

The Creb1 coactivator Crtc1 is required for energy balance and fertility

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The adipocyte-derived hormone leptin maintains energy balance by acting on hypothalamic leptin receptors (Leprs) that act on the signal transducer and activator of transcription 3 (Stat3)^{1–4}. Although disruption of Lepr-Stat3 signaling promotes obesity in mice, other features of Lepr function, such as fertility, seem normal, pointing to the involvement of additional regulators. Here we show that the cyclic AMP responsive element-binding protein-1 (Creb1)-regulated transcription coactivator-1 (Crtc1) is required for energy balance and reproduction—*Crtc1*^{−/−} mice are hyperphagic, obese and infertile. Hypothalamic Crtc1 was phosphorylated and inactive in leptin-deficient *ob/ob* mice, while leptin administration increased amounts of dephosphorylated nuclear Crtc1. Dephosphorylated Crtc1 stimulated expression of the *Cartpt* and *Kiss1* genes, which encode hypothalamic neuropeptides that mediate leptin's effects on satiety and fertility^{5–7}. Crtc1 overexpression in hypothalamic cells increased *Cartpt* and *Kiss1* gene expression, whereas Crtc1 depletion decreased it. Indeed, leptin enhanced Crtc1 activity over the *Cartpt* and *Kiss1* promoters in cells overexpressing Lepr, and these effects were disrupted by expression of a dominant-negative Creb1 polypeptide. As leptin administration increased recruitment of hypothalamic Crtc1 to *Cartpt* and *Kiss1* promoters, our results indicate that the Creb1-Crtc1 pathway mediates the central effects of hormones and nutrients on energy balance and fertility.

Crcs (also known as transducers of regulated CREB activity, or TORCs) are latent cytoplasmic coactivators that shuttle to the nucleus in response to cyclic AMP (cAMP) and calcium signals^{8,9}. Similar to the ubiquitously expressed Crtc2, Crtc1 contains conserved phosphorylation (Ser151) and ubiquitination (Lys575) sites that modulate nuclear shuttling and protein stability^{10,11} (Fig. 1a). However, in contrast to *Crtc2*, *Crtc1* messenger RNA and protein are detected primarily in the brain¹² (Fig. 1a and Supplementary Fig. 1a online).

Under basal conditions, Crtc1 is highly phosphorylated in mouse hypothalamic GT1-7 cells¹³ (Fig. 1b). Exposure to cAMP or a calcium activator triggers Crtc1 dephosphorylation and nuclear translocation, whereas a phosphorylation-defective S151A-mutant Crtc1 is

constitutively nuclear (Supplementary Fig. 1b). Wild-type CRTC1 potentiates cAMP-responsive element (CRE)-luciferase reporter activity in HEK293T cells exposed to the adenylate cyclase activator forskolin or the calcium ionophore A23187, whereas phosphorylation-defective S151A CRTC1 upregulates CRE-luciferase activity even under basal conditions (Fig. 1b). The effects of CRTC1 seem to be CREB1 dependent, because coexpression of a dominant-negative CREB1 polypeptide, called A-CREB¹⁴, disrupts reporter activity in cells exposed to forskolin or A23187 (Fig. 1b).

We evaluated the biological role of Crtc1 by insertional mutagenesis of the *Crtc1* gene with a promoter-less β-galactosidase (β-Geo) gene cassette. By contrast with tissues from control littermates, *Crtc1* mRNA and protein were undetectable in *Crtc1*^{−/−} mice (Fig. 1c). Consistent with its regulation by the *Crtc1* promoter, β-Geo cassette expression in the central nervous system of *Crtc1*-mutant mice mirrored that of endogenous Crtc1 protein (Fig. 1d and Supplementary Fig. 2a,b online). In addition to other brain regions, Crtc1 expression was prominent in arcuate and ventromedial nuclei of the hypothalamus (Fig. 1d).

Crtc1^{−/−} mice were born at the expected mendelian frequency and they were indistinguishable from controls before weaning. Although their linear growth was unimpaired, adult *Crtc1*^{−/−} mice were infertile, and no offspring were obtained from *Crtc1*^{−/−} males or *Crtc1*^{−/−} females mated with wild-type mice (0/6 matings). *Crtc1*^{−/−} female uteri appeared threadlike, with noticeable thinning of the endometrium (Fig. 1e). Although *Crtc1*^{−/−} ovaries had comparable numbers of mature follicles to those of wild-type ovaries, they contained no corpora lutea, markers of ovulation (Fig. 1e). Correspondingly, circulating concentrations of pituitary luteinizing hormone, a key regulator of ovulation, were downregulated in *Crtc1*-mutant mice (Fig. 1e).

Male and female *Crtc1*^{−/−} mice developed persistent obesity beginning at 9 weeks of age on a normal chow diet, and *Crtc1*^{+/-} heterozygotes had intermediate weight gains (Fig. 1f). White adipose mass was increased two- to threefold in *Crtc1*-mutant mice, whereas other tissues were unaffected (Fig. 1f and Supplementary Fig. 3 online). These results indicate that the effects of Crtc1 on body weight are specific to white adipose tissue, affect both males and females and vary with gene dosage.

