

# Shp2 Controls Female Body Weight and Energy Balance by Integrating Leptin and Estrogen Signals

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**In mammals, leptin regulates food intake and energy balance mainly through the activation of LepRb in the hypothalamus, and estrogen has a leptin-like effect in the hypothalamic control of metabolism. However, it remains to be elucidated how estrogen signaling is intertwined with the leptin pathway. We show here that Shp2, a nonreceptor tyrosine phosphatase, acts to integrate leptin and estrogen signals. The expression of a dominant-active mutant (Shp2<sup>D61A</sup>) in forebrain neurons conferred female, but not male, transgenic mice resistance to high-fat diet (HFD)-induced obesity and liver steatosis, accompanied by improved insulin sensitivity and glucose homeostasis. Fed with either HFD or regular chow food, Shp2<sup>D61A</sup> female mice showed dramatically enhanced leptin sensitivity. Microinjection of Shp2<sup>D61A</sup>-expressing adeno-associated virus into mediobasal hypothalamus elicited a similar antiobese effect in female mice. Biochemical analyses showed a physical association of Shp2 with estrogen receptor alpha, which is necessary for the synergistic and persistent activation of Erk by leptin and estrogen. Together, these results elucidate a mechanism for the direct cross talk of leptin and estrogen signaling and offer one explanation for the propensity of postmenopausal women to develop obesity.**

In mammals, leptin regulates food intake and energy balance mainly through activation of the leptin receptor long form (LepRb) and downstream signaling pathways in the hypothalamus, and leptin deficiency causes obesity and diabetes as well as impaired reproductive functions (14, 15, 31). The SH2-tyrosine phosphatase Shp2 was shown to bind directly to activated LepRb by docking on p-Tyr985 in the intracellular domain (23, 37). Genetic ablation of Shp2 in the central nervous system resulted in gross obesity associated with leptin resistance and decreased p-Erk, suggesting a positive role of Shp2 in amplifying leptin signal in the hypothalamus (2, 22, 37).

Estradiol-17 $\beta$  (E<sub>2</sub>), a reproductive hormone, also plays a vital role in energy metabolism and body weight control (13). The deletion of aromatase that catalyzes the formation of estrogen or disruption of estrogen receptor  $\alpha$  (ER $\alpha$ ) leads to more severe age-dependent obesity in females than in males, indicating that estrogen deficiency is responsible for the progression of sexually dimorphic obesity (19, 21). In postmenopausal women or ovariectomized rodents, estrogen deficiency is associated with increased probability of obesity and type 2 diabetes (7, 32). Estrogen replacement in ovariectomized animals suppresses obese development by decreasing food intake and increasing energy expenditure (17). Hormone treatment in postmenopausal women prevents obesity progression and enhances insulin sensitivity and glucose tolerance (33). In contrast, deletion or silencing of ER $\alpha$  in the hypothalamus leads to obesity and metabolic defects in rodents (19, 25). Estrogen has a leptin-like effect in activation of intracellular signals in hypothalamic melanocortin cells (16). However, it remains to be determined how estrogen signaling is intertwined with the leptin pathway.

The positive role of Shp2 in mediating leptin signal in the brain predicted that pharmaceutical enhancement of Shp2 activity locally in the brain would overcome leptin resistance and alleviate

obesity. To test this hypothesis, we generated a transgenic mouse line with expression in forebrain neurons of a dominantly activating (gain-of-function) mutant Shp2<sup>D61A</sup>, which was shown to exhibit dramatically increased phosphatase activity without affecting its SH2-binding activity (26). Characterization of the transgenic mice revealed an unexpectedly critical role of Shp2 in coupling leptin and estrogen signaling. These data fill in a gap in our knowledge about the overlapping function of these two hormones in metabolism and help show why postmenopausal women tend to develop obesity.

## MATERIALS AND METHODS

**Generation of Shp2<sup>D61A</sup> transgenic mice.** Shp2<sup>D61A</sup> mutant was created by site-directed mutagenesis using mouse Shp2 cDNA as a template. The mutant Shp2<sup>D61A</sup> fragment with an hemagglutinin (HA) tag sequence at the C terminus was subcloned into pcDNA3.1 vector. The cytomegalovirus (CMV) promoter in pcDNA3.1 plasmid was replaced by CaMKII $\alpha$  promoter (12) to drive the Shp2<sup>D61A</sup>-HA mutant expression. The engineered construct was injected into the fertilized oocytes of C57BL/6 mice to generate transgenic mouse line in the animal facility of the Sanford Burnham Medical Research Institute (SBMRI). All animal procedures were approved by the University of California, San Diego, and the SBMRI Institutional Animal Care and Use Committees.

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**AAV injection.** The adeno-associated virus (AAV) expression system (Stratagene) was used to produce AAV-green fluorescent protein (GFP) and AAV-Shp2<sup>D61A</sup>-GFP virus. AAV viruses were injected into the mediobasal hypothalamus of mice as previously described (38). Briefly, bilateral injection into the mediobasal hypothalamus was directed by using an ultraprecise stereotax with a resolution of 10  $\mu$ m (Kopf Instruments) to the coordinates of 1.5 mm posterior to the bregma, 5.8 mm below the bregma, and 0.3 mm lateral to the midline. Viruses suspended in cerebrospinal fluid were injected over 10 min through a 26-gauge guide cannula and a 33-gauge injector (Plastics One) connected to a Hamilton Syringe and an infusion pump (WPI Instruments).

**HFD feeding and ovariectomy.** Wild-type (wt) and transgenic mice at age of 8 to 10 weeks were fed with either a 58% kcal high-fat diet (HFD; catalog no. D12331; Research Diets) or a 6% kcal chow diet (catalog no. 8664; Harlan Teklad) for 20 weeks (24). The body weights of mice were monitored weekly. To measure the food intake, the mice were separated into individual cages, and changes in food amounts were recorded daily. Ovariectomy was performed on wt and transgenic female mice at age of 10 to 12 weeks. At 1 week after surgery, the mice were fed either HFD or regular chow diet for 8 weeks, and their body weights were monitored biweekly.

**Metabolic assays.** Serum insulin and leptin levels were measured using commercial kits (catalog no. 90030 and catalog no. INSKR020; Crystal Chem). Estradiol (Cayman), testosterone (Cayman), and corticosterone (Enzo Life Science) were measured by enzyme immunoassay. An insulin tolerance test (ITT) and a glucose tolerance test (GTT) were performed on Shp2<sup>D61A</sup> and wt mice at age of 24 to 26 weeks, after HFD feeding for 16 to 18 weeks. For the ITT, insulin (Humulin R; Eli Lilly) was injected intraperitoneally (1 U/kg [body weight]), and the blood glucose levels were measured with a glucometer (Lifescan One-Touch Basic) at 0, 15, 30, 60, and 120 min. For the GTT, the animals were fasted for 12 to 16 h and injected with glucose (1.5 g/kg [body weight]), and then the blood glucose levels were measured at 0, 15, 30, 60, and 120 min.

For leptin sensitivity, mice fed with HFD or chow diet for 16 weeks were intraperitoneally injected with leptin (1.5  $\mu$ g/kg [body weight]; National Hormone and Peptide Program) twice daily for 3 days (7:30 a.m. and 6:30 p.m.; dose, 1.5  $\mu$ g/kg [body weight]) (24). The mice were separated into individual cages and placed on chow diet. Body weight and food intake were monitored daily for 7 days. O<sub>2</sub> consumption and CO<sub>2</sub> production were measured by using the Oxymax System (Columbus Instruments).

**Cell signaling studies.** Mice at the age of 30 weeks fed a HFD for 20 weeks were fasted for 12 to 16 h. Leptin (1.5  $\mu$ g/kg [body weight]), estradiol-17 $\beta$  (catalog no. 3301 [Calbiochem], 50  $\mu$ M/kg [body weight]), and saline were administered intraperitoneally 1 h before sacrifice. Brain was carefully isolated and homogenized with tissue lysis buffer, and then the lysate was centrifuged at 14,000 rpm for 30 min twice. Lysates containing 50  $\mu$ g of protein were immunoblotted for pY-Stat3 (catalog no. 9131; Cell Signaling), Stat3 (catalog no. 9132; Cell Signaling), pAkt-s473 (catalog no. 9271; Cell Signaling), pAkt-t308 (catalog no. 9275; Cell Signaling), Akt (catalog no. 9272; Cell Signaling), pY-Src and Src (Cell Signaling), pErk1/2 (catalog no. 9101; Cell Signaling), adiponectin (Millipore), and HA (Update). Mouse brain was isolated after perfusion by 4% paraformaldehyde (PFA) for frozen sectioning. Shp2 antibody (syp homemade or sc-280; Santa Cruz Biotechnology), ER $\alpha$  antibody (sc-542; Santa Cruz Biotechnology), and pY-Stat3 antibody were used for immunostaining. Liver was collected for frozen sectioning and then stained by O oil-red or with hematoxylin and eosin (H&E) (4). Adipose tissue was fixed by Z-Fix solution for 24 h to perform H&E staining. Brain samples were collected for frozen sectioning and stained by antibodies.

