**Supplemental Materials and Methods**

**Antibodies and chemicals**

The following antibodies for Western blot were purchased from Cell signaling: phospho-S6K (#9234), S6K (#9202), phospho-4EBP1 (#9456), 4EBP1 (#9452), RagA (#4357), RagC (#3360), mTOR (#2983), Raptor (#2280), phospho-Akt (S473) (#9271), Akt (#9272), phospho-MK2 (#3007), phospho-c-jun (#3270), phospho-Thr-Pro (#9391) and HA-HRP (#2999). Vinculin (#V9264) and Flag (#F3165) were obtained from Sigma. NLK (ab26050) were obtained from Abcam. HA (#MMS-101P) and Myc (#MMS-150P) was from Covance. Raptor antibody used for IP was from Bethyl (#A300-553A). phospho-Raptor-S863 antibody was from Santa Cruz (sc-130214). Antibodies used for immunofluorescence staining: mTOR (#2983), RagC (#3360) were purchased from Cell signaling; LAMP2 (#ab13524 for MEF cells; #ab25631 or HEK293 cells) were obtained from Abcam. Secondary antibodies Alexa Fluor 488, 555, 594, and 647 were obtained from Invitrogen.

Chemicals: SB203580 (#1202), SP600125 (#1496), U0126 (MEK1/2 inhibitor) (#1144) and (5Z)-7-Oxoozaenol (TAK1 inhibitor) (#3604) from Tocris; insulin from Sigma (#I1507); rapamycin from Calbiochem (#53123-88-9); and Torin1 was a generous gift from Dr. David Sabatini (MIT, Massachusetts, USA).

**Cell culture, transfection, lentiviral and retroviral infection**

HEK293, HEK293A, MEF, Neuro-2a, HCT116, Hela and NIH3T3 cells were cultured in high-glucose DMEM (Invitrogen, #11965-092) supplemented with 10%
FBS (Fisher-Scientific, #03600511) and penicillin/streptomycin. Transfections were performed with polyethylenimine. Amino-acid free DMEM was made following the high-glucose DMEM recipe with the exception that all amino acids were omitted. To establish HEK293 cells stably expressing Flag tagged RagA, retroviral constructs of pBABE empty vector or pBABE-Flag-RagA was transfected into HEK293P cells. Viral supernatant was harvested after 48 h and filtered through 0.45-μm filter. Target cells were infected in the presence of 8 μg/ml polybrene. Stable pools were obtained after selection with 2 μg/ml puromycin (InvivoGen, #Ant-pr-5).

To establish HEK293 cells stably expressing HA tagged NLK-WT or -KN, retroviral constructs of pQCXIH empty vector or pQCXIH-HA-NLK-WT or -KN was transfected into HEK293P cells. Virus infection was performed as described above. Stable pools were obtained after selection with 100 μg/ml hygromycin B (Invitrogen, #10687-010).

**Immunoprecipitation (IP) and Western blot**

For IP of NLK or Raptor, cells were lysed in CHAPS lysis buffer [50 mM HEPES (pH 7.4), 2 mM EDTA, 10 mM pyrophosphate, 10 mM glycerophosphate, 10 mM NaF, 100 mM NaCl, 0.3% CHAPS, and protease inhibitors]. For co-IP of Rag complex and Raptor, cells were lysed with Triton lysis buffer [recipe similar as above with an exception of 1% TritonX-100 instead of 0.3% CHAPS]. Primary antibodies for IP were added to lysate and incubated with rotation at 4°C for 2 h, followed by adding Protein A or protein G-Sepharose beads and incubating for additional 1 h. The
resulting beads were washed with lysis buffer 3 times and denatured by the addition of 50 μl of sample buffer. Samples were boiled for 5 min and subjected to Western blot analysis.

**Immunofluorescence staining**

MEF cells were cultured on fibronectin-coated coverslips to the appropriate density and treated as indicated in specific experiments. Cells were fixed with 4% paraformaldehyde for 15 min and then permeabilized with 0.01% Saponin in PBS for 10 min. After blocking in 2% BSA for 1-2 h, slides were incubated with primary antibodies diluted in PBS for 1 h at room temperature. After washing with TBS-Tween 3 times for 5 min, slides were incubated with Alexa Fluor 488, 555 or 647 conjugated secondary antibodies diluted in PBS for 1 h. The slides were then washed with PBS 3 times for 5 min. Slides were mounted with prolong gold antifade reagent with DAPI (#P-36931 from Invitrogen). For staining of HEK293 cells, the protocol was same as above with an exception that cells were permeabilized with 0.1% TritonX-100 in PBS for 10 min. Images were taken on an Olympus FV1000 confocal microscope.

