**Table S1. RPPA screen results.** Below is a list of total and phosphorylated proteins that were significantly downregulated or upregulated following Rab32 knockdown in Hep3B cells.

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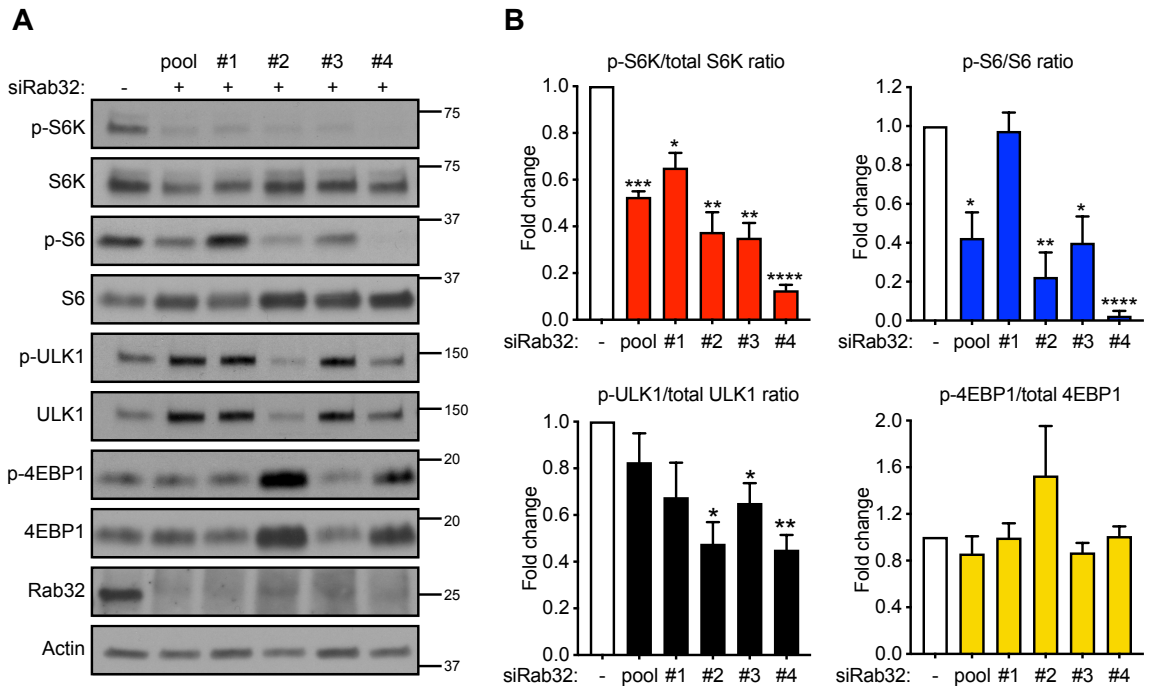


**Figure S1. Rab32 regulates mTORC1 signaling in Hep3B cells.** (A) A representative Western blot of Hep3B cells, as an extension to Fig. 2B, that were treated with siControl, siRab32 pool or four individual siRNAs against Rab32 from the pool (named #1, #2, #3, #4) and analyzed for changes in the downstream mTORC1 substrates p-S6K, S6K, p-S6, S6, p-ULK1, ULK1, p-4EBP1 and 4EBP1. (B) Bar graphs represent fold changes in mTORC1 substrate ratios as means±s.e.m. from n=4 independent experiments from (A). Asterisks denote statistical significance by two-tailed paired Student's *t*-test (* p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001).

