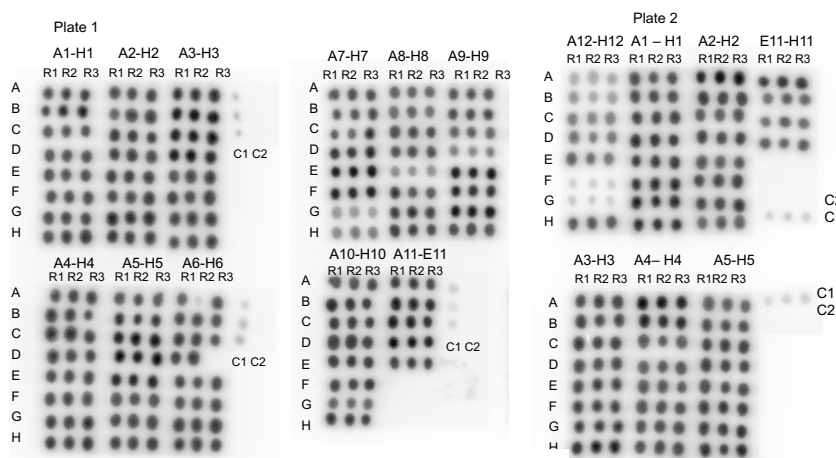


Figure S4.3 Autoradiographs on OPAL assay conducted for pAkt^{T308}. A_x-H_x are sub-library pools of peptides where one amino acid has kept unchanged in the peptide sequence while the rest is randomized with 17 amino acids except serine, threonine and cysteine. Assays were performed in triplicate (as indicated by R1-R3). Controls include: C1 enzyme without substrate peptide, C2 only kinase assay buffer.

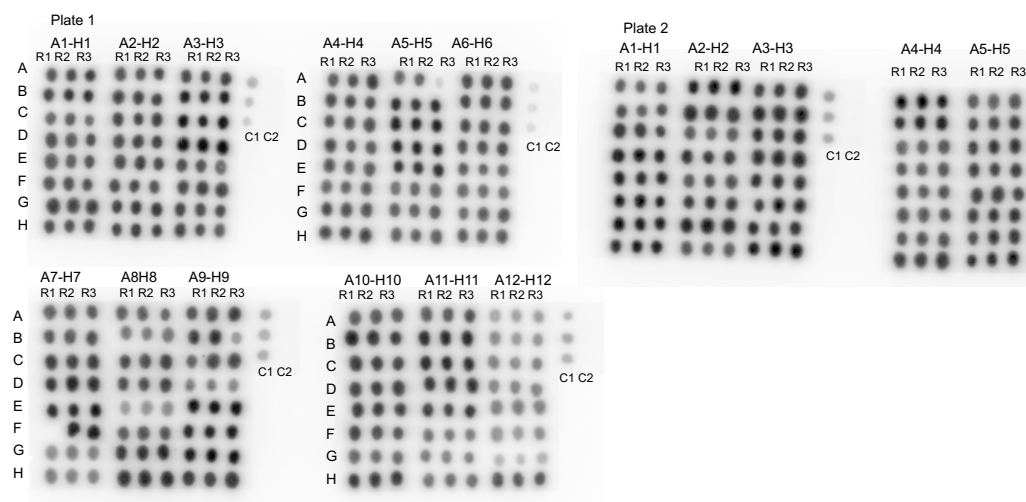


Figure S4.4 Autoradiographs on OPAL assay conducted for ppAkt^{T308, S473}. A_x-H_x are sub-library pools of peptides where one amino acid has kept unchanged in the peptide sequence while the rest is randomized with 17 amino acids except serine, threonine and cysteine. Assays were performed in triplicate (as indicated by R1-R3). Controls include: C1 enzyme without substrate peptide, C2 only kinase assay buffer.

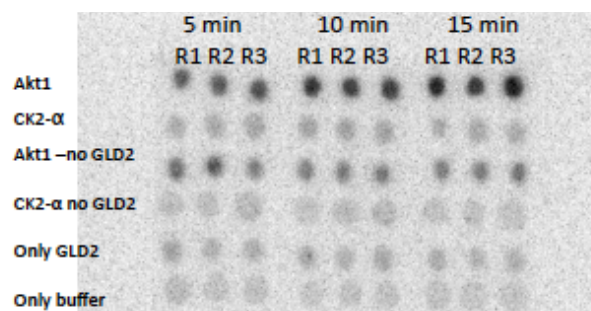


Figure S4.5 Kinase activity assays of Akt1 and CK2- α with GLD2 and γ -[32 P]-ATP as substrates. Spot pictures of the activity assays where the reactions were spotted at 5, 10, 15 min time points. The data are plotted in Figure 4.10.

