

Protein Tyrosine Phosphatase Epsilon Affects Body Weight by Downregulating Leptin Signaling in a Phosphorylation-Dependent Manner

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SUMMARY

Molecular-level understanding of body weight control is essential for combating obesity. We show that female mice lacking tyrosine phosphatase epsilon (RPTPe) are protected from weight gain induced by high-fat food, ovariectomy, or old age and exhibit increased whole-body energy expenditure and decreased adiposity. RPTPe-deficient mice, in particular males, exhibit improved glucose homeostasis. Female nonobese RPTPe-deficient mice are leptin hypersensitive and exhibit reduced circulating leptin concentrations, suggesting that RPTPe inhibits hypothalamic leptin signaling *in vivo*. Leptin hypersensitivity persists in aged, ovariectomized, and high-fat-fed RPTPe-deficient mice, indicating that RPTPe helps establish obesity-associated leptin resistance. RPTPe associates with and dephosphorylates JAK2, thereby downregulating leptin receptor signaling. Leptin stimulation induces phosphorylation of hypothalamic RPTPe at its C-terminal Y695, which drives RPTPe to downregulate JAK2. RPTPe is therefore an inhibitor of hypothalamic leptin signaling *in vivo*, and provides controlled negative-feedback regulation of this pathway following its activation.

INTRODUCTION

High-calorie, fat-rich diets play central roles in the obesity pandemic. The hormone leptin, which is produced in white adipose tissue, is an established regulator of body weight and energy balance (Friedman and Halaas, 1998; Spiegelman and Flier, 2001). Binding of leptin to its receptor in the hypothalamus activates the JAK2 tyrosine kinase, which leads to phosphorylation of the receptor and activation of downstream signaling, including the STAT3, ERK, and PI3-kinase pathways (Myers et al., 2008). Leptin stimulates a class of hypothalamic neurons

to produce pro-opiomelanocortin (POMC), which suppresses food intake and increases energy expenditure. In parallel, leptin inhibits production of the agouti-related protein (AgRP) and neuropeptide Y (NPY) in a separate set of hypothalamic neurons; both peptides stimulate food intake and inhibit energy expenditure (Konner et al., 2009; Morton et al., 2006; Myers et al., 2009). Leptin affects body weight also by targeting neurons outside the hypothalamus (Myers et al., 2009), such as by inhibiting synthesis of serotonin in the brainstem (Yadav et al., 2009). In addition, hypothalamic leptin signaling affects glucose homeostasis by improving peripheral insulin sensitivity (Buettner and Camacho, 2008). Obesity is often associated with leptin resistance, in which the response of the hypothalamic leptin receptor to its ligand is reduced. As a result, most obese humans exhibit elevated concentrations of circulating leptin and respond poorly to the exogenously administered hormone (Myers et al., 2008).

Leptin signaling is under tight control of molecules that target the receptor or its downstream effectors. For example, SOCS3, whose expression is induced by leptin, inhibits signaling by binding JAK2 and the leptin receptor (Myers et al., 2008), while the non-receptor-type tyrosine phosphatase PTP1B inhibits leptin signaling by targeting JAK2. Inactivation of PTP1B in mice causes reduced adiposity and increased sensitivity toward leptin and insulin (Bence et al., 2006; Cheng et al., 2002; Elchebly et al., 1999; Klaman et al., 2000; Xue et al., 2009; Zabolotny et al., 2002). In contrast, the PH and SH2 domain-containing protein SH2B1 supports signaling by the receptor. Accordingly, mice lacking SH2B1 are obese and suffer from hyperglycemia, insulin and leptin resistance, and hyperlipidemia (Ren et al., 2007).

The importance of tyrosine phosphorylation in regulating leptin receptor signaling suggests that protein tyrosine phosphatases (PTPs) (Alonso et al., 2004; Tonks, 2006), which antagonize tyrosine kinase activity, may affect body weight. The SH2 domain-containing phosphatase SHP2 promotes leptin receptor signaling by binding to Y985 of the leptin receptor and thus activating ERK; however, SHP2 also downregulates signaling by inhibiting JAK2/STAT3 activity (Myers et al., 2008; Zhang et al., 2004). The phenotype of mice lacking neuronal SHP2—increased adiposity, decreased leptin sensitivity, and reduced energy expenditure—indicates that overall SHP2 upregulates leptin signaling. PTP1B and SHP2 show that individual PTPs may support or inhibit

signaling by the leptin receptor (Banno et al., 2010; Krajewska et al., 2008; Zhang et al., 2004). PTPs may affect body weight also by regulating other pathways. For example, mice lacking the dual-specificity PTP MKP-1 exhibit enhanced energy expenditure and resistance to diet-induced obesity, despite not showing leptin-related abnormalities (Wu et al., 2006).

In this study we examined whether PTP epsilon, a phosphatase that downregulates insulin receptor signaling in cells (Aga-Mizrachi et al., 2007; Andersen et al., 2001; Nakagawa et al., 2005), helps regulate body weight. We show that female mice lacking PTPe (EKO mice) are protected from weight gain that normally occurs following a high-fat diet (HFD), ovariectomy, or aging. EKO female mice are markedly leptin sensitive; circulating levels of leptin are reduced in obese, ovariectomized, or aged EKO mice, indicating that these mice remain leptin sensitive under physiological conditions associated with leptin resistance. EKO mice of both genders also exhibit improved response to glucose load. At the molecular level RPTPe associates with and dephosphorylates JAK2, thereby downregulating leptin receptor signaling. Leptin-induced phosphorylation of RPTPe at its C-terminal Y695 enhances this process. We conclude that RPTPe is a physiological inhibitor of leptin signaling in the hypothalamus, which acts by targeting JAK2 following activation of the leptin receptor.

RESULTS

Female EKO Mice Are Protected from Challenge-Induced Weight Gain

Mice that genetically lack PTPe (EKO mice) are born in Mendelian ratios, gain weight normally, and maintain proper weight as adults (Tiran et al., 2006, and data not shown). However, at 14–16 months of age, EKO female mice weigh 16% less than matched wild-type (WT) controls; a similar trend exists in male mice (Figure 1A). In order to examine if body weight regulation is abnormal in EKO mice, we subjected young adult (4-month-old) mice to a HFD. When placed on this diet for 11 weeks, female EKO mice gained 55% less weight than matched WT control mice; no similar resistance to HFD was noted in male EKO mice (Figures 1B and 1C). Female rodents gain weight significantly following ovariectomy (e.g., Ainslie et al., 2001). WT and EKO female mice aged 3 months were ovariectomized and followed for 10 weeks. At the end of this period, WT ovariectomized mice added 32% to their starting weight, while no weight gain was observed in ovariectomized EKO mice (Figure 1D). Similar protection from weight gain was observed when groups of WT and EKO mice were ovariectomized at the ages of 7 or 14–16 months (Figure S1, available online, and data not shown). The older group of EKO mice combined the effects of both old age and ovariectomy: these mice weighed less than their WT counterparts prior to ovariectomy, and gained no weight following the procedure (Figure S1). We conclude that female mice lacking PTPe are protected from weight gain that occurs in old age and when body weight homeostasis is challenged following a HFD or ovariectomy.

