Supplementary Information

Rheb Regulates Nuclear mTORC1 Activity Independent of Farnesylation

Authors

Yanghao Zhong[1,2], Xin Zhou[1], Kun-Liang Guan[1,3], Jin Zhang[1,3,4,5,6,*]

Affiliations

[1] Department of Pharmacology, University of California, San Diego, La Jolla, CA, USA

[2] Biomedical Sciences Graduate Program, University of California, San Diego, La Jolla, CA, USA

[3] Moores Cancer Center, University of California, San Diego, La Jolla, CA, USA

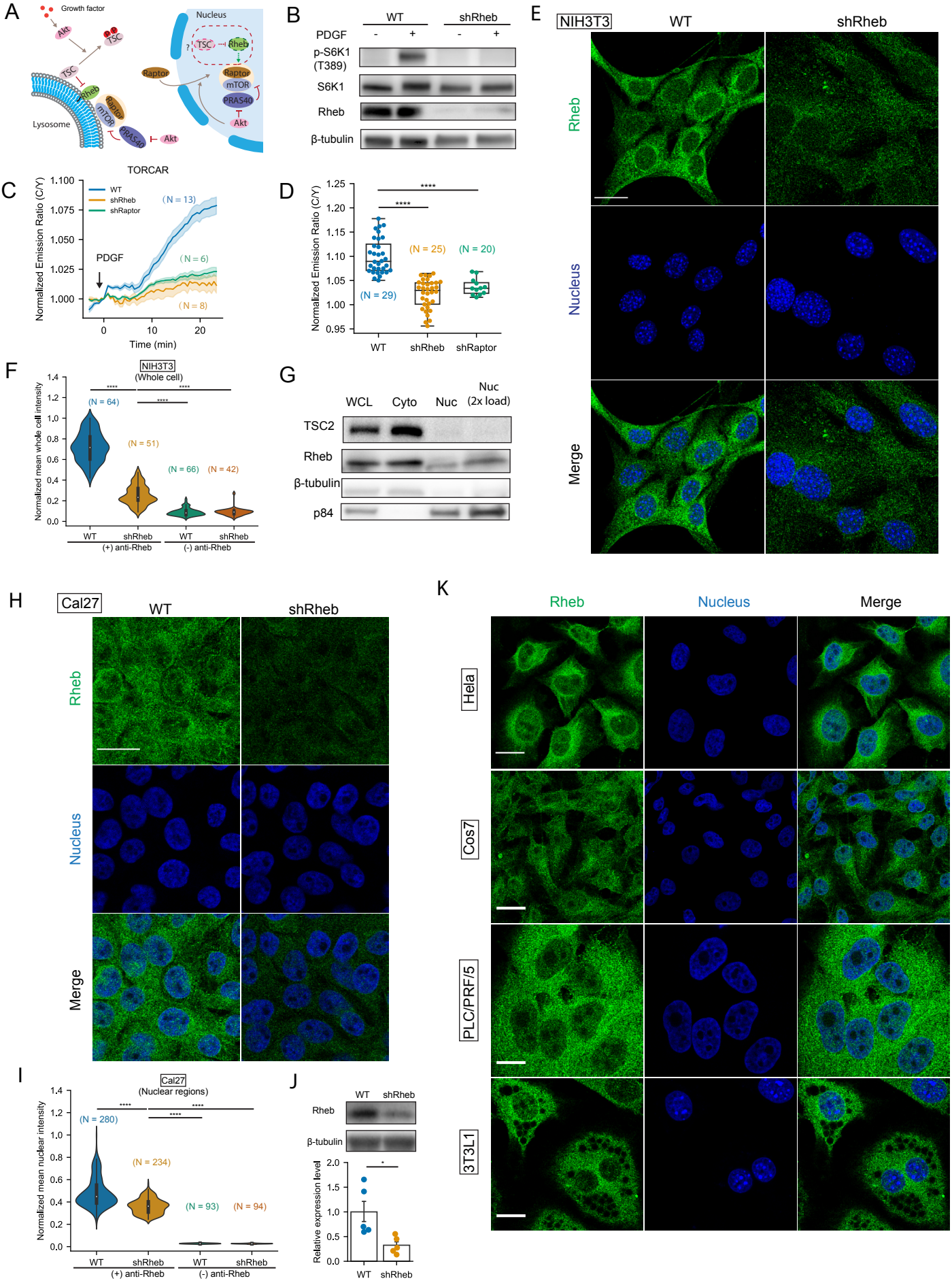
[4] Department of Bioengineering, University of California, San Diego, La Jolla, CA, USA

