Sestrin2 inhibits mTORC1 through modulation of GATOR complexes

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Sestrins are stress-inducible metabolic regulators that suppress a wide range of age- and obesity-associated pathologies, many of which are due to mTORC1 overactivation. Upon various stresses, the Sestrins inhibit mTORC1 activity through an indirect mechanism that is still unclear. GATORs are recently identified protein complexes that regulate the activity of RagB, a small GTPase essential for mTORC1 activation. GATOR1 is a GTPase activating protein (GAP) for RagB whereas GATOR2 functions as an inhibitor of GATOR1. However, how the GATORs are physiologically regulated is unknown. Here we show that Sestrin2 binds to GATOR2, and liberates GATOR1 from GATOR2-mediated inhibition. Released GATOR1 subsequently binds to and inactivates RagB, ultimately resulting in mTORC1 suppression. Consistent with this biochemical mechanism, genetic ablation of GATOR1 nullifies the mTORC1-inhibiting effect of Sestrin2 in both cell culture and Drosophila models. Collectively, we elucidate a new signaling cascade composed of Sestrin2-GATOR2-GATOR1-RagB that mediates stress-dependent suppression of mTORC1 activity.

mTORC1 is a nutrient-sensing metabolic regulator that promotes protein and lipid anabolism and inhibits autophagic catabolism of nutrient deposits, protein aggregates and damaged organelles such as dysfunctional mitochondria1–2. Chronic activation of mTORC1 by overnutrition can result in diverse metabolic pathologies associated with aging, obesity and autophagic defects3. Upon chronic activation of mTORC1 as well as upon diverse environmental stresses, a family of stress-inducible proteins named Sestrins are induced through several stress-responsive transcription factors, such as p53, HIF-1, FoxO and c/EBPβ, and subsequently suppress mTORC1 activation3. In model animals such as Drosophila and mice, Sestrins are shown to be essential for maintaining metabolic homeostasis and preventing age- and obesity-associated pathologies4–6. Many of these pathologies are also suppressed by pharmacological or genetic inhibition of mTORC1/dTORC14–8, suggesting that its ability to suppress mTORC1/dTORC1 activation is central for the Sestrins’ metabolism-regulating role.

One possible mechanism of Sestrins-dependent mTORC1 regulation involves AMPK-activated protein kinase (AMPK)9, which phosphorylates tuberous sclerosis complex 2 (TSC2) and Raptor and thereby inhibits mTORC1 activity9. It has been suggested that Sestrin2, the most thoroughly studied Sestrin isoform, associates with AMPK and promotes its activating phosphorylation by the upstream kinase LKB19. Chemical or shRNA-mediated inactivation of AMPK prevented Sestrin2 from inhibiting mTORC19 although the extent of the effect was varied depending on the types of cells and tissues. For example, Sestrin2 was still able to inhibit mTORC1 in HeLa cells, which do not express LKB1 and therefore exhibit very low amount of AMPK activity10. Therefore, it has been postulated that there could be additional mediators of Sestrin2 that suppress mTORC1 activation.

GATOR is a multiprotein complex that is composed of two subcomplexes called GATOR1 and GATOR210. GATOR1 is composed of three proteins, DEPD5, NPRL2 and NPRL3, whereas GATOR2 possesses five protein components, MIOS, WDR24, WDR59, SEH1L and SEC1310. GATOR1 serves as a GAP for RagB and its close homolog RagA, which are functionally redundant GTPases essential for mTORC1 activation during amino acid-rich conditions11–13, while GATOR2 inhibits the GAP activity of GATOR110. GATOR1 is considered as a tumor suppressor as its absence can lead to constitutive activation of RagB and subsequent elevation of mTORC1 activity. Indeed, many human cancer cell lines have a deficiency in at least one of the three GATOR1 components, and loss of DEPD5 and NPRL2 genes was observed in human glioblastoma and ovarian cancer tissues14–16. The mTORC1-regulating role of GATOR seems to be conserved in Drosophila14,16 and yeasts15–17. Although these
findings implicate that GATOR1 and GATOR2 are important for mTORC1 regulation and tumorigenesis, it is not known how GATOR complexes themselves are physiologically regulated.

