**Table S1.** Primers were used in this work, related to Figure 1.

| Primer | Sequence (5' - 3') |
|---|---|
| Akt (aa 2-459) cloning Forward (F) | CTTAAGGGATCCATGGACTACAAGGACGACGATGACAAGCTCG |
| Akt (aa 2-459) cloning Reverse (R) | CACCATACTAGTGCATCTCCCGTGATGCACTCCATGCTGTCATCTTGGTCAGGTGG |
| Akt (aa 144-459) cloning F | CCATCACGAAAACCTGTATTTTCAGAGCATGAACGAGTTTGAGTACCTG |
| Akt (aa 144-459) cloning R | GCTTCAGGTACTCAAACTCGTTCATGCTCTGAAAATACAGGTTTCGTG |
| Akt (aa 123-459) cloning F | GTCCCACCATCGGGCGCGGATCCATGGGGGCTGAAGAGATGGAGGTGTCCC |
| Akt (aa 123-459) cloning R | GGGACACCTCCATCTCTTCAGCCCCCATGGATCCGCGCCCGATGGTGGGAC |
| Akt-PH domain cloning F | CAGCGGACATATGAGCGACGTGGCTATTGTGAAGGAGGGTTGGCTGC |
| Akt-PH domain cloning R | GCCCCTACTAGTGCATCTCCCGTGATGCACCGGAAGTCCATCTCCTCCTCCTCCTGC |
| PDK1 cloning F | GCTTTAGATCTATGCATCACCATCACCATCACGGTGGCGGTGCCAGGACCACCAGCCAGC |
| PDK1 cloning R | CAAGAAGAATTCCTACTGCACAGCGGCGTCCGGGTGGCTCTGGTATCGC |
| Insert GST into PDK1 plasmid F | GTCCCACCATCGGGCGCGGATCTATGTCCCCTATACTAGGTTATTGG |
| Insert GST into PDK1 plasmid R | GGGATCCCCGGAATTCCCGGGTCATCACCATCACCATCACCATCACC |
| Akt-K142A,H143A,R144A-F | GGCTGAAGAGATGGAGGTGTCCCTGGCCAAGCCC**GCCGCTGCC**GTGACCATGAACGAGTTTGAGTACCTG |
| Akt-K142A,H143A,R144A-R | CAGGTACTCAAACTCGTTCATGGTCAC**GGCAGCGGC**GGGCTTGGCCAGGGACACCTCCATCTCTTCAGCC |
| Akt-Q218A-F | CAGCCCTGAAGTACTCTTTC**GCC**ACCCACGACCGCC |
| Akt-Q218A-R | GGCGGTCGTGGGT**GGC**GAAAGAGTACTTCAGGGCTG |
| Akt-R144A-F | CTGGCCAAGCCCAAGCAC**GCC**GTGACCATGAACGAGTTTGAG |
| Akt-R144A-R | CTCAAACTCGTTCATGGTCAC**GGC**GTGCTTGGGCTTGGCCAG |
| Akt-D274A-F | GAACGTGGTGTACCGG**GCC**CTCAAGCTGGAGAACC |
| Akt-D274A-R | GGTTCTCCAGCTTGAG**GGC**CCGGTACACCACGTTC |
| Akt-K142A,R144A-F | GGAGGTGTCCCTGGCCAAGCCC**GCC**CAC**GCC**GTGACCATGAACGAG |
| Akt-142A,R144A-R | CTCGTTCATGGTCAC**GGC**GTG**GGC**GGGCTTGGCCAGGGACACCTCC |
| Akt-H143A,R144A-F | GGAGGTGTCCCTGGCCAAGCCCAAG**GCCGCC**GTGACCATGAACGAG |
| Akt-H143A,R144A-R | CTCGTTCATGGTCAC**GGCGGC**CTTGGGCTTGGCCAGGGACACCTCC |
| Akt-Deletion in Linker-F | CAGTGACAACTCAGGG*TCCCTGGCCAAGCCCGCCGCTG |
| Akt-Deletion in Linker-R | GGCAGCGGCGGGCTTGGCCAGGGA*CCCTGAGTTGTCACTG |
| Akt-Insert 6 Glycines in linker-F | *GGGGGCGGGGGCGGGGGC*TCACCCAGTGACAACTCAGGG |
| Akt-Insert 6 Glycines in linker-R | *GCCCCCGCCCCCGCCCCC*GCCCGACCGGAAGTCCATCTCC |

DNA oligonucleotide primers used for mutagenesis and cloning. Bold indicates mutated bases, underline indicates restriction enzyme sites, italic indicates insertion and * indicates deletion.

