Science Signaling
AAAS
www.sciencesignaling.org/cgi/content/full/5/223/ra37/DC1

Supplementary Materials for

An ATP-Site On-Off Switch That Restricts Phosphatase Accessibility of Akt

Kui Lin,* Jie Lin, Wen-I Wu, Joshua Ballard, Brian B. Lee, Susan L. Gloor, Guy P. A. Vigers, Tony H. Morales, Lori S. Friedman, Nicholas Skelton, Barbara J. Brandhuber*

*To whom correspondence should be addressed. E-mail: klin@gene.com (K.L.); bbrandhuber@arraybiopharma.com (B.J.B.)

Published 8 May 2012, *Sci. Signal.* **5**, ra37 (2012)
DOI: 10.1126/scisignal.2002618

The PDF file includes:

Fig. S1. Structures of Akt inhibitors used in this study.
Fig. S2. The activity of PP2A or PP1 is inhibited by ATP, ADP, and AMP-PNP, but not by Akt inhibitors.
Fig. S3. ATP-competitive inhibitors block dephosphorylation of Akt2 and Akt3.
Fig. S4. ATP-competitive inhibitors block dephosphorylation of Akt by PP1.
Fig. S5. Scenarios to explain the phosphorylation status of Akt in cells treated with upstream kinase inhibitors before or after treatment with the ATP-competitive inhibitors.
Table S1. Data collection and refinement statistics.
Table S2. Akt1 residues within 4 Å of GDC-0068.
Table S3. LanthaScreen displacement binding assay results.
Table S4. Quantification and statistical analysis of Western blotting data.

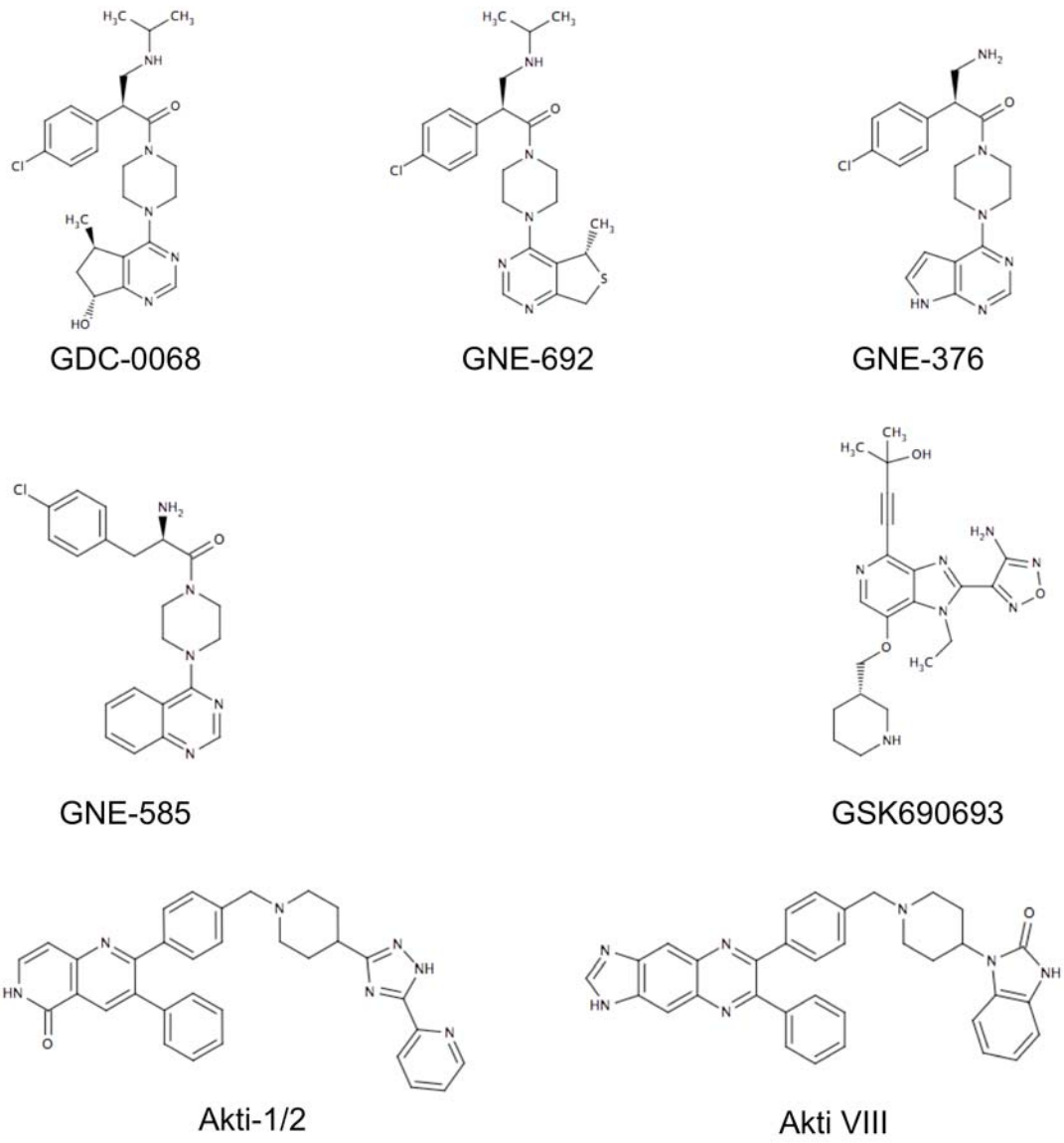

Figure S1. Structures of Akt inhibitors used in this study.

