Supplementary Information File for:

Multifaceted regulation of Akt by diverse C-terminal post-translational modifications

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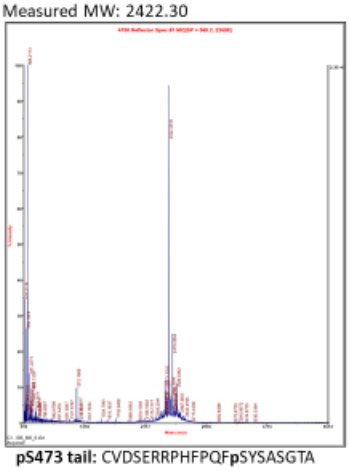
Calculated MW: 2422.49
Measured MW: 2422.30



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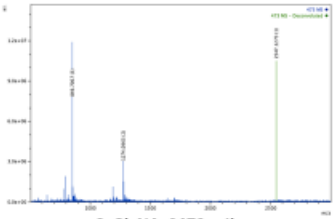

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CVDSERRPHFPQF**O-GlcNAc**SYSASGTA

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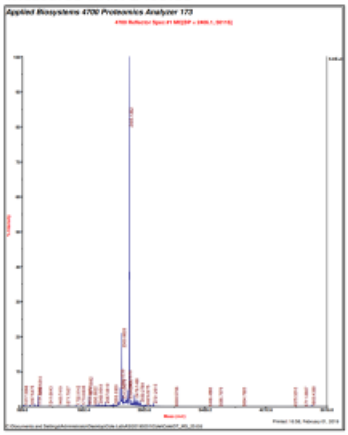

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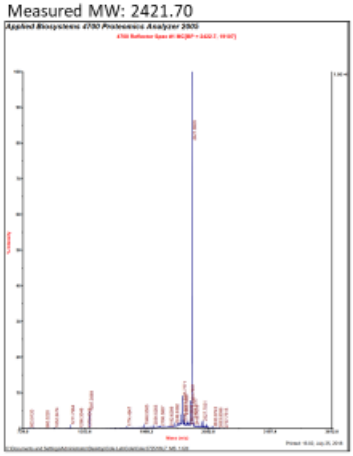

pY474 tail: CVDSERRPHFPQFS**p**YSASGTA

Figure S1: MALDI or ESI spectra of C-tail peptides used for EPL

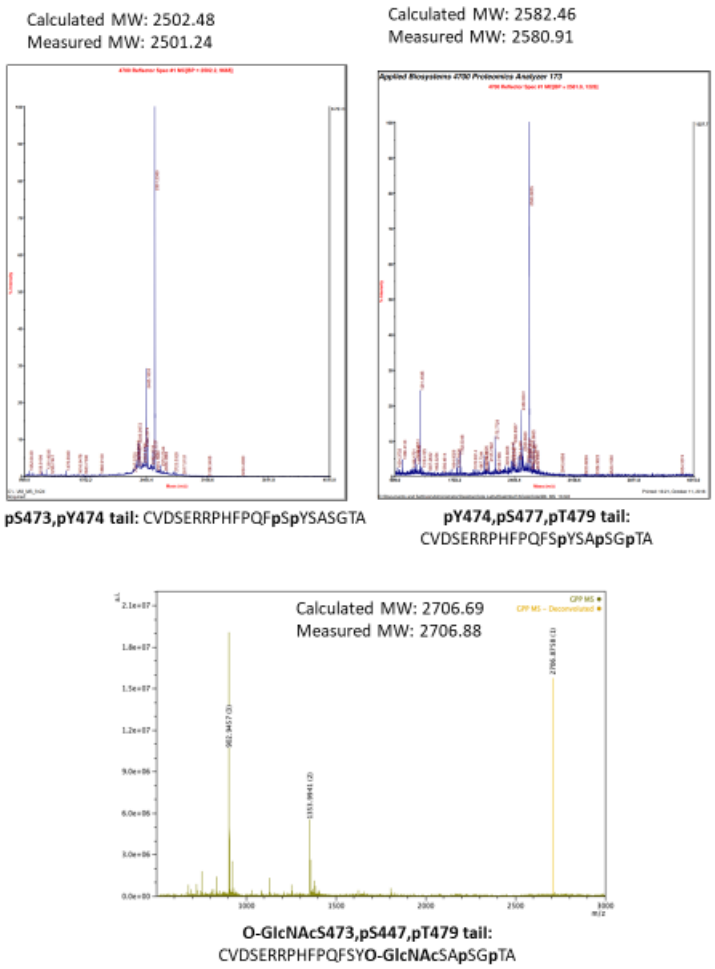

Figure S1: MALDI or ESI spectra of C-tail peptides used for EPL

Peptides with the C-tail sequence of Akt1 (aa460-480) were synthesized as described in the methods section. PTMs for each peptide are indicated in bold. Peptide identities were confirmed using matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) or electrospray ionization mass spectrometry (ESI-MS). Calculated and measured MW values are indicated for each spectrum.

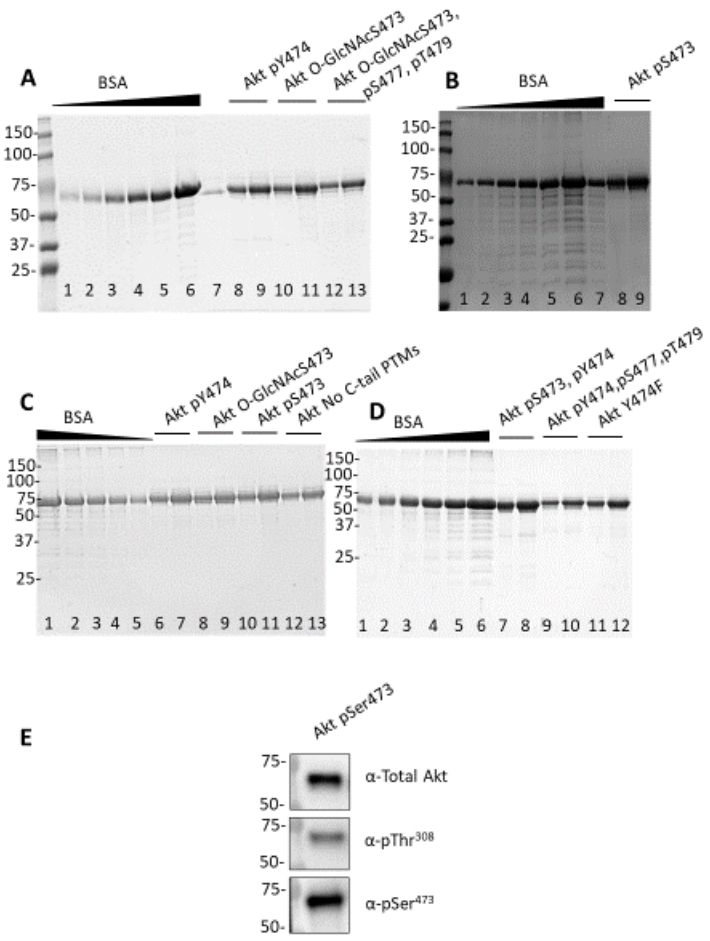

Figure S2: Full length Akt proteins

A-D) Coomassie stained SDS-PAGE of full length semisynthetic Akt proteins with various PTMs. Each Akt sample identified on the label at the top indicates PTMs or mutations at residue(s) on the C-tail of Akt. Proteins were prepared as described in the Methods section. A sample for each Akt protein was run in two adjacent lanes (3 μL and 6 μL) diluted to either 4x or 6x from the original sample concentration. BSA standards are in lanes A1-6, B1-7 (lane 2 and 7 have the same concentration), C1-5 and D1-6 loaded at different concentrations (0.25, 0.5, 1, 1.5, 2 and 3.75 μg). Images were used to determine purity and to measure protein concentration with ImageJ. E) Western blot of full-length Akt showing phosphorylation at Thr308 that was achieved through co-expression with PDK1, as indicated in the Methods section, and phosphorylation at Ser473 through expressed protein ligation.

