SUPPORTING INFORMATION

Supplemental Materials and Methods

cDNA transfection

Cells were transfected with plasmid DNA using PolyJet™ DNA *In Vitro* Transfection reagent (SignaGen Laboratories, #SL100688) according to manufacturer's instructions. Cells were plated in 6-well, 10cm or 15cm culture dishes depending on the experiment. After 24 hrs, cells were transfected with 500ng-5μg of HA, HA-tagged Raptor, HA-tagged LARP1, HA-tagged RHA, FLAG, FLAG-tagged AKAP8L and truncations, PKA Catα, mCherry-tagged PKA Regulatory subunit Iα, Myc-tagged Raptor, and RFP. Media was changed after 4-6 hrs after transfection, and cells were harvested 24-48 hrs later.

Cell lysis and immunoblotting

Cells were washed with 1x PBS and lysed with Laemmli sample buffer (50mM Tris pH 6.8, 2% SDS, 0.025% Bromophenol Blue, 10% glycerol, 5% BME) and boiled for 5 min before separation by 10%-15% SDS-PAGE and transfer to polyvinylidene difluoride membranes (Bio-Rad , #162-0177). Blots were then blocked in 5% milk for 1 hr, probed with primary antibodies and horseradish peroxidase (HRP) conjugated secondary antibodies, and developed with SuperSignal™ West Dura Substrate (Thermo Fisher Scientific, #34075).

Immunoprecipitation

All steps were done on ice or at 4ºC. Cells were washed once with cold 1x PBS then lysed with 0.3% CHAPS (40mM HEPES pH7.5, 120mM NaCl, 1mM EDTA, 10mM Sodium pyrophosphate, 10mM Glycerol-2-phosphate, 50mM NaF, 0.5mM Sodium Orthovanadate, cOmplete™ EDTA free protease tablet (Roche, #40091500), 0.3% CHAPS, milli-Q H_2O). Cells were spun for 10 min at 15,000 rpm to pellet cell debris. 75μL was taken for whole cell lysate, and the remaining lysate was used for immunoprecipitation. Pierce™ Anti-HA (ThermoFisher Scientific, #88836), Pierce™ Protein A/G (ThermoFisher Scientific, #88802), Pierce™ Anti-c-Myc (ThermoFisher Scientific, #20168) or Anti-FLAG (Sigma, #A2220), beads were washed once in 0.3% CHAPS then blocked in 3% Bovine Serum Albumin (BSA) (Fishers Scientific, #BP1600) for at least 1 hr, then washed twice more before adding to cleared cell lysate. Immunoprecipitation was allowed to neutate for 24 hrs at 4ºC then washed three times with 0.3% CHAPS before adding Laemmli sample buffer.

Immunofluorescence

Figure 3A: Mouse embryonic fibroblast (MEF) cells were plated onto coverslips in a 12-well plate (Corning, REF 3513) coated with fibronectin (Sigma, #F4759) for 1 hr. Once adhered, cells were transfected for 24 hrs then processed for confocal imaging. Media was aspirated and cells were washed twice with 1x PBS, then fixed with 4% paraformaldehyde (PFA) (Electron Microscopy Sciences, #15710) for 20 min. Cells were washed twice with 1x PBS, then permeabilized with 0.01% saponin in 1x PBS for 10 min followed by two more 1x PBS washes. Cells were blocked with 2% BSA in 1x TBS-T for 1 hr and washed three times for 5 min each and transferred to a 24-well plate (Corning, REF 3526) Primary antibody was used at 1:800 for anti-DYKDDDDK Tag (Cell Signaling Technology, #14793S) for 1 hr and washed three times with TBS-Tween (TBST) for 5 min. Secondary antibody was used 1:200 Alexa Fluor™ 555 goat anti-rabbit IgG (Invitrogen, #A21428) for 1 hr and washed twice with 1x PBS for 10 min. Cells were then washed with Milli-Q H_2O twice for 10 min and mounted on microscope slides (Fisherbrand, #12-550-15) using ProlongGold with DAPI (Invitrogen, #P36931) overnight. Slides were sealed with nail polish (Maybelline) and imaged using confocal microscopy.

Supplemental Figure 2: HEK293A cells were seeded in 12-well plates on coverslips one day prior to experimentation. Coverslips were pretreated with 5μg/ml of fibronectin (#F4759 from Sigma) at 37°C

for 1 hr, with a quick phosphate-buffered saline (PBS) wash prior to cell seeding. 1μg of FLAG-tagged AKAP8L was transfected for 24 hrs. Cells were briefly washed with 1x PBS and fixed with 4% paraformaldehyde/PBS for 20 min, followed by washing with 1x PBS 3 times for 5 min each. Cells were permeabilized with 0.2% Triton-X/PBS (for GM130 and Lamp2) or cold methanol (at -20°C; for Calreticulin and Cox IV) for 10 min, and then blocked in 2% BSA in TBST or 5% goat serum/0.3% Triton X-100 for 1 hr, followed by washing with TBST 3 times (5 min each). Cells were incubated for 1 hr at room temperature (or overnight at 4°C) with primary antibodies, followed by washing with 1x PBS 3 times (5 min each). Cells were incubated for 1 hr with secondary antibodies, followed by washing with PBS twice (5 min each), then washed with double distilled water twice (5 min each). Coverslips were mounted onto microscope slides using ProLong Gold Antifade Reagent with DAPI. Images were captured with a Zeiss LSM 800 microscope, and exported from Zeiss ZEN imaging software. Individual channels were pseudo-colored using ImageJ prior to assembling figures.

