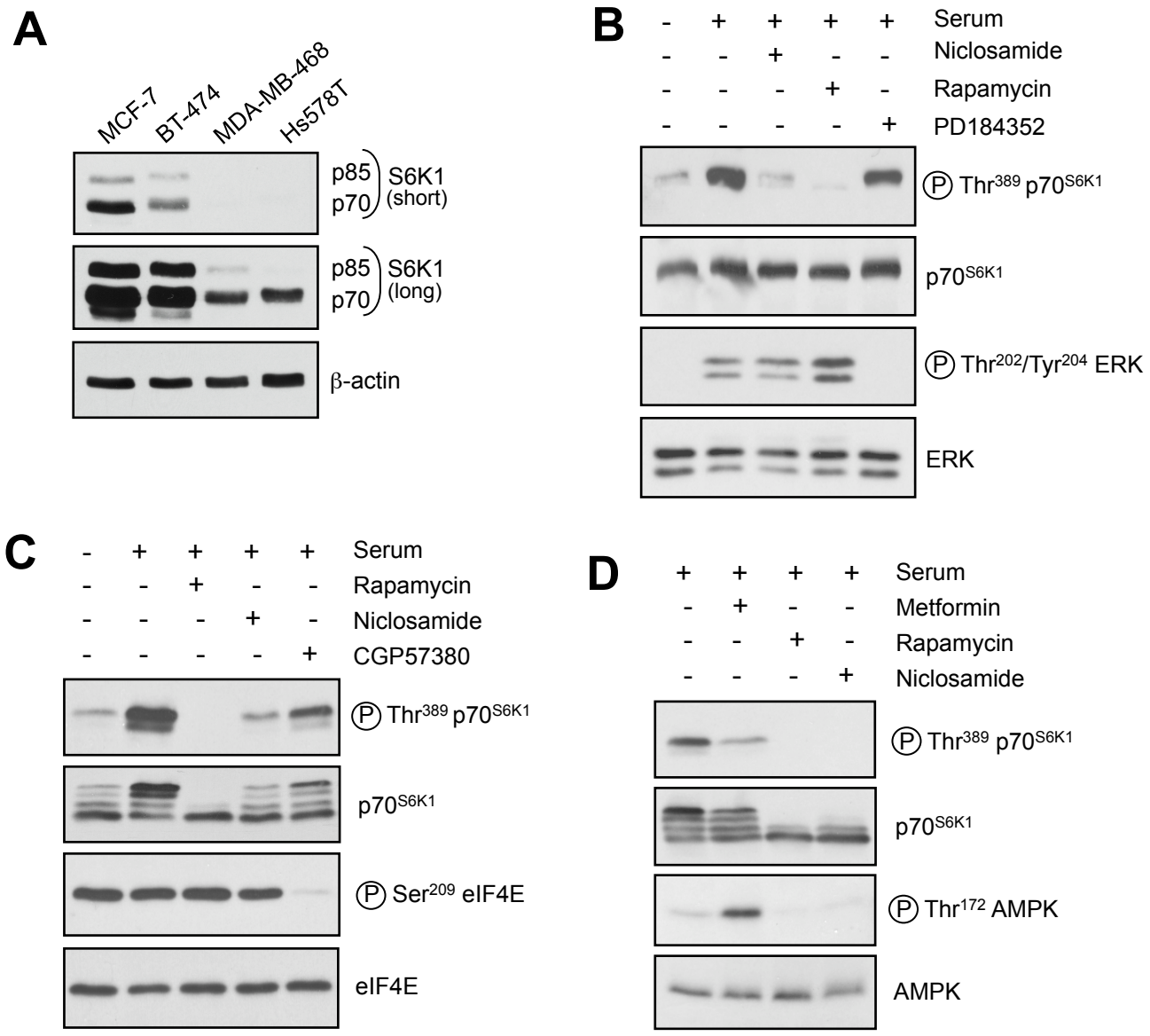
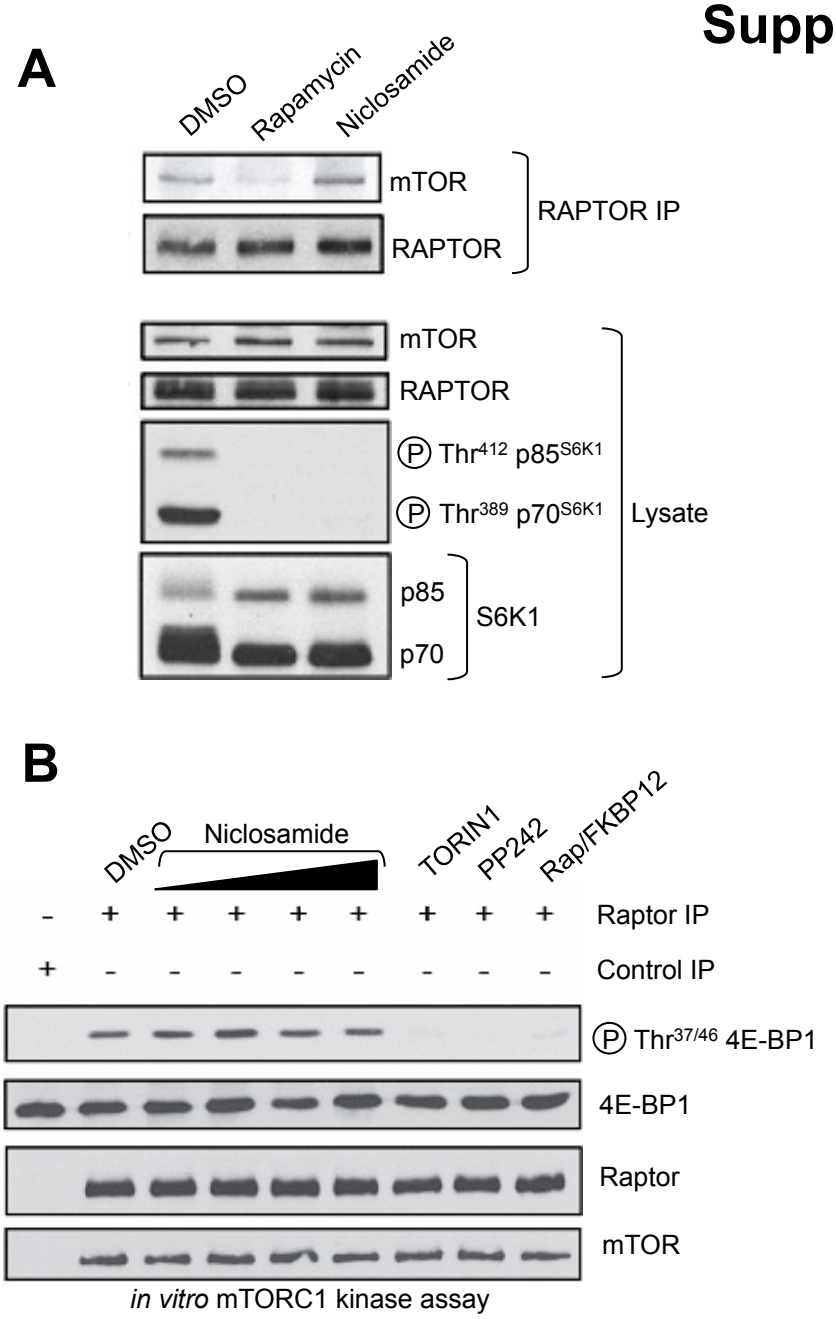
Suppl. Fig. 1



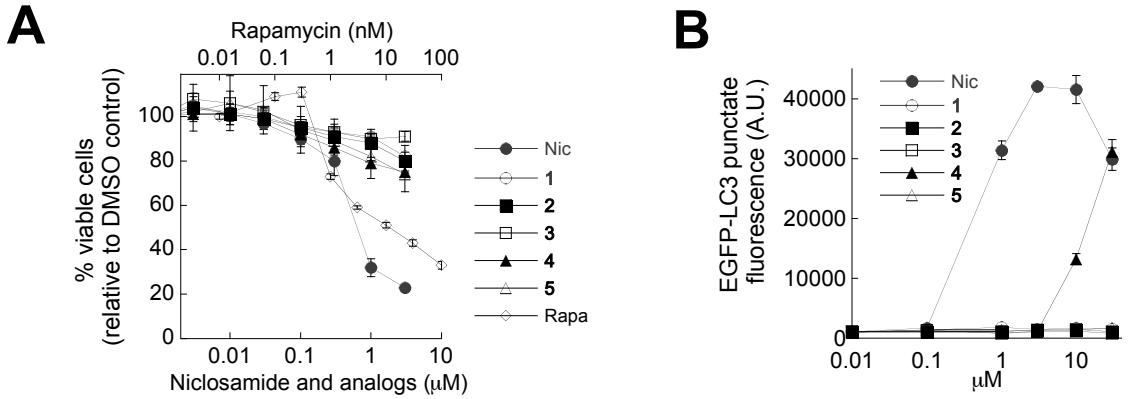
Suppl. Fig.1. Niclosamide inhibits mTORC1 signaling independently of AMPK and MEK/ERK/Mnk signaling. (A) Analysis of S6K1 protein expression levels in human breast cancer cell lines. MCF-7, BT-474, MDA-MB-468 and Hs578T breast cancer cell lines were propagated to near confluence and whole cell lysates prepared. Protein concentration was determined by Bradford assay and 20 µg of protein was resolved by SDS-PAGE and subjected to Western blotting with antibodies against S6K1 and β-actin. (B, C) Niclosamide does not affect MEK/ERK/Mnk signaling. MCF-7 cells propagated to near confluency at which point cells were starved of serum overnight. Cells were then treated for 30 min with 10 µM niclosamide, 100 nM rapamycin, or for 1 h with 10 µM PD184352 or 50 µM CGP57380 prior to stimulation with 10 % (v/v) serum for 15 min. Cell lysates were subjected to immunoblotting