**Purification of GST-LEF1-51-210 and His tagged Raptor fragments from E. Coli**

LEF1-51-210 was subcloned into the pGEX-KG vector with a GST tag. E. Coli strain BL21 bearing the plasmid were cultured until OD₆₀₀=6.0-7.0 and IPTG (0.5 mM) was added to induce protein expression at 30°C for 3 h. Bacteria were lysed
with Triton lysis buffer and sonicated for 5 min with 10 sec interval on ice following 10 sec sonication. Purification of GST protein was performed as described above with a modification at the dialysis step, where the dialysis buffer was 20 mM Tris pH 8.0 and 10% glycerol.

Raptor fragments described in Fig. 6A and Fig. S5B were subcloned into pPEI-His-sumo vector. Protein expression was induced with IPTG (0.5 mM) and bacteria were lysed as described above with an exception that EDTA was omitted in the lysis buffer. Ni-NTA agarose beads (Qiagen, #30210) were added to lysate and incubated with rotation at 4°C for 3 h. Beads were then washed with lysis buffer containing 20 mM imidazole 4 times. His tagged proteins were eluted with lysis buffer containing 200 mM imidazole at 4°C for 30 min, followed by dialysis in 20 mM Tris pH 8.0 and 10% glycerol overnight.
Supplemental Figures

Figure S1.

A. Transfect: HA-S6K +

B. Transfect: HA-S6K +

C. Transfect: Myc-4EBP1 +

D. Transfect: HA-Akt +

E. Transfect: HA-S6K +

F. Transfect: HA-S6K +

G. Sorbitol

H. Sorbitol

I. Sorbitol, Sorbitol+ Sorbitol+Sorbitol+

J. Sorbitol

K. Sorbitol, Sorbitol+ Sorbitol+Sorbitol+
Figure S1. NLK inhibits mTORC1.

(A) Screening for new mTORC1 regulators. Different kinases indicated in the figure were transfected together with S6K into HEK293 cells. Activity of mTORC1 were examined by S6K phosphorylation.

(B) S6K phosphorylation is inhibited by NLK. Empty vector, NLK-WT or -KN was transfected together with S6K into HEK293 cells. Activity of mTORC1 were examined by S6K phosphorylation.

(C) 4EBP1 phosphorylation is inhibited by NLK. Empty vector, NLK-WT or -KN was transfected together with 4EBP1 into HEK293 cells. Activity of mTORC1 were examined by 4EBP1 mobility shift.

(D) NLK does not affect Akt phosphorylation. Empty vector, NLK-WT or -KN was transfected together with Akt into HEK293 cells. Phosphorylation of Akt at Ser473 or Thr308 was detected with specific antibodies.

(E) S6K phosphorylation is not affected by TAK1 or ERK1/2. HA-S6K was co-transfected with NLK, TAK1/TAB1, ERK1, or ERK2. Activity of mTORC1 were examined by S6K phosphorylation.

(F) Verification of EGFP-NLK inhibition on mTORC1. Empty vector, EGFP-NLK-WT or -KN was transfected together with S6K into HEK293 cells. Activity of mTORC1 were examined by S6K phosphorylation.

(G) mTORC1 is inhibited by nutrient deprivation, hyperosmotic and osmotic stress. Cells were deprived of amino acid (1 h), glucose (3 h) or treat with 2-DG (25mM, 30 min), Sorbitol (0.5 M), H\textsubscript{2}O\textsubscript{2} (0.5 mM, 30 min), CoCl\textsubscript{2} (0.5 mM, 4 h) or DTT (2mM,
1 h). Activity of mTORC1 were examined by S6K phosphorylation and 4EBP1 mobility shift.

(H) NLK is activated by stress conditions in MEF cells. MEF cells stably expressing HA tagged NLK were cultured in normal condition (NC) or treated with sorbitol (0.1 M, 0.2 M or 0.5 M, 15 min), H$_2$O$_2$ (0.25 mM, 30 min), DTT (2 mM, 1 h) or amino acid starvation (1 h). Cell lysate was used for in vitro kinase assay as described in Fig. 1D.

(I) Quantification of mTOR dots that show colocalization with lysosomes. 20-25 cells were randomly selected and counted. Star indicates a statistically significant difference.

(J) Hyperosmotic stress induces mTORC1 inhibition is independent on p38 and JNK. Cells were pretreated with DMSO, SB203580 (p38 inhibitor, 10 µM), SP600125 (JNK inhibitor, 20 µM) or both inhibitors for 30 min, then treated with sorbitol (0.2 M) for additional 15 min. Activity of mTORC1 were examined by S6K phosphorylation.