Chapter 5

5 Summary and future perspectives

5.1 Summary

The proto-oncogene Akt/PKB is a key regulatory kinase in the PI3K/Akt cell survival pathway and belongs to the AGC family of kinase (1). There are three isozymes of Akt named Akt1, Akt2 and Akt3. All three isozymes are activated upon growth factor stimulation (2). In Akt1, Thr308 and Ser473 are the key regulatory phosphorylation sites. Following activation by receptor tyrosine kinases at the plasma membrane, phosphoinositide-3-phosphate kinase (PI3K) phosphorylates its immediate downstream target, a lipid second messenger called phosphatidylinositol-4, 5-bisphosphate (PIP₂), converting PIP₂ into phosphatidylinositol-4, 5-triphosphate (PIP₃) (1,3). PIP₃ harbors binding sites for PH domain carrying proteins like Akt and PDK1. Co-localization of PDK1 with Akt at the plasma membrane promotes phosphorylation of Thr308 by PDK1 (1). The second site Ser473 in the regulatory domain of Akt is phosphorylated by mechanistic target of rapamycin complex 2 (mTORC2).

Once the activation via phosphorylation is complete, Akt dissociates from the plasma membrane and is transported to the cytosol and nucleus to phosphorylate additional downstream target substrates (1,3). The mechanism of activation of Akt is well-studied, however, the contribution of each phosphorylation site to the activation of Akt1 remained elusive. Technical difficulties associated with producing single site phosphorylated variants

hindered the characterization of the functionality of each phosphorylation site. This phosphorylation status of Akt1 is clinically relevant as in multiple different types of cancer the level of phosphorylation of each activation site in Akt has been linked with the survival rate of cancer patients (4).

In my initial experiments, my goal was to determine the contribution of each regulatory phosphorylation site to the overall activation of the Akt1 kinase. In this first objective, I used a genetic code expansion strategy in a novel combination with enzymatic phosphorylation to produce site-specifically phosphorylated Akt1 variants (5). Using these two methods, we produced singly phosphorylated Akt1 variants recombinantly in *E. coli* and confirmed the presence of phosphorylation at each site using mass spectrometry and activity assays. We compared the activity of the unphosphorylated, singly phosphorylated and doubly phosphorylated Akt1 variants using radioactive kinase assays with a Akt1 substrate peptide derived from GSK-3 β . The apparent catalytic rate of the kinase when both activation sites are phosphorylated was 1500-fold higher than the unphosphorylated Akt1. The contribution of Thr308 in activation of Akt1 was only reduced from the doubly phosphorylated enzyme by ~3-fold and was significantly more active than pAkt1^{S473}. Live-cell imaging in COS7 cells further confirmed that phosphorylation of Thr308 was sufficient and irreplaceable for Akt activation in cell (5).

The traditional method of using phosphomimetics substitutions, such as Glu and Asp mutations, to mimic phosphorylation was also tested (6). In both the test tube and in mammalian cells, phosphomimetics failed to activate the kinase in comparison to true phosphorylation. Mutation of Thr308Ala produced a kinase dead mutant, which is not active in vitro despite of the phosphorylation at Ser473. In in vitro kinase assays and in COS7 cells, Akt1 Thr308Ser was a partially

activated kinase, revealing a critical interaction between the γ -methyl group of Thr308 and Cys310 in the activation site of Akt1 (5).

Once the contribution of each phosphorylation site on activation of Akt1 was determined, next I investigated the impact of each phosphorylation site on releasing the auto-inhibitory effect of the PH domain of Akt1 (7). I used the same experimental strategies noted above to produce Akt1 variants without the PH domain. Deletion of PH domain increased the enzyme activity of all Akt1 variants. Presence of PH domain has inhibited doubly phosphorylated Akt1 by a factor of 29-fold compared to the non-phosphorylated enzyme and the two singly phosphorylated variants i.e. pAkt1^{T308} and pAkt1^{S473}, were inhibited by factors of 17-fold and 5-fold, respectively.

