Maintenance of Metabolic Homeostasis by Sestrin2 and Sestrin3

Jun Hee Lee,1,6,12,* Andrei V. Budanov,1,8,12 Saswata Talukdar,2 Eek Joong Park,1 Hae Li Park,6 Hwan-Woo Park,6 Gautam Bandyopadhyay,2 Ning Li,1 Mariam Aghajan,1 Insook Jang,6 Amber M. Wolfe,7 Guy A. Perkins,3 Mark H. Ellisman,3 Ethan Bier,4 Miriam Scadeng,5 Marc Foretz,9,10,11 Benoit Viollet,9,10,11 Jerrold Olefsky,2 and Michael Karin1,*

1Laboratory of Gene Regulation and Signal Transduction, Departments of Pharmacology and Pathology
2Department of Medicine, Division of Endocrinology and Metabolism
3National Center for Microscopy and Imaging Research and Department of Neurosciences
4Section of Cell and Developmental Biology
5Department of Radiology
University of California, San Diego, San Diego, CA 92093, USA
6Department of Molecular and Integrative Physiology
7Unit for Laboratory Animal Medicine
University of Michigan, Ann Arbor, MI 48109, USA
8Department of Neurosurgery, Virginia Commonwealth University, Richmond, VA 23298, USA
9INSERM U1016, Institut Cochin, Paris 75014, France
10CNRS, UMR 8104, Paris 75014, France
11Université Paris Descartes, Sorbonne Paris Cité, Paris 75014, France
12These authors contributed equally to this work
*Correspondence: leeju@umich.edu (J.H.L.), karinoffice@ucsd.edu (M.K.)
http://dx.doi.org/10.1016/j.cmet.2012.08.004

SUMMARY

Chronic activation of mammalian target of rapamycin complex 1 (mTORC1) and p70 S6 kinase (S6K) in response to hypernutrition contributes to obesity-associated metabolic pathologies, including hepatosteatosis and insulin resistance. Sestrins are stress-inducible proteins that activate AMP-activated protein kinase (AMPK) and suppress mTORC1-S6K activity, but their role in mammalian physiology and metabolism has not been investigated. We show that Sestrin2—encoded by the Sesn2 locus, whose expression is induced upon hypernutrition—maintains metabolic homeostasis in liver of obese mice. Sesn2 ablation exacerbates obesity-induced mTORC1-S6K activity, glucose intolerance, insulin resistance, and hepatosteatosis, all of which are reversed by AMPK activation. Furthermore, concomitant ablation of Sesn2 and Sesn3 provokes hepatic mTORC1-S6K activation and insulin resistance even in the absence of nutritional overload and obesity. These results demonstrate an important homeostatic function for the stress-inducible Sestrin protein family in the control of mammalian lipid and glucose metabolism.

INTRODUCTION

Overnutrition and sedentary lifestyle are major contributors to the obesity epidemic, which affects one-third of United States adults and up to 1 billion people worldwide (Haslam and James, 2005). As a consequence, obesity-associated diseases, such as diabetes, cardiovascular disease, and nonalcoholic fatty liver disease, have become a major public health problem. Obesity also increases cancer risk, with the most pronounced effect exerted on hepatocellular carcinoma, the prevalent type of liver cancer (Calle et al., 2003). In obese individuals, excessive nutrients are stored as fat in liver and adipose tissues. Accumulation of fat droplets in the liver, a condition known as hepatosteatosis, can eventually lead to chronic liver inflammation (steatohepatitis), liver damage, diabetes, and liver cancer (Angulo, 2002).

mTORC1 is an evolutionarily conserved protein kinase that senses nutrient availability and controls cellular metabolism. Nutritional abundance can lead to chronic mTORC1 activation, thereby enhancing protein and lipid biosynthesis and inhibiting autophagic catabolism (Um et al., 2006; Zoncu et al., 2011). Through its substrate, S6K, mTORC1 causes inhibitory serine phosphorylation of insulin receptor substrates (IRSs), thereby contributing to insulin resistance and subsequent attenuation of insulin-induced AKT activity (Bae et al., 2012; Um et al., 2006). Chronic inhibition of autophagy attenuates clearance of liver lipid droplets (LDs), thereby contributing to hepatosteatosis (Singh et al., 2009). Thus, chronic mTORC1 activation upon persistent hypernutrition and lack of exercise can give rise to insulin resistance and lipid accumulation and eventually drive the pathogenesis of type II diabetes and steatohepatitis (Zoncu et al., 2011). Downregulation of autophagy can also lead to accumulation of damaged mitochondria and reactive oxygen species (ROS), giving rise to tissue degeneration and increased cancer risk (Mizushima and Komatsu, 2011).