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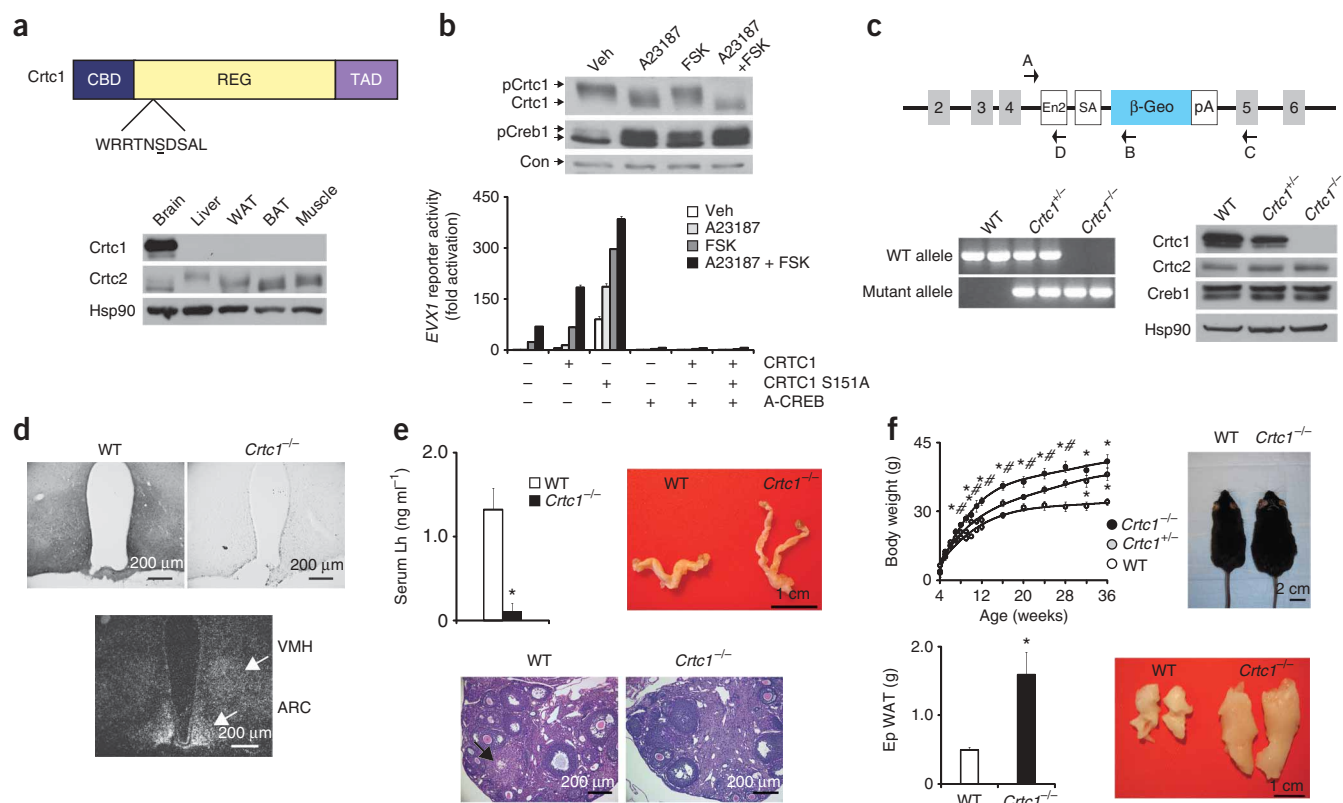


Figure 1 Mice with a knockout of *Crtc1* are obese and infertile. **(a)** Top, *Crtc1* schematic showing Creb1-binding (CBD), regulatory (REG) and transcriptional activation (TAD) domains; regulatory Ser151 phosphorylation site indicated. Bottom, immunoblot of *Crtc1* protein in various tissues. Hsp90, heat shock protein 90, a positive control. **(b)** Top, immunoblot showing effect of calcium ionophore (A23187; 1 μ M) and forskolin (FSK; 1 μ M) on phospho-*Crtc1* (pCrtc1) amounts in GT1-7 cells. Con, Control; Veh, Vehicle. Bottom, effect of wild-type and S151A mutant *CRTC1* on CRE-luc reporter activity in HEK293T cells exposed to FSK and A23187; effect of dominant-negative Creb1 polypeptide A-CREB indicated. $P < 0.05$ for multiple comparisons between the groups. *EVX1*, even-skipped homeobox 1. **(c)** Top, schematic showing *Crtc1* gene disruption through insertion of the gene trap vector pGTO1xf containing engrailed 2 (*En2*) sequences, splice acceptor (SA), β -galactosidase-neomycin resistance (β -Geo) cassette and polyadenylation sequence (pA). Primers used to verify gene trap insertion (A and B) and for genotyping (A, C and D) are indicated. Bottom left, PCR genotyping of wild-type (WT), *Crtc1*^{+/-} and *Crtc1*^{-/-} mice. Bottom right, *Crtc1* protein amounts in brain extracts from WT and *Crtc1*-mutant mice. **(d)** Top, *Crtc1* protein staining in arcuate (ARC) and ventromedial (VMH) nuclei in WT (left) or *Crtc1*^{-/-} mice (right). Bottom, *Crtc1* promoter activity in hypothalamic sections from *Crtc1*^{-/-} mice determined by *in situ* hybridization analysis with β -Geo probe. **(e)** Top, plasma luteinizing hormone (Lh) abundance (left) and uterine morphology (right) in WT and *Crtc1*^{+/-} females ($*P < 0.05$, $n = 3$). Bottom, ovarian sections from WT and *Crtc1*^{+/-} mice. Arrow points to corpus luteum in WT mice. **(f)** Top left, relative weights of WT and *Crtc1*-mutant mice ($*P < 0.05$ compared to WT mice; $\#P < 0.05$ compared to *Crtc1*^{+/-} mice, $n = 6-37$; data are means \pm s.e.m.). Top right, appearance of WT and *Crtc1*^{-/-} littermates at 36 weeks. Bottom left, epididymal fat pad mass in 36-week-old control and *Crtc1*^{-/-} mice ($*P < 0.05$, $n = 6-10$ per group). Bottom right, morphology of epididymal white adipose tissue from 36-week-old WT and *Crtc1*^{-/-} mice. Error bars are means \pm s.e.m.

