Supplementary Table 1

	Peptide mass	14-3-3 isoform	Isoform-specific tryptic peptides
Upper 14-3-3 band (Fig 1F)	1193.56	epsilon	EAAENSLVAYK
	1236.68		HLIPAANTGESK
	1383.71		YLAEFATGNDRK
	1446.55		VAGMDVELTVEER
	1476.01		LICCDILDVLDK
	1819.01		AASDIAMTELPPTHPIR
	2086.96		AAFDDAIAELDTLSEESYK
Lower 14-3-3 band (Fig 1F)	1278.68	delta/zeta	YLAEVAAGDDKK
	1303.83		FLIPNASQAESK
	1417.81		DICNDVLSLLEK
	2039.89		GIVDQSQQAYQEAFEISK
Lower 14-3-3 band (Fig 1F)	1267.54	theta/tau	YLAEVACGDDR
	1319.82		YLIANATNPESK
	1391.05		SICTTVLELLDK
	1531.73		AVTEQGAELSNEER
	2142.04		KQTIDNSQGAYQEAFDISK
	2144.13		TAFDEAIAELDTLNEDSYK
Lower 14-3-3 band (Fig 1F)	1079.79	gamma	YLAEVATGEK
	1642.72		NVTELNEPLSNEER
Lower 14-3-3 band (Fig 1F)	1181.75	alpha/beta	YLSEVASGDNK
	1359.60		YLIPNATQPESK
	1597.92		AVTEQGHELSNEER
	2158.17		QTTVSNSQQAYQEAFEISK
Lower 14-3-3 band (Fig 1F)	1065.72	eta	YLAEVASGEK
	1266.69		NSVVEASEAAYK
	1585.79		AVTELNEPLSNEDR

HA-AS160A was immunoprecipitated from lysate of transfected HEK293 cells that were IGF1-stimulated and extracted in the presence of DSP crosslinker. The immunoprecipitate was denatured and run on a 4-12% Bis-Tris gel, which was stained with Colloidal blue. The two prominent protein bands above the Ig light chain (see similar in Fig 1F) were excised, and peptides were identified by MS/MS analysis of tryptic digests. In addition to the isoform-specific peptides listed in the table, the digests of both protein band contained the peptide DSTLIMQLLR (mass 1204.84), which is common to all the 14-3-3 isoforms detected, and the lower band contained the peptide YDDMAAAMK (mass 1014.45), which is common to alpha/beta and gamma isoforms. The oxidised forms of methionine-containing peptides were also detected. Note that alpha and delta are the phosphorylated forms of 14-3-3 isoforms beta and zeta, respectively (Aitken et al (1995) J. Biol. Chem. 270, 5706-5709).