MCF-7 cells were cultured in Dulbecco modified Eagle medium with 10% fetal bovine serum. PC12<sup>L<sup>ep</sup>Rb</sup> cells were cultured in F-12K medium with 12% horse serum and 3% fetal bovine serum (20). Cells were fixed by 4% PFA for 5 to 10 min. Shp2 primary antibody (sc-7384; Santa Cruz Biotechnology) and ER $\alpha$  primary antibody (sc-542; Santa Cruz Biotech-

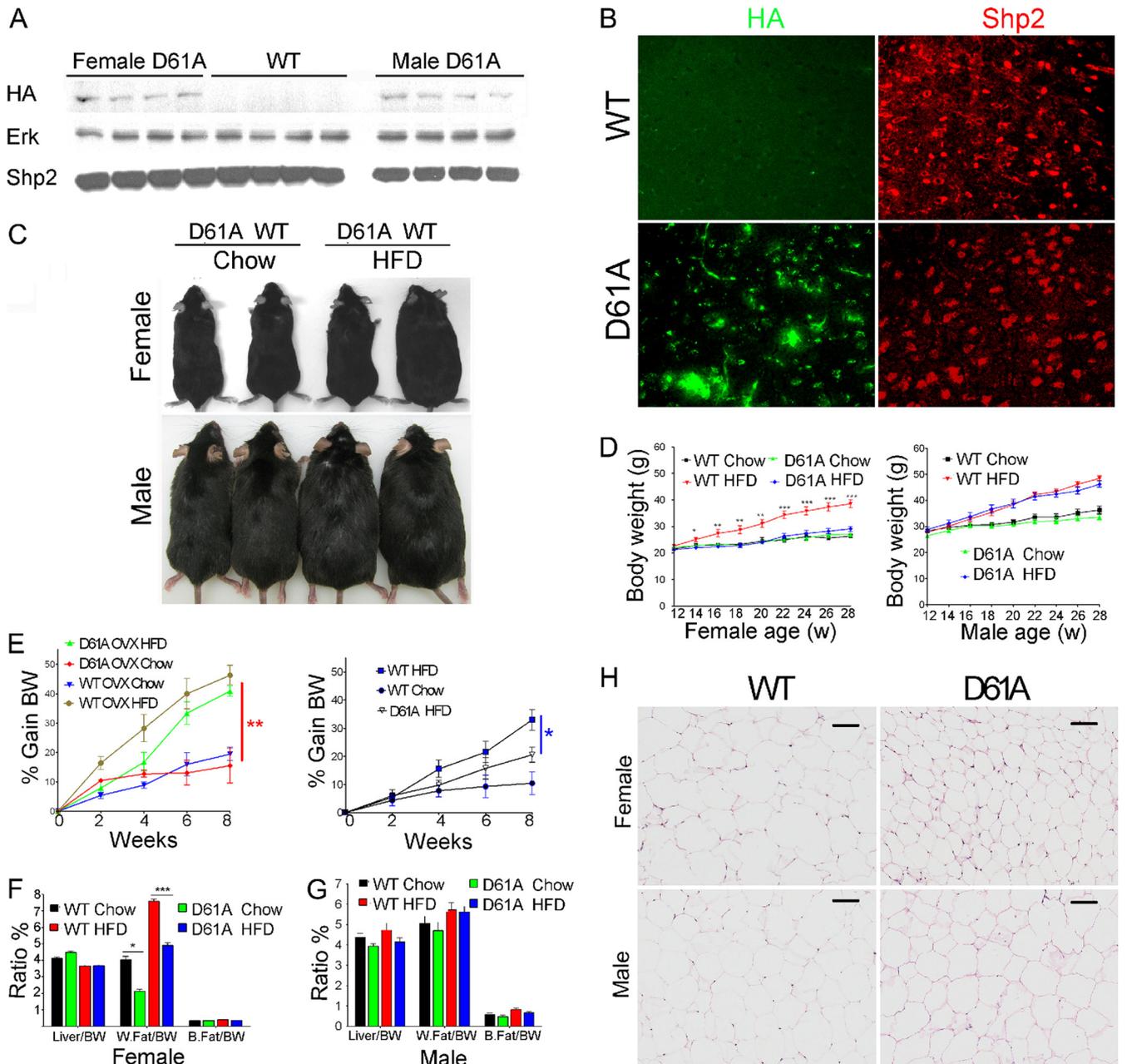
nology) were used to perform immunostaining and immunoblotting. Human ER $\alpha$  small interfering RNA (siRNA; sc-29305; Santa Cruz Biotechnology) and human Shp2 siRNA (catalog no. 1027020; Qiagen) were used to knockdown target gene expression in MCF-7 cells by the Amaxa nucleofector method. After 3 days, the cells were stimulated with hormones, and the cell lysates were analyzed by immunoblotting. pcDNA-Shp2<sup>D61A</sup> construct was transiently transfected into MCF-7 cells. C57BL/6 mice at age of 10 weeks were sacrificed to collect uterus and ovary, and the tissue lysates (1 mg of total proteins) were prepared for immunoprecipitation with antibodies for Shp2 and ER $\alpha$ , followed by immunoblotting with specific antibodies as indicated. Mouse pCMV5-Shp2 and pGEM-ER $\alpha$  were used to express protein by TNT coupled reticulocyte lysate systems (cell-free protein expression, catalog nos. L5061 and L5020; Promega). The translation products were mixed with or without estradiol-17 $\beta$  and subjected to immunoprecipitation assay.

**Statistical analysis.** Data are expressed as means  $\pm$  the standard errors of the mean (SEM). Statistical significance was determined using an unpaired two-tailed Student *t* test and analysis of variance.

## RESULTS

**Shp2<sup>D61A</sup> mice are resistant to HFD-induced obesity in a sex-dimorphic manner.** A dominant-active mutant of tyrosine phosphatase Shp2 (HA-tagged Shp2<sup>D61A</sup>) under the control of *CaMKII $\alpha$*  promoter was selectively expressed in forebrain neurons, including the hypothalamus, and the expression levels were similar in male and female mice, as demonstrated by immunostaining and immunoblotting analyses (Fig. 1A and B). When maintained on regular chow food, the transgenic mice did not show any obvious abnormal phenotype (Fig. 1C). Both wt and transgenic mice, at ages of 8 to 10 weeks, were fed either HFD (58% Kcal) or chow diet (24). After 20 weeks, wt mice of both genders on HFD exhibited significantly increased body weight compared to those fed regular food (Fig. 1D). However, we observed a gender effect of Shp2<sup>D61A</sup> expression in transgenic mice fed with HFD. Shp2<sup>D61A</sup> male mice displayed a dramatic increase of body weight when on HFD compared to chow food, findings very similar to those for the wt controls (Fig. 1D). In contrast, Shp2<sup>D61A</sup> female mice showed only modest levels of body weight increase when placed on HFD (Fig. 1D), suggesting a more prominent effect of Shp2<sup>D61A</sup> expression in female mice for protection against body weight gain/obesity. Ovariectomy of Shp2<sup>D61A</sup> female mice disrupted the resistance to HFD-induced obesity, suggesting that estrogen signal plays an important role in the progression of sex-dimorphic obesity (Fig. 1E). Shp2<sup>D61A</sup> female mice had significantly lower indices of white fat (gonadal) pad to body weight than the wt controls, fed with either HFD or chow diet, although their indices of liver or brown fat versus body weight were normal (Fig. 1F). The indices of male mice did not show significant difference between control and transgenic mice with HFD or chow diet (Fig. 1G). Under the HFD condition, Shp2<sup>D61A</sup> female mice had smaller adipocyte sizes than controls (Fig. 1H), indicating that Shp2<sup>D61A</sup> expression in the brain inhibits obese development. Control and male transgenic mice showed severe hepatic steatosis when fed with HFD, as revealed by Oil Red and H&E staining (Fig. 2A to C). Shp2<sup>D61A</sup> female mice displayed fewer fat droplets in liver than controls on HFD (Fig. 2D to F). Interestingly, even on chow food, Shp2<sup>D61A</sup> female mice showed lower basal levels of liver steatosis than did wt animals (Fig. 2D to F).

