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Sestrin Regulation of TORC1: Is Sestrin a Leucine Sensor?

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Abstract

Sestrins are highly conserved, stress-inducible proteins that inhibit target of rapamycin complex 1 (TORC1) signaling. Following their transcriptional induction, both vertebrate and invertebrate Sestrins turn on the AMP-activated protein kinase (AMPK) which activates the tuberous sclerosis complex (TSC), a key inhibitor of TORC1 activation. However, Sestrin overexpression, on occasion, can result in TORC1 inhibition even in AMPK-deficient cells. This effect has been attributed to Sestrin's ability to bind the TORC1-regulating GATOR2 protein complex, which was postulated to control trafficking of TORC1 to lysosomes. How the binding of Sestrins to GATOR2 is regulated and how it contributes to TORC1 inhibition is unknown. New findings suggest that the amino acid leucine specifically disrupts the association of Sestrin2 with GATOR2, thus explaining how leucine and related amino acids stimulate TORC1 activity. Here we discuss whether and how these findings fit what has already been learned about Sestrin-mediated TORC1 inhibition from genetic studies conducted in fruit flies and mammals.

The Sestrins were discovered in 1999 through a differential display screen for new targets for the transcriptional activity of the tumor suppressor p53 (1). The three mammalian Sestrin isoforms, Sestrin 1–3, are encoded by three separate chromosomal loci, whereas *Drosophila* and *Caenorhabditis elegans* harbor only a single *Sestrin* gene. The abundance of Sestrins is relatively low in resting cells but environmental and metabolic stresses, including DNA damage, oxidative stress, hypoxia, endoplasmic reticulum (ER) stress, energy deprivation and amino acid starvation, induce their expression by activating several transcription factors, including p53, forkhead box O (FoxO), CCAAT-enhancer-binding protein (c/EBP), activating transcription factor 4 (ATF4) and activator protein 1 (AP-1) (Fig. 1A) (1–3). Although Sestrins have been assigned a number of distinct biological functions, their most important and least contentious function is regulation of the nutrient responsive AMPK-TORC1 axis, such that Sestrin accumulation stimulates AMPK and inhibits TORC1 (Fig. 1B) (3). Despite extensive investigation, the mechanism by which vertebrate or invertebrate Sestrins activate AMPK remains obscure, although it has been reported that the Sestrins can

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interact with AMPK or increase the abundance of AMPK subunits (4–6). Nonetheless, genetic experiments in both flies (7) and mice (8, 9) show that metabolic defects caused by Sestrin deficiencies can be corrected by expression of constitutively activated forms of AMPK or treatment with pharmacological AMPK activators. Moreover, AMPK activation accounts for most of the inhibition of TORC1 activity seen upon Sestrin induction, because restoration of AMPK activity results in suppression of dysregulated TORC1 in Sestrin-deficient fly and mouse mutants (7–9). Additional activities attributed to Sestrins include suppression of the accumulation of reactive oxygen species (ROS) (10–12), activation of TORC2 and AKT signaling (13) and inhibition of the cell survival factor X-linked inhibitor of apoptosis protein (XIAP) (14). All mammalian Sestrin isoforms, as well as invertebrate Sestrins, can regulate AMPK, TORC1 and ROS-mediated signaling (3). However, Sestrin overexpression in AMPK-deficient cells can on occasion still attenuate TORC1 activity (15–17). Despite their obscure physiological importance, these findings have led to an extensive search for additional Sestrin-related activities that can explain AMPK-independent inhibition of TORC1 activity.

TORC1 is a nutrient-responsive protein complex containing the protein kinase TOR which is inactivated in response to energy deprivation, in part through activation of AMPK triggered by increased AMP concentrations and insufficient ATP synthesis (18). In addition, specific amino acids, such as glutamine, arginine and leucine, can activate TORC1 through AMPK- and TSC-independent mechanisms. Although this response has been mainly observed in yeast and cultured cells but not in intact animals, a number of different proteins and pathways have been proposed to mediate amino acid-dependent regulation of TORC1 (19–21). The current consensus is that amino acid-generated signals somehow target the RagA/B GTPases that modulate the lysosomal localization and subsequent activation of TORC1. However, additional signaling through the GTPase Rheb (whose activity is inhibited upon AMPK activation) can be a prerequisite for amino acid-induced TORC1 activation (19). Furthermore, several studies have challenged this dogma because glutamine can activate TORC1 in the absence of RagA/B (22), and Rab1A, another GTPase located at the Golgi apparatus, can mediate amino acid sensing independently of RagA/B (23). Although *RagA*-null mouse embryonic fibroblasts (MEFs) do not display lysosomal localization of TORC1, they exhibit persistent TORC1 activity which is not inhibited by glucose or amino acid starvation (24). Depletion of both RagA and RagB in mouse cardiomyocytes also does not diminish TORC1 activity (25), suggesting that RagA/B may be dispensable for physiological TORC1 activation. There is also a conflicting report questioning whether amino acids can alter the GTP/GDP-loading status of RagA/B GTPases (26). Moreover, the physiological function of RagA/B is not confined to amino acid sensing and TORC1 regulation, because genetic studies in mice indicate that RagA/B have a critical role in maintaining lysosomal homeostasis and cardiac functionality independently of amino acids and TORC1 (25). Genetic studies in zebrafish have also concluded that RagA/B have TORC1-independent functions related to lysosomal proteolysis (27). In summary, the involvement of RagA/B in TORC1 regulation is rather contentious, remaining to be demonstrated in intact multicellular organisms.