Antibodies:

Calreticulin
primary: Calreticulin (rabbit, CST #12238, 1:100) + FLAG (mouse, Sigma #F1804, 1:800)
secondary: goat anti-rabbit-555 1:200 + goat anti-mouse-488 1:200

Cox IV
primary: Cox IV (rabbit, CST #3E11, 1:100) + FLAG (mouse, Sigma #F1804, 1:800)
secondary: goat anti-rabbit-555 1:200 + goat anti-mouse-488 1:200

Lamp2
primary: Lamp2 (mouse, Abcam #H4B4, 1:200) + FLAG (rabbit, CST #14793, 1:800)
secondary: goat anti-mouse-488 1:200 + goat anti-rabbit-568 1:200

GM130
primary: GM130 (rabbit, CST #12480, 1:200) + FLAG (mouse, Sigma #F1804, 1:800)
secondary: goat anti-rabbit-568 1:200 + goat anti-mouse-488 1:200

Subcellular fractionation

Cellular fractionation using differential centrifugation was performed according to abcam. In brief, cells were collected in a hypotonic buffer and passed through a 26 gauge syringe to prevent organelle lysing. Cells were incubated in buffer then subjected to differential centrifugation. All steps were done on ice or similar to 4°C.

Immunoprecipitation from nuclear and cytoplasmic fractions

Fractionation and subsequent immunoprecipitation were performed according to the Jove protocol (55) with slight modifications. Fractionation was performed using a kit (Thermo Scientific, REF 78835). HEK293A cells were seeded into 15cm plates overnight. Cells were then transfected with HA-tagged Raptor for 24 hrs. Cells were trypsinized and washed with 1x PBS before using the kit. HA beads were blocked with 3% BSA for at least 1 hr. HA-Raptor was immunoprecipitated overnight at 4°C. Beads were washed as stated in the protocol, with PBST (0.1%), three times. Nuclear and cytoplasmic extracts were loaded at a ratio of 1:2 (V/V) for SDS-PAGE.

siRNA knockdown

Cells were plated and allowed to reach a confluency of about 60%. ON-TARGET plus SMART pool siRNA (Dharmacon, #L-009258-00-0005) against AKAP8L was used at 50nM and transfected using DharmaFECT transfection reagent (Dharmacon, #T-2001-03) for 24-48 hrs.

Starvation protocol

For Supplementary Figure 1, cells were starved of either amino acids for 1 hr or serum for 16 hrs, then stimulated with amino acids for 1 hr or 100nM insulin for 30 mins, respectively. For Supplementary Figure 3E, cells were transfected for 24 hrs before serum removal for 16 hrs and replacement with complete media (+FBS) for 30 min before collection.

^{35}S radiolabeling

All radioactive steps were completed in designated radioactive room or kept in shielded container. Cells were plated into a 6-well plate (Corning Inc., REF 3516). Complete media was removed and cells were washed once with 1x PBS and replaced with media without methionine or cystine (Thermofisher Scientific, #21013024) supplemented with 4mM glutamine for duration of treatment. In the last 10 min of treatment, cells were spiked with a mixture ^{35}S-methionine and ^{35}S-cysteine (PerkinElmer, #NEG772002MC) at 11mCi/mL. Cells were then collected using Laemmli sample buffer. SDS-PAGE and transfer were performed as previously stated. An imaging plate (FujiFilm, #28956475) was placed on top of transferred membrane for 30 min in a closed cassette then imaged using Typhoon phospho imaging software. Film was used to collect final image, left to develop 24-72 hrs in -80°C.

Polysome profiling

Cells were plated in five 15cm dishes and cultured overnight to reach about 70-80% confluency. After treatment, cells were trypsinized and spun down at 1200 rpm for 5 min. Cells were resuspended in ice cold 1x PBS and respun at 4°C, and washed/spun again with 1x PBS containing cycloheximide at 100µgml^{-1}. Resuspend in 800µl of polysome extraction buffer (20mM Tris-HCL pH 7.5, 100mM NaCl, 5mM $MgCl_2$, 0.1% NP-40, and Milli-Q H_2O) containing 100µgml^{-1} cycloheximide, 100 units of RNase inhibitor (Promega #N2511) and a protease inhibitor tablet per sample. Transfer to cold Eppendorf tube and shake gently for 10-30 min. Centrifuge at 15000 rpm for 5 min at 4°C and remove lysates to prechilled tubes. Normalize by protein content using Bradford reagent (Bio-Rad, #5000006) or by RNA concentration using a nanodrop. Load lysates onto previously prepared sucrose density gradients (1ml of sucrose gradient buffer (200mM HEPES pH7.6, 1M KCl, 50mM $MgCl_2$, 100µgml^{-1} cycloheximide, 1x EDTA free protease tablet, 100units/ml RNase inhibitor) and 10-50% sucrose). Gradients were centrifuged in an TH-641 Swinging Bucket rotor (Fisher Scientific, #54295) at 35000 rpm for 2 hrs at 4°C, then sampled using a Biologic LP System with BioFrac Collector and LP Data View Software (Bio-Rad, #7318338) or a profiler from BioComp Instruments. Data was input into GraphPad Prism 8 software to create profile.

Cell size

Cells were plated in triplicate in 100mm x 20mm dishes (Corning Inc., REF 430167). When about 80% or less confluent, cells were washed once with 1x PBS, trypsinized (Sigma, #T3924), neutralized with media, spun down for 3 min x 3000 rpm and resuspended in 1ml of 1x PBS. Cell number and size was measured using a Z2 Coulter Particle Count and Size Analyzer (Beckman Coulter), and processed using Z2 Accucomp software. Final binned-histogram plots were created using GraphPad Prism 8 software.

Cell proliferation

2.0 x 10^4 cells were plated in triplicate in 6-well or 35mm x 10mm (Corning Inc., REF 430166) dishes. Cells were prepared using a 1:1 mixture with Trypan Blue solution (Sigma, #T8154). Cells were counted on days 2, 4 and 6 using counting slides (Bio-Rad, #145-0011) and a Bio-Rad TC20 Automated Cell Counter (Bio-Rad, 1450102).

