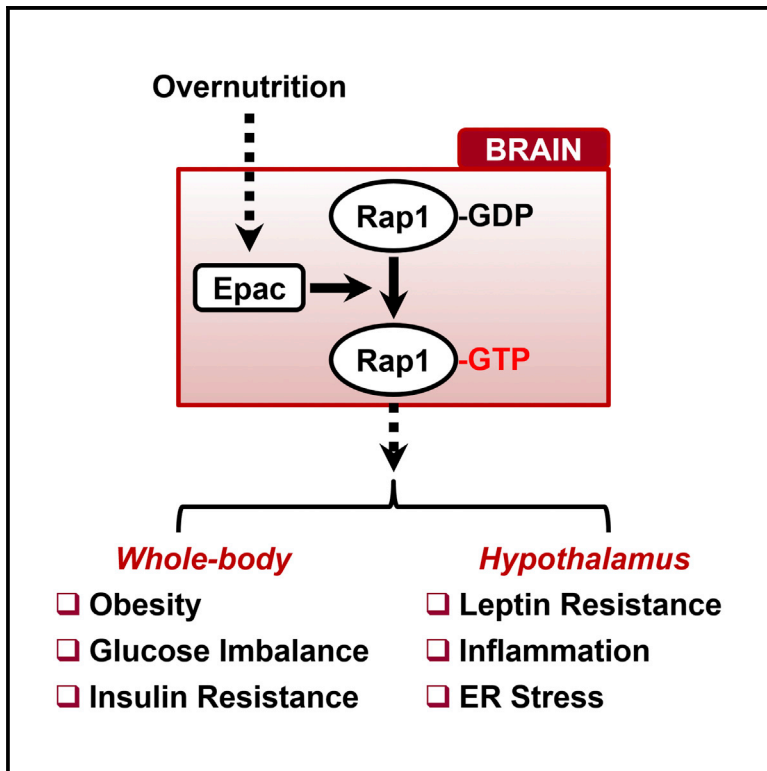


## Neuronal Rap1 Regulates Energy Balance, Glucose Homeostasis, and Leptin Actions

### Graphical Abstract



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### In Brief

The brain is involved in diet-induced obesity and its associated metabolic disturbances. Using mice with neuron-specific deletion of the small GTPase Rap1, Kaneko et al. demonstrate that brain Rap1 plays a central role in dietary obesity, glucose imbalance, peripheral insulin resistance, and central leptin resistance.

### Highlights

- The small GTPase Rap1 in the brain is activated in high-fat-diet-induced obesity
- Loss of neuronal Rap1 protects against diet-induced obesity and glucose imbalance
- Rap1 controls neural leptin sensitivity
- Brain Rap1 interacts with ER stress pathways in leptin resistance and obesity



# Neuronal Rap1 Regulates Energy Balance, Glucose Homeostasis, and Leptin Actions

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## SUMMARY

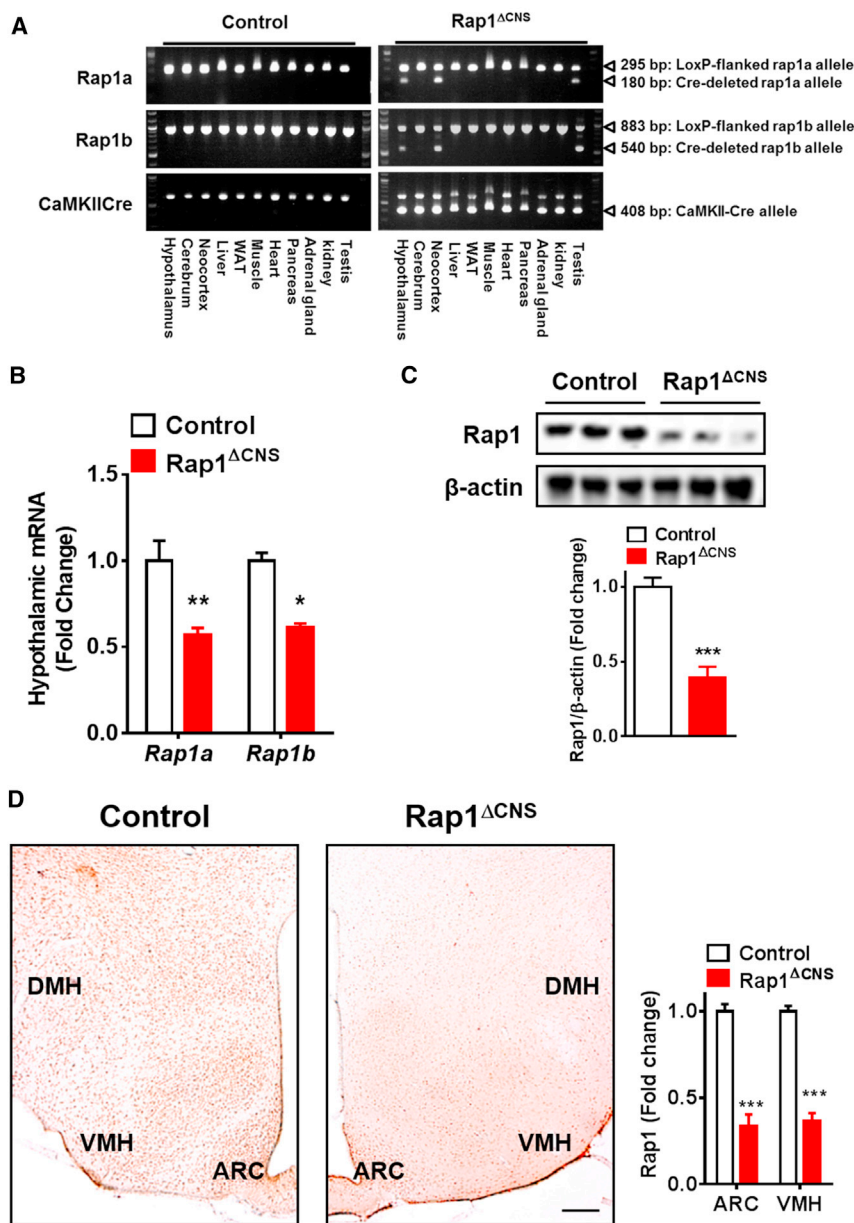
The CNS contributes to obesity and metabolic disease; however, the underlying neurobiological pathways remain to be fully established. Here, we show that the small GTPase Rap1 is expressed in multiple hypothalamic nuclei that control whole-body metabolism and is activated in high-fat diet (HFD)-induced obesity. Genetic ablation of CNS Rap1 protects mice from dietary obesity, glucose imbalance, and insulin resistance in the periphery and from HFD-induced neuropathological changes in the hypothalamus, including diminished cellular leptin sensitivity and increased endoplasmic reticulum (ER) stress and inflammation. Furthermore, pharmacological inhibition of CNS Rap1 signaling normalizes hypothalamic ER stress and inflammation, improves cellular leptin sensitivity, and reduces body weight in mice with dietary obesity. We also demonstrate that Rap1 mediates leptin resistance via interplay with ER stress. Thus, neuronal Rap1 critically regulates leptin sensitivity and mediates HFD-induced obesity and hypothalamic pathology and may represent a potential therapeutic target for obesity treatment.

## INTRODUCTION

The CNS has been long established as robust homeostatic systems for the maintenance of normal body weight and euglycemia (Coll et al., 2007; Dietrich and Horvath, 2013; Morton et al., 2006; Myers and Olson, 2012; Ryan et al., 2012). The crucial role of the CNS in the development of obesity is also becoming increasingly apparent with recent discoveries of obesity susceptibility genes that are often associated with CNS functions (Locke et al., 2015). Obesogenic conditions such as high-fat diet (HFD) feeding cause these CNS homeostatic systems to shift toward positive energy balance, which ultimately leads to obesity (Ryan et al., 2012). However, the neural pathways that actively respond to

HFD feeding and mediate adiposity under overnutrition remain incompletely characterized.

HFD leads to multiple, profound neuropathological changes in hypothalamic nuclei that control body weight (Könner and Brüning, 2012; Morton et al., 2006; Myers et al., 2010; Ryan et al., 2012; Kälén et al., 2015). Hypercaloric feeding rapidly induces neuronal resistance to the actions of leptin, a powerful adipocyte-derived satiety hormone that maintains normal body weight and euglycemia (Frederich et al., 1995; Könner and Brüning, 2012; Morton et al., 2006; Myers et al., 2010; Ryan et al., 2012). Although the detailed mechanisms are still unclear, cellular leptin signaling in the CNS is clearly impaired in rodent models of HFD-induced obesity (Myers and Olson, 2012; Ryan et al., 2012). Thus, defective intracellular leptin signaling in the CNS has been proposed as an underlying cellular mechanism for leptin resistance. Signaling molecules that directly inhibit leptin signaling, including suppressor of cytokine signaling-3 (SOCS-3) (Bjørbaek et al., 1998; Howard et al., 2004; Mori et al., 2004), protein tyrosine phosphatase 1B (PTP1B) (Bence et al., 2006; Cook and Unger, 2002; Zabolotny et al., 2002), and T cell protein tyrosine phosphatase (TCPTP) (Loh et al., 2011), have been identified as crucial mediators of leptin resistance. All of these factors are upregulated in the hypothalamus by HFD-induced obesity (Bjørbaek et al., 1998; Cook and Unger, 2002; Loh et al., 2011; Zabolotny et al., 2002). Moreover, neuron-specific deletion of these inhibitors protects against HFD-induced obesity as well as leptin resistance and insulin resistance (Bence et al., 2006; Howard et al., 2004; Loh et al., 2011; Mori et al., 2004). Thus, SOCS-3 and tyrosine phosphatases collectively contribute to the development of HFD-induced obesity. Obesity induced by HFD is also associated with ER stress and inflammation in the CNS. Recent studies suggest that HFD-induced ER stress and inflammation in the CNS impair hypothalamic control of body weight and glucose balance (Coll et al., 2007; Dietrich and Horvath, 2013; Morton et al., 2006; Myers and Olson, 2012; Ryan et al., 2012; Kälén et al., 2015). Hypothalamic ER stress and inflammation are markedly increased by overfeeding and in multiple obesity models (De Souza et al., 2005; Ozcan et al., 2009; Zhang et al., 2008b). Pharmacologic or genetic induction of ER stress and/or inflammation in the CNS upregulates SOCS-3, PTP1B, and TCPTP expression and causes leptin resistance and



### Figure 1. Validation of Rap1<sup>ΔCNS</sup> Mice

(A) PCR genotyping analyses were performed from several tissues of Rap1<sup>ΔCNS</sup> and control (Rap1-floxed) mice. Cre-deleted alleles are detected only in the hypothalamus, neocortex, and testis.

