

Fig. S1. Non-cell-autonomously regulated phosphorylations. A list of proteins in which phosphorylation was upregulated (A) or downregulated (B) in Ras cells co-cultured with normal cells, compared with Ras cells cultured alone. Abbreviations of protein names are in agreement with the Gene NCBI database. Phosphorylated amino acid residues are marked with bold letters within the peptide sequences. ‘(-)’ – the phosphorylated residue does not have an equivalent one in the human orthologue. ‘H/M ratio’ – heavy to medium ratio of the isotopic labels for a given peptide. ‘N/A’ – not applicable (peptide was not identified or the obtained data were insufficient for quantification).

Fig. S2. Analyses of VASP function. (A) Establishment of MDCK pTR-GFP-Ras^{V12} cells stably expressing His-VASP WT, -VASP S239D, or -VASP S239A. Cell lysates were examined by western blotting with the indicated antibodies. (B) Immunofluorescence of phosphorylated S239 of VASP in MDCK pTR-GFP-Ras^{V12}-VASP WT cells co-cultured with normal cells (left panels) or cultured alone (right panels) on glass. Cells were stained with anti-phospho-S239 VASP antibody (red). Scale bars: 25 μ m. We have noticed that subcellular localization of p-VASP is slightly different depending on the experimental condition; p-VASP is mainly localized at the cell cortex when cells are cultured on glass (supplementary material Fig. S2B), whereas a more cytosolic staining pattern is observed in cells cultured on collagen gels (Fig. 2A), though non cell-autonomous upregulation of p-VASP staining is observed under both experimental conditions. The nature of this discrepancy is not known at present, but it may result from the different immunofluorescence procedure between the two conditions (e.g. fixation time). (C) Analyses of phospho-S239 levels in endogenous VASP proteins in normal cells and Ras cells co-cultured (Mix) or cultured alone and the respective cell lysate were mixed (Alo). Expression of Ras^{V12} was induced by tetracycline treatment for the indicated times, and cell lysates were examined by western blotting with the indicated antibodies. (D) Analyses of phospho-S239 VASP without induction of GFP-Ras^{V12} expression in normal cells and Ras-VASP WT cells co-cultured (Mix) or cultured alone and the respective cell lysate were mixed (Alo). Cells were cultured for 30 h from plating, and cell lysates were examined by western blotting with the indicated antibodies. (E) Comparison of VASP expression between different experimental conditions. Ras cells and Ras-His-VASP WT cells were transfected with control siRNA (Ctrl KD) or VASP siRNA (VASP KD). Cell lysates were analyzed by western blotting with the indicated antibodies. (F) Immunofluorescence of VASP-knockdown MDCK pTR-GFP cells. GFP cells transfected with control siRNA (Ctrl KD) or anti-VASP siRNA (VASP KD) were co-cultured with normal cells on collagen gels. After induction of GFP expression with tetracycline for 24 h, cells were stained with Alexa-Fluor-647-phalloidin (purple). Scale bars: 25 μ m (xy sections), 10 μ m (xz sections). (G) Immunofluorescence of VASP-WT MDCK cells. Ras-VASP WT cells were co-cultured with normal cells or cultured alone for 30 h without induction of GFP-Ras^{V12} expression on collagen gels. Cells were stained with anti-His-tag antibody (green) and Alexa-Fluor-647-phalloidin (purple). Scale bars: 10 μ m. (H) Effect of overexpression of VASP proteins on morphology of Ras cells. Ras cells stably expressing VASP WT, VASP S239D, or VASP S239A were examined after 15 h of tetracycline treatment. Scale bars: 50 μ m. (I) Effect of expression of VASP proteins in VASP-knockdown Ras cells on cell morphology. Ras cells, Ras-VASP WT cells, Ras-VASP S239D cells, or Ras-VASP S239A cells were transfected with control siRNA (Ctrl KD) or VASP siRNA (VASP KD). Planar cell surface was analyzed after 15 h of tetracycline treatment. Data are means \pm s.d. (cells from three independent experiments; $n=1,324$ Ras + Ctrl KD cells, $n=1,293$ Ras + VASP KD cells, $n=1,148$ Ras-VASP WT + VASP KD cells, $n=533$ Ras-VASP S239D + VASP KD cells, $n=530$ Ras-VASP S239A + VASP KD cells); * $P<0.05$, ** $P<0.005$, *** $P<0.001$. (J) Effect of expression of VASP protein in MDCK cells on cell morphology. Planar cell surface was analyzed in MDCK cells, Ras cells, and Ras-VASP WT cells cultured without induction of GFP-Ras^{V12} expression. Data are means \pm s.d. (cells from three independent experiments; $n=1,887$ MDCK cells, $n=2,158$ Ras cells, $n=2,037$ Ras-VASP WT cells). (K) Effect of VASP-knockdown and overexpression of VASP proteins on the initial cell attachment to the substratum. The indicated cell lines were transfected with control siRNA (Ctrl KD) or anti-VASP siRNA (VASP KD) and cultured for 3 days. Cells were then trypsinized and plated on type-I collagen-coated dishes. Following 30 min incubation, unattached cells were washed off, and the remaining cells were stained with crystal violet. Cellular attachment values were obtained from absorbance of the dye trapped in the remaining cells measured in a plate reader at 570 nm. Data are means \pm s.d. from three independent experiments. * $P<0.05$. Values are expressed as a ratio relative to Ras + CTRL KD.

Fig. S3. Analyses of PKA signaling. (A) Analyses of phospho-S239 VASP upon induction of Ras^{V12} expression. Normal or Ras cells were incubated with tetracycline for the indicated times. Cell lysates were examined by western blotting with the indicated antibodies. (B–D) Effect of inhibitors for PKG and/or PKA on phospho-S239 VASP in Ras cells. Ras cells were incubated with tetracycline in the absence (Ctrl) or presence of PKG inhibitor Rp-8-Br-PET-cGMPS (PKG_i) (B), PKA inhibitor H89 (PKA_i) (C), or both inhibitors (PKG_i/PKA_i) (D) for the indicated times. Cell lysates were examined by western blotting with the indicated antibodies. (E) Effect of PKA inhibitors on phospho-S239 VASP in Ras cells. Ras cells were cultured for 4 h with PKA inhibitor KT5720 at the indicated concentrations or with PKA inhibitor H89. Cell lysates were examined by western blotting with the indicated antibodies. (F) Quantification of immunofluorescence intensity of phosphorylated substrates of PKA in Ras cells surrounded by normal cells (RM) or cultured alone (RR) (representative images are shown in Fig. 4F). Cells were incubated with tetracycline for 20 h in the absence (Ctrl) or presence (PKA_i) of PKA inhibitor H89. Data are means \pm s.d. ($n=9$ RM cells, $n=5$ RR cells, $n=5$ RM PKA_i-treated cells, $n=6$ RR PKA_i-treated cells). (G) Quantification of the effect of PKA inhibitor and activator on spreading of Ras cells. Ras cells were cultured with tetracycline for 4 h in the presence of H89 (PKA_i) and/or DBcAMP. Average planar cell surface was measured. Data are means \pm s.d. (cells from three independent experiments; $n=1,589$ Ctrl cells, $n=1,276$ PKA_i-treated cells, $n=1,680$ DBcAMP-treated cells, $n=1,405$ PKA_i/DBcAMP-treated cells); * $P<0.05$, ** $P<0.005$, *** $P<0.001$. (H) Effect of PKA inhibitor or PKA activator on the height of the intercellular lateral membrane between Ras cells. Ras cells were incubated with tetracycline for 4 h in the absence (Ctrl) or presence of PKA inhibitor H89 (PKA_i) or PKA activator DBcAMP (DBcAMP), followed by staining with Alexa-Fluor-647-phalloidin. The length of the lateral membrane was measured in xz confocal sections. Data are means \pm s.d. (cells from three independent experiments; $n=40$ contacts of Ctrl cells, $n=29$ contacts of PKA_i-treated cells, $n=43$ contacts of DBcAMP-treated cells); *** $P<0.001$. (I) Effect of PKA inhibitor on spreading of VASP-knockdown Ras cells. Ras cells were transfected with control siRNA (Ctrl KD) or VASP siRNA (VASP KD) and cultured for 2 days. Cells were then plated and incubated with tetracycline for 4 h in the absence or presence of H89 (PKA_i). Data are means \pm s.d. (cells from three independent experiments; $n=1,373$ Ras + Ctrl KD cells, $n=1,188$ Ras + VASP KD cells, $n=819$ Ras + Ctrl KD PKA_i-treated cells, $n=1224$ Ras + VASP KD PKA_i-treated cells); * $P<0.05$, ** $P<0.005$, *** $P<0.001$.

