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Supplemental Data

Article

Amino Acids Activate mTOR Complex 1 via Ca^{2+}/CaM Signaling to hVps34

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Supplemental Experimental Procedures

Transfections

Escort V Transfection Reagent (Sigma) was used according to the manufacturer's protocol. Transfection of siRNAs was carried out as described previously (Garami et al., 2003) using hVps34 siRNA (920) and Rheb siRNA (Nobukuni et al., 2005) We employed a PKB 1 siRNA (5'-TTGTAGCCAATGAAGGTG CCA-3') to knock down PKB protein levels. Two additional sequences were employed, but neither was as efficient in knocking down PKB protein levels. In addition, a nonsilencing sequence (AATTCTCCGAACGTGTCACGT) and lactose permease (LacZ, 5'-GCGGCT GCCGGAATTTACCTT-3') siRNAs were employed as controls and showed no effect on either S6K1 T389 or PKB S473 phosphorylation. siRNA sequences for CaM included CaM1, TTGCTAATAAGATACAACCAG; CaM2, TACATATTGTTGACTGTCCAT; and CaM3, TTGGTGTTTGCTAGAACCGGG.

hVps34 CaM-Binding Site Mutagenesis

Two sets of primers were generated for producing calmodulin-binding-site hVps34 mutants. The first set of primers was used to replace phenylalanine at position 318 with arginine (5'-GAT CTT GTT TGG AAG **CG**T AGA TAT TAT CTT AC-3''). A second set of primers was used to replace the leucines at positions 330 and 334 with arginines (5'-AAT CAA GAA AAA GCC **AG**G ACA AAA TTC **AG**G AAA TGT GTT AAT TG-3'). Mutagenesis was performed using the QuikChange II Site-Directed Mutagenesis Kit (Stratagene). The hVps34 WT construct, obtained from M. Wymann, University of Basel, was used as a template.

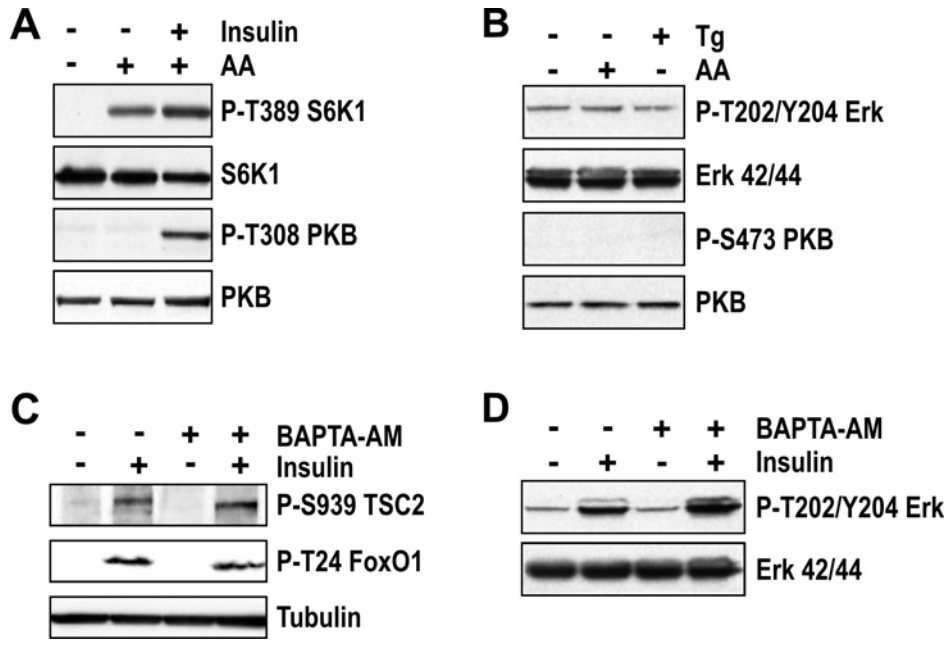

Figure S1. AAs and BAPTA-AM Have No Effect on PKB or Erk Signaling

(A and B) After serum and AA deprivation, HeLa cells were stimulated with AA and/or insulin or Tg as described in Figure 1A and Figure 2C, respectively.