[5] Department of Chemistry & Biochemistry, University of California, San Diego, La Jolla, CA, USA

[6] Lead contact

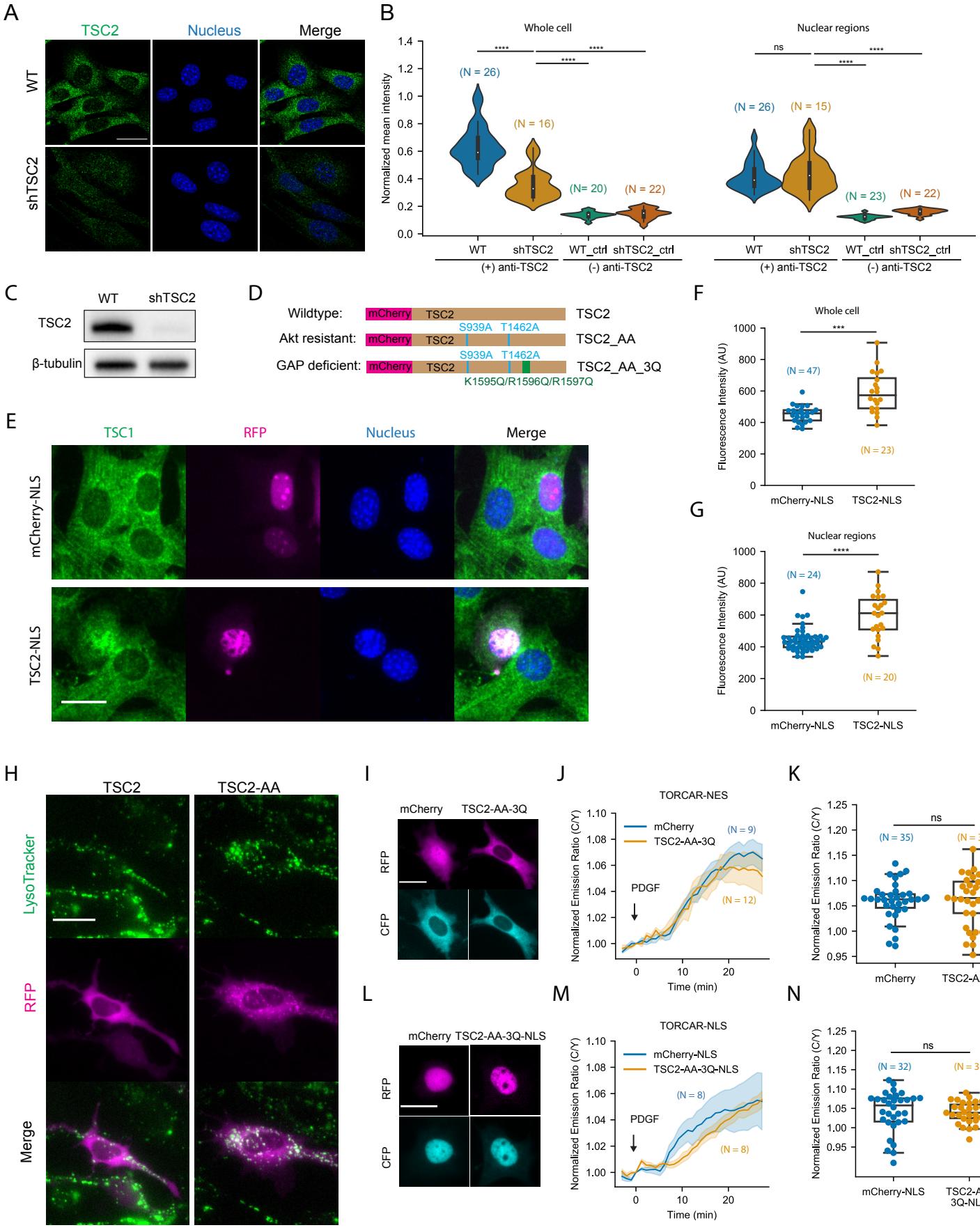
*Correspondence: jzhang32@health.ucsd.edu