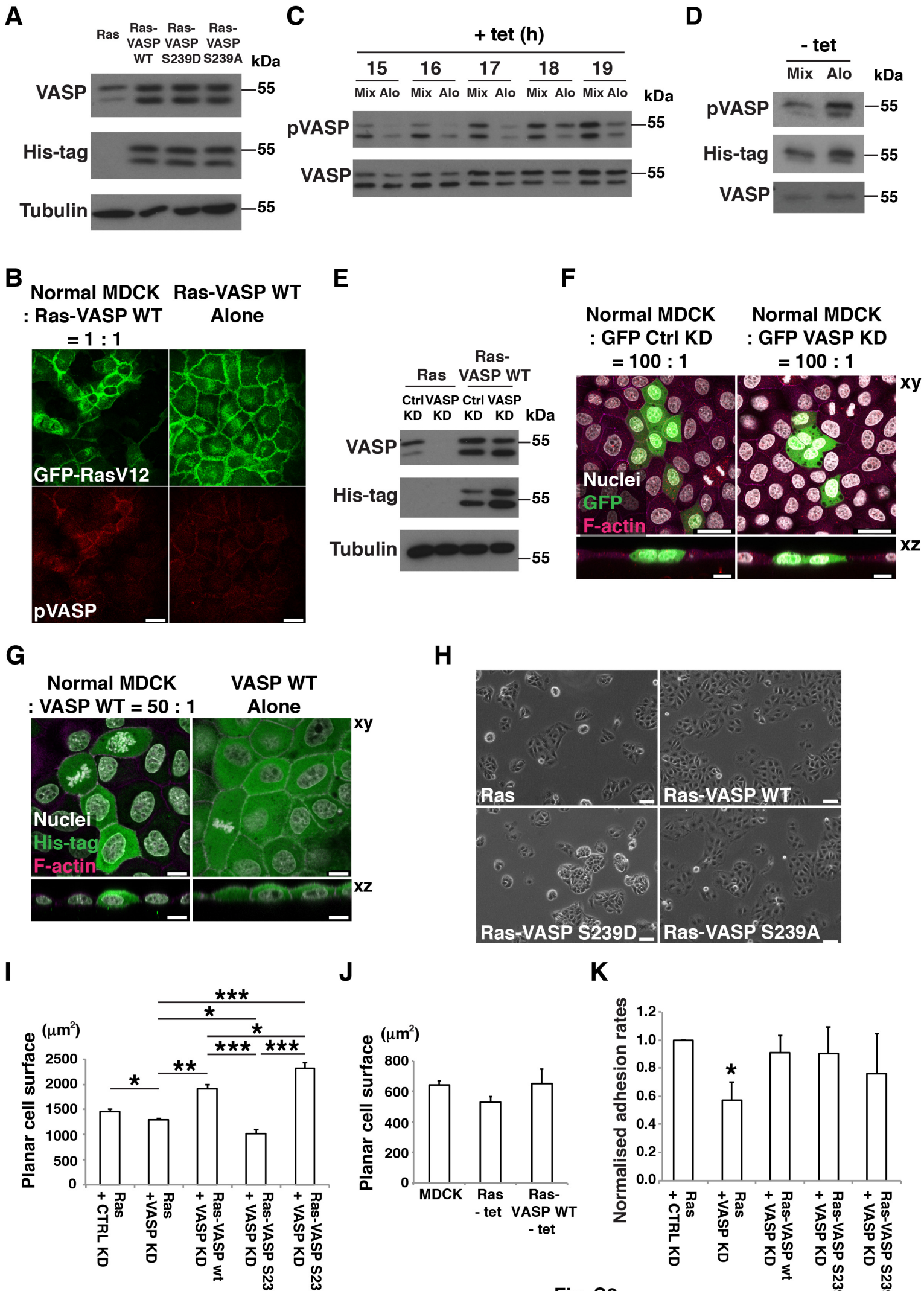
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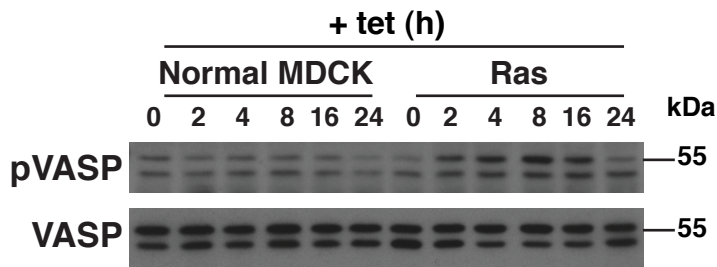
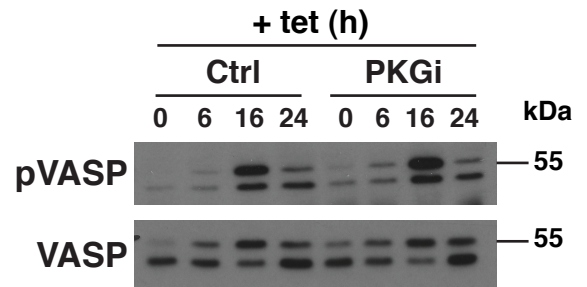
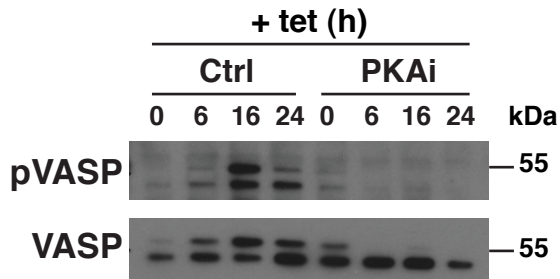
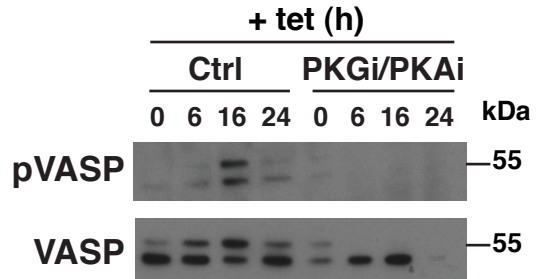
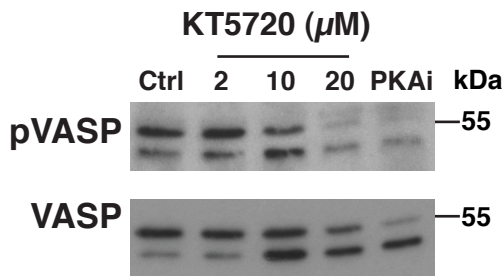
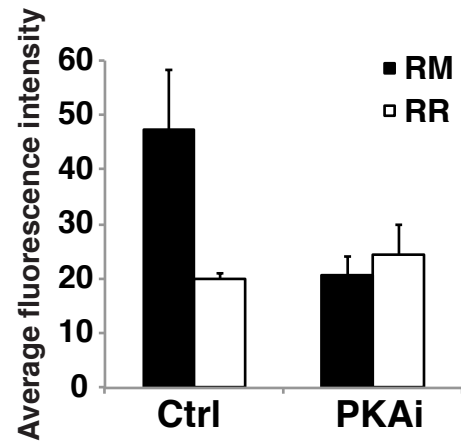
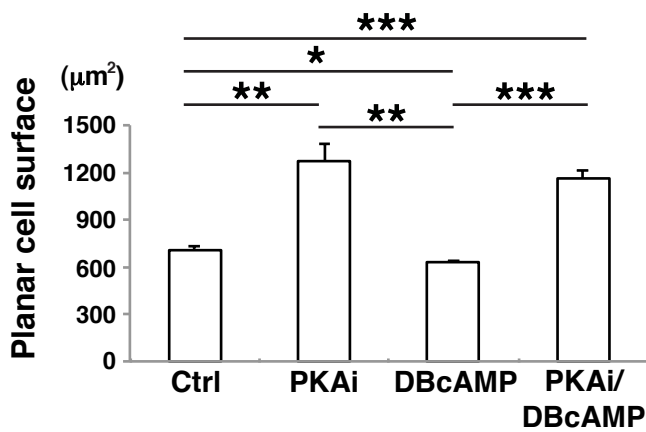
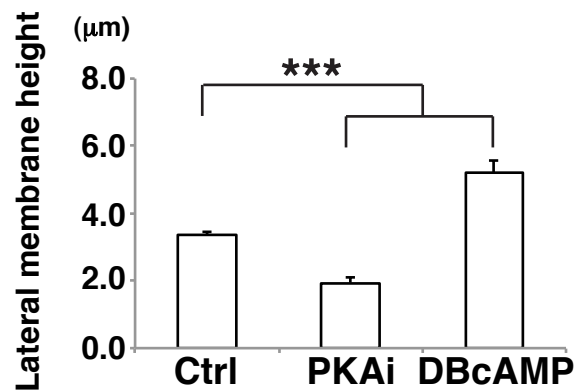
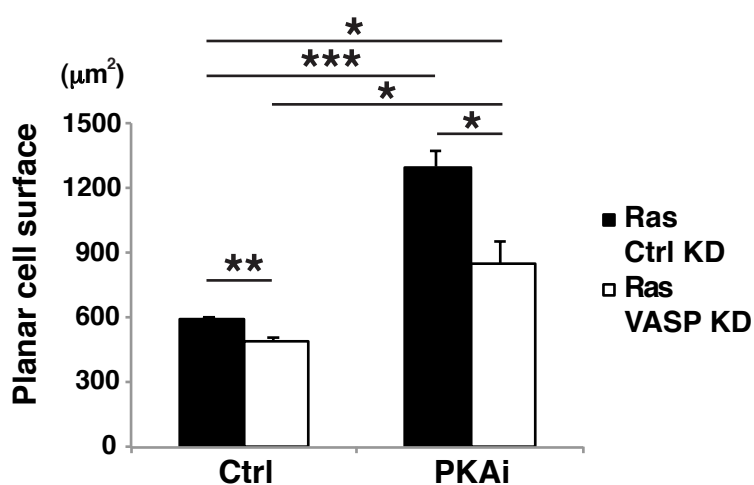
Protein name	Peptide Sequence	Modification within the protein	Analogous modification in human	H/M ratio Exp 1	H/M Ratio Exp2	H/M Ratio Exp3
BAIAP2L1	SIstVDL L TEK	S382	S422	2.4	2.4	2.7
CAMK2D	KPDGVK ES tESSN Tt IED ED VK	T331, T337	S395, T401	N/A	3.1	1.7
CDK1	IGEG ty GVV Y K	T14, Y15	T14, Y15	N/A	4.2	1.7
