Kinase suppressors of Ras 1 and 2 (KSR1 and KSR2) function as molecular scaffolds to potently regulate the MAP kinases ERK1/2 and affect multiple cell fates. Here we show that KSR2 interacts with and modulates the activity of AMPK. KSR2 regulates AMPK-dependent glucose uptake and fatty acid oxidation in mouse embryonic fibroblasts and glycolysis in a neuronal cell line. Disruption of KSR2 in vivo impairs AMPK-regulated processes affecting fatty acid oxidation and thermogenesis to cause obesity. Despite their increased adiposity, ksr2-1- mice are hypophagic and hyperactive but expend less energy than wild-type mice. In addition, hyperinsulinemic euglycemic clamp studies reveal that ksr2-1- mice are profoundly insulin resistant. The expression of genes mediating oxidative phosphorylation is also downregulated in the adipose tissue of ksr2-1- mice. These data demonstrate that ksr2-1- mice are highly efficient in conserving energy, revealing a novel role for KSR2 in AMPK-mediated regulation of energy metabolism.

Kinase suppressor of Ras, metabolism, insulin, energy expenditure
KSR2 Is an Essential Regulator of AMP Kinase, Energy Expenditure, and Insulin Sensitivity

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SUMMARY

Kinase suppressors of Ras 1 and 2 (KSR1 and KSR2) function as molecular scaffolds to potently regulate the MAP kinases ERK1/2 and affect multiple cell fates. Here we show that KSR2 interacts with and modulates the activity of AMPK. KSR2 regulates AMPK-dependent glucose uptake and fatty acid oxidation in mouse embryonic fibroblasts and glycolysis in a neuronal cell line. Disruption of KSR2 in vivo impairs AMPK-regulated processes affecting fatty acid oxidation and thermogenesis to cause obesity. Despite their increased adiposity, ksr2−/− mice are hypophagic and hyperactive but expend less energy than wild-type mice. In addition, hyperinsulinemic-euglycemic clamp studies reveal that ksr2−/− mice are profoundly insulin resistant. The expression of genes mediating oxidative phosphorylation is also downregulated in the adipose tissue of ksr2−/− mice. These data demonstrate that ksr2−/− mice are highly efficient in conserving energy, revealing a novel role for KSR2 in AMPK-mediated regulation of energy metabolism.

INTRODUCTION

Molecular scaffolds, which coordinate the interaction of signaling molecules to affect efficient signal transduction (Burack and Shaw, 2000; Morrison and Davis, 2003), have the potential to serve as organizing nodes for multiple biological inputs. Kinase suppressor of Ras 1 (KSR1) (Kortum and Lewis, 2004; Nguyen et al., 2002) serves as a scaffold for the coordination of signals through the Raf/MEK/ERK kinase cascade. Manipulation of KSR1 reveals its role in regulating the transforming potential of oncogenic Ras, neuronal and adipocyte differentiation, and replicative life span (Kortum et al., 2005, 2006; Kortum and Lewis, 2004; Muller et al., 2000). A related protein, KSR2, has been detected in C. elegans and humans (Channavajhala et al., 2003; Ohmachi et al., 2002). In C. elegans, KSR2 is required for germ line meiotic progression and functions redundantly with KSR1 in excretion, vulva development, and spicule formation. Thus, KSR proteins appear to play critical roles in regulating multiple cell fates.

Cells must sense the nutritional status of the extracellular environment, monitor intracellular energy stores, and integrate that information with intracellular pathways that drive cell fate. The trimeric AMP-activated protein kinase (AMPK) is a critical regulator of energy homeostasis that is activated when the nutritional environment is poor and intracellular ATP levels are low (Hardie, 2007). Under conditions of energy stress, ATP levels fall, and levels of the allosteric activator AMP rise, which promotes binding of the catalytic AMPK α subunit to the γ subunit and protects against dephosphorylation of a critical threonine in the activation loop of the α subunit kinase domain (Sanders et al., 2007). ATP antagonizes the action of AMP on AMPK, making AMPK a sensor of cellular energy stores.

Upon activation, AMPK stimulates metabolic enzymes and induces gene expression programs to promote catabolic activity and inhibit anabolic activity. AMPK stimulates insulin-independent glucose uptake in muscle in response to exercise and hypoxia (Mu et al., 2001), and promotes the β oxidation of long-chain fatty acids by phosphorylating and inhibiting acetyl-CoA carboxylase (ACC), the rate-limiting enzyme of malonyl-CoA synthesis (Ruderman et al., 2003). Malonyl-CoA is both a key substrate for fatty acid synthesis and an inhibitor of carnitine palmitoyltransferase 1 (CPT1), which mediates import of fatty acids into the mitochondria.
acetyl-CoA molecules into the mitochondria for oxidation. By inhibiting ACC, AMPK inhibits the synthesis of fatty acids and promotes their metabolism to generate ATP.

