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Supplemental information

Sestrins Inhibit mTORC1 Kinase Activation Through the GATOR Complex

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Supplemental Data

Figure S1

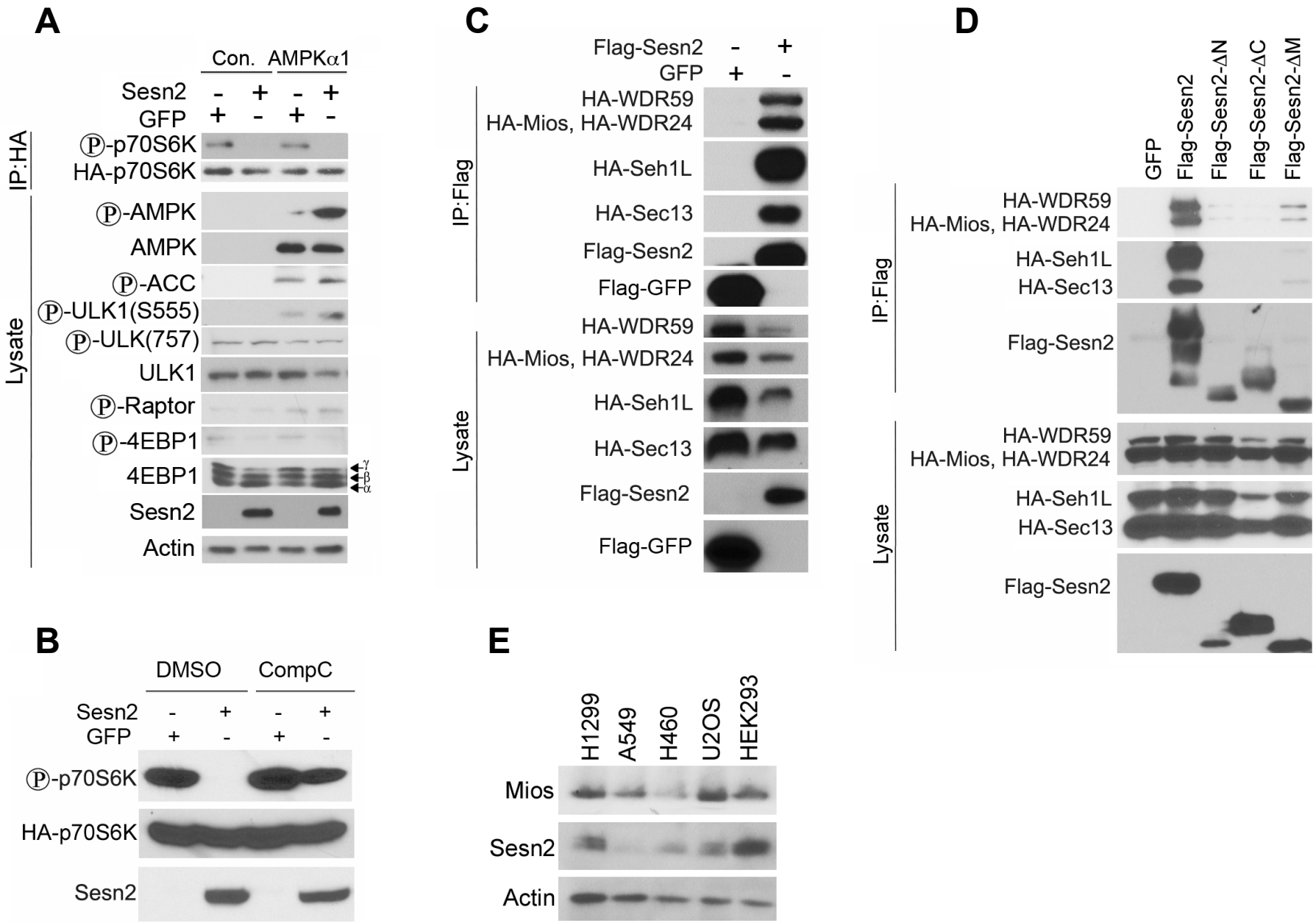


Figure S1, related to Figure 1: Sesn2 inhibits mTORC1 in an AMPK-independent manner and interacts with GATOR2.