Next, I tested the impact of Akt1 phosphorylation status on chemical inhibition. A known PH domain dependent Akt inhibitor, Aki1/2 was used (8). Our results showed that phosphorylation at Ser473 provided resistance to Akt inhibition, increasing the IC₅₀ by 4-fold. These findings highlight the importance of selecting the most pathogenic form of the kinase in designing potent inhibitors for Akt1 (7).

The impact of Akt1 phosphorylation status on substrate selectivity was unknown. A few reports have suggested a role for Akt1 phosphorylation status in modulating substrate selectivity (1,9), yet no systematic investigation has been conducted. We, therefore, used peptide library based approaches to investigate the substrate selectivity of singly and doubly phosphorylated Akt variants.

We found that Akt1 p-status has a global impact on substrate selectivity. The pAkt^{S473} enzyme has a completely different substrate selectivity profile compared to pAkt1^{T308} or ppAkt1. Amazingly, the doubly phosphorylated

enzyme is usually, but not always more active than pAkt1T308. The role of S473 phosphorylation appears to be involve in globally tuning substrate selectivity rather than activating the kinase. From the predicted substrates we verified that the terminal nucleotidyltransferase (germline development 2) Gld2 was phosphorylated at specifically in its N-terminal domain at Ser116. Phosphorylation at Ser116 abolished nucleotide addition activity of GLD2 (10).

5.2 Future directions

5.2.1 Validation of predicted substrates

We developed a new method to produce Akt1 with programmed phosphorylation on each activation site. Although the scientific literature on Akt is vast, previously, very few studies (9) reported on the specific functionality of each phosphorylation site or the relationship between Akt1 phosphorylation status, inhibition and substrate specificity. Most previous studies used protein with mixed or non-homogenous phosphorylation status. Although such a kinase is active it can not reveal the role of each phosphorylation site. Since acidic or alanine substitutions at 308 and 473 alter the activity of this enzyme in the test tube and in cells (5) such substitutions do not report on the activity caused by actual phosphorylation. We have been using a systemic approach to cautiously compare and contrast the phosphorylation site dependent substrate specificity.

The peptide library analysis (Chapter 4) revealed that the phosphorylation status of Akt1 modulates substrate specificity on a global or proteome-wide scale. Based on data from the OPAL screen, we were able to identify potential novel substrates for Akt1. One of the key areas of future directions is validation of these predictions. Although the data I have produced alone is not sufficient to validate

these predictions, *in vitro* activity assays are an essential starting point to prove the ability of Akt1 to phosphorylate a particular substrate. In the cell, the ability of Akt1 to phosphorylate a particular substrate is more complex and will rely on the accessibility, secondary interactions with the kinase, cellular localization and the physiological status of the cell (3). De-phosphorylation of putative Akt1 substrates by phosphatases (3) is another complicating factor.

5.2.2 Targeting Akt1 in cancer treatment

The significance of Akt activation in cancer has been widely studied during the past decades due to the prominent role of Akt in cell growth and survival (11,12). In cancer cells, Akt is found to be over-activated and hyper-phosphorylated, which helps cancer cells to survive (11,13). Therefore, Akt is a prominent target in developing anti-cancer drugs. Clinical trials to test Akt inhibitors are currently in progress (14,15), yet a unique anticancer agent for Akt isozymes has not been successfully designed.

My studies have wide ranging implications for Akt1 drug design as my data suggests that the phosphorylation status of Akt1 impacts its activity, substrate selectivity and its ability to be inhibited. The fact that each Akt1 phospho-form has distinct substrate preferences, indicates that the phospho-forms can be targeted separately. The site-specifically phosphorylated Akt variants are, thus, invaluable tools for drug screening and design. These site-specifically programmed kinase variants will help to make precise inhibitor screening platforms for Akt and ultimately any human kinase of interest.

5.3 Conclusion

Using genetic code expansion and co-expression of a specific upstream kinase, I produced site-specifically phosphorylated Akt1 variants. These variants are indispensable tools that I used to characterize the functionality of each site separately and in combination. These Akt1 phospho-variants were instrumental in my investigations of the activation, auto-inhibition, chemical inhibition and substrate selectivity of Akt1. Isolating the effect of each phosphorylating site allowed a detailed characterization of the individual and unique functional roles of each site in activating a major human kinase linked to cancer.