Supplementary Figure S1



Supplementary Figure S1. Rheb knockdown abolished mTORC1 activity and nuclear specific immunofluorescence staining (Related to Figure 1). (A) A model showing the growth factor stimulated activation of the lysosomal and nuclear mTORC1. Whether the TSC/Rheb axis exists in the nucleus to regulate mTORC1 is not clear. (B) Western blotting data to show that Rheb protein level was reduced in Rheb knockdown stable NIH3T3 cells. Wild type (WT) and Rheb knockdown (shRheb) cells were double starved and stimulated with 50 ng/mL PDGF for 30 min or were unstimulated. Phosphorylation of mTORC1 substrate S6K1 at Thr389 (T389) was examined. Data are representative from three independent experiments. (C-D) Representative averaged time course traces (C) and summary (D) of PDGF-induced maximum responses of TORCAR in double starved NIH3T3 cells. Blue, wild type cells (WT, N = 29 cells from 4 independent experiments); yellow, Rheb knockdown cells (shRheb, N = 25 cells from 3 independent experiments); green, Raptor knockdown cells (shRaptor, N = 20 cells from 3 independent experiments). Shaded areas indicate standard error of the mean (SEM). Box plots show the upper and lower adjacent values, interquartile range and the median. One-way ANOVA with Dunnett's multiple comparisons is used. ****, $p < 0.0001$. (E) Representative confocal images from two independent experiments showing immunofluorescence staining of wild type and Rheb knockdown NIH3T3 cells. Rheb staining is shown in green and nucleus is shown in blue. Scale bar = 20 μm. (F) Quantification of the whole cell immunofluorescence staining of Rheb in wild type (WT) or Rheb knockdown (shRheb) NIH3T3 cells. Control experiments were done by leaving out the primary antibody ((-) anti-Rheb). Intensities were normalized to the maximum intensity. Violin plots show the upper and lower adjacent values, interquartile range and the median. One-way ANOVA with Dunnett's multiple comparisons is used. ****, $p < 0.0001$. Blue, wild type cells, (+) anti-Rheb (N = 64 cells); yellow, Rheb knockdown cells, (+) anti-Rheb (N = 51 cells); green, wild type cells, (-) anti-Rheb (N = 66 cells); orange, Rheb knockdown cells, (-) anti-Rheb (N = 42 cells). Data are representative from two independent experiments. (G) Nuclear fractionation experiment examining the presence of Rheb in the nuclei in NIH3T3 cells. Same proportions of cytosolic (Cyto) and nuclear (Nuc) fractions corresponding to the same amount of whole cell lysate (WCL) were loaded and an extra lane was loaded with doubled amount of the nuclear fraction (2x load). β-tubulin and p84 served as cytosolic marker and nuclear marker, respectively. Data are representative of three independent experiments. (H) Representative confocal images from two independent experiments showing Rheb immunofluorescence staining in wild type (WT) and Rheb knockdown (shRheb) Cal27 cells. Rheb staining is shown in green and nucleus is shown in blue. Scale bar = 20 μm. (I) Quantification of the nuclear signals in (H). Intensities were normalized to the maximum intensity. Control experiments were done by leaving out the primary antibody ((-) anti-Rheb) for wild type (WT) and Rheb knockdown (shRheb) cells respectively. Violin plots show the upper and lower adjacent values, interquartile range and the median. One-way ANOVA with Dunnett's multiple comparisons is used. ****, $p < 0.0001$. Blue, wild type cells, (+) anti-Rheb (N = 280 cells); yellow, Rheb knockdown cells, (+) anti-Rheb (N = 234 cells); green, wild type cells, (-) anti-Rheb (N = 93 cells); orange, Rheb knockdown cells, (-) anti-Rheb (N = 94 cells). Data are representative from two independent experiments. (J) Western blotting to examine Rheb protein level in wild type (WT) and Rheb knockdown (shRheb) Cal27 cells. β-tubulin served as loading control. Quantification is done with five independent blots and unpaired t-test is used. *, $p < 0.05$. (K) Representative immunofluorescence images of Rheb staining in various cell lines including Hela, Cos7, PLC/PRF/5 hepatoma cells and 3T3L1 adipocytes. Data are representative from two independent experiments. Rheb staining is shown in green and nucleus is shown in blue. Scale bars = 20 μm.