EF-2	AG ET R Ft D T R	T57	S57	N/A	2.1	1.4
FUNDC2	K S sQ I P T EV K	S181	S151	3.0	3.1	2.8
Hrnpc	NDK s EEEEQ S SS S L K	S221	S233	N/A	2.8	2.5
LMO7	R K s Y T S D L Q K	S260	S751	N/A	2.1	2.0
MCM2	s D P L T S s P G R	S20, S26	(-), S27	N/A	2.7	1.5
NPM1	s A P G s G S K V P Q K K	S143, S147	S143, (-)	N/A	2.1	2.0
PLEC	G Y s P Y S V S G S G s A A G S R	S4206, S4215	S4613, S4622	3.4	2.1	1.7
PLEC	G Y s P Y S V S G S G s A A G S R	S4206, S4211	S4613, S4616	N/A	2.1	1.5
SERBP1	SK s EEEA H A E D S V M D H H F R	S308	S328	1.7	2.1	1.8
SF3B1	R W D Q T A D Q t P G A t P K	T208, T212	T207, T211	N/A	5.3	5.3
SRP14	K G s V E G F E P S D N K	S45	T45	12.6	4.6	1.6
STRN	F L E S A A D F s D E D E D D D I D G R E K	S164	S245	N/A	1.7	2.0