We performed metabolic studies to determine why *Crtc1*-mutant mice gain more weight than do wild-type mice. Relative to controls, *Crtc1*^{-/-} mice ate more and expended less energy at 12–14 weeks of age, and they became hyperglycemic and hypertriglyceridemic at 9 months of age (Fig. 2a,b). Pointing to the development of insulin resistance, circulating insulin abundance was increased in *Crtc1*^{+/-} mice and was increased to an even greater extent in *Crtc1*^{-/-} homozygotes, who we found to be glucose intolerant by intraperitoneal (i.p.) glucose tolerance testing (Fig. 2b,c). Circulating leptin concentrations were also upregulated in *Crtc1*^{-/-} mice (Fig. 2b).

During feeding, increases in circulating concentrations of leptin as well as insulin and glucose promote satiety and fertility, in part through the activation of arcuate neurons in the hypothalamus^{4,15–17}. Realizing that *Crtc1*^{-/-} mice are hyperphagic, obese and infertile, we wondered whether *Crtc1* is required for the activation of relevant hypothalamic programs in response to feeding signals. Although chronic leptin infusion reduced food intake and body weight in control mice, as has been reported before¹⁸, it had minimal

effects on *Crtc1*^{-/-} mice (Fig. 2d). Arguing against potential effects on leptin bioavailability, chronic leptin infusion promoted Stat3 phosphorylation comparably in arcuate neurons of wild-type and *Crtc1*^{-/-} mice (Fig. 2e).

Consistent with the ability of leptin to increase hypothalamic Stat3 activity, mRNA amounts of proopiomelanocortin, neuropeptide Y and agouti-related peptide, regulatory targets of the *Lepr*-Stat3 pathway, were comparable between *Crtc1*-mutant mice and controls (Supplementary Fig. 4a,b online). Signaling through the downstream melanocortin pathway also seemed normal in *Crtc1* mutants, because i.p. administration of the α -melanocyte-stimulating hormone analog MTII¹⁹ inhibited food intake similarly in wild-type and *Crtc1*^{-/-} mice (Supplementary Fig. 5 online).

We used leptin-deficient *ob/ob* mice to determine whether *Crtc1* activity is disrupted by loss of leptin signaling. Supporting this idea, *ob/ob* mice had increased amounts of phosphorylated, inactive *Crtc1* in the hypothalamus compared to wild-type controls (Fig. 2f). Injection of leptin i.p. increased the amounts of dephosphorylated,