Studies in isolated cells suggest that several molecules may play a role in direct sensing of amino acids and transmitting the signal to TORC1 (Fig. 1B). Three independent groups

reported that the amino acid transporter SLC38A9 functions as an amino acid sensor at the lysosomal membrane (28–30). Although SLC38A9-deficient cells cannot sense arginine, they can still respond to other amino acids, including leucine, implicating the existence of a distinct leucine sensor. One such sensor is the leucyl-tRNA synthetase which functions as a GTPase-activating protein (GAP) for RagD, an antagonist of RagA/B activity (31). In this attractive model, leucine binding to leucyl-tRNA synthetase increases its GAP activity toward RagD, ultimately resulting in increased RagA/B activity. Although this model has been challenged because the RagD-GAP activity of leucyl-tRNA synthetase is relatively weak and another protein (folliculin) may be the preferred GAP for RagD (32), leucyl-tRNA synthetase remains a valid potential participant in leucine sensing because Cdc60, its yeast homolog, interacts with Rag GTPases in a leucine-dependent manner, and is genetically responsible for leucine-dependent activation of yeast TORC1 (33). As in every field, protein function needs to be validated biochemically, physiologically, and genetically.

GAP activity towards Rags (GATOR) is another evolutionarily conserved protein complex that controls TORC1 signaling (34). In mammals, GATOR is composed of two subcomplexes, GATOR1 and GATOR2. GATOR2 is composed of five proteins, WDR59, WDR24, MIOS, SEH1L and SEC13, whereas GATOR1 consists of three subunits, NPRL2, NPRL3 and DEPDC5 (35). GATOR1 functions as a GAP for the RagA/B GTPases, which activate TORC1, whereas GATOR2 is an inhibitor of GATOR1. Affinity purification experiments have shown that Sestrin1, 2, and 3 bind to the GATOR2 subcomplex (16, 17, 36), mainly through its WDR24 and SEH1L subunits (16). Although Sestrin2 does not affect the interaction between GATOR1 and GATOR2 when expressed at moderate amounts (16, 36), Sestrin2 overexpression can induce partial dissociation of GATOR2 from GATOR1, resulting in enhancement of GATOR1 GAP activity (17). The TORC1 inhibitory effect of Sestrin expression is diminished in GATOR1-mutated or silenced cells, suggesting that GATOR1 may have a role in Sestrin-dependent TORC1 inhibition (16, 17, 36). However, the biochemical mechanisms through which GATOR1 functions as a GAP for RagA/B, how GATOR2 inhibits GATOR1, or how Sestrin binding to GATOR2 affects its GATOR1-binding activity and TORC1 regulation is far from clear. Furthermore, the physiological importance of GATOR1/2 regulation by Sestrin2 remains to be determined through rigorous genetic analysis in intact animals. Despite these uncertainties and limitations, two studies by the Sabatini group suggest that Sestrin2 is a leucine sensor (37, 38).

One study shows that the Sestrin2-GATOR2 complex purified from amino acid-starved cells is destabilized upon in vitro incubation with leucine (38). Although Sestrin2 and GATOR2 were almost completely dissociated after addition of a subphysiological concentration (20 μM) of leucine, the GATOR2 subcomplex itself remained intact. Isoleucine and methionine, but not arginine, also disrupted the interaction of Sestrin2 with GATOR2. Considering that leucine, isoleucine and methionine are amphipathic molecules, their effect on Sestrin2-GATOR2 interaction may not be specific. However, the authors of that study showed that Sestrin1 and Sestrin2 themselves bind leucine, whereas no such binding was exhibited by GATOR2 or RagA/C (38). Leucine binding to Sestrin2 also alters its melting temperature, implicating a leucine-induced conformational change. Furthermore, the binding affinity (*Kd*) of Sestrin2 to leucine was determined to be around 20 μM, which is lower than that of leucyl-tRNA synthetase, whose Michaelis constant (K_M) for leucine activation is around 45