Generation of knockout cell lines

The 20 nucleotide guide sequences targeting human AKAP8L were selected from the gene list created by Human Brunello CRISPR knockout pooled library, a gift from David Root and John Doench

(addgene, #73178) and cloned into lentiCRISPR v2 vector (addgene, #52961). The guide sequence targeting Exon 10 and Exon 4 of human *AKAP8L* are shown below:

5'-AAGGAACACTTTAAGTACGT-3' (sgRNA#1)
5'-AGGATAACACCACCAACTAT-3' (sgRNA#2)

The single guide RNAs (sgRNAs) against human AKAP8L or GFP in the lentiCRISPR v2 vector (1500ng) were mixed with the packaging plasmids psPAX2 (900ng) and pMD2.G (600ng) and co-transfected into HEK293A cells using PolyJet™ DNA *In Vitro* Transfection reagent (SignaGen Laboratories, #SL100688) according to manufacturer's instructions. After 48 hrs, virus was collected and applied with Polybrene (Millipore, #TR1003G) to new HEK293A cells. After 24 hrs, virus was removed and replaced with complete media for 24 hrs. After 24 hrs cells were selected using puromycin (5μg/ml; InvivoGen #ant-pr-1). After puromycin selection, cells were single cell sorted (UTSW Flow Cytometry Core, Aria 1) into 96-well plate format into DMEM containing 30% FBS and 50μg/ml^{-1} penicillin/streptomycin. Single clones were expanded, and screened for AKAP8L by protein immunoblotting. Genomic DNA (gDNA) was purified from clones using Quick gDNA prep kit (Zymo Research, #11-317C) and the region surrounding the protospacer adjacent motif (PAM) was amplified with Q5 polymerase (New England BioLabs, #MO492) using the following primers:

(linkers containing EcoRI and Xho1 restriction sites are underlined)

sgRNA#1:
Forward: CCGCTCGAGAAGAGCTCCTCCCCTGTCC
Reverse: CCGGAATTCAACTCGCCCTGCCACTTC

sgRNA#2:
Forward: CCGCTCGAGTTCCTGTGATTGCTGTCCTG
Reverse: CCGGAATTCTGAGGGCACAGTGAAGTCTG

PCR products were purified using the DNA Clean and Concentrator Kit (Zymo Research, #11-305) and cloned into pBluescript II KS+. To determine indels of individual alleles, 10 or more bacterial colonies were expanded and the plasmid DNA extracted, purified and sequenced for AKAP8L genomic DNA (74).

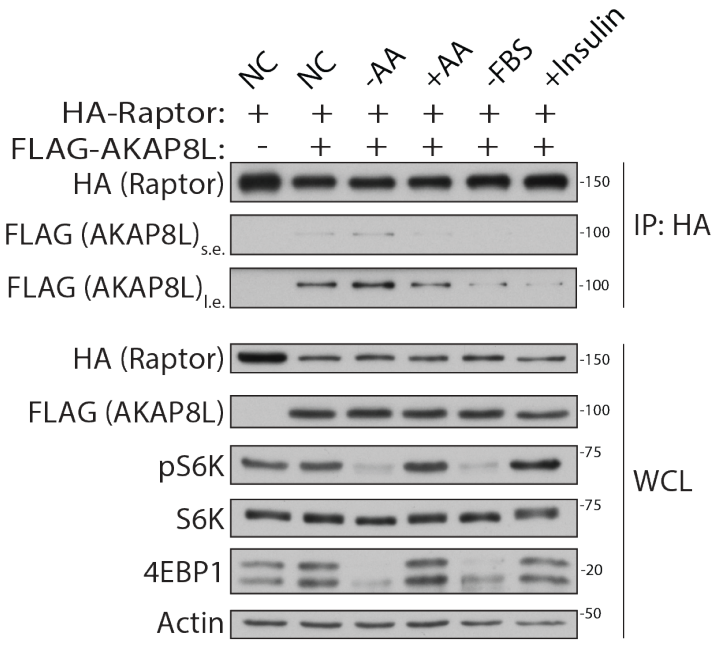
Polysome profile statistics

First, heights of each monosome (80s) and polysome profile peak were normalized between biological replicates. The ratios of individual sample polysomes/monosomes were calculated by: (polysome/monosome)/(averaged control). Relative amounts were then calculated by the averaging the 3 samples of each group. Similar methods were used to calculate relative polysomes and monosomes.