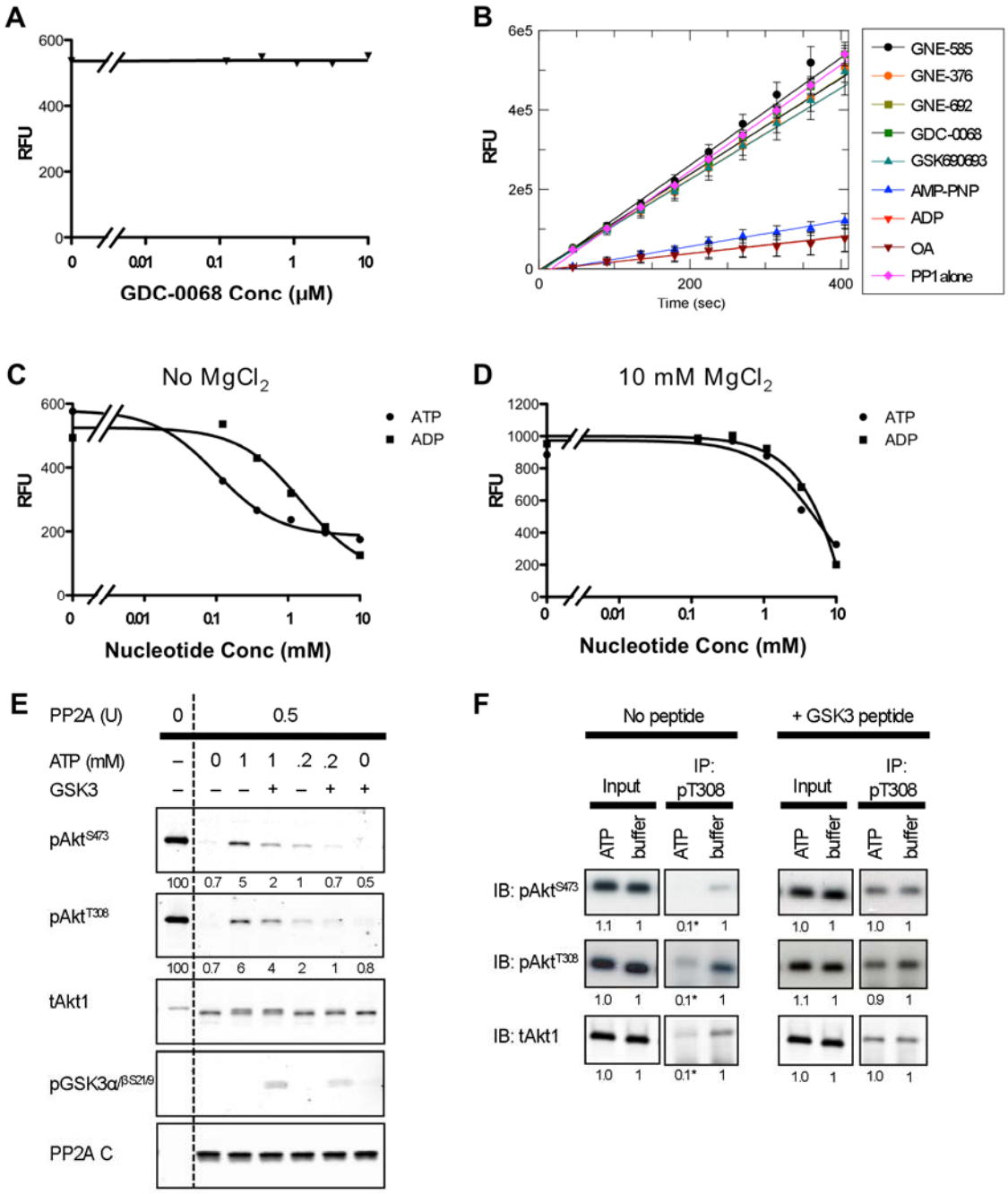

Figure S2. The activity of PP2A or PP1 is inhibited by ATP, ADP, and AMP-PNP, but not by Akt inhibitors. A, Titration of GDC-0068 on PP2A activity measured using the ProFluor Ser/Thr PPase Assay (Promega). **B,** Effects of ATP-competitive inhibitors (10 μM) and nucleotides (10 mM) on PP1 kinetic activity measured using the FDP substrate. Okadaic acid (OA) was used as a positive control. N = 3 replicates. **C,** Titration of ATP and ADP on PP2A activity in the absence of $MgCl_2$. **D,** Titration of ATP and ADP on PP2A activity in the presence of 10 mM $MgCl_2$. **E,** The presence of an Akt substrate reversed the inhibitory effect of ATP on Akt dephosphorylation. Activated His-tagged full-length Akt1 was incubated with 0.5 units of PP2A for 30 min at 30 ^{o}C with or without 5 min pre-incubation with 0.2 or 1 mM ATP, 10 mM $MgCl_2$, 20 ng/μl GSK3