(C and D) HeLa cells were serum and AA deprived as in Figure 1A. BAPATA-AM pretreatment and AAs-stimulations were performed as in Figure 1B and Figure 1A respectively. Cell lysates were examined by Western blot analysis using the indicated antibodies (see Experimental Procedures).

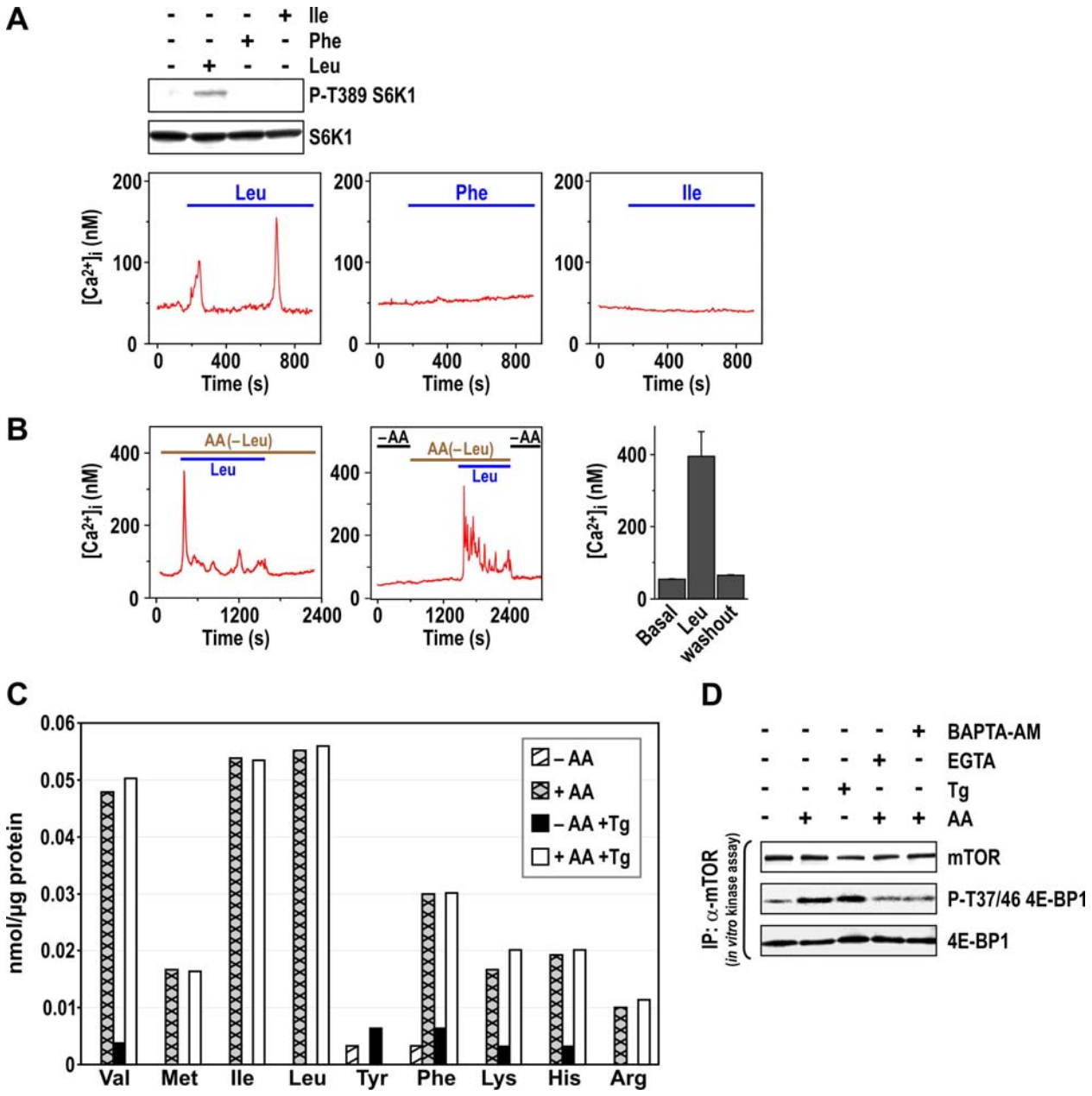

Figure S2. Leucine Alone Increases mTOR Complex 1 Signaling through a Rise in the Levels of $[Ca^{2+}]_i$