In all the above cases, protection of EKO mice from weight gain was associated with reduced body fat content. HFD, ovariectomy, or old age significantly increased the relative weight of abdominal white fat pads of WT female mice; the effect in EKO

mice was either markedly reduced or entirely absent (Figure 1E). Similar results were obtained when the whole-body fat content of elderly (>14 months) WT and EKO female mice was examined pre- and postovariectomy by dual energy X-ray absorptiometry (DEXA) scanning (Figure S1). A trend for lighter abdominal fat pads that did not reach statistical significance was noted in elderly EKO males (Figure 1E). These findings correlated with a smaller size of adipose cells in the fat pads of EKO mice fed a HFD, whose average cross-sectional area was reduced by 25.3% (Figure 1F). No abnormalities were detected in brown adipose tissue of EKO mice (data not shown).

Increased Metabolic Rates of EKO Mice

EKO mice consume the same amount of food as their age- and gender-matched WT counterparts (Figures S2A and S2B), suggesting that the resistance to weight gain in EKO mice may be due to increased energy expenditure. In order to examine this possibility, female WT and EKO mice were examined by indirect calorimetry. Mice were placed in individual cages and their oxygen consumption, CO₂ production, heat production, food consumption, and physical activity were measured continuously for a period of 72 hr. When fed regular chow, EKO mice displayed strong trends for increased consumption of oxygen, increased CO₂ production, and increased heat production (Figures 2A–2C). These trends reached clear statistical significance when the mice were fed high-fat food (Figures 2A–2C). The respiratory exchange ratio (RER), which reflects the relative use of fat or carbohydrates as a source of energy, was similar in both genotypes, although, as expected, the shift from regular lab chow to a HFD lowered this ratio significantly (Figure 2D). The physical activity of WT and EKO mice was similar (Figures S2C and S2D). We conclude that female EKO mice exhibit a pre-existing trend for increased energy expenditure. This difference is strengthened significantly when EKO mice are challenged with a HFD, consistent with the reduced weight gain of these mice in this experimental paradigm.

Decreased Circulating Leptin Concentrations in EKO Mice

In order to determine the basis for resistance to weight gain in female EKO mice, we examined these mice in greater detail. Female EKO mice fed regular chow exhibited a very strong trend for reduced circulating leptin concentrations, both in absolute terms and after normalizing to body weight (Table 1). When WT mice were challenged with a HFD, ovariectomized, or aged past 14 months, their concentrations of circulating leptin increased significantly, consistent with the establishment of a leptin-resistant state. In contrast, leptin concentrations were significantly lower in EKO mice that were similarly challenged (Table 1). Trends for reduced leptin concentrations were found in male EKO mice. Importantly, reduced concentrations of circulating leptin do not simply reflect reduced adiposity of EKO mice, since the results shown in Table 1 were normalized to body weight and since a very strong trend for reduced leptin concentrations was found in nonobese EKO females of normal weight. These results suggest that female EKO mice are leptin hypersensitive.

Improved Glucose Homeostasis in EKO Mice

As both body weight and leptin affect insulin sensitivity, we examined whether EKO mice exhibit altered glucose

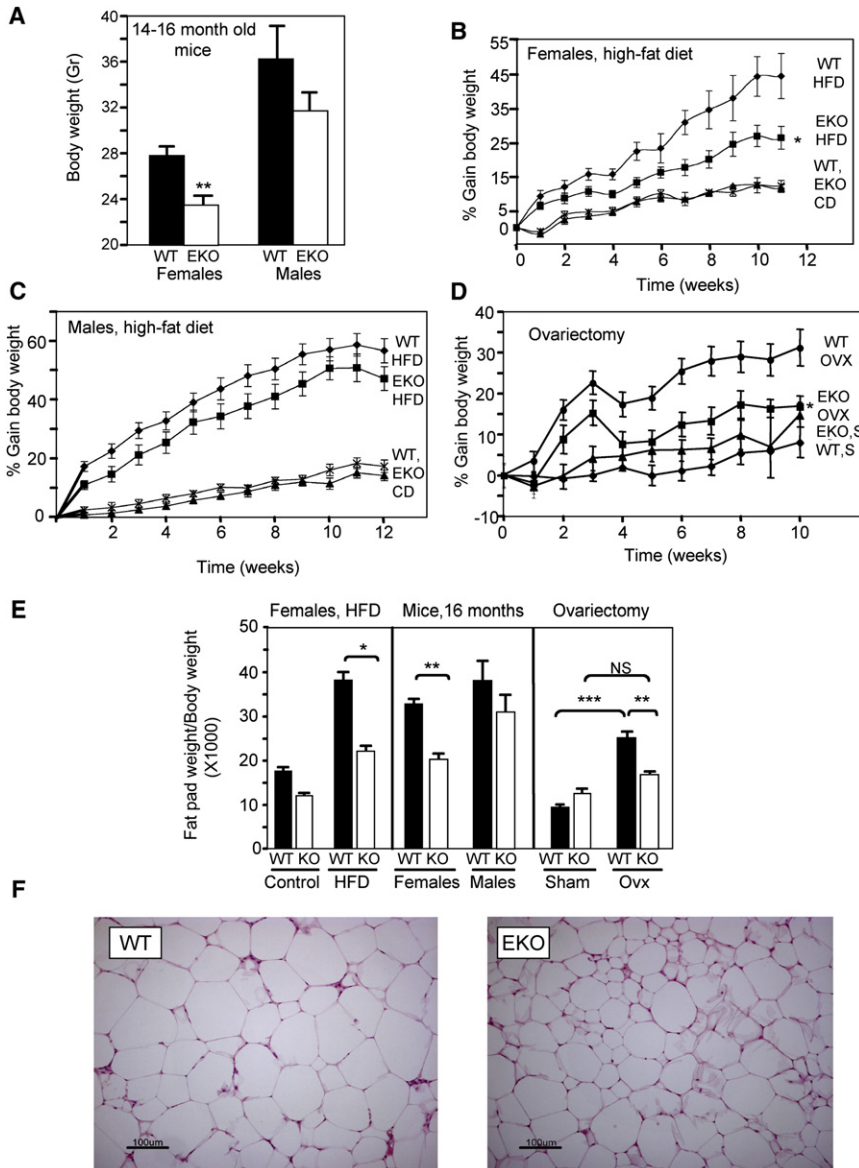


Figure 1. Female EKO Mice Are Protected from Stress-Induced Weight Gain

(A) EKO female mice aged 14–16 months are thinner than their WT counterparts ($n = 12$ per genotype, $**p = 0.0003$ by Student's *t* test).

(B) Four-month-old WT or EKO female mice were fed a HFD or regular chow (CD) for 11 weeks. The starting weights of mice from all experimental groups were similar (mean \pm SE, EKO; HFD, 19.9 ± 0.5 gr; WT, CD, 20.9 ± 0.8 gr; $n = 8$, HFD; $n = 5$, CD). EKO mice fed HFD gain less weight than WT mice fed similar food ($p = 0.004$, by two-way ANOVA with Fisher's Least Significant Difference test).

(C) Similar to (B), performed on WT and EKO male mice. WT HFD and EKO HFD data are not distinct, analyzed as in (B).

(D) Three-month-old female mice were either ovariectomized (OVX) or sham-operated (S). $N = 5$ mice per genotype and treatment. The starting weights of all groups of mice were similar; EKO OVX mice gain less weight than WT OVX mice ($p < 0.0001$, analyzed as in B).

(E) Weights of abdominal fat pads (normalized to body weight) of WT or EKO mice studied in (A)–(D). NS, nonsignificant ($n = 5$ –12 mice per bar; $*p = 0.02$; $**p \leq 0.003$; $***p = 0.0003$ by Student's *t* test).