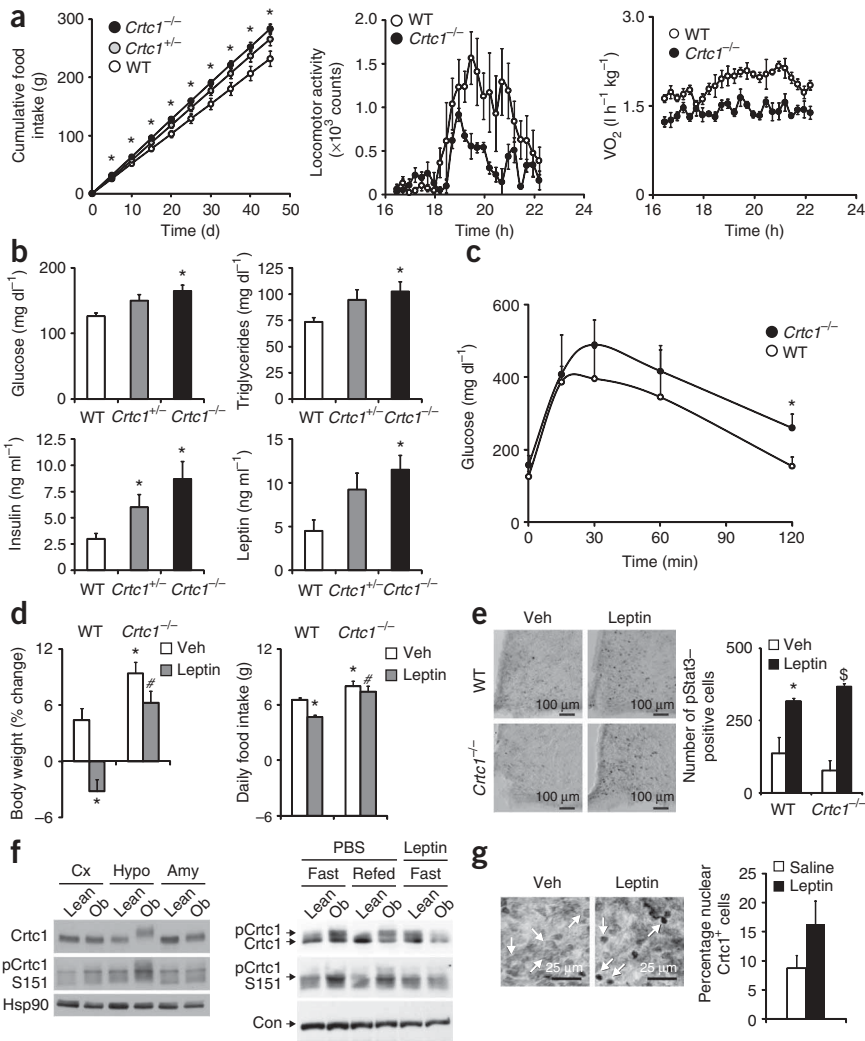


Figure 2 *Crtc1*^{-/-} mice are hyperphagic and have reduced energy expenditure. **(a)** Cumulative food intake over a 45-day interval, beginning at 12 weeks of age, in *Crtc1*^{-/-}, *Crtc1*^{+/-} and WT littermates on a normal chow diet (left; **P* < 0.05, *n* = 7 or 8; data are means ± s.e.m.). Physical activity (center) and oxygen consumption (right) in 14-week-old *Crtc1*^{-/-} and control littermates (*n* = 4 mice per group). **(b)** Circulating glucose (top left), triglyceride (top right), plasma insulin (bottom left) and leptin (bottom right) concentrations in 36-week-old WT, *Crtc1*^{+/-} and *Crtc1*^{-/-} mice (**P* < 0.05, *n* = 5–12 per group). **(c)** Glucose tolerance testing (**P* < 0.05, *n* = 4 or 5) of WT and *Crtc1*^{-/-} mice. **(d)** Effect of subcutaneous leptin infusion (10 d; 300 ng h⁻¹) on body weight (left) and average daily food intake (right) in WT and *Crtc1*^{-/-} mice (**P* < 0.05 compared to vehicle-infused WT mice; #*P* < 0.05 compared to leptin-infused WT mice; *n* = 5–7; data are means ± s.e.m.). **(e)** Left, effect of leptin infusion on phosphorylated Stat3 (p Stat3) staining in arcuate cells of WT and *Crtc1*^{-/-} mice. Right, pStat3-positive cell numbers in leptin-infused WT and *Crtc1*^{-/-} mice (**P* < 0.05 compared to vehicle-infused WT mice; \$*P* < 0.05 compared to vehicle-infused *Crtc1*^{-/-} mice; *n* = 3; data are means ± s.e.m.). **(f)** Left, immunoblot showing amounts of total and pCrtc1 on Ser151 in cortex (Cx), hypothalamus (Hypo) and amygdala (Amy) from lean and *ob/ob* (Ob) mice. Right, effect of leptin or PBS i.p. injection on total and p Crtc1 amounts in hypothalamus from fasted or refed lean and *ob/ob* mice. **(g)** Left, Crtc1 staining in arcuate sections from *ob/ob* mice injected i.p. with leptin (3 μg g⁻¹) or saline control. Arrows point to cytoplasmic Crtc1 staining in control sections and nuclear Crtc1 staining in leptin-treated sections. Right, graph showing percentage of Crtc1⁺ nuclei in hypothalamic sections from PBS- or leptin-treated *ob/ob* mice. Error bars are means ± s.e.m.

nuclear Crtc1 protein in arcuate cells of *ob/ob* mice (Fig. 2g). Consistent with a parallel role for nutrient signaling, i.p. glucose administration also promoted the accumulation of dephosphorylated Crtc1 in the hypothalamus (Supplementary Fig. 6a online). Correspondingly, Crtc1 localized in the nucleus in arcuate cells during *ad libitum* feeding but remained cytoplasmic in other central nervous system regions (Supplementary Fig. 6b).

We performed gene profiling studies to identify hypothalamic genes that contribute to the metabolic and reproductive phenotypes of *Crtc1*-mutant mice. This analysis revealed that mRNAs for the neuropeptide genes cocaine and amphetamine-regulated transcript prepropeptide (*Cartpt*) and *Kiss1* were downregulated in *Crtc1*^{-/-} mice (data not shown). *Cartpt* and *Kiss1* have been found to mediate effects of Lepr signaling on feeding and fertility^{6,7,20,21}. *Cartpt* is expressed with proopiomelanocortin in arcuate neurons, where it inhibits food intake in response to leptin²¹, whereas arcuate *Kiss1* promotes reproductive function by stimulating the secretion of hypothalamic gonadotropin-releasing hormone^{22,23}. Similar to *Crtc1*^{-/-} mice, *Kiss1*-mutant mice have low circulating concentrations of luteinizing hormone, show abnormal uterine morphology and are infertile²⁴. We confirmed that the *Cartpt* and *Kiss1* genes are downregulated in *Crtc1*^{-/-} mice by quantitative PCR (Q-PCR) and *in situ* hybridization analysis (Fig. 3a,b). Hypothalamic staining for

kisspeptin, a cleavage product of the *Kiss1* precursor, was substantially reduced in *Crtc1*^{-/-} arcuate neurons (Fig. 3b). Notably, *Crtc1*-driven β-galactosidase mRNA was co-expressed with *Cartpt* and *Kiss1* neuropeptides in arcuate cells (Fig. 3c).