To avoid the pathogenic consequences of chronic mTORC1 activation, several negative feedback loops have evolved. Using Drosophila as a model organism, we found that chronic dTORC1 activation results in transcriptional activation of the dSesn locus that encodes for Drosophila Sestrin (dSestrin) (Lee et al., 2010).
dSestrin belongs to a highly conserved family of stress-inducible proteins, which in mammals includes Sestrin1, Sestrin2, and Sestrin3 (Budanov et al., 2010). Once induced, the Sestrins potentiate AMPK activation and thereby enhance phosphorylation of tuberous sclerosis complex 2 (TSC2), which in turn attenuates mTORC1 activation and stimulates autophagy (Budanov and Karin, 2008; Mauri et al., 2009). The AMPK-activating and mTORC1-suppressing activities of the Sestrins are conserved between insects and mammals (Lee et al., 2010), but the physiological functions of the mammalian Sestrins remain to be identified. Loss of dSestrin results in diverse age- and obesity-associated pathologies, such as fat accumulation and cardiac dysfunction, all of which are precipitated by activation of dTORC1 (Lee et al., 2010). Given the biochemical similarities between Drosophila and mammalian Sestrins, we postulated that mammalian Sestrins may also have important homeostatic functions during conditions that cause excessive mTORC1 activation. Here we describe such a function for Sestrin2 and Sestrin3 in control of liver insulin resistance and lipid accumulation before and during obesity.

**RESULTS**

**Sestrin2 Deficiency Enhances Obesity-Induced Insulin Resistance and Diabetic Progression**  
Sesn2−/− mice are fully viable and do not display any gross developmental abnormalities (Budanov and Karin, 2008). Body weight as well as glucose homeostasis and insulin responsiveness evaluated by glucose tolerance (GTT) and insulin tolerance (ITT) tests, respectively, were not significantly different between control (Con; either Sesn2+/+ or Sesn2−/−) and Sesn2−/− mice when maintained on normal chow (low-fat diet [LFD]) (Figures S1A–S1C available online).

Expression of Sesn2 mRNA in liver was elevated in mice kept on high-fat diet (HFD), in which 60% of the calories were fat derived, whereas Sesn1 and Sesn3 messenger RNAs (mRNAs) were not affected (Figure S1D). As a result, Sestrin2 protein accumulated in liver of obese mice kept on HFD (Figures S1E–S1G). Loss of Sestrin2 had marginal effects on liver Sesn1 and Sesn3 expression in obese mice (Figures S1G–S1I). Obesity-induced accumulation of Sestrin2 was also observed in skeletal muscle, but not in adipose tissue (Figure S1G). Since Sestrin2 is an obesity-inducible protein, we examined whether Sestrin2 deficiency affected weight gain and glucose homeostasis in mice kept on HFD. As observed in LFD animals, Sesn2−/− mice on HFD did not differ in weight gain (Figure S1J) or food consumption (Figure S1K) from Con mice. Nonetheless, the Sestrin2 deficiency had a pronounced effect on glucose homeostasis and insulin responsiveness in mice kept on HFD. GTT and ITT indicated a significantly higher degree of glucose intolerance (Figures 1A and 1B) and insulin resistance (Figures 1C and 1D) in Sesn2−/− mice relative to their Con counterparts after 3 months of HFD. However, there was no significant defect in insulin secretion during GTT (Figure S1L).

To examine the effect of Sestrin2 ablation in a genetic model of obesity, we intercrossed Sesn2−/− and Lepob/ob mice. As seen with diet-induced obesity, Lepob/ob/Sesn2−/− mice were more glucose intolerant (Figures 1E and 1F) and insulin resistant (Figures 1G and 1H) than Lepob/ob/Sesn2+/− mice. However, the Sestrin2 deficiency had no effect on body weight (Figure S1M) or food consumption (Figure S1N) of Lepob/ob mice. Magnetic resonance imaging (MRI) of Lepob/ob/Sesn2+/− and Lepob/ob/Sesn2−/− mice reaffirmed their similar body mass and visceral and subcutaneous fat pad volumes (Figures S1O and S1P). Nonetheless, at 4 months of age, Lepob/ob/Sesn2−/− mice became severely diabetic, exhibiting much higher fasting blood glucose than Lepob/ob/Sesn2+/− mice (Figure 1I), and started excreting large amounts of glucose in their urine (Figure 1J).

