Supporting information:

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

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Supporting experimental procedures

Bacterial strains and plasmids

The human *akt1* gene was purchased from Harvard PlasmidID repository service (Boston, MA, USA) and subcloned (*NdeI*/*BamH1*) into an IPTG (isopropyl β-D-1-thiogalactopyranoside) inducible T7 promoter driven expression vector (pDS1) (1). In addition, the *akt1* gene was subcloned (*NcoI*/*NotI*) into T7*lac* 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(2). 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) (3). The system is encoded on the pDS-pSer2 plasmid (described previously (1)), 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 μg/ml kanamycin and 100 μg/ml ampicillin. A single colony was used to inoculate 70 ml of LB (with ampicillin 100 μg/ml, 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 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 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 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 μg/ml) was used as the antibiotic to maintain pCDF. In expressing phosphomimetic proteins, ampicillin at a concentration of 100 μg/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 $CaCl_2$) 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 quadruple 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.

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

Supporting Figures

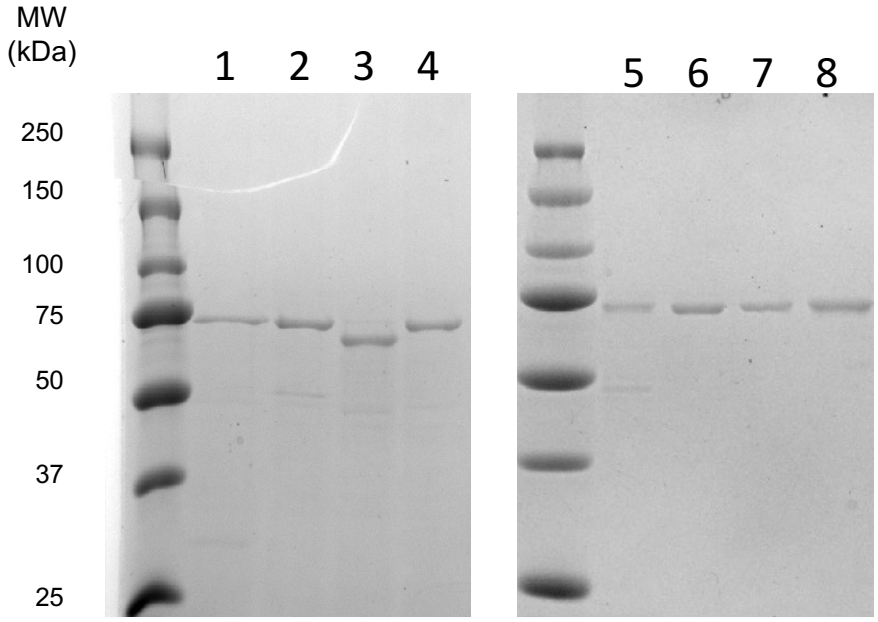

Figure S1. **Production of purified full-length Akt1 and pAkt1 variants.** Following affinity chromatography, purified fractions from size exclusion chromatography are visualized on SDS-PAGE. The Coomassie stained gel shows successful purification of full-length Akt1 variants: un-phosphorylated Akt (lane 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 S4).