(K) Hyperosmotic stress blocks amino acid or glucose stimulated mTORC1 reactivation. Cells were deprived of amino acids (1 h) or glucose (2 h) with or without presence of sorbitol. Amino acids or glucose was then added back to the media for 30 min. Activity of mTORC1 were examined by S6K phosphorylation.
Figure S2. mTORC1 was inhibited by hyperosmotic or oxidative stress in multiple cell lines.

HCT116 (left column), Hela (middle column) or NIH3T3 (right column) cells were treat with sorbitol (0.2 M), NaCl (0.1 M), H₂O₂ (0.25 mM) or menadione (25 mM) for indicated time. Activity of mTORC1 were examined by the phosphorylation of S6K and 4EBP1. Please note that Hela cells do not response to menadione treatment.
Figure S3. Verification of HEK293 cell lines with \textit{Nlk} deletion.

(A) Alignment of \textit{Nlk} KO cell line (Clone 1-8) with WT cells. Clone 1-8 is heterozygous, in which one allele contains one nucleotide insertion, while the other allele contains 11 nucleotides deletion.

(B) Alignment of \textit{Nlk} KO cell line (Clone 2-12) with WT cells. Clone 2-12 is homozygous, in which both alleles contain 2 nucleotides deletion.

(C) Verification of \textit{Nlk} deletion in \textit{Nlk} KO cells by Western blot.
Fig. S4. Stress induced mTORC1 inhibition was compromised in both HEK293 and Neuro-2a cells with Nlk deletion.

(A) Nlk deletion compromised stress induced mTORC1 inhibition. HEK293 WT or Nlk KO cells were treated with sorbitol (0.1 M) for the time points as indicated. Activity of mTORC1 as well as p38 and JNK were examined with specific antibodies.

(B) Nlk deletion compromised NaCl induced mTORC1 inhibition. HEK293 WT or Nlk KO cells were treated with NaCl (50 mM) for the time points as indicated. Activity of mTORC1 as well as p38 and JNK were examined with specific antibodies.
(C) Neuro-2a WT or Nlk KO cells were treated with sorbitol (0.1 M) for the time points as indicated. Deletion of Nlk and activity of mTORC1 were examined with specific antibodies.

(D) Neuro-2a WT or Nlk KO cells were treated with H₂O₂ (0.25 mM) for the time points as indicated. Deletion of Nlk and activity of mTORC1 were examined with specific antibodies.

(F) S6K phosphatase activity is not altered in the Nlk KO cells. HEK293 WT or Nlk KO cells were treated with Torin1 for 10 or 30 min. S6K phosphorylation was examined with specific antibody.

(G) NLK signaling to mTORC1 is independent of JNK. HEK293 WT or Nlk KO cells were pretreated with DMSO or JNK-IN-8 (10 µM) for 30 min. Cells were then kept in normal culture (NC) or treated with sorbitol (0.1 M) for 10 min in the presence of inhibitors. Activity of mTORC1, p38 and JNK were detected with specific antibodies.
Figure S5

A

| Transfect: HA-S6K+ | NC | FBS- | FBS→ Insulin | FBS-→ FBS |
|-------------------|----|------|-------------|-----------|
| Vector            |    |      |             |           |
| NLK-WT            |    |      |             |           |
| NLK-KN            |    |      |             |           |

|                      | p-S6K (S.E.) | p-S6K (L.E.) | HA-S6K | Flag-NLK |
|----------------------|--------------|--------------|--------|----------|
| Vector               |              |              |        |          |
| NLK-WT               |              |              |        |          |
| NLK-KN               |              |              |        |          |

B

|                  | EGFP-RagA | mTOR | LAMP2 | Merge |
|------------------|-----------|------|-------|-------|
| NC               | <image>   | <image> | <image> | <image> |
| Sorbitol         | <image>   | <image> | <image> | <image> |
| H₂O₂             | <image>   | <image> | <image> | <image> |
| AA-              | <image>   | <image> | <image> | <image> |

C

![Colocalization dots chart](chart)

- NC
- Sorbitol
- H₂O₂
- AA-
Figure S5. NLK dampens mTORC1 activation downstream of insulin signaling and Rag complex.

(A) Empty vector, NLK-WT or -KN was transfected together with S6K into HEK293 cells. Cells were starved with serum free media overnight and stimulated with insulin (150 nM, 15 min) or 10% FBS (30 min). mTORC1 activity was examined by S6K phosphorylation.