μM (39). Based on these results, Sestrin2 was proposed to be a leucine sensor that binds GATOR2 only in the absence of leucine, an interaction postulated to inhibit TORC1 activity. To support this proposal, the authors generated Sestrin1, 2 and 3 triple-knockout (Sestrin-null) HEK293 cells using CRISPR-Cas9 methodology. Consistent with previous results (15), Sestrin-null cells did not efficiently shut down TORC1 activity after amino acid starvation. These Sestrin-null cells were reconstituted with various Sestrin2 mutants. The S190W substitution abolished the TORC1-inhibitory function of Sestrin2, whereas two other substitution mutations, L261A and E451A, prevented leucine binding to Sestrin2 and rendered the Sestrin2(L261A/E451A):GATOR2 complex insensitive to leucine. Furthermore, Sestrin2 (L261A/E451A) variants were unable to restore leucine-mediated TORC1 activation in Sestrin-null HEK293 cells (38).

These findings stand in contrast to several other reports, including one from the Sabatini lab itself (16, 17, 36), according to which the Sestrin2:GATOR2 complex is stable and can be isolated from cells cultured in conventional growth media, which contain 300–800 μM of leucine, a concentration that is 15–40 fold higher than its proposed dissociation constant from Sestrin2 (16, 17, 36). The interaction in leucine-rich environments can be reproducibly detected between endogenous Sestrin2 and GATOR2 in various cell types (16, 17, 36). Assuming that Sestrin2 does bind leucine, a more physiologically relevant scenario is that leucine weakens the binding of Sestrin2 to GATOR2, but does not disrupt it completely. Supporting this idea, two earlier papers showed that, although Sestrin2 binds quite well to GATOR2, the interaction becomes stronger upon amino acid starvation (16, 36). Because Wolfson *et al.* used a partially purified Sestrin2:GATOR2 immunocomplex (38), which may contain proteins other than the five GATOR2 components, it is plausible that other proteins or particular posttranslational modifications may further reduce the binding of Sestrin2 to GATOR2 in the presence of leucine.

In the absence of strong in vivo genetic data, additional support for the role of leucine in control of Sestrin2 function came from structural studies showing that Sestrin2 contains a leucine binding pocket and that leucine is required for the proper folding of Sestrin2 (37). Using structural information, the authors identified two other residues, Arg^{390} and Trp^{444}, as being involved in leucine binding. Curiously, however, one of the previously mutated residues, Leu^{261}, does not seem to be part of the leucine binding pocket. Furthermore, two of us (JHL and USC) found that Sestrin2 crystallizes without leucine addition (12) and that the structure thus formed is largely identical to the one generated in the presence of leucine (Fig. 2). These results suggest that leucine binding does not induce a substantial conformational change in Sestrin2. Although we cannot rule out the possibility that the recombinant Sestrin2 protein isolated from *E. coli* and used in our structural studies was already saturated with leucine or a similar ligand, this seems a rather unlikely possibility because the affinity of leucine to Sestrin2 is relatively low (Kd = 20 μM), such that bound leucine should have readily dissociated from Sestrin2 during protein purification and dialysis. Since both of these structures were determined in the crystalline state (12, 37), which may not fully replicate the physiological dilute solution state, we also cannot rule out the possibility that leucine binding may produce effects in local structural dynamics of the leucine-binding pocket in solution. Undoubtedly, additional experiments are needed to rule out or validate an effect of leucine on Sestrin2 structure and function. Considering that

leucine binding alters the melting temperature of Sestrin2 (38), it is plausible that leucine binding could affect Sestrin2 stability. In such a case, it seems likely that leucine should stabilize the protein and increase its abundance, thereby potentiating its effect on AMPK-dependent TORC1 inhibition, unless the leucine-bound form cannot activate AMPK. The effect of leucine binding on Sestrin2-mediated AMPK activation needs to be tested. Although Wolfson *et al.* found that leucine affected the binding of Sestrin2 to GATOR2, they noted that the binding of leucine to Sestrin3 was too weak to have any effect on its interaction with GATOR2 (38). Likewise, the binding of leucine to *Drosophila* Sestrin was also weak (38). Given the high degree of sequence conservation amongst the different Sestrins, these are rather surprising findings. It is possible that Sestrin3 and other Sestrins may have another ligand that is similar to leucine, such as other hydrophobic amino acids or metabolites. Furthermore, although yeast TOR is leucine-responsive, no Sestrin family members are encoded by the yeast genome (3), suggesting an alternative mode of leucine sensing. As described above, leucyl-tRNA synthetase could be that alternative leucine sensor because its role is conserved from mammals to yeast (31, 33). Therefore, if Sestrin2 is indeed a direct leucine sensor, its role seems to be largely mammalian specific and/or restricted to certain experimental conditions.