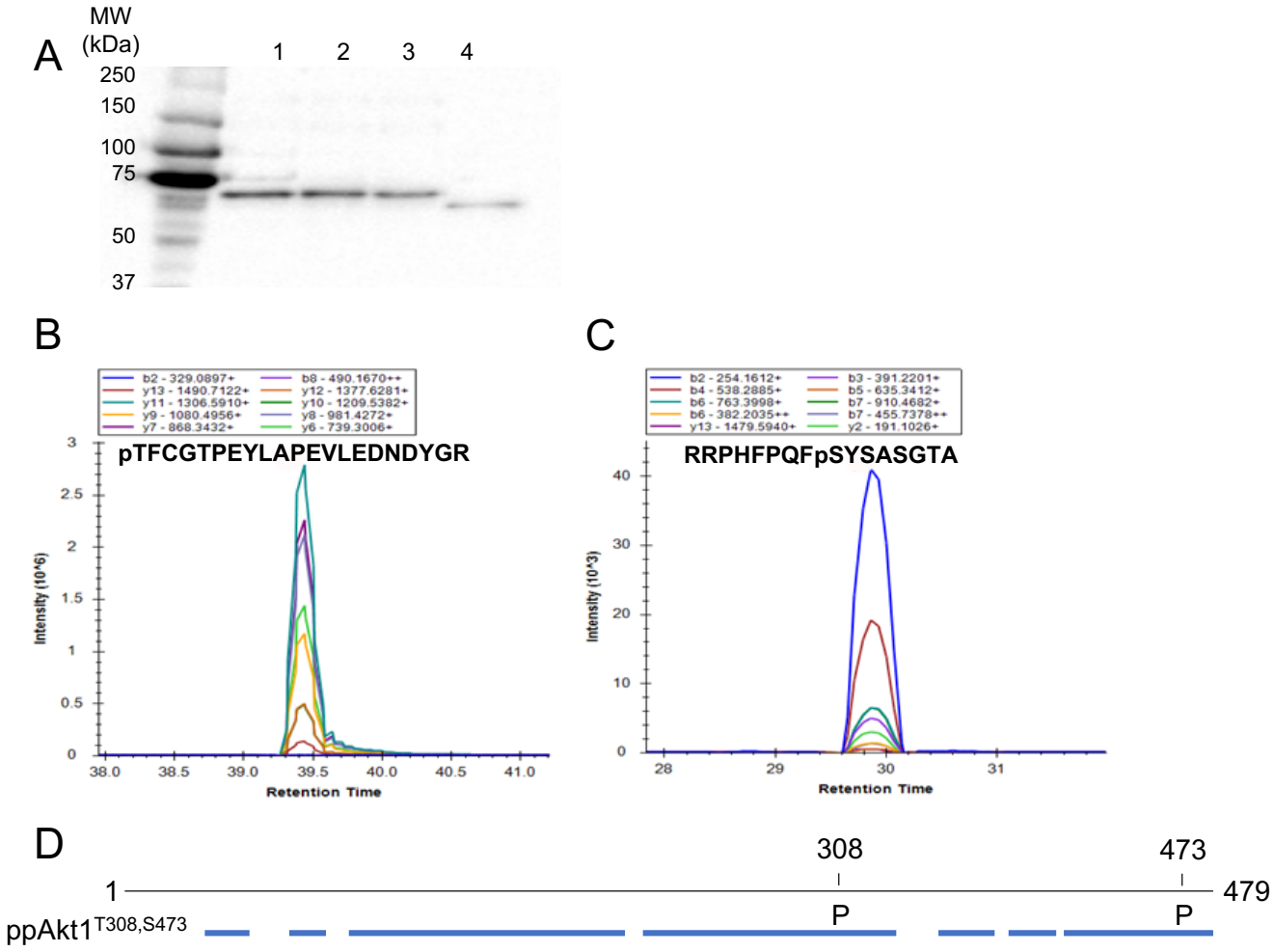

Figure S2. Physical characterization of ppAkt1$^{T308, S473}$. (A) Western blots of His$_6$-tagged un-phosphorylated Akt1 (lane 1), pAkt1^{T308} (lane 2), pAkt1^{S473} (lane 3), and ppAkt$^{T308, S473}$ (lane 4) with anti-his antibody. Mass spectra confirming enzymatically phosphorylated ppAkt1^{T308,S473}. PRM-MS/MS was used to identify peptides with pT308 (B) and pS473 (C) in tryptic-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).