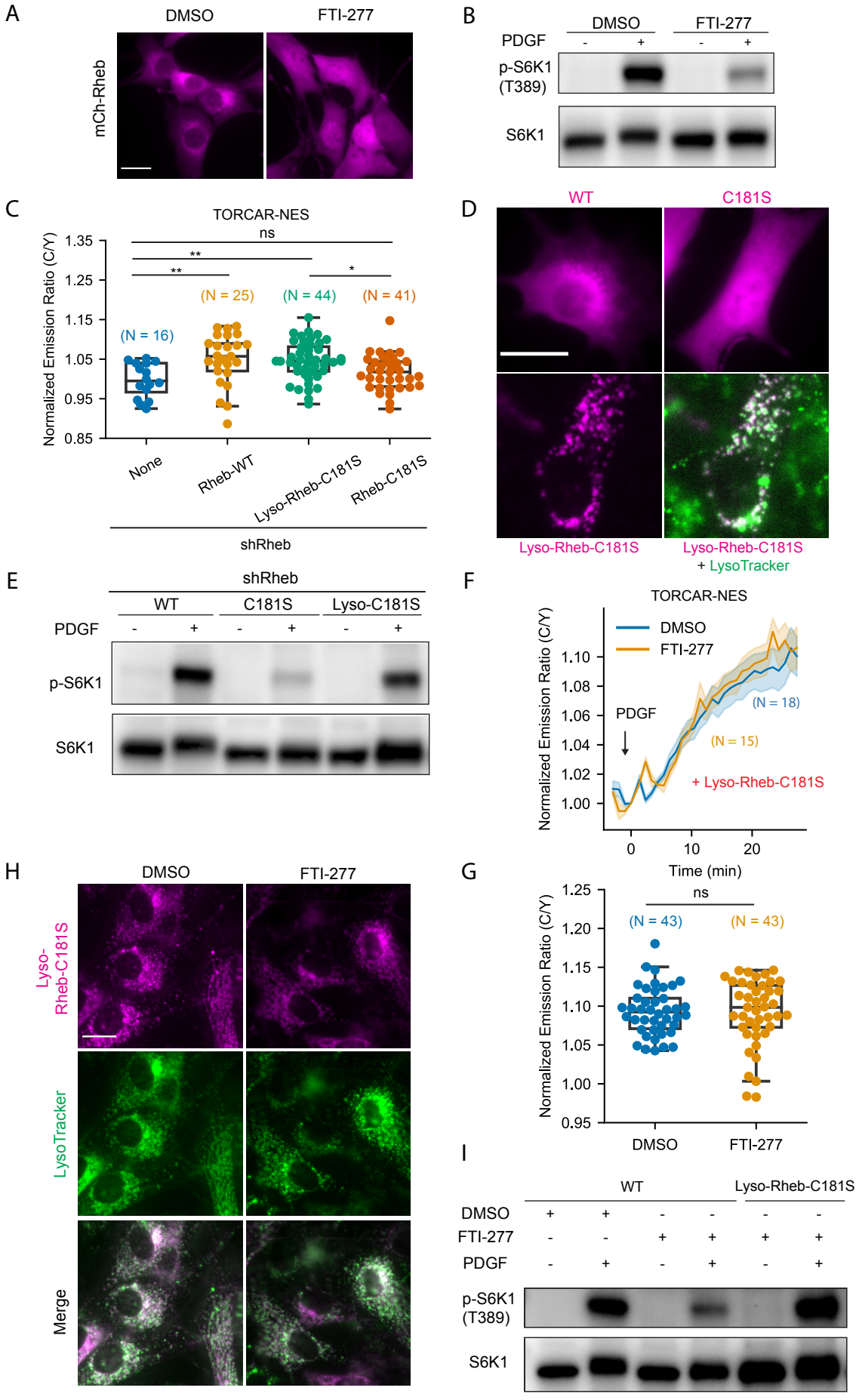
Supplementary Figure S2



Supplementary Figure S2. TSC2 is minimally present in the nucleus in NIH3T3 cells (Related to Figure 2). (A) Representative confocal images from two independent experiments showing TSC2 immunofluorescence staining in wild type (WT) and TSC2 knockdown (shTSC2) NIH3T3 cells. TSC2 staining is shown in green and nucleus is shown in blue. Scale bar = 20 μm. (B) Quantification of the whole cell signals and nuclear signals in (A). Intensities were normalized to the maximum intensity. Control experiments were done by leaving out the primary antibody ((-) anti-TSC2) for wild type and TSC2 knockdown cells respectively. Violin plots show the upper and lower adjacent values, interquartile range and the median. One-way ANOVA with Dunnett's multiple comparisons is used. ****, $p < 0.0001$; ns, not significant. Blue, wild type cells, (+) anti-TSC2 (N = 26 cells for whole cell and N = 26 cells for nuclear regions); yellow, TSC2 knockdown cells, (+) anti-TSC2 (N = 16 cells for whole cell and N = 15 cells for nuclear regions); green, wild type cells, (-) anti-TSC2 (N = 20 cells for whole cell and N = 23 cells for nuclear regions); orange, TSC2 knockdown cells, (-) anti-TSC2 (N = 22 cells for whole cell and N = 22 cells for nuclear regions). Data are representative from two independent experiments. (C) Western blotting to examine TSC2 protein level in wild type (WT) and TSC2 knockdown (shTSC2) NIH3T3 cells. β-tubulin served as loading control. Data are representative of three independent experiments. (D) Schematics of different TSC2 constructs tagged with mCherry. Two Akt phosphorylation sites in wild type TSC2 were mutated to Ala to generate unphosphorylatable mCherry-TSC2-AA. Three residues from 1595-1597 (KRR) were mutated to Gln (QQQ) to generate GAP-deficient mCherry-TSC2-AA-3Q. (E) Representative immunofluorescence images from three independent experiments showing TSC1 staining in NIH3T3 cells overexpressing mCherry-tagged nuclear TSC2 (TSC2-NLS) or nuclear mCherry (mCherry-NLS). TSC1 staining is shown in green, mCherry is labeled as "RFP" and shown in