Realizing that *Cartpt*^{25–27} and *Kiss1* (data not shown) promoters contain conserved Creb1 binding sites, we considered that Crtc1 may regulate both genes via a direct mechanism. Supporting this idea, Creb1 has been shown to promote *Cartpt* gene expression^{25–27}, although its role in *Kiss1* regulation has not been established. In keeping with its effects on Crtc1 dephosphorylation, A23187 treatment increased *Cartpt* and *Kiss1* mRNA levels in GT1-7 cells, and this induction was attenuated after depletion of Crtc1 by RNA interference (RNAi)-mediated knockdown (Fig. 3d). Exposure to A23187 or forskolin also increased *Cartpt* and *KISS1* reporter activities in transient assays (Fig. 4a,b); overexpression of wild-type CRTC1 and, to a greater extent, phosphorylation-defective (S151A) CRTC1 enhanced transcription from both promoters in HEK293T cells (Fig. 4a,b). Consistent with the importance of Creb1, expression of dominant-negative A-CREB inhibitor blocked induction of the *KISS1* reporter by forskolin and A23187 in HEK293T cells (Fig. 4b).

We performed chromatin immunoprecipitation assays (ChIPs) to determine whether Crtc1 and Creb1 regulate *Cartpt* and *Kiss1* genes directly. Creb1 occupied *Cartpt* and *Kiss1* promoters in GT1-7 cells