(B) Constitutively active Rag does not prevent the stress induced mTOR dissociation from lysosomes. EGFP-RagA-QL and HA-RagC-SN were co-transfected into HEK293 cells. Endogenous mTOR and LAMP2 were immunostained with specific antibodies. The EGFP-RagA transfected cells were visualized by EGFP while the un-transfected cells were circled by dashed lines. Scale bars: 10 µm.

(C) Quantification of mTOR co-localization with lysosomes. 15-20 cells with or without EGFP signal were randomly selected and counted. Star indicates a statistically significant difference ($P<0.05$).
Figure S6

(A) Reloading of the samples in Fig. 4A. Whole cell lysate and the corresponding IP samples were loaded side by side. The amount of IP samples were adjusted to make Raptor protein similar to that in cell lysate.

(B) Hyperosmotic and oxidative stress disrupt Raptor-Rag interaction. HEK293T cells stably expressing Flag tagged RagA were treated with sorbitol (0.2 M) or \( \text{H}_2\text{O}_2 \) (0.25 mM) for the time indicated. The interaction between endogenous Raptor and transfected Rag complex was examined by co-immunoprecipitation (IP) with Flag-RagA.

(C) Anchoring Raptor on lysosomes makes mTORC1 resistant to inhibition by NLK. Empty vector, NLK-WT or -KN was transfected into HEK293 cells together with S6K and WT Raptor or Raptor-Rheb. Cells were in normal culture or treated with...
sorbitol (0.1 M) for 15 min. Activity of mTORC1 were examined by S6K phosphorylation.

(D) Hypersmotic stress induces a rapid Raptor phosphorylation. HEK293 cells were treated with sorbitol (0.2 M) for different time points as indicated. Following IP, phosphorylation of Raptor at p-Thr-Pro motif was detected with specific antibody.
Figure S5. NLK phosphorylates Raptor at S863 site.

(A) Raptor is phosphorylated by NLK in vitro. Endogenous mTORC1 complex was immunoprecipitated with Raptor antibody and subjected to in vitro kinase assay with purified NLK kinase. Phosphorylation signal were detected by $^{32}$P-autoradiograph.

(B) Raptor fragments containing residues 741-912 and 741-1000 are phosphorylated by NLK. His tagged fusion proteins of different Raptor fragments were subjected to in vitro

| Name          | Organism | Site | Sequence         |
|---------------|----------|------|-----------------|
| Raptor        | Human    | S863 | TQSAFASPTNKGV   |
| LEF1          | Human    | T155 | HAVHPLTPLITYS   |
| LEF1          | Human    | S166 | YSDEHFSPSHPS    |
| SETDB1        | Human    | T976 | PFSSSEETPKNVA   |
| FOXO1         | Human    | S329 | TISGRLSIPIMTEQ  |
| STAT3         | Mouse    | S727 | TIDLPMSPRTLDS   |
| PERIOD (PER)  | Drosophila | S596 | VMLGIEISPHHDYY  |
| Paxillin      | Mouse    | S126 | FPNKQKSAEPSPT   |
*vitro* kinase assay with purified NLK kinase. Phosphorylation of fragments were detected by $^{32}$P-autoradiograph.

(C) Alignment of Raptor S863 site among different species shows that Raptor S863 is conserved from drosophila to mammals.

(D) Alignment of Raptor S863 and known phosphorylation sites in NLK substrates.
Figure S8

A

B

Cell line: Raptor-WT  Raptor-S863A

C

Sorbitol (0.1 M)

Time (min)  WT  S863A

NC  1, 5  15  60  NC  1, 5  15  60

- p-S6K
- S6K
- p-4EBP1
- Vinculin
**Fig. S8. Generation of Raptor S863A and S863D knockin cell lines.**

(A) Sanger sequencing shows successful mutation in Raptor S863A or S863D knockin cell lines. Sequence graphs show the homozygous mutation in both cell lines. PAM: protospacer adjacent motif.

(B) S863 is the major stress-induced phosphorylation site in Raptor. Cells containing WT or S863A Raptor were treated with sorbitol (0.1 M, 15 min) or \( \text{H}_2\text{O}_2 \) (0.25 mM, 15 min). Following IP, phosphorylation of Raptor at p-Thr-Pro motif was detected with specific antibody.

(C) Effect of sorbitol on mTORC1 activity in wild type and Raptor S863A knockin cells. The samples were similarly treated as those in Fig. 7A & 7B. Phosphorylation of S6K and 4EBP1 were determined by specific antibodies.