## Materials and Methods

Materials

Reagents were obtained from the following sources: HRP-labeled anti-rabbit secondary antibody from Santa Cruz Biotechnology; antibodies to phospho-T389 S6K1, S6K1, Sestrin2, Mios and the FLAG epitope from Cell Signaling Technology; antibodies to the HA epitope from Bethyl laboratories; antibody to raptor from Millipore. FLAG M2 affinity gel and amino acids from Sigma Aldrich; RPMI without leucine, arginine, or lysine from Pierce; DMEM from SAFC Biosciences; XtremeGene9 and Complete Protease Cocktail from Roche; Inactivated Fetal Calf Serum (IFS) and SimplyBlue SafeStain from Invitrogen; amino acid-free RPMI from US Biologicals; [$^3$H]-labeled amino acids from American Radiolabeled Chemicals. The Mios antibody was generously provided by Jianxin Xie (Cell Signaling Technology).

Protein production and purification

Full-length, codon-optimized human Sestrin2 was N-terminally fused with a human rhinovirus 3C protease–cleavable $His_{10}$-$Arg_8$-ScSUMO tag and cloned into a PET-Duet-1 bacterial expression vector. This vector was transformed into *Escherichia coli* LOBSTR (DE3) cells (Kerafast, *30*). Cells were grown at 37 °C to 0.6 OD, then protein production was induced with 0.2 mM IPTG at 18 °C for 12–14 h. Cells were collected by centrifugation at 6,000*g*, resuspended in lysis buffer (50 mM potassium phosphate, pH 8.0, 500 mM NaCl, 30 mM imidazole, 3 mM β-mercaptoethanol (βME) and 1 mM PMSF) and lysed with a cell disruptor (Constant Systems). The lysate was cleared by centrifugation at 10,000*g* for 20 min. The soluble fraction was incubated with Ni-Sepharose 6 Fast Flow beads (GE Healthcare) for 30 min on ice. After washing of the beads with lysis buffer, the protein was eluted in 250 mM imidazole, pH 8.0, 150 mM NaCl and 3 mM βME. The Ni eluate was diluted 1:1 with 10 mM potassium phosphate, pH 8.0, 0.1 mM EDTA and 1 mM dithiothreitol (DTT), and was subjected to cation-exchange chromatography on a 5 ml SP sepharose fast flow column (GE Healthcare) with a linear NaCl gradient. The eluted Sestrin2 was then incubated with 3C protease and dialyzed overnight at 4 °C into 10 mM potassium phosphate, pH 8.0, 150 mM NaCl, 0.1 mM EDTA and 1 mM DTT, followed by a second cation-exchange chromatography run on an SP sepharose fast flow column (GE Healthcare) with a linear NaCl gradient. The protein was further purified via size-exclusion chromatography on a Superdex S200 16/60 column (GE Healthcare) equilibrated in running buffer (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.1 mM EDTA and 1 mM DTT). Selenomethionine (SeMet)-derivatized Sestrin2 was prepared as described previously (*31*) and purified as the native version, except that the reducing-agent concentration (βME and DTT) was 5 mM in all buffers.

Crystallization

Purified Sestrin2 was concentrated to 10 mg/ml and incubated in 2 mM leucine for >1 hour prior to setting crystal trays. Crystals were grown at 18 °C by hanging-drop

vapor diffusion with 1 µl of protein at 10 mg/ml mixed with an equal volume of reservoir solution containing 100 mM MES, pH 6.0, 1.2 M disodium malonate, and 1% (v/v) Jeffamine ED 2001. 1% (v/v) glycerol as an additive slowed nucleation and improved the morphology of the crystals. Selenomethionine-derivatized Sestrin2 crystallized under identical conditions. Crystals were cryoprotected in mother liquor supplemented with 18% (v/v) glycerol.

Data collection and structure determination

Data collection was performed at the Advanced Photon Source end station 24-IDC at Argonne National Lab. All data-processing steps were carried out with programs provided through SBgrid (*32*). Data reduction was performed with HKL2000 (*33*). A complete native dataset was collected to 2.7-Å and a complete SeMet dataset, at the selenium peak wavelength, was collected to 3.0-Å. The phase problem was solved using single-wavelength anomalous dispersion (SAD) and selenium positions (60 total) were determined in HYSS, run as part of the PHENIX AutoSol program (*34*), for the SeMet dataset (space group I23, 5 molecules per asymmetric unit). An interpretable 3.0-Å experimental electron density map was obtained after solvent modification with Parrot from the CCP4 suite (*35*), and manual model building was carried out in Coot (*36*). Subsequent refinement was carried out with the superior 2.7-Å native data set using *phenix.refine*.

Structural analysis

Protein-protein and protein-ligand interfaces were analyzed using PDBePISA (*37*). NCBI's Vector Alignment Search Tool (VAST, *24*) was used to identify structurally related proteins in the PDB. The multiple sequence alignment (MSA) was generated in Jalview (*38*) with the T-Coffee alignment algorithm (*39*). Sequences of Sestrin2 homologs were obtained via NCBI BLAST searches (*40*). All structure figures were made in PyMol (*41*).