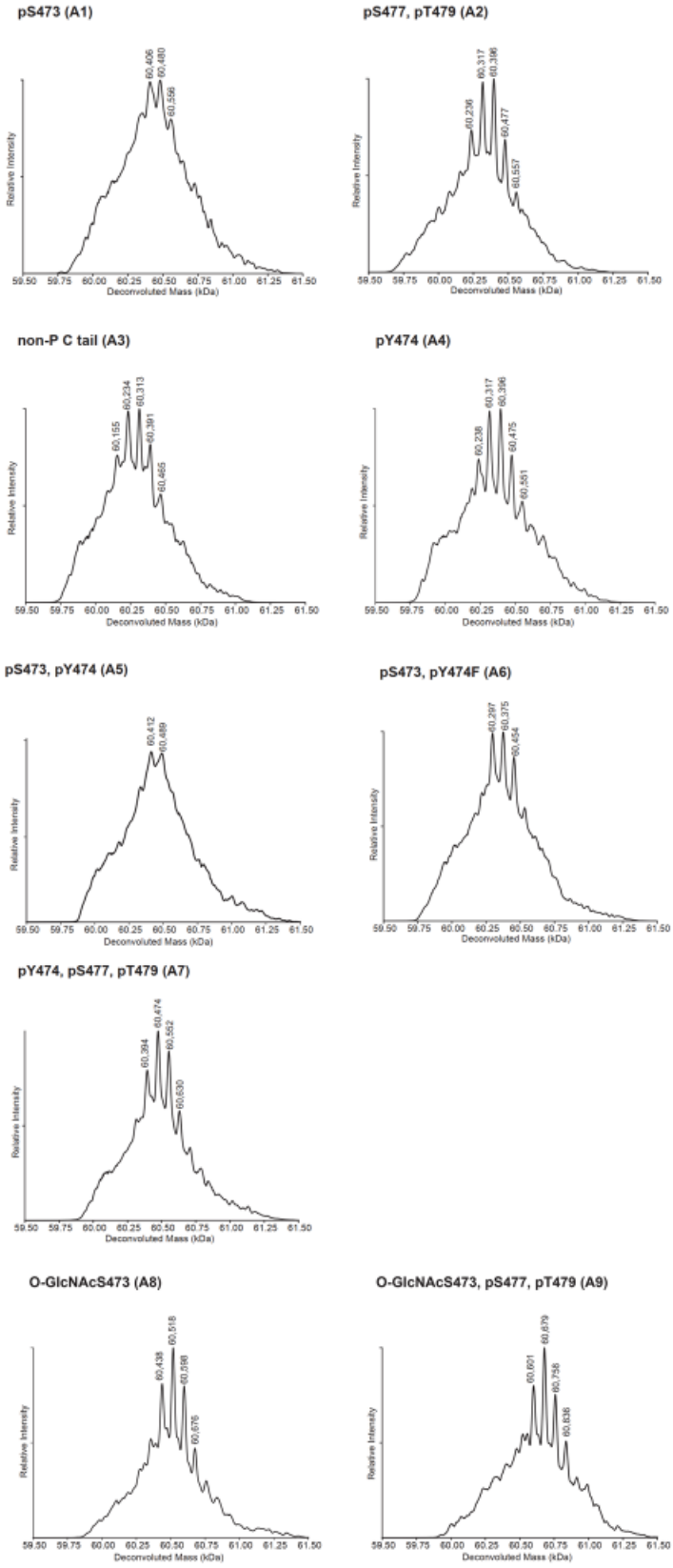

Figure S3: Intact mass analysis of Akt forms

Proteins were analyzed by ESI-LC/MS as described in the Methods, and raw m/z data were processed using Unidec software[1] to obtain the deconvoluted (neutral, average mass) spectra. Note that varying signal-to-noise for each protein limited our ability to fully resolve all peaks. Additionally, the differently modified Akt forms may have different ionization efficiencies and thus these data should be regarded as semi-quantitative. Raw LC/MS data are deposited in Harvard Dataverse (https://doi.org/10.7910/DVN/P6XSPS).

Calculated masses for Akt forms with various numbers of phosphorylations (A1, A2, A3, A4, A5, A7): Akt-2p = 60,158 Da; Akt-3p = 60,238 Da; Akt-4p = 60,318 Da; Akt-5p = 60,398 Da; Akt-6p = 60,478 Da, Akt 7p = 60,558 Da, Akt-8p = 60,638 Da. Calculated masses for Akt Y474F form with and various numbers of phosphorylations (A6): Akt-2p = 60,142 Da; Akt-3p = 60,222 Da; Akt-4p = 60,302 Da; Akt-5p = 60,382 Da; Akt-6p = 60,462 Da, Akt 7p = 60,542 Da, Akt-8p = 60,622 Da. Calculated masses for Akt forms with one O-GlcNAc and various numbers of phosphorylations (A8, A9): Akt-O-GlcNAc-2p = 60,361 Da; Akt-O-GlcNAc-3p = 60,441 Da; Akt-O-GlcNAc-4p = 60,521 Da; Akt-O-GlcNAc-5p = 60,601 Da; Akt-O-GlcNAc-6p = 60,681 Da, Akt-O-GlcNAc-7p = 60,761 Da, Akt-O-GlcNAc-8p = 60,841 Da. All calculated masses assume the presence of 1 N-terminal acetylation.

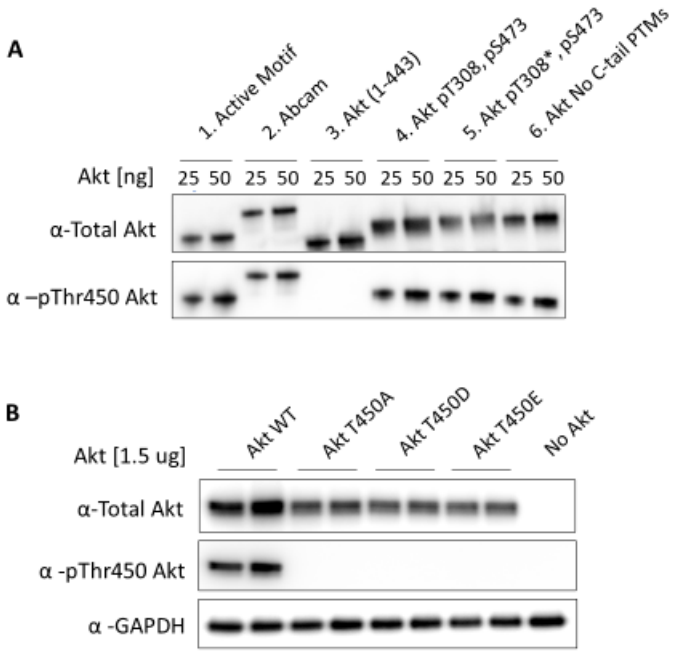

Figure S4: pThr450 phosphorylation

A) Phospho Thr450 levels for commercial and semisynthetic Akt forms that were all produced using a baculovirus expression system. Western blots of total Akt and pT450 shown. Each protein is represented by a pair of lanes since 25 and 50 ng were loaded for each, and the duplicate lanes are labeled as follows. Lane 1, Active Motif recombinant Akt, Lane 2, Abcam GST-full length Akt, Lane 3, recombinant. Akt (aa1-443), Lanes 4-6, full-length semisynthetic Akts: Lane 4. PDK1 phosphorylation *in vivo*, pT308/pS473; Lane 5., PDK1 phosphorylation *in vitro*, pT308/pS473, Lane 6., non-phosphorylated at T308 or S473. Avg. semisynthetic pT450/total Akt is 75+/-20% and for commercial recombinant Akt is 100+/-14% ($p>0.05$). Lane 3 shows anti-pT450 is specific. *denotes T308 phosphorylation by PDK1 in vitro added as previously described[2] B) Further validation of anti-pT450 antibody specificity. Western blot analysis of cell lysates from HCT116 Akt1/2 KO cells transfected with either WT, T450D, T450E, T450A Akt and blotted with anti-Akt (1:2000), anti-pT450 and anti-GAPDH (1:5000).