magenta, and nucleus is shown in blue. Scale bar = 10 μm. (F-G) Quantification of the whole cell signals (F) and nuclear signals (G) in (E). Fluorescence intensities were shown as arbitrary units (AU). Box plots show the upper and lower adjacent values, interquartile range and the median. Unpaired t-test is used. ***, $p < 0.001$; ****, $p < 0.0001$. Blue, cells overexpressing mCherry-NLS (N = 47 cells for whole cell and N = 24 cells for nuclear regions); yellow, cells overexpressing TSC2-NLS (N = 23 for whole cell and N = 20 cells for nuclear regions). Data are representative from three independent experiments. (H) Representative images showing that TSC2-AA partially colocalizes with a lysosomal marker, LysoTracker. mCherry-tagged TSC2 or TSC2-AA mutant were overexpressed in NIH3T3 cells. LysoTracker is shown in green and mCherry is labeled "RFP" and shown in magenta. Data are representative of three independent experiments. Scale bar = 10 μm. (I) Representative fluorescence images from three independent experiments showing the localization of overexpressed constructs in NIH3T3 cells. RFP channel: mCherry and mCherry-TSC2-AA-3Q mutant. CFP channel: TORCAR-NES. Scale bar = 10 μm. (J-K) Representative averaged time course traces (J) and summary (K) of PDGF-induced maximum responses of TORCAR-NES in double starved NIH3T3 cells. Co-expressed constructs: mCherry (blue, N = 35 cells from 3 independent experiments); mCherry-TSC2-AA-3Q (yellow, N = 36 cells from 3 independent experiments). Shaded areas indicate standard error of the mean (SEM). Box plots show the upper and lower adjacent values, interquartile range and the median. Unpaired t-test is used. ns, not significant. (L) Representative fluorescence images from three independent experiments showing the localization of overexpressed nuclearly targeted constructs in NIH3T3 cells. RFP channel: mCherry-NLS and mCherry-TSC2-AA-3Q-NLS mutant. CFP channel: TORCAR-NLS. Scale bar = 10 μm. (M-N) Representative averaged time course traces (M) and summary (N) of PDGF-induced maximum responses of TORCAR-NLS in double starved NIH3T3 cells. Co-expressed constructs: mCherry-