The mechanisms linking dietary nutrients and AMP–regulated energy metabolism to cell fate are incompletely understood. Our analysis of the scaffold KSR2, however, provides a missing link. Here we show that KSR2 interacts with AMPK in a functionally relevant manner in vitro and in vivo. In cultured cells, AMPK–dependent effects on basal glucose uptake, fatty acid oxidation (FAO), and glycolysis are enhanced by KSR2. Gene disruption of ksr2 reduces the glucose-lowering capacity of an AMPK agonist in vivo. The white adipose tissue of ksr2+/− mice demonstrates defective phosphorylation of the AMPK substrate ACC, a key regulator of fatty acid synthesis and oxidation, and a downregulation of genes involved in oxidative phosphorylation. As a consequence of these impaired AMPK–dependent mechanisms, ksr2−/− mice are obese and insulin resistant. In contrast to most other animal models of obesity, ksr2−/− mice are hypophagic and more active. However, they expend less energy than wild-type mice. These data reveal a novel role for KSR2 as a critical regulator of cellular energy balance affecting lipid and glucose metabolism.

RESULTS

KSR1 and KSR2 Interact with AMPK

We used mass spectrometry to identify novel regulators that may affect KSR1 and KSR2 function. Previously reported KSR1–interacting molecules (Muller et al., 2001; Ory et al., 2003; Ritt et al., 2007), including HSP90, HSP70, Cdc37, C-Tak1, and PP2A, were detected in association with KSR2, as were peptidoglycan components of the α1, α2, β1, and γ1 subunits of AMPK (Figure 1A). This interaction was verified by the coprecipitation of endogenous AMPK α1 subunit in immunoprecipitates of KSR2, but not in immunoprecipitates of KSR1 (Figure 1B). When both AMPKα2 and KSR1 were overexpressed, an interaction between the two proteins could be detected. However, when compared for their relative ability to precipitate endogenous or ectopic AMPKα2, KSR2 consistently precipitated more AMPK than did KSR1 (Figure 1C). To determine the sites required for interaction with AMPK, full-length and truncated versions of KSR1 and KSR2 were expressed in 293T or COS-7 cells, and their ability to precipitate endogenous AMPK or the ectopic AMPKα2 subunit was tested. Deletion analysis indicated that the CA3 region contributes to the interaction of the AMPKα2 subunit with KSR1 (Figures 1D and 1E) and that the CA3 region and amino acids unique to KSR2 intervening between the CA2 and CA3 region (Dougherty et al., 2009) each contributed to the ability of KSR2 to interact with the AMPKα2 subunit (Figures 1F and 1G). The observation that KSR2 retains additional sequences contributing to its interaction with AMPK may explain its ability to precipitate a proportionally greater amount of AMPK.

KSR2 Promotes Glucose and Fatty Acid Metabolism

We tested whether mutations that impaired the interaction of AMPK with KSR1 or KSR2 affected ERK activation. The ΔCA3 mutation severely affected maximal activation of ERK by KSR proteins as observed previously for KSR1 (Michaud et al., 1997) (Figure 2A and see Figure S2A available online). However, KSR2Δ327–392 effects on ERK activation were equivalent to KSR2 (Figure 2A). These data indicate it is unlikely ERK activation plays a role in mediating the effects of KSR2 on AMPK signaling.

AMPK stimulates glucose uptake and the oxidation of fatty acids by mitochondria, thereby promoting ATP synthesis (Ruderman et al., 2003). To test whether KSR proteins affect these catabolic functions of AMPK, we generated ksr2−/− mice (Figure S1) and ksr2−/− mouse embryonic fibroblasts (MEFs). We measured glucose uptake in ksr2−/− and ksr1−/− MEFs and in null MEFs expressing their respective cognate ksr transgenes. Though MEFs do not express detectable KSR2 (data not shown), expression of ectopic KSR2 in ksr2−/− MEFs increased basal glucose uptake 5-fold (Figure 2B), and expression of KSR1 in ksr1−/− MEFs increased basal glucose uptake 2-fold (Figure S2B). Deletion of the CA3 region (KSR2.ΔCA3) modestly impaired basal glucose uptake, but deletion of sequences between the CA2 and the CA3 regions (KSR2.Δ327–392) decreased KSR2–induced glucose uptake by 50% (Figure 2B). Deletion of the CA3 region (KSR1.ΔCA3) was sufficient to significantly disrupt the effects of KSR1 on basal glucose uptake (Figure S2B).