Here we show that Sestrin2 is a physiological regulator of GATOR complexes. By physically interacting with GATOR2, Sestrin2 releases GATOR1 from GATOR2-mediated inhibition. GATOR1 then inhibits RagB GTPase and subsequently prevents mTORC1 activation by amino acids. These results illustrate a new mechanism through which Sestrin2 negatively regulates mTORC1 and describe one way in which GATOR activity is regulated in mammalian and insect cells.

**Results**

Sestrin2 can regulate mTORC1 through an AMPK-independent pathway. To test the necessity of AMPK in Sestrin2-mediated mTORC1 control, we treated AMPK-null mouse embryonic fibroblasts (Ampkα1−/−/Ampkα2−/− MEFs) with Sestrin2-overexpressing adenoviruses (Ad-SESN2). Strikingly, AMPK-null MEFS were still capable of downregulating mTORC1-dependent phosphorylation of p70 S6 kinase (S6K) upon Sestrin2 expression as much as WT MEFS (Fig. 1A). Thus, expression of truncated Sestrin2 proteins were unable to bind GATOR2, constitutive AMPK activation can inhibit chronic mTORC1-activated AMPKγ and constitutive AMPK activation can inhibit chronic mTORC1, AMPK is not solely responsible for Sestrin2-induced mTORC1 suppression.

Identification of GATOR2 components as Sestrin2-binding proteins. To identify new mediators of Sestrin2 function, we conducted a tandem affinity purification (TAP)-mass spectrometry (MS) experiment. In the experiment, there were only six proteins, namely MIOS, WDR24, WDR59, SEH1L, SEC13 and PPM1A, whose unique peptide sequences were represented in the Sestrin2-interacting proteome more than three times (Fig. 1B). Because each of these proteins showed a very weak to hardly detectable physical interaction with Sestrin2 when co-expressed in human embryonic kidney 293 (HEK293) cells (Fig. S1A), we initially judged that the interactions between Sestrin2 and these proteins were insignificant. However, after realizing that five of these proteins form a protein complex named GATOR2, we hypothesized that an intact GATOR2 complex may be required for stable interaction with Sestrin2. Indeed, when all GATOR2 components were co-expressed with Sestrin2 in HEK293 cells, all five proteins were strongly co-immunoprecipitated (IPed) with Sestrin2 (Fig. 1C). This specific interaction was also observed in an in vitro pull-down assay (Fig. S1B). Endogenous GATOR2 components were also co-IPed with endogenous Sestrin2 in mouse liver tissues (Fig. 1D) and embryonic fibroblasts (MEF) (Fig. 1E and S2), confirming the existence of endogenous physical association between Sestrin2 and GATOR2.

GATOR1 does not interact with Sestrin2. Sestrin1, a closely related homolog of Sestrin2 with the same ability to suppress mTORC1, also interacted with GATOR2 complex in HEK293 cells (Fig. S3A). We thought that one of the subdomains in Sestrin1 and Sestrin2, which were identified through a phylogenetic analysis, could be solely responsible for binding to GATOR2 (Fig. S3B). However, truncated Sestrin2 proteins were unable to bind GATOR2, suggesting that only intact full-length Sestrin2 can physically interact with GATOR2 (Fig. S3C). We were also curious if Sestrins can also interact with GATOR1, another GATOR subcomplex loosely bound to GATOR2. However, when co-expressed with Sestrin2 and GATOR2 in HEK293 cells, the three GATOR1 components, DEPDC5, NPRL2 and NPRL3, were not found in the anti-Sestrin2 IP complex that contained all GATOR2 components (Fig. S3D). Consistent with this result, none of the GATOR1 components were represented in the total of ~599 peptides identified from the TAP-MS experiment described above. Therefore, Sestrin2 specifically associates with GATOR2 but not GATOR1.