Supplementary Table 2

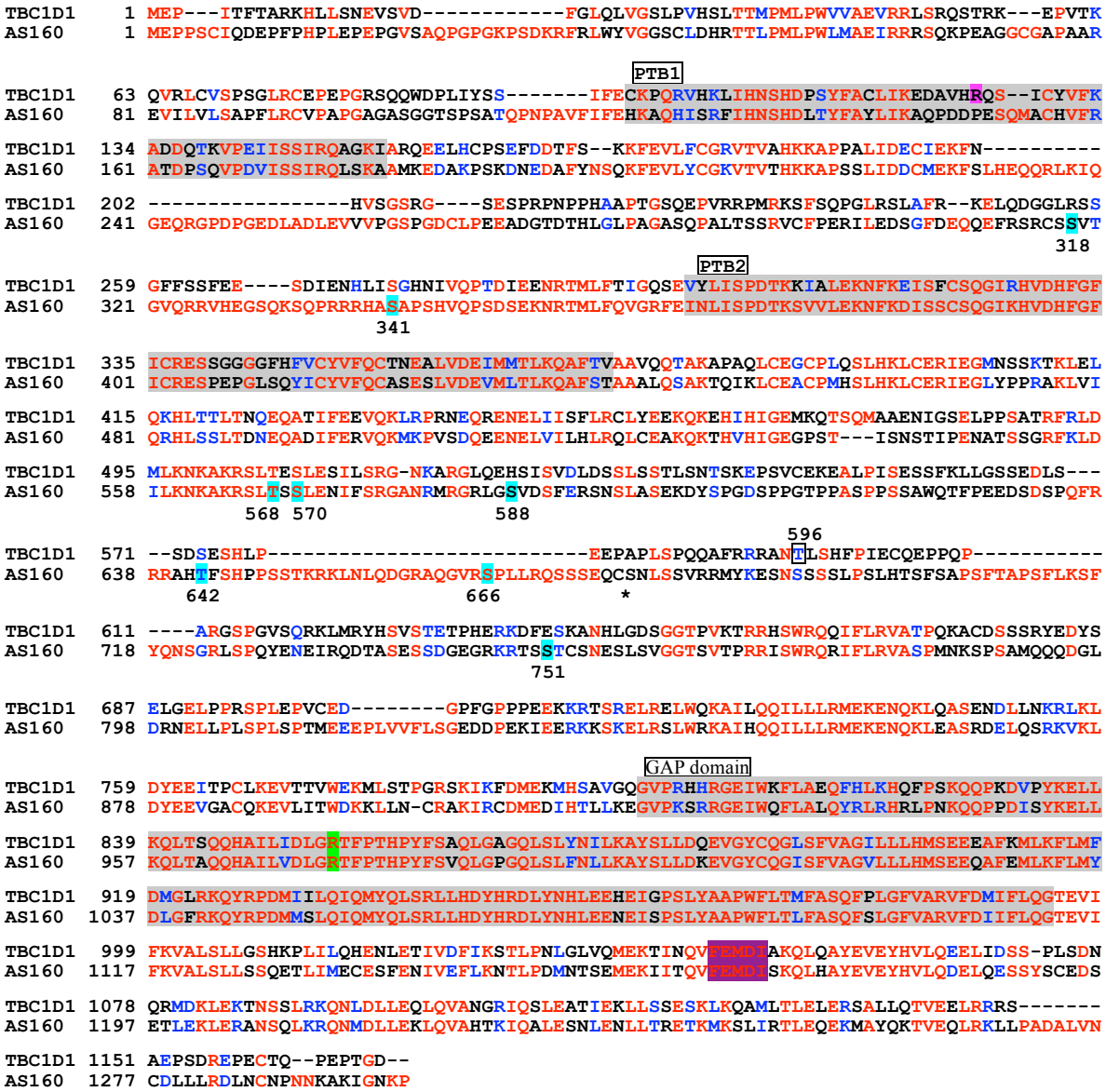
Mass	Sequence of phophopeptide	Sample detected in	Method of detection
552.96	HApSAPSHVQPSDSEK (S341)	A, B, C, D	MRM
717.35	SLTSpSLENIFSR (S570)	A, B, C, D, E	PIS, MRM
545.23	LGpSVDSFER (S588)	A, C, D	PIS, MRM
588.86	AQGVRpSPLLR (S666)	A, B, C, D, E	PIS, MRM
1054.09	TSpSTCSNESLSVGGTSVTPR (S751)	B, D	MRM

Endogenous AS160 was immunoprecipitated from lysate (20 mg) of HEK293 cells that were serum-starved (D), serum-fed (E), IGF1-stimulated (C), IGF1-stimulated in the presence of LY294002 (B) or treated with Calyculin A (A). The immunoprecipitated AS160 was denatured and run on a 4-12% Bis-Tris gel. The AS160 bands were cut out, digested with trypsin and the digests were analysed on an Applied Biosystems 4000 Q-TRAP mass spectrometer. Phosphopeptides were detected using a precursor ion scan (PIS). A Multiple Reaction Monitoring Analysis (MRM) was also performed to specifically look for known phosphorylated sites on AS160. In addition to the phosphopeptides in Table 1, the phosphopeptide AHpTFSHPPSSTK (in which pT corresponds to phosphorylated Thr642) was detected in GFP-TAP tag purified AS160 from a calyculin A-treated HEK293 cell line stably expressing GFP-TAP-AS160, and HA-AS160 isolated from IGF1-stimulated cells. Residue numbers correspond to the AS160B, long form of the protein (see Methods), though we do not know which splice variant generated the endogenous AS160 isolated here.