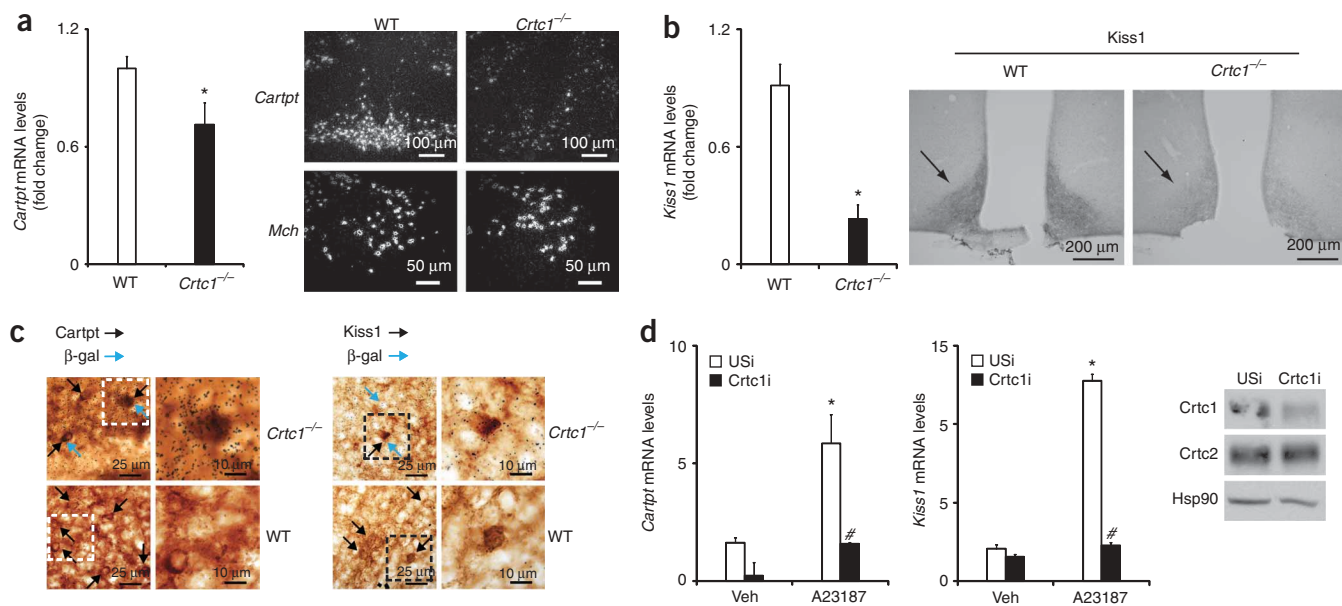


Figure 3 Reduced hypothalamic expression of anorexigenic and reproductive neuropeptide genes in *Crtc1*^{-/-} mice. **(a)** Q-PCR (left; **P* < 0.05, *n* = 3) and *in situ* hybridization (right) analysis of *Cartpt* mRNA amounts in WT and *Crtc1*^{-/-} mice. *In situ* hybridization analysis of melanin-concentrating hormone (*Mch*) mRNA in WT and *Crtc1*^{-/-} mice also shown as a positive control. **(b)** Q-PCR (left; **P* < 0.05, *n* = 5 or 6) of *Kiss1* in hypothalami of *Crtc1*^{-/-} and control littermates. Right, kisspeptin staining in arcuate sections from WT and *Crtc1*^{-/-} mice with antiserum to kisspeptin-10. **(c)** Combined immunohistochemistry for *Cartpt* (left) or *Kiss1* (right) and *in situ* hybridization for *Crtc1* promoter-driven β -gal in colchicine-treated *Crtc1*^{-/-} mice and control littermates. Black arrows indicate cells with positive immunostaining. Blue arrows indicate cells positive for β -gal mRNA. Images to the right are higher magnifications of the boxed regions in the adjoining images to the left. **(d)** Effect of A23187 exposure (1 μ M; 2 h) on mRNA amounts for *Cartpt* (left) and *Kiss1* (center) in control (USi) and *Crtc1*-depleted (Crtc1i) GT1-7 cells (**P* < 0.05 compared to vehicle-treated cells expressing unspecific siRNA; #*P* < 0.05 compared to A23187-treated cells expressing unspecific RNAi; data are means \pm s.e.m.). Right, immunoblot showing effect of RNAi-mediated *Crtc1* knockdown on *Crtc1* protein amounts relative to control cells expressing unspecific siRNA (USi).

under basal conditions and after exposure to forskolin or A23187 (Fig. 4c). *Crtc1* occupancy of the *Cartpt* and *Kiss1* promoters was low under basal conditions (when *Crtc1* is sequestered in the cytoplasm) and increased after exposure to cAMP or calcium activator (when dephosphorylated *Crtc1* shuttles to the nucleus and binds Creb1; Supplementary Fig. 1b). Consistent with its effect on nuclear *Crtc1* protein amounts in arcuate neurons, i.p. leptin administration also enhanced recruitment of hypothalamic *Crtc1* to *Cartpt* and *Kiss1* promoters in *ob/ob* mice (Fig. 4d). Taken together, these results indicate that Creb1 and *Crtc1* regulate *Cartpt* and *Kiss1* gene expression through a direct mechanism.

On the basis of the importance of *Crtc1* and Creb1 for transcriptional induction by cAMP and calcium signals, we wondered whether they are required for the effects of leptin on neuropeptide gene expression. Exposure to leptin increased *Cartpt* and *KISS1* reporter activities synergistically with forskolin in *Lepr*-expressing HEK293T cells, and these effects were augmented by CRTC1 overexpression (Fig. 4e,f). Similar to its effects on cAMP and calcium signaling, A-CREB inhibitor blocked induction of the *KISS1* promoter in cells treated with leptin (Fig. 4f).

Our results indicate that *Crtc1* is activated by hormonal and nutrient signals in the hypothalamus, where it promotes energy balance and fertility by enhancing Creb1 activity over relevant genes. Like leptin-deficient *ob/ob* mice, *Crtc1*^{-/-} females have abnormal uterine morphology and low circulating luteinizing hormone levels²⁸. However, *Crtc1*-mutant mice are only moderately obese, potentially reflecting compensatory effects of other *Crtc* family members.

In addition to its effects on Stat3, leptin has also been reported to modulate cation channel activity²⁹ and inhibit the activity of

the AMP-activated kinase¹⁷. Given the ability of calcium and AMP-activated kinase pathways to regulate *Crtc1* activity, we imagine that these pathways may also mediate the stimulatory effects of leptin on *Crtc1*.

Obesity risk in humans has a strong genetic component, which is thought to involve heterozygous loss-of-function mutations in genes that, individually, may show only modest phenotypic changes. The presence of hyperphagia, increased adiposity and insulin resistance in *Crtc1*^{+/-} mice suggests that mutations in the *Crtc1* gene may also promote the development of obesity in humans. Future studies of *Crtc1* gene mutations in affected populations should provide further insight.

METHODS

Mice. We housed mice in a temperature-controlled environment under a 12-h light-dark cycle with free access to water and a standard rodent chow diet (Lab Diet 5001). We obtained approval for mouse studies from the Salk Institute Institutional Animal Care and Use Committee. We obtained mouse embryonic stem cells containing an insertional gene trap in the *Crtc1* gene (XK522; 129/Ola mouse strain) from BayGenomics^{30,31}. We injected embryonic cells into C57BL/6 blastocysts to generate chimeric mice. We backcrossed heterozygous mice with C57BL/6 mice for three generations and then intercrossed them to obtain homozygous *Crtc1*^{-/-} mice. We obtained male C57BL/6J, BKS.Cg-*m* +/+ *Lepr^{db}/J* and B6.V *LepOb* mice from Jackson Laboratories.

Genotyping. We prepared genomic DNA from tail biopsies as previously described³². We verified insertion of the pGT01xf cassette by sequence analysis with primers A (5'-GCATCCCTAGCTCTCACTCAGTTAC-3') and B (5'-GCG CGTACATCGGGCAAATAA-3'). We genotyped *Crtc1*-mutant mice by PCR amplification of wild-type and mutant alleles. We amplified the wild-type allele