**Shp2<sup>D61A</sup> expression reduces food intake and enhanced energy expenditure.** To examine the energy balance, we monitored

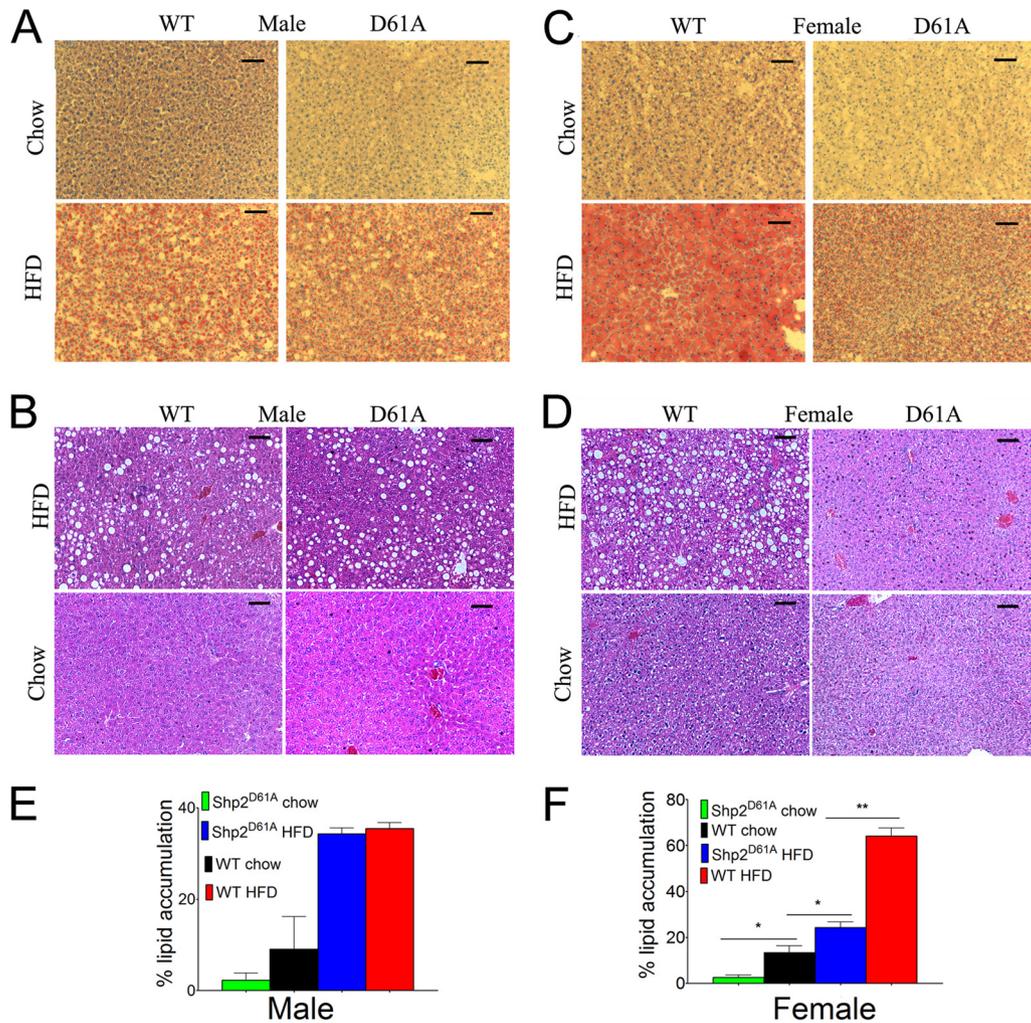


**FIG 1** Shp2<sup>D61A</sup> expression confers resistance to HFD-induced obesity in female mice. (A and B) The expression of Shp2<sup>D61A</sup> in the hypothalamus area of transgenic mice was examined by anti-HA and anti-Shp2 immunoblotting (A) and immunostaining (B). Similar levels of HA-tagged Shp2<sup>D61A</sup> were detected in the brains of male and female mice. D61A: Shp2<sup>D61A</sup>. Scale bar, 50  $\mu$ m. (C) Representatives of wt or transgenic female and male mice that were fed on HFD or chow diet for 20 weeks. (D) Body weights of wt versus Shp2<sup>D61A</sup> mice on either HFD or chow diet. The data are expressed as means  $\pm$  the SEM ( $n = 10$  to 15; \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$ ). (E) Female mice at the ages of 10 to 12 weeks were ovariectomized. After 1 week, the mice were fed on either HFD or regular chow food. The body weights were monitored biweekly. The percentages of weight gain are shown. The data are expressed as means  $\pm$  the SEM ( $n = 4$  to 6; \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ). (F and G) Index of gonadal fat pad to body weight. The ratios of brown adipose tissue or liver to body weight were compared between control and transgenic male or female mice on chow diet or HFD. The data are expressed as means  $\pm$  the SEM ( $n = 11$  to 15; \*,  $P < 0.05$ ; \*\*\*,  $P < 0.001$ ). (H) Representative image of adipocyte sizes on HFD, with H&E staining. Scale bar, 100  $\mu$ m.

food intake and found a significant decrease of food consumption by Shp2<sup>D61A</sup> female mice, compared to controls, when fed HFD (Fig. 3A). After being placed on a HFD for 4 weeks, Shp2<sup>D61A</sup> female mice showed increased heat production compared to wt mice in a HFD (Fig. 3B), and Shp2<sup>D61A</sup> female mice showed significantly more O<sub>2</sub> consumption and CO<sub>2</sub> release than did con-

trols (Fig. 3C to F). These data suggest that Shp2<sup>D61A</sup> expression in female mice attenuated HFD-induced obesity due to enhanced energy expenditure and decreased food intake.

**Shp2<sup>D61A</sup> female mice show improved insulin sensitivity and glucose homeostasis.** Blood insulin and fasting glucose levels were similarly elevated in wt and Shp2<sup>D61A</sup> male mice fed with