Sestrin2 binding to GATOR2 attenuates formation of the GATOR1-GATOR2 complex. Because the Sestrin2-GATOR2 complex does not show any evidence of physical association with GATOR1, it is plausible that Sestrin2 can inhibit the binding of GATOR1 to GATOR2. Indeed, when co-transfected with GATOR1 and GATOR2 in HEK293 cells, Sestrin2 diminished the association between GATOR1 and GATOR2 (Fig. 2A, 2B) in a dose-dependent manner (Fig. S4). Sestrin2 itself was found in the anti-GATOR2 IP complex (Fig. 2B and S4) but not in the anti-GATOR1 IP complex (Fig. 2A). In vitro addition of Sestrin2 protein to immunopurified GATOR1 and GATOR2 protein components (Fig. 2C) also reduced the amount of GATOR2 components pulled-down with NPRL2 (GATOR1) antibody (Fig. 2D). Sestrin2 overexpression also attenuated formation of endogenous GATOR1-GATOR2 supercomplex; although there is a substantial association between NPRL2 and WDR24 in normal liver, adenovirus-mediated overexpression of Sestrin2 in mouse liver reduced the amount of NPRL2 co-IPed with WDR24 from liver lysates (Fig. 2E). We have recently shown that tunicamycin (Tm)-induced ER stress inhibits mTORC1 by inducing endogenous Sestrin2 (Fig. 2F). Thus, we tested whether ER stress could modulate GATOR signaling through Sestrin2. In WT mice, Tm-induced Sestrin2 showed strong association with WDR24 (Fig. 2F), and less NPRL2 was bound to WDR24 upon Tm treatment (Fig. 2F). However, Tm-induced decrease in NPRL2-WDR24 association was not observed in Sestrin2−/− mice (Fig. 2F), indicating that GATOR1/GATOR2 complexes are indeed a target of endogenous Sestrin2 during hepatic ER stress. These results collectively suggest that increased expression of Sestrin2 can promote release of GATOR1 from the GATOR1-GATOR2 complex by competing with GATOR1 for the association with GATOR2.

Sestrin2 potentiates GATOR1’s GAP activity towards RagB. As GATOR1 is an inhibitor of GATOR1, Sestrin2-mediated release of GATOR1 from the GATOR1-GATOR2 complex can direct the GAP activity of GATOR1 towards its target RagB. To test this possibility, we tested if Sestrin2 can regulate RagB in cells. Although transfected GST-tagged RagB is predominantly in a GTP-bound state under normal culture conditions, Sestrin2 co-expression significantly elevated the level of RagB-GDP while decreasing the level of RagB-GTP (Fig. 3A). Sestrin2 also promoted hydrolysis of GTP bound to endogenous RagB protein (Fig. 3B), suggesting that Sestrin2 indeed controls RagB. Interestingly, the amount of NPRL2, a GATOR1 component, co-IPed with RagB was substantially increased when Sestrin2 was expressed (Fig. 3C), and the amount of RagB co-IPed with NPRL2 was also increased upon Sestrin2 expression (Fig. 3D), suggesting that Sestrin2 enhanced the association between GATOR1 and RagB. When co-transfected with GATOR1, GATOR2 and GST-tagged RagB/C in HEK293 cells, Sestrin2 increased the amount of NPRL2 and DEPDC5 proteins pulled down with GST-tagged RagB/C proteins (Fig. 3E). These data collectively indicate that Sestrin2 regulates RagB activity by directing the GAP activity of GATOR1 towards RagB.

Sestrin2 regulates subcellular mTOR localization. Rag GTPases regulate mTORC1 partially by regulating its subcellular localization. In the presence of amino acids, RagB/GTP recruits mTORC1 to the lysosomal surface where mTORC1 can be activated by RagB, as well as by other GTPases such as Rheb. It is thought that RagB is activated in the presence of sufficient amino acids; however, from the experiments described above, we found that Sestrin2 can inhibit RagB even in the presence of sufficient amino acids. Consequently, Sestrin2 expression expelled mTOR from the lysosomal surface (Fig. 4A). To determine whether GATOR complex regulation is
critical for the Sestrin2’s role in regulating mTOR localization, we silenced NPRL2, an essential subunit of GATOR1 required for its GAP activity. Sestrin2 was unable to control mTOR localization in NPRL2-silenced condition; NPRL2-silenced cells showed constitutive lysosomal localization of mTOR regardless of Sestrin2 expression (Fig. 4B). These results suggest that GATOR complexes are essential for Sestrin2-mediated control of subcellular mTOR localization.