(B) Hypothalamic Rap1 mRNA levels were measured using qPCR analyses (n = 3/group). All error bars are SEM.

(C) Rap1 western blot and densitometric quantification of hypothalamic Rap1. β-Actin was used as a loading control (n = 4/group).

(D) Representative images of brain slices from Rap1<sup>ΔCNS</sup> mice and control mice stained for Rap1. Left: immunohistochemistry images. Right: quantification of immunohistochemistry (n = 4/group).

\*p < 0.05, \*\*p < 0.01, \*\*\*p < 0.001 compared with control mice based on t tests in (B)–(D). Scale bar, 200 μm. See also Figures S1 and S2.

ever, the contributions of CNS Rap1 to energy balance and glucose homeostasis are largely unknown. In the present study, we investigated the role of CNS Rap1 in the regulation of whole-body energy and glucose metabolism by producing and characterizing mice with targeted deletion of *Rap1a* and *Rap1b*, the genes encoding Rap1, selectively in forebrain neurons.

## RESULTS

### Loss of Neuronal Rap1 Protects against Diet-Induced Obesity

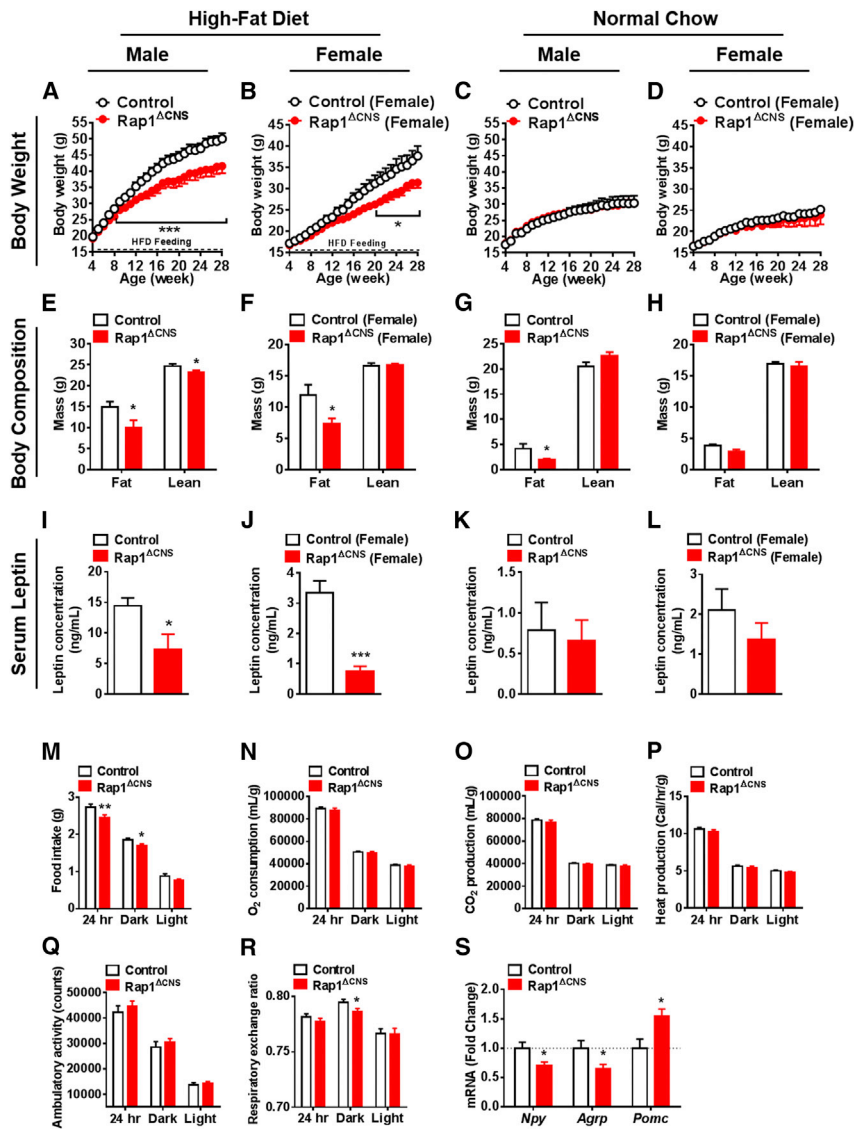
We first investigated whether Rap1 is expressed in the hypothalamus. Consistent with previous work (Kim et al., 1990; Pan et al., 2008), quantitative real-time PCR showed that *Rap1a* and *Rap1b* are expressed in various tissues, including the CNS, and that both mRNAs are abundant in the hypothalamus (Figure S1A). We further examined the hypothalamic distribution of Rap1 by immunohistochemistry analyses. Rap1 is expressed throughout

obesity (Cakir et al., 2013; Hosoi et al., 2008; Zhang et al., 2008b). On the contrary, manipulations that alleviate hypothalamic ER stress or reduce hypothalamic inflammation ameliorate cellular leptin resistance and obesity in animals (Kleinridders et al., 2009; Milanski et al., 2009; Ozcan et al., 2009; Schneeberger et al., 2013; Zhang et al., 2008b). Although HFD feeding clearly elicits hypothalamic dysfunction, promoting obesity, the underlying molecular signaling pathways are poorly understood.

The Ras-like small GTPase Rap1 is a crucial regulator of multiple cellular processes, including adhesion, polarity, and proliferation, in non-neuronal cells (Gloerich and Bos, 2011). In the CNS, Rap1 has diverse roles in an array of neuronal functions from neuronal excitability, synaptic plasticity, and neuronal polarity to memory and learning (Spilker and Kreutz, 2010). How-

the mediobasal hypothalamus, including in multiple nuclei that regulate whole-body metabolism such as the arcuate (ARC), ventromedial (VMH), and dorsomedial (DMH) nuclei (Figure S2A; Figure 1D). We then asked whether CNS Rap1 is activated in HFD-induced obese mice. As shown in Figure S2B, the active (guanosine triphosphate [GTP]-bound) form of Rap1 is increased in the brain of HFD-induced obese mice compared with lean control mice. Total Rap1 levels were not changed in response to HFD (Figure 7A). These results and a previous study showing that Rap1 activity is increased in lean mice after acute HFD feeding (Fukuda et al., 2011) strongly suggest that Rap1 is involved in the metabolic responses to HFD feeding.

We thus explored whether CNS Rap1 contributes to diet-induced obesity and associated metabolic disturbances. To



**Figure 2. Loss of Neuronal Rap1 Protects against Diet-Induced Obesity**

(A–L) Metabolic phenotype of Rap1<sup>ΔCNS</sup> mice. Shown are weekly body weight of HFD-fed males (A) and females (B) (n = 10–17/group), body composition in HFD-fed male (E) and female (F) mice at 20 weeks of age, and serum leptin levels in HFD-fed male (I) and female (J) mice at 24 weeks of age. The HFD was initiated at 4 weeks of age. Also shown are weekly body weight of normal chow-fed males (C) and females (D) (n = 9–12/group), body composition in normal chow-fed male (G) and female (H) mice at 20 weeks of age, and serum leptin levels in normal chow-fed male (K) and female (L) mice at 24 weeks of age.

(M–R) Metabolic profile of 2-week HFD-fed male Rap1<sup>ΔCNS</sup> mice (n = 8/group) on food intake (M), O<sub>2</sub> consumption (N), CO<sub>2</sub> production (O), heat production (P), ambulatory activity (Q), and respiratory exchange ratio (R) during 24-hr dark or light cycles. Note that mice at 7 weeks of age had comparable body weight (control: 23.41 ± 0.7 g versus Rap1<sup>ΔCNS</sup>: 23.07 ± 0.7 g, p > 0.05, t tests), fat mass (control: 4.41 ± 0.7 g versus Rap1<sup>ΔCNS</sup>: 3.16 ± 0.2 g, p > 0.05, t tests), and lean mass (control: 16.86 ± 0.4 g versus Rap1<sup>ΔCNS</sup>: 17.27 ± 0.5 g, p > 0.05, t tests) at the time of the CLAMS study.

(S) Hypothalamic mRNA expression of the feeding-related neuropeptide genes. Hypothalami were collected from HFD-fed male mice at 28 weeks of age (n = 6/group). qPCR analyses were performed to measure mRNAs.