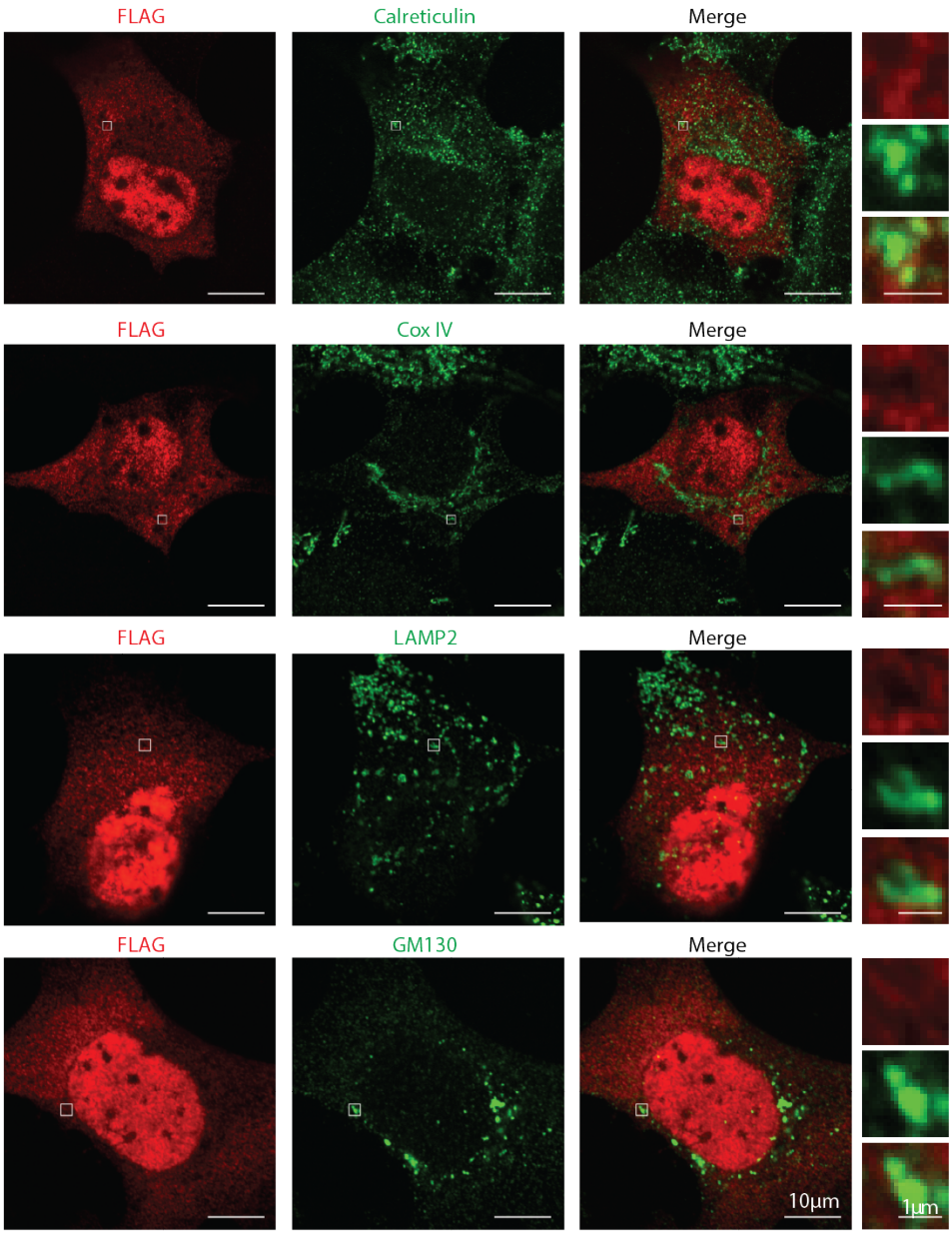
Statistics

Diagrams with individual data points are represented as the mean of three technical replicates ± standard deviation (SD). Significance was analyzed using Student's T-test. The T-test function was performed in Excel, two tails and unpaired. Significance (*P*): * = < 0.05, ** = < 0.01, *** = < 0.001.