5.4 References

1. Manning, B. D., and Cantley, L. C. (2007) AKT/PKB signaling: navigating downstream. *Cell* **129**, 1261-1274
2. Hanada, M., Feng, J., and Hemmings, B. A. (2004) Structure, regulation and function of PKB/AKT--a major therapeutic target. *Biochim Biophys Acta* **1697**, 3-16
3. Manning, B. D., and Toker, A. (2017) AKT/PKB Signaling: Navigating the Network. *Cell* **169**, 381-405
4. Gallay, N., Dos Santos, C., Cuzin, L., Bousquet, M., Simmonet Gouy, V., Chaussade, C., Attal, M., Payrastre, B., Demur, C., and Recher, C. (2009) The level of AKT phosphorylation on threonine 308 but not on serine 473 is associated with high-risk cytogenetics and predicts poor overall survival in acute myeloid leukaemia. *Leukemia* **23**, 1029-1038
5. Balasuriya, N., Kunkel, M. T., Liu, X., Biggar, K. K., Li, S. S., Newton, A. C., and O'Donoghue, P. (2018) Genetic code expansion and live cell imaging reveal that Thr-308 phosphorylation is irreplaceable and sufficient for Akt1 activity. *J Biol Chem* **293**, 10744-10756
6. Dissmeyer, N., and Schnittger, A. (2011) Use of phospho-site substitutions to analyze the biological relevance of phosphorylation events in regulatory networks. *Methods in molecular biology* **779**, 93-138
7. Balasuriya, N., McKenna, M., Liu, X., Li, S. S. C., and O'Donoghue, P. (2018) Phosphorylation-Dependent Inhibition of Akt1. *Genes (Basel)* **9**

8. Barnett, S. F., Defeo-Jones, D., Fu, S., Hancock, P. J., Haskell, K. M., Jones, R. E., Kahana, J. A., Kral, A. M., Leander, K., Lee, L. L., Malinowski, J., McAvoy, E. M., Nahas, D. D., Robinson, R. G., and Huber, H. E. (2005) Identification and characterization of pleckstrin-homology-domain-dependent and isoenzyme-specific Akt inhibitors. *Biochem J* **385**, 399-408
9. Jacinto, E., Facchinetti, V., Liu, D., Soto, N., Wei, S., Jung, S. Y., Huang, Q., Qin, J., and Su, B. (2006) SIN1/MIP1 maintains rictor-mTOR complex integrity and regulates Akt phosphorylation and substrate specificity. *Cell* **127**, 125-137
10. Chung, C., Balasuriya, N., Manni, E., Liu, X., Li, S.-C., O'Donoghue, P., and Heinemann, I. U. (2019) Gld2 activity is regulated by phosphorylation in the N-terminal domain. *RNA Biol*, DOI:10.1080/15476286.15472019.11608754
11. Altomare, D. A., and Testa, J. R. (2005) Perturbations of the AKT signaling pathway in human cancer. *Oncogene* **24**, 7455-7464
12. Gonzalez, E., and McGraw, T. E. (2009) The Akt kinases: isoform specificity in metabolism and cancer. *Cell Cycle* **8**, 2502-2508
13. Bellacosa, A., Kumar, C. C., Di Cristofano, A., and Testa, J. R. (2005) Activation of AKT kinases in cancer: implications for therapeutic targeting. *Adv Cancer Res* **94**, 29-86
14. Nitulescu, G. M., Margina, D., Juzenas, P., Peng, Q., Olaru, O. T., Saloustros, E., Fenga, C., Spandidos, D., Libra, M., and Tsatsakis, A. M. (2016) Akt inhibitors in cancer treatment: The long journey from drug discovery to clinical use (Review). *International journal of oncology* **48**, 869-885
15. Tamura, K., Hashimoto, J., Tanabe, Y., Kodaira, M., Yonemori, K., Seto, T., Hirai, F., Arita, S., Toyokawa, G., Chen, L., Yamamoto, H., Kawata, T., Lindemann, J., and Esaki, T. (2016) Safety and tolerability of AZD5363 in Japanese patients with advanced solid tumors. *Cancer chemotherapy and pharmacology* **77**, 787-795