(F) Sections of paraffin-embedded abdominal white fat pads of WT and EKO mice fed a HFD were stained with hematoxylin and eosin and analyzed using the Osteoll software package (Bioquant, Nashville, TN). The mean cross-sectional area of EKO cells was reduced by 25.3% relative to WT cells (cross-sectional areas [mean \pm SE, in square microns], WT, 4559.1 ± 119.7 [$n = 1501$ cells]; EKO, 3635.6 ± 69.7 [$n = 1708$ cells]; $p = 1.2 \times 10^{-12}$ by Student's *t* test). In this and in all subsequent figures, error bars represent SE.

homeostasis. Fasting concentrations of glucose and insulin were similar in plasma of WT and EKO mice of both genders that were fed regular chow (Table 1). Exposing EKO and WT mice to a HFD for 12 weeks significantly increased their fasting concentrations of circulating glucose and insulin; however, glucose and insulin concentrations in male EKO mice fed a HFD were markedly reduced relative to WT mice, suggesting insulin hypersensitivity (Table 1). When challenged with an intraperitoneal glucose load, EKO male mice displayed significantly improved responses when fed regular or high-fat food. Similar but weaker findings were noted in female EKO mice (Figures 3A–3D). Injection of insulin resulted in a rapid and similar drop in circulating glucose concentrations in both WT and EKO mice; however, glucose concentrations remained lower for a longer time period in male EKO mice fed a HFD (Figures 3E–3H). Intraperitoneal injection of insulin resulted in a more

pronounced increase in phosphorylation of AKT in liver and muscle tissue of EKO male mice (Figures 3I and 3J), confirming the insulin hypersensitivity of these mice. Overall, these results indicate that EKO

EKO Mice Are Leptin Hypersensitive

Reduced circulating leptin concentrations correlated most closely with the weight gain resistance phenotype of EKO mice; both were most prevalent in EKO females that had been challenged metabolically. Since reduced leptin concentration in circulation may reflect increased sensitivity of leptin signaling in the hypothalamus, we examined this issue in greater depth. PTPe mRNA is found in the arcuate, paraventricular, and ventromedial hypothalamic nuclei in mice (Figures 4A–4F), in which leptin acts. Dual in situ hybridization for PTPe and the leptin receptor in the arcuate nucleus revealed colocalization of both mRNAs in cells in this region (Figures 4G–4I); most of the cells that expressed the leptin receptor coexpressed PTPe. Protein blot

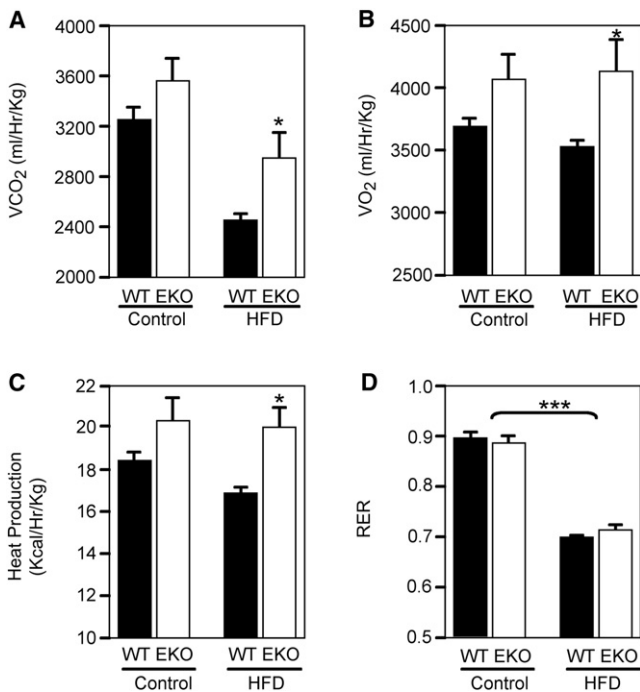


Figure 2. EKO Female Mice Exhibit Increased Whole-Body Energy Expenditure by Indirect Calorimetry

Four-month-old WT or EKO female mice were fed a HFD or regular chow (control), and their production of carbon dioxide (VCO_2), oxygen consumption (VO_2), and heat production were analyzed for 72 hr ($p \leq 0.037$, $n = 6$ per genotype and treatment). HFD lowered the respiratory exchange rate (RER) in both genotypes ($p < 0.0001$). Feeding and mobility of WT and EKO mice were similar (Figure S2). Data are from the night, active phase.

analysis of dissected hypothalamic tissue revealed strong expression of the receptor-type form of PTPe, RPTPe; this form is entirely absent from hypothalami of EKO mice (Figure 4J). No compensatory changes were found in expression of RPTP alpha, a PTP that is closely related to RPTPe, or of PTP1B or SHP2, which affect hypothalamic leptin signaling (Figure 4J). No obvious compensatory changes in expression of other

PTPs were noted in EKO mouse hypothalamus (Figure S3A). RPTPe is therefore present in the hypothalamic nuclei that respond to leptin and participate in regulating body weight. Of note, expression of RPTPe protein in adipose tissue, where leptin is produced, is almost undetectable (Figure S3B).

In order to determine if EKO female mice are hypersensitive to leptin, we stimulated them with leptin in acute and chronic administration protocols. Four-month-old female WT and EKO mice of similar weight were injected intraperitoneally with various doses of leptin or with vehicle alone (PBS). After 30–45 min the mice were sacrificed and hypothalamic STAT3 phosphorylation was examined as readout for leptin receptor activation (Figures 4K and 4L). Basal phosphorylation of STAT3, which was similar in hypothalami of WT and EKO mice injected with PBS, increased significantly following injection of leptin. However, leptin-induced STAT3 phosphorylation was 38% stronger in EKO hypothalami (Figure 4L), confirming their leptin hypersensitivity. Similar results were obtained in mice fed a HFD (data not shown), indicating that leptin hypersensitivity persists also in obese EKO animals. In contrast, male EKO and WT mice responded similarly in this system (Figure 4L). Chronic administration of leptin to mice for several days reduced food intake in female EKO mice more significantly than similarly treated WT mice (Figure 4M); a trend for more significant loss in body weight was observed in EKO mice (data not shown). We conclude, therefore, that female EKO mice are leptin hypersensitive. Importantly, the hypothalami of WT and EKO mice that had not been stimulated with leptin contained similar amounts of mRNAs for the leptin receptor and for peptides that it regulates (NPY, AGRP, POMC) (Figure S4). Leptin hypersensitivity of EKO mice therefore most likely originates in aberrant regulation of leptin receptor signaling following its activation and not, for example, in increased expression of the receptor. Collectively, the above findings indicate that leptin hypersensitivity precedes the body weight phenotype of EKO mice and is not caused by it.