We then tested whether KSR2 or KSR1 affected cellular fatty acid metabolism. The rate of oxygen consumption (OCR) was used as an index of oxidative phosphorylation (Wu et al., 2007). OCR in ksr2−/− or ksr1−/− MEFs was compared to OCR in ksr2−/− or ksr1−/− MEFs expressing full-length KSR2 or KSR1, respectively. In both ksr2−/− and ksr1−/− MEFs, palmitate had little or no ability to stimulate oxygen consumption. However, the reintroduction of KSR2 (Figure 2C) or KSR1 (Figure S2C) restored oxidation of palmitate. As with glucose uptake, disrupting the interaction of KSR2 or KSR1 with AMPK prevented restoration of FAO (Figure 2C and Figure S2C).

The neuroblastoma X glioma hybrid cell line NG108–15 expresses endogenous KSR2 (Dougherty et al., 2009). An shRNA was constructed to knock down KSR2 expression in these cells. In culture, neuronal cell lines in general, and NG108–15 cells in particular, synthesize ATP primarily from glycolysis (Ray et al., 1991). Since AMPK activation promotes glycolysis (Marsin et al., 2000), we examined the ability of KSR2 knockdown with and without the coexpression of a constitutively active AMPK construct to regulate glycolysis in NG108–15 cells. As an index of lactate production, extracellular acidification was markedly reduced in these cells when RNAi was used to inhibit KSR2 expression. However, expression of a constitutively active AMPK (Stein et al., 2000) restored glycolysis to levels seen in control cells (Figure 2D).

Phosphorylation of Thr172 on the effector loop of AMPK promotes its activity (Stein et al., 2000). In MEFs, the presence or absence of KSR2 did not affect AMPK Thr172 phosphorylation (data not shown), suggesting that AMPK signaling may require KSR2 for proper spatial regulation similar to its effect on ERK (Dougherty et al., 2009). However, in NG108–15 cells, loss of KSR2 inhibits the phosphorylation of AMPK on Thr172 in response to AICAR and modestly impairs phosphorylation of the AMPK substrate ACC (Figure 2E). These data support the conclusion that KSR2 is an AMPK regulator. To determine the relative impact of KSR1 and KSR2 on AMPK function and fatty acid metabolism in vivo, we examined the metabolic phenotype of ksr1−/− and ksr2−/− mice.
Figure 1. KSR1 and KSR2 Interact with AMPK

(A) AMPK-related peptides detected from endogenous proteins co-precipitating with KSR2. KSR2-associated proteins were isolated and peptide fragments were detected by mass spectrometry.
Loss of ksr2 Causes Obesity

KSR2 protein is detectable by western blot only in brain (Figure S1C). Therefore, we determined the relative abundance of ksr2 mRNA in various tissues (Figure 3A). Consistent with western blot analysis, ksr2 mRNA is approximately 100-fold more abundant in brain than in skeletal muscle or liver. 

mRNA is detectable at low levels in adipose tissue. In contrast, ksr1 mRNA is expressed strongly in skeletal muscle but present at approximately one-third that level in brain and 5% of that level in adipose tissue. ksr1−/− mice have been previously reported to have enlarged adipocytes but normal amounts of all adipose tissues (Kortum et al., 2005). Further analysis revealed no altered response to an AMPK agonist (Figure S3) and no overt metabolic defects (Figure S4 and Table S1) in ksr1−/− mice.

To test the role of KSR2 in the regulation of AMPK-mediated signaling, we targeted exon 4 within the ksr2 locus for deletion (Figure S1). The ksr2 null allele was transmitted through the germline, and heterozygous intercrosses yielded all three genotypes in a ratio close to the expected Mendelian distribution (relative ratios ksr2+/+ 1, ksr2+/− 2.25, ksr2−/− 0.91; n = 441). During development in utero and at birth, ksr2−/− mice were identical in size and weight to wild-type and ksr2+/− mice (Figure 3B, left panel). However, while nursing, ksr2−/− mice grew at approximately 50% the rate observed in...
Thirty-two percent of ksr2−/− mice (31 of 98) failed to survive until weaning. Premature death was not due to the failure of ksr2−/− pups to nurse properly, as all mice had milk in their stomachs upon necropsy. The addition of foster mothers did not improve survival. Furthermore, nutrient absorption was identical in wild-type and ksr2−/− mice (data not shown). We measured the growth rate of surviving ksr2−/− mice and observed that the ksr2−/− mice attained body weights similar to wild-type and ksr2+/− littermates 6–10 weeks after birth (Figure 3C). At 20–24 weeks of age, ksr2−/− mice exceeded the body weight of their wild-type and ksr2+/− littermates and became obese (Figure 3B, I).
KSR2 Regulates AMPK and Energy Expenditure