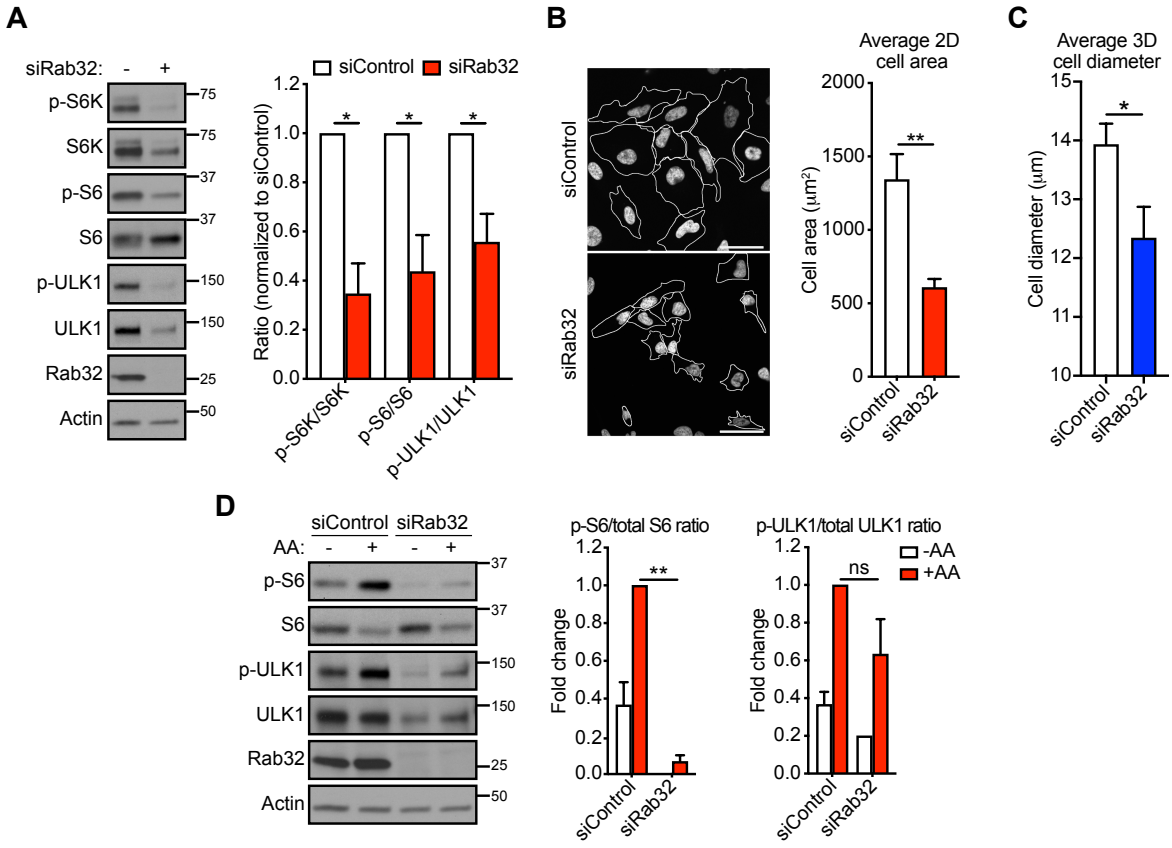


**Figure S2. Rab32 regulates mTORC1 signaling in HeLa cells.** (A) HeLa cells were treated with siControl or siRab32 and analyzed for the mTORC1 substrates p-S6K, S6K, p-S6, S6, p-ULK1 and ULK1. The bar graph represents fold changes in phospho/total protein ratios from n=4 independent experiments. (B) Graph and representative images depict a reduction in average 2D cell area in Rab32 siRNA versus control siRNA treated HeLa cells (n=4 independent experiments). Shown are representative pictures. Scale bar, 50 μm. (C) Graph of the average 3D cell diameter of trypsinized control or Rab32 siRNA treated HeLa cells from n=3 independent experiments. (D) HeLa cells that were treated with either Control or Rab32 siRNA were starved, re-stimulated with AAs and analyzed for mTORC1 substrates. The bar graphs represent fold changes in phospho/total protein ratios from at least n=3 independent experiments. Data for all panels are represented as means±s.e.m. and asterisks denote statistical significance by two-tailed paired Student's *t*-test (* p<0.05, ** p<0.01).