using the indicated antibodies. (D) Niclosamide inhibits mTORC1 signaling independently of AMPK. MCF-7 were cultured in complete media. When cells reached approximately 80% confluency, the medium was replaced with fresh growth medium with or without 10 mM metformin for 8 h or with 100 nM rapamycin or 10 µM niclosamide for 1 h. Cells were then lysed and lysates analysed by immunoblotting using the indicated antisera.

Suppl. Fig. 2

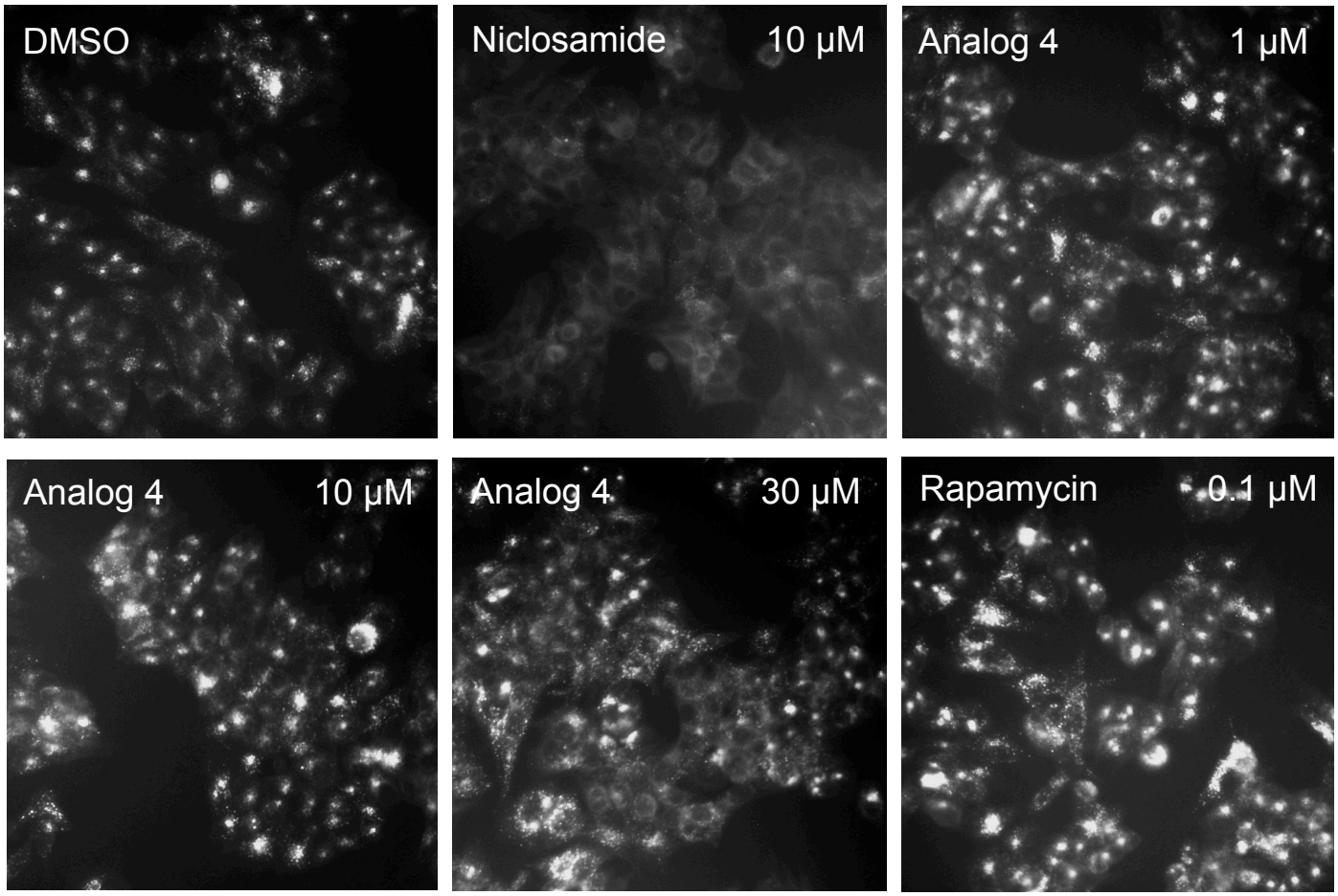


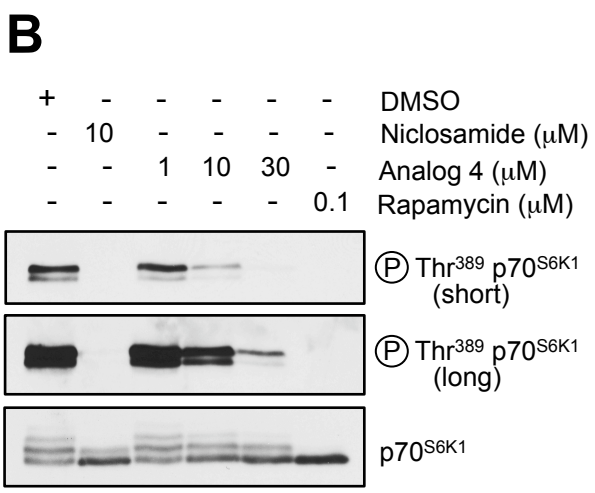
Suppl. Fig. 2. Niclosamide does not disrupt mTOR-raptor binding nor does it affect mTORC1 catalytic activity *in vitro*. (A) MCF-7 cells cultured to 80% confluence, starved of serum overnight and then pre-treated with 10 μM nicosamide or 30 nM rapamycin for 1 h prior to stimulation with 10% FBS for 45 min. Cells were then lysed in CHAPS lysis buffer as described in (Kim *et al.