KSR2 Promotes AMPK Signaling In Vivo

We tested whether an interaction between endogenous KSR2 and AMPK could be detected similar to that observed when KSR2 was overexpressed in COS-7 cells (Figure 1). Brain lysates from wild-type and ksr2+/− mice were precipitated with antibodies against the AMPKα subunit and probed for KSR2 on western blot. KSR2 was detected in anti-AMPKα immunoprecipitates from WT brain but not detected in anti-AMPKα immunoprecipitates from ksr2−/− brain or from wild-type brain immunoprecipitated with a nonimmune antibody (Figure 3G).

To determine whether ksr2−/− mice have defects in AMPK signaling, we tested the ability of an intraperitoneal injection of the AMPK agonist AICAR to lower blood glucose. Wild-type mice showed a rapid and sustained reduction in blood glucose in response to the AMPK agonist AICAR that was not altered by deletion of ksr1 (Figure S3). However, disruption of ksr2 in mice delayed the onset of the drop in blood glucose and reduced the extent of the decrease (Figure 3H). Given the effect of AICAR in ksr2−/− mice, the phosphorylation state of AMPK and ACC was examined in white adipose tissue. While wild-type mice showed a rapid response to the AMPK agonist AICAR to lower blood glucose, wild-type adipose tissue remained unphosphorylated, that was not altered by deletion of ksr1.

Figure 4. ksr2−/− Mice Are Hypothermic and Hypophagic

(A) Hematoxylin and eosin staining of BAT from WT (left) and ksr2−/− (right) mice.

(B) Rectal temperature in 5- to 6-month-old male and female WT (light bars, n = 8 for each sex) and ksr2−/− mice (dark bars, n = 6 males, n = 11 females) during light (1 p.m.) and dark (9 p.m.) cycles (left panel).

(C) UCP1 mRNA levels in BAT (left panel) in 9- to 10-month female WT (light bars, n = 11) and ksr2−/− mice (dark bars, n = 5). UCP1 protein levels (right panel) in 8-month-old WT and ksr2−/− female mice.

(D) Average daily food intake in six WT males (age 11.5 ± 1.0 weeks), seven WT females (age 10.6 ± 1.1 weeks), five ksr2−/− males (age 11.4 ± 0.6 week), and five ksr2−/− females (age 10.5 ± 1.0 week).

(E) Serum leptin concentrations in 5- to 6-month-old WT and ksr2−/− mice.

(F) Twenty-four hour food intake following control PBS or 5 mg/kg leptin injections in WT and ksr2−/− mice. n = 5 for each genotype.

(G) Neuropeptide mRNA expression in 8- to 9-month-old female WT and ksr2−/− mice. n = 7 for each genotype.

(B–G) Results are shown as the mean ± SD. *p < 0.05; **p < 0.01; ***p < 0.001.
KSR2 Regulates AMPK and Energy Expenditure

KSR2 Coordinates OXPHOS Gene Expression

The observation that KSR2 expression regulated oxidative metabolism in vitro (Figure 2C) led us to examine gene expression in the white adipose tissue of ksr2−/− and wild-type mice. Gene Set Enrichment Analysis (GSEA) (Subramanian et al., 2005) revealed that gene sets previously identified as downregulated in obese mouse models and in some humans with abnormal glucose tolerance are regulated by ksr2 (Figure S7A, Table S2). Strongest among these was a gene set previously shown to be downregulated in ob/ob mice (Nadler et al., 2000). A notable number of sets that included genes for oxidative metabolism were also downregulated in ksr2−/− mice. A subset of oxidative phosphorylation genes coregulated across different mouse tissues (OXPHOS-CR) was identified previously (Mootha et al., 2003). This gene set corresponds to two-thirds of the