**Sesn2−/− Mice Display Defective Insulin Responsiveness in Liver and Adipose Tissue**

To better understand the basis for defective glucose homeostasis and insulin responsiveness in obese Sesn2−/− mice, we performed hyperinsulinemic-euglycemic clamp studies (Figures S2A–S2C). Upon insulin infusion, Sesn2−/− mice kept on HFD exhibited a significantly lower glucose infusion rate (GIR), confirming the presence of whole-body insulin resistance (Figure 2A). However, Sesn2−/− mice did not differ from Con mice with respect to insulin-stimulated glucose disposal rate (IS-GDR), indicative of muscle insulin responsiveness (Figure 2B). However, suppression of hepatic glucose production (HGP) during the hyperinsulinemic-euglycemic clamp was substantially reduced in Sesn2−/− mice (Figure 2C). Consequently, more than 80% of the effect of Sestrin2 loss on GIR reduction was attributable to the decreased HGP suppression, while the contribution of insulin-induced glucose uptake changes in peripheral tissues was minimal and not statistically significant. These indications suggest that defective glucose homeostasis in Sesn2−/− mice is largely due to decreased hepatic insulin sensitivity.

We also examined insulin responsiveness in adipose tissue. Suppression of serum free fatty acids (FFAs) during the hyperinsulinemic-euglycemic clamp studies provides an indirect assessment of adipocyte insulin sensitivity. Although FFA suppression was modestly reduced in Sesn2−/− mice, the difference did not reach significance (Figures S2D and S2E). To more directly measure adipose tissue insulin responsiveness, we measured insulin-induced glucose uptake by primary adipocytes isolated from Con and Sesn2−/− mice kept on HFD. Whereas glucose uptake in Con adipocytes was stimulated 2.5-fold by insulin, Sesn2−/− adipocytes exhibited markedly reduced insulin responsiveness (Figures S2F and S2G), suggesting that Sestrin2 controls insulin sensitivity also in adipose tissue.

**Compromised Insulin-Stimulated PI3K-AKT Signaling in Liver of Sesn2−/− Mice**

AKT is an important effector of insulin signaling in liver and adipose tissue, mainly controlling glucose and lipid metabolism (Manning and Cantley, 2007). To examine whether Sestrins, which control AMPK and mTORC1 activity (Budanov and Karin, 2008; Lee et al., 2010), also modulate insulin-induced AKT activation, we examined AKT phosphorylation in livers of Con and Sesn2−/− mice kept on HFD before and after insulin administration. In Con liver, insulin treatment induced AKT phosphorylation at Thr308 and Ser473, the two sites responsible for AKT activation. However in Sesn2−/− livers, insulin-induced AKT phosphorylation at both sites was substantially reduced.
AKT activation results in inhibitory phosphorylation of forkhead box O (FoxO) family transcription factors, including FoxO1, leading to reduced expression of genes that encode gluconeogenic enzymes such as phosphoenolpyruvate carboxykinase (PEPCK). In Sesn2−/− mice, AKT-dependent FoxO1 phosphorylation was decreased (Figure 2D), and Pepck mRNA amounts were elevated relative to Con mice (Figure S2H). These results indicate that Sesn2 is required for maintenance of insulin-induced AKT activation and its sequelae during obesity.

Consistent with the hyperinsulinemic-euglycemic clamp studies showing that muscle insulin responsiveness is not affected by loss of Sesn2 (Figure 2B), insulin-induced AKT phosphorylation was largely similar between Con and Sesn2−/− muscle tissue (Figures S2I and S2J). In Sesn2−/− adipose tissue, insulin-induced AKT phosphorylation was slightly reduced relative to Con (Figures S2K and S2L).

To find out the mechanism through which insulin-induced AKT signaling is downregulated in Sesn2−/− mouse liver, we analyzed activities of signaling components that act upstream of AKT. We found that insulin-stimulated phosphoinositide 3-kinase (PI3K) activity in liver of Sesn2−/− mice was lower than that of Con liver (Figures 2F and 2G). Insulin-induced tyrosine phosphorylation of IRS-1 was also reduced in Sesn2−/− liver compared to Con liver (Figure 2H), suggesting that insulin resistance in Sesn2−/− liver occurs upstream or at the level of IRS-1. Interestingly, Ser632 phosphorylation of IRS-1 (corresponding to Ser636 in human IRS-1), which opposes IRS-1 tyrosine phosphorylation (Boura-Halfon and Zick, 2009), was significantly higher in Sesn2−/− liver both before and especially after insulin treatment (Figures 2H and 2I).

