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Ventromedial hypothalamic primary cilia control energy and skeletal homeostasis

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Abstract

Dysfunction of primary cilia is related to dyshomeostasis, leading to a wide range of disorders. The ventromedial hypothalamus (VMH) is known to regulate several homeostatic processes, but those modulated specifically by VMH-primary cilia are not yet known. In this study, we identify VMH-primary cilia as an important organelle that maintains energy and skeletal homeostasis by modulating the autonomic nervous system. We established loss-of-function models of primary cilia in the VMH by either targeting IFT88 (IFT88 KOSF-1) using steroidogenic factor 1-Cre (SF1-Cre) or injecting an adeno-associated virus Cre (AAV-Cre) directly into the VMH. Functional impairments of VMH-primary cilia were linked to decreased sympathetic activation and central leptin resistance, which led to marked obesity and bone density accrual. Obesity was caused by hyperphagia, decreased energy expenditure, and blunted brown fat function, as well as associated with insulin and leptin resistance. The effect of bone density accrual was independent from obesity, as it was caused by the decreased sympathetic tone resulting in increased osteoblastic and decreased osteoclastic activities in the IFT88 KOSF-1 and VMH-primary cilia knock-down mice. Overall, our current study identifies VMH-primary cilia as a critical hypothalamic organelle that maintaining energy and skeletal homeostasis.
**Introduction**

Homeostatic regulation in the central nervous system (CNS) is a complex process that is critical for systemic physiological balance. Therefore, identifying the brain systems integrating internal and external signals is paramount to understand how the CNS maintains body homeostasis under diverse conditions. The hypothalamus is a vital center for body functions, including energy and skeletal homeostasis. Its functions include receiving and integrating complex peripheral signals, and responding to them using humoral factors, autonomic nerves, neuronal and behavioral components. Recently, hypothalamic primary cilia, a solitary antenna-like extension of the plasma membrane, was suggested as an important organelle for the regulation of body homeostasis (1, 2). Although these studies suggested the role of primary cilia in the arcuate nucleus of the hypothalamus (ARC) and melanocortin pathway-mediated weight regulation, the physiological roles of primary cilia in different hypothalamic nuclei remains poorly understood. Moreover, much less is known about the mechanism by which hypothalamic primary cilia mediate diverse homeostatic functions.

The ventromedial hypothalamus (VMH) is an oval-shaped hypothalamic nucleus located directly above the ARC that has been identified as a site for regulation of body weight and glucose homeostasis (3). Early studies have shown that VMH lesions induced parasympathetic tone, leading to hyperinsulinemia and obesity (4, 5). Later, electrical stimulation experiments confirmed that the VMH is involved in the regulation of not only parasympathetic, but also sympathetic nerve activity (6). Furthermore, research has shown that the VMH is engaged in other various homeostatic regulations, such as skeletal homeostasis, mood behaviors, and reproductive function (7-9).
Interestingly, a recent report showed that primary cilia are expressed in the VMH, and that the length of VMH-primary cilia is dynamically changed by metabolic conditions, in which shorter cilia are dominant in metabolically unfavorable conditions such as obesity and leptin resistance (10). Though these dynamic changes suggest that VMH-primary cilia might play key roles in the maintenance of body homeostasis, there is no report yet on functional roles of VMH-primary cilia in regulating body homeostasis.

The intraflagellar transport 88 (IFT88) is a core member of IFT machinery (IFT B complex) that carries ciliary building blocks along microtubules during the assembly and maintenance of the cilium (11). Previously, conditional deletion of IFT88 was used to address cell- or tissue-specific primary cilia function (1, 12). In the present study, in order to gain insight into the functional roles of primary cilia in the VMH, we generated VMH-specific primary cilia knock-out (KO) mice by crossing SF1-Cre and floxed IFT88 (IFT88fl/fl) mice, and investigated the homeostatic role of primary cilia in this nucleus. We also administrated bilateral adeno-associated virus Cre (AAV-Cre) in IFT88fl/fl mice, specifically targeting the VMH, to overcome any potential peripheral effect of the SF1-Cre line.

