

CRTC1 in Mc4r-expressing cells is required for peripheral metabolism and systemic energy homeostasis

Running title: CRTC1 in Mc4r-expressing cells and metabolism

Haruka Miyamori M.S.^{1,*}, Takumi Yokokawa Ph.D.^{1,*}, Motoki Miyakita M.S.¹, Kazuki Ozaki B.S.¹, Tsuyoshi Goto Ph.D.¹, Kazuo Inoue Ph.D.¹, and Shigenobu Matsumura Ph.D.²

¹Division of Food Science and Biotechnology, Graduate School of Agriculture, Kyoto University, Kyoto, Japan.

²Department of Nutrition, Osaka Metropolitan University, Osaka, Japan

*Equal contribution to this work

Corresponding author: Takumi Yokokawa

Division of Food Science and Biotechnology, Graduate School of Agriculture, Kyoto University.

Kitashirakawa Oiwake-cho, Sakyo-ku, Kyoto 606-8502, Japan.

Phone: +81-075-753-9382; E-mail: yokokawa.takumi.8w@kyoto-u.ac.jp

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Abstract

Melanocortin-4-receptor (Mc4r) is a G protein-coupled receptor (GPCR) that controls systemic energy balance by regulating food intake and energy expenditure. Although the detailed molecular mechanism remains unclear, the activation of cAMP signaling in Mc4r-expressing cells reportedly suppresses food intake and increases energy expenditure. cAMP-responsive element-binding protein-regulated transcriptional co-activator-1 (CRTC1) is selectively expressed in neuronal cells and participates in transcriptional control, thereby contributing to neuronal plasticity and energy homeostasis. Considering the cAMP-dependent regulation of CRTC1 activity, CRTC1 in Mc4r-expressing cells may contribute to energy balance regulation through the melanocortin pathway. In this context, we examined the physiological contribution of CRTC1 in Mc4r-expressing cells to energy metabolism. In this study, mice with CRTC1 deficiency in Mc4r-expressing cells exhibited 1) modest obesity, glucose intolerance, insulin resistance, hyperinsulinemia, and hyperlipidemia; 2) decreased systemic energy expenditure and thermogenesis; 3) suppression of melanocortin agonist-induced adaptation of energy expenditure and food intake; 4) impaired thermogenic programs and oxidative pathway in brown adipose tissue and skeletal muscle; and 5) enhanced lipogenic programs in the liver and white adipose tissue. These results provide novel insights into the molecular mechanisms underlying the regulation of energy balance by the melanocortin system.

Article Highlights

- The detailed molecular mechanisms by which Mc4r and CRTC1 regulate energy homeostasis remain unclear.
- CRTC1 deficiency in Mc4r-expressing cells elicits obesity and decreases energy expenditure.

- CRTC1 deficiency in Mc4r-expressing cells perturbs peripheral metabolism.
- CRTC1 in Mc4r-expressing cells contributes to systemic energy homeostasis.

Melanocortin-4 receptor (Mc4r), a G protein-coupled receptor (GPCR) expressed widely in the central nervous system such as the paraventricular nucleus of the hypothalamus and dorsal motor nucleus of the vagus (1,2), is a critical coordinator of whole-body energy homeostasis by the melanocortin system. Conventional Mc4r knockout mice exhibit hyperphagia, decreased energy expenditure, and obesity (3,4). Pharmacological activation of the melanocortin system suppresses food intake and increases energy expenditure in a Mc4r-dependent manner (5,6). In humans, MC4R mutations are associated with obesity (7). However, the detailed molecular mechanisms by which Mc4r regulates food intake and energy expenditure remain unclear. As Mc4r is a G_s-linked GPCR, its activation promotes cAMP synthesis (1). Mice with G_sα deficiency in Mc4r-expressing cells demonstrated obesity with hyperphagia, decreased energy expenditure, insulin resistance, and impaired thermogenesis (8). We recently demonstrated that chemogenetic G_s signal activation in Mc4r-expressing cells increased energy expenditure and suppressed food intake (9). Therefore, a downstream target of the Mc4r/cAMP axis may participate in metabolic regulation by the melanocortin system.

cAMP-responsive element-binding protein (CREB)-regulated transcriptional co-activators (CRTC), also known as a transducer of regulated CREB activity (TORCs), are involved in synaptic plasticity, energy homeostasis, and obesity [reviewed in (10)]. CRTCs, which have been identified as transcriptional regulators of CREB (11), shuttle from the cytoplasm to the nucleus in response to cAMP and calcium signaling, and subsequently regulate the activity of transcription factors (12,13). CRTCs comprise three members, CRTC1–3, which are expressed in various tissues (11). CRTC2 and 3 are ubiquitously expressed in the brain and peripheral tissues, whereas CRTC1 is specifically detected in the brain (14). In *Caenorhabditis elegans*, *crtc-1* is involved in metabolism and lifespan (15,16). Systemic CRTC1 knockout mice exhibit increased food intake,

reduced energy expenditure, and obesity under a regular diet (17). Furthermore, CRTCL polymorphisms are associated with body mass index and fat mass in humans (18). These results suggest that CRTCL regulates systemic energy homeostasis; however, the underlying molecular mechanisms remains unclear.