RPTPe Downregulates Leptin Receptor Signaling

The increase in hypothalamic STAT3 phosphorylation following stimulation of EKO mice with leptin strongly suggests that

Table 1. Concentrations of Circulating Leptin, Glucose, and Insulin in WT and EKO Mice

		Females				Males		
		Control	HFD	Old Age	OVX	Control	HFD	Old Age
Plasma leptin (pg/ml per gr BW)	WT	92.1 ± 13.7	168.9 ± 35.9	403.2 ± 51.8	439.8 ± 63.1	77.4 ± 18.5	743 ± 120	720 ± 39
	EKO	51.8 ± 11.4 (0.053)	76.9 ± 10.1 (0.014)	180.6 ± 19.9 (0.0006)	229.3 ± 83.8 (0.045)	59.5 ± 7.2 (NS)	629 ± 128 (NS)	412 ± 178 (NS)
Fasting glucose (mg/dl)	WT	57.3 ± 2.3	70.1 ± 2.4			69.2 ± 4.9	102.2 ± 6.5	
	EKO	55.9 ± 2.4 (NS)	67.4 ± 4.4 (NS)			65.9 ± 2.7 (NS)	80.8 ± 4.4 (0.014)	
Fasting insulin (ng/ml)	WT	0.37 ± 0.11	0.79 ± 0.21			0.29 ± 0.06	1.47 ± 0.27	
	EKO	0.37 ± 0.05 (NS)	0.51 ± 0.10 (NS)			0.35 ± 0.09 (NS)	0.80 ± 0.24 (0.033)	

Plasma leptin concentrations in 4-month-old mice that were fed regular chow (control), fed a HFD for 12 additional weeks (HFD), were ovariectomized and followed for 10 additional weeks (OVX), or were 14–17 months old (old age). Fasting serum and insulin were measured in 4-month-old mice fed regular chow (control) and in mice fed high-fat food for 5–7 additional weeks. All mice were fasted for 16–22 hr prior to sampling. Data are mean ± SE, $n = 4$ –17 mice per genotype, gender, and treatment. P values compare the EKO value with the WT value above it. NS, nonsignificant.

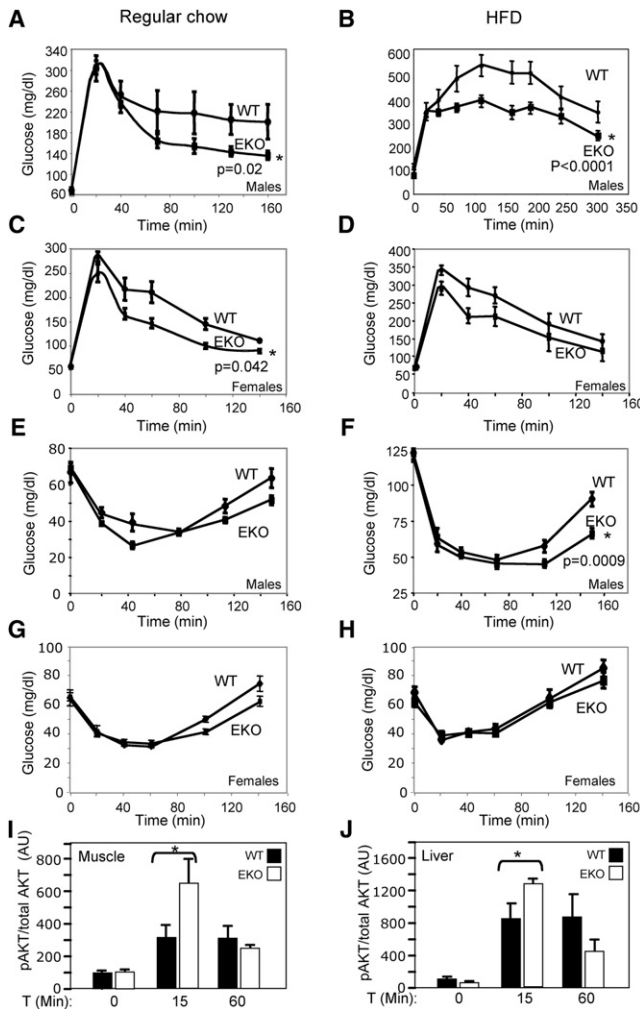


Figure 3. Glucose Metabolism in EKO Mice

(A–D) Glucose tolerance test. Eight-week-old WT or EKO mice were injected IP with glucose (1.5 gr/Kg body weight); blood glucose concentrations were measured at the indicated times.

(E–H) Mice were injected with insulin (0.75 U/Kg body weight); blood glucose was followed as above. Mice were fed regular lab chow (A, C, E, G) or a HFD for 5–7 weeks before the experiment (B, D, F, H). Differences between WT and EKO graphs marked with asterisks are significant by two-way ANOVA with Fischer’s Least Significant Difference test.

(I and J) Male mice fed regular chow were injected IP with insulin. Phosphorylation of AKT at T308 was analyzed by protein blotting. AKT phosphorylation at Ser473 was similar in both genotypes (data not shown). Shown are mean + SE; *p ≤ 0.044 by Student’s t test, n = 5–10 mice per bar.

RPTPe downregulates leptin signaling by targeting STAT3 or elements further upstream, such as JAK2 or the leptin receptor itself. In agreement, expression of RPTPe in 293T cells significantly decreased leptin-induced phosphorylation of STAT3 (at Y705), JAK2 (at Y1007/1008), and AKT/PKB (at T308) (Figures 5A–5C), sites that are required for activation of their respective molecules. Presence of RPTPe also decreased JAK2-induced phosphorylation of the leptin receptor (Figure S5A). As expected, the catalytically inactive, substrate-trapping mutant D302A RPTPe did not reduce phosphorylation of JAK2 and STAT3 in

similar experiments (Figure S5B). Purified RPTPe, but not its inactive mutant D302A RPTPe, dephosphorylated purified JAK2 when the two proteins were mixed in vitro (Figure 5D and Figure S5C), suggesting that JAK2 is a substrate of RPTPe. JAK2 and RPTPe formed a stable complex in 293T cells in the absence of the leptin receptor (Figure 5E); D302A RPTPe also bound JAK2 (Figure S5D). Purified RPTPe and JAK2 physically associate in vitro, suggesting that this association is direct (Figure S5C). Although presence of the leptin receptor or its activation is not strictly required for the RPTPe–JAK2 association, the receptor does coprecipitate with the JAK2–RPTPe complex (Figure 5E). Furthermore, in some cases (e.g., Figure 5E), presence of the receptor strengthened somewhat the association between RPTPe and JAK2. We conclude that JAK2 and RPTPe are present in the same molecular complex, and that RPTPe downregulates leptin receptor signaling by dephosphorylating JAK2. Yet, we cannot rule out at the present time the possibility that RPTPe also targets the leptin receptor or STAT3.

Leptin-Induced C-Terminal Phosphorylation of RPTPe Increases Its Ability to Target JAK2

The physiological role of PTPe can be affected by phosphorylation at its C-terminal tyrosine (Y695 in RPTPe = Y638 in the non-receptor isoform, cyt-PTPe; Berman-Golan and Elson, 2007; Granot-Attas et al., 2009). In order to examine if leptin stimulation induces phosphorylation of RPTPe in the hypothalamus, we injected young adult nonobese WT female mice with leptin, isolated hypothalamic tissue at various times afterwards, and examined phosphorylation of endogenous RPTPe at Y695. Basal phosphorylation of RPTPe was observed in these studies; phosphorylation increased significantly following leptin injection, reaching maximal levels after 60 min and declining thereafter (Figures 6A and 6B). Leptin-induced phosphorylation of RPTPe in WT mice was more prominent in mice fed a HFD than in mice fed regular food (Figure 6B). This finding is consistent with RPTPe playing a role in establishing the leptin-resistant state in obese mice, as discussed below.