Supplementary Table 3

	Control	GVRpSP
SF	1.2	1.9
Unstimulated	1	1
IGF1	1.36	2.3
LY + IGF1	1.21	1.1
Calyculin A	1.36	2.8

This table shows the change in the quantity of the phosphopeptide containing phosphoSer666 (GVRpSP) between the various samples, that is serum-fed (SF), unstimulated, IGF1-stimulated (IGF1), IGF1-stimulated in the presence of LY294002 (LY+IGF1) and calyculin A-treated. The lowest intensity recorded for each peptide was recorded as one and all other intensities recorded for that peptide in different samples were normalised against this value. Three unphosphopeptides were also analysed in the experiment to control for loading, for which one set of data is shown as a representative example.



Supplementary Fig 1: Sequence alignment of human TBC1D1 and long form of AS160 generated using ClustalW (http://www.ebi.ac.uk/cgi-bin/clustalw) and Boxshade (http://www.ch.embnet.org/software/BOX_form.html). Identical residues are in red type, similar residues in blue, and differences in black. Residues that have been found to be phosphorylated in human AS160 are shaded in turquoise with residue numbers indicated. The R125W mutation site in TBC1D1 (Stone et al., 2006) is shaded in pink, and the FEMDI sequences in violet. The threonine residue (Thr596) in TBC1D1 that may be analogous to Thr642 in AS160 is boxed. The PTB domains (residues 96 to 153 and 301 to 373 in TBC1D1; 121 to 180 and 367 to 439 in AS160) and GAP domain (residues 800 to 994 in TBC1D1; 918 to 1112 in AS160) are indicated by grey shading with the active site R in green. In the main text, we call this long form, which has been identified in skeletal muscle cDNA libraries, AS160B. AS160A is a shorter form that lacks residues 679 to 733 (at the location marked *), which are derived from expression of exon 11 (NLSSVRRMYKESNSSSSLPSLHTSFSAPSFTAPSFLKSFYQNSGRLSPQYENEIS). Antibodies for immunoprecipitation and Western blotting of human and mouse AS160 were raised in sheep against the synthetic peptide KAKIGNKP (near the C-terminus of AS160).

Supplementary Fig 2 on next page: Specificity of the phospho-specific antibodies raised against the eight phosphorylated sites that were identified in AS160 (and anti-PAS)

A. Phospho-specific antibodies were raised against the seven phosphorylated sites that were identified in endogenous AS160 extracted from HEK293 cells (Supplementary Table 2)**.** HEK293 cells were transfected with HA-AS160A with the wild-type sequence and with single Ser/Thr to Ala mutations as indicated along the top of the figure. Crude lysates (60 μg) were probed for binding to the antibodies indicated at the side. The control lane represents lysates of untransfected HEK293 cells.