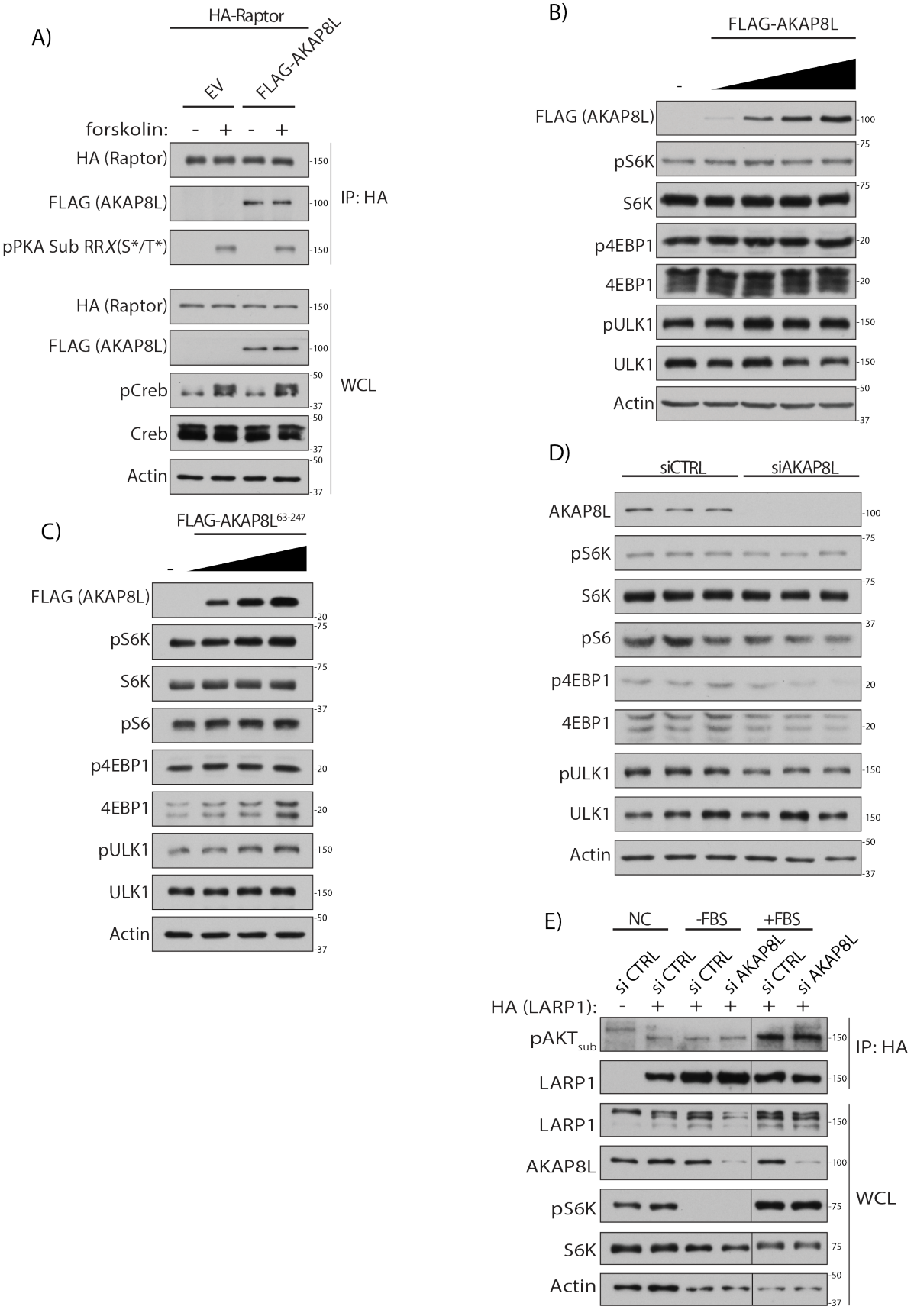
SUPPLEMENTAL FIGURE TITLES AND LEGENDS



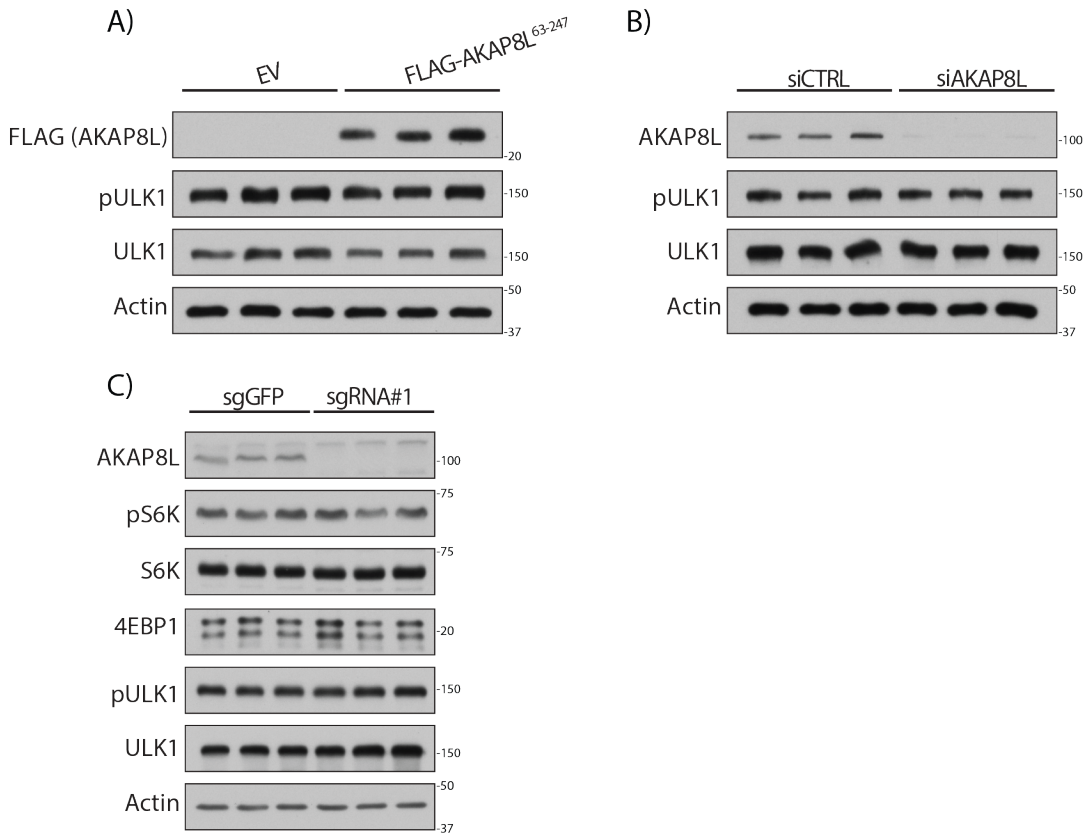
Supplemental Figure 1. AKAP8L interacts with Raptor under amino acid starvation conditions. Co-immunoprecipitation of FLAG-tagged AKAP8L and HA-tagged Raptor. Cells were either starved of amino acids (-AA) for 1 hr or serum (-FBS) for 16 hrs, then stimulated with amino acids (+AA) for 1 hr or 100 nM insulin for 30 mins, respectively. NC= normal condition. FBS = fetal bovine serum. IP = immunoprecipitation. WCL = whole cell lysate. s.e. = short exposure. l.e. = long exposure.



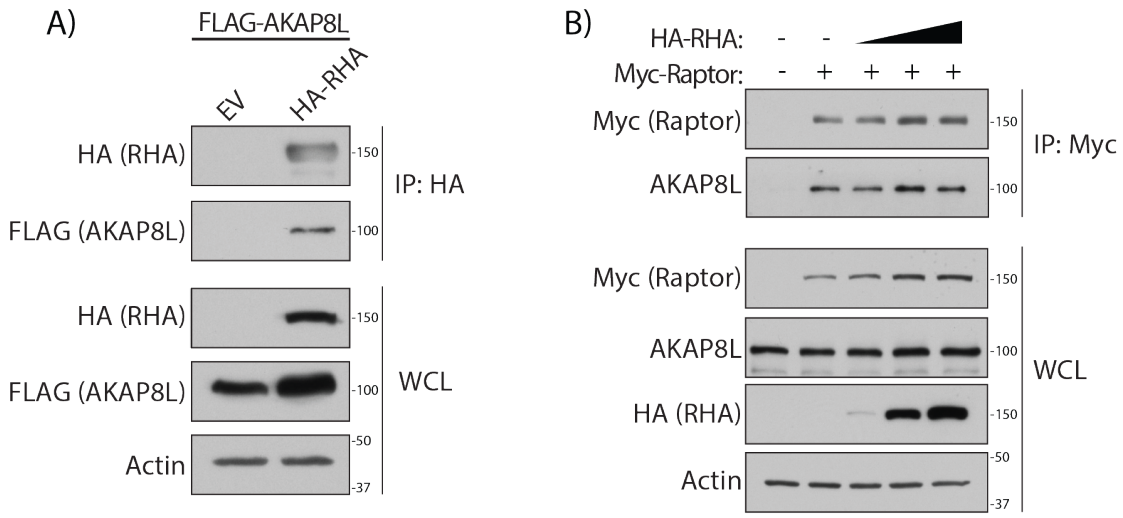
Supplemental Figure 2. AKAP8L does not co-localize with some known organelle markers. FLAG-tagged AKAP8L was transfected in human embryonic kidney 293A (HEK293A) cells for 24 hrs. Immunofluorescence staining was performed to analyze the localization of FLAG-tagged AKAP8L (red) and different organelle markers (green; Calreticulin for endoplasmic reticulum, Cox IV for mitochondria, LAMP2 for lysosomes, and GM130 for Golgi apparatus). Higher magnification images of the depicted area shown on the right. Scale bar, 10μm. Scale bar of each inset, 1μm.