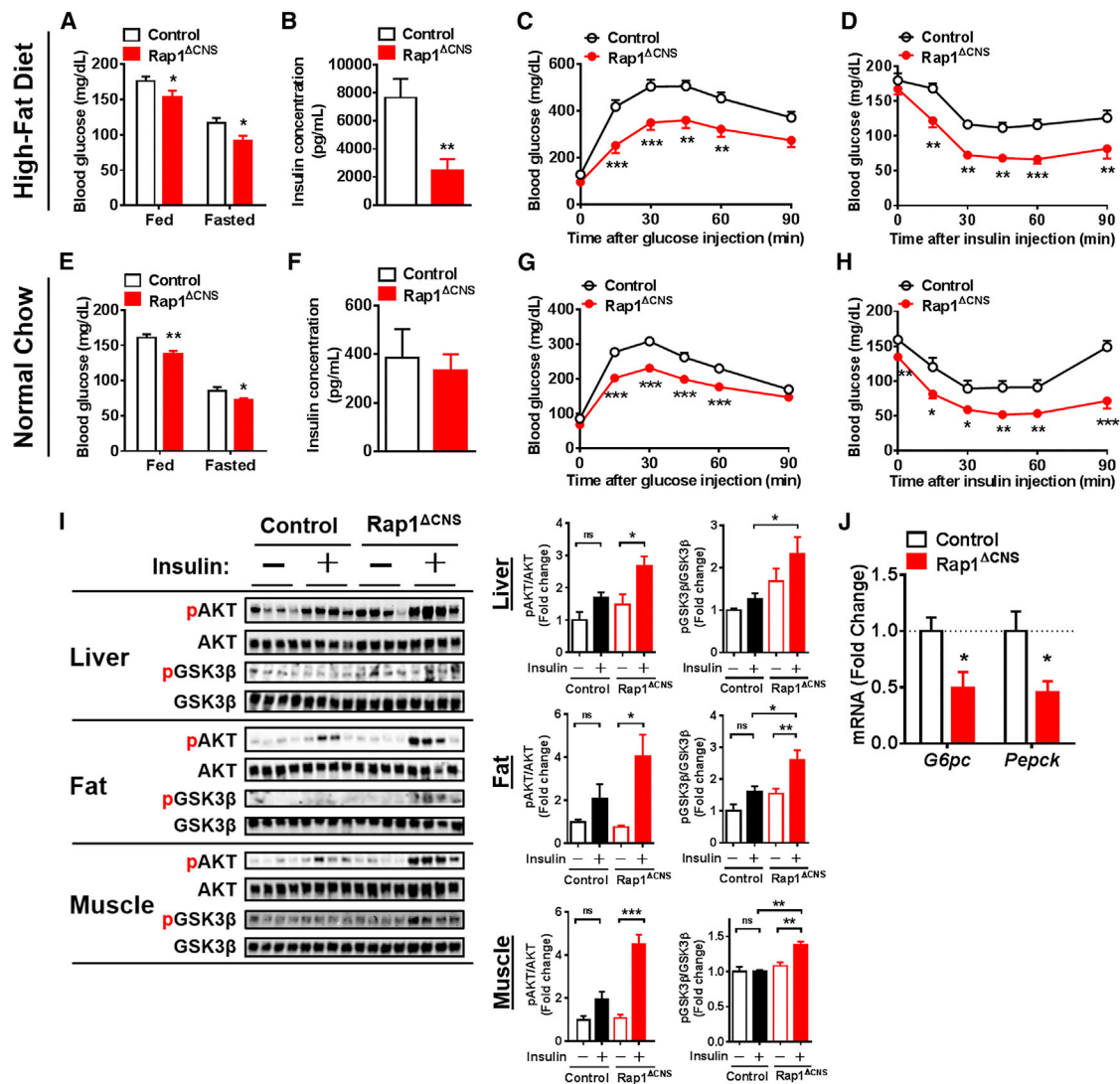
\*p < 0.05, \*\*p < 0.01, \*\*\*p < 0.001 for two-way ANOVA followed by Sidak's multiple comparisons tests in (A) and (B) or t tests in (E)–(H), (J), (M), (R), and (S). All error bars are SEM. See also Figures S3–S5.

produce mice Rap1-null in the forebrain (Rap1<sup>ΔCNS</sup>) (Pan et al., 2008), we crossed double-floxed *Rap1a* and *Rap1b* (*Rap1*) mice to mice harboring the *CaMKIIαCre* driver, which express Cre recombinase in postnatal forebrain neurons in the CNS (Minichiello et al., 1999). We confirmed CNS-specific recombination of the floxed alleles (Figure 1A), forebrain (including hypothalamus) deletion of *Rap1* mRNAs (Figure 1B), and selective depletion of Rap1 protein from forebrain regions, including the hypothalamus (Figure 1C). Rap1 protein levels were also significantly reduced in multiple hypothalamic nuclei (Figure 1D).

Using this Rap1<sup>ΔCNS</sup> mouse model, we examined whether Rap1 has a role in the CNS regulation of energy and glucose homeostasis in vivo. Male Rap1<sup>ΔCNS</sup> and control male mice (the double-floxed mice of *Rap1a* and *Rap1b*) were placed on a HFD starting at 4 weeks of age to test whether loss of Rap1 protects against diet-induced obesity. The HFD-fed male Rap1<sup>ΔCNS</sup> mice showed markedly reduced body weight gain (Figure 2A), signifi-

cantly lower adiposity (Figure 2E), and reduced serum leptin levels (Figure 2I) compared with controls. In contrast, Rap1<sup>ΔCNS</sup> and control male mice fed a normocaloric chow diet exhibited similar body weight, adiposity, and serum leptin levels (Figures 2C, 2G, and 2K). Female Rap1<sup>ΔCNS</sup> mice under HFD feeding also demonstrated lower body weight and adiposity than female controls (Figures 2B, 2D, 2F, 2H, 2J, and 2L), suggesting no sexual dimorphism in Rap1 function. We used male mice only for subsequent experiments.

We then investigated the basis for the leaner phenotype of HFD-fed Rap1<sup>ΔCNS</sup> mice by directly assessing energy balance in open-circuit indirect calorimetry cages. Although the body weight and adiposity of Rap1<sup>ΔCNS</sup> mice did not diverge from control animals after 2 weeks of HFD (Figures S3A and S3B), Rap1<sup>ΔCNS</sup> mice exhibited hypophagia (Figure 2M) associated with increased hypothalamic expression of anorexigenic neuropeptide POMC mRNA and decreased expression of orexigenic neuropeptide NPY and AgRP mRNAs (Figure 2S; Figure S5B). In contrast, no difference in energy expenditure (oxygen consumption, carbon dioxide production, locomotor activity, or thermogenesis) was observed between Rap1<sup>ΔCNS</sup> and control mice (Figures 2N–2Q). Notably, Rap1<sup>ΔCNS</sup> mice showed a lower



**Figure 3. Improved Glucose Homeostasis in Rap1<sup>ΔCNS</sup> Mice**

(A–D) Glucose homeostasis parameters of Rap1<sup>ΔCNS</sup> or control mice fed a high-fat diet for 24 weeks (n = 7–14/group). Shown are glucose (A), serum insulin levels (B), glucose tolerance test (GTT) (C), and insulin tolerance test (ITT) (D).

(E–H) Glucose profile of age- and body weight-matched lean cohorts (control: 21.23 ± 0.91 g versus Rap1<sup>ΔCNS</sup>: 21.78 ± 0.78 g, p > 0.05 based on t tests) at 7 weeks of age (n = 7–12/group). Shown are glucose (E), serum insulin levels (F), GTT (G), and ITT (H).

(I) Cellular insulin sensitivity (n = 4/group). Shown are western blot (left) and quantification (right) of Akt (Thr<sup>308</sup>) and GSK-3β (Ser<sup>9</sup>) phosphorylation in the liver, fat, and muscle 10 min after a bolus injection of insulin (1 U/kg, intraperitoneal [i.p.]) or saline into Rap1<sup>ΔCNS</sup> or control mice fed an HFD for 24 weeks.

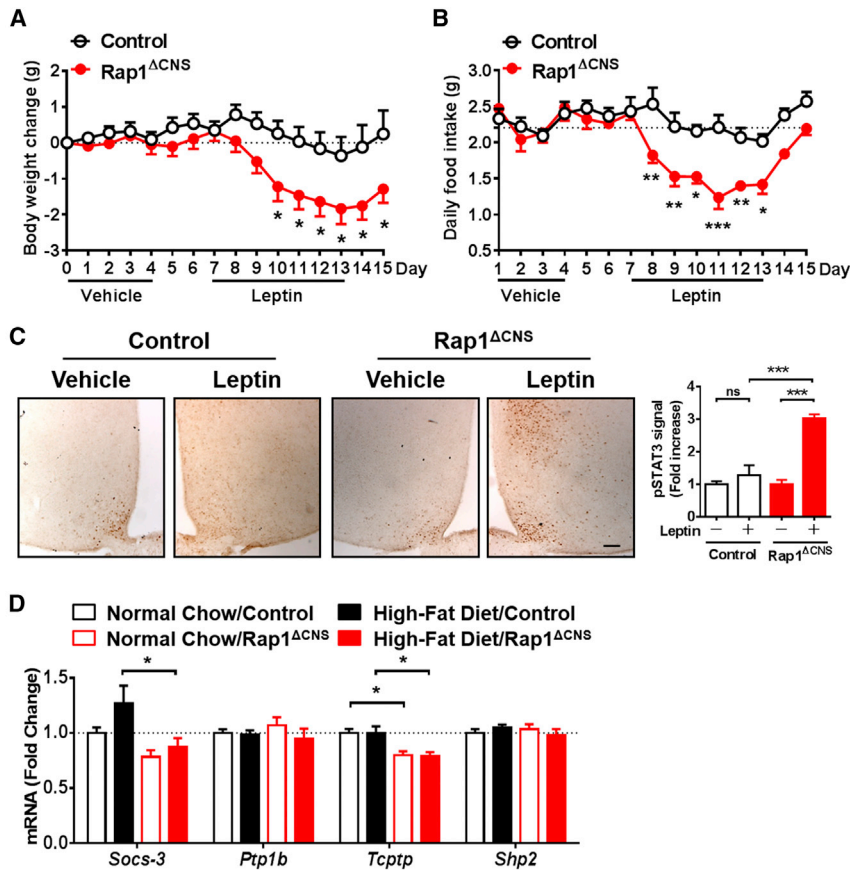
(J) qPCR analysis of hepatic mRNA expression of genes encoding *G6pc* and *Pepck* of 24-week HFD-fed Rap1<sup>ΔCNS</sup> mice (n = 6/group).