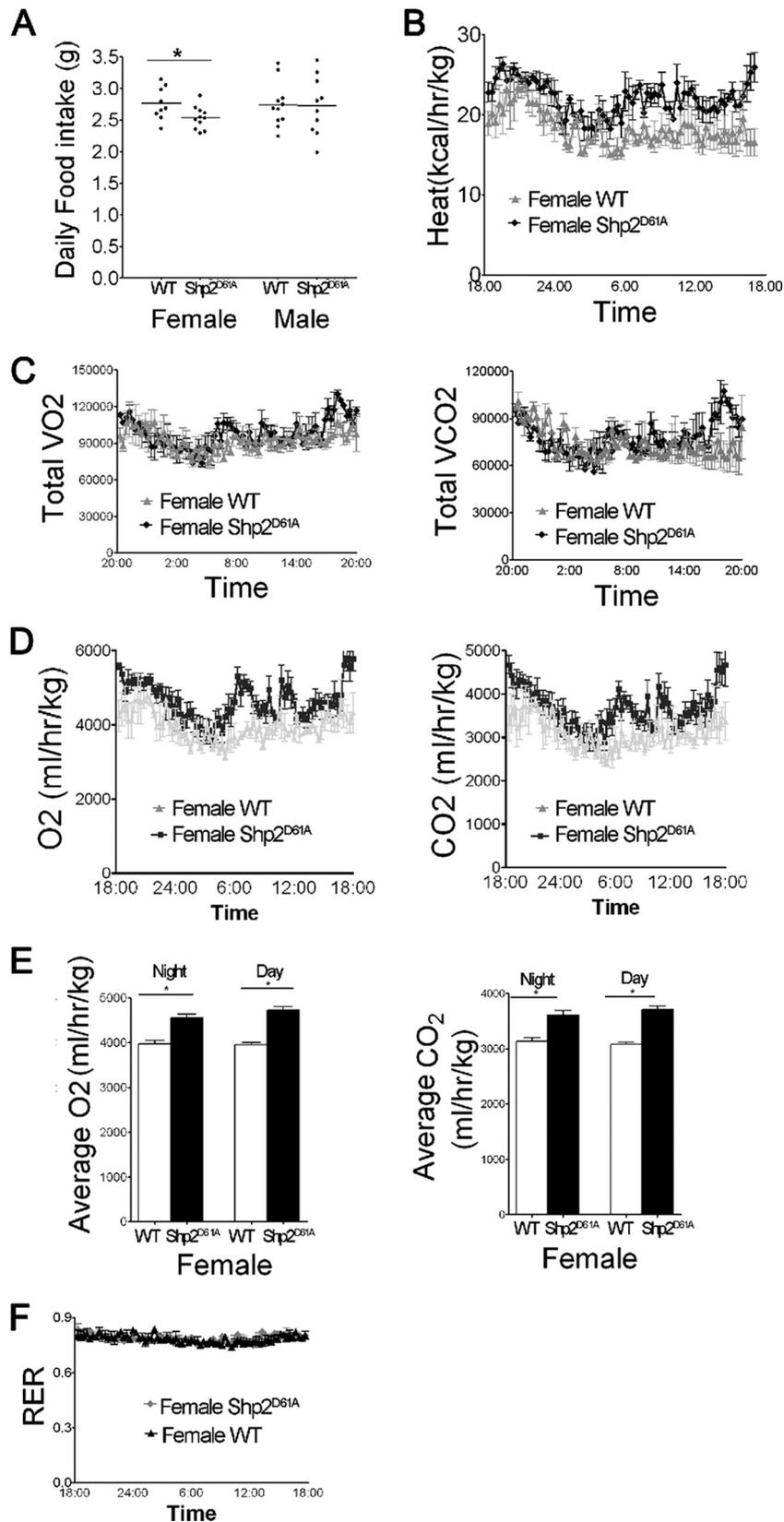


**FIG 2**  $Shp2^{D61A}$  expression suppresses liver steatosis. (A and B) Liver steatosis of male mice shown using Oil Red and H&E staining, respectively. (C and D) Fatty livers of female mice are shown using Oil Red and H&E staining, respectively. (E and F) Hepatic lipid accumulation was quantified for male and female mice on chow or HFD. Scale bar, 50  $\mu$ m. ( $n = 3$  to 5; \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ). D61A,  $Shp2^{D61A}$ .

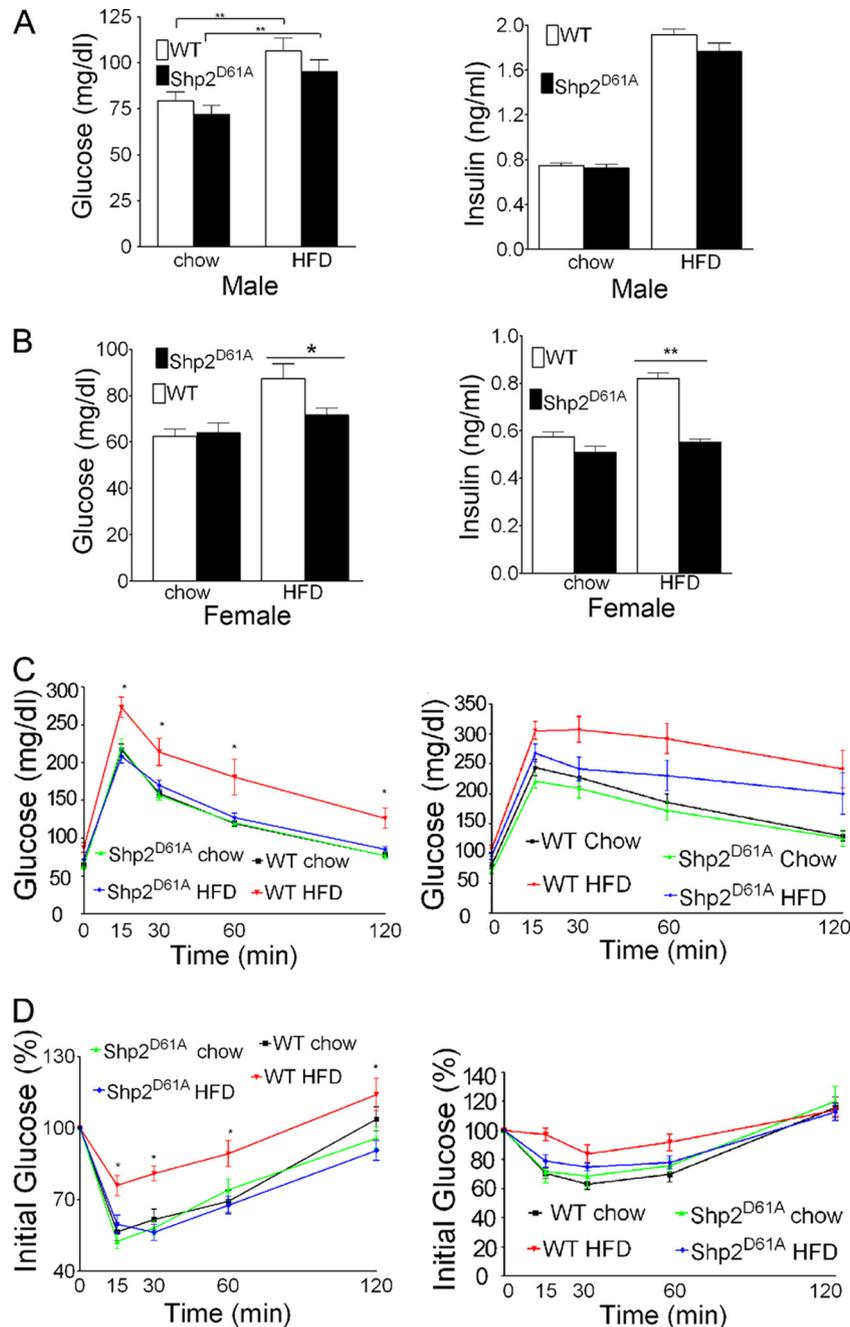
HFD compared to mice fed regular food (Fig. 4A), suggesting the development of obesity-related hyperglycemia and insulin resistance. In contrast,  $Shp2^{D61A}$  female mice on HFD exhibited normal or slightly increased insulin and glucose concentrations (Fig. 4B). We performed GTTs and ITTs on mice at the ages of 26 to 28 weeks. The wt mice on HFD showed a decreased response to a glucose load (Fig. 4C and D), indicating the status of HFD-induced glucose intolerance. In contrast,  $Shp2^{D61A}$  female mice on either a regular diet or HFD showed normal glucose tolerance levels compared to wt controls on chow diet (Fig. 4C). After being paced on HFD,  $Shp2^{D61A}$  female mice were more insulin sensitive than wt mice (Fig. 4D). The insulin sensitivity was similar between  $Shp2^{D61A}$  female mice fed a HFD or chow diet and wt controls on regular food (Fig. 4D). After on HFD for 16 weeks,  $Shp2^{D61A}$  male mice also exhibited mildly improved glucose and insulin sensitivity compared to wt male mice (Fig. 4C and D). Thus,  $Shp2^{D61A}$  expression in forebrain neurons prevents obesity-related insulin resistance and glucose intolerance.

**$Shp2^{D61A}$  enhances leptin sensitivity.** We investigated whether the antiobese phenotype is due to increased leptin sensitivity in

$Shp2^{D61A}$  transgenic mice. As shown in Fig. 5A and B, the leptin levels of control and male transgenic mice on HFD were significantly elevated compared to the same groups on a chow diet, indicating a hyperleptinemia status.  $Shp2^{D61A}$  female mice showed significantly lower leptin levels than wt female mice on HFD (Fig. 5B), suggesting an increased leptin sensitivity in female transgenic animals. To further determine leptin sensitivity, we performed intraperitoneal injection of leptin twice daily for 3 days and monitored the body weight changes continuously for 7 days (24). Either on HFD or chow diet, wt mice underwent similar percentages of body weight loss upon leptin injection (Fig. 5C and D). However,  $Shp2^{D61A}$  transgenic female mice displayed more significant weight loss after leptin administration compared to female control mice for the first 3 days while receiving leptin (Fig. 5C). Over the 3 days after leptin injection, the body weight of  $Shp2^{D61A}$  female mice on HFD remained at low levels compared to wt female mice on HFD (Fig. 5C). The same dose of leptin administration caused a similar weight loss between wt and  $Shp2^{D61A}$  male mice fed with HFD or chow diet (Fig. 5D). Thus,  $Shp2^{D61A}$  expression enhanced the leptin sensitivity in female mice, resulting in protec-



**FIG 3** Shp2<sup>D61A</sup> female mice displayed reduced food intake and increased energy expenditure. (A) The daily food intake of mice on HFD. The data are expressed as means  $\pm$  the SEM. \*,  $P < 0.05$ . (B) Heat production was measured for wt and Shp2<sup>D61A</sup> female mice on HFD. The data are expressed as means  $\pm$  the SEM. (C) O<sub>2</sub> consumption and CO<sub>2</sub> production of wt versus Shp2<sup>D61A</sup> female mice on HFD. Total O<sub>2</sub> consumption and CO<sub>2</sub> production data were shown without normalization. (D to F) O<sub>2</sub> consumption and CO<sub>2</sub> production of wt versus Shp2<sup>D61A</sup> female mice on HFD. The data normalized by the whole body weight are expressed as means  $\pm$  the SEM. \*,  $P < 0.05$ .