Curriculum Vitae

Nileeka Balasuriya BSc, MSc

Post-secondary education and degrees:

University of Peradeniya, Peradeniya,
Sri Lanka
2004-2008, BSc

Dalhousie University, Halifax, Nova Scotia,
Canada
2009-2011, MSc

University of Western Ontario, London, Ontario,
Canada
2014-2019, PhD

Honours and Awards

1. **Canadian Cancer Society travel award** (valued \$2000): Awarded to attend the “protein kinases and protein phosphorylation” conference held in Cambridge, UK (August 6-11, 2017)
2. **Queen Elizabeth II Graduate Student Scholarship** (valued \$15000): Biochemistry Department, Schulich School of Medicine and Dentistry University of Western Ontario-2015/2016
3. Selected as one of the **top 5 nominees** to represent Canada in the **Lindau Nobel Laureate Meeting 2016**
4. **Gold category award** and cash prize: Canadian Student Health Research Forum 2016, Winnipeg, Manitoba
5. **Ontario Graduate Student Scholarship** (valued \$15000): Biochemistry Department, Schulich School of Medicine and Dentistry, University of Western Ontario-2017/2018

6. **Represented** the student group from **Schulich School of Medicine and Dentistry** participated in the Canadian Student Health Research Forum 2016 which gathers the top 5% PhD students across the Canadian medical schools
7. **Top hundred poster:** London Health Research Day (2016)
8. **Schulich Graduate Scholarship** (Valued \$2000 per year) Biochemistry Department, Schulich School of Medicine and Dentistry, University of Western Ontario (2015-2017)
9. **International Union of Nutritional Sciences (IUNS) Travel Fund** Agricultural Institute of Canada Foundation (AICF) - 2010/11: Awarded to attend the Proceedings of the 7th International Conference of Functional Foods for Prevention and Management of Metabolic Syndrome. Dallas, Texas, US. Dec. 2-4, 2010
10. **Entrance Scholarship** (valued \$5000): Faculty of Agriculture, Dalhousie University – 2009-2011
11. **Best Oral Presentation:** Graduate Research Day, 2010
12. **Gold Medal:** Best academic performance of the B.Sc. Food Science and Technology degree, University of Peredaniya, Sri Lanka (2004-2008)
13. **Gold Medal:** Best performance in food quality control – B.Sc. Food Science and Technology degree, University of Peredaniya, Sri Lanka (2004-2008)

Related Work Experience

Teaching assistant: Biochemistry laboratory 3380G, Biochemistry Department, Western University (2015-2018)

Graduate research assistant: Biochemistry Department (O'Donoghue laboratory) Western University (2014-2018)

Research associate: Department of Engineering, Faculty of Agriculture, Dalhousie University (2014)

Research assistant: Atlantic Poultry Research Center, Dalhousie University (2013)

Research assistant: Wild Blueberry Research Laboratory, Faculty of Agriculture, Dalhousie University (2013)

Visiting research scientist: Faculty of Agriculture, Dalhousie University and University of Prince Edward Island (2012)

Graduate research assistant: Natural Product Chemistry Laboratory, Tree Fruit Bioproduct Group, Faculty of Agriculture, Dalhousie University (2009-2012)

Teaching assistant: Functional foods and nutraceuticals, Faculty of Agriculture, Dalhousie University (2010)

Demonstrator: Department of Applied Nutrition, Wayamba University of Sri Lanka (2009)

Demonstrator: Department of Food Science and Technology, Faculty of Agriculture, University of Peradeniya, Sri Lanka (2008)

Research associate: Chemical and Microbiological Laboratories, Industrial Technology Institute, Colombo, Sri Lanka (2007-2008)

Publications

Manuscripts

1. Chung, C., **Balasuriya, N.**, Manni, E., Liu, X., Li, S.-C., O'Donoghue, P., and Heinemann, I. U. (2019) Gld2 activity is regulated by phosphorylation in the N-terminal domain. *RNA Biol*, DOI:10.1080/15476286.15472019.11608754
2. **Balasuriya, N.**, McKenna, M., Liu, X., Li, S. S. C., and O'Donoghue, P. Phosphorylation-dependent inhibition of Akt1. *Genes (Basel)* **2018** 9(9), 450
3. **Balasuriya, N.**, Kunkel, M., Biggar, K., Li, S., Newton, A., O'Donoghue, P. 2018. Genetic code expansion and live cell imaging reveal that Thr308 phosphorylation is irreplaceable and sufficient for Akt1 activity. *Journal of Biological Chemistry*. **2018**, 293, 10744-10756.
4. Ejike, C. E. C. C., Collins, S. A., **Balasuriya, N.**, Swanson, A.K., Mason, B., Udenigwe, C. C. Prospects of microalgae proteins in producing peptide-based functional foods for

promoting cardiovascular health. *Trends in Food Science and Technology*. **2017**, 59, 30-36.