A)
HA-Raptor
EV
FLAG-AKAP8L
forskolin: - + - +
HA (Raptor)
FLAG (AKAP8L)
pPKA Sub RRX(S*/T*)
IP: HA
HA (Raptor)
FLAG (AKAP8L)
pCreb
Creb
Actin
WCL
B)
FLAG-AKAP8L
FLAG (AKAP8L)
pS6K
S6K
p4EBP1
4EBP1
pULK1
ULK1
Actin
C)
FLAG-AKAP8L63-247
FLAG (AKAP8L)
pS6K
S6K
pS6
p4EBP1
4EBP1
pULK1
ULK1
Actin
D)
siCTRL
siAKAP8L
AKAP8L
pS6K
S6K
pS6
p4EBP1
4EBP1
pULK1
ULK1
Actin
E)
NC
-FBS
+FBS
si CTRL
si CTRL
si CTRL
si AKAP8L
si CTRL
si AKAP8L
HA (LARP1): - + + + + +
pAKTsub
LARP1
IP: HA
LARP1
AKAP8L
pS6K
S6K
Actin
WCL

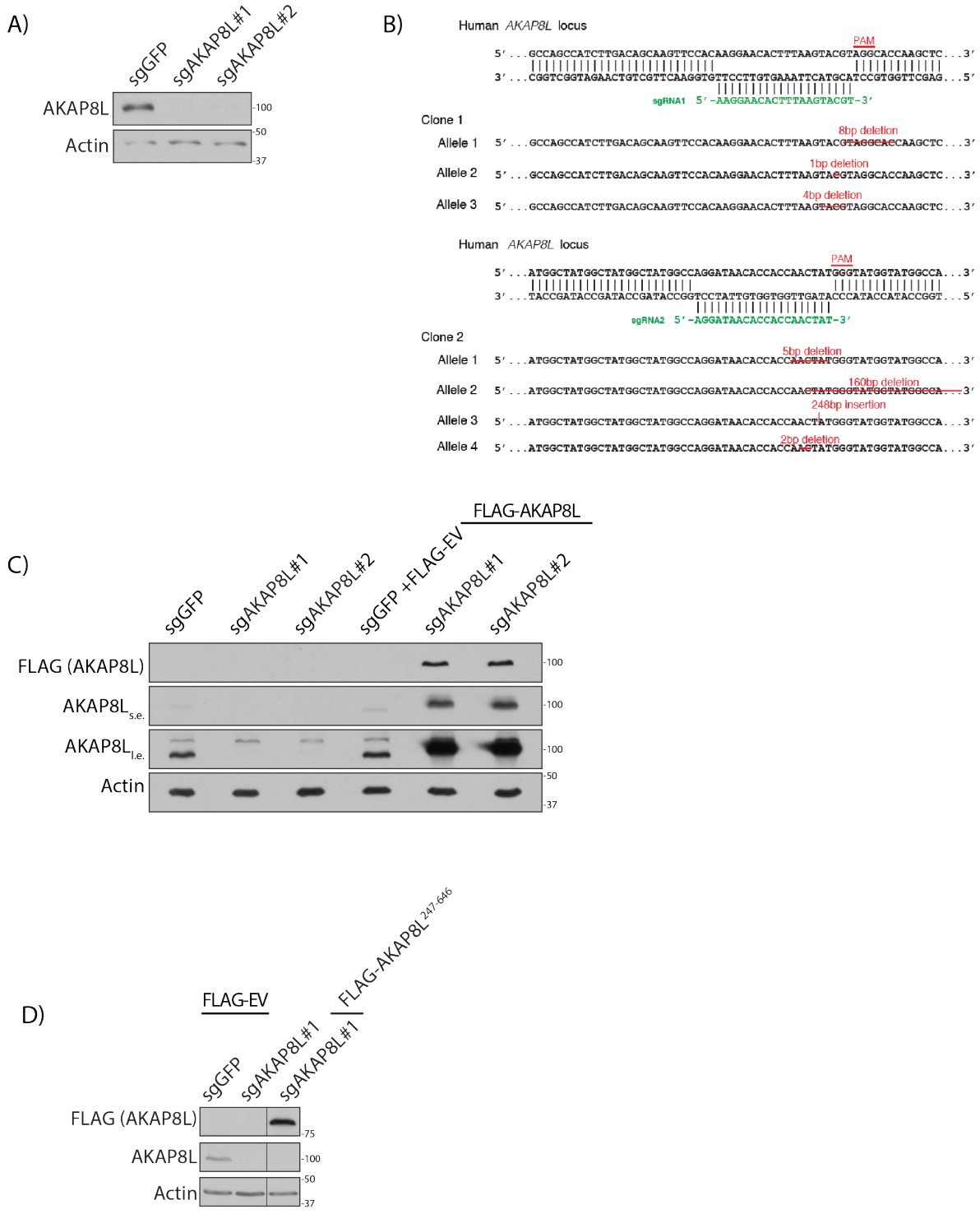
Supplemental Figure 3. AKAP8L does not regulate Raptor Ser791 phosphorylation or the phosphorylation of known mTORC1 substrates. (A) Forskolin does not alter AKAP8L-Raptor binding. Co-immunoprecipitation of FLAG-tagged AKAP8L with HA-tagged Raptor treated with or without forskolin. Phospho-specific antibody against PKA substrate motif RR*X*(S*/T*) was used to probe for the phosphorylation of Raptor. The phosphorylation of CREB at Ser 133 was used as a positive control for forskolin treatment. IP = immunoprecipitation. WCL = Whole cell lysate. **(B)** AKAP8L expression doesn't alter the phosphorylation of known mTORC1 substrates. Overexpression of FLAG-tagged AKAP8L (0.5μg, 1.0μg, 2.0μg, 3.0μg) to analyze phosphorylation status of known mTORC1 substrates (S6K, 4EBP1, ULK1). **(C)** AKAP8L amino acid region 63-247 expression doesn't alter the phosphorylation of known mTORC1 substrates. Overexpression of FLAG-tagged AKAP8L 63-247 (1.0μg, 3.0μg, 5.0μg) to analyze phosphorylation status of known mTORC1 substrates (S6K, 4EBP1, ULK1). **(D)** siRNA knockdown of AKAP8L does not alter phosphorylation status of known mTORC1 substrates (S6K, 4EBP1, ULK1). siRNA (50nM) against AKAP8L was transfected for 24 hrs before collection. siCTRL = control siRNA. **(E)** siRNA knockdown of AKAP8L does not alter phosphorylation status of LARP1. HA-tagged LARP1 was transfected 24 hrs before siRNA treatment (24hrs) and serum starvation (16 hrs) followed by stimulation with compete media for 30 mins. Phospho-AKT substrate R*XX*(S*/T*) was used to analyze the phosphorylation of immunoprecipitated HA-tagged LARP1. NC = normal condition. FBS = fetal bovine serum.