Sestrin2 is not present on the lysosomal membrane. Rag GTPases were shown to be present on the lysosomal membrane. Thus, we were interested in subcellular localization of Sestrin2. Interestingly, Sestrin2 was almost completely excluded from lysosomal membrane (Fig. 4C). Consistent with the strong physical association between Sestrin2 and GATOR2 (Fig. 1), WDR24, a GATOR2 component, showed substantial co-localization with Sestrin2 (Fig. 4D), while also excluded from lysosomal membrane (Fig. 4E). Because GATOR1 was reported to be present on the lysosomal membrane when overexpressed, and because Sestrin2 indirectly regulates the association between GATOR1 and RagB, it is possible that Sestrin2 changes the subcellular localization of GATOR1 through displacement of GATOR2. Indeed, although endogenous DEPDC5, a GATOR1 component, does not show significant lysosomal localization under normal culture conditions, a substantial amount of DEPDC5 accumulated on the lysosomal membrane when Sestrin2 was overexpressed (Fig. 4F, arrows). Overexpressed Sestrin2 itself was not co-localized with lysosomally-accumulated DEPDC5 (Fig. 4G). These results collectively indicate that Sestrin2 indirectly regulates RagB-mTORC1 signaling by modulating GATOR complexes.
Sestrin2 regulates mTORC1 activity through GATOR regulation. Then we determined whether GATOR regulation is essential for Sestrin2-mediated inhibition of mTORC1 activity. As previously reported, Sestrin2 expression in HEK293 cells suppressed phosphorylation of mTORC1 downstream targets including p70 ribosomal protein S6 kinase (S6K) and eukaryotic translation initiation factor 4E-binding protein (4E-BP) (Fig. 5A). The phosphorylation of ribosomal protein S6, which is mediated by S6K, was also downregulated by Sestrin2 expression (Fig. 5A). RNAi-mediated silencing of NPRL2, which completely abrogates GATOR1 activity, nullified Sestrin2’s effect on mTORC1-dependent phosphorylation events (Fig. 5A), supporting the notion that GATOR complexes mediate Sestrin2-induced suppression of mTORC1.

**Drosophila Sestrin regulates cell growth through GATOR regulation.** Sestrin2-mediated inhibition of mTORC1 activity is conserved in Drosophila and overexpression of mammalian Sestrin results in inhibition of dTORC1. Because components of GATOR1/2 complexes are also well conserved in Drosophila, we investigated whether GATOR regulation is also important for Drosophila Sestrin (dSesn)-mediated cell growth control in Drosophila. Especially, we focused on Drosophila wing epithelium where dSesn- and dRagB-mediated control of dTORC1 and cell growth has been well characterized. 

**Figure 2 | Sestrin2 decreases GATOR1-GATOR2 binding.** (A and B) Sestrin2 destabilizes physical interaction between GATOR1 and GATOR2 components as indicated. NPRL2, a GATOR1 component, or WDR24, a GATOR2 component, was IPed using Flag antibody in (A) and (B), respectively. Input (WCL) and IP complex were analyzed by IB. (C) Purification of Sestrin2, GATOR1 and GATOR2 proteins, Sestrin2 and HA-GATOR1/2 complexes were purified from transformed E. coli and transfected HEK293 cells, respectively. (D) Sestrin2 inhibits assembly of GATOR1-GATOR2 supercomplex in vitro. As indicated, the purified proteins were mixed and incubated together. GATOR1 was pulled down from the mixture using NPRL2 antibody. GATOR1 and co-purified GATOR2 was detected through IB with HA antibody. (E) Sestrin2 overexpression decreases GATOR1-GATOR2 association in mouse liver. 10³ pfu of adenoviruses expressing GFP or Sestrin2 were injected into two-month-old WT mice through the tail vein. After 4 days, liver lysates were prepared, and endogenous GATOR2 was IPed with WDR24 antibody. WCL and IP complex were analyzed by IB. (F) Sestrin2 is required for the effect of tunicamycin (Tm) on GATOR1-GATOR2 interaction. Two-month-old WT or dSesn+/− mice were injected with Tm (500 mg/Kg i.p.) as described. After 24 hrs, liver lysates were prepared, and GATOR2 was IPed with WDR24 antibody. WCL and IP complex were analyzed by IB. Cropped gel images are used in this figure and the gels were run under the same experimental conditions.
mTORC1 regulation by Sestrin-family proteins is highly conserved between mammals and Drosophila. GATOR2 downregulation can correct autophagy defects of dSesn-null flies. Because dSesn-mediated suppression of chronic dTORC1 activation is important for proper autophagy, dSesn-null mutant flies exhibited an autophagy defect and associated degenerative phenotypes in the skeletal muscle. We questioned if GATOR2 misregulation is physiologically responsible for endogenous dSesn's autophagy-controlling function. Interestingly, the autophagy downregulation observed in dSesn-null skeletal muscle was nearly completely suppressed by the hypomorphic mutation of GATOR2 components (transheterozygotic mutation of Wdr24 and Sec13), as manifested by restoration of the dAtg8-II/I ratio (Fig. 5D, 5E). This genetic interaction result suggests that one of Sestrin’s main physiological functions is the suppression of GATOR2.