(A) HeLa cells were deprived of serum and AAs, as in Figure 1A then stimulated for 30 min with Leu (210 mg/L), Phe (132 mg/L) or Ile (210 mg/L), 2x the concentrations present in DMEM. Western blot analyses were performed using the indicated antibodies. The traces are representative $[Ca^{2+}]$i responses in fura-2–loaded HeLa cells stimulated with Leu, Phe or Ile under conditions similar to those described in Figure 2B, except that the phenylalanine experiments were carried out in the presence of 5 mM extracellular Ca^{2+}. Positive $[Ca^{2+}]_i$ signals were seen in 360 cells stimulated with Leu, but were not observed with Phe or Ile (>200 cells examined for each).

(B) Left panel, Hela cells were cultured in Leu-free DMEM containing all other amino acids and 10% dialyzed FCS, and then stimulated with Leu (210 mg/L) during the period indicated by the blue bar. Middle panel, HeLa cells were cultured in AA-free DMEM with 10% dialyzed FCS, followed by the re-addition of 2x AA without Leu (brown bar), and then Leu in the continuing presence of all AAs (blue bar). At the end of the experiment all AAs were withdrawn. Right panel, shows mean $[Ca^{2+}]_i$ levels under basal conditions before Leu addition, at the peak response to Leu, and following 15 min Leu withdrawal (values are mean ± SEM, n=219 cells).

(C) HeLa cells were treated with AAs and/or 150 µM Tg for 30 min, as described in Figure 2C, washed twice in ice-cold PBS, lysed in 500 µl dH_2O, and sonicated. Lysates were centrifuged at 13,000 rpm at 4ºC for 10 min to remove cell debris. Salicylic acid was then added to the supernatant to a final concentration of 2% w/v and left on ice for 30 min. After centrifugation at 13,000 rpm at 4ºC for 10 min, the supernatants were subjected to amino acid analysis using an automated Hitachi Amino Acid Analyzer. The precipitate was used for protein determination after solubilization in 1N NaOH. Amino acid contents were normalized to the protein content of each sample. For amino acid measurements, the baseline was set up to > 2.2, meaning values which were above 2.2 were only taken for plotting the bar diagram.

(D) Cell treatments were performed as in Figure 2C. Lysate preparation, mTOR immunoprecipitation and in vitro mTOR Complex 1 kinase activity assays were done as described by Sancak et al. (2007). The result shown is a representation of three independent experiments.

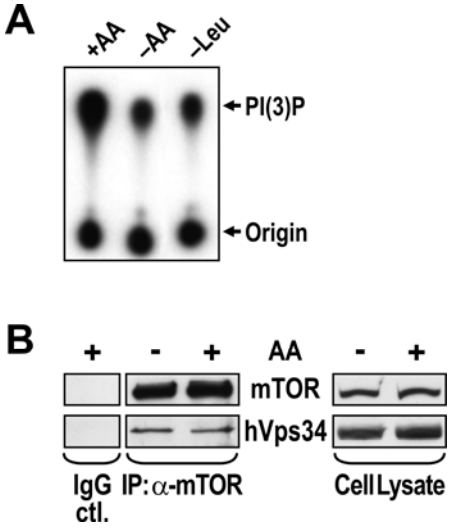

Figure S3. Leucine Withdrawal Inhibits hVps34 and Endogenous hVps34 Immunoprecipitates with mTOR

(A) AA-stimulated cells were deprived of either all AAs or Leu alone as in Figure 3D and Figure S2B, respectively. The hVps34 activity assay was performed as in Figure 3D. Equal input of hVps34 in all samples was determined by protein concentrations.

(B) Endogenous mTOR was immunoprecipitated from either AA-deprived or 2x AA-stimulated HeLa cells as described in Figure 4C.