Leptin-induced phosphorylation of RPTPe was observed also in 293 cells expressing the leptin receptor and JAK2; leptin-induced phosphorylation was prevented by omission of exogenous JAK2 (Figure 6C). Together with phosphorylation of purified RPTPe at Y695 by purified JAK2 in vitro (Figure 6D), these results indicate that JAK2 can phosphorylate RPTPe downstream of the activated leptin receptor. Importantly, while WT RPTPe induced dephosphorylation of JAK2 in cells treated with leptin, its nonphosphorylatable mutant Y695F RPTPe did so less efficiently (Figure 6E); this suggests that phosphorylation of RPTPe at Y695 is important for its ability to act upon JAK2. WT RPTPe was also more capable of reducing STAT3 phosphorylation (Figure 6E). We conclude that activation of the leptin receptor induces phosphorylation of RPTPe at Y695, thus increasing the ability of RPTPe to dephosphorylate JAK2 as part of a negative feedback regulatory mechanism for hypothalamic leptin signaling.

DISCUSSION

The pre-existing leptin hypersensitivity in young female EKO mice that precedes their obesity-resistant phenotype, and the

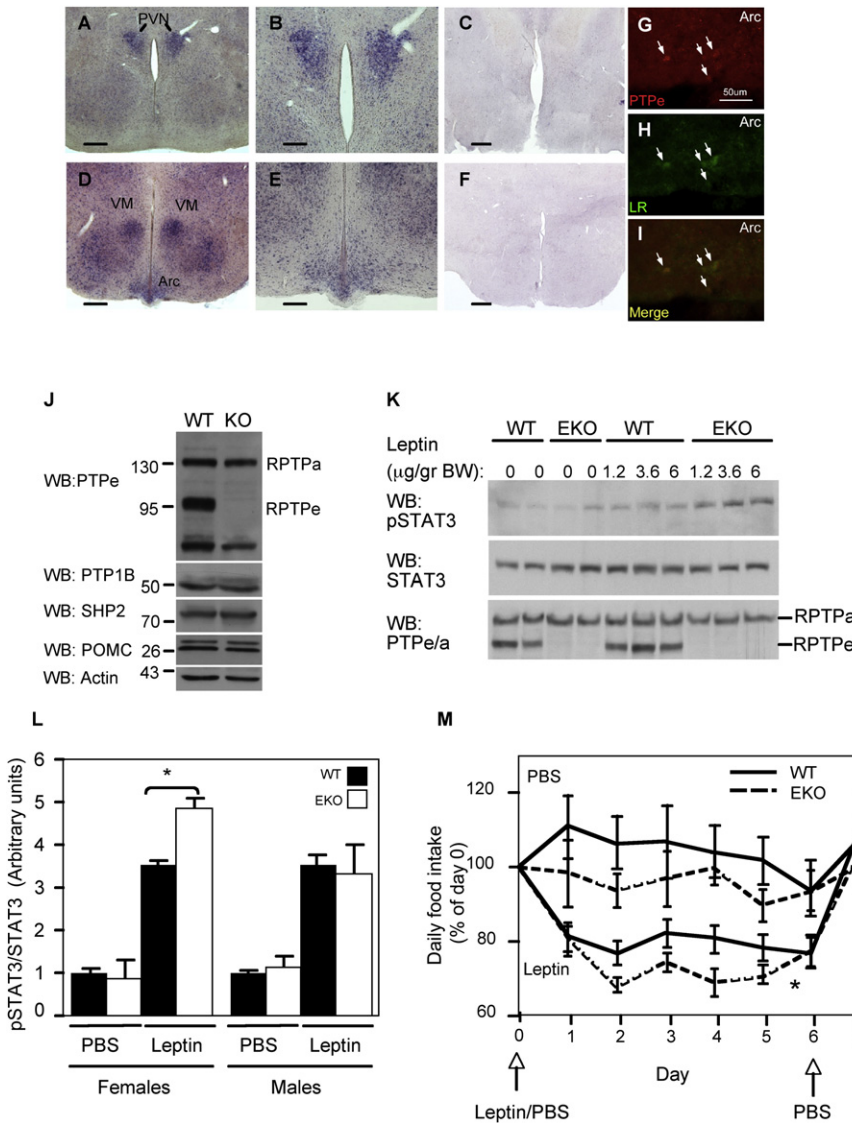


Figure 4. Female EKO Mice Are Leptin Hypersensitive

(A–I) PTPe is expressed in the hypothalamus. WT mouse brain was hybridized to an antisense (A, B, D, E) or sense (negative control, C and F) PTPe cRNA probe. (A)–(C), Bregma –0.82 mm; (D)–(F), Bregma –1.46 mm. (B) and (E) show higher magnifications of (A) and (D), respectively. (G)–(I) show double in situ analysis in the arcuate nucleus using probes for PTPe (red) and leptin receptor (green) mRNAs. PVN, paraventricular nucleus. VM, ventromedial nucleus. Arc, arcuate nucleus. Bars, 250 μm (A, C, D, F) or 125 μm (B and E).

(J) RPTPe protein is expressed in the hypothalamus. Hypothalamic tissue from 2-month-old female WT and EKO mice was analyzed by protein blotting. POMC serves as a hypothalamic marker. (K) Acute leptin treatment: 4-month-old female WT or EKO mice were fasted for 16 hr and then injected intraperitoneally with vehicle (PBS) or leptin at the indicated doses. Hypothalami were collected 30 min later.

(L) Female and male WT and EKO mice were injected with leptin (5 μg/gr body weight) or PBS; hypothalami were collected 45 min later. N = 11–12 mice per bar. *p = 0.036 by Student's t test.

(M) Chronic leptin treatment: following an adaptation period, 4-month-old female mice were injected with PBS or leptin twice daily starting on day 0. From day 6 all mice were injected with PBS. Shown is the daily food intake relative to day 0. The graphs for leptin-treated WT and EKO mice are distinct (p < 0.05) by two-way ANOVA with Fischer's Least Significant Difference test. N = 8–12 mice per genotype and treatment.

relatively low concentrations of circulating leptin when these mice are metabolically challenged strongly suggest that RPTPe downregulates leptin receptor signaling in the hypothalamus. High-fat food, ovariectomy, and old age are associated with weight gain, increased concentrations of circulating leptin, and decreased sensitivity to leptin (Ainslie et al., 2001; Carrascosa et al., 2009; Kelesidis et al., 2010). The pre-existing leptin hypersensitivity of EKO mice counteracts at least in part the leptin-resistant state that is induced in WT mice in these situations, and most likely leads to reduced weight gain that is observed in EKO mice. RPTPe is therefore part of the molecular mechanism that induces leptin resistance when mice are challenged as above.

Our molecular studies strongly suggest that RPTPe inhibits leptin receptor signaling by targeting the downstream kinase JAK2 in a manner supported by phosphorylation of RPTPe at Y695. RPTPe coimmunoprecipitates with JAK2 and dephosphorylates it at Y1007/1008. Y1007 is located within the activa-

tion loop of JAK2, and its phosphorylation is required for activation of the kinase (Feng et al., 1997). Dephosphorylation of JAK2 at this site by RPTPe is then consistent with RPTPe inhibiting leptin receptor signaling and with the leptin sensitivity phenotype of EKO mice. The issues of whether RPTPe targets additional tyrosine residues in JAK2 and whether RPTPe targets the leptin receptor directly require further studies.