crosstide fusion protein (Cell Signaling Technologies), or both ATP and the GSK3 crosstide fusion protein. Phosphorylated Akt, total Akt1, phosphorylated GSK3, and abundance of PP2A C subunit were determined by IB. **F**, Effect of GSK3 peptide substrate on ATP's protection of phospho-epitope accessibility. Phospho Akt/total Akt ratios were calculated as percentage (E) or fold (F) of the values in untreated controls. *, $P < 0.05$ compared to untreated control; N = 3 Western blots.

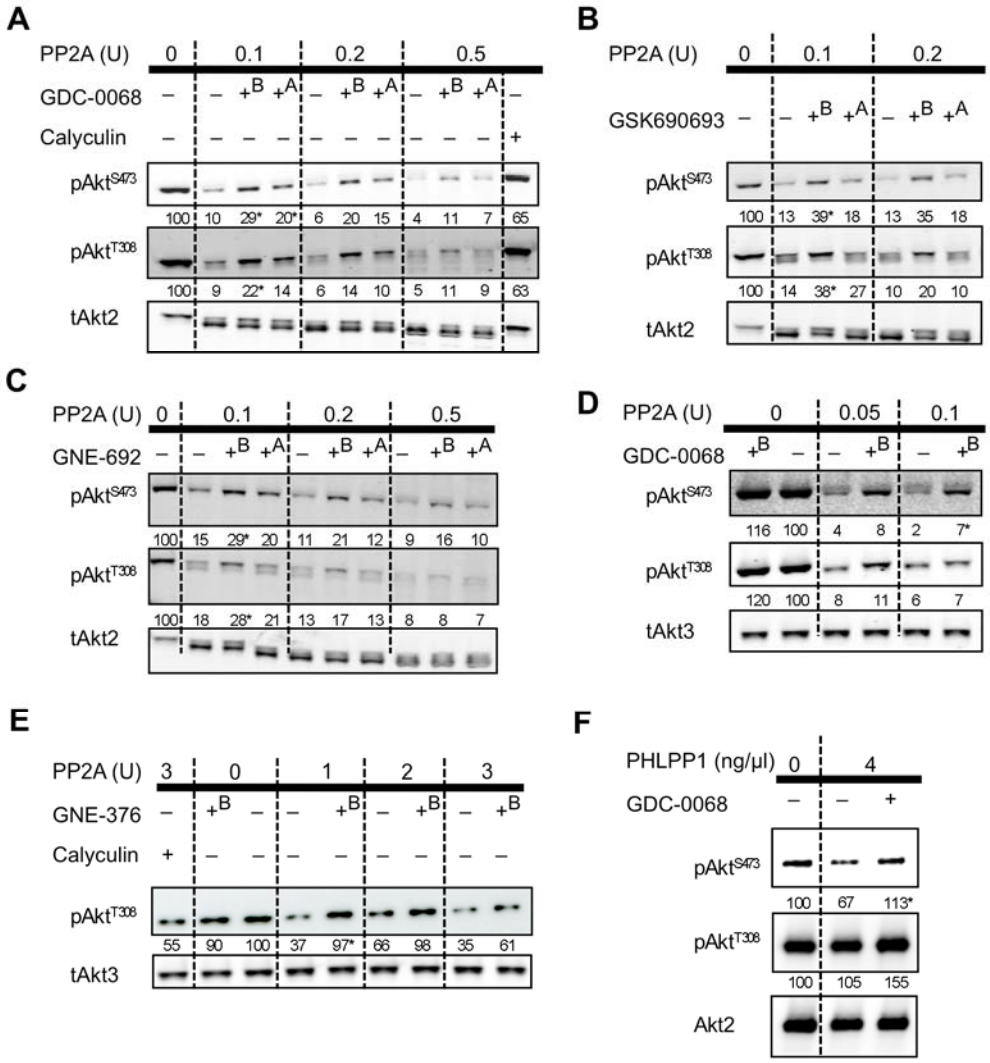

Figure S3. ATP-competitive inhibitors block dephosphorylation of Akt2 and Akt3. A-C, Activated His-Akt2 was incubated with the indicated units of PP2A in the absence or presence of 10 µM GDC-0068 (**A**), GSK690693 (**B**), or GNE-692 (Compound 36 in ref. 16) (**C**). **D-E,** Activated His-Akt3 was incubated with the indicated units of PP2A in the absence or presence of 10 µM GDC-0068 (**D**), or GNE-376 (Compound 56 in ref. 17) (**E**). Compounds were either pre-incubated with Akt for 20 min (^{B}) or added simultaneously (^{A}) with PP2A. Phosphatase reactions were terminated with SDS sample buffer and phosphorylated and total Akt were determined by immunoblots (IB). **F,** Activated His-Akt2 was incubated with indicated concentration of recombinant PHLPP1 in the absence of presence of 10 µM GDC-0068. Phospho Akt/total Akt ratios were calculated as percentage of the values in untreated (Akt only with no phosphatase or compound) controls. *, $P < 0.05$ compared to Akt treated with phosphatase only without compounds. N = 3 Western blots. Note that the labels "pAkt^{S473}" and "pAkt^{T308}" correspond to the positions of the phosphorylated residues in Akt1 and were also used to denote the equivalent residues in Akt2 (Ser^{474} and Thr^{309}, respectively) and Akt3 (Ser^{472} and Thr^{305}, respectively).