VASP	K V s K Q E E A S G G P P V P K	S242	S239	3.2	3.3	1.4
VIM	E T N L D s L P L V D T H S K R	S430	S430	N/A	2.5	2.5
ZO1	s V A s S Q P P K P T K	S166, S169	S175, S178	N/A	3.2	1.5

B

Protein name	Peptide Sequence	Modification within the protein	Analogous modification in human	H/M ratio Expl	H/M Ratio Exp2	H/M Ratio Exp3
COL17A1	A H s P A S T L P N s P G S T F E R	S85, S93	S85, S93	0.24	0.12	0.22
EML4	A s P s P Q P S S Q P L Q I H R	S144, S146	S144, S146	N/A	0.60	0.30
FOSL2	s P P A S G L Q P L R	S115	S200	0.42	0.44	0.28
Foxk1	S A P A s P T H P G L M s P R	S234, S242	S420, S428	N/A	0.50	0.35
JUNB	D A t P P V s P I N M E D Q E R	T247, S251	S255, S259	N/A	0.18	0.14
LARP1	S L P T T V P E s P N Y R	S657	S774	N/A	0.49	0.27
MCT1	s K E s L Q E A E K	S200, S203	S210, S213	N/A	0.24	0.13
MRCKβ	H S T P S N S S N P S G P P s P N s P H R	S1693, S1696	S1690, S1693	0.33	0.50	0.29
PDXDC1	V Q G T G V t P P Q t P S G T R	T721, T725	T687, T691	N/A	0.20	0.19
SPEN	H S s F H E E D D P V G s P R	S1209, S1219	S1268, S1278	N/A	0.37	0.24
SQSTM1	L T P V s P G G s S T E D R	S245, S249	S272, S276	0.79	0.34	0.31
SQSTM1	L T P V s P G G s S T E D R	S245	S272	0.73	0.48	0.43
SRP72	T V S S P P T S P R P G s A A t A S A S T S N I I P P R	S630, T633	S630, S633	N/A	0.44	0.29
TNS1	R M s V G D R	S1370	S1393	0.63	0.14	0.23
TXLNA	E Q G C E G P G A Q S P G s P R	S530	S515	0.69	0.41	0.32
ZC3HC1	S Q D A t C S P G S E Q A E R s P G I V S R	T336, S347	T333, S344	N/A	0.29	0.36

Fig. S1
Anton et al.



A**B****C****D****E****F****G****H****I****Fig. S3**