\*p < 0.05, \*\*p < 0.01, \*\*\*p < 0.001 for t tests in (A), (B), (E) and (J); two-way ANOVA followed by Sidak's multiple comparisons tests in (C), (D), (G), and (H); or one-way ANOVA followed by Tukey's multiple comparison test in (I). All error bars are SEM. See also Figure S6.

respiratory quotient than controls, indicating the preferential use of fat as an energy source (Figure 2R). Thus, decreased food intake and preferential oxidation of fat as an energy substrate likely contributes to decreased adiposity in neuronal Rap1-null mice under hypercaloric feeding. In chow-fed lean mice, food intake (Figure S4A), energy expenditure (Figures S4B–S4F), and mRNA levels of feeding-related hypothalamic neuropeptides (Figure S5A) did not differ significantly between genotypes. These findings suggest that CNS Rap1 plays a crucial role in mediating diet-induced body weight gain and adiposity.

### Improved Glucose Balance and Peripheral Insulin Sensitivity in Rap1<sup>ΔCNS</sup> Mice

Consistent with the leaner body weight phenotype, Rap1<sup>ΔCNS</sup> mice displayed significantly lower levels of blood glucose and insulin than control animals under HFD feeding (Figures 3A and 3B), suggesting that mice lacking Rap1 in the CNS have increased peripheral insulin sensitivity. Indeed, HFD-fed Rap1<sup>ΔCNS</sup> mice showed enhanced glucose tolerance (Figure 3C) and improved insulin sensitivity (Figure 3D). This higher glucose-tolerant and insulin-sensitive phenotype was



**Figure 4. Leptin Sensitivity Is Increased in Rap1<sup>ΔCNS</sup> Mice**

(A and B) Male mice were maintained on a high-fat diet for 8 weeks and injected with leptin (3 mg/kg, twice per day, i.p.) or vehicle during the indicated period. Body weight (A) and food intake (B) were measured every day. Age- and body weight-matched cohorts were used (n = 8/group).

(C) Leptin (3 mg/kg, i.p.) was administered to the indicated mice (n = 3/group). Left: representative immunohistochemistry images for pSTAT3. Scale bar, 100 μm. Right: quantification of immunohistochemistry.

(D) Hypothalamic expression of genes involved in leptin resistance in Rap1<sup>ΔCNS</sup> and control mice. Hypothalami were collected from age-matched normal chow or 4-week HFD-fed male mice at 12 weeks of age (after high-fat diet feeding, control: 36.77 ± 1.0 g versus Rap1<sup>ΔCNS</sup>: 34.59 ± 0.6 g, p > 0.05 based on t tests; after normal chow feeding, control: 32.06 ± 1.7 g versus Rap1<sup>ΔCNS</sup>: 29.78 ± 1.3 g, p > 0.05 t tests) (n = 4–5/group).

\*p < 0.05, \*\*p < 0.01, \*\*\*p < 0.001 for two-way ANOVA followed by Bonferroni's multiple comparisons tests in (A) and (B) or one-way ANOVA followed by Tukey's multiple comparison test in (C) and (D). All error bars are SEM. See also Figures S3 and S7.

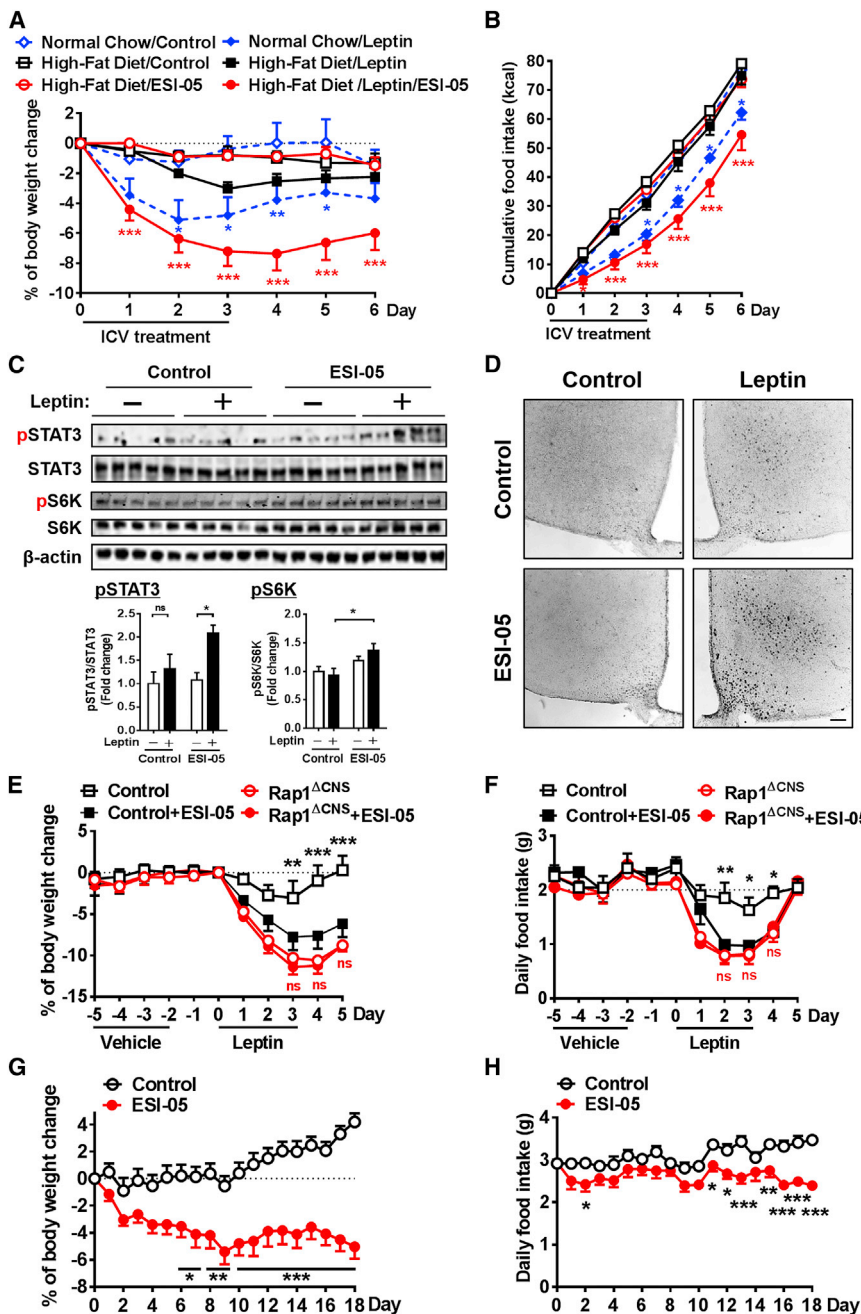
also observed in age- and weight-matched Rap1<sup>ΔCNS</sup> cohorts maintained on a normocaloric diet (Figures 3E–3H), suggesting that Rap1 deficiency in the CNS influences insulin/glucose balance regardless of body weight and adiposity. In further support of improved insulin sensitivity, insulin signaling was significantly enhanced in the liver, muscle, and fat of HFD-fed Rap1<sup>ΔCNS</sup> mice, as assessed by western blot analyses using phospho-specific antibodies to Akt and Gsk3β (Figure 3I), the central mediators of insulin signaling (Manning and Cantley, 2007). We also observed that hepatic insulin-independent phosphorylation of Akt was significantly increased in normal chow-fed Rap1<sup>ΔCNS</sup> mice (Figure S6). In agreement with enhanced hepatic insulin sensitivity, hepatic expression levels of the gluconeogenic genes phosphoenolpyruvate carboxykinase (Pepck) and glucose-6-phosphatase (G6pc) were significantly reduced in HFD-fed Rap1<sup>ΔCNS</sup> mice compared with HFD-fed controls (Figure 3J). Collectively, these findings suggest that, in addition to its role in body weight regulation, neuronal Rap1 regulates glucose balance and peripheral insulin sensitivity.

#### Enhanced Leptin Sensitivity in Neuronal Rap1-Deficient Mice

Mice with genetic ablation of neuronal Rap1 exhibit traits suggestive of enhanced leptin sensitivity, including decreased circulating leptin levels (Figure 2I), hypophagia (Figure 2M), and

altered levels of leptin-regulated hypothalamic neuropeptides (Figure 2S). We therefore tested whether Rap1 is required for the development of HFD-induced leptin resistance. Rap1<sup>ΔCNS</sup> and control mice were

placed on an HFD (60% fat) for 8 weeks, beginning at 2 months of age, to induce leptin resistance. We did not observe any significant difference in body weight (28.02 ± 0.71 g for Control versus 27.61 ± 0.62 g for Rap1<sup>ΔCNS</sup>, n = 8/group, p > 0.05, t test), fat mass (4.76 ± 0.45 g versus 3.45 ± 0.42 g, n = 8/group, p > 0.05, t test), or lean mass (20.82 ± 0.44 g versus 21.72 ± 0.31 g, n = 8/group, p > 0.05, t test) between the two groups after 8 weeks of HFD feeding (Figures S3C and S3D), probably because of the late onset of HFD challenge. Using these age- and body weight-matched cohorts, we then assessed the anorectic response to leptin by injecting Rap1<sup>ΔCNS</sup> and control mice with leptin twice daily. Although control mice developed leptin resistance (Figures 4A and 4B), Rap1<sup>ΔCNS</sup> mice responded to leptin with body weight reduction and suppression of food intake (Figures 4A and 4B). Further, cellular leptin sensitivity, as demonstrated by leptin-induced phosphorylation of STAT3, a marker of activated leptin signaling (Bates et al., 2003; Gao et al., 2004; Metlakunta et al., 2008; Vaisse et al., 1996), was significantly enhanced in Rap1<sup>ΔCNS</sup> mice but absent in controls under HFD conditions (Figure 4C). Also, hypothalamic Socs-3 and Tcptp were significantly lower in Rap1<sup>ΔCNS</sup> mice than in controls (Figure 4D). In addition to its effect under an HFD diet, Rap1 deficiency potently enhanced leptin actions under normal caloric conditions (Figure S7). Therefore, deletion of CNS Rap1 enhances cellular leptin signaling and protects against leptin resistance.