Sestrin2 Attenuates Chronic mTORC1 Activation during Obesity

As Ser632 phosphorylation of IRS-1 is mediated through S6K (Um et al., 2004), an mTORC1 target, we postulated that Sestrin2 regulates insulin signaling sensitivity through the AMPK-mTORC1-S6K axis. Therefore, we examined whether
mTORC1-S6K signaling is affected by Sestrin2 loss. As previously reported (Um et al., 2004), mTORC1-dependent activatory S6K phosphorylation at both Thr389 and Ser411 (Ali and Sabatini, 2005) was modestly elevated in livers of Con mice kept on HFD compared to LFD-kept mice (Figures 3A and 3B). However, in liver of Sesn2−/− mice kept on HFD, the increase in S6K phosphorylation compared to LFD-fed Sesn2−/− and even HFD-kept Con liver was much larger (Figures 3A and 3B), suggesting that Sestrin2 attenuates chronic liver mTORC1-S6K signaling upon nutritional overload. The chronic upregulation of S6K was found in both basal and insulin-stimulated liver tissues of Sesn2−/− mice kept on HFD (Figure 3C). However, there were no significant changes in S6K phosphorylation between livers of Con and Sesn2−/− mice kept on LFD (Figures 3A and 3B), and this is consistent with the low expression of Sestrin2 in lean mice (Figures S1D–S1G) and the absence of metabolic abnormalities in Sesn2−/− mice kept on LFD (Figures S1A–S1C).

We previously showed that Sestrins negatively regulate the mTORC1-S6K axis by potentiating AMPK activation (Budanov and Karin, 2008; Lee et al., 2010). Livers of Sesn2−/− mice kept on HFD exhibited lower activatory AMPK phosphorylation (Figures 3C and 3D) and decreased AMPK catalytic activity (Figures 3E and S3A). Livers of Sesn2−/−/Lepob/ob mice also exhibited lower AMPK activity than Sesn2+/−/Lepob/ob livers (Figures 3F and S3B). The decrease in AMPK activity was particularly striking because total AMPK amounts increased upon Sestrin2 loss in both HFD- and Lepob-induced obese mice (Figures 3C, S3A, and S3B), suggesting that low AMPK and high mTORC1 activity in obese Sestrin2-deficient livers led to a compensatory increase in AMPK protein expression. AMPK-dependent phosphorylation of acetyl-CoA carboxylase (ACC) at Ser79 was modestly reduced in Sestrin2-deficient obese livers (Figure S3C). Conversely, autophosphorylation of mTOR at Ser2481, an indicator of mTORC1 catalytic activity (Soliman et al., 2010), was higher in Sesn2−/−/Lepob/ob livers than in Sesn2−/−/Lepob/ob livers (Figure S3D). Correspondingly, S6K catalytic activity, which is directly stimulated by mTORC1, was elevated in Sesn2−/−/Lepob/ob livers relative to Sesn2+/−/Lepob/ob livers (Figures 3G and S3E). S6K-mediated inhibitory
phosphorylation of IRS-1 at Ser632 and Ser635 (Um et al., 2004) was also substantially elevated in Sesn2+/− obese livers (Figures S3F–S3I). Thus, Sestrin2 is a positive regulator of AMPK activation in the obese liver that is required for keeping mTORC1 activity suppressed.

Sestrin2 loss modestly decreased liver AMPK activity in lean mice (Figure S3J). However, mTORC1 activity, monitored by its target S6K, was similar between Con and Sesn2+/− mice (Figure S3K), and insulin-induced AKT activation in liver (Figure S3L) and in adipose tissue (Figure S3M) was also comparable between lean Con and Sesn2+/− mice. These results suggest that, in the absence of nutritional overload or obesity, conditions that increase Sestrin2 expression, lower AMPK activity caused by loss of basal Sestrin2 expression is insufficient to cause mTORC1 hyperactivation and insulin resistance.

**AMPK Activation Restored Insulin Sensitivity in Sesn2+/− Mice**

If Sestrin2’s protective effect against obesity-induced insulin resistance is exerted through modulation of AMPK and mTORC1 activities, restoration of AMPK activity or suppression of chronic mTORC1 should restore insulin signaling to obese Sesn2+/− mice. Therefore, we attempted to suppress chronic mTORC1 activity in Sesn2+/− mice using rapamycin. Although rapamycin administration suppressed mTORC1-S6K signaling in Sesn2+/− liver, it failed to restore blood glucose homeostasis in obese Sesn2+/− mice (data not shown). However, it was recently shown that long-term rapamycin administration to mice can suppress mTORC2, which is critical for AKT activation and insulin responsiveness (Lamming et al., 2012). Therefore, we used AICAR (5′-AMP), which can effectively suppress hepatic mTORC1 activity through AMPK activation without affecting mTORC2 (Reiter et al., 2005). Although AICAR administration caused only a slight reduction in basal hyperglycemia and glucose intolerance (Figures S4A and S4B), it significantly restored insulin responsiveness of HFD-kept Sesn2+/− mice (Figure 4A), suggesting that AICAR-mediated AMPK reactivation can correct the insulin resistance phenotype caused by Sestrin2 loss. Indeed in the liver, AICAR also restored AMPK activity and suppressed mTORC1-S6K activity (Figure 4B), and restored insulin-induced AKT activation (Figures 4C and S4C). Furthermore, liver-specific transduction of adenoviruses expressing constitutively active AMPK (Ad-AMPK(C)) (Figure S4D) was sufficient to suppress insulin resistance in HFD-kept Sesn2+/− mice (Figures 4D and 4E). These data strongly suggest that Sestrin2 maintains hepatic insulin sensitivity by promoting AMPK activation in liver.