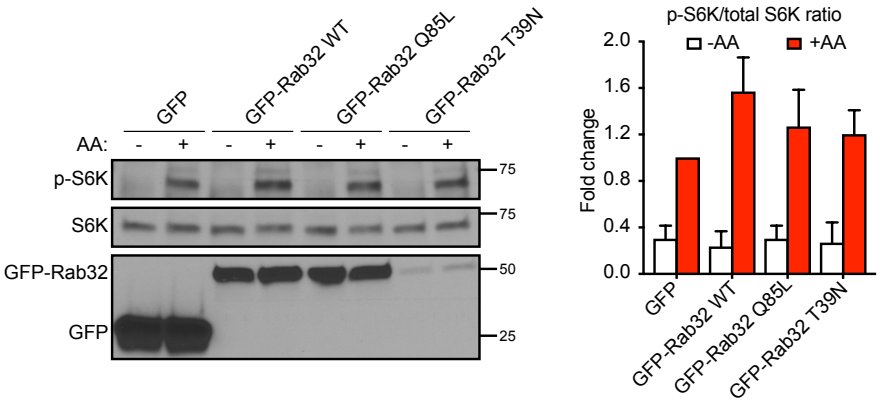


**Figure S3. Overexpression of Rab32 does not potentiate mTORC1 signaling after AA manipulation.** A representative Western blot of Hep3B cells that were transfected with GFP control or GFP-tagged WT, Q85L or T39N Rab32 plasmids and subjected to 50 min AA starvation followed by 15 min AA re-feeding as described in Fig. 3. The lysates were analyzed for p-S6K and S6K. The bar graph represents fold changes in phospho/total protein ratios as means±s.e.m. from n=3 independent experiments.