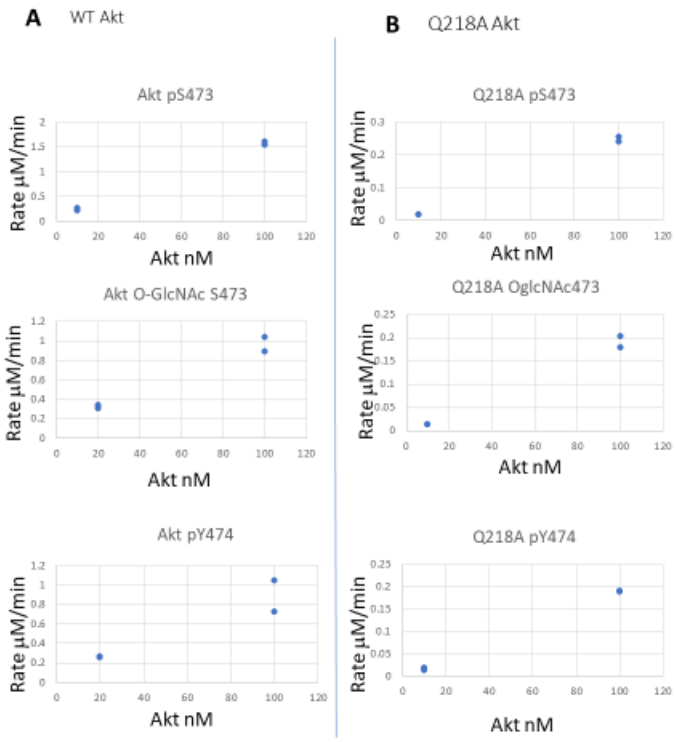

Figure S5: Enzymatic analysis of Full length Akt proteins

Kinase assays were performed to obtain activity versus concentration plots for WT and Q218A full length Akt proteins with pS473, O-GlcNAcS473, pY474 to demonstrate that the reaction velocities were approximately linear with respect to Akt concentrations. (n=2)

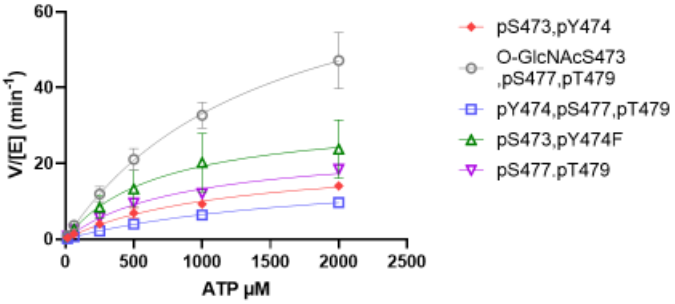

Figure S6: Kinetic characterization of Akt proteins

Steady-state kinetic plots for v/[E] versus [ATP] with 20 µM GSK3 peptide substrate. The obtained enzymatic parameters k_{cat}, $K_{m,}$ and k_{cat}/K_m are shown in Figure 2B, 10 nM of Akt was used for each of these assays (n=2).

Name	C-tail PTMs	Semisynthetic full length Akt with phospho T308	Peptide K_m (μM)
A4	pY474		2.6±0.8
A8	O-GlcNAcS473		2.2±0.3

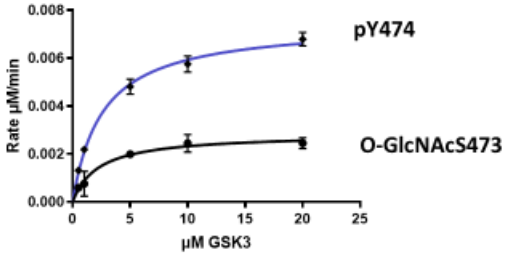

Figure S7: Peptide Km measurements for Akt proteins

Table summarizes the K_m of Akt with O-GlcNAcS473 and pY474 to GSK3 peptide substrate. Kinase assays are described in detail in the methods section and were performed using a range from 0-20 μM GSK3 peptide (n=2). Data plots for each assay are shown below.

Semisynthetic full length Akt with phospho T308	kcat/Km (min^{-1} μM^{-1})	
	WT	Q218A
pS473	0.24	0.0086
pY474	0.044	0.0089
O-GlcNAc S473	0.053	0.0082
No C-tail PTMs	0.003	0.0076

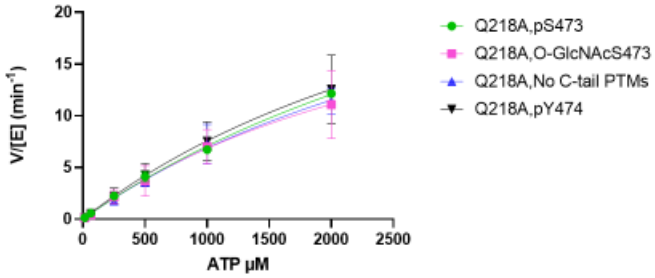

Figure S8: Kinetic characterization of Q218A Akt proteins

Kinase assays of Q218A Akt proteins with pS473, pY474, O-GlcNAcS473 and No C-tail PTMs were performed to obtain k_{cat}/K_m values for comparison with WT Akt with corresponding C-tail PTMs, values summarized in table. Steady-state kinetic plots of v/[E] versus [ATP] with 20 μM GSK3 peptide substrate are shown.

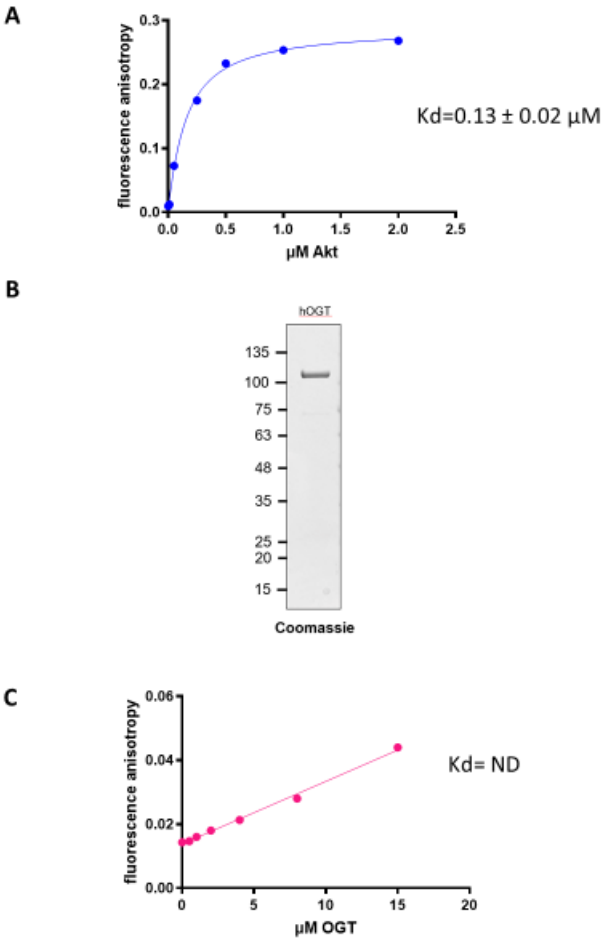

Figure S9: Fluorescence Anisotropy binding assays

(A) Fluorescence anisotropy assay to measure the K_d of fluorescein-labeled soluble PIP3 (di-C6) and Akt with O-GlcNAc S473. (B) Coomassie stained SDS-PAGE showing human O-Linked N-Acetylglucosamine Transferase (OGT) used for fluorescence anisotropy experiment. (C) Fluorescence anisotropy to measure the K_d of fluorescein-labeled soluble PIP3 (di-C6) and OGT. Measurements (n = 3) were fit to quadratic binding equation and K_d values shown ± SE.