* [2002] *Cell* **110**, 163-175) and mTORC1 isolated from cells by immunoprecipitation with anti-raptor antibody. mTOR association with raptor was monitored by resolving immunoprecipitates on SDS-PAGE and probing with antibodies specific for raptor and mTOR. Lysates were analysed for S6K1 phosphorylation to confirm that niclosamide and rapamycin both efficiently blocked mTORC1 signaling. (B) *In vitro* catalytic activity of mTORC1 was measured by incubating bacterially-expressed purified recombinant human 4E-BP1 with mTORC1 immunoprecipitates from MCF-7 cells for 15 min at 30°C in the presence of increasing concentrations (0.1, 0.5, 3, 30 μM) of niclosamide, 20 nM TORIN1, 3 μM PP242 or 10 μM rapamycin/FKBP12 complex. The reactions were were analysed by immunoblotting using the indicated antisera.

Suppl. Fig. 3



Suppl. Fig. 3. (A) MCF-7 cells were treated with the indicated concentrations of rapamycin or niclosamide and analogs for 48 h and viable cells were determined using the MTT assay. (B) MCF-7 cells expressing EGFP-LC3 were treated with the indicated concentrations of niclosamide or analogs for 4 h in complete media containing 10% (v/v) FBS and the formation of EGFP-LC3 puncta was measured using automated fluorescence microscopy as described in the *Experimental Procedures* section.





Suppl. Fig. 4. The ability of niclosamide analog 4 to extrude protons from the lysosome correlates with the ability of this drug to inhibit mTORC1 signaling. (A) MCF-7 cells were seeded in complete media and propagated for 48 h, at which point lysotracker red dye was added directly to the conditioned media at the final concentration of 100 nM. Cells were incubated with the dye for 1 h 30 min. Then, fresh complete medium containing either 0.1% (v/v) DMSO, 10 μM niclosamide, 100 nM rapamycin or the indicated concentrations of analog 4. Lysotracker staining was monitored by wide-field fluorescence microscopy as described in the *Experimental Procedures* section. (B) Cells imaged in (A) were then lysed and lysates analysed for mTORC1 activation by SDS-PAGE/ Western blot using the indicated antisera.