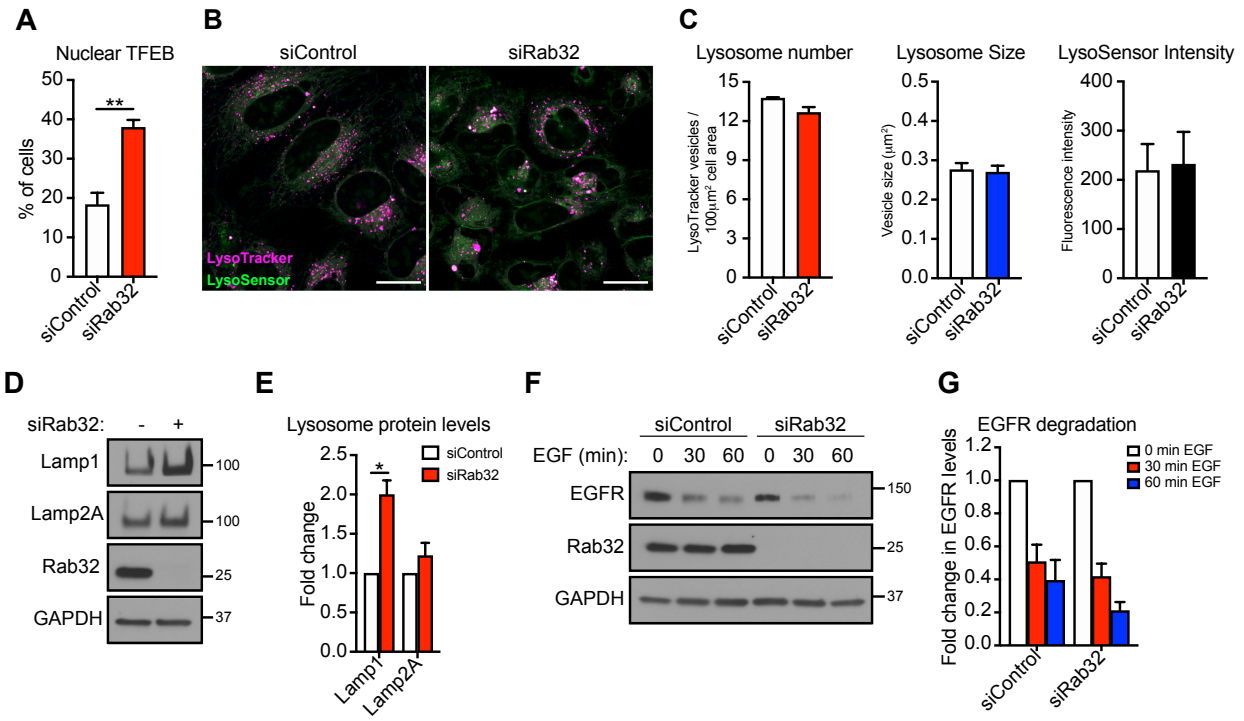


**Fig. S4. Loss of Rab32 increases nuclear TFEB localization and lysosome biogenesis in HeLa cells.** (A) Bar graph depicting a significant increase in percentage of cells with nuclear TFEB-GFP localization after Rab32 knockdown in HeLa cells; n=551 siControl and n=400 siRab32 cells counted across n=3 independent experiments. (B) Representative confocal images of HeLa cells that were treated with respective siRNAs and labeled with Lysotracker Deep Red (magenta) and LysoSensor (green) dyes. Scale bar, 10 μm. (C) Bar graphs representing quantification of 333 siControl and 375 siRab32 HeLa cells across n=3 independent experiments. (D) Representative Western blot of HeLa cells treated for 72 hrs with control or Rab32 siRNA and analyzed for lysosomal proteins Lamp1 and Lamp2A. (E) The bar graph represents fold changes in lysosomal proteins after Rab32 knockdown from n=4 independent experiments. (F) Representative Western blot of HeLa cells that were treated with respective siRNAs for 72 hrs and serum starved for 4 hrs in the presence of 50 □g/ml cycloheximide followed by 50 ng/ml EGF treatment for indicated time points and analyzed for EGFR degradation. (G) The bar graph depicts the rate of EGFR degradation (normalized to GAPDH and to *t*=0 min EGF treatment for both siControl and siRab32). All data are presented as means±s.e.m. and

asterisks denote statistical significance by two-tailed paired Student's *t*-test (* $p<0.05$, ** $p<0.01$).