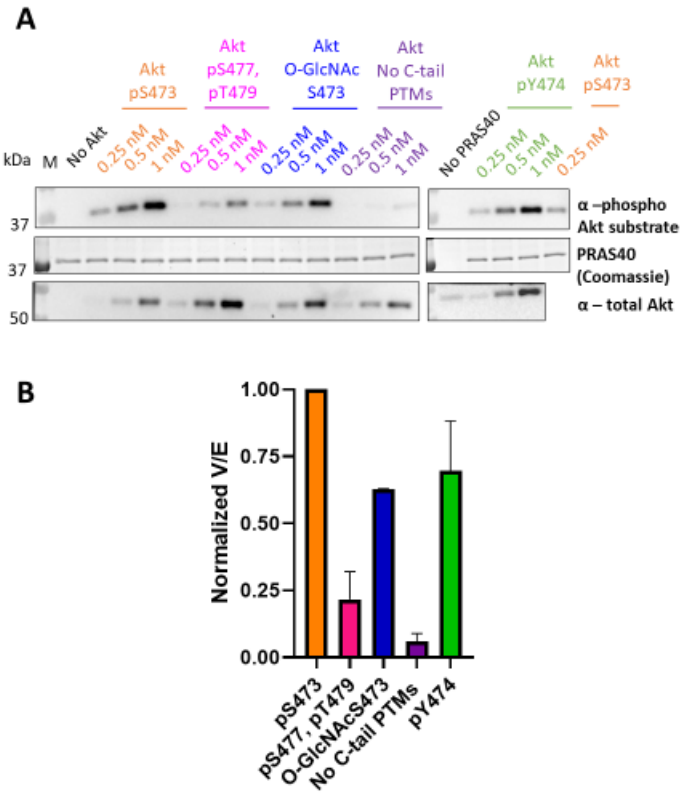

Figure S10: Akt with various C-tail PTMs can phosphorylate PRAS40

A) Western blot analysis of kinase assays using PRAS40 as a substrate for Akt proteins with different C-tail PTMs including A1, A2, A3, A4, and A8. Akt range of concentrations employed were 0.25, 0.5 and 1 nM. Assays were carried out using 1 µM PRAS40 at 30 °C for 5 minutes. The membrane was blotted with phospho Akt substrate antibody (R/KXR/KXXS*/T*) and quantification by densitometry showed linearity in all cases. 160 ng of PRAS40 was loaded per lane. Note that pSer473 was loaded as a control on both gels to facilitate comparison of the separate Western blots. (B) Bar plot of V/E values calculated for the forms of Akt measured and normalized to Akt with pSer473 (A1). Bars represent span of the average values from replicate measurements. (n=2).

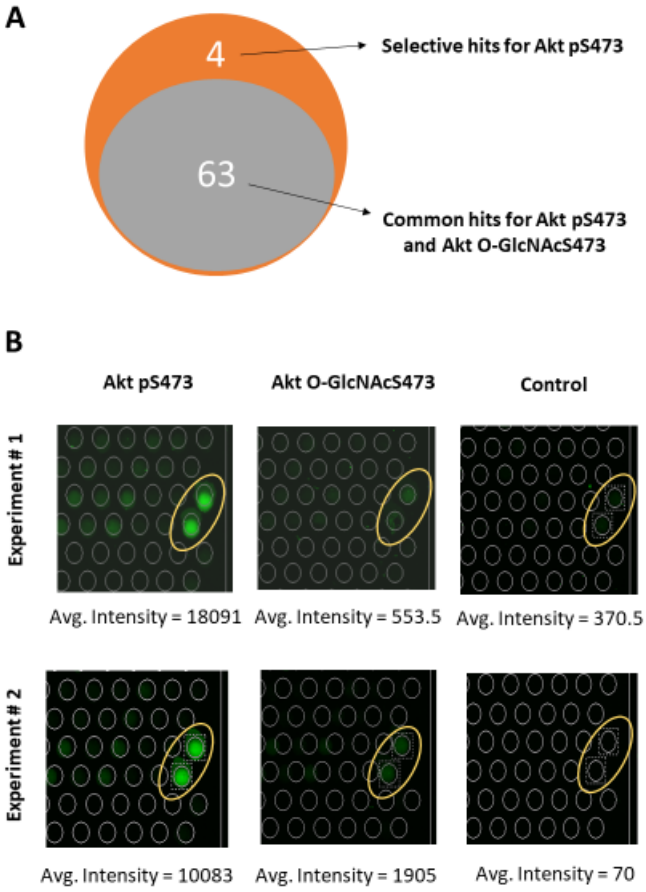

Figure S11: Analysis of potential Akt substrates identified by protein microarrays.

A) Venn diagram representation of protein substrates identified as hits for both Akt pSer473 and O-GlcNAcSer473 (63) and selective hits for pSer473 (4). (B) Highlighted section of the protein microarrays analyzed for the PPM1H region. Images obtained from GenePix of protein microarrays showing PPM1H phosphorylation by pSer473 Akt (A1), O-GlcNAcSer473 Akt (A8) and control (no Akt). The average intensity of signal is indicated for each pair of spots. Both replicates are shown. Raw data for all microarrays was deposited in Gene Expression Omnibus (GEO) and can be accessed through GSE181983.

A

Common hits	pS473	O-GlcNAc S473	MLF2	8381	20952
ABI1	11451	14989	MOK_frag	9704	6878
AMBRA1	8012	6860	NEDD4L	6403	3629
APOE	21203	16693	NKD1	9704	11466
ARHGAP9	10731	9743	NRIP2	25861	15739
ARHGEF16	18118	11469	OSBPL11	5264	7362
ARHGEF28	11704	4829	OXSR1	7841	5504
BAG6	12208	9071	PCGF2	54672	20813
BC104209_frag	8765	2244	POP4	6915	2362
BTRC	16970	25244	POP7	36623	41687
C19orf60	11917	7805	PPP1R11	28130	10873
C1orf174	19107	9939	PRKAR1A	12519	22490
CCT2	9765	12434	PSRC1	9530	11084
CGGBP1	11603	8071	PYCRL	12719	7698
CMTM8	7676	7277	Q6nv61	7592	4396
CNKSR3	8636	6376	RAP1GDS1	7008	3415
CTDP1	6046	2958	REPS1	11539	8017
EFHC1	4031	6596	RUVBL2	10638	23905
FAM168A	6526	8505	SFMBT2	11883	8835
FAM175A	7303	9687	SHANK2	13679	6872
FXN	4651	13720	SHQ1	13542	6792
GAB1	40356	24617	SLFN5	12170	11843
GAB2	13214	15794	SMARCC1	20593	8829
GMPPA	10738	7676	SORBS1	19637	27454
GPT2	10673	10289	SPATA33	9097	4174
HEATR3	19422	16230	STOX1	19985	3812
ISCA2	19011	24634	TBCE	10487	7903
ITPKB	28286	23100	URI1	8929	11269
KLRC1	19223	27670	USP50	8822	3849
MAGEB1	21621	8193	WDR53	5882	6716
MARK4	9322	7084	YIF1B	15718	6599
MEF2D	44534	38883	ZC3HAV1	14578	6348

B

p473 selective hits	pS473	O-GlcNAc S473
PPM1H	14087	1230
NCKIPSD	6486	1020
OFD1_frag	15273	4028
TRIT1	9889	3722

Figure S12: List of common hits from protein microarrays

Data from GenePix was analyzed as described in Methods section. Numbers on the columns correspond to the average intensity for each pair (duplicate experiments) is shown for each microarray assayed with either Akt pS473 or O-GlcNAcS473. This value was initially used to identify proteins with high intensity but ultimately visual inspection of the scanned microarrayed confirm if a specific protein was a selective hit or not. A) Table contains list of 63 proteins in alphabetical order that are hits for both Akt with phospho and O-GlcNAc at Ser473. B) Table contains list of 4 proteins that are selective hits for pS473 Akt.