ksr2−/− mice show elevated lipolysis, free fatty acids, triglycerides (Figure 6A), and fasting insulin levels (Figure 6B). Adiponectin, resistin, PAI-1, IGF-1, and thyroxin were not significantly altered by disruption of ksr2. MCP-1 was significantly elevated in male but not female ksr2−/− mice (Figure S6). These data suggested that elevated endogenous insulin levels might be compensating for peripheral insulin resistance caused by the disruption of ksr2. We therefore performed hyperinsulinenic euglycemic clamps on wild-type, ksr1−/−, and obese ksr2−/− mice. Glucose homeostasis in ksr1−/− mice mirrored that of wild-type mice (Table S1). In contrast, ksr2−/− mice maintained comparable blood glucose concentrations only at glucose infusion rates that ranged between 20%–50% of that observed in wild-type mice (Figure 6C). Though hepatic glucose production (HGP) was reduced in ksr2−/− mice in comparison to wild-type mice, the ability of insulin to suppress HGP in the null mice was markedly suppressed (Figure 6D). Similarly, whole-body glucose turnover, glycolysis, and glycogen production were reduced in knockout mice (Figure 6E). These data are consistent with markedly reduced glycolytic content in the livers and glycolytic muscle of ksr2−/− mice (Figure 6F). To estimate insulin-stimulated glucose uptake in individual tissues, 2-deoxy-D-[1-14C] glucose was administered as a bolus (10 mCi) 75 min after the start of clamp. In comparison to wild-type mice, ksr2−/− mice showed profound insulin resistance in skeletal muscle (gastrocnemius) and both white (epididymal) and brown adipose tissue (Figure 6G). In combination with the effect on HGP, these data reveal that disruption of ksr2 impairs glucose uptake at the major sites of insulin action.

To test whether the effect of KSR2 disruption had a cell-autonomous effect on insulin-stimulated glucose uptake, we examined glucose uptake in isolated EDL muscles from wild-type and ksr2−/− mice. Insulin significantly stimulated glucose uptake in the isolated muscle from either genotype to a similar degree (Figure 6H). In contrast, the AMPK agonist AICAR had a small but significant impact on glucose uptake in wild-type EDL muscle but no effect on EDL muscle from ksr2−/− mice. These data suggest that KSR2 has a direct effect on AMPK-stimulated glucose uptake in EDL muscle. However, the insulin resistance observed in skeletal muscle of ksr2−/− mice appears to be a non-cell-autonomous effect, perhaps secondary to the obesity and impaired lipid metabolism caused by KSR2 disruption (Bergman et al., 2006).
genes encoding the oxidative phosphorylation biochemical pathway. The OXPHOS-CR gene set is reported to be downregulated in skeletal muscle of some humans with impaired glucose tolerance and correlated with changes in total body metabolism. We examined the expression of a custom gene set corresponding to the mouse orthologs (mOXPHOS-CR) and determined that this set was significantly downregulated (p < 0.001, NES = -2.39, FDR < 0.001) in the white adipose tissue of ksr2/−/− mice. Eighty-three percent (20 of 24) of the genes demonstrated lower expression in ksr2/−/− mice in comparison to wild-type mice (Figure S7). The transcriptional coregulator PGC1α is a potent regulator of OXPHOS-CR genes (Mootha et al., 2003). Microarray analysis revealed that PGC1α is also markedly decreased in the white adipose tissue of ksr2/−/− mice (Figure S7). These data demonstrate that the AMPK regulator KSR2 plays a potent role in controlling the expression of

Figure 5. ksr2/−/− Mice Are More Active but Expend Less Energy Than WT Mice
(A) Oxygen consumption (left), carbon dioxide production (middle), and RQ (right) in WT and ksr2/−/− mice.
(B) Cumulative locomotor activity in WT and ksr2/−/− mice.
(C) Spontaneous (middle) and cumulative (right) energy expenditure in male and female WT and ksr2/−/− mice.
(D) Oxygen consumption (upper panel) and core body temperature of female WT and ksr2/−/− mice at 32°C.
(A–D) Six WT males (age 11.5 ± 1.0 weeks), seven WT females (age 10.6 ± 1.1 weeks), five ksr2/−/− males (age 11.4 ± 0.6 week), and five ksr2/−/− females (age 10.5 ± 1.0 weeks). (D) Four WT (age 30.7 ± .02 weeks) and three ksr2/−/− (32.6 ± .07 weeks) female mice. (A–D) Results are shown as the mean ± SD. *p < 0.05; **p < 0.01; ***p < 0.001, unpaired, two-tailed t test or (C) two-way ANOVA with Bonferroni’s post hoc test.
PGC1α-dependent gene programs whose alteration may contribute to impaired glucose tolerance in ksr2−/− mice.

**DISCUSSION**

In this study, we show that the molecular scaffold KSR2 is an essential regulator of AMPK activity controlling cellular thermogenesis, fat oxidation, and glucose metabolism. These data suggest a model (Figure 7) whereby a decrease in AMPK function impairs FAO and increases lipid storage, contributing to obesity. These findings reveal a novel pathway regulating energy expenditure and glucose metabolism, the elucidation of which may facilitate therapeutic intervention in obesity.