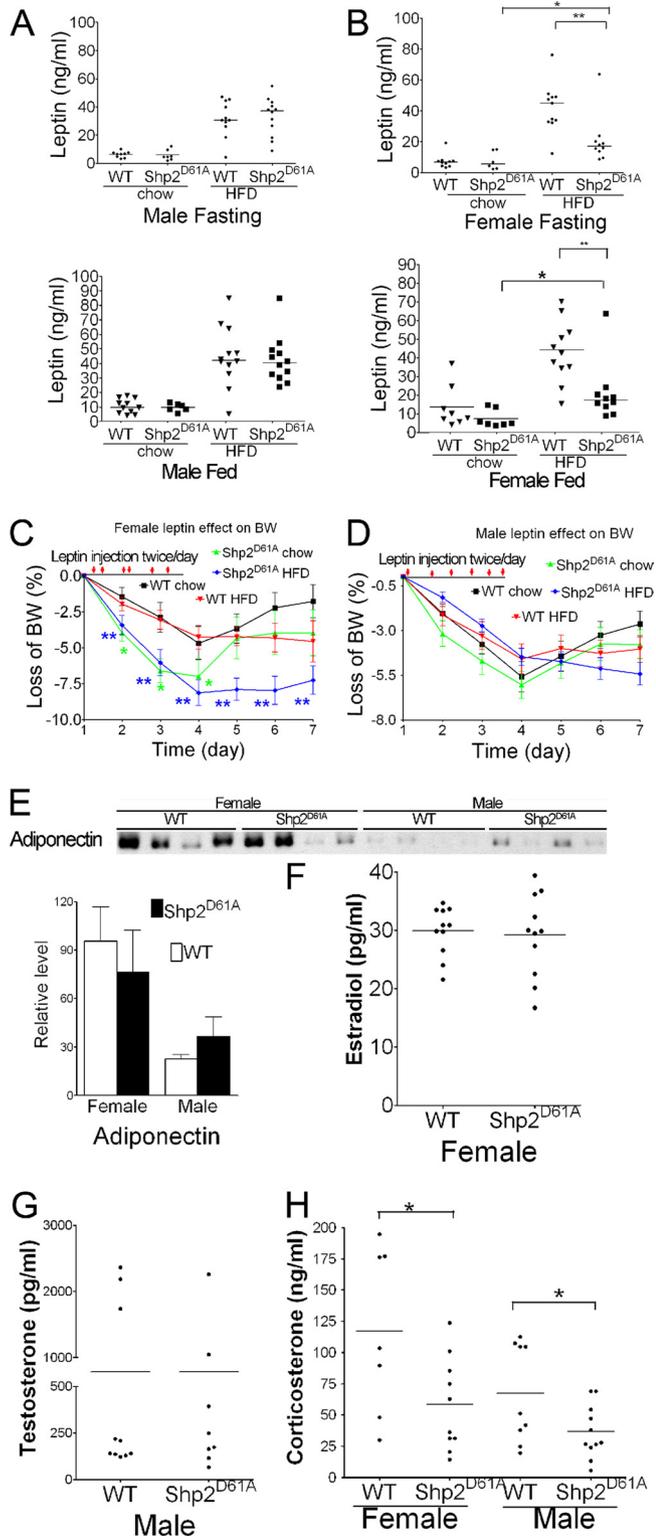
**FIG 4** Shp2<sup>D61A</sup> expression improves metabolic parameters. (A) Serum insulin and blood glucose levels were compared between Shp2<sup>D61A</sup> and wt male mice, fed HFD or chow diet. The data are expressed as the means  $\pm$  the standard deviation (SD). (B) Blood glucose and serum insulin levels were measured for control and transgenic female mice on HFD compared to chow diet. The data are expressed as means  $\pm$  the SD. (C) GTT in wt versus Shp2<sup>D61A</sup> mice on HFD or chow diet. The data are expressed as means  $\pm$  the SD. Left panel, female mice; right panel, male mice. (D) ITT in wt versus Shp2<sup>D61A</sup> mice on HFD or chow diet. The data are expressed as means  $\pm$  the SD. Left panel, female; right panel, male mice. ( $n = 11$  to 15, \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ).

tion from HFD-induced obesity and associated hyperglycemia, hyperinsulinemia, and hyperleptinemia.

Under HFD conditions, the level of adiponectin was higher in female than in male animals (Fig. 5E). There was no difference between wt and Shp2<sup>D61A</sup> groups in both genders, suggesting that adiponectin does not play a role in resistance to HFD-induced obesity in this model. To investigate whether sex hormones are involved in obesity development, we examined the serum levels of

estrogen and testosterone. No significant difference was observed between wt and Shp2<sup>D61A</sup> mice (Fig. 5F and G). However, the level of corticosterone was significantly decreased in the Shp2<sup>D61A</sup> group of both genders, compared to the wt control (Fig. 5H), suggesting that corticosterone is not a major factor in the sex-dimorphic development of obesity.

**A Shp2/ER $\alpha$  complex orchestrates leptin and estrogen signals.** The results described above suggest a biochemical connec-



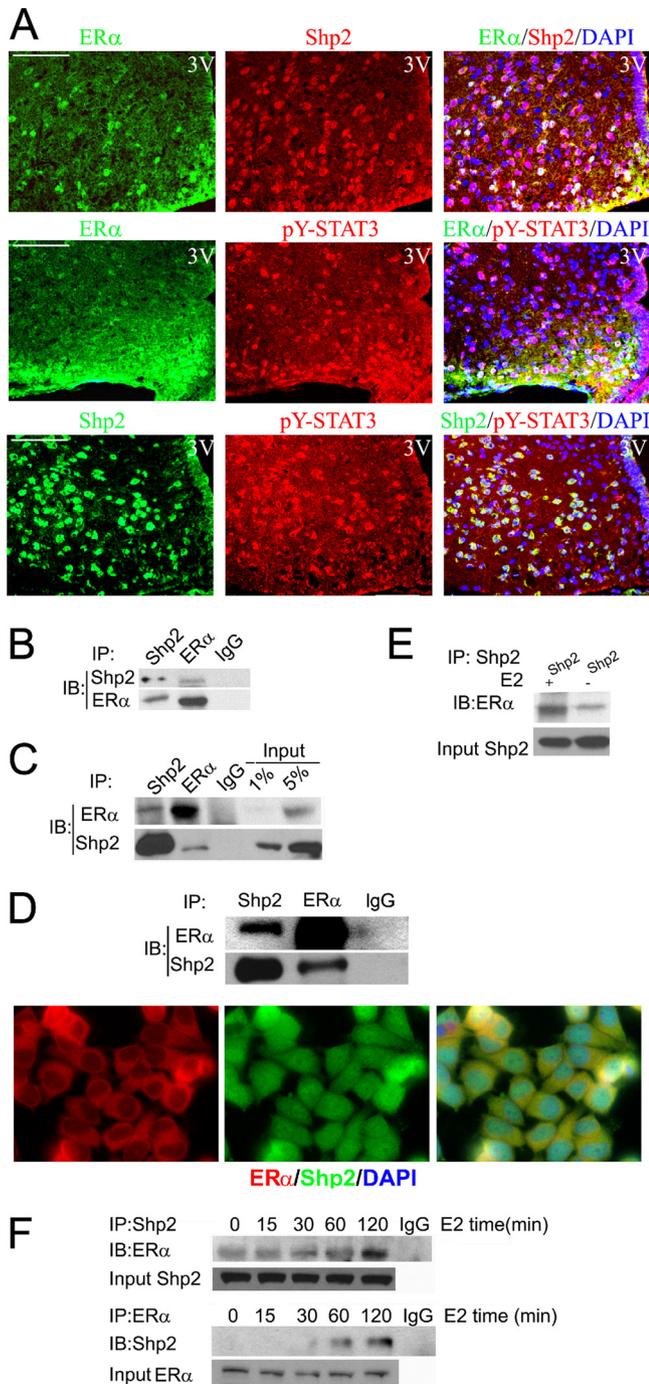
**FIG 5** Expression of Shp2<sup>D61A</sup> increases leptin sensitivity. After mice were fed a HFD or a chow diet for 20 weeks, the serum leptin levels were measured. (A) Serum leptin levels were compared between wt and Shp2<sup>D61A</sup> male mice on a HFD or chow diet. (B) Serum leptin amounts were compared between wt and Shp2<sup>D61A</sup> female mice on a HFD or chow diet. (C) Leptin was injected intraperitoneally twice daily for 3 days. The body weights of female mice were measured daily for 7 days. The data are expressed as means  $\pm$  the SD. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$ . (D) Leptin was injected intraperitoneally twice daily for 3 days. Body weight of