Cell lysis and immunoprecipitation

Cells were rinsed one time with ice-cold PBS and immediately lysed with Triton lysis buffer (1% Triton, 10 mM β-glycerol phosphate, 10 mM pyrophosphate, 40 mM Hepes pH 7.4, 2.5 mM MgCl2 and 1 tablet of EDTA-free protease inhibitor [Roche] (per 25 ml buffer). The cell lysates were cleared by centrifugation at 13,000 rpm at 4°C in a microcentrifuge for 10 minutes. For anti-FLAG-immunoprecipitations, the FLAG-M2 affinity gel was washed 3 times with lysis buffer. 30 µl of a 50/50 slurry of the affinity gel was then added to clarified cell lysates and incubated with rotation for 2 hours at 4°C. Following immunoprecipitation, the beads were washed 4 times with lysis buffer containing 500 mM NaCl. Immunoprecipitated proteins were denatured by the addition

of 50 µl of sample buffer and boiling for 5 minutes as described (*42*), resolved by 8%–16% SDS-PAGE, and analyzed by immunoblotting.

For co-transfection experiments in HEK-293T cells, 2.5 million cells were plated in 10 cm culture dishes. Twenty-four hours later, cells were transfected using the polyethylenimine method (*43*) with the pRK5-based cDNA expression plasmids indicated in the following amounts: 200 ng FLAG-Sestrin2, 200 ng FLAG-Sestrin2-Nterm, 200 ng FLAG-Sestrin2-Cterm, 200 ng HA-Sestrin2-Cterm, and 600 ng metap2. For in vitro dissociation experiments, 20 ng of HA-Sestrin2 wild-type or mutant were transfected into cells stably expressing FLAG-WDR24. The total amount of plasmid DNA in each transfection was normalized to 5 µg with empty pRK5. Thirty-six hours after transfection, cells were lysed as described above.

For experiments that required amino acid starvation or restimulation, cells were treated as previously described (*44*). Briefly, cells were incubated in leucine free RPMI for 50 minutes and then restimulated with leucine for 10 minutes.

Leucine binding assay

5 million HEK-293T cells were plated in a 15 cm plate four days prior to the experiment. Forty-eight hours after plating, the cells were transfected via the polyethylenimine method with the pRK5-based cDNA expression plasmids indicated in the figures in the following amounts: 12 µg FLAG-metap2, 5 µg FLAG-Sestrin2, 5 µg FLAG-Sestrin2-Nterm, 5 µg FLAG-Sestrin2-Cterm, 5 µg HA-Sestrin2-Cterm,12 µg HA-GST-Rap2A, 7 µg HA-Sestrin2 wild-type or mutant. The total amount of plasmid DNA in each transfection was normalized to 20 µg total DNA with empty-PRK5. Forty-eight hours after transfection cells were lysed as previously described. If multiple samples of the same type were represented in the experiment, the cell lysates were combined, mixed, and evenly distributed amongst the relevant tubes.

Anti-FLAG or Anti-HA magnetic beads (Pierce) were blocked by rotating in 1 µg/µl bovine serum albumin (BSA) for 20 minutes at 4 °C, then washed twice in lysis buffer and resuspended in an equal volume of lysis buffer. 30 µl of bead slurry was added to each of the clarified cell lysates and incubated as previously described. Post-IP, the beads were washed as previously and incubated for one hour on ice in cytosolic buffer (0.1% Triton, 40 mM HEPES pH 7.4, 10 mM NaCl, 150 mM KCl, 2.5 mM MgCl2) with the appropriate amount of [$^3$H]-labeled amino acids and cold amino acids. At the end of one hour, the beads were aspirated dry and rapidly washed three times with cytosolic buffer. The beads were aspirated dry again and resuspended in 80 µl of cytosolic buffer. Each sample was mixed well and three 10 µl aliquots were separately quantified using a TriCarb scintillation counter (PerkinElmer). This process was repeated in pairs for each sample, to ensure similar incubation and wash times for all samples analyzed across different experiments.

For each sample, an immunoprecipitation was performed in parallel. After washing four times as previously described, the proteins were eluted in lysis buffer + 500

mM NaCl and 1 mg/ml HA peptide (for 1 hour at 30 °C) or FLAG-peptide (1 hour at 4°C). The eluted proteins were denatured by the addition of sample buffer and boiling for 5 minutes as described, then resolved by 8%–16% SDS-PAGE and analyzed with Coomassie blue stain.

In vitro Sestrin2-GATOR2 dissociation assay

HEK-293T cells stably expressing FLAG-WDR24 were transfected with HA-Sestrin constructs as described above. 48 hours after transfection, cells were starved for all amino acids for 50 minutes, lysed and subjected to anti-FLAG immunoprecipitation as described previously. The GATOR2-Sestrin2 complexes immobilized on the FLAG beads were washed twice in lysis buffer with 500 mM NaCl, then incubated for 10 minutes in 1 mL of cytosolic buffer with the indicated concentrations of leucine. The amount of GATOR2 and Sestrin2 that remained bound was assayed by SDS-PAGE and immunoblotting as described previously.

Differential Scanning Fluorimetry

The DSF (a.k.a Thermofluor) assays were performed according to the LightCycler 480 instruction manual. Briefly, 5X Sypro orange dye and Sestrin2 at 4 μM were combined with or without leucine or arginine (at the indicated concentrations) in thermal shift buffer (100 mM Tris pH 7.4, 100 mM NaCl, and 1 mM DTT) in a volume of up to 10 μl in one well of a LightCycler Multiwell 384-well plate. Each condition was tested in triplicate. The plate was subjected to a protocol in which the temperature increased from 20° to 85°C at 0.06°C/second. Fluorescence was recorded and plotted over time, and melting temperatures were calculated as described in the LightCycler 480 instruction manual. Briefly, the negative first derivative of the curve shown (change in fluorescence/change in temperature) was plotted against the temperature. The peak (i.e., lowest point on this curve) reflects the melting temperature. Each reported melting temperature is the mean ± SD for three replicates from one experiment.