Given the aforementioned evidence, CRTCL in Mc4r-expressing cells is probably a downstream target of the Mc4r/cAMP axis and to modulate melanocortin system-dependent metabolic regulation. We hypothesized that CRTCL in Mc4r-expressing cells is essential for the integrity of energy metabolism. Recently, we showed that deletion of CRTCL in Mc4r-expressing cells elicits hyperphagia and, subsequently, obesity and systemic insulin resistance under high-fat or high-sucrose diet conditions (19). In contrast, the present study shows that under normal diet conditions, deletion of CRTCL in Mc4r-expressing cells induces modest obesity before the onset of hyperphagia. Furthermore, we demonstrate that CRTCL in Mc4r-expressing cells regulates systemic energy expenditure, thermogenesis and peripheral energy metabolism. These results provide novel insights into the molecular mechanisms underlying the regulation of energy balance by the melanocortin system.

Research Design and Methods

Animals

All animal care procedures were approved by the Animal Research Committee of Kyoto University. Mice were housed at 23 ± 2 °C under a 12-hour light-dark cycle and provided standard laboratory chow (MF; Oriental Yeast, Tokyo, Japan) and water *ad libitum*, unless otherwise noted.

As previously described (19), to generate mice selectively lacking CRTCL in Mc4r-expressing cells, we crossed *Crtc1*-flox mice (20) with Mc4r-2a-Cre mice (Stock No. 030759;

Jackson Laboratory, Bar, Harbor, ME, USA) (21). *Crtc1*-flox mice lacking the Cre transgene served as controls (Con). Although the female *Mc4r*-*CRTC1* KO mice were fertile, all mice used in the experiments were bred by crossing male *Crtc1*-flox/flox; *Mc4r*-Cre and female *Crtc1*-flox/flox mice. All experiments were performed with littermates. Mice were group-housed unless otherwise noted and were euthanized by decapitation or cervical dislocation.

Cold exposure experiment

For biochemical analyses, 6-week-old male mice were exposed to 5 °C for 3 h and then sacrificed. Interscapular BAT (iBAT), inguinal WAT (iWAT), and perigonadal WAT (pgWAT) were rapidly excised, frozen in liquid nitrogen, and stored at –80 °C until analysis.

To measure the rectal temperature, 7–8-week-old male mice were individually housed and exposed to 5 °C for 3 h. The rectal temperature was measured using a PTM1 portable temperature monitor and RET-3 probe (Physitemp, Clifton, NJ).

Melanotan-II experiment

To measure the food intake response to a melanocortin agonist, single-caged 10–12-week-old male mice were intraperitoneally injected with melanotan-II (MTII; 5 mg/kg body weight; Toronto Research Chemicals, Toronto, ON, Canada) or saline 30 min before lights out. The accumulated food intake was measured for 2 and 4 h. One week after the first experiment, a crossover experiment was performed. To evaluate MTII-induced adaptation of oxygen consumption and respiratory exchange ratio (RER), 7–9-week-old male mice were intraperitoneally injected with MTII (10 mg/kg body weight) or saline using a crossover design.

Metabolic measurement using indirect calorimetry was performed at 28 °C, after which average oxygen consumption and RER were calculated 1–5 h after the injection.

Metabolic analyses

Oral glucose tolerance test, insulin tolerance test, blood analyses, and indirect calorimetry were performed as previous described (19) (Supplemental materials).

Biochemical analyses

Gene and protein expression levels were analyzed using real-time PCR and western blotting, as previous described (22,23) (Supplemental materials).

Statistical analysis

All statistical analyses were performed using GraphPad Prism 10 software (GraphPad Software, San Diego, CA, USA). Comparisons between two groups were performed using two-tailed Welch's *t*-tests or the Mann–Whitney test, as appropriate. Comparisons between two factors were performed using two-way ANOVA followed by Šídák's multiple comparison test or Fisher's least significant difference test. The statistical significance was set at $P < 0.05$.

Data and Resource Availability

The data and resources generated and/or analyzed during the current study are available from the corresponding author upon reasonable request.