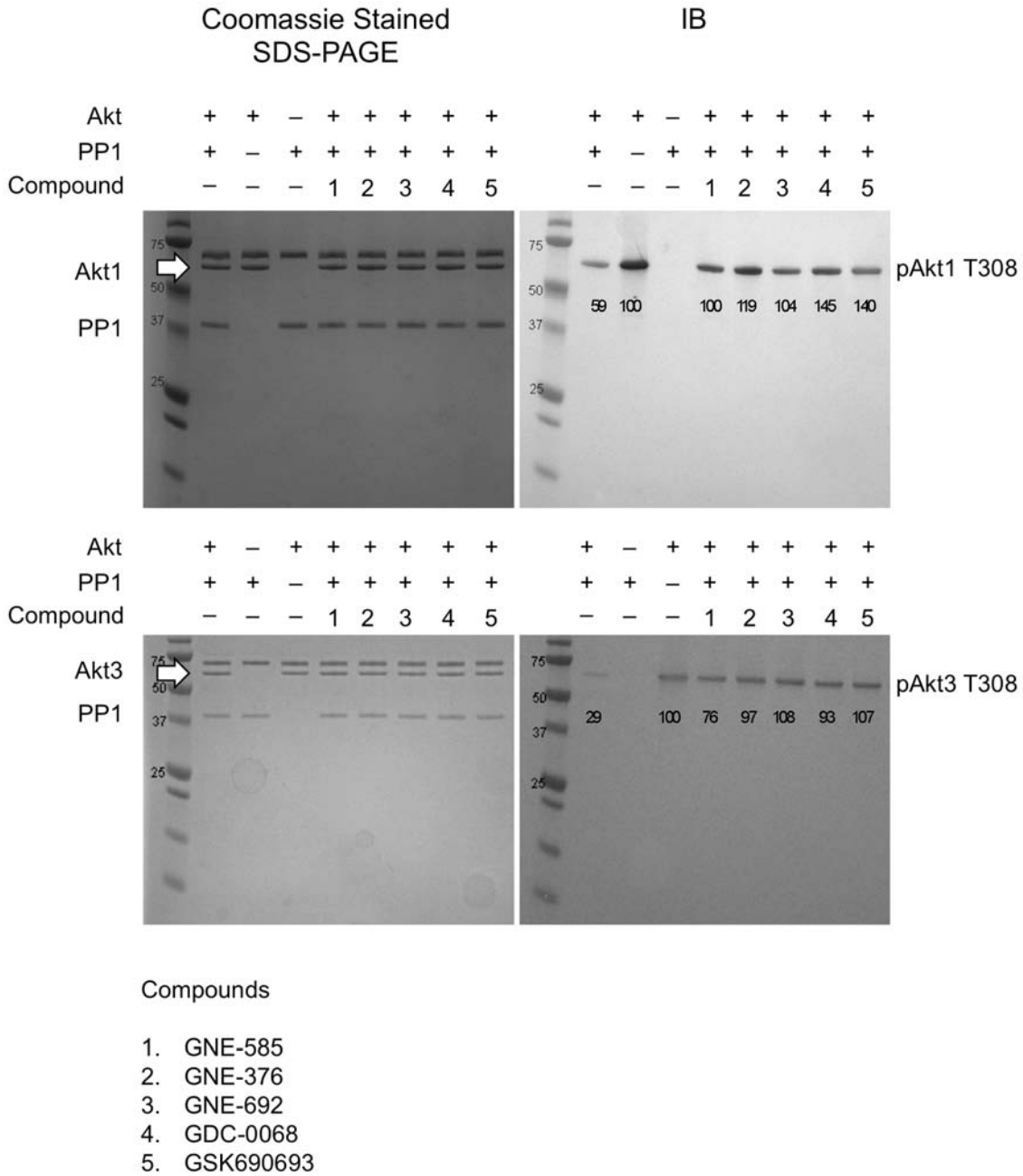

Figure S4. ATP-competitive inhibitors block dephosphorylation of Akt by PP1. 1 μM recombinant Akt proteins were incubated with an equal molar concentration of PP1A enzyme overnight at 4°C in PP1 activity assay buffer. Inhibitor concentrations were fixed at 10 μM. Reactions were subjected to SDS-PAGE and then either stained with Coomassie blue or transferred to nitrocellulose membrane and phosphorylation of Thr^{308} determined by IB. For Akt1, N = 3 Western blots; for Akt3, N = 2 Western blots. Phospho Akt/total Akt ratios were calculated as percentage of the values in untreated(Akt only with no phosphatase or compound) controls. Note that the labels "pAkt^{S473}" and "pAkt^{T308}" correspond to the positions of the phosphorylated residues in Akt1 and were also used to denote the equivalent residues in Akt2 (Ser^{474} and Thr^{309}, respectively) and Akt3 (Ser^{472} and Thr^{305}, respectively).