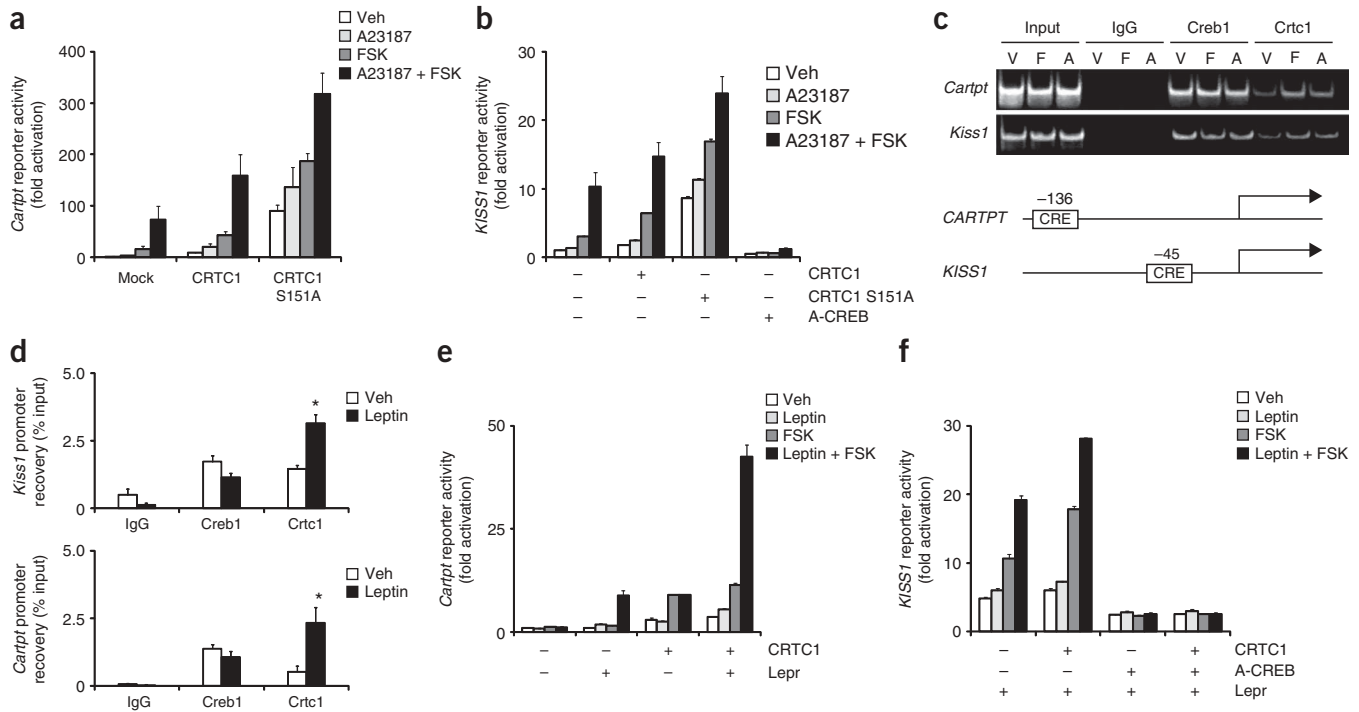


Figure 4 Crtc1 regulates hypothalamic *Cartpt* and *Kiss1* gene expression. **(a,b)** Transient assay showing effect of FSK and A23187 on *Cartpt*-luciferase **(a)** and *KISS1*-luciferase **(b)** reporters in HEK293T cells expressing WT or phosphorylation-defective S151A CRTC1. Effect of dominant negative A-CREB polypeptide is also shown. $P < 0.05$ for multiple comparisons between the groups. **(c)** Top, chromatin immunoprecipitation (ChIP) assay of GT1-7 cells showing effect of FSK (F; 1 μ M), A23187 (A; 1 μ M) or vehicle (V) on Creb1 and Crtc1 occupancy of the *Cartpt* and *Kiss1* promoters. Bottom, schematic showing conserved Creb binding sites in the *CARTPT* and *KISS1* promoters. **(d)** ChIP assay of hypothalamic tissue from *ob/ob* mice injected i.p. with PBS (Veh) or leptin (3 μ g g^{-1}) for 1 h. Hypothalamic Creb1 and Crtc1 occupancy of the *Cartpt* and *Kiss1* promoters in control and leptin-injected mice is indicated. $P < 0.05$ for Creb1 and Crtc1 occupancy compared to IgG control. $*P < 0.05$ compared to hypothalami from vehicle-treated *ob/ob* mice. **(e,f)** Transient assay of HEK293T cells with *Cartpt* **(e)** or *KISS1* **(f)** luciferase reporters. Effect of leptin (100 nM) on reporter activity in control and Lepr-expressing cells shown; co-treatment with FSK (1 μ M) is indicated. Expression of WT or phosphorylation-defective S151A CRTC1 is also indicated. Effect of dominant-negative A-CREB on reporter activity is shown. $P < 0.05$ for multiple comparisons between the groups. Error bars are means \pm s.e.m.

with primers A and C (5'-ATTCTCATATACCTCTCTTCTGGTGC-3'), and we amplified the mutant allele with primers A and D (5'-GCATGAATCAACTTTGGAGACATGCG-3').

Plasmids and drugs. We generated the *KISS1* luciferase reporter by cloning the human *KISS1* promoter region spanning -1141 to +8 base pairs from the start of exon 1, into pGL2 (Promega). The EVX-1 luciferase reporter and CRTC1 expression constructs have been previously described³³. We constructed lentiviruses encoding U6 promoter-driven interfering RNAs directed against the *Crtc1* sequence, 5'-GGTCCCTGCCCAACGTGAAC-3', as previously described³⁴. We purchased forskolin (Sigma), A23187 (Calbiochem) leptin (R&D Systems) and MTII (Bachem) from the respective manufacturers. We used dimethyl sulfoxide (Sigma) as a diluent and vehicle control for forskolin and A23187. We used sterile PBS as a diluent and vehicle control for leptin and MTII.