Results

Mc4r-CRTC1 KO mice exhibit modest obesity, glucose intolerance, insulin resistance, and hyperlipidemia under normal chow conditions

To understand the role of CRTC1 in Mc4r-expressing cells in energy homeostasis, we generated Mc4r-expressing cell-specific CRTC1 knockout (Mc4r-CRTC1 KO) mice by crossing Mc4r-Cre knock-in mice (21) to *Crtc1*-flox mice as per our previous study (19). When fed standard chow, male and female Mc4r-CRTC1 KO mice showed a significant increase in body weight after 12 and 15 weeks of age, respectively (Fig. 1A and B). At 26 weeks of age, male and female mutant mice were 25% and 37% heavier than their sex-matched control littermates, respectively. These male and female mutant mice displayed increases in BAT, liver, iWAT, and pgWAT weight compared with those of control littermates (Fig. 1C and D). The mutant mice showed significant or marginal increases in skeletal muscle weight (Fig. 1E and F). Systemic glucose intolerance and insulin resistance were also observed in both male and female mutant mice at 24 weeks of age (Fig. 1G and H). These mutant mice exhibited higher levels of serum insulin, total cholesterol, and TG but not NEFA (Fig. 1I). These results suggest that CRTC1 in Mc4r-expressing cells contributes to energy homeostasis as well as glycemic and lipid control.

Melanocortin signaling directly regulates insulin secretion (24), glucose tolerance (25), and circulating cholesterol (26). To investigate whether CRTC1 in Mc4r-expressing cells is associated with glycemic control and blood lipid profiles independently of obesity, we assessed glucose tolerance, insulin sensitivity, and blood lipid levels before the obesity onset. Predictably, 6-week-old Mc4r-CRTC1 KO mice did not show a significant change in body weight and wet weights of pgWAT, iWAT, BAT, liver, and skeletal muscles compared with that in control mice (Fig. 2A–C). These mutant mice did not exhibit any significant changes in glucose tolerance or insulin sensitivity (Fig. 2D and E). Furthermore, serum insulin, triglyceride, NEFA, and cholesterol levels

did not significantly change between the mutant and control mice at 6 weeks of age under fed and fasting state (Fig. 2F and G). These results suggest that glucose intolerance, insulin resistance, and hyperlipidemia observed in mutant mice are epiphenomena of obesity.

Mc4r-CRTC1 KO mice show decreased melanocortin agonist-induced food intake suppression and late-onset hyperphagia

We investigated whether increased energy intake caused obesity in Mc4r-CRTC1 KO mice. Mc4r-CRTC1 KO mice ingested a similar amount of food as control mice at 6 weeks of age (Fig. 3A). In contrast, compared with control mice, Mc4r-CRTC1 KO mice demonstrated increased daily food intake at 19 weeks of age (Fig. 3B). To detect slight differences, we further measured food intake in Con and Mc4r-CRTC1 KO mice after melanocortin agonist (MTII) or saline injection and found that the mutant mice showed decreased MTII-induced suppression of accumulated food intake 4 h after injection (Fig. 3C). To evaluate causal relationships between obesity and hyperphagia, we measured food intake and body weight over a period of 8–12 weeks of age. In male KO mice, a significant increase in body weight preceded that in food intake (Fig. 3D and E). In another cohort, 8-week-old male KO mice demonstrated a significant increase in pgWAT weight without significant body weight gain (Fig. 3F). Female KO mice did not exhibit any increase in food intake and body weight by 12 weeks of age, a time when adipose tissue weight was significantly increased (Fig. 3G and H). These results suggest that the onset of obesity precedes hyperphagia in KO mice. Taken together, deletion of CRTC1 in Mc4r-expressing cells decreases food intake suppression through melanocortin system activation and causes late-onset hyperphagia.

Basal and melanocortin agonist-induced energy expenditure is reduced before the onset of obesity in Mc4r-CRTC1 KO mice

To investigate whether CRTC1 in Mc4r-expressing cells is involved in energy expenditure, we measured whole-body basal metabolism using indirect calorimetry. At 7–8 weeks of age, basal oxygen consumption in Mc4r-CRTC1 KO mice significantly decreased, with no significant changes in RER and motor activity (Fig. 3I). To determine whether CRTC1 in Mc4r-expressing cells participates in melanocortin system-mediated regulation of energy expenditure, we measured oxygen consumption and RER in Con and Mc4r-CRTC1 KO mice after MTII or saline injection. Two-way ANOVA showed that CRTC1 deficiency in Mc4r-expressing cells decreased oxygen consumption (Fig. 3J), which was consistent with the baseline analysis (Fig. 3I). Both genotypes demonstrated MTII-induced enhancement of oxygen consumption and a decline in RER 1–3 h after injection, whereas Mc4r-CRTC1 KO mice displayed significant suppression of MTII-induced adaptation of oxygen consumption and RER 3–5 h after injection (Fig. 3J). These results suggest that CRTC1 in Mc4r-expressing cells contributes to MTII-induced late-phase adaptations of energy expenditure, especially lipid oxidation. To clarify the physiological machinery underlying the decreased energy expenditure in the mutant mice, we assessed thermogenic capacity by measuring rectal temperature before and after 3 h of cold exposure. The basal rectal temperature of mutant mice was marginally lower than that of Con mice (Fig. 3K). After 3-hour cold exposure, mutant mice exhibited significantly lower rectal temperature than that in Con mice (Fig. 3K), suggesting the importance of CRTC1 in Mc4r-expressing cells in the thermogenic pathway. Taken together, before the onset of obesity and hyperphagia, CRTC1 deletion in Mc4r-expressing cells elicits a decline in energy expenditure and thermogenesis, both of which are likely to contribute to the onset and development of obesity.