**Sestrin2 Loss Aggravates Hepatosteatosis Caused by Dietary or Genetic Obesity**

Decreased AMPK and increased mTORC1 activities are associated with fat accumulation (Kahn et al., 2005; Laplante and Sabatini, 2009), and Sestrin is important for prevention of fat droplet accumulation in the Drosophila fat body (Lee et al., 2010), an equivalent of the mammalian liver (Søndergaard, 1993). Indeed, Sestrin2 deficiency aggravated hepatosteatosis caused by dietary or genetic obesity. In lean mice, liver triglycerides were only slightly elevated in Sesn2+/− livers compared to Con livers (Figure 5A). However, when mice were kept on HFD, liver triglycerides were substantially higher in Sesn2+/− mice than in Con livers (Figure 5A). Sestrin2+/− mice also exhibited increased LD accumulation revealed by oil red O staining (Figure 5B). Transmission electron microscopy (EM) showed that the size of LDs was increased upon loss of Sestrin2 under HFD.
conditions (Figures 5C and 5D). Livers of Sesn2−/−/Lepob−/− mice also contained higher amounts of triglycerides than Sesn2+/−/Lepob−/− livers (Figure 5A).

Diminished autophagy, as a result from Sestrin2 loss, AMPK inhibition, and mTORC1 activation (Chan, 2009; Egan et al., 2011; Maiuri et al., 2009), can contribute to accumulation of LDs in liver (Singh et al., 2009). EM analysis revealed that in livers of Con mice on HFD, many LDs were associated with vesicles surrounded by double membranes (Figures 5C and S5A). Such structures were barely detectable on the much larger LDs in Sesn2−/− hepatocytes of mice on HFD (Figures 5C and S5A). Based on previous reports, these structures most likely represent autophagic vesicles (AVs) engaged in LD digestion (Singh et al., 2009). Consistent with this, immunofluorescence imaging of LDs and the AV marker LC3 showed a considerable colocalization of both structures in Con liver but not in Sesn2−/− liver from mice kept on HFD (Figure S5D).

Although the association of LD with AV was dramatically decreased in livers of Sesn2−/− HFD-kept mice, the difference between LFD-kept Con and Sesn2−/−/Lepob−/− mice was much less prominent (Figure S5C).

Lower AMPK activity in liver could also result in decreased mitochondrial biogenesis and reduced β-oxidation of fatty acids, which contributes to lipid accumulation (Kahn et al., 2005), and reduced mitochondrial content was confirmed by quantitative PCR of mitochondrial DNA (Figures S5E and S5F). In addition, Sestrin2 deficiency resulted in reduced expression of two mitochondrial biogenesis marker genes, Tfam and Nrf1 (Figures 5F and S5G). Consistent with these results, β-oxidation of palmitic acid by liver homogenates was also reduced upon Sestrin2 deficiency in Lepob−/− mice (Figure 5G), and hepatic AMPK restoration through AICAR or Ad-AMPK CA suppressed liver triglyceride accumulation (Figures S4E and S4F).

Chronic AMPK downregulation and mTORC1 activation can promote processing and nuclear translocation of the lipogenic transcription factor SREBP (Li et al., 2011; Porstmann et al., 2008). Correspondingly, Sesn2−/−/Lepob−/− livers contained more processed SREBP than Sesn2+/−/Lepob−/−/Lepob−/− livers (Figure 5A). Consistent with these results, β-oxidation of palmitic acid by liver homogenates was also reduced upon Sestrin2 deficiency in Lepob−/− mice (Figure 5G), and hepatic AMPK restoration through AICAR or Ad-AMPK CA suppressed liver triglyceride accumulation (Figures S4E and S4F).