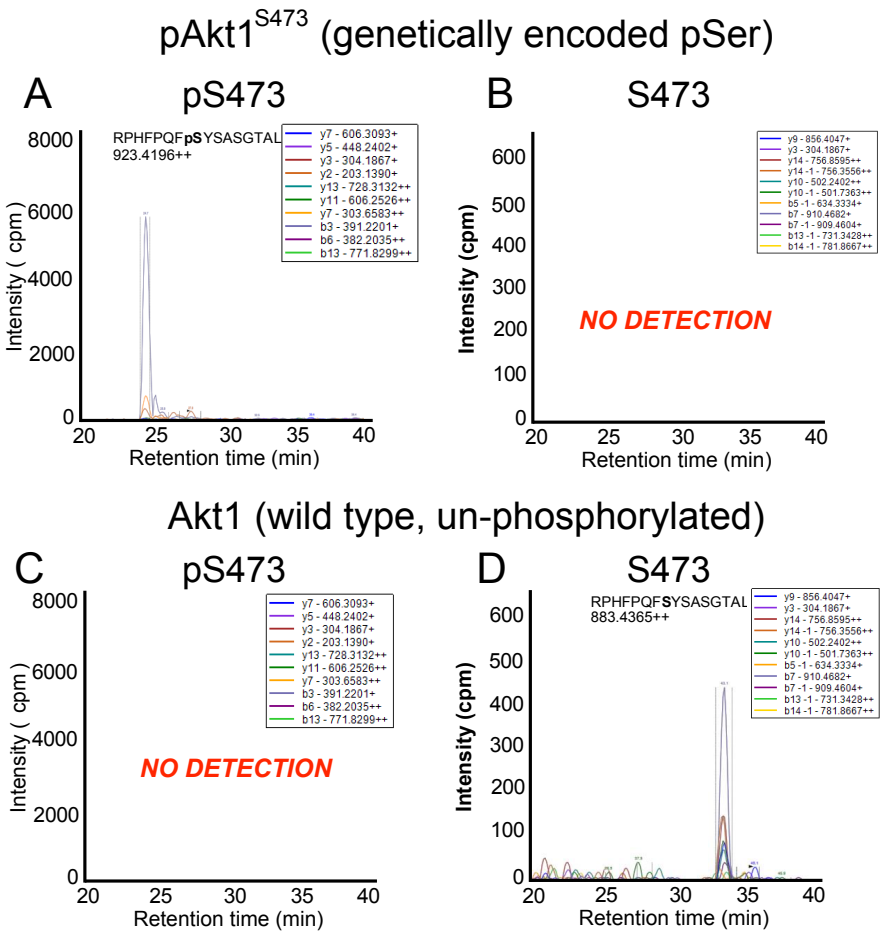

Figure S3. **Mass spectra confirming quantitative, genetically encoded pS473 in Akt1.** MRM-MS/MS was used to identify pS473 (A) but not Ser473 in a tryptic digested sample of pure pAkt1^{S473} (A, B). MRM-MS/MS also identified a peptide containing S473 (D) but not pS473 (C) in samples of purified and un-phosphorylated Akt1 (C, D).