Mc4r-CRTC1 KO mice demonstrate an altered thermogenic program in BAT

To explore the molecular mechanism by which CRTC1 in Mc4r-expressing cells regulates energy expenditure, we conducted biochemical analyses of peripheral tissues at 6 weeks of age. First, we investigated whether Mc4r-CRTC1 KO mice showed an impaired thermogenic pathway in BAT. Six-week-old Mc4r-CRTC1 KO mice exhibited a decreasing trend in *Ucp1* and *Irf4* expression compared with that in Con mice in the fed state (Fig. 4A). After 17 h of fasting, the mutant mice showed a significant decrease in *Prdm16* and a marginal decrease in *Cidea* expression (Fig. 4E). Furthermore, mutant mice showed marginal and significant decreases in the expression of *Cs*, a mitochondrial marker, in BAT under fed (Fig. 4B) and fasting conditions (Fig. 4F), respectively. We also found that mutant mice exhibited significant decreases in the protein levels of UCP1 and CS (Fig. 4D). Since deletion of *Prdm16* elicits whitening of BAT (27), we next assessed WAT-selective marker expression. We found no significant changes in the gene expression of WAT-selective markers (*Agt* and *Retn*) under the fed (Fig. 4C) and fasting (Fig. 4G) conditions. These results suggest that CRTC1 in Mc4r-expressing cells partially contributes to the thermogenic program in BAT.

To investigate the role of CRTC1 in Mc4r-expressing cells in the acute thermogenic response, we measured the thermogenic gene and protein expression in the BAT of both genotypes after 3 h of cold exposure. Two-way ANOVA analysis showed that cold exposure increased cold-responsive gene expression (*Ucp1*, *Ppargc1a*, and *Dio2*) and UCP1 protein levels, whereas Mc4r-CRTC1 deletion selectively decreased the gene and protein expression of UCP1 (Fig. 4 H and I). Furthermore, the gene and protein expression of UCP1 in mutant mice was significantly lower than that in Con mice after cold exposure (Fig. 4 H and I). These results further support the

possibility that CRTC1 in Mc4r-expressing cells modulates the thermogenic program.

Mc4r-CRTC1 KO mice partially exhibit decreased oxidative metabolism- and thermogenesis-related molecules in skeletal muscles

The melanocortin system also regulates energy metabolism in skeletal muscles (28), which contributes to enhanced systemic energy expenditure. Therefore, we investigated whether CRTC1 in Mc4r-expressing cells is responsible for the expression of metabolism-related molecules in soleus and plantaris muscles, both of which are oxidative and glycolytic fiber-rich, respectively. We found no significant changes in the expression of glucose (*Slc2a4/Glut4* and *Hk2*), lipid metabolism-related genes (*Cd36*, *Cpt1b*, and *Acadm*), and mitochondrial markers (*Cox4i1* and *Cs*) in soleus muscle (Fig. 5A), whereas significant or marginal decreases in *Hk2*, *Cd36*, *Cox4i1*, and *Cs* were noted in plantaris muscles of Mc4r-CRTC1 KO mice (Fig. 5E). Consistent with mRNA levels, protein levels of CS were significantly decreased in the mutant mice (Fig. 5H). Total *Ppargc1a* expression was decreased in plantaris but not soleus muscle (Fig. 5 B and F). Isoform-specific analysis revealed a decrease in the expression of *Ppargc1a-b* and *-c*, both of which are positively regulated by sympathetic nerve activity and subsequent β 2-adrenergic stimulation (29,30), in soleus muscle (Fig. 5B). In contrast, an insignificant change was noted for *Ppargc1a-b* and *-c* in plantaris muscle (Fig. 5F). This inconsistency may be due to these low expression levels in glycolytic fibers. Considering the hypothalamic melanocortin system modulates the thermogenic program in skeletal muscle (31), we assessed the expression of uncoupling and thermogenic genes and found that the expression of the thermogenic genes *Atp2a1* and *Atp2a2* was decreased in the soleus muscle of the mutant mice (Fig. 5C). In plantaris muscle (Fig. 5G), no significant decrease in thermogenic gene expression was observed, although *Atp2a1* mRNA levels

were slightly, but not significantly, lower. Unexpectedly, we observed a marginal or significant decrease in the protein levels of Sarcoplasmic/endoplasmic reticulum calcium ATPases (SERCAs) in plantaris but not in soleus muscle (Fig. 5 D and H). This inconsistency may be because of the masking of small differences due to technical issues, considering levels of SERCAs protein levels in soleus muscle are relatively variable. Taken together, these results suggest that CRTTC1 in Mc4r-expressing cells partially regulates the oxidative pathway and thermogenic program in the skeletal muscle.