Cell lines and tissue culture

HEK-293T cells were cultured in DMEM 10% IFS supplemented with 2 mM glutamine. All cell lines were maintained at 37°C and 5% CO2.

Plasmid preparation

For production of cell lines stably expressing wild-type and mutant Sestrin2 at levels close to endogenous expression, constructs were cloned into the pLJC5 lentiviral construct containing the UBC promoter as previously described.

Generation of CRISPR/Cas9 genetically modified cells

HEK-293T cells with lacking of all three Sestrins were generated as previously described (*20*). Briefly, guide RNAs were cloned into the pX330 vector and transiently transfected into HEK-293T cells transfected with 250 ng shGFP pLKO, 1 ug of the pX330 guide construct, and 500 ng of empty pRK5 using XtremeGene9. cells were single cell sorted with a flow cytometer into the wells of a 96-well plate containing 150 µl of DMEM supplemented with 30% IFS. Cells were grown for two weeks and the resultant colonies were trypsinized and expanded. Clones were validated for loss of the relevant protein via immunoblotting. Sestrin1-3 triple null cells were generated by sequential knock-out of the individual Sestrins.

Lentivirus production and lentiviral transduction

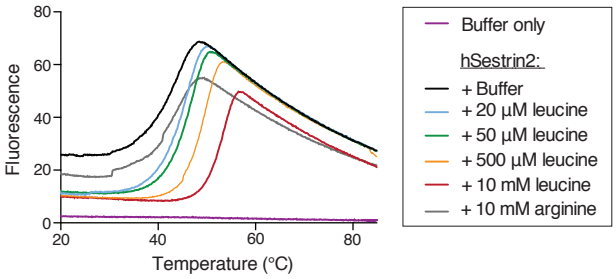
Lentiviruses were produced by transfection of viral HEK-293T cells with either pLJM1-GFP or pLJC5-FLAG-Sestrin2 (wild-type or mutant) constructs in combination with the VSV-G envelope and CMV ΔVPR packaging plasmids. Twenty-four hours after transfection, the media was changed to DMEM with 20% IFS. Forty-eight hours after transfection, the virus-containing supernatant was collected from the cells and passed through a 0.45 µm filter. Target cells were plated in 6-well plates containing DMEM 10% IFS with 8 µg/mL polybrene and infected with virus containing media. Twenty-four hours later, the media was changed to fresh media containing puromycin for selection.

Statistical analysis

Two-tailed t tests were used for comparison between two groups. All comparisons were two-sided, and P values of less than 0.001 were considered to indicate statistical significance.

# Figure S1

**A**




| Sample | Tm (°C) | Shift (°C) |
|---|---|---|
| Sestrin2 + Buffer | 44.3 ± 0.22 | |
| + 20 µM leucine | 46.1 ± 0.33 | 1.8 |
| + 50 µM leucine | 47.2 ± 0.11 | 2.9 |
| + 500 µM leucine | 49.9 ± 0.06 | 5.6 |
| + 10 mM leucine | 53.5 ± 0.18 | 9.1 |
| + 10 mM arginine | 44.8 ± 0.54 | < 1 |

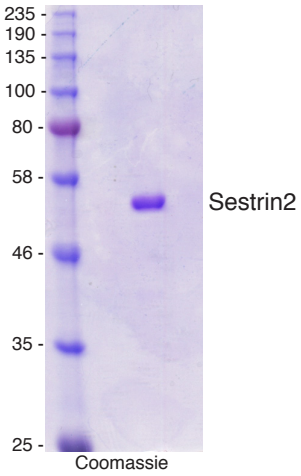


**Fig. S1**

**A**) Differential Scanning Fluorimetry with bacterially purified Sestrin2. Purified Sestrin2 was incubated with Sypro orange dye with or without leucine or arginine. Upon heating the sample the change in fluorescence was captured and used to calculate melting temperatures (Tm) under the indicated conditions. Values are Mean ± SD from 3 replicates. The protein was analyzed by SDS-PAGE followed by Coomassie blue staining.
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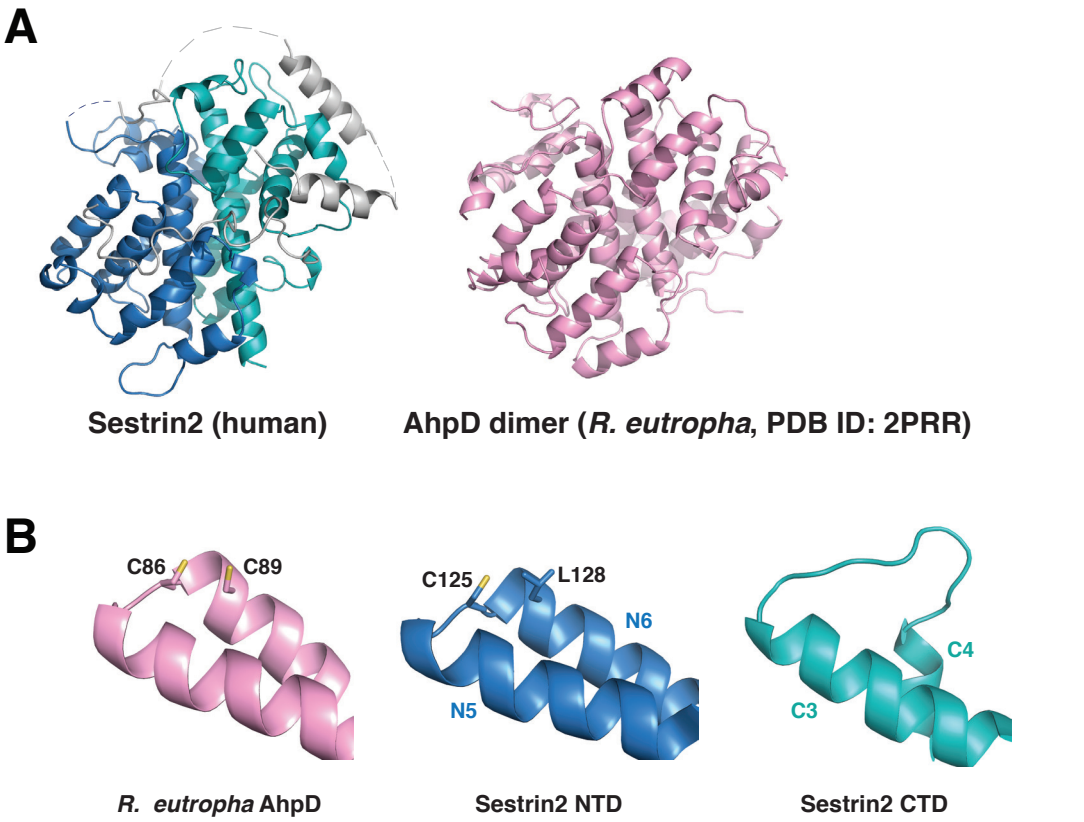
# Figure S2