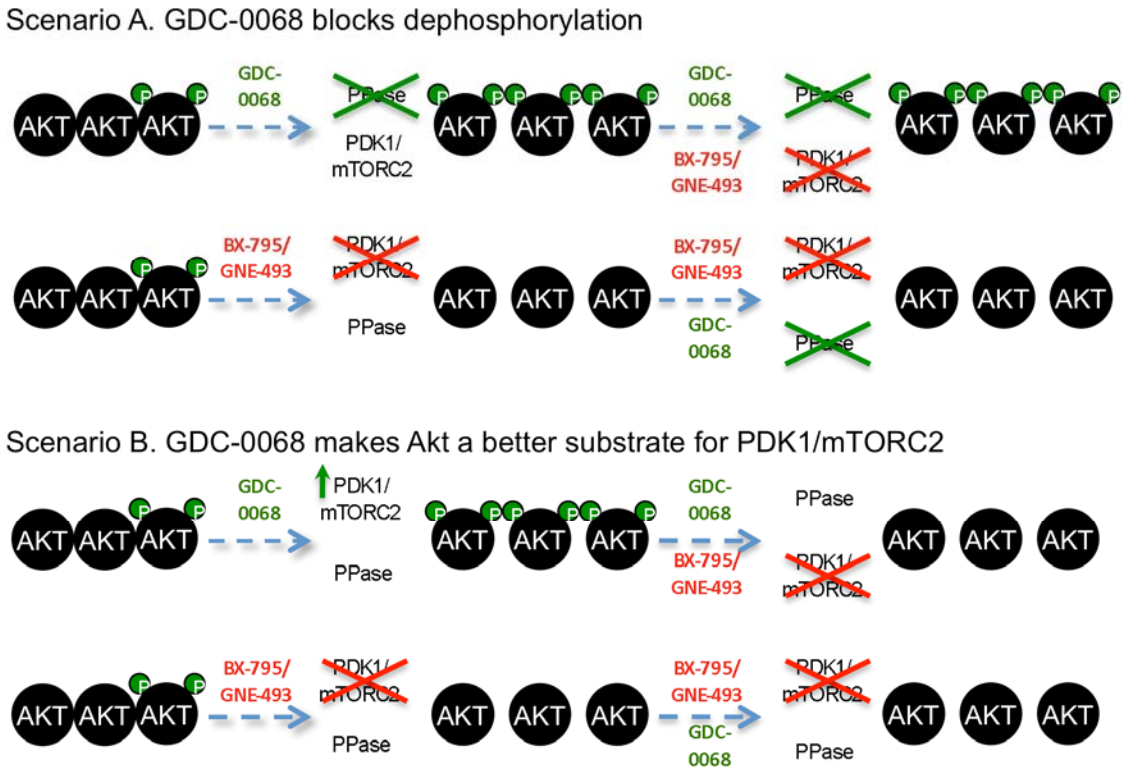

Figure S5. Scenarios to explain the phosphorylation status of Akt in cells treated with upstream kinase inhibitors before or after treatment with the ATP-competitive inhibitors. Scenario A: If the increase in phosphorylation of Akt observed in cells is primarily due to protection from dephosphorylation, pretreatment of cells with an ATP-competitive compound such as GDC-0068 should lock Akt in the phosphorylated state that will remain phosphorylated even when its upstream kinases PDK1 (which phosphorylates Thr^{308}) and mTORC2 (which phosphorylates Ser^{473}) are subsequently inhibited (such as by the PDK1 inhibitor BX-795 or dual PI3K/mTOR inhibitor GNE-493). In contrast, if cells are pretreated with inhibitors of PDK1 or mTORC2 and Akt then becomes dephosphorylated by phosphatases, subsequent treatment with an ATP-competitive inhibitor should not promote the increase in phosphorylated Akt in the continued presence of upstream kinase inhibitors. Scenario B: If the increase in phosphorylated Akt is due to phosphorylation by upstream kinases, either pretreatment with an ATP-competitive inhibitor followed by treatment with inhibitors of the upstream kinases, or pretreatment with inhibitors of the upstream kinases followed by treatment with an ATP-competitive inhibitor, should lead to dephosphorylation of Akt due to persistent phosphatase activity.