It remains unclear to what extent specific tissues contribute to the metabolic defects caused by KSR2 disruption. Ex vivo experiments suggest a role for KSR2 in adipose tissue and muscle. However, KSR2 is detectable by western blot only in the brain, and mRNA profiling detects much lower levels of KSR2 in muscle, liver, and adipose tissue. These data suggest that important actions of KSR2 on AMPK are probably exerted within the central nervous system.

Resting metabolic rate is the major contributor to obligatory energy expenditure and is strongly associated with fat-free mass (Cunningham, 1991). However, fat-free mass accounts for only 70%–80% of the variability in resting metabolic rate (Sparti et al., 1997). Genetically determined differences in the ability of organisms to consume oxygen to make ATP have been proposed as an explanation for at least some of the remaining variability (Harper et al., 2008). The reduced oxygen consumption and energy efficiency of ksr2−/− mice identify ksr2 as a novel genetic determinant of metabolic rate. To some extent, decreased energy expenditure in ksr2−/− mice could be ascribed to the decrease in UCP1 expression, which might contribute to the 1.5°C drop in rectal temperature of ksr2−/− mice. The reduced rectal temperature of ksr2−/− mice therefore suggests that ksr2 is essential for the physiological regulation of resting thermogenesis.
The regulation of AMPK by KSR2 plays a critical role in determining whether fatty acids are oxidized in mitochondria to generate ATP or are stored as triglycerides. Our in vivo and ex vivo data demonstrate that deletion of KSR2 in white adipose tissue impairs the ability of AMPK to phosphorylate ACC, which should inhibit FAO and promote triglyceride storage (Ruderman et al., 2003). This defect provides a simple explanation for the increased fat mass of ksr2−/− mice (Brommage et al., 2008). Accordingly, ampkα2−/− mice share traits with ksr2−/− mice. On a high-fat diet, ampkα2−/− mice demonstrate elevated body weight, morbidly increased adipose mass, and adipocyte hypertrophy without an increase in food intake relative to control mice (Villena et al., 2004). Similar to ksr2−/− mice, deletion of the AMPK α2 subunit causes insulin resistance and AICAR intolerance (Vollet et al., 2003). Also consistent with our findings, loss of AMPK signaling that results from hepatic disruption of the AMPK kinase, LKB1, causes hyperglycemia and glucose intolerance while not impairing insulin action (Shaw et al., 2005). Thus, KSR2 may affect cell-autonomous energy homeostasis in multiple tissues by directly modulating lipid and glucose metabolism via AMPK, while behavioral phenotypes of KSR2-deficient mice such as hypophagia and hyperactivity are likely compensatory responses for body set point defense.

We performed microarray analysis on mRNA isolated from wild-type and ksr2−/− adipose tissue to identify genetic pathways affected by the deletion of KSR2. GSEA of microarray data identified curated gene sets that are significantly regulated by the presence or absence of KSR2 (Figure S7 and Table S2). Grouping the highest ranking (p = 0.000; FDR ≤ 0.001; FWER ≤ 0.045) gene sets according to overlapping genes identifies three general cellular functions regulated by KSR2. In white adipose tissue, KSR2 appears most potently affect genes regulating adipocyte differentiation, genes involved in oxidative phosphorylation, and genes affecting the metabolism of branched-chain amino acids and short-chain lipids. KSR2-dependent regulation of genes controlling adipocyte differentiation (e.g., Nadler Obesity Down, IDX TSA Up Cluster 6, and TNFα Down gene sets) plays a role in the increased adipocyte cell number observed in ksr2−/− mice. Decreased expression of genes involved in oxidative phosphorylation (e.g., Electron Transport, Mootha VoxPhos, and Mitochondria gene sets) likely contributes to the decreased ability of ksr2−/− mice to metabolize lipid and carbohydrate. Gene sets for branched chain amino acid catabolism and propionate, butanoate, and pyruvate metabolism represent key pathways that generate key substrates (e.g., acetyl-CoA, succinyl-CoA) to the TCA cycle and fatty acid synthesis. The regulation of these pathways by KSR2 suggests a role for the scaffold in controlling both the availability of these key substrates and the enzymes necessary for their metabolism. However, future experiments will be required to determine whether these changes in gene expression are mediated by the action of KSR2 in adipose tissue or in another tissue with prominent expression, like the brain.