Data are presented as means ± standard error, p values were calculated with a Student’s t test. See also Figure S4.
Sestrin2 and Liver Metabolic Homeostasis

Concomitant Loss of Sestrin2 and Sestrin3 Causes Spontaneous Insulin Resistance

Unlike dSesn-null flies that show spontaneous metabolic abnormalities (Lee et al., 2010), lean Sesn2−/− mice do not develop spontaneous hepatosteatosis or glucose homeostasis defects. This may be due to the existence of multiple mammalian Sesn homologs that compensate for Sestrin2 loss. To examine this possibility, we generated Sesn3−/− mice from a Sesn3-targeted embryonic stem cell (ESC) line (Friedel et al., 2007). Sesn3−/− mice were intercrossed with Sesn2−/− mice to generate Sesn2−/−/Sesn3−/− (DKO) mice (Figure S6A). Although the DKO mice did not exhibit spontaneous obesity (Figure S6B) or hepatosteatosis (Figure S6C) on LFD, they exhibited higher S6K phosphorylation (Figures 6A, 6B, and S6D–S6G) and lower FoxO3a, GSK3, and TSC2 phosphorylation (Figure S7A, S7D, and S7E) than Con mice. These results suggest that both Sestrin2 and Sestrin3 are required for proper blood glucose homeostasis under normal conditions.

Sestrins Promote AKT Phosphorylation through Inhibition of mTORC1

Finally, using cultured cells, we determined whether Sestrins can regulate insulin-Pi3K-AKT signaling in a cell autonomous manner. In Sesn2−/− mouse embryonic fibroblasts (MEFs), both basal (Figure 7A) and insulin-stimulated (Figure 7B) AKT phosphorylation at Thr308 and Ser473 was reduced relative to Sesn2+/+ MEFs. When overexpressed in MCF7, H1299, and HepG2 cells, Sestrin2 increased AKT phosphorylation even without insulin stimulation (Figures 7C, S7A, and S7B). The same effect was exhibited by Sestrin1, Sestrin3, and dSestrin (Figures S7A and S7C), indicating that the AKT regulatory function is conserved throughout the Sestrin family. Overexpression of AKT downstream targets including FoxO3a, GSK3, and TSC2, as well as the lipid kinase activity of PI3K, was also increased upon Sestrin2 expression (Figures S7A and S7C), indicating that the AKT regulatory function is conserved throughout the Sestrin family. Overexpression of AKT downstream targets including FoxO3a, GSK3, and TSC2, as well as the lipid kinase activity of PI3K, was also increased upon Sestrin2 expression (Figures S7A and S7C), indicating that the AKT regulatory function is conserved throughout the Sestrin family. Overexpression of AKT downstream targets including FoxO3a, GSK3, and TSC2, as well as the lipid kinase activity of PI3K, was also increased upon Sestrin2 expression (Figures S7A and S7C).
of AKT signaling and blood sugar homeostasis is evolutionarily conserved.

DISCUSSION

The AMPK-mTORC1 axis is a well-established and conserved central regulator of cellular metabolism (Kahn et al., 2005; Zoncu et al., 2011). In cultured mammalian cells subjected to genotoxic stress and in Drosophila, the AMPK-mTORC1 axis output is modulated by expression of Sestrin proteins (Budanov and Karin, 2008; Chen et al., 2010; Lee et al., 2010). However, given the existence of three mammalian Sesn genes, whose protein products exhibit conserved biochemical functions (Budanov et al., 2004; Budanov and Karin, 2008), a physiological role for Sestrins in metabolic regulation could previously be observed only in Drosophila (Lopez et al., 2010). Early experiments suggested that the Sestrins regulate information flow through the AMPK-mTORC1 axis during genotoxic or oxidative stress, which result in their transcriptional upregulation via p53 (Budanov and Karin, 2008) and FoxO3A (Nogueira et al., 2008). The current results, however, indicate that the mammalian Sestrins also have important physiological functions that are not limited to acute stress conditions. Here we show that Sestrin2 and Sestrin3 have physiologically relevant homeostatic functions both before and during obesity in the control of insulin signaling and blood glucose metabolism. This function is probably exerted through Sestrins’ ability to promote AMPK activation and suppress mTORC1-S6K signaling (Budanov and Karin, 2008).

The glucose homeostasis disorder observed in obese Sesn2−/− mice is mainly due to hepatic insulin resistance, which is reversible by AMPK reactivation through AICAR and adeno-virus-mediated transduction. AMPK reactivation also strongly suppressed chronic mTORC1-S6K activation and hepatosteatosis in livers of obese Sesn2−/− mice while it promoted insulin-induced AKT activation. Therefore, chronic downregulation of AMPK activity upon Sestrin2 deficiency results in enhanced mTORC1 activation, thereby attenuating insulin-induced AKT activity. This effect is presumably due to the phosphorylation of IRS proteins at their negative regulatory sites by the mTORC1 kinase or that AMPK regulation in tissues other than liver may also be important. Therefore, future studies should examine whether AMPK-related kinases are also regulated by the Sestrins and whether the function of Sestrin2 in other organs, such as adipose tissue, makes a contribution to the observed Sesn2−/− metabolic phenotypes. Interestingly, ectopic expression of Sestrin2 and other Sestrin family members induces AKT phosphorylation, even in cells that are not acutely stimulated with insulin. Sestrin-dependent activation of AKT is also observed in Drosophila and appears to be mediated via AMPK and TSC2, whose activity can potentiate activation of mTORC2, the AKT Ser473 kinase. Clearly, the ability of Sestrins to promote