A
Sestrin2 (human)
AhpD dimer (R. eutropha, PDB ID: 2PRR)
B
C86
C89
C125
L128
N6
N5
C4
C3
R. eutropha AhpD
Sestrin2 NTD
Sestrin2 CTD

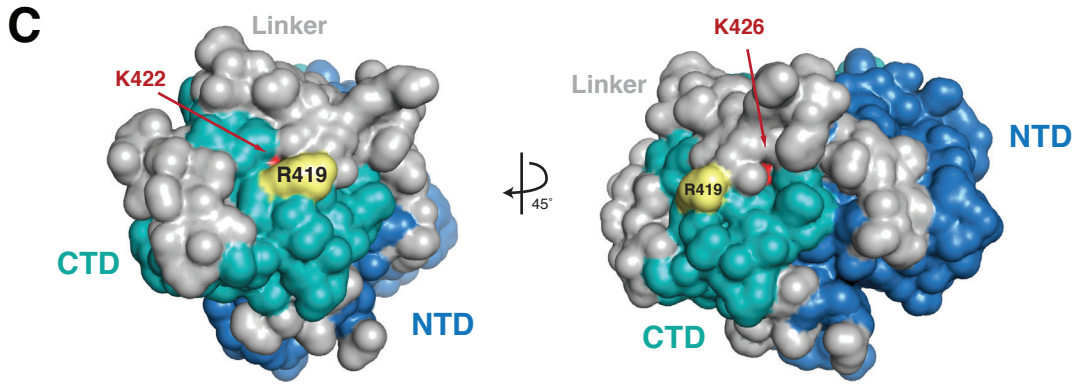
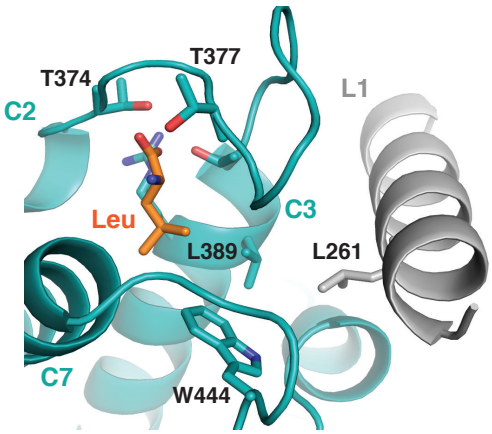

C
Linker
K422
R419
CTD
NTD
45°
K426
Linker
R419
NTD
CTD

**Fig. S2**

**A**) Side-by-side comparison of the human Sestrin2 structure from this study and *R. eutropha* AhpD (PDB ID: 2PRR).
**B**) Close up view of the active site cysteine residues in *R. eutropha* AhpD and the corresponding regions in the N- and C- terminal domains of human Sestrin2. Helix numbers and relevant residues are labeled.
**C**) Solvent exposed surface view of Sestrin2, with the reported "RKK" motif highlighted. Arg419 (yellow) is exposed while Lys422 and Lys426 (red) are buried