**Figure 5. ESI-05 Reverses Leptin Resistance in HFD-Induced Obese Mice**

(A and B) ESI-05 enhances leptin-induced body weight reduction (A) and food intake suppression (B). Leptin (2 μg) or vehicle was i.c.v.-infused with or without ESI-05 (0.2 nmol) to HFD-fed obese C57BL/6 mice (HFD for 5 months, n = 8–10/group) or lean normal chow-fed C57BL/6 mice (n = 5/group) (twice per day for 3 days).

(C) Western blot images (top) and quantification (bottom) of hypothalamic STAT3 (Tyr<sup>705</sup>) and S6K (Thr<sup>389</sup>) phosphorylation 1 hr after a bolus injection of leptin (2 μg, i.c.v.) or saline into HFD-fed mice that received ESI-05 (0.2 nmol, i.c.v.) or vehicle 3 hr before leptin injection (n = 5/group).

(D) Representative immunohistochemistry images of hypothalamic pSTAT3. HFD-fed obese mice received ESI-05 (2 nmol, i.c.v.) or vehicle, followed 3 hr later by i.c.v. injection of leptin (2 μg) for 1 hr. Scale bar, 100 μm.

(E and F) Effect of ESI-05 on leptin sensitivity in Rap1<sup>ΔCNS</sup> mice. Shown are body weight change (E) and food intake (F). HFD-fed obese control or Rap1<sup>ΔCNS</sup> mice (HFD for 5 weeks, n = 5–7/group) received i.c.v. injections of leptin (2 μg) with or without ESI-05 (0.2 nmol) twice a day over 3 days. (G and H) Effect of ESI-05 on body weight and food intake in HFD-induced obese mice. Shown are body weight change (G) and food intake (H). HFD-fed obese C57BL/6J mice (HFD for 16 weeks, n = 10/group) received i.c.v. injections of ESI-05 (0.2 nmol for days 1–14, 1 nmol for days 15–18) once a day.

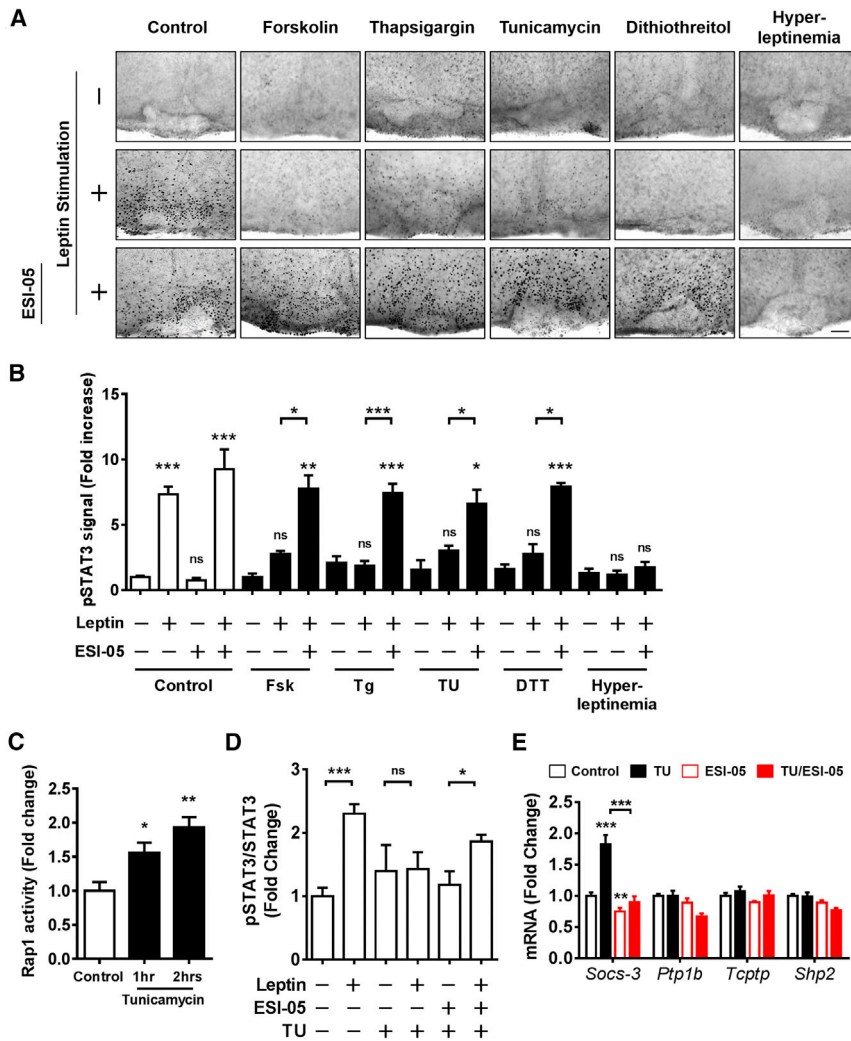
\*p < 0.05, \*\*p < 0.01, \*\*\*p < 0.001 for two-way ANOVA followed by Tukey's multiple comparison test in (A), (B), (E), and (F) or Sidak's multiple comparison test in (C), (G) and (H). All error bars are SEM.

**ESI-05 Reverses Leptin Resistance in HFD-Induced Obesity**

To assess the translational value of CNS Rap1, we investigated the effects of a well-established selective inhibitor of Epac2, ESI-05 (Rehmann, 2013; Tsalkova et al., 2012). Epac2 is one of the two members of exchange protein directly activated by cAMP (Epac) that serves as GTP/protein diphosphate (GDP) exchange factors for Rap1. Epac2 is predominantly expressed throughout the brain and in the adrenal gland in humans (Kawasaki et al., 1998) and in mice (Figure S1B). We infused leptin, the selective Epac2 inhibitor ESI-05, or both into the brains of

wild-type HFD-induced obese mice. Co-administration of ESI-05 markedly sensitized leptin-responsive neurons, as indicated by restoring leptin-induced suppression of food intake, reduction of body weight (Figures 5A and 5B), and phosphorylation of the independent leptin signaling mediators STAT3 and S6K (Figures 5C and 5D). Notably, ESI-05 restored leptin sensitivity to a similar degree in normocaloric-fed lean mice

receiving leptin alone (Figures 5A and 5B). To confirm Rap1 mediation of ESI-05 effects, we repeated these experiments in Rap1<sup>ΔCNS</sup> mice. Consistent with mediation by Rap1, ESI-05 did not enhance leptin sensitivity in Rap1<sup>ΔCNS</sup> mice (Figures 5E and 5F). Next, we investigated whether ESI-05 has this anti-obesity effect when centrally administered alone to HFD-induced hyperleptinemic and leptin-resistant obese mice. Central daily infusion of ESI-05 (0.2 nmol/brain/day) significantly reduced the body weight and food intake of HFD-induced obese mice (Figures 5G and 5H). In contrast, the body weight of vehicle-treated control obese mice exhibited no changes during



**Figure 6. Rap1 Mediates Leptin Resistance Conferred by Chemically Induced ER Stress**

(A) Effect of ESI-05 on multiple forms of leptin resistance in organotypic brain slices. The slices were incubated with either forskolin (Fsk, 20  $\mu$ M), thapsigargin (Tg, 30  $\mu$ M), TU (30  $\mu$ M), DTT (1 mM), or a high dose of leptin (hyperleptinemia, 120 nM) in the presence or absence of ESI-05 (50  $\mu$ M) for 6 hr and then stimulated with leptin (120 nM, 60 min). Leptin-induced pSTAT3 is shown. Scale bar, 100  $\mu$ m.

(B) Quantification of hypothalamic pSTAT3 (n = 3–21/group) in organotypic brain slices.

(C) Activation of brain Rap1 by chemically induced ER stress. Lean C57BL/6 mice were administered tunicamycin (10  $\mu$ g, i.c.v.) for the indicated period (n = 5–6/group). Proteins were extracted from the treated brains, and Rap1 activity was measured.

(D) ESI-05 blocks ER stress-induced leptin resistance in vivo. Tunicamycin (10  $\mu$ g, i.c.v.) was injected with or without ESI-05 (0.2 nmol, i.c.v.) into the brain of lean C57BL/6 mice. Three hours later, leptin (5  $\mu$ g, i.c.v.) was administered to the mice (n = 4–5/group). The hypothalami were collected 60 min after leptin injection and subjected to western blot analysis using pSTAT3 antibodies.

(E) Relative mRNA expression of *Socs-3*, *Ptp1b*, *Tcptp*, and *Shp2* in brains of mice centrally receiving tunicamycin (10  $\mu$ g) with or without ESI-05 (0.2 nmol) for 4 hr (n = 12–13/group).

\*p < 0.05, \*\*p < 0.01, \*\*\*p < 0.001 for one-way ANOVA followed by Tukey's multiple comparison test in (B)–(E). All error bars are SEM.