Mc4r-CRTTC1 KO mice demonstrate altered lipid metabolism in the liver

As Mc4r reportedly modulates hepatic metabolism (26,32,33), we investigated whether CRTTC1 in Mc4r-expressing cells is related to hepatic metabolism. First, we assessed the expression levels of glucose and lipid metabolism-related genes in the fed state at 6 weeks of age. We found no significant differences in the expression of genes related to lipid synthesis and oxidation between Con and Mc4r-CRTTC1 KO mice (Fig. 6 A and B). Further investigation revealed increasing trends of *Pparg* and *Srebf1* gene expression in the mutant mice (Fig. 6C); these genes encode transcription factors that regulate the expression of genes involved in lipid metabolism (34–36). Hence, to minimize the effect of feeding, we conducted a biochemical analysis of the liver after 17 h of starvation. We found comprehensive upregulation of lipid synthesis- and VLDL secretion-related genes in the Mc4r-CRTTC1 KO mice compared with that in Con mice (Fig. 6 E and F). The mutant mice showed increased gene expression of related transcription factors *Pparg*, *Mlxipl/Chrebpa*, *Nr1h3/LxRa*, and *Nr1h2/LxRβ* (Fig. 6H). Although 6-week-old KO mice exhibited no significant change in the levels of lipogenic proteins under both fed and fasting condition (Fig. 6 D and I), we observed a significant increase in the lipogenic

protein levels in the liver of 26-week-old KO mice (Fig. 6J). The mutant mice also exhibited enhanced expression of lipid oxidation-related (Fig. 6G) and mitochondrial (Supplementary Fig. 1B) genes. In contrast, lacking CRTC1 in Mc4r-expressing cells did not significantly change the protein expression levels of mitochondrial markers under both fed and fasting states (Supplementary Fig. 1 A and C). Taken together, these results indicate that CRTC1 deficiency in Mc4r-expressing cells elicits lipogenic responses in the liver.

CRTC1 in Mc4r-expressing cells partially regulates lipid metabolism-related molecules in WAT

The melanocortin system regulates lipid metabolism in visceral WAT (33,37); thus, we conducted biochemical analyses of pgWAT. First, we measured the expression of lipolysis-related molecules. Mc4r-CRTC1 KO mice demonstrated increased gene expression of *Pnpla2*, but not *Lipe*, encoding ATGL and HSL, respectively (Fig. 7A). In contrast, HSL did not differ significantly, [whereas](#) ATGL decreased significantly in female KO mice at the protein level (Fig. 7D). Given that only gene expression increases in WAT from humans with obesity (38), a specific increase in *Pnpla2* mRNA may stem from an initial adipocyte expansion response. We next investigated whether CRTC1 in Mc4r-expressing cells is involved in the lipogenic pathway. Mc4r-CRTC1 KO mice exhibited significant upregulation of *Lpl*, *Slc2a1*, and *Dgat1* expression and a marginal increase in *Fasn* expression in pgWAT (Fig. 7B). Furthermore, CRTC1 deletion in Mc4r-expressing cells increased the gene expression of *Srebf* and *Mlxipl*, both of which are transcription factors regulating lipogenic gene expression (Fig. 7C). At protein levels, male, but not female, mice exhibited a comprehensive increase in lipogenic protein expression (Fig. 7D). Considering the fact that female mice preferentially store fat in subcutaneous adipose tissue compared to male mice, we also measured the lipogenic protein expression in subcutaneous adipose tissue; however,

we did not detect significant change in lipogenesis- and lipolysis-related protein levels (Supplementary Fig. 2). Collectively, these results suggest that CRTC1 in Mc4r-expressing cells partly regulates the lipid metabolism in visceral WAT in a sex-dependent manner, which may be involved in the delay of the obesity onset in female KO mice; however, causal relationships remain unclear.