# Figure S3

**A**


T374
T377
L1
C2
Leu
C3
L389
L261
C7
W444

**B**

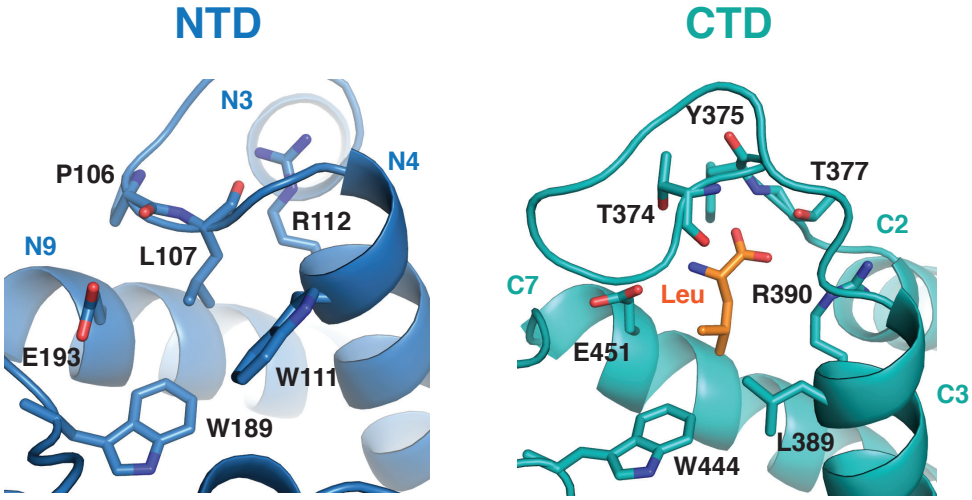
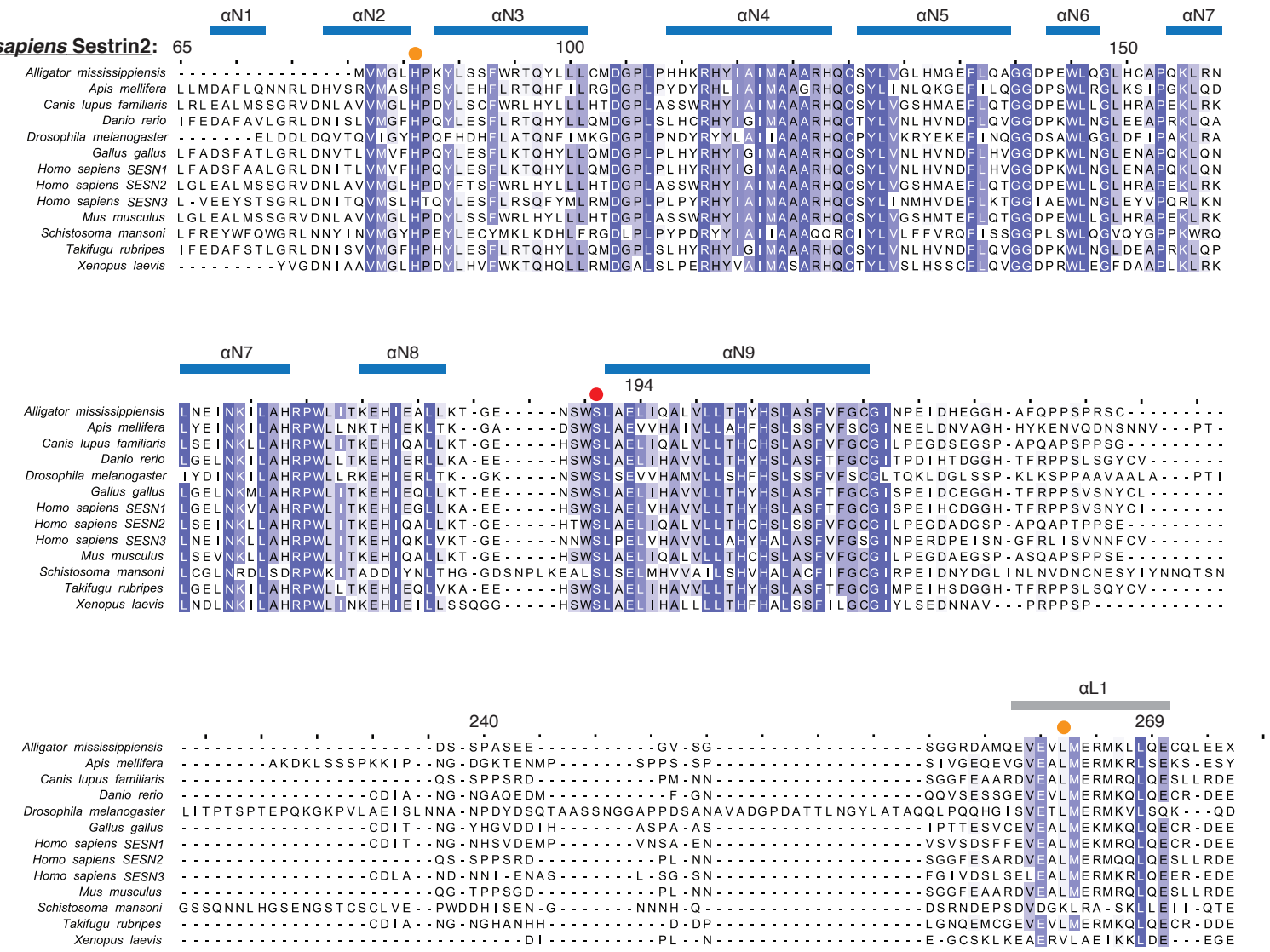