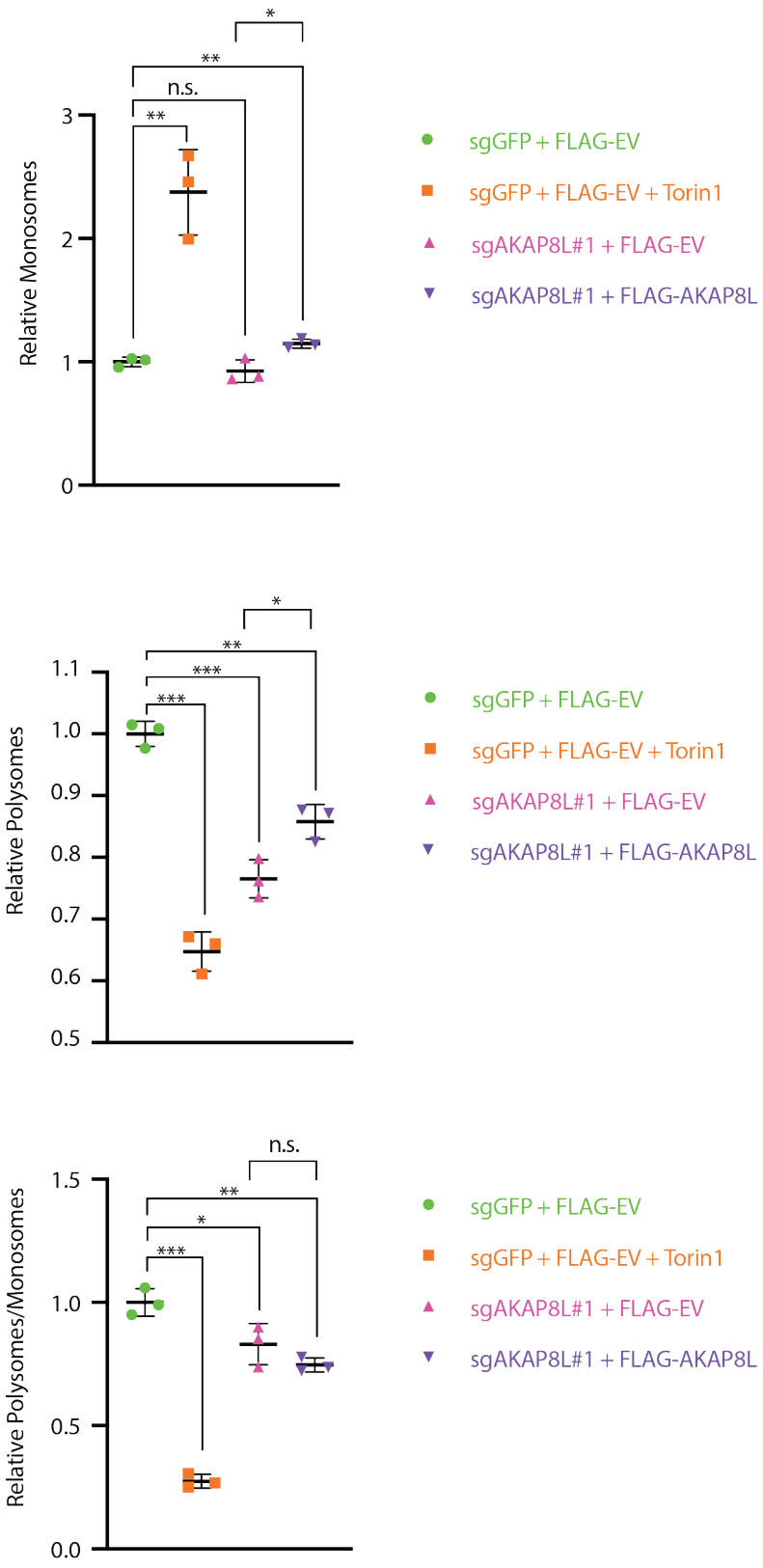
Supplemental Figure 4. AKAP8L does not regulate the phosphorylation of ULK1 and other mTORC1 substrates. (A) AKAP8L (amino acids 63-247) doesn't alter the phosphorylation of ULK1. The phosphorylation status of ULK1 was analyzed after the overexpression of either EV or FLAG-tagged AKAP8L 63-247 for 24hrs. **(B)** Depletion of AKAP8L doesn't alter the phosphorylation of ULK1. The phosphorylation status of known mTORC1 substrate ULK1, after siRNA knockdown of AKAP8L. siCTRL = control siRNA. **(C)** Depletion of AKAP8L does not change the phosphorylation of some mTORC1 substrates. HEK293A control (sgGFP) or HEK293A AKAP8L knockout (sgAKAP8L) cells were analyzed (in triplicates) for the phosphorylation status of known mTORC1 substrates.