Discussion

We found that Mc4r-CRTC1 KO mice exhibited 1) modest obesity, glucose intolerance, insulin resistance, hyperinsulinemia, and hyperlipidemia; 2) decreased systemic energy expenditure; 3) suppression of melanocortin agonist-induced adaptation of energy expenditure and food intake; 4) impaired thermogenic programs and oxidative pathway in BAT and skeletal muscle; and 5) altered lipid metabolism in the liver and pgWAT. In a previous study (19), when fed a high-fat diet, mice with CRTC1 deficiency in Mc4r-expressing cells showed hyperphagia within a few days of starting a high-fat diet and subsequently became obese, which suggested that CRTC1 in Mc4r-expressing cells suppresses excessive fat intake and thereby controls body weight. Similarly, mice lacking CRTC1 in SF1-expressing cells (20) and CRTC1/2 in Sim1-expressing cells (39) exhibited hyperphagia and obesity when feeding a high-fat diet. Considering that systemic CRTC1 knockout mice demonstrated increased food intake, decreased energy expenditure, and obesity under normal diet conditions (17), CRTC1 is suggested to control body weight even under normal diet conditions and to modulate energy expenditure via regulating peripheral tissue metabolism; however, these mechanisms underlying these effects remains unclear. In this context, this study demonstrates that CRTC1 in Mc4r-expressing cells modulates peripheral metabolism and systemic energy expenditure and contributes to body weight control

under normal diet conditions. This is the first study to elucidate the detailed molecular mechanism for the regulations of systemic energy expenditure by CRTCL.

In addition to decreasing the basal energy expenditure, CRTCL deficiency in Mc4r-expressing cells impaired the MTII-induced suppression of food intake, enhancement of energy expenditure, and decrease in RER. These results suggest that CRTCL in Mc4r-expressing cells is required for metabolic adaptation by the melanocortin system. Notably, the effect of CRTCL deficiency in Mc4r-expressing cells on MTII-induced metabolic adaptations was limited immediately after MTII injection, becoming evident 3–5 h after injection, indicating that CRTCL in Mc4r-expressing cells contributes to maintaining, but not to eliciting, metabolic regulation by the melanocortin system. CRTCL is a transcriptional co-activator of CREB, which regulates the expression of immediate early genes and subsequently participates in synaptic plasticity (40). Furthermore, hypothalamic CREB transcriptionally controls the expression of neuropeptides regulating energy metabolism, such as corticotropin-releasing hormone (41,42). Considering the delayed contribution of CRTCL/CREB owing to its requirement for transcription and translation, it is reasonable to assume that CRTCL selectively participates in the late phase of MTII-induced metabolic adaptations. Given that CRTCL is involved in the melanocortin system, its polymorphisms might have to be considered in the clinical use of Mc4r-targeting drugs such as setmelanotide.

CRTCL deficiency in Mc4r-expressing cells impaired thermogenic programs, oxidative pathway, and lipid metabolism in peripheral tissues. Furthermore, the mutant mice exhibited decreased expression of *Ppargc1a-b* and *-c* isoforms, both of which are upregulated by the sympathetic nervous system (30). The melanocortin system regulates the peripheral metabolism via the sympathetic or parasympathetic nervous system (33,37,43–45). Moreover, G_s signaling in

Mc4r-expressing cells regulates the thermogenic program and energy expenditure (8,9). Therefore, CRTC1 in Mc4r-expressing cells may modulate melanocortin system-induced autonomic regulation and the subsequent peripheral metabolism, which at least partly contributes to energy expenditure and body weight control. To elucidate the physiological mechanism, further studies that directly assess autonomic involvement are required.

Mc4r-CRTC1 KO mice demonstrated enhanced lipogenic gene expression in the liver, especially in the fasting state. Pharmacological blockade of the central melanocortin system increased lipogenic genes in the liver (33). Therefore, CRTC1 may partly participate in the melanocortin system/hepatic lipid metabolism axis. As opposed to the fasting state, we failed to detect a comprehensive enhancement of lipid metabolism-related gene expression in the livers of KO mice in the fed state. Considering that these hepatic gene expressions are highly responsive to feeding and subsequent blood parameter responses (46), variation due to feeding might have masked the differences between genotypes. Alternatively, CRTC1 in Mc4r-expressing cells might have no or limited contribution to hepatic metabolism in the fed state, suggesting that the molecule specifically participates in fasting-induced acute transcriptional response in the liver. Furthermore, unlike other peripheral tissues, we fail to detect significant change in lipogenic and mitochondrial proteins in the liver at 6 weeks of age, although 26-week-old KO mice showed an increase in lipogenic proteins. Therefore, although further studies are required, the contribution of the hepatic lipogenesis and mitochondrial dysfunction to the onset of obesity in the mutant mice may be limited.

As a limitation, the difference of detectability between used methods (for instance, food intake, body weight, indirect calorimetry, and biochemical analyses) may mask the causal relationships between these indices. Studies using systemic Mc4r knockout mice, which exhibit

overt early-onset obesity, inconsistently suggest the causal relationships between hyperphagia, energy expenditure, and obesity [reviewed in (47)]. The modestly obesogenic phenotype of Mc4r-CRTC1 KO enables us to conduct physiological and biochemical analyses before the onset of obesity and hyperphagia. These results suggest that impairment of peripheral metabolism preceded the onset of obesity and hyperphagia in Mc4r-CRTC1 KO mice. However, although increases in body weight and adipose tissue mass statistically precede that in food intake, increases in these factors appear simultaneous, especially in male mice. Therefore, considering the difference of detectability, our data do not completely rule out a contribution of hyperphagia to the obesity onset in KO mice.