NTD
CTD
N3
P106
N4
R112
N9
L107
E193
W111
W189
Y375
T377
T374
C2
C7
Leu
R390
E451
C3
L389
W444

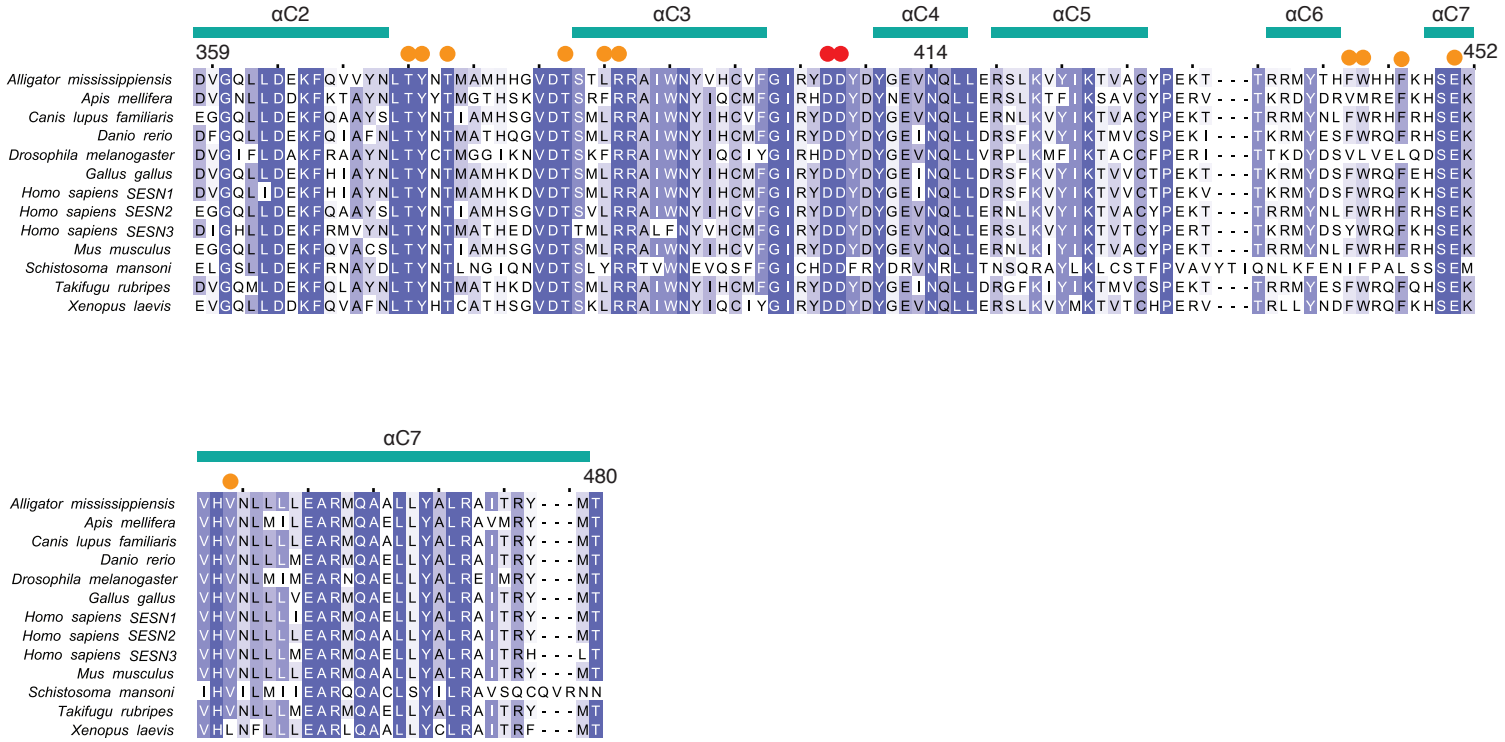
**Fig. S3**

**A**) PyMol view of Linker helix L1 packed against the side of the leucine-binding pocket, with residue Leu261 in close proximity to the bound leucine (orange).
**B**) Side by side comparison of the leucine binding pocket (right, CTD) and the corresponding location in the N-terminal domain (left, NTD).

# Figure S4

## A


αN1
αN2
αN3
αN4
αN5
αN6
αN7
H. sapiens Sestrin2:
65
100
150
Alligator mississippiensis
Apis mellifera
Canis lupus familiaris
Danio rerio
Drosophila melanogaster
Gallus gallus
Homo sapiens SESN1
Homo sapiens SESN2
Homo sapiens SESN3
Mus musculus
Schistosoma mansoni
Takifugu rubripes
Xenopus laevis
αN7
αN8
αN9
194
αL1
240
269
αL2
306
αC1
343



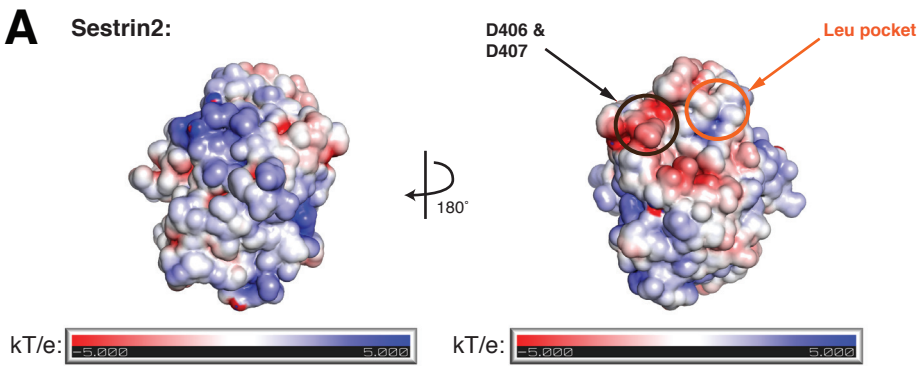

GATOR2 binding

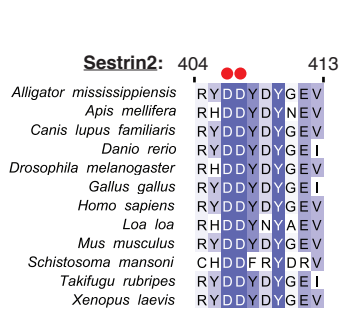
Leucine binding

**Fig S4**

**A**) Expanded Multiple Sequence Alignment of Sestrin2 homologues from various organisms. Positions are colored white to blue according to increasing sequence identity. Residues involved in GATOR2 binding (red) and leucine binding (orange) are indicated.