KSR1 and KSR2 are best known for their function as scaffolds for the Raf/MEK/ERK signaling cassette, facilitating the activation of Raf and MEK (Dougherty et al., 2009; Kortum and Lewis, 2004; Nguyen et al., 2002). The interaction of AMPK with these molecular scaffolds raises the intriguing possibility that KSR proteins function not only as scaffolds, but also as components of an energy and nutrient sensor that couples information about the nutritional environment and intracellular energy status of a cell to a kinase cascade with potent effects on cell proliferation, differentiation, and survival. The discovery that C-TAK1/MARK3/Par1α, a member of the AMPK kinase family, phosphorylates KSR1 and inhibits its ability to promote the activation of MEK by Raf (Muller et al., 2001) supports this concept. In complex with KSR2, AMPK might provide similar means to restrict energy-intensive proliferative stimuli emanating from activated ERK when ATP is limited. ksr2−/− mice might thus develop impaired metabolic homeostasis due to an inability to respond appropriately to energy deficits and curb ERK signaling when the nutritional environment is inadequate.

Consistent with that paradigm, our results demonstrate that disruption of ksr2 causes obesity through a reduction in cellular energy consumption despite hypophagia. Therefore, our observations reveal ksr2−/− mice to be a novel model of obesity with potential relevance to obesity-related dysregulation of glucose metabolism. That molecular scaffolds regulating the activation of Raf, MEK, and ERK can have a profound effect on fat accumulation suggests that factors affecting energy balance may have previously unappreciated roles on MAP kinase signaling. In particular, future studies focusing on cell-type-specific mechanisms of KSR2/AMPK interactions may provide important insight into novel mechanisms regulating physiological control of energy storage and expenditure with implications for glucose homeostasis.

**EXPERIMENTAL PROCEDURES**

**Mice**
ksr1−/− mice were described previously (Kortum et al., 2005; Nguyen et al., 2002). Standard gene-targeting techniques and homologous recombination were used to generate ksr2−/− mutant mice. The Institutional Animal Care and Use Committee (University of Nebraska Medical Center, Omaha, NE) approved all studies. Animals were maintained on a 12 hr light/dark schedule (light on at 0600) and had free access to laboratory chow (Harlan Teklad LM 485) and water. All in vivo analyses were performed on mice of 3–7 months of age.

**Cells**
ksr1−/− and ksr2−/− MEFs were generated from day 13.5 embryos and immortalized by 379 protocol as described (Kortum and Lewis, 2004) or by
expression of 5V40 large T antigen. Expression of KSR1, KSR2, and co-
responding mutants in MEFS was also measured as described previously (Kor-
tum and Lewis, 2004). NG108-15 cells, COS-7, and 293T cells were obtained
from ATCC.

Immunoprecipitation and Immunoblots
Immunoprecipitation was performed on postnuclear membranes with anti-
bodies to the FLAG, Pyo, and Myc epitope tags as described previ-
ously (Kortum and Lewis, 2004; Ritt et al., 2007). Antibodies for AMPKα, phospho-
Thr172 AMPK, ACC, and phospho-Ser79 ACC, UCP1, and GAPDH were
from Cell Signaling Technologies. Anti-KSR2 antibody 1G4 was from Abnova.
Anti-α-tubulin antibodies were from Santa Cruz.

Glucose Uptake and ERK Assays
Glucose uptake was measured with 2-deoxy-[2,6,3-H]glucose in the presence or absence of 20 μM cytochalasin B and 200 μM phloretin as described previ-
ously (Chaika et al., 1999). ERK phosphorylation was quantified on the Odyssey
system (LI-COR) with anti-phospho-ERK1/2 (Cell Signaling No. 9106) and anti-
ERK1 (Santa Cruz Biotechnology, sc-93) primary antibodies and goat anti-
mouse Alexa Fluor 680 (Innoven) and goat anti-rabbit IRDye 800 (Rockland)
as secondary antibodies as described (Kortum and Lewis, 2004).

Fatty Acid Oxidation and Glycolysis Assays
FAO and glycolysis were determined by measuring OCR and extracellular
acidification rate, respectively, in cultured cells using the XF24 Analyzer (Sea-
horse Bioscience) (Wu et al., 2007).

KSR2 shRNA
A short hairpin targeting the nucleotides for amino acids 868-874 of mouse
KSR2 was cloned into the lentiviral MISSION pLKO.1-puro vector. Puro-
mycin-resistant cells were selected using 2 μg/ml puromycin (Sigma).

ksr1 and ksr2 mRNA Quantification
Total RNA was isolated from selected mouse tissues using Tri-Reagent
(Molecular Research Center, Inc.), ksr1, ksr2, GusB, and Tbp were simulta-
neously quantified from 5 μg of total RNA using Quantigene 2.0 Plex gene
sets (Panomics), following the manufacturer’s recommendations. Total RNA
was hybridized to custom probe set 21121 for 24 hr at 54°C while shaking at
900 RPM with an orbital diameter of 3 mm before signal amplification and
quantification using a Luminex 200 instrument. The median fluorescent inten-
sity of at least 50 beads was used to determine the average gene expression
for tissue samples from three mice performed in duplicate.