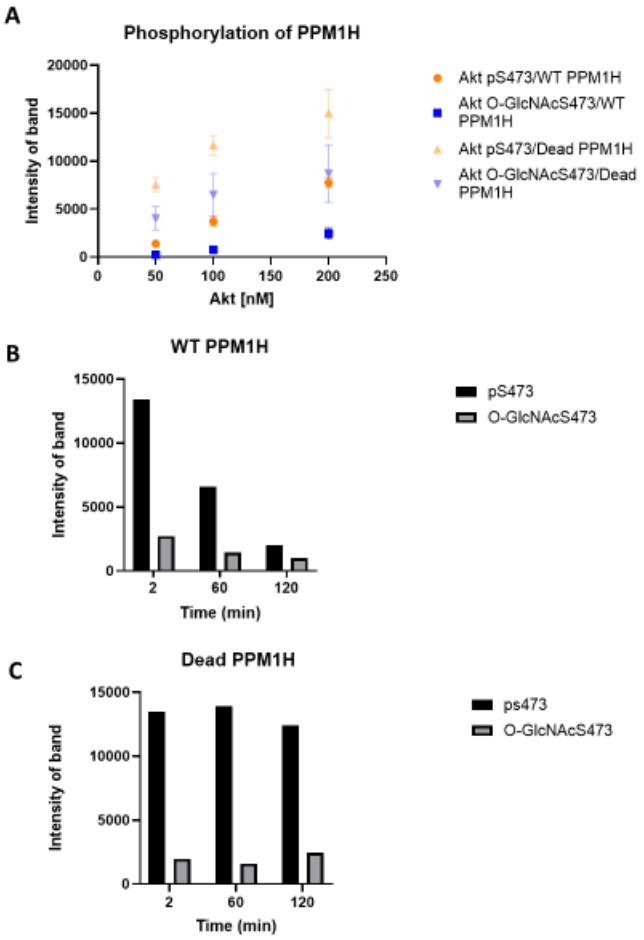

Figure S13: Quantification of PPM1H phosphorylation

A) Quantification of Akt phospho substrate western blots showed concentration dependent phosphorylation of WT and Dead (H153D) PPM1H by Akt with pS473 or O-GlcNAcS473.(Figure 5A). B and C) Quantification of relative western blot intensity with respect to control for kinase assays with preincubation of Akt and either WT PPM1H (B) or Dead (H153D) PPM1H (C) performed for 2, 60 and 120 minutes at 22 °C as shown in Figure 6A or Figure 6B, respectively. (n=2)

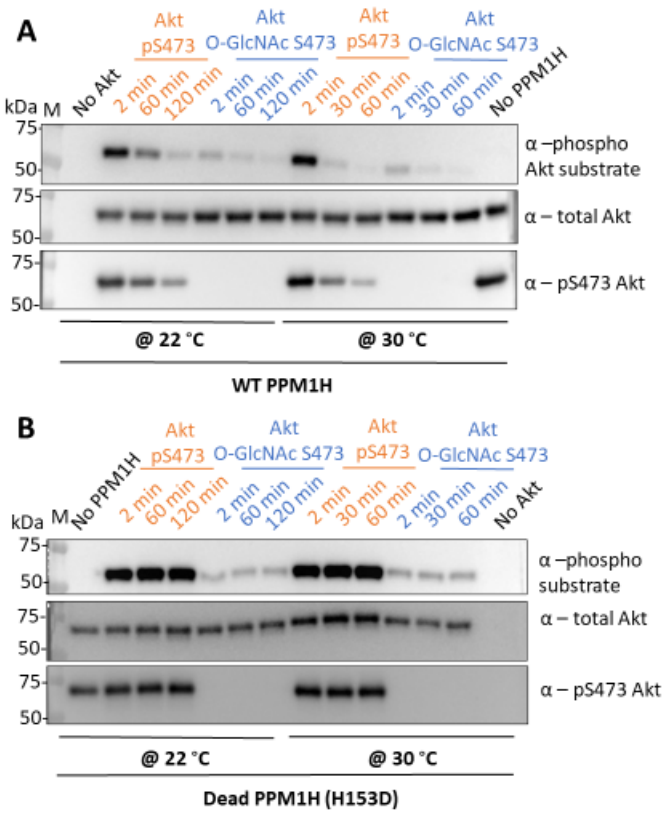

Figure S14: Effects of pre-incubation with PPM1H on Akt's activity

Western blot analysis of kinase assays using pSer473 Akt (A1) and O-GlcNAcSer473 Akt (A8) at 100 nM and 1 µM of (A) WT PPM1H or (B) Dead (H153D) PPM1H as a substrate. Preincubation of Akt forms and PPM1H in the absence of ATP was performed for 2, 60 and 120 min at 22 °C or 2, 30 and 60 min at 30 °C. Subsequently, the kinase reaction was initiated by adding 2 mM ATP. Assays were carried out 30 °C for 20 minutes. 250 ng of PPM1H was loaded per well. Membranes were blotted with phospho Akt substrate antibody (R/KXR/KXXS*/T*) (1:1000), total Akt antibody (1:5000) and phosho Ser473 antibody (1:5000). Assays were performed at least twice (n=2).

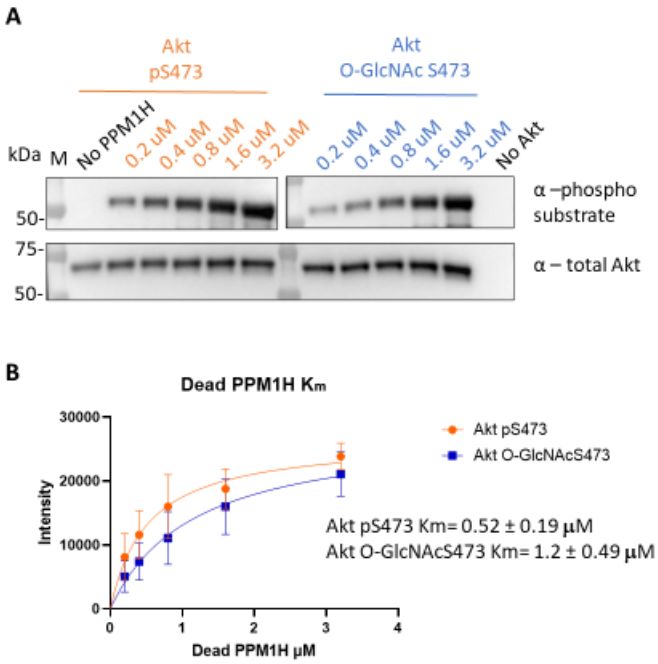

Figure S15: Kinase assays to measure Akt K_m for PPM1H.

A) Western blots showing kinase assays of Akt with pS473 and O-GlcNAc S473 using catalytically inactive PPM1H (H153D). B) Plot of quantification of phospho substrate antibody band intensities. K_m for Akt phospho and O-GlcNAc Ser473 are shown with standard errors. (n=2)

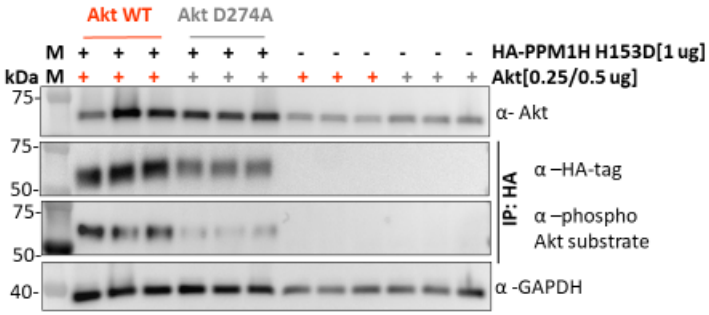

Figure S16: Transfection of Akt WT and D274A and PPM1H to HCT116 Akt1/2 KO cells

Western blot analysis of HCT116 Akt1/2 KO cells transfected with either WT (left) or D274A (catalytically dead, right) Akt, with or without PPM1H showing three different replicates. Anti-Akt (1:2000) and Anti-GAPDH (1:5000) were blotted from cell lysates. Anti-HA-tag (1:1000) and Anti-phospho Akt substrate (1:1000) were blotted from lysates that were immunoprecipitated with HA-tag magetic beads (see Methods)