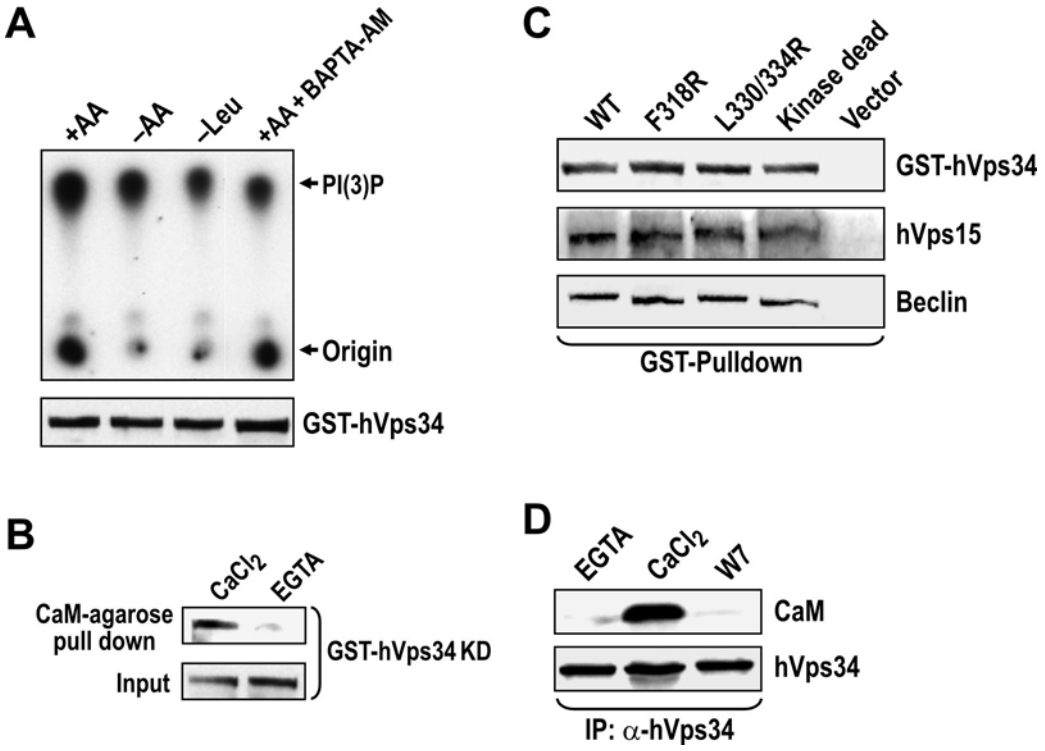

Figure S4. Effects of AA Withdrawal and Leucine Withdrawal or BAPTA-AM Treatment on GST-hVps34 and the Interaction of Beclin and hVps15 with hVps34 CaM-Binding Mutants

(A) HEK 293 cells expressing WT GST-hVps34 were stimulated with AAs and then either deprived of AAs or Leu or treated with BAPTA-AM as in Figure S2B or Figure 3D, respectively. The hVps34 activity assay was performed as in Figure 6B .

(B) Calmodulin interaction with ectopically expressed KD GST-hVps34. Expression levels of KD GST-hVps34 and its ability to interact with CaM beads was as described in Figure 6A.

(C) WT, KD, and CaM-binding mutants of hVps34 were ectopically expressed in HEK 293 cells. Post 48 hrs transfection cells were harvested and the expressed proteins were immunoprecipitated as in (Figure 6A). GST-immunoprecipitates were analyzed by Western blotting with the indicated antibodies (see Experimental Procedures).

(D) hVps34 was immunoprecipitated from lysates of cells treated with AAs in the presence of 0.5 mM $CaCl_2$ or 2 mM EGTA, as described in Figure 6D. The immune complexes generated were first washed as described by Nobukuni et al. (2005), except the final washes were carried out as follows: Lanes 1, 2 mM EGTA; Lane 2, 0.5 mM $CaCl_2$ and Lane 3, 100 μM W7. All washes were performed 3x 20 min at 22ºC. Western blot analyses were performed using the indicated antibodies (see Experimental Procedures).