Figure 6. Ablation of Sestrin2 and Sestrin3 Causes mTORC1 Upregulation and Spontaneous Insulin Resistance

(A and B) Sesn2−/−/Sesn3−/− DKO mice exhibit elevated mTORC1-dependent S6K phosphorylation. Livers and skeletal muscles from three 4-month-old Con (Sesn2−/−) and DKO mice kept on LFD were analyzed by immunoblotting for S6K phosphorylation on Thr389.

(C and D) Livers and skeletal muscles were collected from Con and DKO mice, after 6 hr of fasting, before (+) or 10 min after (+) insulin injection (0.8 U/kg body weight), homogenized and analyzed by immunoblotting with the indicated antibodies.

(E and F) After 6 hr of fasting, 4-month-old Con and DKO mice kept on LFD were subjected to ITT (E, n = 3) and area-under-curve analysis (F). Data are presented as means ± standard error. p value was calculated with a Student’s t test. See also Figure S6.
AMPK activation, suppress mTORC1-S6K signaling and activate AKT is not cell type specific and therefore takes place in many different tissues.

It should be noted that unlike Sesn2−/− mice, mice with hepato-cyte-specific TSC1 ablation do not experience aggravated hepatosteatosis during obesity (Yecies et al., 2011). This is probably due to the different mechanisms of action of TSC1 and Sestrin2; unlike TSC1, Sestrin2 activates AMPK, which can control lipid metabolism in mTORC1-independent ways (Kahn et al., 2005). For example, AMPK phosphorylates and inactivates ACC, reducing fatty acid synthesis and indirectly stimulating mitochondrial import and oxidation of fatty acids. AMPK also phosphorylates and activates ULK1/ATG1, which initiates autophagy (Egan et al., 2011). In addition, AMPK can promote mitochondrial biogenesis, which can increase β-oxidation (Cantó et al., 2009). These metabolic outputs of AMPK are decreased upon Sestrin2 deficiency, and as a result Sesn2−/− livers exhibit diminished β-oxidation and aggravated hepatosteatosis during obesity. In addition, Sestrin2 deficiency resulted in the absence of LD-associated autophagosomes, which are presumably the major pathway for LD digestion and utilization in hepatocytes (Singh et al., 2009). Another contributor could
be SREBP1, whose processing was modestly enhanced by Sestrin2 loss, although lipogenic gene expression and de novo lipogenesis in the Sesn2−/− liver were not significantly increased. Regardless of the relative contribution of these molecular conduits to hepatosteatosis in Sesn2−/− mice, it is unlikely that hepatosteatosis per se is responsible for the metabolic defects in these mice, as certain strains of mice can exhibit fatty liver without marked metabolic abnormalities (Farese et al., 2012). Further supporting this idea, Sesn2−/−/Sesn3−/− mice exhibit hepatic insulin resistance without displaying any signs of aberrant fat accumulation in liver.

Our results indicate that Sestrin2 is the only Sestrin protein that is inducible upon hypernutrition and obesity in mouse liver. Therefore, although Sesn2−/− mice continue to express Sestrin1 and Sestrin3, they are clearly more insulin resistant and glucose intolerant than control mice when rendered obese. As a result, Sesn2−/−/Lepob/ob mice become extremely diabetic, while Sesn2+/−/Lepob/ob mice display relatively mild insulin resistance. In addition, obese Sesn2−/− mice accumulate more lipids in their livers than Con mice. These pronounced phenotypes, however, are unlikely to be due to unique biochemical functions of Sestrin2 that are not shared with Sestrin1 and Sestrin3. Cell culture studies indicate that all three mammalian Sestrins and dSestrins can potentiate AMPK activation, and the defects exhibited by Sesn2−/− mice are consistent with reduced AMPK activity and subsequent increase in mTORC1 activation. Indeed, combined Sestrin2 and Sestrin3 deficiency causes insulin resistance in nonobese mice and alters mTORC1-S6K signaling in both liver and muscle. It is also worth mentioning that ATM/p53 signaling mutations in humans and mice, which strongly reduce hepatic expression of Sestrin1-Sestrin3, are associated with insulin resistance and aberrant glucose metabolism (Armanita et al., 2010; Bar et al., 1978; Schneider et al., 2006). Given their induction by genotoxic stress and their ability to control glucose and lipid homeostasis, the Sestrins may represent the missing link between DNA damage and metabolism.