**Table S2.** Amino acid sequence of synthetic peptides used in this study, related to Figure 1.

| ID | Sequence |
|---|---|
| Non-p C tail | CVDSERRPHFPQFSYSASGTA |
| 3p-Ser$^{473}$, Ser$^{477}$, Thr$^{479}$ C tail | CVDSERRPHFPQFpSYSApSGpTA |
| pSer$^{477}$/pThr$^{479}$ C tail | CVDSERRPHFPQFSYSApSGpTA |
| pSer$^{473}$ C tail | CVDSERRPHFPQFpSYSASGTA |
| pSer$^{477}$C tail | CVDSERRPHFPQFSYSApSGTA |
| pThr$^{479}$ C tail | CVDSERRPHFPQFSYSASGpTA |
| S473D C tail | CVDSERRPHFPQFDYSASGTA |
| Fluorescein-non-p C tail | CVDSERfKPHFPQFSYSASGTA |
| Fluorescein-pSer$^{473}$ C tail | CVDSERfKPHFPQFpSYSASGTA |
| pSer$^{477}$/pThr$^{479}$, Phe472Bpa, H468K(Biotin) C tail | CVDSERRPK(Biotin)FPQBSYSApSGpTA |
| GSK3-Biotin for kinase assay | RSGRARTSSFAEPGGK(Biotin) |
| GSK3 for crystal | GRPRTTSFAE |
| ATP-GSK3 bisubstrate | GRPRTT-Dap(ATP)-FAE |

fK: fluorescein coupled to N-ε-lysine

Dap: L-2,3-diaminopropionic acid

K(Biotin): Biotin coupled to N-ε-lysine

B: p-Benzoyl-L-phenylalanine

**Table S3.** Data collection and refinement statistics (molecular replacement), related to Figures 3 and 4.

| | AKT1 144-480<br>PDB ID 6BUU | AKT1 123-480<br>PDB ID 6CO1 |
|---|---|---|
| **Data collection** | | |
| Space group | $p2_1$ | $p2_1$ |
| Cell dimensions | | |
| $\alpha, \beta, \chi$ (Å) | 86.62,56.26,<br>91.83 | 86.32,56.09,<br>92.02 |
| $\alpha, \beta, \gamma$ (°) | 90.00,105.10,<br>90.00 | 90.00,104.56,<br>90. |
| Resolution (Å) | 88.67-<br>2.40(2.44-2.40) | 28.82-<br>2.12(2.17-2.12) |
| $R_{sym}$ or $R_{mergeRmeas}$ | 0.084 (0.18) | 0.101 (0.76) |
| *Rpim* | 0.046 (0.11) | 0.042 (0.31) |
| *CC1/2* | 0.953 | 0.996 |
| $I / \sigma I$ | 28.39(3.65) | 11.3 (2.0) |
| Completeness (%) | 94.40(61.9) | 98.50(81.8) |
| Total reflections | 96,011 | 326,363 |
| Unique reflections | 31,906 | 48,316 |
| Redundancy | 3.0(1.9) | 6.8 (6.6) |
| | | |
| **Refinement** | | |
| Resolution (Å) | 88.67-2.40 | 89.07-2.12 |
| No. reflections | 28,033 | 45,932 |
| $R_{work}$ / $R_{free}$ | 0.18/0.22<br>(0.21/0.29) | 0.18/0.23<br>(0.24/0.30) |
| No. atoms | | |
| Protein | 5,553 | 5,558 |
| Ligand/ion | 6 | 16 |
| Water | 231 | 504 |
| *B*-factors | | |
| Protein | 39.25 | 43.4 |
| Ligand/ion | 39.2 | 71.2 |
| Water | 33.3 | 45.7 |
| R.m.s. deviations | | |
| Bond lengths (Å) | 0.016 | 0.016 |
| Bond angles (°) | 0.002 | 0.002 |

*Number of crystals for each structure should be noted in footnote. *Values in parentheses are for highest-resolution shell. It has 96.86 in preferred regions with 2.04% in allowed regions; no incorrect chiral volumes are detected

**Table S4.** Analysis of MS/MS data by Crossfinder for FL-Akt1Phe472Bpa, pThr$^{308}$, 2pSer$^{477}$,Thr$^{479}$ with UV treatment, related to Figure 6.