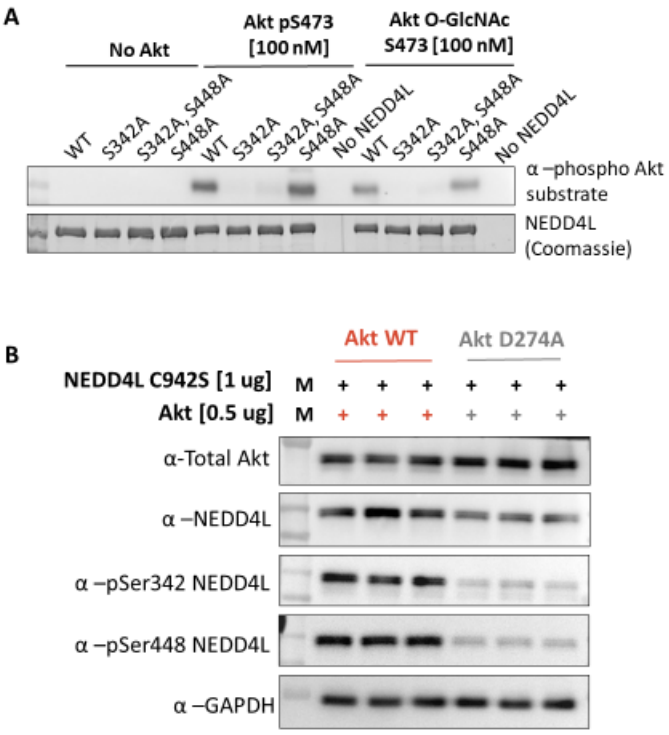

Figure S17: NEDD4L phosphorylation by Akt

A) Western blot of kinase assays using WT, S342A, S448A and S342A/S448A NEDD4L as substrate for Akt with phospho or O-GlcNAc at S473. Note that phospho Akt substrate antibody fails to recognize phosphorylation at S448, but site-specific antibodies allowed us to show this site can also be phosphorylated by Akt. (Figure 7A). B) Western blot analysis of HCT116 Akt1/2 KO cells transfected with either WT (left) or D274A (catalytically dead, right) Akt and NEDD4L showing three different replicates. Anti-Akt(1:2000), pS342 NEDD4L (1:2000), NEDD4L (1:2000), pS448 NEDD4L (1:3000), Anti-GAPDH (1:5000). Data from this image is represented in Figure 8C.

Name	Oligonucleotide 5' to 3'
NEDD4L S342A-F	CAAGGTTGAGGTCATGCGCAGTCACCGACGCAG TTGCAG
NEDD4L S342A-R	CTGCGTCGGTGACTGCGCATGACCTCAACCTTGA GGAGG
NEDD4L S428A-F	GGCCTCGTAGCCTCGCCTCGCCAACAGTAACTTT ATCTGCCCC
NEDD4L S428A-R	GATAAAGTTACTGTTGGCGAGGCGAGGCTACGA GGCCGGC
PPM1H S124A-F	CCAAGAGACGGTCCGCCCTTCCCAATGGGGAAG GG
PPM1H S124A-R	CCCATTGGGAAGGGCGGACCGTCTCTTGGATGA GTTCC
Akt T450A_F	CCCAGATGATCACCATCGCACCACCTGACCAAGATG ACAGC
Akt T450A_R	CATCTTGGTCAGGTGGTGCGATGGTGATCATCTGGG CCGTG
Akt T450D_F	CCCAGATGATCACCATCGACCCACCTGACCAAGATG ACAGC
Akt T450D_R	CATCTTGGTCAGGTGGGTCGATGGTGATCATCTGGG CCGTG
Akt T450E_F	CCCAGATGATCACCATCGAGCCACCTGACCAAGATG ACAGC
Akt T450E_R	CATCTTGGTCAGGTGGCTCGATGGTGATCATCTGGG CCGTG

Table S1: Primers used to mutate NEDD4L and PPM1H

WT NEDD4L and PPM1H were expressed and purified along with mutants S342A, S448A, S342A/S448A (NEDD4L) and S124A (PPM1H). For pT450 validation experiments in HCT116 Akt 1/2 KO we made the following mutations T450A, T450D, T450E using the primers with sequences indicated above.

METHODS

Peptide synthesis

Peptides were synthesized using standard Fmoc solid-phase strategies with preloaded Wang resins as solid support. Those peptides corresponding to residues 460-480 of Akt (CVDSERRPHFPQFSYSASGTA) were synthesized on a Prelude peptide automated synthesizer (Protein Technologies, Inc.) and mono-benzyl protected phospho-residues were installed manually. Residues were double coupled using 5 equivalents of Fmoc-protected amino acids in dimethylformamide (DMF), 4.8 equivalents of 1-[Bis(dimethylamino)methylene]-1H-1,2,3-triazolo[4,5-b]pyridinium 3-oxid hexafluorophosphate (HATU) in 0.4 M N-Methylmorpholine (1.5 hours for standard amino acids and 3 hours for phospho-amino acids)[2]. GSK3 peptide substrate corresponding to residues 14-27 of GSK3 (RSGRARTSSFAEPGGK) was synthesized using N-ε-biotin-lysine Wang resin (Iris Biotech GmbH) as described previously[2]. O-GlcNAcylated peptides were synthesized by coupling reactions that utilize Fmoc-protected amino acids (5 equiv.), 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate) (5 equiv.) and *N*,*N*-diisopropylethylamine (10 equiv.) in dimethylformamide incubated for 1 h. For glycosylated serine or threonine residues, 2 equiv. pentafluorophenyl-activated monomer in 3 mL of dimethylformamide were coupled overnight[3,4]. Peptides were cleaved from resin with trifluoroacetic acid: water: triisopropylsilane (95:2.5:2.5, v/v/v) or trifluoroacetic acid: phenol: water: thioanisole:1,2-ethanedithiol (82.5:5:5:5:2.5, v/v/v/v/v) for 2.5 to 4 hours at room temperature, then precipitated with cold diethyl ether and purified using reverse-phase C18 HPLC using a gradient of water: acetonitrile containing 0.05% trifluoroacetic acid. Pure fractions (>90%) were combined and concentrated on a rotavap, lyophilized, and stored at −20 °C until use. The correct peptide structures were confirmed using matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) or electrospray ionization mass spectrometry (ESI-MS). Peptide concentrations were determined by weight or amino acid analysis.

Molecular Cloning and Mutagenesis

The human Akt gene from pcDNA3-Flag-HA-6xHis-Akt (Addgene, # 9021) was subcloned into a modified pFastBac1 plasmid containing the gyrase MxeIntein and chitin binding domain (CBD). QuickChange PCR (Agilent Technologies) was used to produce Akt mutants as described previously[2]. Akt (aa2-459)-MxeIntein-CBD fusion protein was expressed in SF9 insect cells. All proteins expressed were based on human sequences. DNA primers used to mutate NEDD4L and PPM1H as well as those used to mutate Akt at T450 are shown in Table S1. The open reading frames of all wild-type (WT) and mutant constructs were verified by DNA sequencing.

Kinase assays using GSK3 peptide substrate

The kinase parameters of semisynthetic Akt constructs were determined by phosphorylating N-ε-biotin-lysine GSK3 peptide (RSGRARTSSFAEPGGK) in radiometric reactions as previously described[2,5]. Radiometric kinase assays were carried out in 1.5 mL plastic tubes in a 25 µL reaction mixture containing 50 mM HEPES pH 7.5, 10 mM $MgCl_2$, 1 mM EGTA, 2 mM DTT, 1 mM sodium orthovanadate, 0.5 mg/ml BSA (kinase reaction buffer), 0.42 µCi ^{32}P γ-ATP

and varying amounts of biotinylated GSK3 peptide substrate (0-20 μM) and ATP (0-2 mM). Reactions were initiated by the addition of Akt and were performed at 30 °C for 10 min. The kinase reactions were quenched by adding 20 μL of 100 mM EDTA and then 10 μL of 10 mg/ml Pierce avidin (Thermo Scientific) was added to each sample and incubated for 20 min at room temperature. Samples were transferred to centrifugal filtration units (Nanosep 10kDa cutoff, PALL) and washed five times with 100 μL of washing buffer (0.5 M sodium phosphate, 0.5 M NaCl, pH 8.5). The filtration units were placed in 10 mL scintillation fluid and beta emission was measured using a Beckman liquid scintillation counter (Beckman LS6500). Background levels without Akt or peptide substrate were in the range of 300-600 cpm and points including enzyme and substrate were above background. All measurements were made at least twice and replicates generally agreed within 20%.