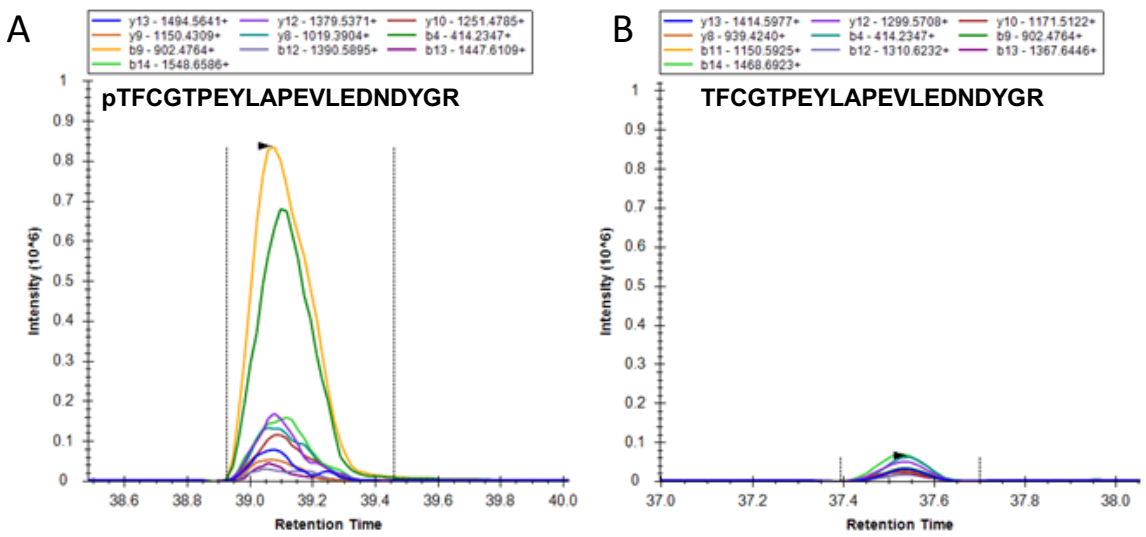

Figure S4. Physical characterization of pAkt1^{T308}. Mass spectra (A) confirming enzymatically phosphorylated pAkt1^{T308}. PRM-MS/MS was used to identify peptides in tryptic digested samples of pAkt1$^{T308.}$ Abundant occurrence of peptide containing pThr308 (A) and relatively very low levels of unphosphorylated form of peptide containing T308 (B) were identified.

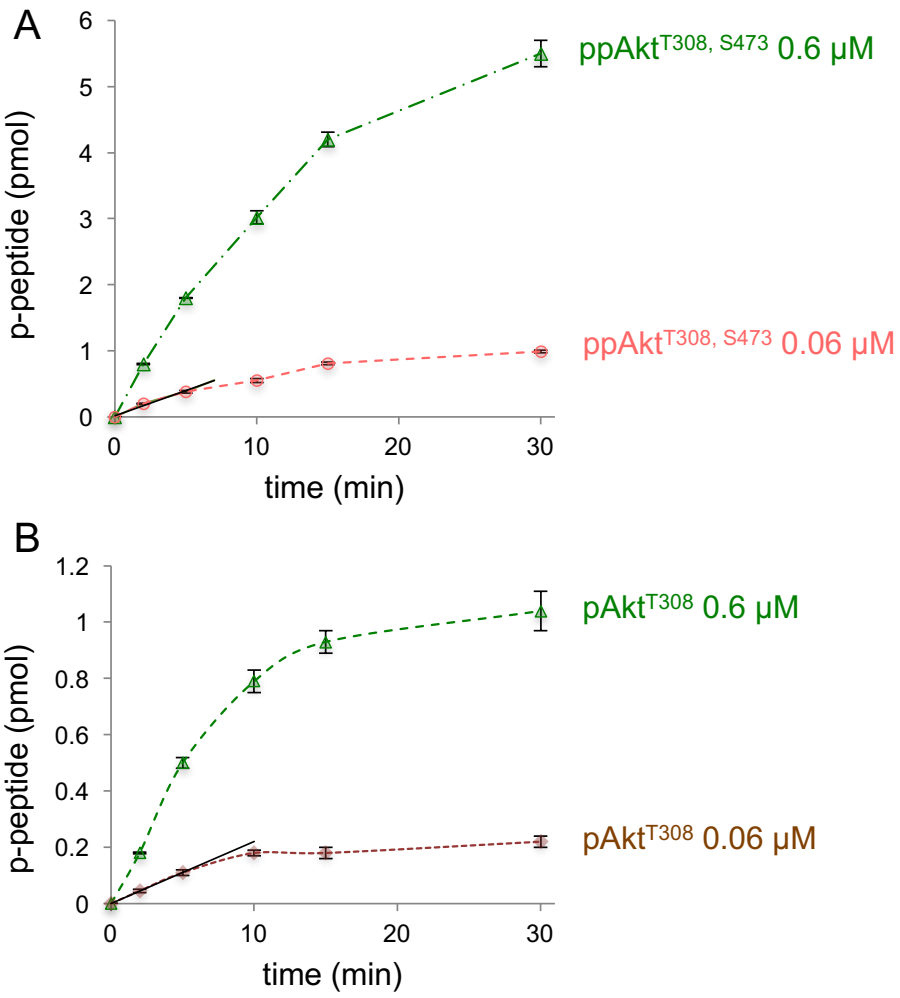

Figure S5. Activity of pAkt1^{T308} variants at reduced enzyme concentrations. A) ppAkt1 and B) pAkt1^{T308} enzyme concentrations were reduced by 10-fold in order to provide a sufficient linear phase to measure the reaction velocity accurately.