NLS (blue, N = 32 cells from 3 independent experiments); mCherry-TSC2-AA-3Q-NLS (yellow, N = 31 cells from 3 independent experiments). Shaded areas indicate standard error of the mean (SEM). Box plots show the upper and lower adjacent values, interquartile range and the median. Unpaired t-test is used. ns, not significant.

Supplementary Figure S3



Supplementary Figure S3. Farnesylation-enabled membrane is required for canonical mTORC1 activation (Related to Figure 3 and Figure 4). (A) mCherry-Rheb expression was induced by 100 ng/mL doxycycline in Rheb knockdown NIH3T3 cells with stable reconstitution of mCherry-Rheb. Cells were treated with DMSO or 5 μM FTI-277 overnight and Rheb localization was assessed by live cell imaging. Scale bar = 10 μm. Images are representative from three independent experiments. (B) Western blotting analysis to show FTI-277 inhibits PDGF-induced S6K1 phosphorylation. Wild type (WT) NIH3T3 cells were treated with DMSO or 5 μM FTI-277 overnight, double starved and stimulated with 50 ng/mL PDGF or were unstimulated. Phosphorylation of mTORC1 substrate S6K1 at Thr389 (T389) was examined. Data are representative from three independent experiments. (C) Summary of PDGF-induced maximum responses of TORCAR-NES in double starved Rheb knockdown (shRheb) cells transfected with different Rheb constructs. Rheb knockdown cells with no Rheb reconstitution served as a negative control (None, blue, N = 16 cells from 3 independent experiments); Rheb knockdown cells expressing wild type Rheb (Rheb-WT, yellow, N = 25 cells from 3 independent experiments); Rheb knockdown cells expressing lysosomally targeted Rheb-C181S (Lyso-Rheb-C181S, green, N = 44 cells from 6 independent experiments); Rheb knockdown cells expressing Rheb-C181S (Rheb-C181S, orange, N = 41 cells from 6 independent experiments). Box plots show the upper and lower adjacent values, interquartile range and the median. One-way ANOVA with Bonferroni's multiple comparisons is used. *, $p < 0.05$; **, $p < 0.01$; ns, not significant. (D) Representative fluorescence images from three independent experiments showing the localization of different Rheb constructs in Rheb knockdown cells stably expressing wild type mCherry-Rheb (WT), mCherry-Rheb-C181S (C181S) and Lyso-mCherry-Rheb-C181S (Lyso-Rheb-C181S). Lyso-Rheb-C181S showed partial colocalization with LysoTracker, the lysosome marker. Scale bar = 10 μm. (E) Western blotting to assess the role of Rheb membrane association in mTORC1 activity. Cells in (D) were induced with 100 ng/mL doxycycline, double starved and stimulated with 50 ng/mL PDGF for 30 min or were unstimulated. Phosphorylation of mTORC1 substrate S6K1 at Thr389 (T389) was examined. Data are representative from three independent experiments. (F-G) Representative averaged time course traces (F) and summary (G) of PDGF-induced maximum responses of TORCAR-NES in double starved NIH3T3 cells overexpressing Lyso-Rheb-C181S and treated with DMSO (blue, N = 43 cells from 3 independent experiments) or farnesyltransferase inhibitor FTI-277 at 5 μM overnight (yellow, N = 43 cells from 3 independent experiments). Shaded areas indicate standard error of the mean (SEM). Box plots show the upper and lower adjacent values, interquartile range and the median. Unpaired t-test is used. ns, not significant. (H) Representative images from three independent experiments showing that the localization of lysosomally targeted Lyso-Rheb-C181S was not affected by FTI-277 treatment in a Lyso-Rheb-C181S stable line. Cells were treated with DMSO or 5 μM FTI-277 overnight and Lyso-Rheb-C181S localization was assessed by live cell imaging. LysoTracker is shown in green and Lyso-Rheb-C181S is shown in magenta. (I) Western blotting analysis to show Lyso-Rheb-C181S rescued mTORC1 activity in the presence of FTI-277. Wild type (WT) NIH3T3 cells or NIH3T3 cells stably expressing Lyso-Rheb-C181S were treated with DMSO or 5 μM FTI-277 overnight, double starved and stimulated with 50 ng/mL PDGF or were unstimulated. Phosphorylation of mTORC1 substrate S6K1 at Thr389 (T389) was examined. Data are representative from three independent experiments.