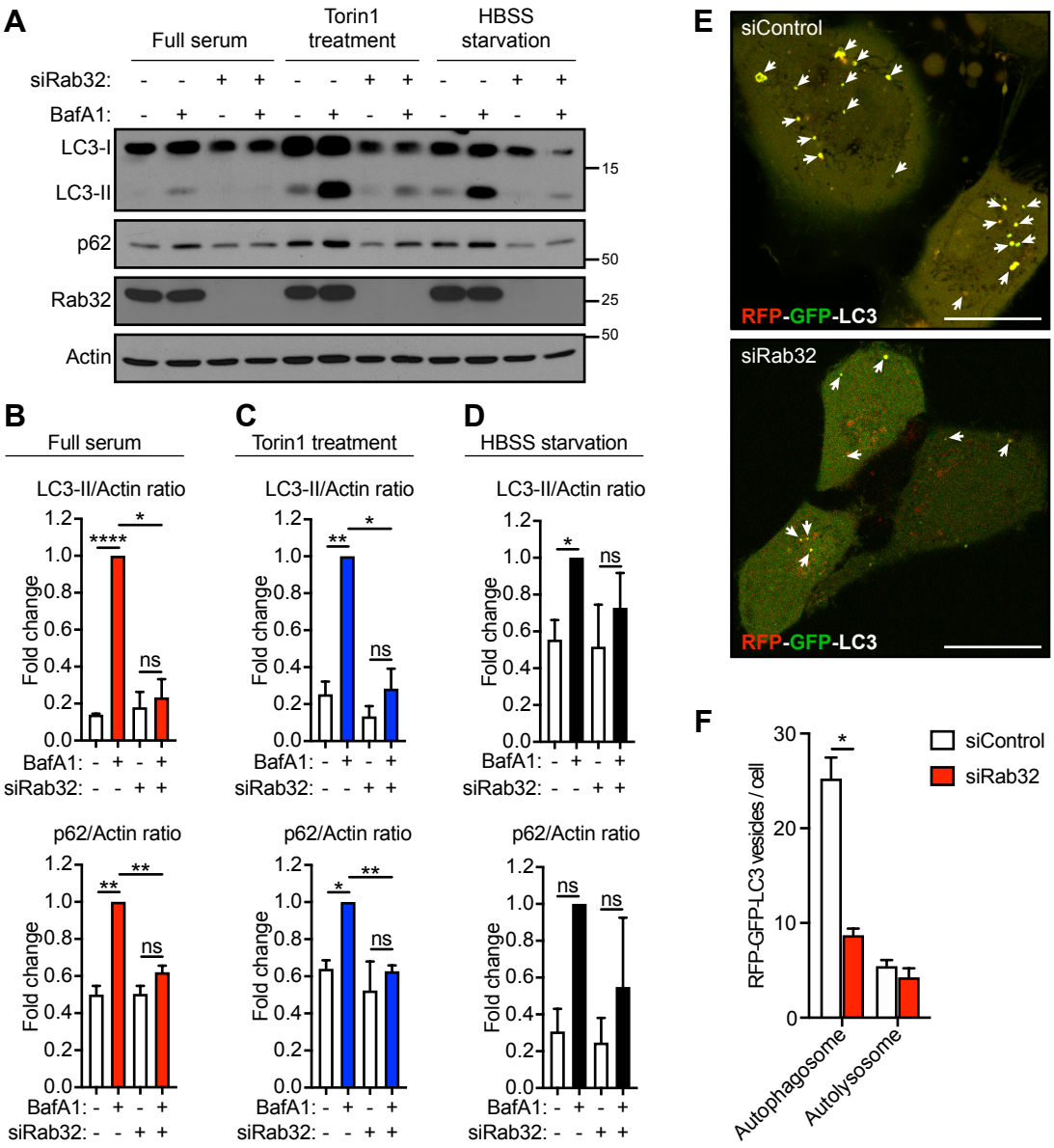


**Fig. S5. Rab32 knockdown attenuates autophagy in HeLa cells.** (A) Western blot of HeLa cells treated for 72 hrs with control or Rab32 siRNA followed by 2 hr treatment with the lysosome inhibitor Bafilomycin A1 (BafA1, 100 nM) under basal, full serum conditions (10% FBS), in the presence of mTOR inhibitor (Torin1, 1 μM), or under HBSS starvation conditions and analyzed for changes in the autophagic proteins LC3 and p62. (B-D) Bar graphs represent fold changes of LC3-II and p62 protein levels (normalized to siControl + BafA1) and indicate a reduction in autophagic flux. (E) Representative images of RFP-GFP-LC3 tandem fluorescence reporter in HeLa cells treated with control or Rab32 siRNA

for 72 hrs. $RFP^+/GFP^+$ fluorescence depicts autophagosomes ("yellow") while $RFP^+$ only fluorescence depicts autolysosomes ("red"). Total n=50 cells for siControl and n=68 cells for siRab32 condition were counted across n=3 independent experiments. White arrows point to examples of RFP-GFP-LC3 vesicles. Scale bars, 20 μm. (F) The bar graph represents a significant decrease in average autophagosome vesicles per cell after Rab32 depletion. All data are presented as means±s.e.m. from n=3 independent experiments and asterisks denote statistical significance by two-tailed paired Student's *t*-test (* $p<0.05$, ** $p<0.01$, *** $p<0.001$).