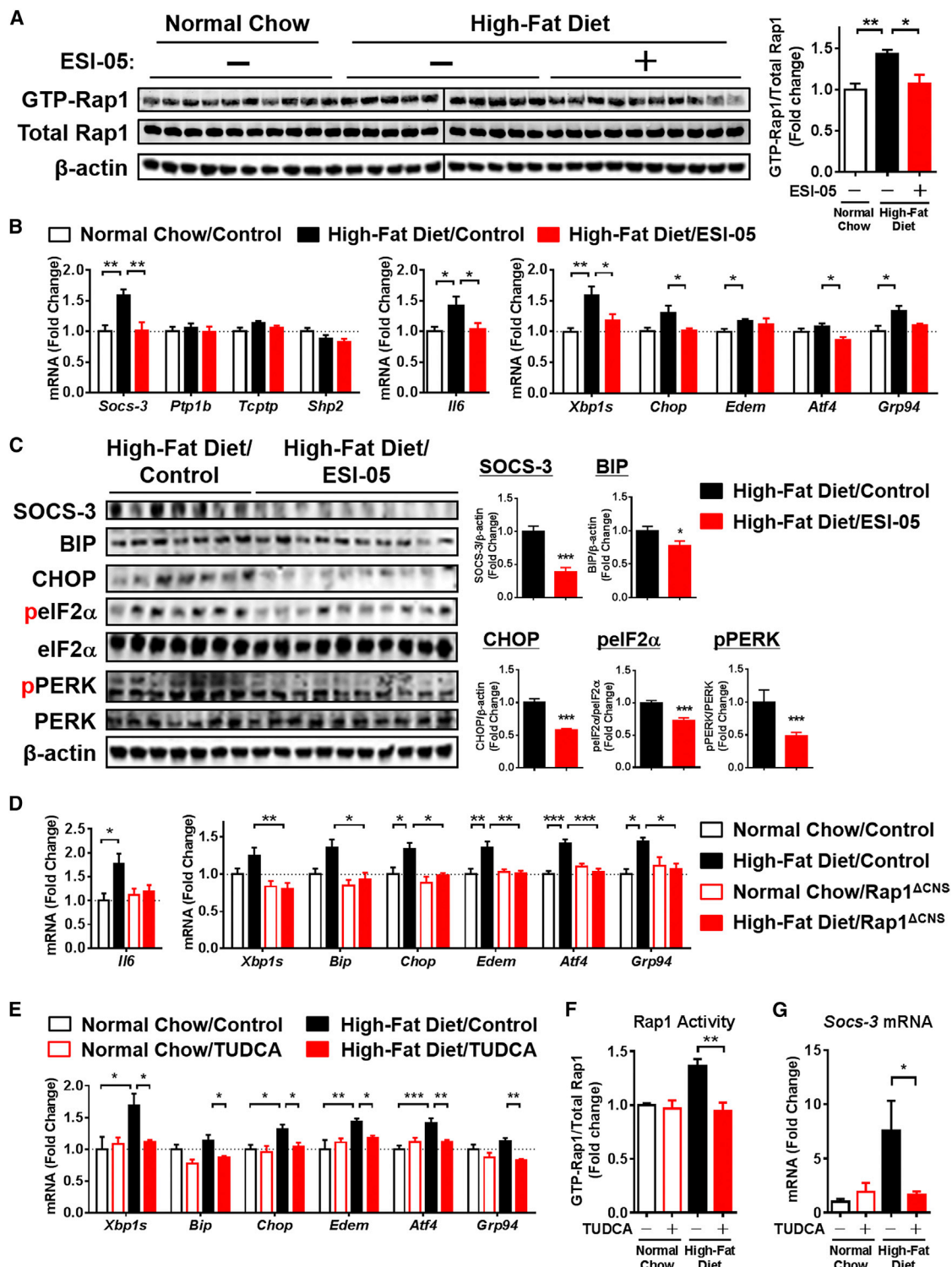
the course of the experiment (Figures 5G and 5H). Thus, chronic administration of ESI-05 alone is indeed able to decrease the body weight of HFD-induced obese mice (vehicle versus ESI-05, p < 0.05). Collectively, these findings demonstrate that Epac2 inhibition reverses leptin resistance and reduces body weight in HFD-induced obese mice.

### Rap1 Is Required to Mediate Leptin Resistance Conferred by Chemically Induced ER Stress

We next sought to determine potential underlying mechanisms by which central Epac-Rap1 signaling contributes to leptin resistance. Cellular leptin resistance can be caused by multiple mechanisms that include ER stress and hyperleptinemia (Fredrich et al., 1995; Köner and Brüning, 2012; Morton et al., 2006; Myers et al., 2010; Ozcan et al., 2009; Ryan et al., 2012; Zhang et al., 2008b), which prompted us to explore potential interactions between Epac signaling and putative leptin resistance-inducing factors. First, we modeled leptin resistance by treating organotypic brain slices with pharmacological agents that induce cellular leptin resistance. Similar to previous

observations (Fukuda et al., 2011; Williams et al., 2014), leptin-induced phosphorylation of STAT3 was blocked by treatment with the ER stress inducers tunicamycin (TU), thapsigargin, and DTT (Figures 6A and 6B), whereas leptin stimulated STAT3 phosphorylation in controls (Figures 6A and 6B). Strikingly, pretreatment with ESI-05, a selective Epac2 inhibitor (Tsalkova et al., 2012), abolished ER stress-induced leptin resistance in slices (Figures 6A and 6B). ESI-05 also blocked cellular leptin resistance induced by forskolin, which activates Epac-Rap1 signaling (de Rooij et al., 1998; Fukuda et al., 2011; Figures 6A and 6B). ESI-05 had negligible effects on leptin resistance resulting from treatment with high-dose leptin (mimicking hyperleptinemia) (Figures 6A and 6B). ESI-05 alone did not stimulate leptin-dependent STAT3 phosphorylation (Figure 6B). To further confirm the effect of ESI-05 in vivo, we chemically induced ER stress in the brain of lean C57BL/6 mice by the injection of TU. TU treatment increased GTP-bound (active) Rap1 in the brain (Figure 6C). Inhibition of CNS Epac2 prevented hypothalamic leptin resistance and *Socs-3* induction triggered by centrally injected TU in mice (Figures 6D and 6E), confirming our ex vivo findings. Interestingly, other key factors involved in leptin resistance, such as negative regulators of leptin signaling (PTP1B and TCPTP) and a positive regulator, SHP2 (Zhang et al., 2004), remained unaltered





**Figure 7. Blockade of Rap1 Signaling in the CNS Protects Mice from HFD Induction of Hypothalamic ER Stress and Il-6**

(A) Western blot images (left) and quantification (right) of the amount of the active form of Rap in the brain of lean mice or HFD-induced obese mice that received ESI-05 (0.2 nmol, i.c.v., twice a day for 3 days) or vehicle (n = 10/group).

(B) Relative mRNA expression of *Socs-3*, *Ptp1b*, *Tcptp*, *Shp2*, *Il6*, *Xbp1s*, *Chop*, *Edem*, *Atf4*, and *Grp94* in the hypothalamus of ESI-05-treated HFD-induced obese mice or lean control mice. Mice were maintained on an HFD or a normal chow for 33 weeks and received ESI-05 (0.2 nmol, i.c.v., twice a day) or vehicle for 3 days (n = 9/group).

(legend continued on next page)

(Figure 6E). These findings suggest that Epac2 participates in ER stress-induced leptin resistance.

### Reciprocal Connection between Rap1 and ER Stress in the CNS under Overnutrition

Because HFD-induced obese mice exhibited both increased CNS Rap1 activity (Figure 7A; Figure S2B) and ER stress (Ozcan et al., 2009; Won et al., 2009; Zhang et al., 2008b; Figure 7B), we next investigated whether Rap1 is involved in cellular processes that mediate HFD-induced ER stress. To test this, we manipulated CNS Rap1 activity by either pharmacologic inhibition using ESI-05 or by brain-specific *Rap1* deletion (in  $Rap1^{\Delta CNS}$  mice). Central delivery of ESI-05 into the brain of wild-type HFD-induced obese mice significantly suppressed elevated Rap1 activity in the CNS (Figure 7A). Treatment with ESI-05 also reversed the increased expression levels of ER stress marker genes (*Xbp1s*, *Chop* and *Atf4*) and elevated *Il-6* and *Socs-3* in the hypothalamus of HFD-induced obese mice (Figure 7B). In addition, ESI-05 significantly reduced hypothalamic phosphorylation of the core components of the unfolded protein response (UPR), PERK, and eIF2 $\alpha$  (Walter and Ron, 2011; Figure 7C). These responses were almost completely recapitulated in  $Rap1^{\Delta CNS}$  mice. We challenged  $Rap1^{\Delta CNS}$  mice and age- and body weight-matched controls with HFD for 4 weeks and measured hypothalamic expression levels of ER stress markers and *Il-6*. After 4 weeks of HFD feeding, there were no significant differences in body weight and adiposity between the two groups. Nonetheless, quantitative real-time PCR revealed significant increases in ER stress markers (*Chop*, *Edem*, *Atf4*, and *Grp94*) and pro-inflammatory cytokine *Il-6* in the hypothalamus of control mice after HFD challenge, whereas an HFD failed to upregulate the classical markers of UPR activation and *Il-6* in  $Rap1^{\Delta CNS}$  mice (Figure 7D). This suggests that Rap1 deficiency in the CNS prevents HFD induction of ER stress and pro-inflammatory cytokine *Il-6*. In contrast, a reduction in ER stress with the chemical ER chaperone tauroursodeoxycholic acid (TUDCA) (Ozcan et al., 2006) restored Rap1 activity to normal levels and reduced ER stress markers (Figures 7E and 7F). *Socs-3* was markedly reduced in the hypothalamus of TUDCA-treated (Figure 7G) and ESI-05-treated (Figure 7B) obese mice. These findings revealed a previously unrecognized mechanistic link between ER stress and Epac-Rap1 signaling in mediating hypothalamic leptin resistance.