Supplementary Figure S4

Fig. S1B

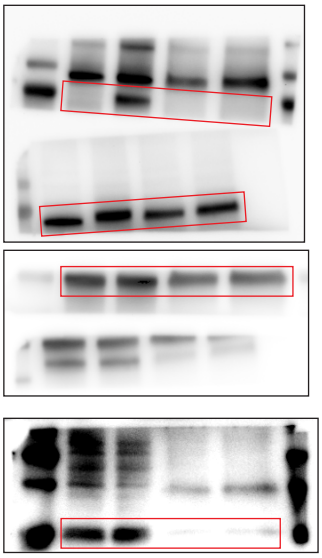


Fig. S1F

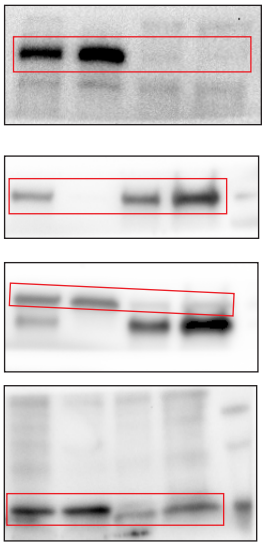
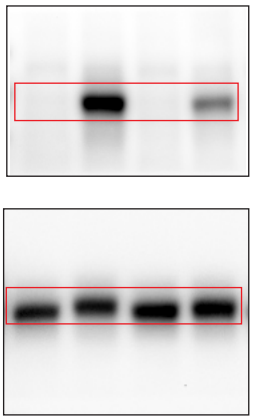


Fig. S1J

Fig. S2C

Fig. S3B



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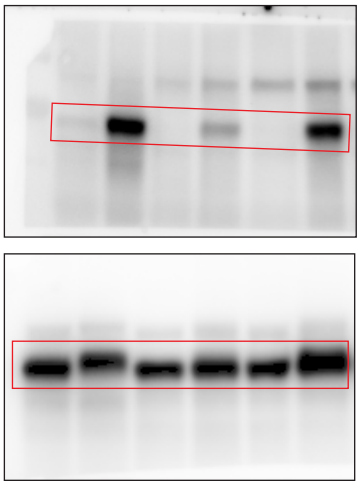
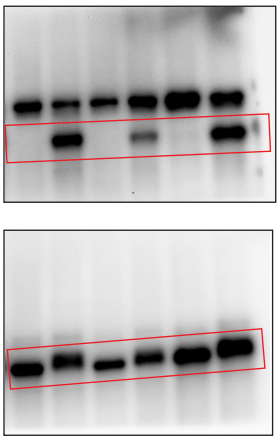


Fig. S3I



Supplementary Figure S4. Uncropped blots shown in this study (Related to immunoblotting section in STAR Methods).