In summary, the present study revealed that CRTC1 in Mc4r-expressing cells modulates peripheral energy metabolism and maintains systemic energy homeostasis. These results provide novel insights into the molecular mechanisms underlying the regulation of energy balance by the melanocortin system.

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Conflicts of interest

The authors declare no conflicts of interest, financial or otherwise.

Author contributions

Conceptualization: H.M. and T.Y.; formal analysis: H.M. and T.Y.; investigation: H.M., T.M., K.O., and T.Y.; writing—original draft preparation: T.Y.; writing—review and editing: H.M. and T.G.; supervision: K.I. and S.M.; funding acquisition: T.Y. All authors have read and agreed to the published version of the manuscript. T.Y. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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Figure Legends

Figure 1. Mc4r-CRTC1 KO mice demonstrate obesity, glucose intolerance, insulin resistance, and hyperlipidemia. (A) Body weight curves of male (left) and female (right) Con and Mc4r-CRTC1 KO mice ($n = 10\text{--}15$ per group). Representative images of male Con and Mc4r-CRTC1 KO mice (B), their organs (C), and skeletal muscle (E) at 26 weeks of age. Organ (D) and skeletal muscle (F) weights of 26-week-old male Con and Mc4r-CRTC1 KO mice ($n = 10\text{--}13$ per group). (G) Glucose tolerance tests in male (left) and female (center) Con and Mc4r-CRTC1 KO mice at 24 weeks of age, with area under the curve (AUC; right) ($n = 6\text{--}9$ per group). (H) Insulin tolerance tests in male (left) and female (center) Con and Mc4r-CRTC1 KO mice at 24 weeks of age with AUC (right) ($n = 6\text{--}9$ per group). (I) Serum insulin, triglyceride (TG), cholesterol, and non-esterified fatty acid (NEFA) levels in male and female Con and Mc4r-CRTC1 KO mice at 26 weeks of age ($n = 6\text{--}10$ per group). Values are mean $\pm$ SEM; dot plots represent individual data points. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

Figure 2. Mc4r-CRTC1 KO mice did not show glucose intolerance, insulin resistance, or hyperlipidemia before the onset of obesity. (A) Body weights of male and female Con and Mc4r-CRTC1 KO mice at 6 weeks of age ($n = 9\text{--}13$ per group). (B–C) Skeletal muscle and organ weights of 6-week-old male Con and Mc4r-CRTC1 KO mice ($n = 9\text{--}13$ per group). (D) Glucose tolerance tests in male (left) and female (center) Con and Mc4r-CRTC1 KO mice at 6 weeks of age, with area under the curve (AUC; right) ($n = 5\text{--}8$ per group). (E) Insulin tolerance tests in male (left) and female (center) Con and Mc4r-CRTC1 KO mice at 6 weeks of age, with AUC (right) ($n = 5\text{--}8$ per group). (F) Fed serum insulin, triglyceride (TG), cholesterol, and non-esterified fatty acid (NEFA) levels in male and female Con and Mc4r-Crtc1 KO mice at 6 weeks of age ($n = 9\text{--}13$ per group).

(G) Fasting serum insulin, TG, cholesterol, and NEFA levels in male Con and Mc4r-CRTC1 KO mice at 6 weeks of age ($n = 7-9$ per group). Values are mean $\pm$ SEM; dot plots represent individual data points. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

Figure 3. Energy balance in Con and Mc4r-CRTC1 KO mice. (A–B) Food intake was measured in male and female Con and Mc4r-CRTC1 KO mice at 6 and 19 weeks of age ($n = 6-9$ per group). (C) Food intake after MTII (percentage versus saline) in male Con and Mc4r-CRTC1 KO mice at 10–12 weeks of age ($n = 14$ per group). (D–E) Food intake and body weight of male Con and Mc4r-CRTC1 KO mice were subsequently measured for 8–12 weeks of age and then adipose tissue weights were measured ($n = 5-6$ per group). (F) Body weight and adipose tissue weight of male Con and Mc4r-CRTC1 KO mice at 8 weeks of age ($n = 11-12$ per group). (G–H) Food intake and body weight of female Con and Mc4r-CRTC1 KO mice were subsequently measured for 8–12 weeks of age and then adipose tissue weights were measured ($n = 5$ per group). (I) Oxygen consumption, respiratory exchange ratio (RER), and motor activity of male and female Con and Mc4r-CRTC1 KO mice were measured using indirect calorimetry at 7–8 weeks of age ($n = 6-9$ per group). (J) Average oxygen consumption and RER in male Con and Mc4r-CRTC1 KO mice were measured 1–3 and 3–5 h after saline or MTII injection ($n = 5$ per group). (K) Rectal temperature was measured before and after 3 hours of cold exposure ($n = 7$ per group). Values are mean $\pm$ SEM; dot plots represent individual data points. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ vs saline. MTII, melanotan-II.