Suppl. table 1. Effect of niclosamide on the activity of 95 protein kinases.

	Percentage of activity remaining			Percentage of activity remaining	
Kinase	**10 µM**	**1 µM**	**Kinase**	**10 µM**	**1 µM**
PDK1§	103±4	98±4	MELK	105±42	85±31
RSK1§	105±13	120±25	NUAK1	91±5	101±3
RSK2§	93±2	70±28	CK1	109±12	104±3
PKBα§	118±8	110±8	CK2	102±2	103±15
PKBβ§	95±6	86±8	DYRK1A	86±4	98±3
SGK1§	96±3	98±9	DYRK2	79±6	94±5
S6K1§	85±4	78±9	DYRK3	82±1	104±10
PKA§	102±0	96±2	NEK2a	116±13	114±13
ROCK2§	105±3	94±1	NEK6	90±11	81±1
PRK2§	110±4	101±15	IKKβ	96±4	91±18
PKCα§	101±11	98±6	IKKε	105±7	85±18
PKCζ§	107±17	101±6	TBK1	100±3	110±7
MSK1§	104±14	101±5	PIM1	77±6	93±1
MKK1	111±9	119±28	PIM2	85±3	79±8
ERK1	97±26	89±12	PIM3	79±1	108±0
ERK2	104±9	101±6	SRPK1	30±3	66±31
JNK1	98±6	98±15	eEF2K	79±6	105±23
JNK2	116±6	109±19	HIPK1	117±1	101±2
JNK3	84±7	88±9	HIPK2	114±4	103±3
p38α MAPK	99±16	99±19	HIPK3	113±2	104±13
p38β MAPK	116±3	112±6	PAK2	95±11	97±9
p38γ MAPK	105±1	98±17	PAK4	73±2	82±6
p38δ MAPK	101±5	93±1	PAK5	108±0	105±2
ERK8	92±7	97±9	PAK6	115±12	132±7
PKD1	110±5	99±11	MST2	115±9	101±2
MNK1	103±8	100±1	MST4	132±17	125±9
MNK2	112±25	104±18	GCK	95±2	106±19
MAPKAP-K2	101±3	105±8	MINK1	63±1	97±18
MAPKAP-K3	85±5	101±3	MLK1	107±1	116±12
PRAK	74±5	73±5	MLK3	107±2	110±2
CAMKKβ	108±16	105±10	IRAK4	113±8	114±2
CAMK1	85±32	95±12	RIPK2	95±11	99±15
SmMLCK	60±7	89±7	TTK	81±7	88±16
PHK	101±6	109±4	Src	104±3	99±2
CHK1	111±7	102±2	Lck	96±20	91±24
CHK2	83±2	101±12	CSK	112±10	118±2
GSK3β	109±18	98±8	YES1	117±11	112±7
CDK2-cyclinA	105±1	98±5	IGF-1R	110±1	110±11
PLK1	86±1	95±6	IR	109±1	117±6
Aurora A	100±14	97±5	IRR	91±1	109±1
Aurora B	112±8	109±9	HER4	115±5	101±7
LKB1	107±7	108±5	FGF-R1	107±17	105±2
AMPK	107±14	104±9	VEGF-R	114±19	106±15
MARK2	101±7	103±2	EPH-A2	82±9	110±13
MARK3	99±1	106±11	EPH-B3	115±14	106±13
MARK4	95±7	96±9	SYK	108±1	98±2
BRSK1	100±11	110±3	BTK	90±19	114±5
BRSK2	90±12	82±18			

Suppl. Table. 1. Effect of niclosamide on the *in vitro* catalytic activity of 95 protein kinases. *In vitro* kinase assays were carried out in the presence of 10 μM or 1 μM niclosamide as described in the *Experimental Procedures* section. Values are presented as percentage of remaining kinase activity in reactions with niclosamide relative to control incubations with DMSO. Standard deviation (S.D.) of two independent reactions are shown. § Denotes AGC kinase family members. Abbreviations used in this table: AMPK, AMP-activated protein kinase; BRSK1, brain-specific kinase 1; BRSK2, brain-specific kinase 2; BTK, Bruton's tyrosine kinase; CaMK1, calmodulin-dependent kinase 1; CaMKKβ, calmodulin-dependent kinase kinase beta; CDK2-cyclinA, cyclin-dependent kinase 2-cyclin A; CHK1, checkpoint kinase 1; CHK2, checkpoint kinase 2; CK1, casein kinase 1; CK2, casein kinase 2; CSK, C-terminal Src kinase; DYRK1A, dual-specificity tyrosine-phosphorylated and -regulated kinase 1A; DYRK2, dual-specificity tyrosine-phosphorylated and -regulated kinase 2; DYRK3, dual-specificity tyrosine-phosphorylated and –regulated kinase 3; eEF2K, eukaryotic elongation factor 2 kinase; EPH-A2, ephrin-A2; EPH-B3, ephrin-B3; ERK1, extracellular-signal regulated kinase 