tion of Shp2 with an estrogen signaling component downstream of the leptin receptor in control of energy metabolism in the hypothalamus (Fig. 1E). Consistently, it has been shown previously that estrogen has a leptin-like effect in the hypothalamus for the control of energy balance, although the mechanism is not fully understood (16). To probe the underlying mechanism for the gender effect of the Shp2 transgene expression, we explored a putative role of Shp2 in coupling leptin and estrogen signals. We used a leptin-stimulated pY-Stat3 signal to locate LepRb-positive cells. Intracellular colocalization of Shp2, ER $\alpha$ , and pY-Stat3 was detected in the hypothalamus by immunofluorescent staining (Fig. 6A). Using ovary and uterus tissue or brain lysates, we demonstrated that Shp2 and ER $\alpha$  were coimmunoprecipitated with each other in both ways (Fig. 6B and C), indicating the formation of a Shp2/ER $\alpha$  complex *in vivo*. Immunostaining analysis also showed colocalization of Shp2 and ER $\alpha$  in MCF-7 breast cancer cells (Fig. 6D). To test whether Shp2 association with ER $\alpha$  is stimulated by estrogen, we performed an immunoprecipitation assay *in vitro*. Treatment of cells with E<sub>2</sub> enhanced the binding of Shp2 with ER $\alpha$ , suggesting a stimulatory effect by estrogen (Fig. 6E). In MCF-7 cells, the binding of Shp2 and ER $\alpha$  was greatly enhanced 30 min after E<sub>2</sub> treatment (Fig. 6F). Therefore, Shp2/ER $\alpha$  is linked with leptin and estrogen signaling.

**Shp2 enhances leptin and estrogen action.** We determined whether leptin and estrogen operate in concert to activate intracellular signaling pathways. Stimulation by leptin or estrogen induced rapid phosphorylation of Erk, Src, Stat3, and Akt (Fig. 7A), and combined treatment with leptin and estrogen dramatically enhanced the phosphorylation signal of Erk, indicating a synergistic effect in cell signaling (Fig. 7A). No dramatic change in the phosphorylation levels of Src were detected after leptin or estrogen stimulation (Fig. 7A). We injected leptin and/or estrogen into wt and Shp2<sup>D61A</sup> mice placed on HFD for 20 weeks. Phosphorylation of Erk, Akt, and Stat3 was significantly increased after 1 h of leptin or estrogen administration compared to mice given a saline injection (Fig. 7B). Compared to controls, transgenic mice of both genders displayed higher basal level of p-Erk, p-Akt, and p-Stat3. Consistently, the transgenic mice exhibited higher sensitivity to leptin and estrogen than wt animals and possessed lower circulating levels of leptin (Fig. 5A). Moreover, the overall phosphorylation levels of Erk, Akt, and Stat3 were higher in female than in male mice, suggesting that leptin and estrogen operate in concert in regulating Erk, Akt, and Stat3 pathways *in vivo* (1, 5, 17, 18, 36, 37).

We further investigated the cross talk of leptin and estrogen signaling in MCF-7 cells that express both LepRb and ER $\alpha$ . Knockdown of Shp2 or ER $\alpha$  expression by RNA interference significantly decreased the basal levels of p-Erk (Fig. 7C). Shp2 knockdown delayed induction of p-Erk from 15 to 30 min by estrogen stimulation (Fig. 7C). Combined stimulation of estrogen with leptin partially restored the reaction time of p-Erk to 15 min. Interestingly, ER $\alpha$  knockdown also caused a delayed induction of

male mice was measured daily for 7 days. The data are expressed as means  $\pm$  the SD. (E) Immunoblotting analysis was performed to evaluate serum levels of adiponectin after 20 weeks on HFD. The lower panel gives the relative intensities of the adiponectin bands in the immunoblot. (F to H) Serum levels of estrogen, testosterone, and corticosterone measured after 20 weeks on a HFD.



**FIG 6** Shp2 couples leptin and estrogen signaling in hypothalamus. (A) Shp2, ER $\alpha$ , and pY-STAT3 signals were detected by immunostaining with specific antibodies in the hypothalamus area after leptin injection. Scale bar, 50  $\mu$ m. (B and C) Brain (B) and uterus (C) tissues were used to perform coimmunoprecipitation assay with specific antibodies to Shp2 and ER $\alpha$ . (D) MCF-7 cell lysate was prepared for coimmunoprecipitation with specific antibodies to ER $\alpha$  or Shp2 and subjected to immunoblotting as shown. Immunofluorescent staining for Shp2 and ER $\alpha$  in MCF-7 cells. (E) *In vitro* translation system using estradiol-17 $\beta$  (E2) enhanced binding of Shp2 and ER $\alpha$  *in vitro*. (F) E2-induced association of Shp2 with ER $\alpha$  in MCF-7 cells.

p-Erk signal by leptin, similar to Shp2 knockdown; the combined treatment of leptin and estrogen did not rescue the delay in p-Erk induction. In contrast, expression of Shp2<sup>D61A</sup> in MCF-7 cells resulted in sustained Erk activation by leptin and estrogen (Fig. 7D), indicating a coordinated regulation by Shp2 of the leptin and estrogen signaling pathways.

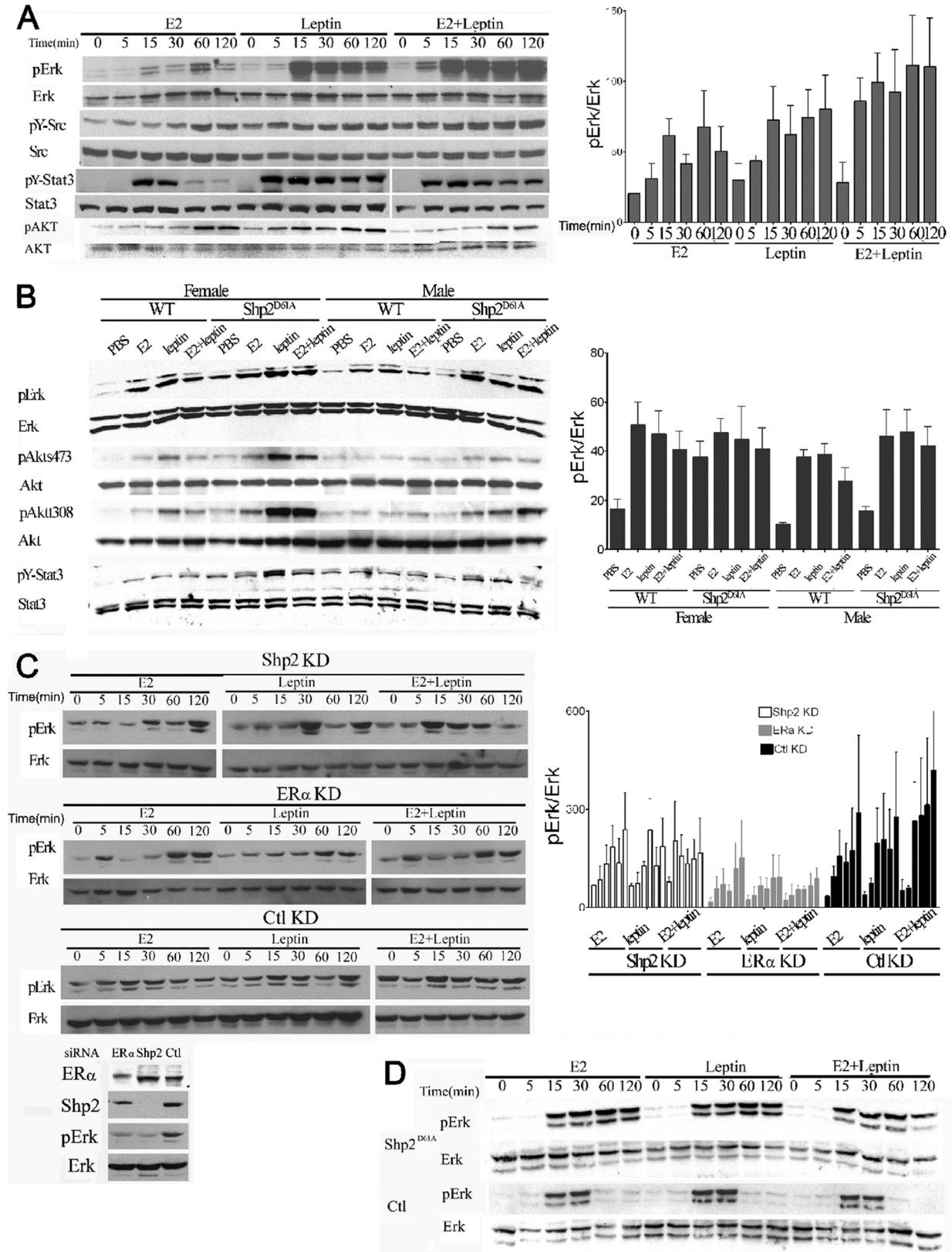
**Hypothalamic injection of AAV-Shp2<sup>D61A</sup> causes a similar phenotype as the transgenic mice.** As described above, Shp2<sup>D61A</sup> was expressed in forebrain neurons in the transgenic mice generated in the present study. To determine whether hypothalamus is indeed the target site for Shp2<sup>D61A</sup>, we generated AAV-Shp2<sup>D61A</sup> virus and specifically administered the AAV-GFP control or AAV-Shp2<sup>D61A</sup> virus into the mediobasal hypothalamus of wt C57BL/6 mice at age of 8 weeks (38) (Fig. 8A). The female mice injected with AAV-Shp2<sup>D61A</sup> had body weights similar to those of the control group on chow diet. Fed with HFD, the body weights of AAV-Shp2<sup>D61A</sup> female mice were significantly decreased compared to those for the controls (Fig. 8B and C). Consistent with the transgenic male animals, the antiobese effect was not detected in male mice that received AAV-Shp2<sup>D61A</sup> when placed on an HFD. AAV-Shp2<sup>D61A</sup> injection suppressed animal food intake compared to the control group (Fig. 8D). AAV-Shp2<sup>D61A</sup>-injected female mice had less fat mass than control mice injected with AAV-GFP (Fig. 8E and F). AAV-Shp2<sup>D61A</sup> female mice displayed a higher rate of O<sub>2</sub> consumption and CO<sub>2</sub> production (whether or not normalized by lean mass weight) (Fig. 8G and H). Furthermore, the injection of AAV-Shp2<sup>D61A</sup> also enhanced glucose handling capability of experimental animals (Fig. 8I). Thus, hypothalamic expression of Shp2<sup>D61A</sup> attenuated HFD-induced obese development by increasing energy expenditure and improving glucose homeostasis in a sex-dimorphic manner.