Figure 4. Mc4r-CRTC1 KO mice display partial impairment of the thermogenic program in BAT. (A–C) Gene expression of thermogenic, mitochondrial, and WAT-selective markers in iBAT from

fed mice ($n = 9\text{--}11$ per group). (D) Representative immunoblots and expression levels of UCP1 and CS in iBAT from fed mice ($n = 7\text{--}8$ per group). (E–G) Gene expression of thermogenic, mitochondrial, and WAT-selective markers in iBAT from fasted mice ($n = 9\text{--}10$ per group). (H and I) Thermogenic gene expression and UCP1 protein expression in iBAT with or without 3-hour cold exposure ($n = 8$ per group). Values are mean $\pm$ SEM; dot plots represent individual data points. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, $****P < 0.0001$. BAT, brown adipose tissue; WAT, white adipose tissue; iBAT, interscapular brown adipose tissue.

Figure 5. Effect of CRTCL1 deficiency in Mc4r-expressing cells on metabolic gene expression in skeletal muscle. (A–C) Expression of glucose and lipid metabolism-related genes, mitochondrial genes, PGC-1 α isoforms, uncoupling molecules, and thermogenic genes in soleus muscle ($n = 9\text{--}11$ per group). (D) Representative immunoblots and expression levels of CS and SERCA1, and SERCA2 in soleus muscle ($n = 7\text{--}8$ per group). (E–G) Expression of glucose and lipid metabolism-related genes, mitochondrial genes, PGC-1 α isoforms, uncoupling molecules, and thermogenic genes in plantaris muscle ($n = 9\text{--}11$ per group). (H) Representative immunoblots and expression levels of CS and SERCA1, and SERCA2 in plantaris muscle ($n = 7\text{--}8$ per group). Values are mean $\pm$ SEM; dot plots represent individual data points. $*P < 0.05$.

Figure 6. Effect of CRTCL1 deficiency in Mc4r-expressing cells on lipid metabolism-related molecule expression in liver. (A–C) The expression levels of metabolic genes (lipid synthesis and oxidation, and related transcription factors) in the liver of fed mice ($n = 8\text{--}12$ per group). (D) Representative immunoblots and expression levels of lipogenic proteins in the liver of 6-week-old fed mice ($n = 7\text{--}8$ per group). (E–H) The expression levels of metabolic genes (lipid synthesis,

VLDL secretion, lipid oxidation, and related transcription factors) in the liver of fasted mice ($n = 9-10$ per group). (I) Representative immunoblots and expression levels of lipogenic proteins in the liver of 6-week-old fasted mice ($n = 9-10$ per group). (J) Representative immunoblots and expression levels of lipogenic proteins in the liver of 26-week-old fed mice ($n = 8$ per group). Values are mean $\pm$ SEM; dot plots represent individual data points. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

Figure 7. CRTC1 deficiency in Mc4r-expressing cells enhances lipogenic programs in pgWAT. (A–C) The mRNA levels of lipolysis- and lipogenesis-related genes and related transcription factors in pgWAT from Con and Mc4r-CRTC1 KO mice ($n = 9-12$ per group). (D) Representative immunoblots and expression levels of lipolysis- and lipogenesis-related proteins in pgWAT of 6-week-old mice ($n = 8-11$ per group). Values are mean $\pm$ SEM; dot plots represent individual data points. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$. pgWAT, perigonadal white adipose tissue.

Supplementary Figure 1. Effect of CRTC1 deficiency in Mc4r-expressing cells on mitochondrial marker expression in liver. (A) Representative immunoblots and expression levels of mitochondrial proteins in the liver of 6-week-old fed mice ($n = 7-8$ per group). (B) The expression levels of mitochondrial genes in the liver of fasted mice ($n = 9-10$ per group). (C) Representative immunoblots and expression levels of mitochondrial proteins in the liver of 6-week-old fasted mice ($n = 9-10$ per group). Values are mean $\pm$ SEM; dot plots represent individual data points. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

Supplementary Figure 2. Effect of CRTC1 deficiency in Mc4r-expressing cells on lipid metabolism-related proteins in iWAT. Representative immunoblots and expression levels of lipolysis- and lipogenesis-related proteins in iWAT of 6-week-old female mice ($n = 8$ per group). Values are mean $\pm$ SEM; dot plots represent individual data points.