Activation of the leptin receptor in vivo in the mouse hypothalamus and in cultured cells induces phosphorylation of RPTPe at its C-terminal Y695; JAK2 participates in this process, either alone or in concert with other kinases. Phosphorylation of RPTPe has clear physiological consequences, since the nonphosphorylatable Y695F RPTPe is less able to dephosphorylate JAK2 downstream of the leptin receptor. Leptin-induced phosphorylation of RPTPe is increased in obese, leptin-resistant WT mice (Figure 6B), suggesting that hypothalamic RPTPe is recruited more strongly to downregulate leptin receptor signaling in obese mice. This finding is consistent with a role for RPTPe in establishing the leptin-resistant state in obesity in WT mice, and with reduced leptin resistance in obese PTPe-deficient mice. Collectively, the above data support a model (Figure 6G) by which JAK2 and RPTPe associate constitutively prior to activation of the leptin receptor; it is possible that RPTPe helps downregulate leptin

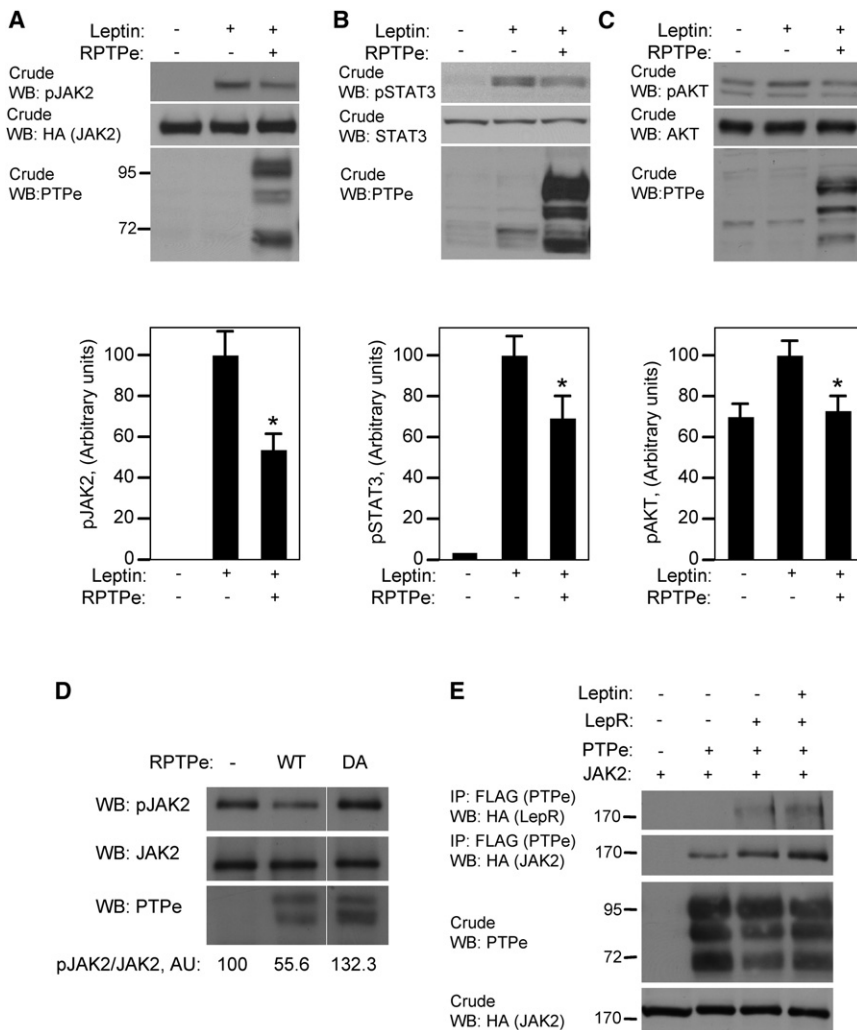


Figure 5. RPTPe Reduces Phosphorylation of JAK2 and STAT3 and Associates with JAK2

(A) (Top) 293T HEK cells expressing the leptin receptor, HA-tagged JAK2, and FLAG-RPTPe were stimulated (+) or not (–) for 5 min with 100 nM leptin and analyzed for JAK2 phosphorylation at Y1007/1008. (Bottom) Bar diagram summarizing results of four similar experiments; *p < 0.05 by Student's t test.

(B and C) 293 cells expressing the leptin receptor and RPTPe, showing phosphorylation of endogenous STAT3 at Y705 or endogenous AKT/PKB at T308.

(D) PTPe dephosphorylates JAK2 in vitro. Purified PTPe intracellular domain (WT or D302A [DA]) and beads carrying autophosphorylated JAK2 were incubated at 30°C for 30 min and analyzed by SDS-PAGE. JAK2 was completely dephosphorylated after 60 min incubation (Figure S5C).

(E) RPTPe and JAK2 physically associate in cells. 293T HEK cells expressing leptin receptor, JAK2, and FLAG-RPTPe were stimulated (+) or not (–) for 5 min with 100 nM leptin. RPTPe was immunoprecipitated, and presence of associated JAK2 and leptin receptor was determined.

molecules (e.g., Grb2 [Toledano-Katchalski and Elson, 1999]), one or more of which might facilitate its activity toward JAK2.

C-terminal phosphorylation of PTPe downstream of activated membranal receptors induces this phosphatase to regulate other signaling events as well. Phosphorylation of RPTPe downstream of activated Neu/ErbB2 in mouse mammary tumor cells induces it to

dephosphorylate and activate Src, which is required for maintaining full transformation of these cells (Berman-Golan and Elson, 2007; Gil-Henn and Elson, 2003). Phosphorylation of the nonreceptor isoform of PTPe, cyt-PTPe, at the same site following integrin activation in osteoclasts is required for full activation of Src, for proper organization of podosomes in these cells and for bone degradation (Chiusaroli et al., 2004; Granot-Attas et al., 2009). A general signaling module then emerges in which activation of a membranal receptor results in C-terminal phosphorylation of PTPe, which induces PTPe to perform a physiological role. This study links this module with regulation of body weight and provides the first example where phosphorylation of PTPe inhibits, rather than supports, the transduced signal.

Our findings indicate that EKO mice of both genders exhibit improved glucose homeostasis. This result is expected in older EKO mice and in EKO mice that were fed a HFD or were ovariectomized due to their relatively reduced adiposity. However, similar findings in young male and female EKO mice of normal weight imply that this effect is independent of the weight phenotype. Furthermore, increased activation of AKT in liver and muscle following injection of insulin to EKO mice implies that

signaling already at this stage. Following receptor activation, RPTPe is phosphorylated at Y695, at least in part by JAK2; phosphorylation directs RPTPe to dephosphorylate JAK2 at Y1007/1008, thus providing negative feedback that returns activated leptin signaling to baseline. RPTPe has potent autodephosphorylating activity (Berman-Golan and Elson, 2007), which may counter its phosphorylation by JAK2 and limit its effect. In the absence of RPTPe, the extent of leptin resistance induced by obesity is reduced, and mice gain less weight as compared to their WT obese counterparts. The precise molecular mechanism by which phosphorylation of RPTPe directs this PTP to act upon JAK2 is not clear at the present time. Y695F RPTPe and WT RPTPe can each be found in a molecular complex with JAK2 and the leptin receptor (Figure 6F). Moreover, WT and Y695F RPTPe are catalytically active, and the Y695E mutant of RPTPe, which mimics in part phosphorylation of RPTPe at Y695, exhibits unaltered kinetic properties toward para-nitrophenylphosphate (PNPP) (Berman-Golan and Elson, 2007). This information suggests that phosphorylation at Y695 affects neither the ability of RPTPe to associate with JAK2 nor its enzymatic activity. Phosphorylation does, however, allow RPTPe to bind other

PTPe downregulates insulin receptor signaling. Previous findings by us and by others have shown that PTPe downregulates insulin receptor signaling in CHO cells (Andersen et al., 2001), in hepatocytes (Nakagawa et al., 2005), and in muscle cells (Aga-Mizrachi et al., 2007). This study extends these results to the whole-animal level by showing that lack of PTPe improves regulation of blood glucose homeostasis and insulin response in liver and muscle. Nonetheless, improved insulin sensitivity in EKO liver may be due to altered rates of insulin secretion or might be related to leptin hypersensitivity, as leptin regulates insulin-mediated hepatic glucose production (Buettner and Camacho, 2008). Further studies, including tissue-specific deletion of PTPe, are required to address this issue. The neuronal insulin receptor is a potent regulator of body weight. However, we believe that altered insulin signaling does not cause the body weight phenotype of EKO mice, since this phenotype is found mainly in EKO females, while improved glucose metabolism is found in EKO mice of both genders.