Several additional findings pose further obstacles on the road for Sestrin2 to win the title of a leucine sensor. First, genetic analysis showed that the single Sestrin protein encoded by the *Drosophila* genome is as capable of regulating AMPK and TORC1 activities in vivo and in vitro, as the mammalian Sestrins (7). Second, all of the original cell culture studies conducted by at least two different laboratories on Sestrin1 and 2 (4) or Sestrin3 (40), which demonstrated their ability to activate AMPK and inhibit TORC1, were done under normal culture conditions, namely in the presence of leucine and other amino acids. Therefore, there is little evidence that leucine affects Sestrin function in cells other than amino acid-starved triple Sestrin-null HEK293 cells and mouse fibroblasts. Furthermore the genetic analysis of Sestrin function in *Drosophila* or mice revealed that Sestrins are fully capable of inhibiting TORC1 activity in vivo under amino acid-replete conditions (7–9, 13). Although it needs to be determined whether direct binding of Sestrin to leucine has a physiological role in vivo, all Sestrins can activate AMPK and inhibit TORC1 in intact animals in the presence of physiological amounts of leucine. Even if one postulates that AMPK activation does not contribute to the TORC1 inhibitory activity of Sestrins, it should be acknowledged that Sestrin-mediated TORC1 inhibition can take place in the presence of amino acids.

Another important aspect of Sestrin biology is the requirement of stress to induce *Sestrin* expression in both cells (4, 40) and tissues (4, 8, 9). Leucine starvation results in the transcriptional induction of Sestrin2 due to activation of the protein kinase GCN2 and its target transcription factor ATF4 (41). GCN2 is a physiological sensor for most amino acids, including leucine and glutamine, whose function is conserved from yeast to mammals (42, 43). Therefore, glutamine starvation, which does not affect physiological leucine concentrations, inhibits TORC1 through GCN2-mediated Sestrin2 induction (41), as seen in other studies in which Sestrin abundance has been increased by exposure to genotoxic or oxidative stress (1–3). Thus, it remains to be clarified whether direct leucine binding indeed impairs the mTORC1-inhibiting function of Sestrin2 under these stresses.

Finally, Wolfson *et al.* suggested that Sestrin2 inhibition could be used to increase muscle protein synthesis by activating mTORC1 (38). This proposal is contradicted by numerous genetic studies performed using various model organisms, including worms, flies and mice. Such studies show that loss of Sestrin in muscle results in or facilitates various pathologies, including autophagy defects, accumulation of damaged mitochondria, oxidative muscle damage and myofiber degeneration (6, 7, 44). Furthermore, *Sestrin* knockout mice are predisposed to various systemic metabolic defects, including fat accumulation and insulin resistance (8, 9, 13). Therefore, the use of systemic Sestrin2 inhibitors proposed by these authors would be expected to accelerate age-associated muscle degeneration and other metabolic derangements, rather than alleviate them. The assessment of the therapeutic value of Sestrin inhibitors requires proper genetic analysis of Sestrin-deficient animal models, all of which are readily available. So far, the genetic studies mentioned above indicate that Sestrin induction is needed for stress reduction, a function that does not seem to be opposed by physiological leucine concentrations.

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Gloss

Environmental and metabolic stresses, such as DNA damage and nutrient deprivation, induce the expression of evolutionarily conserved proteins called Sestrins. In both flies and mammals, Sestrin inhibits the nutrient-responsive protein kinase complex Target of Rapamycin Complex 1 (TORC1), which regulates the biosynthesis of macromolecules including proteins, lipids, and nucleic acids. The mechanisms through which Sestrins inhibit TORC1 activity are both indirect, depending on activation of the adenosine monophosphate (AMP)-activated protein kinase (AMPK), and direct, mediated through interaction with the TORC1 regulating protein complex GATOR. New findings suggest that the ability of Sestrins to interact with GATOR is regulated by the amino acid leucine. Here we discuss whether and how this finding fits what has already been learned about the physiological functions of Sestrin in mammals and insects.