Food intake and indirect calorimetry. We housed mice individually for 3 d before measuring food intake or calorimetry. We monitored locomotor activity, oxygen consumption and carbon dioxide production with a Comprehensive Lab Animal Monitoring System (Columbus)³². We administered saline, leptin (2 μ g g^{-1}) and MTII (2 μ g g^{-1}) by i.p. injection 30 min before the onset of the dark cycle.

Nutrient and hormone measurements. We collected blood from the tail vein into EDTA-coated capillary tubes (StatSpin). We measured blood glucose and triglyceride levels with a OneTouch Ultra glucometer (LifeScan) and a Cardio-Chek PA analyzer. We determined insulin (Mercodia) and leptin (Alpco) abundance according to the manufacturers' protocols. We obtained luteinizing

hormone levels through the University of Virginia Center for Research in Reproduction Ligand Assay and Analysis Core. For glucose tolerance testing, we fasted mice for 16 h and then injected them with glucose (2 mg g^{-1} ; i.p.).

Subcutaneous infusion. We filled osmotic minipumps (0.25 μ l h^{-1} , Alzet) with 0.45- μ m-filtered sterile PBS or leptin (1.2 mg ml^{-1}) and implanted the primed pumps subcutaneously into isoflurane-anesthetized 7–8-week-old male *Crtc1*^{-/-} and wild-type littermates. We measured body weight and food intake daily for 10 d.

Immunohistochemistry and *in situ* hybridization. We performed immunohistochemistry and *in situ* hybridization experiments as previously described³⁵. To optimize cellular labeling for *Cartpt* and *Kiss1*, we injected mice intracerebroventricularly with 5 μ l sterile colchicine (2 mg ml^{-1}) as previously described³⁶.

Cell culture, transfections and luciferase assays. We cultured GT1-7 cells (a gift from P. Mellon) and HEK293T cells (American Type Culture Collection) in DMEM (Mediatech) containing 10% FBS (HyClone), 100 μ g ml^{-1} penicillin-streptomycin and 1 mM pyruvate (Mediatech). We performed transient transfections of GT1-7 cells with Eugene HD (Roche). We transfected HEK293T cells with Lipofectamine 2000 (Invitrogen). We determined luciferase and β -galactosidase activities as previously described³³.

RNA isolation and quantitative real-time PCR. We determined mRNA levels by Q-PCR analysis with the LightCycler 480 (Roche) as previously described^{10,11}.

Gene-profiling experiments. We fasted male 12-week-old wild-type and *Crtc1*^{-/-} mice (three of each genotype) overnight for 16–18 h and then fed them a normal chow diet for 6 h. We performed gene-profiling experiments on

total hypothalamic RNAs with an Affymetrix Mouse Genome 430 array as previously described³⁷.

Chromatin immunoprecipitation assays. We plated GT1-7 cells in 15-cm plates and treated them as indicated in **Figure 4c**. For mouse experiments, we dissected hypothalamic tissue from mice after the indicated manipulations. We prepared chromatin and performed immunoprecipitations as previously described³⁷.

Western blots. We performed immunoblots for Crtc1, Crtc2, Creb1 and phosphorylated Creb1 Ser133 as previously described^{10,11} with antibodies 6939E1, 6865, 244 and 5322, respectively (Salk Institute). We obtained antibodies for phosphorylated (Ser151) Crtc1 from Cell Signaling. We used immunoblots for Hsp90 (Santa Cruz Biotechnology) or nonspecific immunoreactive bands as negative controls.

Cell counting. We quantified phosphorylated Stat3 staining as well as nuclear and cytoplasmic Crtc1 staining in hypothalamic sections with an independent observer in a blinded manner. We counted five to eight matched arcuate-containing sections in whole-brain series from independent experiments.

Statistical analyses. Data presented are means \pm s.e.m. We performed statistical analyses with SigmaStat (Systat). We determined statistical differences for one factor between two groups or more than two groups with an unpaired Student's *t*-test or an analysis of variance (ANOVA) with a *post-hoc* test, respectively. We determined statistical differences for two factors between more than two groups with a two-way ANOVA with a *post-hoc* test. We considered values of $P < 0.05$ to be statistically significant.

Accession codes. Microarray data have been deposited in the National Center for Biotechnology Information Gene Expression Omnibus and are accessible through the accession number GSE12209.

Note: Supplementary information is available on the Nature Medicine website.

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AUTHOR CONTRIBUTIONS

J.Y.A., M.D.C., H.I., P.E.S. and M.M. contributed to the experimental design of this study. J.Y.A., M.D.C., H.I. and C.M.A. performed the mouse experiments. J.Y.A. and N.G. conducted cell culture and biochemical analyses. J.Y.A., N.G. and C.M.A. performed the immunohistochemical staining and *in situ* hybridization experiments. M.D.C. and J.X. developed the Crtc1 and pCrtc1 (Ser151) antisera, respectively. J.Y.A., N.G. and H.I. analyzed the data. M.M. and J.Y.A. wrote the manuscript.

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