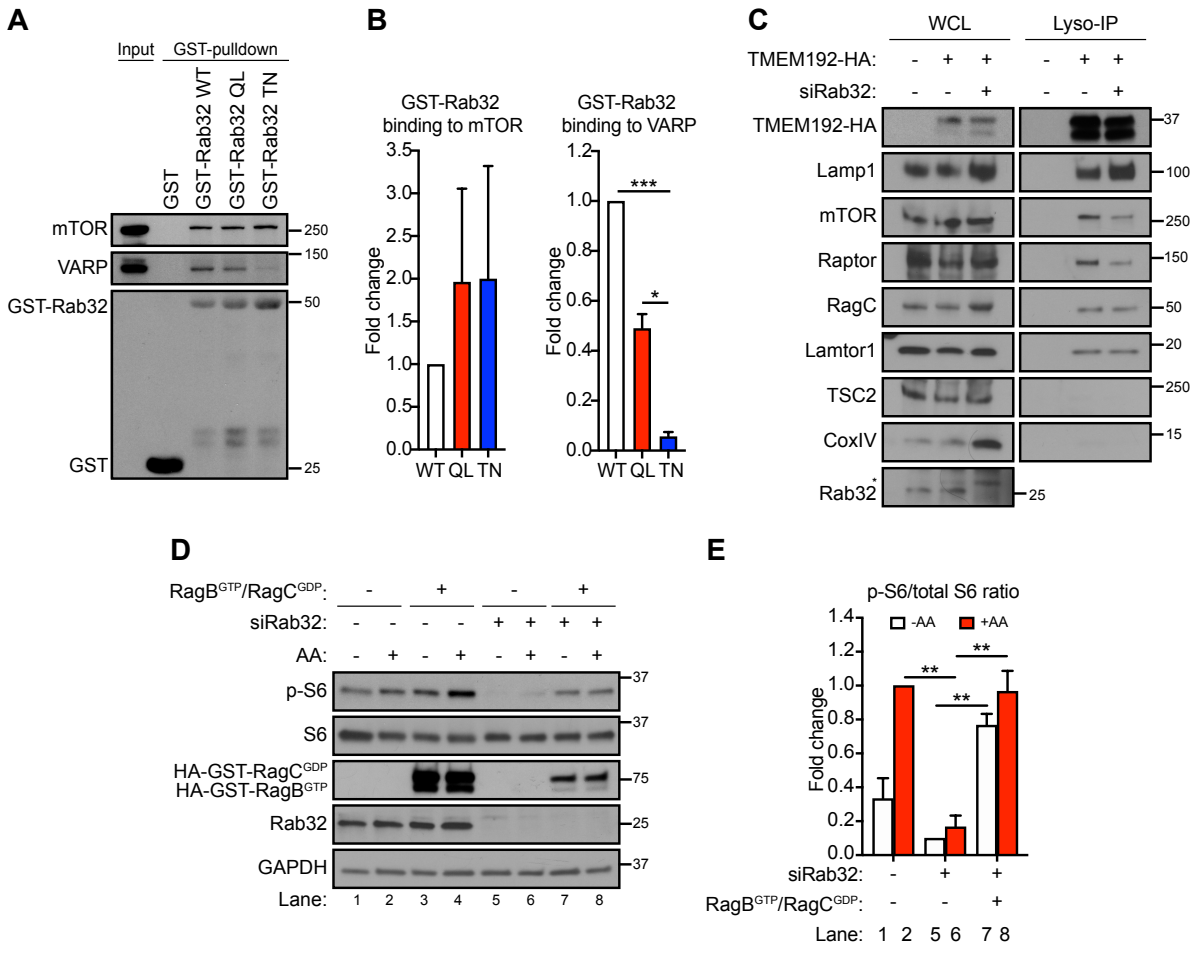


**Fig. S6. Rab32 binds to mTOR and regulates mTOR association with lysosomes in HeLa cells.** (A) GST-pulldown assay using purified Rab32 proteins (WT, Q85L or T39N) incubated with HeLa cell lysates and analyzed for binding to mTOR and Rab32's effector VARP (n=3 independent experiments). (B) Bar graphs depicting quantification of GST-Rab32 pulldown to mTOR or VARP from (A) (normalized to GST-Rab32 WT). (C) Representative Western blot of biochemically-isolated lysosomes (Lyso-IP) from control or Rab32 knockdown HeLa cells shows the relative association of mTOR, Raptor, RagC and Lamtor1 with lysosomes; WCL – whole cell lysates (n=2 independent experiments) (D) Representative Western blot of HeLa cells that were treated with either siControl or siRab32 for 72 hrs followed by expression of active Rag heterodimers ($RagB^{GTP}/RagC^{GDP}$) and subjected to AA starvation and re-stimulation as described in Fig. 3. (E) The bar graph represents a significant decrease in p-S6/S6 ratio after Rab32

knockdown which is rescued by expressing active Rag heterodimers. Quantification represents n=3 independent experiments. (B,E) Asterisks denote statistical significance by two-tailed paired Student's *t*-test (* $p<0.05$, ** $p<0.01$, *** $p<0.001$).