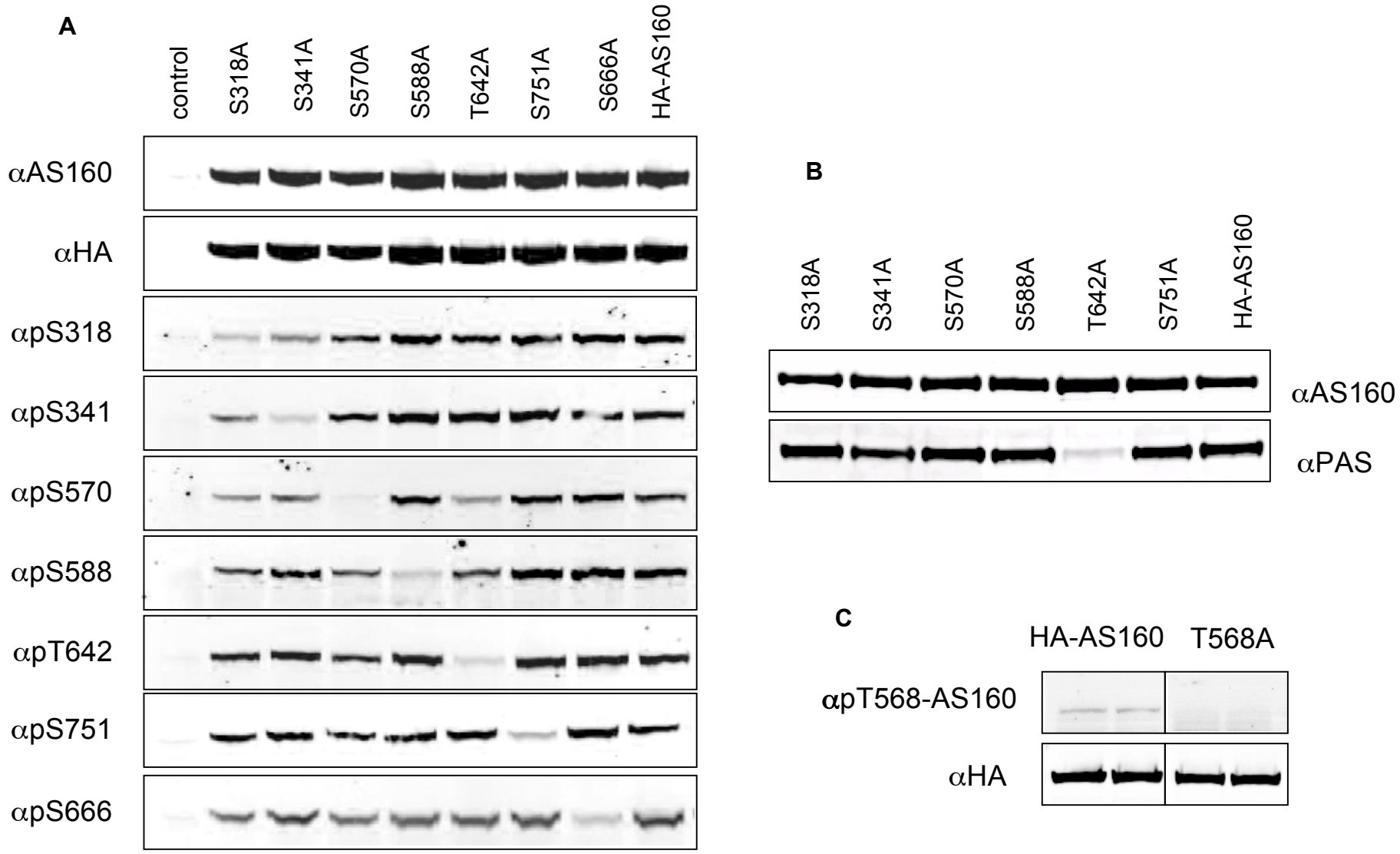
This figure shows that when tested on HA-AS160 extracted from serum-grown cells, the signals for each of the phosphospecific antibodies was markedly reduced or abolished when the appropriate residue was mutated to Ala. For the pSer666-AS160 and pSer751-AS160 antibodies, the signals were unaffected by mutation at any of the other phosphorylation sites. However, interdependency was revealed between pSer318 and pSer341, in that Ser318Ala-AS160 displayed a reduced recognition by the pSer341-AS160 antibody and vice versa. These data and earlier results (Fig 3B) indicate a hierarchy in which phosphorylation of Ser318 supports enhanced phosphorylation of Ser341. Moreover, the recognition by the pThr642-AS160 antibody was slightly decreased in the Ser570Ala mutant. Similarly, the pSer570-AS160 signal was abolished by Ser570Ala mutation as expected, and was also decreased in the Ser318Ala-AS160, Ser341Ala-AS160 and Thr642Ala-AS160, indicating that phosphorylation of Ser570 was enhanced by phosphorylation at these three residues.