Table S1. Data collection and refinement statistics

	Akt1:GDC-0068	Akt1:GSK3β peptide: MnAMP-PNP
Data collection		
Space group	P2(1)2(1)2(1)	P2(1)
Cell dimensions		
a, *b*, *c* (Å)	47.03, 57.95, 151.61	86.64, 55.85, 91.40
α, β, γ (°)	90.0, 90.0, 90.0	90.0, 103.43, 90.0
Resolution (Å)	30.0-2.0(2.11-2.0)	30.0-2.8 (2.95-2.8)
R_{sym} or R_{merge}	0.089 (0.192)	0.104 (0.317)
$I / \sigma I$	11.0 (3.8)	5.7 (2.4)
Completeness (%)	91.4 (60.0)	99.4 (96.6)
Redundancy	6.0 (4.3)	3.4 (2.9)
Refinement		
Resolution (Å)	30.0 – 2.0	30.0 – 2.8
No. reflections	24,899	21,128
R_{work} / R_{free}	17.3/23.6	22.1/28.7
No. atoms	3047	5555
Protein	2697	5390
Ligand/ion	32	66
Water	318	100
B-factors		
Protein	35.8	47.2
Ligand/ion	32.2	64.8
Water	44.0	34.3
R.m.s. deviations		
Bond lengths (Å)	0.021	0.007
Bond angles (°)	1.756	1.106

*Values in parentheses are for highest-resolution shell.
One crystal was used to collect data for each structure.

Table S2. Akt1 residues within 4 Å of GDC-0068

N-Lobe Residue	C-Lobe Residue
Leu^{156}	Glu^{234}
Gly^{157}	Glu^{278}
Lys^{158}	Met^{281}
Gly^{159}	Thr^{291}
Gly^{162}	
Lys^{163}	
Val^{164}	
Ala^{177}	
Lys^{179}	
Leu^{181}	
Thr^{211}	
Met^{227}	
Glu^{228}	
Tyr^{229}	
Ala^{230}	
Phe^{438}	

Table S3. Lanthascreen displacement binding assay results

Compound	Inactive AKT-1 IC_{50} (nM)	Inactive AKT-2 IC_{50} (nM)	Active AKT-1 IC_{50} (nM)	Active AKT-2 IC_{50} (nM)
GNE-692	569.7 ± 46.5	1500 ± 300	2.9 ± 0.3	39.2 ± 7.9
GDC-0068	1100 ± 100	2600 ± 700	36.9 ± 11.9	145.1 ± 18.9

All data are average of $N \geq 3$ experiments.

Table S4. Quantification and statistical analysis of Western blotting data

Figure 1A

Cell Line	GDC-0068 (μM)	pAkt T308		pAkt S473	
		Mean	SD	Mean	SD
PC3-NCI	DMSO	1.0	0.05	1.0	0.10
	0.001	1.1	0.06	1.1	0.10
	0.01	**1.8**	0.15	**1.4**	0.13
	0.1	**6.3**	0.83	**4.2**	0.28
	1	**12.3**	1.19	**8.8**	0.39
	10	**9.0**	0.98	**3.1**	0.26
MCF7-neo/HER2	DMSO	1.0	0.20	1.0	0.44
	0.001	1.1	0.20	0.6	0.30
	0.01	**3.1**	0.66	1.2	0.26
	0.1	**15.1**	3.47	**7.9**	0.86
	1	**34.1**	5.22	**19.7**	1.69
	10	**29.6**	4.29	**12.0**	1.67

Cell Line	GDC-0068 (μM)	pPRAS40 T246		pFoxO3a T32		pS6 S235/236	
		Mean	SD	Mean	SD	Mean	SD
PC3-NCI	DMSO	1.0	0.05	1.0	0.38	1.0	0.12
	0.001	1.0	0.02	1.0	0.25	1.0	0.09
	0.01	0.9	0.05	0.8	0.28	0.7	0.17
	0.1	**0.7**	0.01	0.8	0.12	0.8	0.21
	1	**0.2**	0.05	**0.1**	0.06	**0.5**	0.12
	10	**0.0**	0.01	**0.0**	0.00	**0.2**	0.07
MCF7-neo/HER2	DMSO	1.0	0.41	1.0	0.17	1.0	0.11
	0.001	1.1	0.49	1.0	0.46	1.0	0.01
	0.01	1.1	0.46	0.8	0.15	1.1	0.12
	0.1	0.6	0.06	**0.5**	0.12	1.1	0.12
	1	**0.3**	0.08	**0.1**	0.07	0.7	0.12
	10	**0.0**	0.01	**0.0**	0.01	**0.4**	0.13

Fig. 1A: Phosphorylated to total protein ratios were calculated as fold over DMSO treated control; $P \leq 0.05$ compared to DMSO control are indicated in bold.