## DISCUSSION

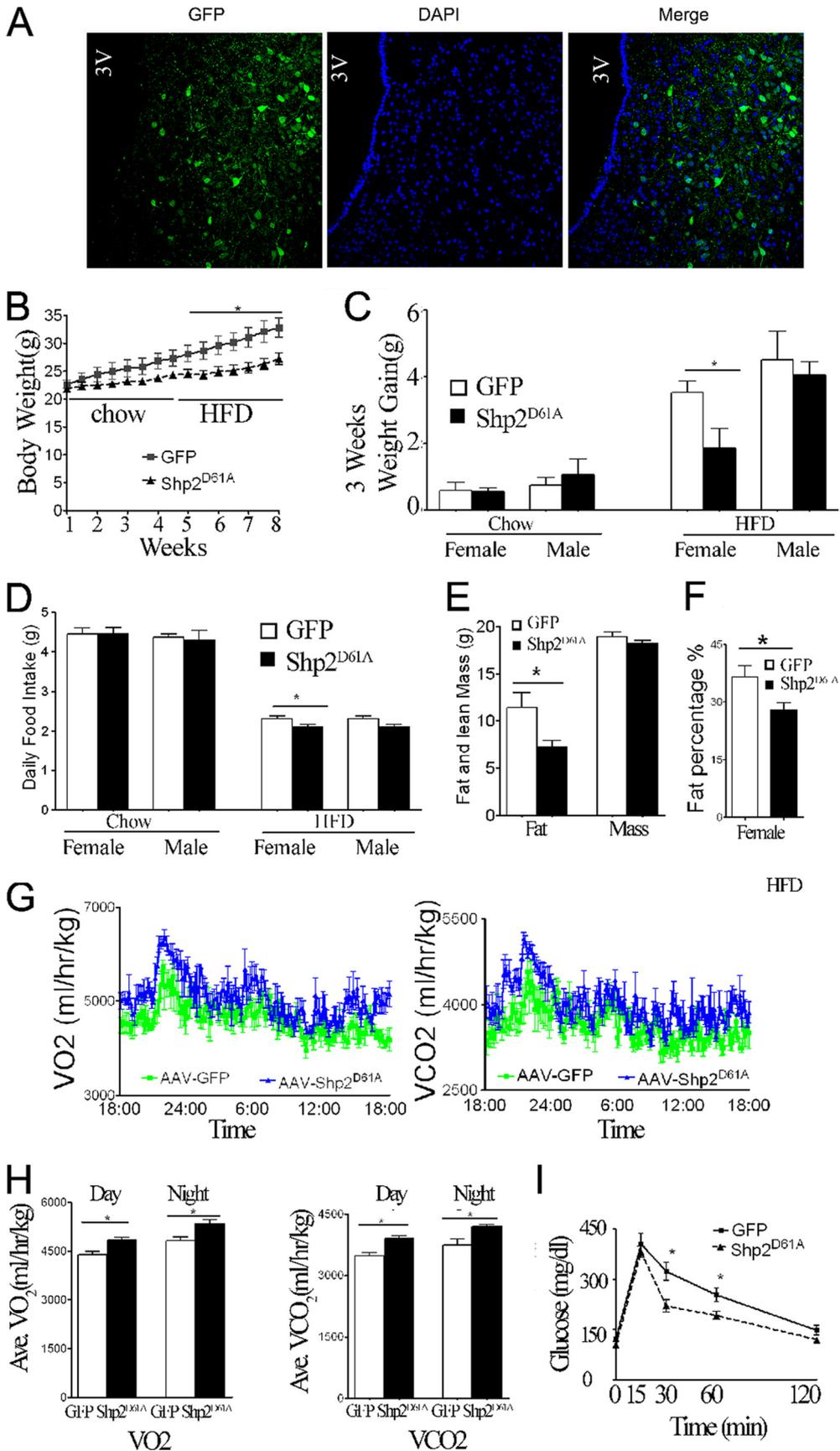
In previous experiments, we showed that selective ablation of Shp2 in the brain resulted in leptin resistance and early onset obesity, suggesting a positive role of this enzyme in leptin signal relay in the hypothalamus (22, 37). In the present study, we have demonstrated that enhancing Shp2 activity locally in the hypothalamus increases leptin activity in the control of energy metabolism. As expected, expression of a dominant-active mutant of Shp2 in forebrain neurons decreases adiposity, increases energy expenditure, and impairs food intake in transgenic mice. Although the Shp2<sup>D61A</sup> mutant is more widely expressed in forebrain neurons, including the hypothalamus in the transgenic mouse, we further demonstrate that a similar antiobese phenotype is induced by the injection of AAV-Shp2<sup>D61A</sup> virus into mediobasal hypothalamus. Together, these data strongly suggest that the hypothalamus is indeed the primary acting site for Shp2 in the transgenic mouse line for the regulation of leptin signal.

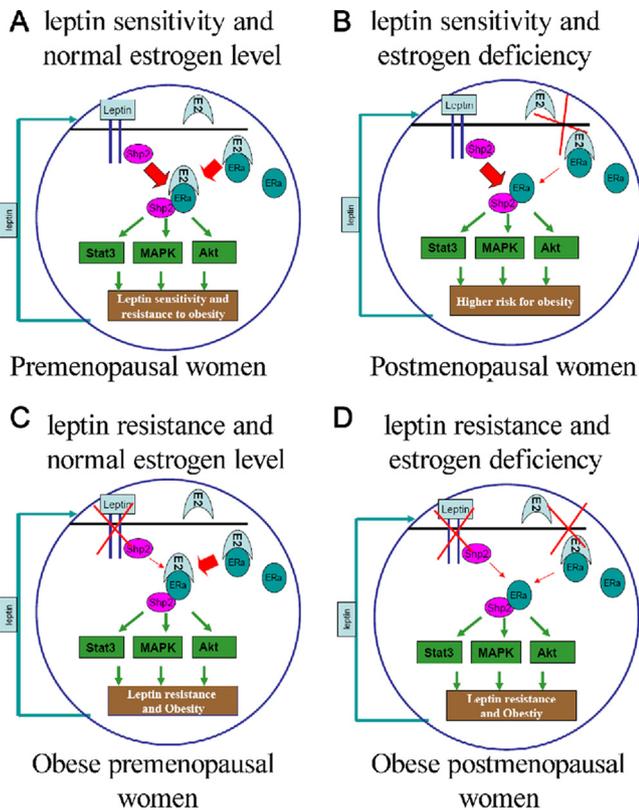
However, it is interesting that the antiobese effect of Shp2<sup>D61A</sup> mutant is much more prominent in female than in male mice. Consistent with the sex-dimorphic phenotype, we have found that Shp2 is physically associated with the estrogen receptor ER $\alpha$  and that the association is enhanced by estrogen stimulation. Thus, Shp2, a cytoplasmic signaling molecule, acts to mediate the direct coupling of signaling events triggered by a metabolic hormone leptin and a sex hormone estrogen in the hypothalamus (Fig. 9). Expression of Shp2<sup>D61A</sup> mutant in male mice induced a mild effect on insulin signaling and glucose homeostasis, suggesting that the HPA axis is involved in the control of glucose metabolism.