1; ERK2, extracellular-signal regulated kinase 2; ERK8, extracellular-signal regulated kinase 8; FGF-R1, fibroblast growth factor receptor; GCK, germinal centre kinase; GSK3β, glycogen synthase kinase 3 beta; HER4, V-erb a erythroblastic leukaemia viral oncogene homologue 1; HIPK1, homoedomain interacting protein kinase 1; HIPK2, homeodomain interacting protein kinase 2; HIPK3, homeodomain interacting protein kinase 3; IGF-1R, insulin-like growth factor 1 receptor; IKKβ, inhibitory κB kinase; IKKε, inhibitory κB kinase-related kinase ε; IR, insulin receptor; IRAK4, interleukin-1 receptor associated kinase 4; IRR, insulin-related receptor; JNK1, c-Jun N-terminal kinase 1; JNK2, c-Jun N-terminal kinase 2; JNK3, c-Jun N-terminal kinase 3; Lck, lymphocyte cell-specific tyrosine protein kinase; LKB1, liver-deleted kinase 1; MAPKAP-K2, mitogen-activated protein kinase (MAPK)-activated protein kinase 2; MAPKAP-K3, MAPK-activated protein kinase 3; MARK2, microtubule-affinity-regulating kinase 2; MARK3, microtubule-affinity-regulating kinase 3; MARK4, microtubule-affinity-regulating kinase 4; MELK, maternal embryonic leucine-zipper kinase; MINK1, misshapen-like kinase 1; MKK1, MAPK kinase 1; MLK1, mixed lineage kinase 1; MLK3, mixed lineage kinase 3; MNK1, MAPK-integrating protein kinase 1; MNK2, MAPK-integrating protein kinase 2; MSK1, mitogen- and stress-activated protein kinase 1; MST2, mammalian homologue STE20-like kinase 2; MST4, mammalian homologue STE20-like kinase 4; NEK2a, NIMA (never in mitosis in *Aspergillus nidulans*)-related kinase 2a; NEK6, NIMA-related kinase 6; NUAK1, SnF1-like kinase; p38α MAPK, p38 alpha mitogen-activated protein kinase; p38β MAPK, p38 beta mitogen-activated protein kinase; p38γ MAPK, p38 gamma mitogen-activated protein kinase; p38δ MAPK, p38 delta mitogen-activated protein kinase; PAK2, p21-activated protein kinase 2; PAK4, p21-activated protein kinase 4; PAK5, p21-activated protein kinase 5; PAK6, p21-activated protein kinase 6; PDK1, 3-phosphoinositide-dependent protein kinase 1; PHK, phosphorylase kinase; PIM1, provirus integration site for Moloney murine leukaemia virus kinase 1; PIM2, provirus integration site for Moloney murine leukaemia virus kinase 2; PIM3, provirus integration site for Moloney murine leukaemia virus kinase 3; PKA, protein kinase A; PKBα, protein kinase B alpha; PKBβ, protein kinase B beta; PKCα, protein kinase C alpha; PKCζ, protein kinase C zeta; PKD1, protein kinase D1; PLK1, polo-like kinase 1; PRAK, p38 MAPK-regulated activated kinase; PRK2, PKC related kinase 2; RIPK2, Rho-interacting protein kinase 2; ROCK2, Rho-dependent protein kinase 2; RSK1, ribosomal S6 kinase 1; RSK2, ribosomal S6 kinase 2; S6K1, S6 kinase 1; SGK1, serum and glucocorticoid-regulated kinase 1; SmMLCK, smooth muscle myosin light-chain kinase; Src, sarcoma; SRPK1, serine/arginine protein kinase 1; SYK, spleen tyrosine kinase; TBK1, TANK (tumor-necrosis-factor-receptor-assciated factor associated-nuclear-factor κB activator)-binding kinase 1; TTK, tau tubulin kinase; VEGF-R1, vascular endothelial growth factor receptor 1; YES1, Yamaguchi sarcoma viral oncogene homologue 1.