(A) Sesn2 inhibits mTORC1 in an AMPK-independent manner in immortalized MEF. Immortalized *AMPK$\alpha^{-/-}$* MEF were co-transfected with HA-p70S6K- together with either Sesn2- or control GFP-expressing constructs in the presence or absence of AMPKα1-expressing plasmid. 48 hrs later cells were lysed, HA-p70S6K was immunoprecipitated with anti-HA beads and phosphorylation and expression of the corresponding proteins were analyzed by immunoblotting.

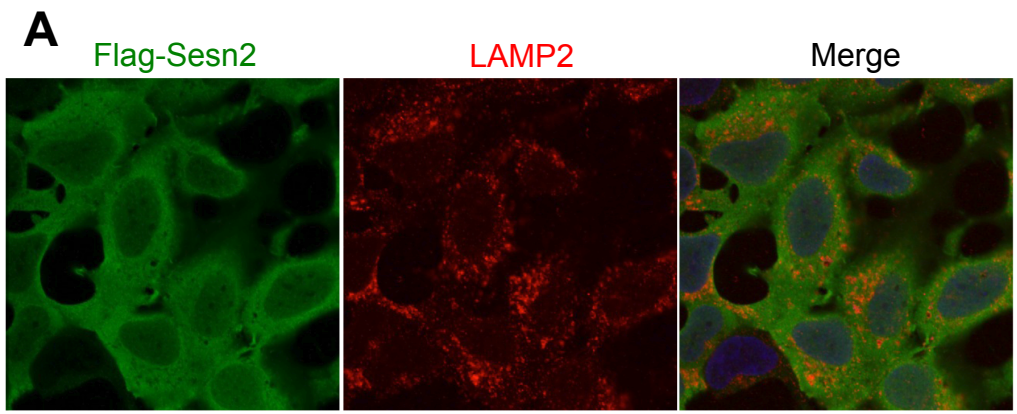
(B) Inhibition of AMPK has a partial effect on mTORC1 inhibition by Sesn2. HEK293T cells were co-transfected with HA-p70S6K- together with either GFP- or Sesn2- expressing constructs in the presence or absence of an AMPK inhibitor compound C (10μM) or DMSO control. 48 hrs later cells were lysed, HA-p70S6K was immunoprecipitated with anti-HA beads. Phosphorylation of HA-p70S6K and expression of HA-p70S6K and Sesn2 were analyzed by immunoblotting with the indicated antibodies.

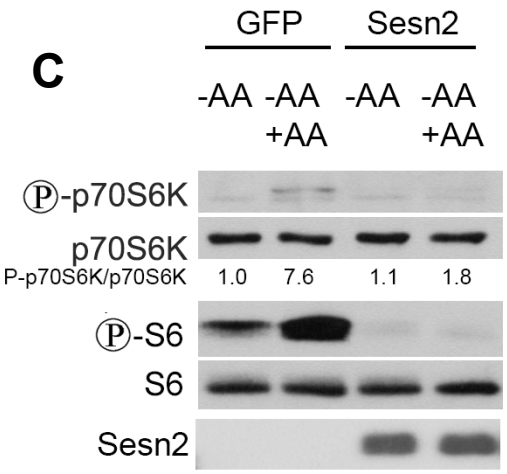
(C) Flag-Sesn2, but not Flag-GFP interacts with GATOR2. The constructs expressing either Flag-Sesn2 or Flag-GFP were co-transfected with HA-tagged GATOR2-expressing constructs into HEK293T cells, immunoprecipitated with anti-Flag beads and the proteins were analyzed by immunoblotting with anti-HA or anti-Flag antibodies.