PTPe affects body weight primarily in female mice. An opposite gender preference was described in one of the studies of PTP1B-deficient mice, and was attributed either to diet or to the genetic background of the mice used (Klaman et al., 2000). While these factors may affect EKO mice, other possibilities exist. For example, estrogen regulates energy status by affecting leptin sensitivity in the brain (Gao and Horvath, 2008; Gao et al., 2007), raising the possibility that interactions between estrogen and leptin signaling may amplify the body weight phenotype of EKO mice in females. Gender-specific differences exist also in the relative importance of signaling by leptin or insulin to body weight regulation, with leptin playing a more prominent role in females (Clegg et al., 2003; Shi et al., 2009). Further studies are required to address this issue.

Mice lacking PTP1B fed high-fat food exhibited increased energy expenditure and decreased adiposity, gained less weight, and were more insulin sensitive than WT mice (Elchebly et al., 1999; Klaman et al., 2000). Further studies showed that PTP1B-deficient mice are leptin hypersensitive and that PTP1B dephosphorylates JAK2 (Cheng et al., 2002; Zabolotny et al., 2002), and that these phenotypes were due at least partly to PTP1B regulating leptin signaling in brain neurons (Banno et al., 2010; Bence et al., 2006; Xue et al., 2009). The apparent similarity in the roles of PTP1B and RPTPe in leptin signaling may reflect the need for tight control of this critical signaling pathway by multiple PTPs. Nonetheless, both PTPs differ markedly in terms of structure and regulation, leaving open the possibility that they may regulate leptin signaling under different physiological circumstances and thus perform complementary roles. Administration of leptin to obese humans generally fails to reduce body weight due to obesity-induced leptin resistance (Keesidid et al., 2010). Targeted inhibition of those PTPs, such as RPTPe, which inhibit leptin receptor signaling and which are important for establishing leptin resistance in the obese state, may increase the usefulness of treating obesity with leptin.

EXPERIMENTAL PROCEDURES

Mice

Gene-targeted mice lacking all forms of PTPe (EKO mice, C57Bl/6 genetic background) were used (Peretz et al., 2000). Mice were kept in a pathogen-

free facility and fed regular chow (2018SC; Harlan Teklad, Madison, WI, USA; 18% of calories from fat) and water ad libitum. On occasion mice were fed high-fat food (diet 03307 [58.6% of calories from fat], Harlan Teklad; or diet D12492 [60% of calories from fat], Research Diets, New Brunswick, NJ). For indirect calorimetry, mice were fed either diet D12492 or a matched control diet (diet 12450B, 10% calories from fat; Research Diets). All experiments were approved by the Institutional Animal Care and Use Committee of the Weizmann Institute in accordance with Israeli law.

Reagents

The following cDNAs were used: mouse WT and Y695F RPTPe (Toledano-Katchalski and Elson, 1999), D302A RPTPe (Gil-Henn and Elson, 2003), and HA-tagged human JAK2 and leptin receptor (generous gifts of Dr. Michel Tremblay, McGill University, Canada). The following monoclonal antibodies were used: anti-FLAG (clone M2, Sigma-Aldrich, St. Louis, MO, USA), POMC (Acris antibodies, Herford, Germany), PTP1B (clone 15, BD Biosciences, San Jose, CA), actin (clone C4, MP Biochemicals, Illkirch, France), antioxidantized PTP (Persson et al., 2004) (clone 335636, R&D Systems), and STAT3 and pY705 STAT3 (clones 124H6 and 3E3, respectively, Cell Signaling Technology, Danvers, MA). Polyclonal antibodies used included the following: PTPe/PTPa (Elson and Leder, 1995), pY695 RPTPe (Berman-Golan and Elson, 2007), anti-HA (probe Y11, Santa Cruz Biotechnology, Santa Cruz, CA), JAK2 and pY1007/1008 JAK2 (Millipore, Billerica, MA, USA), and AKT and pThr308 AKT (Cell Signaling Technology). Leptin was purchased from Protein Laboratories Rehovot, Ltd., Rehovot, Israel.

Metabolic Rate and Physical Activity

Indirect calorimetry, locomotor activity, and food and water intake were measured using the Labmaster system (TSE Systems, Bad Homburg, Germany). Mice were placed in individual cages with a light/dark cycle of 12 hr. Following 48 hr adaptation, the system determined O₂ consumption, CO₂ production, heat production, RER, and food and water consumption for each mouse individually for a further 72 hr. A photobeam-based monitoring system was used to track animal movement (including rearing and climbing).

Glucose and Insulin Tolerance Tests

Mice were fasted overnight and injected IP with either glucose (1.5 gr/kg body weight) or insulin (0.75 U/kg body weight; Novo Nordisk, Denmark). Whole venous blood was obtained from the orbital sinus. Blood glucose concentrations were measured with an automated glucometer (Freestyle, Abbott Diabetes Care, Alameda, CA). Leptin and insulin were measured by ELISA (Chrysal Chem, Downers Grove, IL).

Leptin Stimulation

For acute stimulation, mice were housed individually, starved overnight, and injected IP with leptin (5 μg/gr body weight). Mice were sacrificed by cervical dislocation and their hypothalami were dissected and snap frozen in liquid nitrogen. For chronic stimulation mice were housed individually and injected i.p. with PBS twice daily. After return of body weight and food consumption to baseline (after 7–9 days), PBS was replaced with leptin (1 μg/gr body weight per injection, 2 μg/gr body weight per day); control mice continued to receive PBS injections. After 6 days, all mice received PBS. Body weights and food consumption were measured daily.

Tissue Culture

293 cells were grown in Dulbecco's modified Eagle's medium (Sigma-Aldrich) supplemented with 10% fetal calf serum (Invitrogen), 4 mM glutamine, 50 U/ml penicillin, and 50 μg/ml streptomycin at 37°C, 5% CO₂. Cells were transfected using the calcium phosphate method (Chen and Okayama, 1988).