Kinase assays using protein substrates

Kinase assays were carried out in 1.5 mL microcentrifuge tubes in a 25 μL reaction mixture containing 50 mM HEPES pH 7.5, 10 mM $MgCl_2$, 1 mM EGTA, 2 mM DTT, 1 mM sodium orthovanadate, 0.2 mg/mL BSA (kinase reaction buffer), 2 mM ATP, 1 μM of substrate and varying amounts of Akt (indicated in figure legends). Reactions were initiated by the addition of Akt and were performed at 30 °C for 5 or 20 minutes. The kinase reactions were quenched by adding 8.3 μL of 4x SDS loading buffer and boiled for 5 minutes. Samples were loaded in a 12% SDS-PAGE (ng of protein loaded are indicated in each figure legend) and transferred onto a nitrocellulose membrane as described in our Western blot protocol above. Akt phospho-substrate antibody (K/RXK/RXXS*/T*) was diluted 1:1000, Akt (pan) antibody 1:5000 (CST C67E7) and phoshoSer473 antibody 1:5000 (CST), all in 5% BSA solution.

Expression and purification of the C-terminally truncated Akt

The C-terminally truncated Akt (aa 2-443) with the N-terminal Strep-tag was expressed using the baculovirus-insect cell system described above. The cells from 1 L culture were resuspended in the lysis buffer and lysed in a 40 ml Dounce homogenizer, and then clarified as described above. The resulting lysate was passed through a column containing 5 mL Strep-Tactin resin (IBA Lifesciences), and the resin was washed five times with 5 mL of washing buffer (50 mM HEPES pH 8.0, 300 mM NaCl, 1 mM EDTA). The C-terminally truncated Akt was eluted with 20 ml of the washing buffer containing 2.5 mM desthiobiotin and 1 mM PMSF, and then further purified by size exclusion chromatography on a Superdex 75 column (Cytiva) with the Akt storage buffer. The purified fractions were combined, concentrated, and then stored at -80 °C with a final concentration of 1 mg/mL.

Kinase assays on protein microarrays

HuProt v3.2 human protein microarrays (CDI Laboratories) were used to identify potential substrates for Akt with different PTMs in the C-tail (pSer473, O-GlcNAcS473)[6]. Microarrays were blocked in TBS-T containing 5% BSA for 1 hour at room temperature in a slow shaker, removed from TBS-T and incubated for 90 minutes at 30 °C with 0.5 μM Akt, 5 mM ATP, 0.08 mg/mL BSA and 1 mM DTT in 120 μL reaction buffer (50 mM HEPES pH 7.5, 12 mM $MgCl_2$, 1 mM EGTA and 0.01% NP-40). The reaction was quenched by the addition of buffer containing:

50 mM HEPES pH 7.5, 1 mM EGTA and 0.01% NP-40, 100 mM EDTA and 500 mM NaCl, and washed 3 times for 10 minutes in a slow shaker. Subsequent washes were performed for 10 min and 3 times for each of the following conditions: 1% SDS in reaction buffer, 1% SDS in TBS-T and TBS-T. Microarrays were incubated in TBS-T containing 5% BSA with Akt phosphorylated substrate antibody recognizing K/RXK/RXXS*/T* (Lifespan #S-C63885) in a 1:500 dilution overnight at 4 °C. Microarrays were washed 4 times for 10 minutes with TBS-T and incubated with secondary antibody Alexa-Fluor 555 (Thermo Fisher Scientific # A21428) at 1:2000 in TBS-T with 5% BSA for 90 minutes. Then, microarrays were washed 4 times for 10 minutes with TBS-T, water. Microarrays were centrifuged at 1000 rpm for 2 minutes and allowed to dry for 5 minutes away from the light. Then, they were scanned in a GenePix 4400 Microarray Scanner (Molecular Devices). Raw data were processed to determine positive hits, which were selected based on signal intensity (value of the median foreground intensity minus the median background intensity) using a cutoff value of 3 standard deviations (s.d.) above the median. Since experiments were carried out twice and each microarray has a pair of spots that represent each protein, 3 out of the 4 spots had to display a signal above the threshold for a protein to be considered a positive hit. Proteins that also appeared as positive hits in the control microarrays with no Akt were eliminated from the final list. Selective hits were so-classified when they only appeared in either the Akt phosphoS473 or O-GlcNAcSer473 microarrays, but not in the control microarray without Akt. Computationally identified selective hits were also visually inspected to compare the intensity of the spots and verify if in fact the protein was preferentially phosphorylated by either form of Akt. Values representative of the raw data were uploaded in GEO (accession number pending) and normalized according to CDI recommendations. We used the following formula: $z = [\alpha - \alpha(avg)] / \alpha(std)\ \alpha(avg)]$; where α is the signal intensity (median foreground intensity minus median background intensity), $\alpha(avg)$ is the average signal intensity and $\alpha(std)$ is the standard deviation for all spots on the microarray.

Expression and Purification of PRAS40, PPM1H, NEDD4L and OGT

PRAS40: *E. coli* BL21 Codon Plus or Rosetta pLysS were transformed with GST-PRAS40, kindly provided by Dr. Do-Hyang Kim. PRAS40 was expressed in by inoculating 1 L of LB media with 100 mg/mL Amp with 5 mL of an overnight culture. Cells were cultured in a floor shaker at 37 °C until the OD reached 0.6 then induced with 0.5 mM IPTG and cultured in a floor shaker at 12 °C for 20 h. Cells were harvested and spun at 5000 xg, for 10 min at 4 °C.

Cells were resuspended in lysis buffer (50 mM HEPES pH 7.5, 150 mM NaCl, 1 mM EDTA, 10% glycerol, 0.1% Triton X-100, 1mM PMSF, 5 mM DTT) containing one dissolved protease inhibitor tablet (Thermo Fisher Scientific) and lysed by passing the lysate through a french press 3 times. The lysate was centrifuged (17,500g, 40 min, 4 °C) and the supernatant was passed through a column with 3 mL glutathione resin (Thermo Fisher Scientific) at 4 °C. The resin was washed 10 times with storage buffer (50 mM HEPES pH 7.5, 150 mM NaCl, 2 mM beta-mercaptoethanol, 0.5 mM PMSF, 1 mM EDTA, 10% glycerol) to remove Triton X-100 and then eluted with 50 mM glutathione in 10 mL storage buffer. GST was cleaved using PreScission Protease and samples were dialyzed overnight in a 12-14 kDa cutoff membrane (Spectrum Chemical) in 1 L of 50 mM HEPES pH 7.5, 100 mM glycine, 0.5 mM PMSF, 10% glycerol, 0.5 mM PMSF, 5 mM DTT. To

remove the cleaved GST tag, proteins were loaded onto glutathione resin twice. Then, protein was concentrated by ultrafiltration in 10 kDa cutoff Amicon to <200 µL then aliquoted, frozen, and stored at –80 °C.

NEDD4L: *E. coli* BL21 Codon Plus or Rosetta pLysS were transformed with pGEX-6P-2 GST- NEDD4L with a C2 (1-153aa) and (356-375aa) deletion. NEDD4L was expressed by inoculating 1 L of LB media with 100 mg/mL Amp with 5 mL of an overnight culture. Cells were cultured in a floor shaker at 37 °C until the OD reached 0.6 and then induced with 0.5 mM IPTG and cultured in a floor shaker at 16 °C for 18 hours. Cells were harvested and spun at 5000g, for 10 minutes at 4 °C. Cells were resuspended in lysis buffer (25 mM Tris-HCl pH 8.0, 250 mM NaCl, 1 mM PMSF and 1x Pierce (Thermo Fisher Scientific) protease inhibitor tablet), transferred to a 50 mL conical and lysed by passing the lysate through a French press 3 times. Lysed cells were spun at 15,000g for 40 minutes at 4 °C and the clear lysate was loaded on a column with 2 mL of glutathione resin. The resin was washed with 20 mL lysis buffer with 0.1% Triton twice and with 10 mL lysis buffer without Triton five times. NEDD4L protein was eluted from the column with 10 mL elution buffer (10 mL lysis buffer with 50 mM glutathione). PreScission Protease was added before dialysis (5 µL of 500 U stock) and samples were dialyzed in a 12-14 kDa cutoff membrane (Spectrum Chemical) overnight into 25 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM TCEP and buffer was changed for an additional 3 hours on a stir plate at 4 °C. To remove the cleaved GST tag, proteins were passed through glutathione resin twice. Then, the protein was concentrated by ultrafiltration using a 30 kDa cutoff Amicon filter to less than 1 mL and purified by FPLC using a size exclusion column Superdex 200 (Cytiva). A single monomer peak was observed at 12 mL and fractions were collected and concentrated to <200 µL then aliquoted, frozen, and stored at –80 °C.