# Figure S5


A
Sestrin2:
D406 &
D407
Leu pocket
180˚
kT/e:
-5.000
5.000
kT/e:
-5.000
5.000


B
Sestrin2: 404
413
Alligator mississippiensis RYDDYDYGEV
Apis mellifera RHDDYDYNEV
Canis lupus familiaris RYDDYDYGEV
Danio rerio RYDDYDYGEI
Drosophila melanogaster RHDDYDYGEV
Gallus gallus RYDDYDYGEI
Homo sapiens RYDDYDYGEV
Loa loa RHDDYNYAEV
Mus musculus RYDDYDYGEV
Schistosoma mansoni CHDDFRYDRV
Takifugu rubripes RYDDYDYGEI
Xenopus laevis RYDDYDYGEV

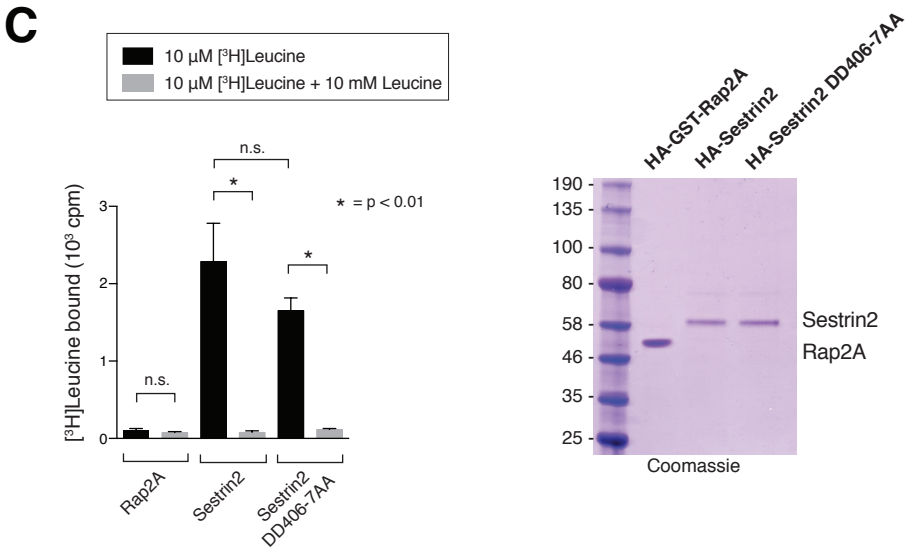

C
10 μM [3H]Leucine
10 μM [3H]Leucine + 10 mM Leucine
n.s.
*
* = p < 0.01
*
n.s.
[3H]Leucine bound (10³ cpm)
0
1
2
3
Rap2A
Sestrin2
Sestrin2 DD406-7AA
HA-GST-Rap2A
HA-Sestrin2
HA-Sestrin2 DD406-7AA
190
135
100
80
58
46
35
25
Sestrin2
Rap2A
Coomassie

**Fig S5**

**A**) Two views of Sestrin2 with the solvent exposed surface colored by electrostatic potential. The location of the leucine-binding pocket (orange circle) and a nearby negative charge cluster (black circle) are indicated.
**B**) Multiple sequence alignment of Showing the conserved residues Asp406 and Asp407.
**C**) Sestrin2 DD407-7AA still binds leucine. Binding assays were performed and immunoprecipitates analyzed as in Figure 2C. The indicated proteins purified from HEK-293T cells and used in the [$^3$H]-leucine binding assay were analyzed by SDS-PAGE followed by Coomassie blue staining. Equal volumes of each elution were loaded.