### DISCUSSION

Overnutrition is associated with reduced sensitivity to key metabolic hormones such as leptin and insulin, and insensi-

tivity to these hormones is fundamental to the pathogenesis of metabolic disease. Although it is clear that the CNS participates in the biological responses to obesogenic conditions, the detailed neurobiological pathways remain unclear. In this study, we provide compelling genetic and pharmacological evidence that Rap1 in the CNS acts as a key component of the mechanistic pathway linking overnutrition to obesity and metabolic disorders. Mice with Rap1 deficiency in the CNS gained significantly less body weight and adiposity during HFD feeding compared with HFD-fed control mice and were resistant to HFD-induced systemic glucose imbalance, central leptin resistance, and peripheral insulin resistance. In contrast,  $Rap1^{\Delta CNS}$  mice showed little effect on body weight, energy expenditure, and hypothalamic regulation of feeding-related neuropeptides under normocaloric feeding. A specific role for Rap1 in metabolic dysfunction under a hypercaloric diet is further supported by current and previous findings that CNS Rap1 activity in HFD-fed mice is higher than in chow-fed ones. The decrease in adiposity in HFD-fed  $Rap1^{\Delta CNS}$  mice is likely due to hypophagia without changes in energy expenditure because motor activity and thermogenesis were unchanged. This unaltered energy expenditure in  $Rap1^{\Delta CNS}$  mice compared with controls is in accord with a previous study reporting that leptin does not actually enhance energy expenditure in mice but only prevents the decrease associated with leptin-induced hypophagia (Halaas et al., 1997). Additionally,  $Rap1^{\Delta CNS}$  mice have a decreased respiratory quotient, suggesting a preferential use of fat as an energy source. This may also contribute to the reduced adiposity gain during HFD feeding. Consistent with reduced feeding behavior,  $Rap1^{\Delta CNS}$  mice also had reduced mRNA levels of the orexigenic neuropeptides AgRP and NPY and increased mRNA encoding the anorexigenic neuropeptide POMC. As shown in Figures 1C and 1D, Rap1-expressing cells were retained in the hypothalamus of  $Rap1^{\Delta CNS}$  mice (*Rap1a<sup>fl/fl</sup>*, *Rap1b<sup>fl/fl</sup>*, *CaMKII $\alpha$ -Cre*). This is consistent with previous reports showing that Ca<sup>2+</sup>/calmodulin-dependent protein kinase II $\alpha$  (CaMKII $\alpha$ ) expression in the forebrain is almost exclusively restricted to excitatory, glutamatergic neurons and absent from GABAergic neurons and non-neuronal cells (Liu and Murray, 2012). Thus,  $Rap1^{\Delta CNS}$  mice are a partial knockdown model of CNS Rap1 and could potentially underestimate the role of CNS Rap1 in controlling metabolism. Collectively, these results suggest that CNS Rap1 plays a critical role in the development of HFD-induced obesity, whereas suppression can protect against obesity and metabolic disruption by controlling food intake and maintaining leptin and insulin sensitivity.

(C) Western blot images (left) and quantification (right) of SOCS-3, BIP, CHOP, eIF2 $\alpha$ , PERK,  $\beta$ -actin, and phosphorylated forms of eIF2 $\alpha$  and PERK in the hypothalamus of HFD-induced obese mice that were given ESI-05 (0.2 nmol, i.c.v., twice a day for 10 days) or vehicle (n = 7–10/group).

(D) Relative mRNA expression of *Il-6*, *Xbp1s*, *Bip*, *Chop*, *Edem*, *Atf4*, and *Grp94* in the hypothalamus of  $Rap1^{\Delta CNS}$ ,  $Rap1^{\Delta CNS}$ , or control animals were challenged with an HFD for 4 weeks. Age- and body weight-matched cohorts were used (n = 4–5/group).

(E) Relative mRNA expression of *Xbp1s*, *Bip*, *Chop*, *Edem*, *Atf4*, and *Grp94* in the brains of the mice. The hypothalami were collected from HFD-fed obese or normal chow-fed mice (HFD for 62 weeks, n = 4–7/group) that received vehicle or TUDCA (2.5  $\mu$ g, i.c.v.) for 3 days. Hypothalamic mRNA levels were determined by qPCR analyses.

(F and G) A chemical chaperone decreased both Rap1 activity (F) and mRNA expression of *Socs-3* (G) in the brain of HFD-induced obese mice. The brain samples were collected from HFD-fed obese or normal chow-fed mice (HFD for 62 weeks, n = 4–7/group) that received TUDCA (2.5  $\mu$ g, i.c.v.) for 3 days and subjected to a Rap1 assay and qPCR analyses.

\*p < 0.05, \*\*p < 0.01, \*\*\*p < 0.001 for one-way ANOVA followed by Tukey's multiple comparison test in (A), (B), and (D)–(G) or t tests in (C). All error bars are SEM.

In addition to its effect on energy balance, deletion of *Rap1a* and *Rap1b* from forebrain neurons resulted in markedly improved glucose tolerance, enhanced systemic insulin sensitivity, and increased cellular insulin signaling in skeletal muscle, adipose tissue, and liver under HFD feeding. Consistent with improved hepatic insulin sensitivity, *Rap1<sup>ΔCNS</sup>* mice exhibit reduced hepatic expression of the key gluconeogenic enzymes PEPCK and G6pc. Improved systemic glucose balance appears to occur independently of *Rap1* deletion-induced suppression of body weight and adiposity because age-, body weight- and adiposity-matched lean *Rap1<sup>ΔCNS</sup>* mice also displayed lower glucose levels and improved glucose and insulin tolerance under normocaloric diet feeding. Thus, these data suggest a previously unrecognized role for neuronal *Rap1* in controlling peripheral insulin sensitivity without changes in body weight. The molecular mechanisms mediating this effect are unclear but could involve greatly reduced hypothalamic SOCS-3 in neuronal *Rap1*-deficient mice because selective SOCS-3 deletion in VMH SF1 neurons (Zhang et al., 2008a) or leptin receptor-expressing cells (Pedroso et al., 2014) improves glucose and insulin tolerance and enhances peripheral insulin sensitivity without affecting body weight. However, additional mechanisms are likely involved because *Socs-3* expression alone did not induce glucose imbalance in mice when expressed in leptin-responsive neurons (Reed et al., 2010).

The robust effects of forebrain-specific *Rap1* deletion are likely mediated by enhanced hypothalamic sensitivity to leptin. *Rap1<sup>ΔCNS</sup>* mice fed an HFD have lower serum leptin levels than HFD-fed control mice, implying enhanced leptin sensitivity. We further demonstrate that pharmacological inhibition of CNS *Rap1* signaling also improves multiple indices of leptin sensitivity and protects mice against development of leptin resistance. *Rap1<sup>ΔCNS</sup>* mice failed to develop resistance to the anorectic and cellular actions of exogenous leptin under HFD feeding conditions. Notably, *Rap1<sup>ΔCNS</sup>* mice displayed enhanced leptin actions regardless of diet type, further supporting the primary role of *Rap1* in controlling neuronal leptin sensitivity. Although leptin sensitivity was increased in *Rap1<sup>ΔCNS</sup>* mice under both normal chow and HFD conditions, we observed that food intake and preferential oxidation of fat were only affected in mice under hypercaloric feeding. The lack of the effects during a normocaloric diet may be due to the lower concentration of serum leptin levels in chow-fed mice, which may not be high enough to induce reduced food intake and increased preferential oxidation of fat even in *Rap1*-deficient mice. Remarkably, reduced *Rap1* activity in the CNS, either by genetic deletion or by pharmacologic inhibition, resulted in suppression of two direct endogenous inhibitors of cellular leptin signaling, SOCS-3 and TCPTP, as well as inhibitory mechanisms such as inflammatory signals and UPR pathways. Because these inhibitors and inhibitory pathways appear to limit cellular leptin signaling under overnutrition (Bence et al., 2006; Bjørbaek et al., 1998; Cook and Unger, 2002; De Souza et al., 2005; Howard et al., 2004; Loh et al., 2011; Mori et al., 2004; Ozcan et al., 2009; Zabolotny et al., 2002; Zhang et al., 2008b), reductions associated with *Rap1* deficiency likely account, at least in part, for enhanced leptin sensitivity in *Rap1<sup>ΔCNS</sup>* mice. These results collectively

support our hypothesis that neuronal *Rap1* is a major regulator of leptin sensitivity and acts as a mediator of leptin resistance in obesity.

One of the most important questions arising from this study concerns how overnutrition leads to the activation of *Rap1* in the CNS. *Rap1* can be activated (converted from the GDP- to the GTP-bound form) by at least five distinct classes of guanine nucleotide exchange factors (GEFs) (Spilker and Kreutz, 2010). Previous studies reported that the GEFs *Epac1* and *Epac2* attenuate cellular leptin signaling in cultured cells and brain slices (Fukuda et al., 2011; Sands et al., 2006). However, the biological significance of this effect remains controversial because, although one study showed resistance to diet-induced obesity in *Epac1*-null mice (Yan et al., 2013), another reported augmented diet-induced obesity and glucose imbalance in *Epac1* global knockout mice (Kai et al., 2013). In contrast to these conflicting data on the role of *Epac1* in the control of whole-body energy and glucose balance, our findings suggest that brain *Epac2* is likely involved in *Rap1* activation and its effects on hypothalamic functions in HFD-induced obesity. Interestingly, *Rap1* activity is elevated in the brain of HFD-induced obese mice compared with that of normal chow fed-lean mice in the absence of changes in the total protein levels of *Rap1* (Figure 7A), *Epac1*, and *Epac2* (Figure S2C), indicating that HFD activation of *Epac-Rap1* signaling seems to be mediated via post-translational modification on *Epac*. Considering that *Epac* is directly activated by cyclic AMP (cAMP), one implication of this result is that *Rap1* could be activated via G protein-coupled receptors (GPCRs) that act through either *G<sub>s</sub>* or *G<sub>i</sub>* to modulate the cAMP-*Epac* pathway (Neves et al., 2002). Because CNS *Rap1* activity is increased by HFD, it is interesting to speculate that a GPCR ligand is produced in response to HFD that links overnutrition to *Rap1* activation in the CNS. Identification of such a circulating factor is a critical step for the development of agents that modulate CNS *Rap1*, possibly as pharmacological treatment for eating disorders, obesity, and metabolic diseases.