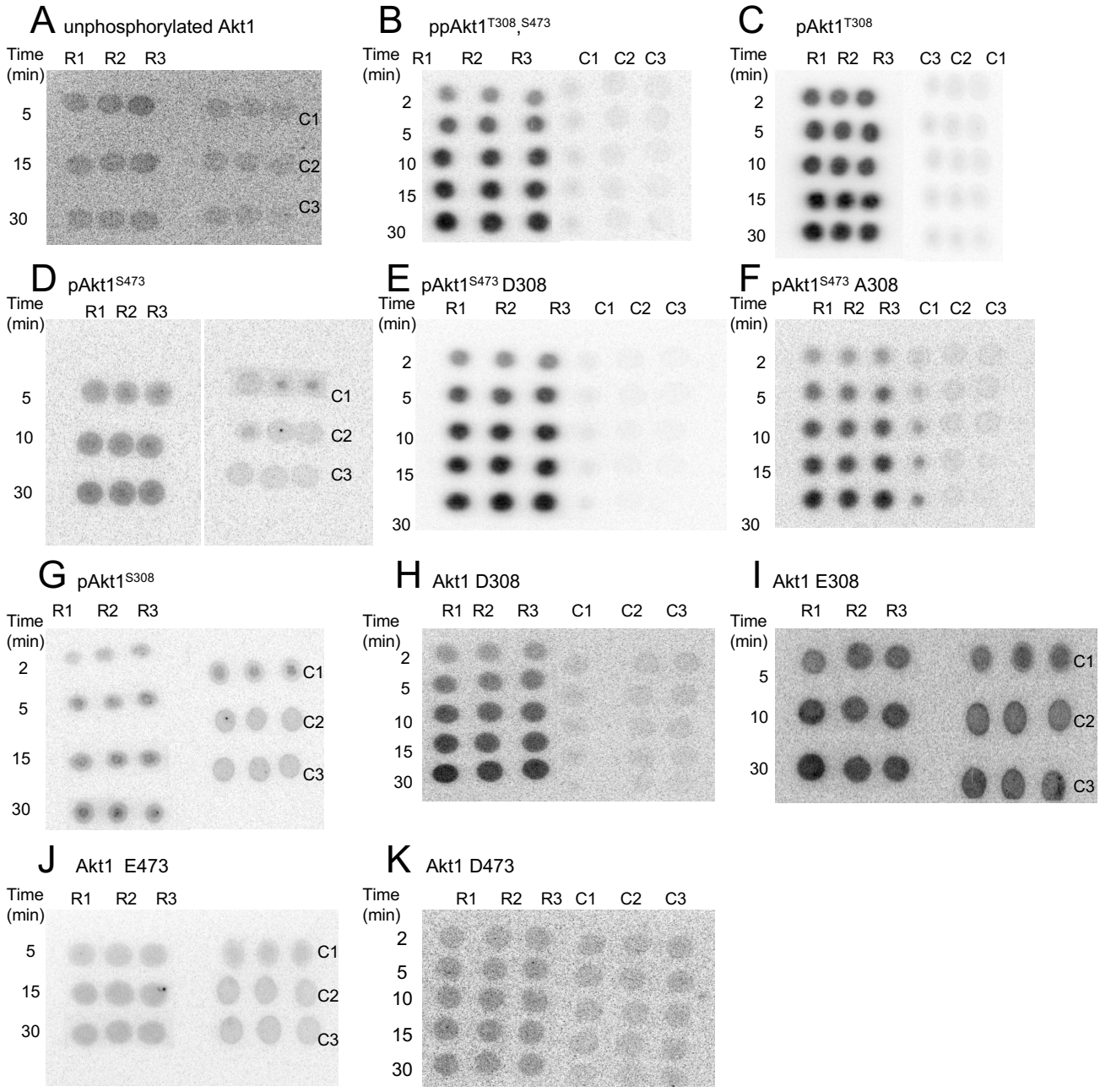

Figure S6. Autoradiographs of γ-[^{32}P]-ATP kinase assays with pAkt1 variants. Time courses are shown for reactions catalyzed by (A) unmodified Akt1, (B) ppAkt1^{T308,S473}, (C) pAkt1^{T308} (D) pAkt1^{S473} (E) pAkt1^{S473} D308 (F) pAkt1^{S473} A308 (G) pAkt1^{S308} (H) Akt1^{D308} (I) Akt1^{E308} (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.

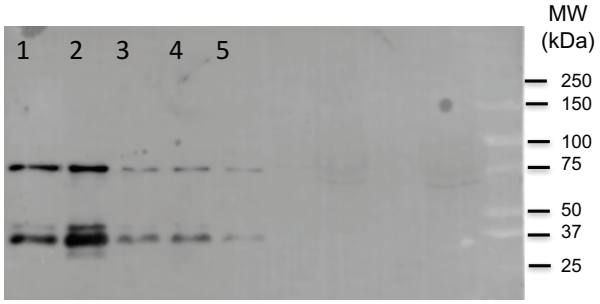

Figure S7. Production of pAkt1^{S308}. Fractions from affinity chromatography are visualized on SDS-PAGE by anti-His immunoblot. Elution gradient of pAkt1^{S308} (lanes 1-5) with increasing imidazole concentrations.