Quantitative PCR
RNA was extracted from BAT and hypothalamus using TRIzol reagent
(Innoven) according to the manufacturers’ instructions. After subsequent
DNase treatment, reverse transcriptions were performed using SuperScript
III (Innoven) and Oligo-dT20 primers (Innoven). Real-time PCR for UCP1
in BAT and neurones in hypothalamus, and the ribosomal housekeeping
gene L32 were performed on a Bio-Rad Cycler using Q SYBR Green Supermix
(Biorad). Relative quantification of the target transcript in comparison to a refer-
tance transcript was calculated from the real-time PCR efficiencies and the
crossing point deviation of the target sample versus its control (Pfaffl, 2001).

Body Composition and Adipocyte Size
Body composition was measured using nuclear magnetic resonance tech-
nology (NMR, EchoMRI, Quantitative Magnetic Resonance Body Composition
Analyzer, Echo Medical Systems, LLC, Houston, TX). Total adipose tissue from
each depot was excised, and the wet weight was determined. Abdominal,
subcutaneous, and BAT were fixed in Bouin’s fixative, sectioned in a micro-
tome, and stained with hematoxylin and eosin. Adipocyte cross-sectional
area was determined from photomicrographs of epididymal fat pads using
iPLab software (Scanalytics Inc., Fairfax, VA) (Kortum et al., 2005).

In Vivo Metabolic Phenotype Analysis
Food intake was measured daily manually over 5 consecutive days in freely
feeding mice. Total energy expenditure, locomotor activity, and RQ (relative
rates of carbohydrate versus fat oxidation) of mice were determined by indirect
calorimetry using a customized 32 cage indirect calorimetry system (TSE
Systems, Midland, MI). The mice were placed in the calorimetry system cages
for up to 6 days and nights, with at least 24 hr for adaptation before data
recording. For oxygen consumption measurements at thermoneutral condi-
tions, the environmental temperature requiring the least energy for organisa-
mal heating or cooling processes was determined using indirect calorimetry within
a climate control system. Thermoneural zone for ksr2−/− and wild-type mice
was determined. Oxygen consumption analysis was then performed to ascer-
tain that the thermogenetic phenotype was not an artifact resulting from
relative cold exposure of ksr2−/− and wild-type mice.

Metabolite Assays
Blood glucose was measured with an Ascensia Glucometer Elite (Fisher Sci-
entific), Plasma insulin was measured with the Mouse Insulin Elisa Kit (Crystal-
Chem, Chicago, IL) using mouse standards. Serum-free fatty acids were
measured colorimetrically (Roche). Plasma triglycerides and glycerol were
measured using the GPO-Trinder colorimetric assay kit (Sigma). Plasma leptin
was measured using the Rat Leptin RIA kit (Linco Research, St Louis, MO).
Glycogen content was analyzed with the Glucose HK assay (Sigma). For
measurement of lipolysis, mice were fasted overnight for 12 hr. Subcutaneous
fat was excised and minced in Krebs-Ringer bicarbonate buffer at 37°C for
3 hr, and a sample of media was assayed for glycerol content using Free
Glycerol Reagent (Sigma).

Ex Vivo Culture of White Adipose Tissue Explants
Subcutaneous fat pads were removed from 7-month-old wild-type and ksr2−/−
mice, prepared, and treated as described (Gaidhu et al., 2009).

Ex Vivo Glucose Uptake
Glucose uptake was measured in isolated EDL muscle from wild-type and
ksr2−/− mice using 2-deoxy-[2,6,3-H]glucose and 14Cmannitol as described
previously (Sakamoto et al., 2005).

Statistical Analysis
Data are expressed as mean ± s.e.m. Differences between two groups were
assessed using the unpaired two-tailed t test and among more than two
groups by analysis of variance (ANOVA).

ACCESSION NUMBERS
The microarray data have been deposited at the National Center for Biotech-
nology Information (NCBI) under Gene Expression Omnibus (GEO) accession
number GSE17923.

SUPPLEMENTAL DATA
Supplemental Data include Supplemental Experimental Procedures, seven
figures, and two tables and can be found with this article online at http://

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