Discussion
Sestrins link environmental stresses to inhibition of mTORC1. For instance, Sestrin2 suppresses mTORC1 activity in response to DNA damage, ER stress, nutritional stress or energetic stress. Sestrin-mediated suppression of mTORC1 activity is important for maintaining cellular and organismal homeostasis during stress. Loss of Sestrin2 in mice or its homologue in Drosophila causes chronic upregulation of mTORC1/dTORC1 that results in diverse age- and obesity-associated pathologies. The current study provides a molecular mechanism through which Sestrins inhibit mTORC1 activity. Under normal condition, GATOR1 and GATOR2 form a GATOR supercomplex in which GATOR1’s GAP activity is inhibited by GATOR2. With GATOR1’s activity suppressed, RagB is activated by its guanylate exchange factor (GEF) Ragulator and can recruit mTORC1 to the lysosomal surface. On the lysosomal surface, mTORC1 is activated by its association with RagB and Rheb and can phosphorylate downstream targets such as S6K and 4E-BP. However, in response to environmental stresses, Sestrin2 is induced and associates with GATOR2 to form a Sestrin2-GATOR2 complex, thereby freeing GATOR1 to become an active GAP that directly binds to RagB and enhances its GTPase activity (Fig. 5G). GTP hydrolysis results in inactivation of RagB (RagB-GDP), releas-
ing mTORC1 from the lysosomal surface and causing mTORC1 inactivation. Sestrin2-dependent AMPK activation provides an additional mechanism for mTORC1 suppression through regulation of TSC2-Rheb and Raptor. Although AMPK is dispensable for Sestrin2-mediated mTORC1 inhibition in MEF cells, the AMPK-dependent mechanism could at least partially contribute to the mTORC1 regulation in certain physiological contexts, such as during DNA damage.

RagB GTPase signaling provides a molecular conduit that allows amino acids to regulate mTORC1. However, other types of regulatory inputs, such as growth factors and environmental stresses also modulate mTORC1 signaling and are thought to act through a TSC2-Rheb and Raptor module. Although AMPK is dispensable for Sestrin2-mediated mTORC1 inhibition in MEF cells, the AMPK-dependent mechanism could at least partially contribute to the mTORC1 regulation in certain physiological contexts, such as during DNA damage.

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expression (Fig. 2B and S4). As Sestrin2 is known to inhibit mTORC1 signaling when robustly expressed upon stresses, it is plausible that the GATOR1/2-dissociating activity of Sestrin2 is only observed when its amount far exceeds the level of GATOR1/2. Importantly, we have shown that, when either stress-induced or ectopically-expressed, Sestrin2 can strongly diminish the binding between endogenous GATOR1 and GATOR2 components (Fig. 2E, 2F).