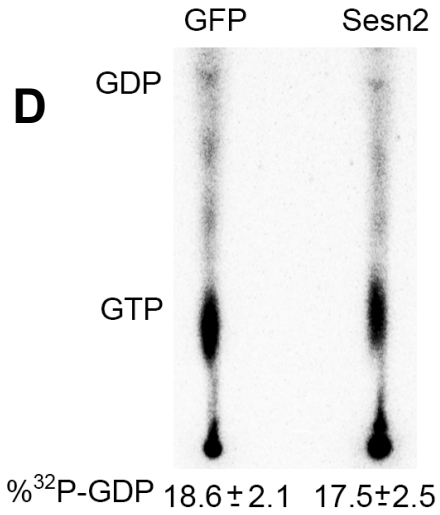
(D) Deletion of N-terminus, C-terminus or middle part of Sesn2 deteriorates interaction between Sesn2 and GATOR2. HEK293T cells were co-transfected with HA-GATOR2-expressing constructs together with the constructs expressing Flag-tagged truncated Sesn2 mutants. The complexes were immunoprecipitated with anti-Flag beads and analyzed by immunoblotting as in Figure 1B.

(E) Expression of Sesn2 and Mios in different cancer cell lines as in Figure 1F.

Figure S2


A
Flag-Sesn2
LAMP2
Merge


C
GFP
Sesn2
-AA
-AA +AA
-AA
-AA +AA
Ⓟ-p70S6K
p70S6K
P-p70S6K/p70S6K
1.0
7.6
1.1
1.8
Ⓟ-S6
S6
Sesn2