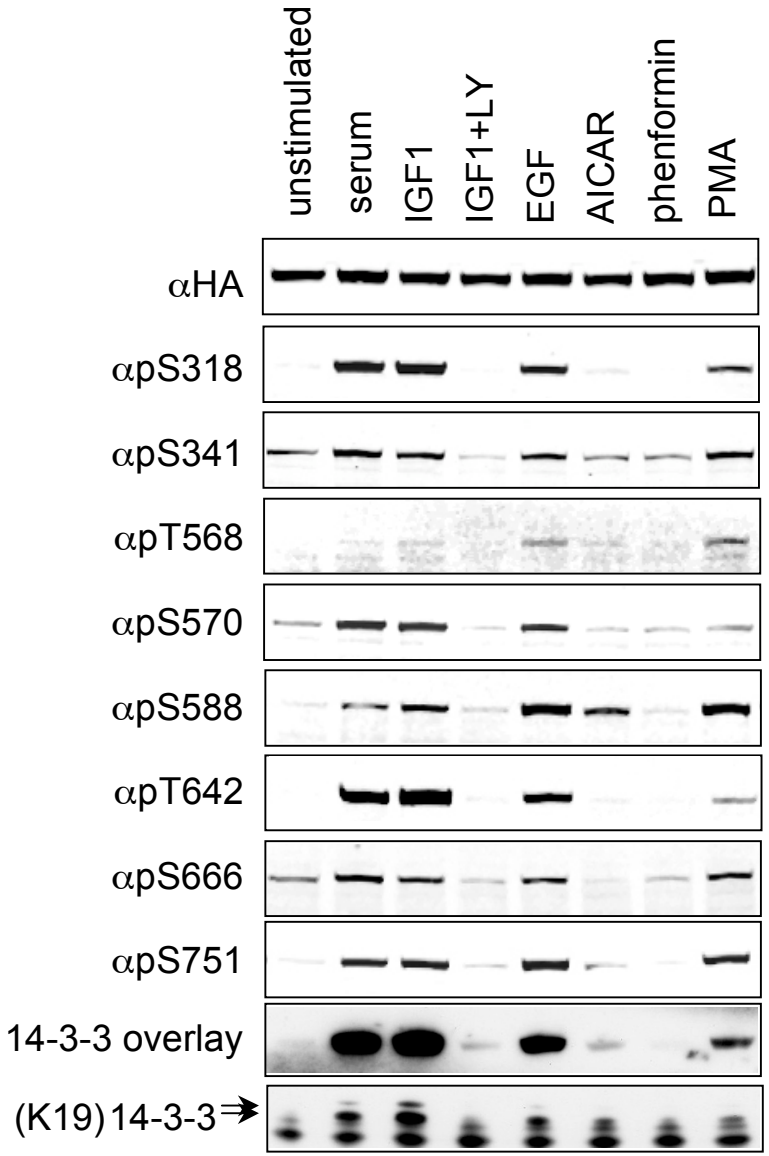
B. Specificity of anti-PAS towards sites on AS160: As for A, except that blots were probed with anti-AS160 and anti-phospho (Ser/Thr)-Akt/PKB substrate (PAS) antibody (Cell Signaling Technology).

The PAS antibody, which recognises sequences phosphorylated on a generic Akt/PKB phosphomotif, has been used in several studies to assess phosphorylation of AS160. This figure shows that recognition of AS160 by anti-PAS was largely abolished by the Thr642Ala mutation indicating that phosphoThr642 is the prime anti-PAS recognition site on AS160.

C. Phospho-specific antibodies against the eighth site (pThr568) that was first identified in tryptic digests of in vitro phosphorylated AS160 (Fig 5) and then in HA-AS160 extracted from PMA-stimulated HEK293 cells (Fig 6 and Supplementary Fig 3). The figure shows that the pThr568 antibody signal was abolished by mutation of Thr568 to Ala.

Supplementary Fig 2





Supplementary Fig 3: Phosphorylation of all eight sites on AS160 in response to a wider range of stimuli in HEK293 cells, and effects of protein kinase inhibitors

HEK293 cells transfected with HA-AS160A were stimulated as indicated. Crude lysates (60 μg per lane) were analysed by Western blotting using phospho-specific-AS160 antibodies (Supplementary Fig 2A, C) and 14-3-3 overlays, as indicated. In addition, co-precipitated 14-3-3s were identified using the K19 antibody.