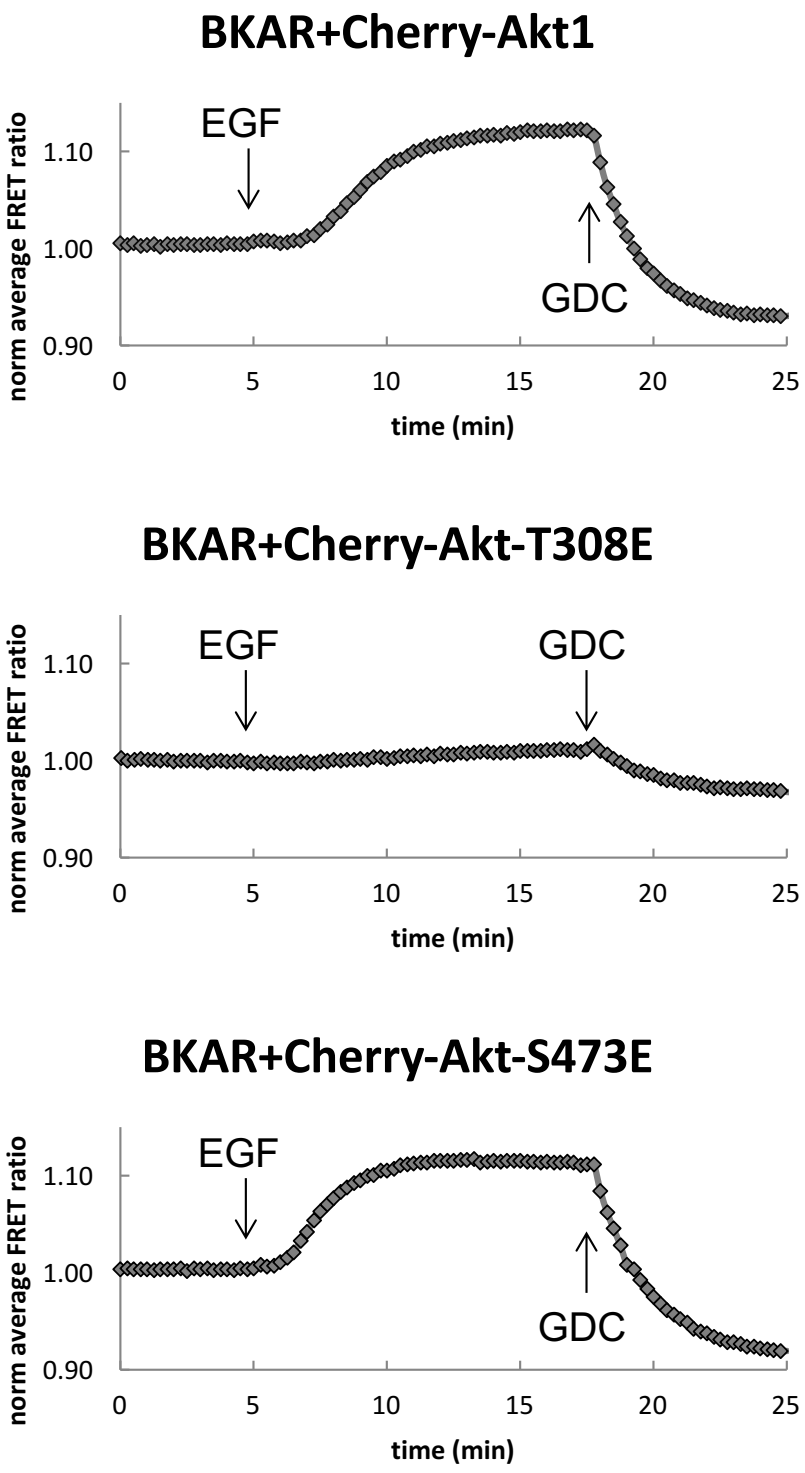

Figure S8. Cellular activity of Akt1 variants with glutamate substitutions. COS-7 cells co-expressing BKAR and equal levels of the indicated Cherry-tagged Akt were imaged and the CFP/FRET ratio examined following EGF stimulation and Akt inhibition with GDC 0068 (GDC). Mutation of Thr308 to Glu resulted 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.