| ID | M, calc | m/z, obs | charge | type | score | Sequence 1 | Sequence 2 |
|---|---|---|---|---|---|---|---|
| 39 | 829.5274 | 415.7703 | 2 | linear | 909 | SLLSGLLK | |
| 18 | 925.5386 | 463.7767 | 2 | linear | 1172 | HPFLTALK | |
| 26 | 974.5108 | 488.2626 | 2 | linear | 823 | LENLMLDK | |
| 43 | 1243.583 | 415.5349 | 3 | linear | 552 | TFHVETPEER | |
| 48 | 1272.606 | 637.3105 | 2 | linear | 1232 | VTMNEFEYLK | |
| 5 | 1346.712 | 449.9113 | 3 | linear | 509 | DLKLENLMLDK | |
| 15 | 1622.825 | 812.4213 | 2 | linear | 763 | FFAGIVWQHVYEK | |
| 9 | 1660.831 | 831.4239 | 2 | linear | 1377 | EEWTTAIQTVADGLK | |
| 50 | 1683.891 | 562.305 | 3 | linear | 815 | VTMNEFEYLKLLGK | |
| 16 | 1750.92 | 438.7379 | 4 | linear | 750 | FFAGIVWQHVYEKK | |
| 10 | 1788.926 | 597.3161 | 3 | linear | 1289 | EEWTTAIQTVADGLKK | |
| 7 | 1848.886 | 925.4506 | 2 | linear | 625 | EAPLNNFSVAQCQLMK | |
| 8 | 1864.881 | 622.6346 | 3 | linear | 1016 | EAPLNNFSVAQCQLMK | |
| 17 | 1940.952 | 971.4856 | 2 | linear | 1304 | FYGAEIVSALDYLHSEK | |
| **36** | **1972.914** | **658.6458** | **3** | **linear** | **1465** | **RPKFPQBSYSASGTA** | |
| 37 | 2118.974 | 707.3323 | 3 | linear | 682 | SGSPSDNSGAEEMEVSLAKPK | |
| 42 | 2445.08 | 816.035 | 3 | linear | 2033 | TFCGTPEYLAPEVLEDNDYGR | |
| **399** | **2594.194** | **649.5563** | **4** | **xlink** | **1933** | **RPKFPQbSYSASGTA** | **DGATmK** |
| **403** | **2594.194** | **865.7386** | **3** | **xlink** | **1144** | **RPKFPQbSYSASGTA** | **DGATMk** |
| **400** | **2610.188** | **653.555** | **4** | **xlink** | **1307** | **RPKFPQbSYSASGTA** | **DGATmK** |
| **404** | **2610.188** | **653.555** | **4** | **xlink** | **1294** | **RPKFPQbSYSASGTA** | **DGATMk** |

uncross-linked peptide 1 highlighted red, cross-linked peptide1-peptide 2 highlighted blue

**Table S5.** Analysis of MS/MS data by Crossfinder for FL-Akt1Phe472Bpa, pThr$^{308}$, 2pSer$^{477}$,Thr$^{479}$ without UV treatment, related to Figure 6.

| ID | M, calc | m/z, obs | charge | type | score | Sequence 1 | Sequence 2 |
|---|---|---|---|---|---|---|---|
| 18 | 925.5386 | 463.7766 | 2 | linear | 617 | HPFLTALK | |
| 21 | 952.4689 | 477.2415 | 2 | linear | 829 | ITDFGLCK | |
| 40 | 957.6224 | 479.8182 | 2 | linear | 974 | SLLSGLLKK | |
| 43 | 1243.583 | 415.535 | 3 | linear | 779 | TFHVETPEER | |
| 49 | 1288.601 | 645.3076 | 2 | linear | 1354 | VTMNEFEYLK | |
| 2 | 1304.655 | 653.3366 | 2 | linear | 978 | CLQWTTVIER | |
| 29 | 1404.769 | 703.3926 | 2 | linear | 1522 | LFELILMEEIR | |
| 30 | 1420.764 | 474.5957 | 3 | linear | 1185 | LFELILMEEIR | |
| 41 | 1498.837 | 500.62 | 3 | linear | 1635 | TERPRPNTFIIR | |
| 45 | 1525.908 | 509.6439 | 3 | linear | 930 | TLGPEAKSLLSGLLK | |
| 20 | 1581.761 | 528.2612 | 3 | linear | 1794 | HRVTMNEFEYLK | |
| 15 | 1622.825 | 406.7131 | 4 | linear | 1502 | FFAGIVWQHVYEK | |
| 31 | 1626.779 | 407.7019 | 4 | linear | 833 | LGGGSEDAKEIMQHR | |
| 9 | 1660.831 | 554.618 | 3 | linear | 970 | EEWTTAIQTVADGLK | |
| 16 | 1750.92 | 876.4673 | 2 | linear | 3504 | FFAGIVWQHVYEKK | |
| 10 | 1788.926 | 895.4699 | 2 | linear | 1198 | EEWTTAIQTVADGLKK | |
| 33 | 1801.921 | 601.6483 | 3 | linear | 689 | LSPPFKPQVTSETDTR | |
| 7 | 1848.886 | 925.4509 | 2 | linear | 1863 | EAPLNNFSVAQCQLMK | |
| 8 | 1864.881 | 622.6347 | 3 | linear | 1294 | EAPLNNFSVAQCQLMK | |
| 14 | 1923.006 | 642.0099 | 3 | linear | 888 | EVIVAKDEVAHTLTENR | |
| 17 | 1940.952 | 971.4832 | 2 | linear | 1810 | FYGAEIVSALDYLHSEK | |
| **36** | **1972.914** | **987.4648** | **2** | **linear** | **1917** | **RPKFPQBSYSASGTA** | |
| 37 | 2118.974 | 707.3317 | 3 | linear | 645 | SGSPSDNSGAEEMEVSLAKPK | |
| 44 | 2886.404 | 963.1439 | 3 | linear | 2342 | TFHVETPEEREEWTTAIQTVADGLK | |

uncross-linked peptide 1 highlighted red