**FIG 7** Shp2 enhances leptin and estrogen signals. (A) Leptin and E2 induced phosphorylation of Erk1/2, Src, Stat3, and Akt in PC12<sup>LepR<sup>b</sup></sup> cells. The right panel shows p-Erk quantification ( $n = 3$ ). (B) Leptin and E2 stimulated phosphorylation of Erk1/2, Akt, and Stat3 in the brains of Shp2<sup>D61A</sup> mice. The right panel shows p-Erk quantification ( $n = 4$  to 5). (C) Knockdown of Shp2 or ER $\alpha$  inhibited p-Erk1/2 induction by leptin or estrogen. Cells incubated in medium with 1 to 2% serum were treated with either or both of the two hormones. The right panel shows p-Erk quantification ( $n = 3$ ). (D) Cells starved in serum-free medium were treated with leptin and/or estrogen for the induction of p-Erk1/2 signals.





**FIG 9** Model for concerted leptin and estrogen signaling in hypothalamic regulation of energy balance. (A) Young women and girls have normal levels of leptin and estrogen that act in concert in the hypothalamus to regulate energy metabolism. (B) Even with normal leptin levels, postmenopausal women have increased risk for development of obesity and leptin resistance, due to estrogen deficiency. (C) Obese young women with normal estrogen production may develop obesity due to leptin resistance. (D) Obese postmenopausal women have defects in leptin and estrogen signaling due to leptin resistance and estrogen deficiency.

In addition to the well-known role in control of metabolism and energy balance, leptin also regulates reproductive functions (13), indicating the overlap of downstream signaling pathways elicited by leptin and sex hormones. Rodents lacking leptin (*ob/ob*) or leptin receptor (*db/db*) are infertile (34, 39). Leptin injection restores the fertility of *ob/ob* mice (3, 8). Characterization of genetically engineered mouse models also showed that leptin mediates reproductive function in the forebrain (11, 29). Deficient signaling downstream of leptin receptor leads to various obese phenotypes in a sex-biased manner. On the other hand, estrogen, a sex hormone, plays a critical role in gender development, repro-

ductive physiology, and the female mammary gland. Importantly, experimental data *in vivo* and *in vitro* have provided evidence that estrogen signaling modulates fat accumulation and body weight by binding to ER $\alpha$  (2, 19, 27). In rodents, siRNA-mediated silencing of ER $\alpha$  in the hypothalamus area resulted in development of obesity and other metabolic disorders (17, 25). Consistently, ovariectomized rats deficient for estrogen develop obesity and leptin resistance, which can be reversed by E2 replacement therapy (9). A recent report indicates that a mutant ER $\alpha$  retaining the activity for noncanonical signaling (lacking the DNA-binding ability to the estrogen response element [ERE]) is sufficient to mediate the antiobese effect of ER $\alpha$  in energy homeostasis (28). However, it remains to be elucidated how the estrogen and leptin signals are coupled in regulation of energy metabolism.

Data presented here suggest that the Shp2/ER $\alpha$  complex, acting downstream of LepRb, mediates the concerted regulation of cytoplasmic signaling pathways by leptin and estrogen. Ovariectomy of Shp2<sup>D61A</sup> mice disrupted their resistance to HFD-induced obesity. Consistently, ablation of ER $\alpha$  or aromatase in mice leads to the development of age-dependent obesity and female animals displayed more severe weight gain, indicating that the estrogen signaling pathway is associated with sexually dimorphic obesity (19). The Shp2 effect was amplified in the Shp2<sup>D61A</sup> mutant molecule, since it has elevated catalytic activity due to the removal of the autoinhibitory mechanism. Therefore, expression of Shp2<sup>D61A</sup> allowed us to observe a dramatic difference between male and female mice in the present study. One prominent effect for the integration of leptin and estrogen signaling by Shp2 is to mediate stronger and more sustained activation of the Erk pathway, and it may also affect signaling events through Stat3 and Akt (Fig. 7B). Therefore, transgenic mice showed enhanced sensitivity to leptin and estrogen stimulation. Compared to wt controls, Shp2<sup>D61A</sup> mice exhibited lower circulating levels of leptin and higher basal levels of p-Erk, reinforcing the notion that Shp2 acts to amplify leptin signal. However, caution needs to be taken in interpretation of results from transgenic mice in which a constitutively active molecule is expressed in the forebrain region. In the present study, Shp2<sup>D61A</sup> was expressed in transgenic mice at lower level than the wt protein. Therefore, we are reasonably confident that the transgenic phenotype is not an artificial result but reveals a physiological function of Shp2.

Based on the experimental data presented here and previously known overlapping functions between leptin and estrogen, we propose a model illustrating the mechanism for development of obesity in postmenopausal women due to estrogen deficiency and leptin resistance (Fig. 9). Epidemiological data have also suggested a higher risk for the development of breast cancer in obese women

**FIG 8** Hypothalamic injection of AAV-Shp2<sup>D61A</sup> prevents HFD-induced obesity by inhibiting food intake and increasing energy expenditure. C57BL/6 mice (chow-fed, ~3 months old) with matched sex and body weight were randomized and bilaterally injected with AAV-Shp2<sup>D61A</sup> or the control AAV-GFP into the mediobasal hypothalamus. After viral injection, the mice were maintained on chow feeding for 3 to 4 weeks and then switched to HFD feeding. (A) Brain sections across the mediobasal hypothalamus were prepared from AAV-GFP injected mice, and the GFP distribution in hypothalamus sections was examined by GFP immunostaining. DAPI (4',6'-diamidino-2-phenylindole) staining revealed the nuclei of all cells in the sections. 3V, third ventricle. Scale bar, 50  $\mu$ m. (B) Body weights of AAV-Shp2<sup>D61A</sup>-injected female mice versus AAV-GFP-injected female mice. (C) Body weight gains of AAV-Shp2<sup>D61A</sup>-injected mice versus AAV-GFP-injected mice on HFD. The data are expressed as means  $\pm$  the SD. \*,  $P < 0.05$ . (D) Average daily HFD intake of AAV-Shp2<sup>D61A</sup>-injected mice versus AAV-GFP-injected mice. The data are expressed as means  $\pm$  the SD. \*,  $P < 0.05$ . (E) Fat and lean masses of AAV-Shp2<sup>D61A</sup>-injected mice versus AAV-GFP-injected female mice. (F) Fat percentages of AAV-Shp2<sup>D61A</sup>-injected female mice versus AAV-GFP-injected female mice with HFD. The data are expressed as means  $\pm$  the SD. \*,  $P < 0.05$ . (G and H) O<sub>2</sub> consumption and CO<sub>2</sub> production of AAV-Shp2<sup>D61A</sup>-injected female mice versus AAV-GFP-injected female mice on HFD. O<sub>2</sub> consumption and CO<sub>2</sub> production data were normalized by lean mass of mice. The data are expressed as means  $\pm$  the SD. \*,  $P < 0.05$ . (I) GTT in AAV-Shp2<sup>D61A</sup>-injected female mice versus AAV-GFP-injected female mice on HFD. The data are expressed as means  $\pm$  the SD. \*,  $P < 0.05$ .

because of an elevated estrogen level (6, 30). Increased serum levels of leptin were frequently detected in breast cancer patients (35), and oncogene-induced mammary tumorigenesis was suppressed in mice deficient for leptin or leptin receptor (10). Notably, overexpression of Shp2 has been detected in breast cancer cells (40). Elucidating Shp2 action in linking leptin and estrogen signaling in breast tumorigenesis may provide novel therapeutic target for improving prognosis of breast cancer patients worldwide.

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