**EXPERIMENTAL PROCEDURES**

**Mice and Diets**

Sesn2−/− mice (Budanov and Karin, 2008) were backcrossed to the C57BL/6 strain for at least seven generations. Sesn3−/− mice on the C57BL/6 background were generated from ESCs obtained from EUCOMM. These cells were created by targeted gene trap approach and contain reporter-tagged ORF. Mice also in the C57BL/6 background were purchased from the Jackson Lab. Mice were maintained in filter-topped cages and were given free access to autoclaved regular chow diet (LFD) or HFD (composed of 59% fat, 15% protein, 26% carbohydrates based on caloric content; Bio-Serv) and water at University of California, San Diego (UCSD) or University of Michigan (UM) according to National Institutes of Health (NIH) and institutional guidelines. All animal studies were overseen by the University Committee on Use and Care of Animals (UCUCA) at UM or by the Institutional Animal Care and Use Committee (IACUC) at UCSD.

**Mammalian Cell Culture and Biochemical Assays**

MCF7-tet OFF Sesn2F cells were described (Budanov and Karin, 2008). Immortalized Sesn2−/− and Sesn2−/− fibroblasts were generated by infection of MEFs with a lentiviral vector expressing ARFI shRNA. Immortalized Ampkα1−/−/Ampkα2−/− fibroblasts were described (Laderoute et al., 2006), and Tsc2−/− and Tsc2−/− immortalized fibroblasts were obtained from D. Kwiatkowsky. Cell culture conditions, MCF7-tet OFF system, and infection with retroviral vectors were described (Budanov and Karin, 2008). Immunoblotting, RNA analysis, immune complex kinase assay, lipid metabolism assays, and adenoviral procedures are described in the Supplemental Experimental Procedures.

**Evaluation of Glucose Homeostasis**

For glucose and insulin tolerance tests, mice were starved for 6 or 12 hr as indicated. Blood was drawn from a tail nick at the indicated time points after i.p. injection of glucose (1 g/kg body weight) or insulin (0.65 U/kg body weight), and blood glucose was instantly measured with a one-touch ultra glucose meter. Hyperinsulinemic-euglycemic clamps were performed with an infusion of 12 mIU insulin/kg body weight per minute as described (Oh et al., 2010). Glucose uptake assay in primary adipocytes and serum insulin measurement were done as described (Oh et al., 2010). Glucose in urine was quantified as in the Supplemental Experimental Procedures.

**Histology**

EM and fluorescence staining were done as described in the Supplemental Experimental Procedures. Oil red O staining of frozen liver sections (Park et al., 2010) and immunostaining of Drosophila imaginal discs (Lee et al., 2010) were described.

**Statistical Analysis**

Data are presented as means ± standard error or means ± standard deviation as indicated in legends. p values were calculated with a Student’s t test or two-way ANOVA as indicated.

**SUPPLEMENTAL INFORMATION**

Supplemental Information includes Supplemental Experimental Procedures and seven figures and can be found with this article online at http://dx.doi.org/10.1016/j.cmet.2012.08.004.

**ACKNOWLEDGMENTS**

We thank D. Kwiatkowski (Harvard), R. Shaw (Salk Institute), J.E. Dixon (UCSD), J.L. Guan, S. Pletcher, R.A. Miller, A. Saltiel (UM), Developmental Studies Hybridoma Bank, Cell Signaling Technology, Santa Cruz Biotechnology, Biomiga, Stenecell, and Abgent for reagents and access to lab equipment. We thank M. Kim for suggestions and constructive criticism, and A. Chen, M. Lu, H. Ogata, C.R. Sanchez, and K. Kim for technical assistance. Work was supported by grants and fellowships from the NIH (CA118165, CA155120, ES006376, and P42-ES010337 to M.K., DK082080 to A.B., P30-DK034933, P30-CA46592, P30-AG024824, and P30-AQ013283 to J.H.L., and P41-RR004050 and P30-CA23100 to M.H.E.), Ellison Medical Foundation (AG-SS-2440-10 to M.K. and AG-NS-0932-12 to J.H.L.), American Diabetes Association (7-08-MN-29 to M.K.), Human Frontier Science Program Organizati (LT00653/2008-L to J.H.L.), American Association for the Study of Liver Diseases/American Liver Foundation (to J.H.L. and E.J.P.), and Natural Sciences and Engineering Research Council of Canada (to E.J.P.), M.K. is an American Cancer Society Professor.

Received: May 26, 2011

Revised: July 26, 2012

Accepted: August 9, 2012

Published online: September 4, 2012

**REFERENCES**