Although our results indicate that the level of Sestrin2 expression is important for proper GATOR1/2 modulation and mTORC1 inhibition, it is possible that the molecular relationship between Sestrin2 and GATOR2 is affected by factors other than the stoichiometric Sestrin2 levels. The aforementioned recent papers showed that Sestrin2 can mediate mTORC1 inhibition upon amino acid starvation, a stress that does not induce transcriptional Sestrin2 upregulation30–32, and that the association between Sestrin2 and GATOR2 is enhanced upon amino acid starvation30. It is certainly possible that Sestrin2 is subjected to a post-translational regulation that regulates its GATOR-modulating activity. For example, it has been recently shown that Sestrin2 can be phosphorylated by ULK1, a protein kinase that is activated upon starvation33.

Methods
Antibodies and Reagents. Sestrin2 antibodies were formerly described4. Lentiviruses were generated and amplified in the Vector Core facility at the University of Michigan (UM).

Adenoviruses. shRNA adenoviruses were constructed using the BLOCK-iT Adenoviral RNAi Expression System (K5941-90, Invitrogen) according to the manufacturer’s instructions. Target sequences against mouse genes including Wdr24 (GCACCAGATGGATGAGAATCT), NPRL2 (GGGTTCACCTTTAGATGTTCT), Depdc5 (GGGGTGATCCAGCTCTGATTCTT), Nprl2 (GGGGTGATCCAGCTCTGATTCTT), and Nprl2 (GGGGTGATCCAGCTCTGATTCTT) were designed using the BLOCK-iT RNAi Designer (Invitrogen).

Cell culture and Transfection. HEK293 and MEF cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM, Invitrogen) containing 10% fetal bovine serum (FBS) and penicillin/streptomycin at 37°C in 5% CO2.
as previously described. Cells were harvested 24 hrs after transfection for biochemical assays.

TAP-MS Analysis. TAP-MS analysis was performed according to the method that we have formerly described. In brief, MCF10A cells were stably infected with pBARE-Flag-SBP-Sestrin2 retroviruses. Cells were then cultured to 80% confluency and lysed with buffer containing 1 mg/ml CHAPS. Lysates were incubated with Glutathione-Sepharose 4B bead (Amersham). The immunocomplexes conjugated to a protein G/A bead (Calbiochem). GST-tagged proteins were pulled down with glutathione-Sepharose 4B bead (Amersham). The immunocomplexes were then washed four times with the lysis buffer and analyzed through IP by the indicated antibodies.

Immunoprecipitation (IP). Cell and tissue lysates were prepared in a lysis buffer containing 0.3% CHAPS and protease inhibitor cocktail (Roche), and IPed with anti-HA (A2095, Sigma) or anti-Flag (A2220, Sigma) agarose bead or other antibodies containing 0.3% CHAPS and protease inhibitor cocktail (Roche), and IPed with anti-RagB or anti-RagC antibodies (Invitrogen) for 30 min and counterstained with DAPI (Invitrogen). Samples were analyzed using a Fluoview 500 laser confocal microscope (Olympus).

Drosophila and Mice. w1118 ap-GAL4 and UAS-D Seymour strains were described. UAS-Npr2 (LG4481) was generated by Transgenic RNAi project (TRiP) at Harvard Medical School and obtained from the Bloomington stock center. Null mutant strains of Drosophila and Mice were maintained in filter-topped cages and were given free access to autoclaved regular Chow diet and water at the University of Michigan (UM) according to the NIH and institutional guidelines. All animal studies were approved and overseen by the University Committee on Use and Care of Animals at UM.
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Author contributions

J.S.K. and S.H.R. performed mammalian cell culture experiments and biochemical analyses. S.H.R. performed immunostaining experiments. M.Kim performed Drosophila experiments. H.W.P. performed mouse experiments. I.A.S. and H.P. assisted with immunoblotting experiments. U.S.C. provided the purified Sestrin2 proteins. W.W., K.L.G. and M.Karin provided the TAP-MS data. J.S.K., S.H.R., I.A.S., H.P. and J.H.L. prepared the manuscript. J.H.L. developed and directed the research project. All authors discussed the experimental results and reviewed the manuscript.

Additional information

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Corrigendum: Sestrin2 inhibits mTORC1 through modulation of GATOR complexes

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The results presented in Fig. 1B were produced by Wei Wang, Kun-Liang Guan and Michael Karin. The authors neglected to indicate that the same results were also reported by Parmigiani et al. (reference 31), but in that paper the relevant data are not shown.

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