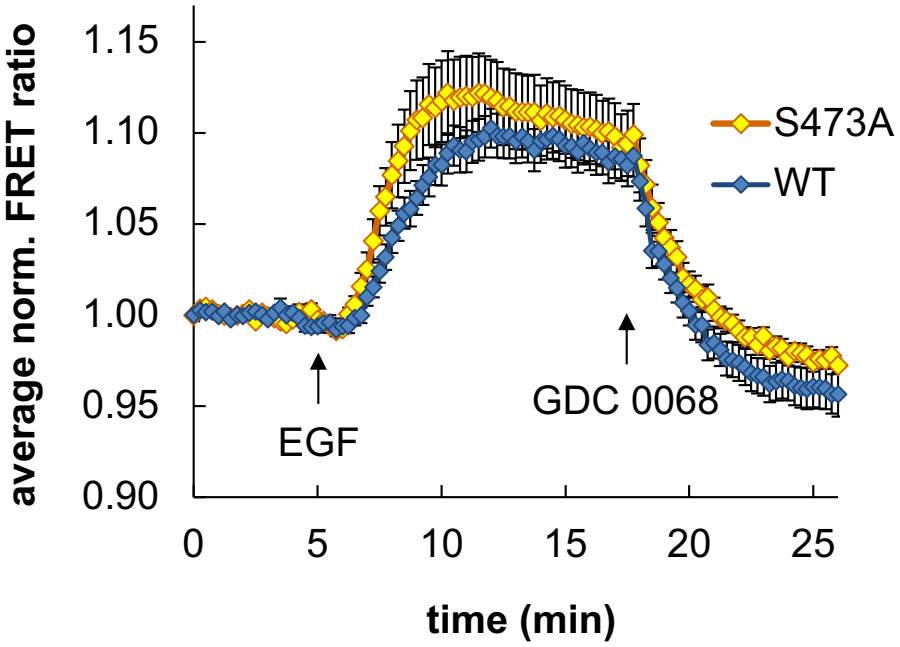

Figure S9. Cellular activity of phospho-ablated Akt1 variant S473A. Serum-starved COS7 cells expressing BKAR with minimally detectable levels of Cherry-tagged Akt were imaged during stimulation with EGF followed by treatment with the Akt inhibitor GDC 0068. The average FRET ratios for WT (blue) and S473A (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.