Figure 1B

PDK1 (ng)	GDC-0068 (μM)	pAkt T308	
		Mean	SD
0	0	100	
5	0	226	36.1
	1[a]	253	2.1
	10[a]	235	27.6
	1[b]	194	2.8
	10[b]	190	14.8
10	0	291	38.2
	1[a]	321	58.0
	10[a]	325	121.6
	1[b]	278	17.0
	10[b]	265	7.8

Figure 1C

PP2A (U)	GDC-0068 (μM)	pAkt S473		pAkt T308	
		Mean	SD	Mean	SD
0	0	100		100	
0.1	0	16	9.2	16	7.1
	10	**51**	10.6	**57**	15.1

Figure 1D

PP2A (U)	GDC-0068 (μM)	pAkt S473		pAkt T308	
		Mean	SD	Mean	SD
0	0	100		100	
0.1	0	18	6.1	22	6.7
	10[B]	**61**	14.8	**64**	17.0
	10[A]	**39**	9.9	28	28.3

Figure 1E

PP2A (U)	GDC-0068 (μM)	pMEK	
		Mean	SD
0	0	100	
0.1	0	5	1.7
	10[B]	4	1.0
	10[A]	5	1.7

Figure 1F

PP2A (U)	Akti-1/2 (μM)	pAkt S473	
		Mean	SD
0	0	100	
0.1	0	13	3.8
	10	**5**	1.4

Fig. 1, B to F: Phosphorylated to total protein ratios were calculated as percentage of untreated controls; $P \leq 0.05$ compared to phosphatase only lanes are shown in bold.

Figure 2A

2nd	1st	pAkt T308		pAkt S473	
		Mean	SD	Mean	SD
DMSO	DMSO	1.0		1.0	
	GSK690693	10.4	0.77	3.9	0.29
	GNE-692	15.2	1.69	4.4	0.49
	GDC-0068	10.9	0.09	3.8	0.03
GNE-493	DMSO	0.1	0.01	0.5	0.02
	GSK690693	25.3	0.15	6.3	0.04
	GNE-692	29.9	1.81	5.7	0.34
	GDC-0068	29.4	1.29	5.7	0.25
DMSO	DMSO	1.0		1.0	
GSK690693		8.0	0.16	2.4	0.05
GNE-692		7.4	0.12	2.4	0.04
GDC-0068		7.6	0.06	2.4	0.02
DMSO	GNE-493	0.1	0.00	0.2	0.00
GSK690693		0.2	0.00	0.3	0.00
GNE-692		0.3	0.05	0.1	0.03
GDC-0068		0.1	0.03	0.1	0.02

Figure 2B

2nd	1st	pAkt T308		pAkt S473	
		Mean	SD	Mean	SD
DMSO	DMSO	1.0		1.0	
	GSK690693	7.7	1.04	3.1	0.42
	GNE-692	7.5	1.85	3.0	0.74
	GDC-0068	8.0	1.34	3.0	0.49
BX-795	DMSO	0.1	0.00	0.3	0.01
	GSK690693	4.7	0.08	4.7	0.08
	GNE-692	7.0	0.29	4.3	0.18
	GDC-0068	6.1	0.40	4.2	0.28
DMSO	DMSO	1.0		1.0	
GSK690693		11.1	0.49	2.2	0.10
GNE-692		13.4	2.34	2.3	0.41
GDC-0068		10.3	1.96	2.5	0.48
DMSO	BX-795	0.1	0.04	0.3	0.08
GSK690693		0.8	0.43	1.2	0.14
GNE-692		1.6	0.70	0.8	0.07
GDC-0068		1.6	0.77	0.8	0.07

Fig. 2, A and B: Phosphorylated to total Akt ratios were calculated as fold of DMSO treated controls (which was set as 1) for each treatment.

Figure 3E

	GDC-0068	OA	pAkt T308				pAkt S473			
			p/t MyrAkt1		p/t endo		p/t MyrAkt1		p/t endo	
			Mean	SD	Mean	SD	Mean	SD	Mean	SD
Vec	–	–			0.3	0.02			0.3	0.08
	+	–			8.4	1.59			5.8	2.06
	–	+			0.5	0.07			1.8	0.24
MyrAkt1	–	–	4.2	1.28	0.2	0.16	3.0	1.31	0.3	0.28
	+	–	19.9	6.62	5.6	2.86	21.9	8.94	3.2	2.78
	–	+	13.4	4.08	0.5	0.50	11.3	5.86	2.7	3.73
MyrAkt1 -R273A	–	–	**1.0**	0.31	0.5	0.21	**0.7**	0.23	0.6	0.21
	+	–	**7.9**	2.46	14.5	5.81	20.6	10.13	5.5	1.79
	–	+	**2.2**	0.63	0.7	0.39	4.3	1.43	2.0	0.33
MyrAkt1 -S473A	–	–	**0.2**	0.03	0.4	0.17			0.4	0.12
	+	–	**10.1**	1.39	12.4	1.06			7.9	2.27
	–	+	**0.6**	0.09	0.7	0.12			3.2	0.72
mock	–	–			0.3	ND			0.3	ND
	+	–			11.0	ND			4.5	ND
	–	+			0.8	ND			2.3	ND

Fig. 3E: Phosphorylated to total (p/t) Akt ratios were calculated for both MyrAkt1 and endogenous (endo) Akt. $P \leq 0.05$ compared to wild-type MyrAkt1 under the same conditions are indicated in bold.