Our *ex vivo* studies revealed a previously unidentified link between *Rap1* and ER stress, the relevance of which was substantiated *in vivo*. These findings imply that reciprocal interaction perpetuates CNS ER stress and *Rap1* activation during overnutrition, subsequently leading to leptin resistance, and predict that inhibition of *Epac-Rap1* signaling breaks this link. Suppression of CNS ER stress produces anti-obesity benefits (Hosoi et al., 2014; Liu et al., 2015; Ozcan et al., 2009); therefore, suppression of *Epac-Rap1* signaling might also produce benefits by enhancing leptin sensitivity and improving energy balance. Our data strongly support this view. Central administration of ESI-05 to HFD-induced obese mice significantly reversed ER stress, expression of pro-inflammatory *Il-6*, and upregulation of the endogenous leptin inhibitor SOCS-3 in the hypothalamus. The effect of ESI-05 is sufficiently robust to restore leptin sensitivity to that of healthy lean mice. ESI-05 treatment is associated with prolonged weight loss maintenance even after treatment cessation. This distinguishing property of ESI-05 makes it more attractive as a potential anti-obesity therapy. Although ESI-05 alone showed its effect within a few days after the onset of treatment (Figures 5G and 5H), an acute anorectic effect was

only observed with ESI-05 plus a supraphysiological dose of leptin (5  $\mu$ g). This may be due to differences in the concentration of leptin in the brain. The cerebrospinal fluid leptin concentration (<0.5 ng/ml) was reported to be one to two orders of magnitude lower than serum levels (Schwartz et al., 1996). In addition, increasing the concentration of exogenous leptin in the brain causes a dose- and time-dependent decrease in body weight and food intake (Halaas et al., 1997; Rahmouni et al., 2002). Therefore, we speculate that the exogenous leptin given to the brain sensitized by ESI-05 causes a more robust and immediate reduction in body weight and food intake than endogenous leptin. Nevertheless, it is important to note that ESI-05 alone caused a body weight reduction in HFD-induced obese mice. Apart from its action in the brain, Epac2 was reported to be involved in insulin secretion in pancreatic  $\beta$  cells (Song et al., 2013; Zhang et al., 2009). Further studies will thus need to clarify whether systemic inhibition of Epac2 affects whole-body glucose balance. Additional evidence that Rap1 is the molecule that mediates the therapeutic benefit of ESI-05 comes from our experiments using Rap1<sup>ΔCNS</sup> mice. The lack of ESI-05 effects on leptin sensitivity in Rap1<sup>ΔCNS</sup> mice strongly suggests that the effect is via Rap1 and also rules out potential off-target effects of ESI-05. Most importantly, ESI-05 infusion alone causes weight loss in diet-induced obese and hyperleptinemic mice, likely by reversing leptin insensitivity associated with HFD-induced upregulation of SOCS-3. Collectively, these results demonstrate the potential of ESI-05 as a leptin sensitizer and provide insight into the promising, translational value of the Rap1 pathway.

## EXPERIMENTAL PROCEDURES

### Mice and Diets

Mice were used for all experiments. C57BL/6 mice were obtained from The Jackson Laboratory. Rap1<sup>loxp/loxp</sup>/Rap1b<sup>loxp/loxp</sup> mice were provided by Dr. Alexei Morozov (Pan et al., 2008). All genetically modified mice were backcrossed to the C57BL/6 (The Jackson Laboratory) background more than six times. Rap1<sup>ΔCNS</sup> mice were generated in the following breeding strategy: double-floxed male mice of *Rap1a* and *Rap1b* were crossed to the female CaMKII $\alpha$  Cre driver (line 159) (Minichiello et al., 1999). We used only female CaMKII Cre mice to obtain cohorts because CaMKII Cre is expressed in the testis (Figure 1A; Minichiello et al., 1999) and the male germline can produce offspring that carries the Cre allele in all tissues. From these matings, we produced mice with deletion of *Rap1a* and *Rap1b* in Cre-expressing neurons and control mice with floxed *Rap1a* and *Rap1b* genes. All mice were maintained under 12:12 hr light-dark cycle conditions and in a temperature-controlled environment with ad libitum access to water and a normal diet (Pico Lab 5V5R) or high-fat diet (60% kcal fat, Research Diet, D12492). The care of all animals and procedures conformed to the Guide for the Care and Use of Laboratory Animals of the NIH and were approved by the Institutional Animal Care and Use Committee of Baylor College of Medicine (AN-6076).

### Physiological Measurements

Body weight was measured weekly. Blood samples were collected via the saphenous vein from 4 hr-fasted mice. Serum blood was isolated after centrifugation (5000  $\times$  g for 10 min) at 4°C and stored at -80°C. Blood glucose was measured by using a One Touch Ultra blood glucose meter. Plasma leptin and insulin were analyzed with a Milliplex MAP mouse metabolic hormone magnetic bead panel kit. Glucose tolerance tests were performed on overnight-fasted mice. D-glucose (1.5 g/kg) was injected intraperitoneally, and blood glucose was measured at indicated time points. Insulin tolerance tests were performed on 4 hr-fasted mice. Insulin (1 U/kg) was injected intraperitoneally, and blood glucose was measured at indicated time points.

### Body Composition and Energy Expenditure Measurements

Whole-body composition was measured using nuclear magnetic resonance imaging (EchoMRI). Body weight- and body composition-matched 5-week-old control and Rap1<sup>ΔCNS</sup> mice were fed on a high-fat diet. Two weeks later, metabolic assessment was performed at 7 weeks of age. Mice were first acclimatized to the metabolic cages and housed individually for 3 days before measurements were taken. Metabolic parameters, including O<sub>2</sub> consumption, CO<sub>2</sub> production, respiratory exchange ratio, heat production, ambulatory activity, and food intake, were determined by using the Columbus Instruments comprehensive lab animal monitoring system (CLAMS).

### Leptin Sensitivity Test

Mice were singly housed and acclimatized for 1 week prior to the study. Body weight- and body composition-matched 15-week-old control and Rap1<sup>ΔCNS</sup> mice were placed on a high-fat diet for 8 weeks. Both groups were injected intraperitoneally with vehicle (Dulbecco's PBS [dPBS], Sigma-Aldrich, D8537) twice a day (5 p.m. and 9 a.m.) for 4 consecutive days. Three days after the last vehicle treatment, mice were injected intraperitoneally with leptin (3 mg/kg, Harbor-UCLA Research and Education Institute) twice a day for 6 consecutive days. Food intake and body weight were measured daily. Similarly, we performed a leptin sensitivity test for ESI-05 treatment mice. Intracerebroventricular (i.c.v.) surgery was carried out on mice fed a high-fat diet, and then they were singly housed. One week after the i.c.v. surgery, the mice were injected with vehicle, leptin (2  $\mu$ g/mouse), ESI-05 (0.2 nmol/mouse), or leptin/ESI-05 twice a day. Body weight and food intake were measured daily.

### Organotypic Brain Slice Culture

Hypothalamic slices were made essentially as described before (Fukuda et al., 2011). Briefly, C57BL/6 mice pups, 8–11 days old, were decapitated, and the brains were quickly removed. Hypothalamic tissues were blocked and sectioned in a depth of 250  $\mu$ m on a vibratome (VT1000S, Leica) in chilled Gey's balanced salt solution (Invitrogen) enriched with glucose (0.5%) and KCl (30 mM). The coronal slices containing the arcuate nucleus were then placed on Millicell-CM filters (Millipore, pore size 0.4  $\mu$ m, diameter 30 mm) and maintained at an air-medium interface in minimum essential media (Invitrogen) supplemented with heat-inactivated horse serum (25%, Invitrogen), glucose (32 mM), and GlutaMAX (2 mM, Invitrogen). Cultures were typically maintained for 10 days in standard medium, which was replaced three times a week. After 10 days, the slices were used for experiments.

### Cannula Implantation and Treatments

Mice were anesthetized with isoflurane and positioned in a stereotaxic frame. The skull was exposed, and a 26-gauge single stainless steel guide cannula (C315GS-5-SPC, Plastics One) was implanted into the third cerebral ventricles (-0.9 mm from the bregma,  $\pm$  0.5 mm lateral, -2.5 mm from the skull). The cannula was secured to the skull with screws and dental cement. After i.c.v. cannulation, the mice were housed singly and given at least 1 week to recover. On experimental days, the mice were infused with 1  $\mu$ l of each solution: vehicle (dPBS or DMSO), leptin (2  $\mu$ g/mouse), ESI-05 (0.2 nmol/mouse, Axxora, BLG-M092-05), leptin/ESI-05, TU (10  $\mu$ g/mouse, EMD Millipore, 654380), TU/ESI-05, or TUDCA (2.5  $\mu$ g/mouse, EMD Millipore, 580549).

### Detection of GTP-Rap1 by Rap1 Pull-Down Assay

A Rap1 pull-down assay was performed using the Active Rap1 pull-down and detection kit (Thermo Fisher Scientific) according to the manufacturer's recommendation. After brain samples were dissected out, samples were snap-frozen and subsequently stored at -80°C. Proteins were extracted by the provided lysis/wash buffer with protease cocktail inhibitor, and then lysates were centrifuged at 16,000  $\times$  g for 15 min at 4°C. The protein concentration was determined with BCA protein assay reagent (Pierce, 23225) with BSA as standard. Equal amounts of protein were subjected to affinity precipitation of GTP-Rap1 by using the Active Rap1 pull-down and detection kit. The amount of Rap1 was assessed by performing western blotting with the provided antibody (1:1,000). To assess the levels of total Rap1 or  $\beta$ -actin, cell extract was directly applied to western blotting without a pull-down assay.

## Statistics

The data are presented as mean  $\pm$  SEM. Statistical analyses were performed using GraphPad Prism for a two-tailed unpaired Student's *t* test or one- or two-way ANOVA followed by post hoc Tukey's, Bonferroni's, or Sidak's tests. *p* < 0.05 was considered to be statistically significant.

## 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.celrep.2016.08.039>.

## AUTHOR CONTRIBUTIONS

M.F. conceived the study. K.K. and M.F. designed the experiments. K.K., P.X., E.L.C., S.S.C., and A.N. performed the experiments. A.M. and Y.X. contributed reagents and intellectually assisted with the Rap1<sup>ACNS</sup> mouse studies. K.K., P.X., and M.F. analyzed the data and interpreted the results. The major part of the manuscript was written by M.F. with some help from K.K. All authors approved the final version of the manuscript.

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