5. **Balasuriya, N.**, Rupasinghe, H. P. V., Sweeney, M., McCarron, S. and Gottschall-Pass, K. Antihypertensive effects of apple peel extract on spontaneously hypertensive rats. *Pharmacologia*. **2015**, 6(8), 371-376.

6. **Balasuriya, N.** and Rupasinghe, H. P. V. Antihypertensive properties of flavonoid rich apple peel extract. *Food Chemistry*. **2012**, 135, (4) 2320-2325.

7. **Balasuriya, B.W.N.** and Rupasinghe, H. P.V. Plant flavonoids as angiotensin converting enzyme inhibitors in regulation of hypertension. *Functional Foods in Health Disease*. **2011**, 5, 172-188.

Book Chapters:

Rupasinghe, H. P. V., **Balasuriya, N.**, Wang Y., Prevention of type 2 diabetes by polyphenols of fruits. In book *Nutritional and Antioxidant Therapies: Treatments and Perspectives*. 2017. DOI: 10.1007/978-3-319-67625-8_18.

Abstracts of conferences attended: PhD

International

1. Functional characterization of differentially activated oncogenic kinase Akt1. Protein kinases and protein phosphorylation. Federation of American Societies of Experimental Biology. Cambridge, UK. August, 2017. (Poster presentation)
2. Activating the oncogenic kinase Akt1 by genetically encoded phosphorylation. International Union of Molecular Biology and Biochemistry. Vancouver, BC. July, 2016. (Poster presentation)

Local

1. Differentially activated oncogenic kinase Akt1. London Health Research Day. London, ON. March, 2017. (Poster presentation)
2. Reassignment of the genetic code using phosphoserine in synthesizing differentially activated Akt1. Canadian Student Health Research Forum. University of Manitoba, Winnipeg, June, 2016. (Poster presentation)

3. Synthesis of differentially activated oncogenic kinase Akt1 by site-specific incorporation of phosphoserine. London Health Research Day. London, ON. March, 2016. (Poster presentation)

Abstracts of conferences attended: MSc

International

1. Antihypertensive effects of apple peel extract (APE) on spontaneously hypertensive rats. Nileeka Balasuriya, H.P. Vasantha Rupasinghe, Marva Sweeney, Shelby McCarron and Katherine Gottschall-Pass. 17th World Congress of Food Science. Montreal, Canada. August 17-21, 2014. (Poster presentation)

2. Antihypertensive properties of fruit bioactives. Nileeka Balasuriya and Vasantha Rupasinghe. Proceedings of the Plant Canada Conference. St. Mary's University. Halifax, Canada. July 17 – 21, 2011. (Oral presentation)

3. Inhibition of angiotensin converting enzyme by wild berry extracts in vitro. Nileeka Balasuriya and Vasantha Rupasinghe. Proceedings of the 8th Annual Conference of Natural Health Products Research Society – Joining Forces for a Healthier Tomorrow. Montreal, Canada. May 24 – 27, 2011. (Oral presentation)

4. Inhibition of angiotensin converting enzyme in vitro by apple skin extract, selected flavonoids and their metabolites. Nileeka Balasuriya and Vasantha Rupasinghe. Proceedings of the 7th International Conference of Functional Foods for Prevention and Management of Metabolic Syndrome. Dallas, Texas, US. Dec. 2-4, 2010. (Oral presentation)

Local

1. Effects of flavonoid-rich apple peel extract on biomarkers of oxidative stress in spontaneously hypertensive rat kidneys. Iain McPhee, Nileeka Balasuriya, Shelby McCarron, H.P. Vasantha Rupasinghe, Marva Sweeney-Nixon and Katherine Gottschall-Pass. AUBC Conference. University of Prince Edward Island, 2014. (Poster presentation)