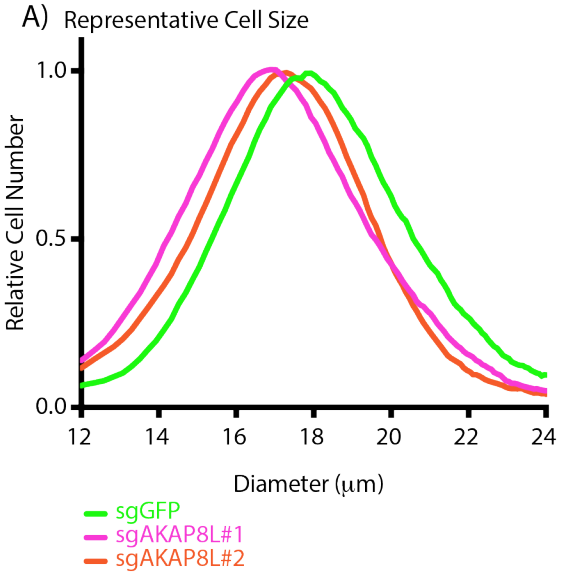
Supplemental Figure 5. AKAP8L interacts with RNA helicase A. (A) AKAP8L interacts with RNA helicase A (RHA). Co-immunoprecipitation of FLAG- tagged AKAP8L with HA-tagged RHA. **(B)** RHA does not affect binding between Raptor and AKAP8L. Co-immunoprecipitation of FLAG-tagged AKAP8L and increasing titrations of HA-tagged RHA (1.0μg, 3.0μg, 5.0μg) with MYC-tagged Raptor. IP = immunoprecipitation. WCL = Whole cell lysate.

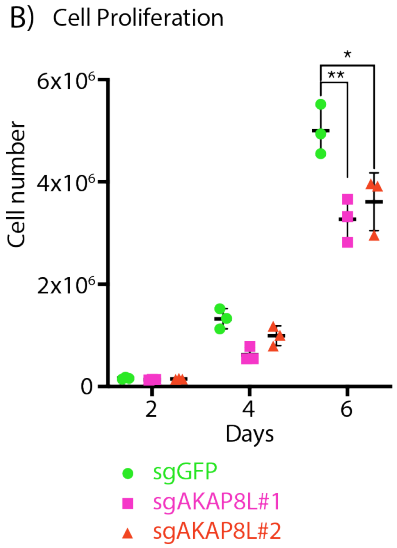


Supplemental Figure 6. Generation of AKAP8L knockout and rescue cell lines. (A) AKAP8L protein level diminished in AKAP8L KO HEK293A cells. AKAP8L KO HEK293A cells were generated using CRISPR/Cas9 genome editing. Single clones were expanded and screened for AKAP8L by protein immunoblotting. **(B)** Sequencing of AKAP8L KO HEK293A cells revealed 3 alleles with indels in clone 1 and 4 alleles with indels in clone 2. To determine the indels of individual alleles, at least 10 bacterial colonies were expanded, then bacterial plasmid was extracted, purified and sequenced for AKAP8L genomic DNA. **(C)** Reconstituted AKAP8L KO HEK293A cells express FLAG-tagged AKAP8L. Generation of AKAP8L KO cells stably overexpressing full-length FLAG-tagged AKAP8L. s.e. = short exposure. l.e. = long exposure. **(D)** Reconstituted AKAP8L KO HEK293A cells express FLAG-tagged AKAP8L amino acid region 247-646. Generation of AKAP8L KO cells stably overexpressing FLAG-tagged AKAP8L 247-646.

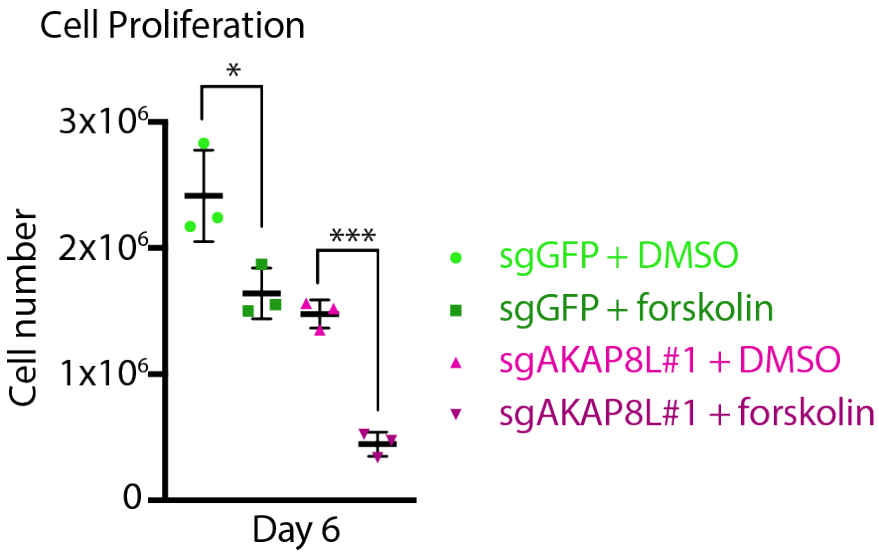


Supplemental Figure 7. Monosome and polysome regulation by AKAP8L. Relative amounts of monosomes (top), polysomes (middle) and polysome/monosome ratios (bottom). Significance (*P*): * = < 0.05, ** = < 0.01, *** = < 0.001. Values are displayed as means ± SD. Significance was analyzed using Student's T-test.





Supplemental Figure 8. AKAP8L regulates cell size and proliferation. (A) AKAP8L KO HEK293A cells have a smaller diameter than control cells (sgGFP). The size of AKAP8L KO cells were measured using a coulter counter. Samples with closest value to mean plotted as a representative image. Significance (*P*): sgGFP vs. sgAKAP8L#1 = < 0.05, sgGFP vs. sgAKAP8L#2 = < 0.01. **(B)** AKAP8L KO HEK293A cells proliferate slower than control cells (sgGFP). AKAP8L KO cells were counted on the indicated days using Trypan Blue and a BioRad automated cell counter. Values are displayed as means ± SD. Significance was analyzed using Student's T-test. Number of biological repeats is $n \geq 3$. Number of technical repeats of each sample is $n = 3$ per experiment. Significance (*P*): * = < 0.05, ** = < 0.01, *** = < 0.001.



Supplemental Figure 9. Activation of cAMP decreases cell proliferation independent of AKAP8L. Control and AKAP8L KO cells were counted on day 6 using Trypan Blue and a BioRad automated cell counter. Media was changed every day with or without 10μM forskolin and 200μM IBMX. Values are displayed as means ± SD. Significance was analyzed using Student's T-test. Number of technical repeats of each sample is $n = 3$.