D
GFP
Sesn2
GDP
GTP
%32P-GDP
18.6±2.1
17.5±2.5

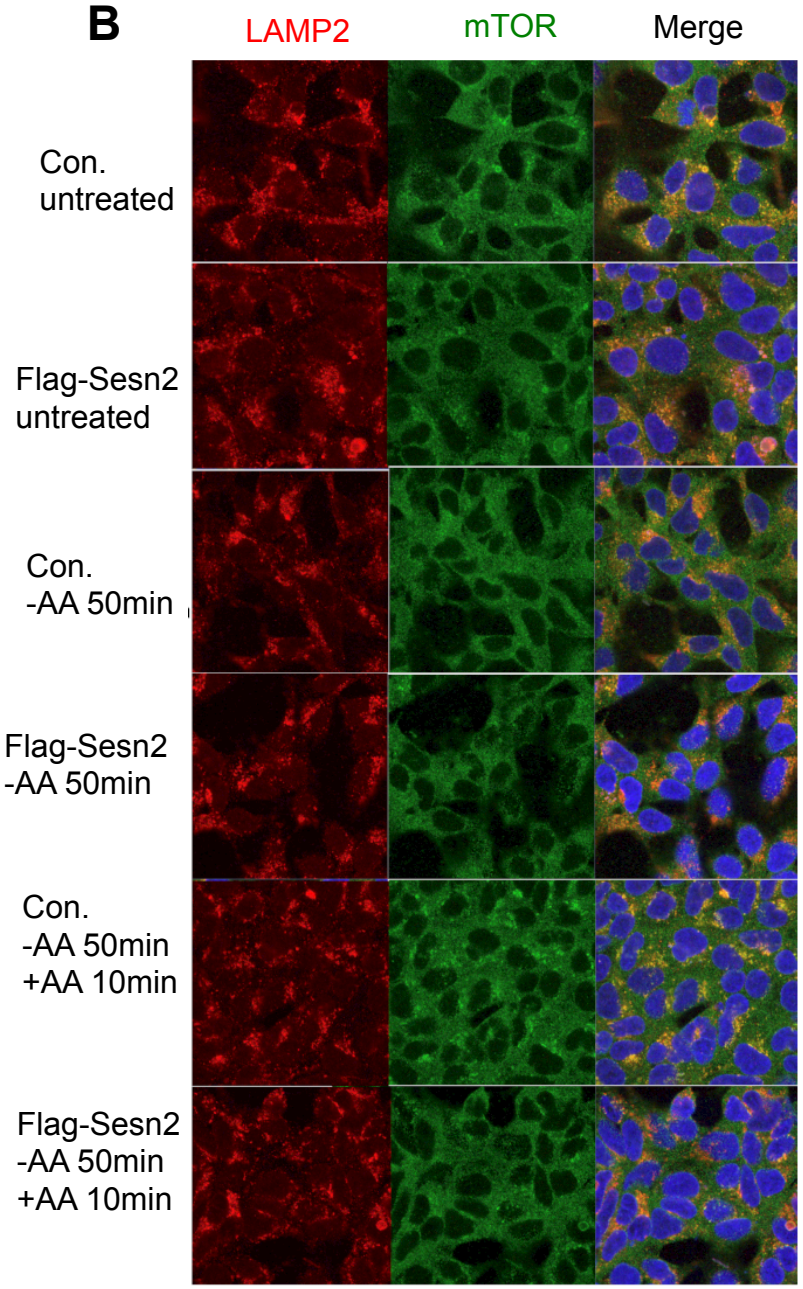

B
LAMP2
mTOR
Merge
Con.
untreated
Flag-Sesn2
untreated
Con.
-AA 50min
Flag-Sesn2
-AA 50min
Con.
-AA 50min
+AA 10min
Flag-Sesn2
-AA 50min
+AA 10min

Figure S2, related to Figure 4: the Effects of ectopic expression of Sesn2 on mTORC1 localization, activation of mTORC1 by AA and RagB GDP/ GTP Loading.

(A) Sesn2 does not co-localize with the lysosomes. Flag-Sesn2 was ectopically expressed with a high efficiency in HEK293T cells as in Figure 4A and the cells were stained with anti-Flag and anti-LAMP2 antibody followed by immunoflourescent analysis.

(B) Sesn2 suppresses mTOR localization in control conditions and after stimulation with AA. HEK293T cells were infected with either Flag-Sesn2-expressing lentiviral construct or control pLU vector, incubated with AA-free medium for 50 min and re-stimulated with AA for 10 min followed by immunoflourescent analysis with anti-mTOR and anti-LAMP2 antibodies as in Figure 4A.

(C) Sesn2 suppresses mTORC1 activation by AA in H1299 cells. H1299 cells infected with either Sesn2- or control GFP-expressing lentiviral constructs were incubated in AA-free medium for 50 min and re-stimulated with AA for 10 min. Phosphorylation and expression of the corresponding proteins were analyzed by immunoblotting.

(D) Sesn2 overexpression does not affect RagB GDP/GTP loading. HEK293T cells were infected with either Flag-Sesn2- or control GFP- expressing lentiviruses and metabolically labeled with ^{32}P for 4 hrs. RagB was immunoprecipitated with anti-RagB antibody, guanine nucleotides were extracted and separated by thin-layer chromatography followed by visualization on Typhoon 9410 phosphoimager. Radioactive signals were quantified by ImageQuant software. The values shown are averages ± SD of three experiments.

Supplemental Experimental Procedures

Cell Culture, Transfection, Infection and Treatment

Lung adenocarcinoma H1299, A549, H460, osteosarcoma U2OS, and kidney epithelial HEK293 and 293T cells were cultured in high-glucose DMEM containing 10% FBS and penicillin/streptomycin. All transfections were performed with Lipofectamine and Plus reagents (Life Technologies). Infections with lentiviral vectors were performed as described (Budanov and Karin, 2008). For AA starvation and re-stimulation experiments, cells were incubated in AA-free medium and re-supplied with a mix of all AA for the next 10 min.