PPM1H: *E. coli* BL21 Codon Plus or Rosetta pLysS were transformed with pET15b 6HIS SUMO PPM1H WT or H153D, which were kindly provided by Dr. Dario Alessi. PPM1H was expressed by inoculating 1 L of LB media (Sigma Aldrich) with 100 µg/mL Amp with 5 mL of an overnight culture. Cells were cultured in a floor shaker at 37 °C until the OD reached 0.6 and then induced with 0.5 mM IPTG and cultured in a floor shaker at 16 °C for 18 hours. Cells were harvested and spun at 5,000g, for 10 min at 4 °C. Cells were resuspended in lysis buffer (50 mM Tris/HCl pH7.5, 150 mM NaCl, 0.1% Triton, 2 mM $MnCl_2$, 0.5 mM TCEP (tris(2-carboxyethyl)phosphine)), 1 mM Pefabloc (4-(2-aminoethyl)-benzene-sulfonyl fluoride) and 1 mM benzamidine) transferred to a 50 mL conical and lysed by passing the lysate through a french press 3 times. Lysed cells were spun at 15,000g for 40 min at 4 °C and the clear lysate was loaded on a column with 2 mL of cobalt-agarose resin (Amintra Cobalt NTA Affinity Resin, Expedeon). The column was washed with 40 mL of high salt wash buffer (50 mM Tris/HCl pH 7.5, 500 mM NaCl, 2 mM $MnCl_2$, 0.03% Brij 35, 20 mM imidazole, 0.5 mM TCEP), 10 mL low salt wash buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 2 mM $MnCl_2$, 10 mM Imidazole, 0.03% Brij 35, 0.5 mM TCEP) and protein was eluted with 10 mL elution buffer (10 mL low salt wash buffer with 500 mM imidazole) in two rounds, letting 5 mL elution buffer incubate on the resin for 10 min before slow elution. SENP sumo-protease was added before dialysis (5 µL) and samples

were dialyzed using a 12-14 kDa cutoff membrane overnight into 50 mM Tris-HCl pH 7.5, 150 mM NaCl, 2 mM $MnCl_2$, 0.5 mM TCEP and buffer was changed for an additional 3 hours on a stir plate at 4 °C. To remove cleaved His-SUMO tag, proteins were passed through a Co-NTA column twice. Then, the protein was concentrated by ultrafiltration using a 10 kDa cutoff Amicon filter to less than 1 mL and purified through FPLC using a Superdex75 (Cytiva). A single peak was observed at 12 mL and fractions were collected and concentrated to less than 200 µL and then aliquoted, frozen, and stored at −80 °C.

O-Linked N-Acetylglucosamine Transferase (OGT): OGT was prepared as previously described[7]. Chemically competent BL21(DE3) cells (GoldBio) were transformed by heat shock with pET24b-ncOGT plasmid encoding a hexahistidine-tagged ncOGT (nucleocytoplasmic OGT isoform). After selection on kanamycin plates (50 µg/mL final concentration), a single colony was inoculated to sterile Luria broth medium containing kanamycin and allowed to grow for 16 hours at 37°C. 10 mL of overnight culture were added to 500 mL of Terrific broth (EMD) supplemented with kanamycin, and cultured at 250 rpm at 37 °C. After reaching an OD_{600} of 0.6, the culture was cooled to 16°C. Expression was induced by adding IPTG to a final concentration of 0.5 mM and shaking at 16°C for 20 hours. Bacteria were pelleted by centrifugation (6000*g*, 10 mins, 4 °C) and the resulting pellets were resuspended in 10 mL of lysis buffer (100 mM Tris-HCl, 1 M NaCl, 1 mM Trition X-100, 2mM PMSF, 1 tablet Roche Complete Mini, EDTA-free Protease Inhibitor Cocktail Tablet, pH 7.5). Solid lysozyme was then added to a final concentration of 5 mg/mL and the slurry was left on ice for 30 min. The slurry was further homogenized by tip sonication (40% amplitude, 10 s pulse duration, 30 s off, 6 cycles) on ice. The lysate was cleared by centrifugation (20000*g*, 30 min, 4 °C) and added to 1 mL of pre-washed Co-NTA agarose beads (GoldBio). The beads were incubated with lysate for 1 hour at 4°C with gentle agitation. The lysate was allowed to drain through gravity-flow columns, and subsequently washed with 100 mL of wash buffer (25 mM Tris HCl, 0.5 M NaCl, 1 mM DTT, 20 mM imidazole, pH 7.5). Bound proteins were eluted using 5 mL of elution buffer (25 mM Tris HCl, 0.5 M NaCl, 1 mM DTT, 200 mM imidazole, pH 7.5). Fractions containing purified OGT were dialyzed (10 kDa MW cutoff) to 50 mM HEPES, 150 mM NaCl, 1 mM DTT, pH 7.5 for 2 hours before replacing the dialysis buffer and allowing further dialysis at 4°C overnight. Protein was then concentrated (Amicon Ultra 50 kDa cutoff, Millipore) and exchanged to storage buffer (50 mM HEPES, 150 mM NaCl, 1 mM DTT, 0.1 mM PMSF, 10% glycerol, pH 7.5) to a final concentration of 2.5 mg/mL. Protein was stored at −80°C before use. Typical yield: 1 mg/L Terrific broth at >90% purity by SDS-PAGE (see Figure S7B).

Fluorescence anisotropy

To determine the binding affinity of O-GlcNAcSer473 Akt and OGT with phosphatidyl inositol-3,4,5-triphosphate (PIP3), different amounts of Akt were mixed with 50 nM fluorescein-labeled soluble PIP3 (Cayman Chemical) in binding buffer (50 mM HEPES pH 7.5, 150 NaCl, 2 mM DTT, 0.05 mg/ml ovalbumin) and incubated at room temperature for 30 minutes. Fluorescence anisotropy spectra were recorded using a Multi-Mode Microplate Reader (Biotek

Instruments) at 23 °C. The K_d values were determined by fitting the data to the quadratic binding equation below, as previously reported[2,8,9].

b=K_d + *X* + Fixed

Y= Y0 - [(Y0 - Ymax)/(2*Fixed)] * [b - sqrt(b^2 - 4**X**Fixed)]

where X is the ligand concentration and Y is the anisotropy unit. Fixed represents the concentration of the fluorophore (0.05). All measurements were performed three times.

Western blots

After transferring proteins from SDS-PAGE to membranes using an iBlot2 device (ThermoFisher Scientific), the membranes were blocked at RT for 1 hour in 5% BSA in TBS-T buffer. Membranes were incubated with primary antibodies: Akt (pan) (CST 11E7), RL2 (ThermoFisher Scientific MA1-072), Akt pS473 (Abcam ab81283), Akt pT308 (CST #9275), Akt p450 (CST D25E6), Anti-pTyr (4G10 05-321x). Refer to figure legends or specific methods for titer information. overnight at 4 °C, washed 3 times of 5 min with TBS-T buffer, and then incubated with secondary HRP-linked antibody (rabbit CST #7074, or mouse CST #7076). Membranes were developed with Amersham ECL Western blotting detection reagents (Cytiva) or Clarity ECL Western Blotting Substrates (BioRad) and imaged using a GeneSys imaging. system.

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