Figure 4A

		pAkt S473		pAkt T308		tAkt	
		Mean	SD	Mean	SD	Mean	SD
WCL	DMSO	1.0		1.0		1.0	
	OA	3.5	0.58	3.6	0.82	0.8	0.20
	GSK690693	3.8	0.68	10.0	1.73	1.0	0.08
	GDC-0068	3.5	0.70	14.0	2.68	0.9	0.14
IP: FLAG	DMSO	1.0		1.0		1.0	
	OA	3.3	0.93	2.3	0.03	1.0	0.11
	GSK690693	3.7	0.38	6.4	0.77	1.2	0.30
	GDC-0068	2.8	0.57	7.0	0.15	1.1	0.15
IP: pS473	DMSO	1.0		1.0		1.0	
	OA	3.9	0.08	3.7	0.23	4.0	0.17
	GSK690693	2.1	0.51	1.1	0.12	2.0	0.33
	GDC-0068	1.6	0.55	1.7	0.36	2.3	0.32
IP: pT308	DMSO	1.0		1.0		1.0	
	OA	2.8	0.17	1.8	0.24	1.9	0.09
	GSK690693	1.0	0.23	1.7	0.08	1.5	0.15
	GDC-0068	0.9	0.03	1.3	0.55	1.6	0.18
IP: Akt	DMSO	1.0	0.00	1.0		1.0	
	OA	4.5	0.56	2.1	0.32	0.9	0.13
	GSK690693	3.0	0.27	4.0	0.37	0.9	0.06
	GDC-0068	4.2	0.85	18.8	3.97	1.2	0.33

Figure 4B

		pS473		pT308		tAkt1	
		Mean	SD	Mean	SD	Mean	SD
Input	DMSO	1.0		1.0		1.0	
	GDC-0068	1.2	0.31	1.1	0.24	1.1	0.30
	GSK690693	0.9	0.22	1.0	0.06	0.9	0.22
	GNE376	0.9	0.19	1.0	0.13	0.9	0.20
	Akti-1/2	0.9	0.22	1.1	0.27	1.0	0.31
IP: His	DMSO	1.0		1.0		1.0	
	GDC-0068	1.7	0.19	2.3	0.09	1.6	0.30
	GSK690693	1.4	0.11	1.7	0.05	1.4	0.24
	GNE376	1.6	0.23	1.9	0.10	1.4	0.20
	Akti-1/2	1.6	0.18	1.7	0.07	1.8	0.38
IP: pS473	DMSO	1.0		1.0		1.0	
	GDC-0068	0.2	0.03	0.3	0.27	0.3	0.11
	GSK690693	0.5	0.13	0.3	0.18	0.2	0.08
	GNE376	0.2	0.06	0.3	0.20	0.3	0.18
	Akti-1/2	2.0	0.04	1.9	0.09	2.9	0.78
IP: pT308	DMSO	1.0		1.0		1.0	
	GDC-0068	0.1	0.05	0.3	0.31	0.2	0.03
	GSK690693	0.3	0.09	0.5	0.26	0.4	0.08
	GNE376	0.2	0.03	0.4	0.29	0.3	0.09
	Akti-1/2	1.5	0.05	0.9	0.08	1.0	0.12

Fig. 4, A and B: Phosphorylated and total Akt signals were calculated as fold of DMSO control.

Figure 5D

		pAkt S473		pAkt T308		tAkt1	
		Mean	SD	Mean	SD	Mean	SD
Input	Buffer	1.0		1.0		1.0	
	AMPPNP	0.9	0.12	1.0	0.20	1.0	0.31
	ATP	1.0	0.24	1.1	0.08	1.0	0.32
IP: His	Buffer	1.0		1.0		1.0	
	AMPPNP	1.2	0.12	1.0	0.41	1.1	0.33
	ATP	1.3	0.34	1.4	0.46	1.1	0.37
IP: pS473	Buffer	1.0		1.0		1.0	
	AMPPNP	1.0	0.22	0.7	0.13	0.7	0.07
	ATP	1.0	0.12	0.6	0.18	0.7	0.08
IP: pT308	Buffer	1.0		1.0		1.0	
	AMPPNP	0.2	0.09	0.1	0.06	0.1	0.07
	ATP	0.1	0.07	0.1	0.08	0.1	0.05
IP: Akt1	Buffer	1.0		1.0		1.0	
	AMPPNP	0.3	0.13	0.5	0.38	0.4	0.14
	ATP	0.2	0.05	0.4	0.10	0.4	0.16

Figure 5E

		pS473		pT308		tAkt1	
		Mean	SD	Mean	SD	Mean	SD
Input	DMSO	1.0		1.0		1.0	
	ADP	0.7	0.18	0.8	0.29	0.7	0.13
IP: His	DMSO	1.0		1.0		1.0	
	ADP	1.5	0.25	1.5	0.51	1.7	0.28
IP: pS473	DMSO	1.0		1.0		1.0	
	ADP	2.8	0.72	8.3	1.39	10.0	2.12
IP: pT308	DMSO	1.0		1.0		1.0	
	ADP	6.9	1.36	5.2	1.01	15.9	2.20
	DMSO	1.0		1.0		1.0	
IP: Akt1	ADP	6.6	0.75	6.3	1.25	14.6	1.56

Fig. 5, D and E: Phosphorylated and total Akt signals were calculated as fold of buffer or DMSO controls.

All data represented as means ± standard deviation (SD) (based on three experiments).