Cell Lysis, Immunoprecipitation and Immunoblot Analyses

For ordinary immunoblot analysis cells were lysed in RIPA-SDS buffer as previously described (Budanov et al., 2002). For immunoprecipitation analysis cells were lysed in 0.3% NP40 or 0.3%CHAPS buffer as previously described (Budanov and Karin, 2008). The lysates were incubated with the mix of indicated antibodies and protein A:G Sepharose beads for 4 hrs (or alternatively with anti-Flag or anti-HA beads). After centrifugation the beads were washed 4 times with the lysis buffer. Total lysates and immunoprecipitated proteins were resolved by SDS-PAGE, transferred onto PVDF membranes and probed with the relevant antibodies. The antibodies used for the experiments are: anti-Flag and anti-Actin from Sigma, anti-Sesn2 from Proteintech and Santa Cruz, anti-GFP and anti-GAPDH from Santa Cruz, anti-LAMP2 from Abcam, anti-RagB from Novus Biological, Anti-phospho(T389)-p70S6K, anti-phospho(S235/236)-S6, anti-phospho(S65)-4EBP1, anti-phospho(T172)-AMPKα, anti-phospho(S79)-ACC, anti-phospho(S792)-Raptor, anti-phospho(S555)-ULK1, anti-phospho(757)-ULK1, anti-ULK1, anti-HA, anti-Myc, anti-RagA, anti-Mios, anti-AMPKα, anti-TSC2, anti-Raptor, anti-p70S6K, anti-S6 and anti-4EBP1 were from Cell Signaling Inc.

Constructs

HA-Tagged GATOR2-expressing plasmids were from Addgene, HA-p70S6K, pLU, pLU-Flag-Sesn2, pLU-Flag-Sesn2-ΔN,-ΔC,-ΔM and pLU-GFP were previously described (Budanov and Karin, 2008; Lee et al., 2013). To generate pLU-Flag-GFP construct Flag-GFP cDNA was generated by PCR and cloned into pLU vector.

Immunocytochemistry

HEK293T cells were plated on coverslips, treated, washed with PBS and fixed with 4% paraformaldehyde. Cells were permeabilized with 0.3% Triton X-100 and incubated with primary antibodies overnight. After 3 washes with PBS cells were incubated with Alexa Fluor-conjugated secondary antibodies: Alexa Fluor 555 Donkey Anti-Mouse IgG and Alexa Fluor 488 Donkey Anti-Rabbit IgG (Life technologies) and analyzed on Zeiss LSM700 confocal microscope.

Analysis of RagB GDP/GTP Content

Sesn2- or GFP-infected HEK293T cells were metabolically labeled with 0.5mCi ^{32}P-orthophosphate (Perkin Elmer) for 4 hrs, washed with PBS and lysed in 0.3% CHAPS buffer. RagB was immunoprecipitated with anti-RagB antibody (Novus Biologicals) as reported previously (Sancak et al., 2008), guanine nucleotides were extracted in elution buffer (2mM EDTA, 1mM GDP, 1mM GTP, 0.2% SDS, 5mM DTT) at 65^{o}C for 5 min, loaded on polyethylenimine (PEI) cellulose plate and resolved by thin layer chromatography in a chamber saturated with 0.75M KH_2PO_4 pH 3.4 as described byCastro et al., 2005). The plates were visualized by Typhoon 9410 phosphoimager. Radioactive signals were quantified by ImageQuant software. The values shown are averages ± SD of three experiments.

TAP-Tag Protein Complex Isolation and Mass Spectrometry.

MCF10A cells were infected with either pBabe-SBP-Flag-Sesn2-puro or control pBabe-puro retroviral constructs and selected with puromycin. 20 subconfluent 15 cm dishes were lysed in 40 ml of NP40 buffer (0.3%NP40, 50mM Tris-HCl pH=7.5, 150mM NaCl, protease and phosphatase inhibitors) and immunoprecipitated with 200mkl of anti-Flag beads for 3 hrs, following washing and elution with Flag-peptide. In the second step of immunoprecipitation, the solutions were incubated with 100mkl of streptavidin resin (GE Healthcare 17-5113-01) for 3 hrs, washed 3 times with the lysis buffer and 3 times with NH_4HCO_3 and eluted with 4mM biotin. The proteins were digested with trypsin, dried and dissolved in 10% acetonitrile and 0.8% of formic solution. The peptide mix was analyzed by a tandem liquid chromatography and mass spectrometry using Eksigent 2DnanoLC coupled with LTQ-Orbi-Trap mass spectrometer. MS/MS spectra were compared with human International Protein Index database from the European Bioinformatic Institute.

Supplemental References

Castro, A.F., Rebhun, J.F., and Quilliam, L.A. (2005). Measuring Ras-family GTP levels in vivo--running hot and cold. Methods *37*, 190-196.