# Figure S6

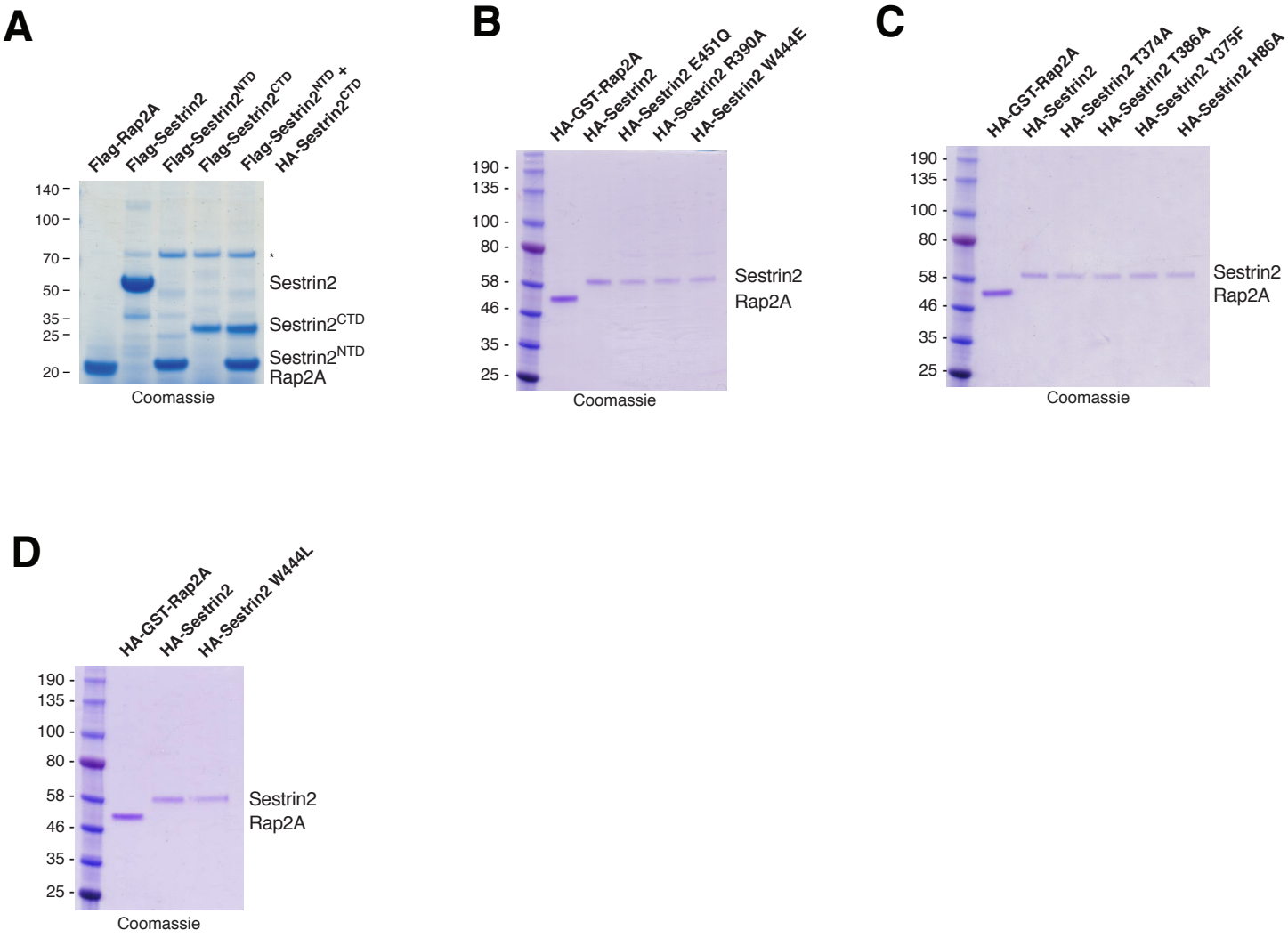

A
Flag-Rap2A
Flag-Sestrin2
Flag-Sestrin2NTD
Flag-Sestrin2CTD
Flag-Sestrin2NTD +
HA-Sestrin2CTD
140
100
70
50
35
25
20
*
Sestrin2
Sestrin2CTD
Sestrin2NTD
Rap2A
Coomassie
B
HA-GST-Rap2A
HA-Sestrin2
HA-Sestrin2 E451Q
HA-Sestrin2 R390A
HA-Sestrin2 W444E
190
135
100
80
58
46
35
25
Sestrin2
Rap2A
Coomassie
C
HA-GST-Rap2A
HA-Sestrin2
HA-Sestrin2 T374A
HA-Sestrin2 T386A
HA-Sestrin2 Y375F
HA-Sestrin2 H86A
190
135
100
80
58
46
35
25
Sestrin2
Rap2A
Coomassie
D
HA-GST-Rap2A
HA-Sestrin2
HA-Sestrin2 W444L
190
135
100
80
58
46
35
25
Sestrin2
Rap2A
Coomassie

**Fig S6**

**A-D**) The indicated proteins purified from HEK-293T cells and used in the [$^3$H]-leucine binding assay in Figures (A) 1E, (B) 2C, (C) 3C, and (D) 4B were analyzed by SDS-PAGE followed by Coomassie blue staining. Equal volumes of each elution were loaded.

# Table S1. Data collection and refinement statistics

| Protein | Sestrin2 + Leu native | Sestrin2 + Leu SeMet derivative |
|---|---|---|
| **Organism** | *Homo sapiens* | *Homo sapiens* |
| **PDB ID** | 5DJ4 | |
| **Data collection** | | |
| **Space group** | I23 | I23 |
| **a, b, c (Å)** | 293.03, 293.03, 293.03 | 292.99, 292.99, 292.99 |
| **α, β, γ (°)** | 90.0, 90.0, 90.0 | 90.0, 90.0, 90.0 |
| **Wavelength (Å)** | 0.9792 | 0.9792 |
| **Resolution range (Å)** | 92.67 – 2.70 (2.75 – 2.70) | 207.17 – 3.0 (3.07 – 3.0) |
| **Total reflections** | 4,575,519 | 3,363,435 |
| **Unique reflections** | 114,283 | 162,883 |
| **Completeness (%)** | 100 (100) | 100 (100) |
| **Redundancy** | 40.0 (39.4) | 20.6 (20.4) |
| **Anomalous completeness (%)** | - | 99.9 |
| **$R_{sym}$ (%)** | 15.3 (>100) | 16.3 (>100) |
| **$R_{p.i.m.}$ (%)** | 3.8 (59.7) | 4.7 (38.7) |
| **I/σ** | 24.5 (1.4) | 21.7 (2.5) |
| **$CC_{1/2}$ (%)** | 99.8 (65.6) | 99.9 (90.5) |
| **Refinement** | | |
| **Resolution range (Å)** | 92.66 – 2.70 (3.45-2.70) | |
| **$R_{work}$ (%)** | 19.6 | |
| **$R_{free}$ (%)** | 22.3 | |
| **Number of Reflections:** | | |
| **Total** | 114,277 | |
| **$R_{free}$ reflections** | 2,001 | |
| **Number of non-hydrogen atoms** | 14,945 | |
| **Protein atoms** | 14,830 | |
| **Water atoms** | 115 | |
| **R.m.s. deviations:** | | |
| **Bond lengths (Å)** | 0.011 | |
| **Bond angles (°)** | 1.09 | |
| **Average B factors (Å$^2$):** | | |
| **Protein** | 57.6 | |
| **Leu** | 49.6 | |
| **Water** | 45.6 | |
| **Wilson B-factor (Å)** | 57.3 | |
| **Ramachandran (%):** | | |
| **Favored (%)** | 97.0 | |
| **Allowed (%)** | 2.5 | |
| **Outlier (%)** | 0.5 | |
| **Clashscore** | 6.02 | |
| **Molprobity score** | 2.35 | |
| **Molprobity percentile** | 92$^{nd}$ | |