Fig. 1A

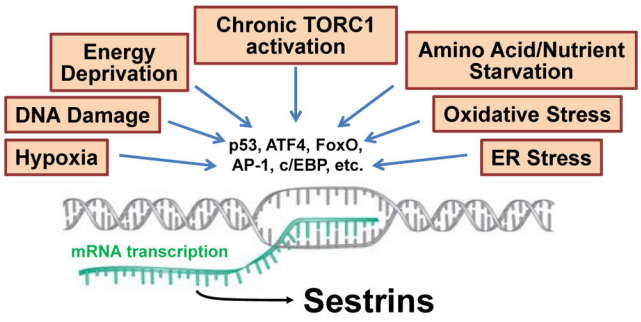

Fig. 1B

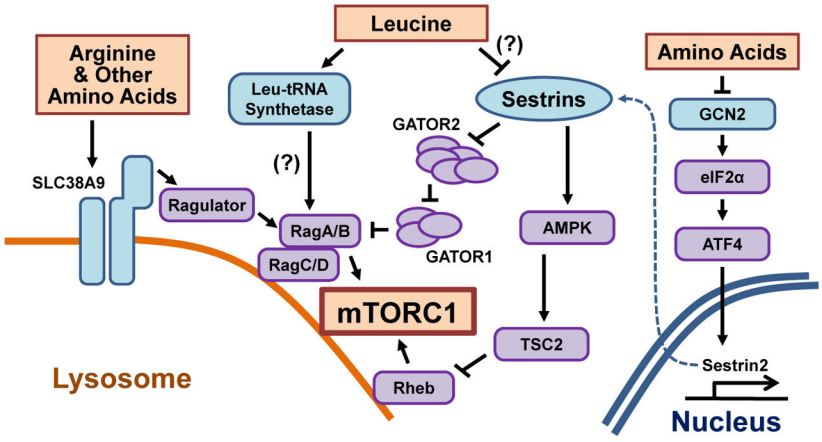

Fig 1. The impact of Sestrins on amino acid-dependent TORC1 signaling

(A) Sestrins are transcriptionally induced in response to diverse types of environmental stress. (B) Once induced, Sestrins inhibit TORC1 signaling through activation of AMPK and modulation of the GATOR complexes. Two papers (37, 38) suggest that amino acid leucine disrupts the Sestrin2-GATOR2 interaction, thereby modulating the ability of Sestrin2 to inhibit TORC1 signaling. Additional molecules, such as leucyl-tRNA synthetase, GCN2 and SLC38A9, also play a role in mediating amino acid-dependent TORC1 regulation.

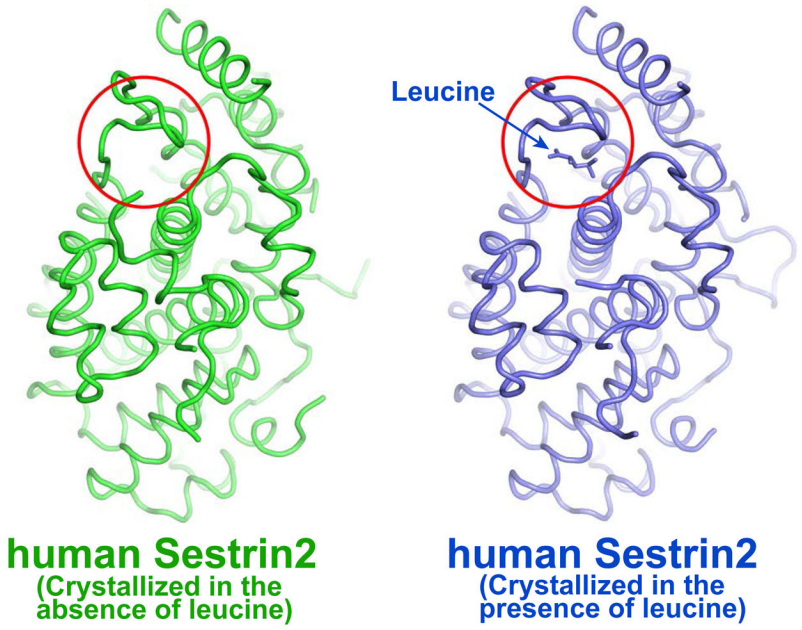

Fig 2. Structure comparison of human Sestrin2 in the absence (green, PDB ID: 5CUF) or presence (blue, PDB ID: 5DJ4) of leucine

The leucine binding pocket is marked by the red circle. The calculated R.M.S. difference between the two structures is 0.70 Å based on the FATCAT server (http://fatcat.burnham.org/fatcat/). The illustration of protein structures was generated with PYMOL (Delano Scientific, LLC).