Protein Analysis

Samples were lysed in buffer A (50 mM Tris-HCl [pH 7.5], 150 mM NaCl, 1% Nonidet P-40), supplemented with protease inhibitors (1 mM N-[α-aminoethyl] benzene-sulfonyl fluoride, 40 μM bestatin, 15 μM E64, 20 μM leupeptin, 15 μM pepstatin; Sigma-Aldrich) and 0.5 mM sodium pervanadate. Immunoprecipitation (Berman-Golan and Elson, 2007) and SDS-PAGE and protein blotting (Gil-Henn and Elson, 2003) were performed as described. Lysates to be analyzed with the antioxidantized PTP antibody (Persson et al., 2004) were treated

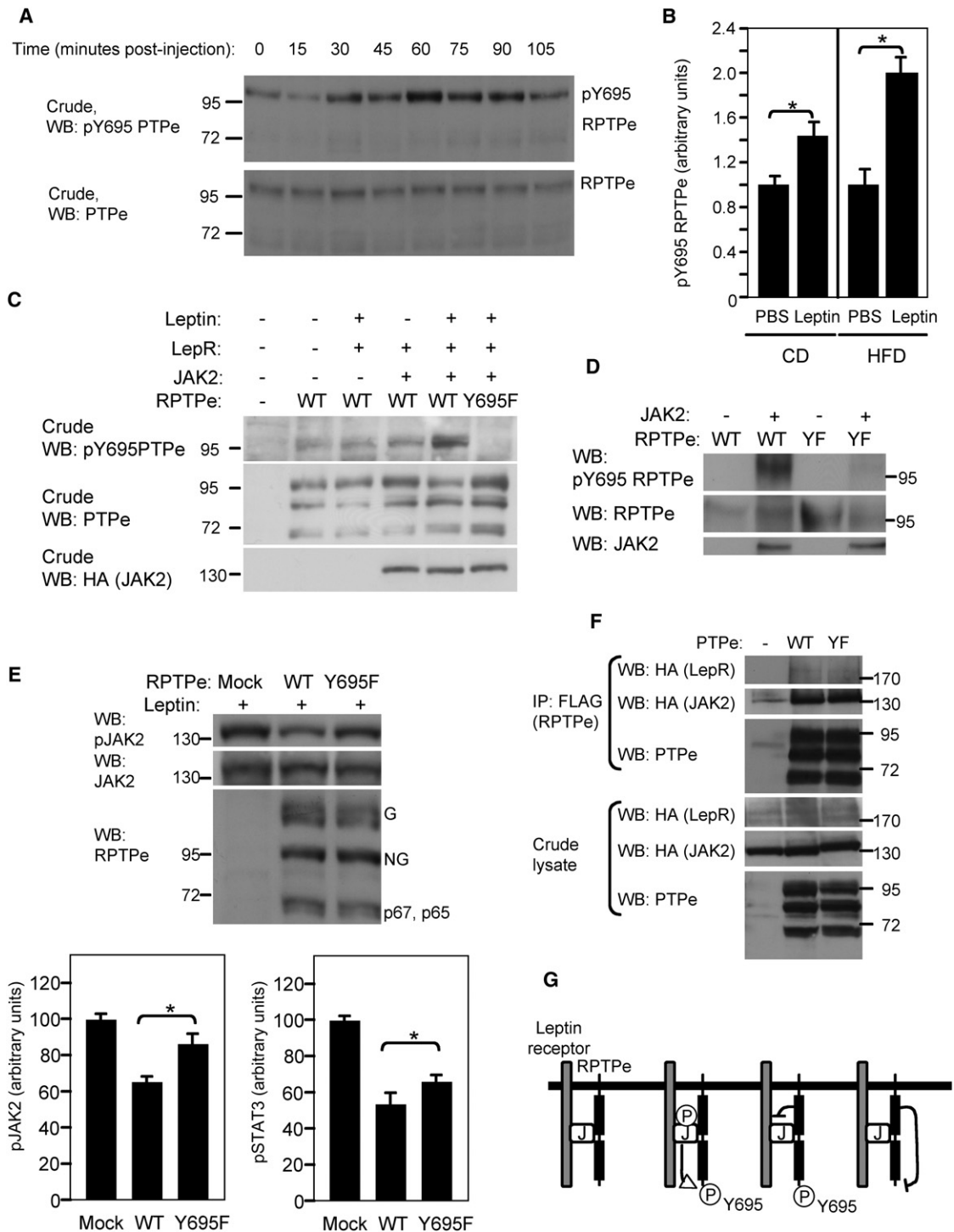


Figure 6. RPTPe Is Phosphorylated at Y695 Following Activation of the Leptin Receptor

(A) Four-month-old female WT mice were injected IP with leptin (5 μ g/gr body weight). Hypothalami were isolated at the indicated times and analyzed with a pY695-PTPe specific antibody.

(B) Bar diagram showing increased phosphorylation of RPTPe at Y695 at t = 60 min following leptin injection in mice fed chow diet (CD) or fatty food (HFD). N = 7–10 mice per bar; *p \leq 0.0065 by Student's t test.

(C) 293T cells were transfected with cDNAs for the leptin receptor, JAK2, and WT or Y695F RPTPe, and stimulated with 100 nM leptin for 5 min as indicated. pY695 PTPe was determined by protein blotting.

(D) Purified JAK2 phosphorylates purified RPTPe at Y695. Purified RPTPe (WT or Y695F [YF]) and JAK2 were incubated for 30 min at 37°C and assayed for RPTPe phosphorylation by protein blotting.

with 0.5 mM sodium pervanadate for 10 min prior to SDS-PAGE. Purification of PTPe from 293 cells was performed as described (Sines et al., 2007). HA-JAK2 was immunoprecipitated from 293 cells with an HA antibody; phosphorylated JAK2 was isolated from cells pretreated with leptin. For in vitro dephosphorylation of pJAK2, 30 ng of PTPe and 25 μ l of beads carrying pY JAK2 were incubated at 30°C for 30–60 min in a buffer consisting of 50 mM MES (pH 7.3), 0.5 mM dithiothreitol, 0.5 mg/ml bovine serum albumin. For in vitro phosphorylation of PTPe, 30 ng of PTPe and 25 μ l of beads carrying JAK2 were incubated at 37 degrees for 30 min in a buffer consisting of 60 mM HEPES (pH 7.6), 5 mM MgCl₂, 5 mM MnCl₂, 0.125 mM sodium peroxyvanadate, 100 μ M ATP, 1.25 mM DTT.

Statistics

Data were analyzed using a two-tailed Student's t test or by ANOVA as indicated, with significance set at $p = 0.05$. Data are shown as mean \pm SE.

SUPPLEMENTAL INFORMATION

Supplemental Information includes five figures, Supplemental Experimental Procedures, and Supplemental References and can be found with this article at doi:10.1016/j.cmet.2011.02.017.

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(E) Phosphorylation of RPTPe at Y695 downstream of the leptin receptor enhances the ability of RPTPe to downregulate JAK2 and STAT3 phosphorylation. (Top) 293T cells expressing the leptin receptor, JAK2, and WT or Y695F RPTPe were stimulated with 100 nM leptin for 5 min. pY1007/1008 JAK2 levels were analyzed by protein blotting. (Bottom) Reduced ability of Y695F RPTPe to dephosphorylate JAK2 (left) and STAT3 (right) (mean \pm SE, JAK2, * $p = 0.011$, $n = 7$ repeats; STAT3, * $p = 0.043$, $n = 5$ repeats).

(F) Phosphorylation of RPTPe at Y695 is not required for its association with JAK2 or the leptin receptor. 293 cells expressing HA-JAK2, HA-leptin receptor, and either WT or Y695F RPTPe were stimulated with 100 nM leptin for 5 min and lysed. RPTPe was precipitated, and presence of associated JAK2 and the leptin receptor was determined by protein blotting.

(G) RPTPe inhibits leptin receptor signaling via dephosphorylating JAK2. (1) JAK2 and RPTPe exist in a complex that also includes the leptin receptor. (2) Upon activation of the receptor, JAK2 autophosphorylates and transphosphorylates the receptor (data not shown) and RPTPe at Y695. (3) Phospho-RPTPe dephosphorylates JAK2 and downregulates leptin receptor signaling. (4) RPTPe autodephosphorylates, thus reducing its ability to act upon JAK2. The points of contact between JAK2 and RPTPe are hypothetical. J, JAK2.

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