We found that normal primary cilia function in the VMH is required for maintaining normal sympathetic nerve activity. Specific deletion of the primary cilia using the Cre-loxP system or bilateral AAV-Cre injection into the VMH caused metabolic dysregulations and abnormal skeletal homeostasis. Thus, this study suggests that VMH-primary cilia play critical roles in regulating energy and skeletal homeostasis through modulation of the sympathetic nervous system.
Results

Specific deletion of primary cilia in the VMH.

To examine the physiological role of primary cilia in the ventromedial hypothalamus (VMH), we used the conditional knock-out (KO) system and disrupted intraflagellar transport (IFT) machinery by deleting the *Ift88* gene, an essential factor for primary cilia formation, specifically in the SF-1 neurons of the VMH. Breeding *IFT88*fl/fl with SF1-Cre mice resulted in mice with deficiency of primary cilia solely in those neurons (Figure 1, A and B) (13, 14). While the number of neurons expressing primary cilia and the length of the cilia were comparable in the paraventricular nucleus of the hypothalamus (PVN), dorsomedial hypothalamus (DMH), lateral hypothalamus (LH), and arcuate nucleus of the hypothalamus (ARC) between WT and IFT88 KO SF-1 mice, these factors were markedly reduced in the VMH of IFT88 KO SF-1 mice (Figure 1, A and B and Supplementary Figure 1).

Deletion of IFT88 did not affect overall brain morphology, including brain width and length (Supplementary Figure 2, A and B). Neuronal cytoarchitecture of the VMH and expression of SF-1 in the VMH were also intact in IFT88 KO SF-1 mice (Supplementary Figure 2, C-E). In addition, the numbers and soma size of SF-1 neurons were comparable between WT and IFT88 KO SF-1 mice (Supplementary Figure 2, F-H).

Altogether, these results indicated that the VMH of IFT88 KO SF-1 mice was intact and the neurons were viable. Next, we performed a co-expression analysis of primary cilia and SF-1 via immunohistochemistry on peripheral tissues, which express eGFP under the regulatory elements of the SF-1 gene through bacterial artificial chromosome (BAC) transgene (15). For this method, we used the antibodies somatostatin receptor 3
(SSTR3), acetylated tubulin (Ac-Tub), and adenyl cyclase III (ACIII), which are all well-known markers of primary cilia. Consistent with results from previous studies (16-18), primary cilia in the SF1-expressing peripheral cells, such as the pituitary gonadotrophs, adrenal cortex, ovary theca, and testis Leydig cell, were not detected (Supplementary Figure 3, A-D). The pituitary and adrenal glands, ovary, and testis were anatomically intact, and circulating corticosterone, aldosterone, and estradiol (E2) levels were also comparable between WT and IFT88 KO<sub>SF-1</sub> mice (Supplementary Figure 4, A-H) (19). Furthermore, comparable litter size and litter per month, coupled with no differences in organ structure and steroid hormone levels, confirmed that the IFT88 KO<sub>SF-1</sub> mice have normal HPA and HPG axes (Supplementary Table 1). These results indicate that the deletion of IFT88 in the SF-1 neurons of the VMH using SF1-Cre leads to specific primary cilia deletion only in those neurons, without developmental or detectable hormonal changes in the brain and the periphery.

*Primary cilia in the VMH are required to maintain normal body weight.*

Although the VMH has been identified as a brain site for energy homeostasis (19-21), it is not known whether VMH-primary cilia play a role in the regulation of whole-body energy homeostasis. To address this question, body weight was monitored weekly in male and female IFT88 KO<sub>SF-1</sub> mice. Normal chow (NC)-fed IFT88 KO<sub>SF-1</sub> mice displayed significantly increased body weight compared to WT littermates (Figure 1C and Supplementary Figure 5A). Similarly, IFT88 KO<sub>SF-1</sub> male mice fed a high-fat diet (HFD) showed an obese phenotype (Supplementary Figure 6A). Dual-energy X-ray absorptiometry (DEXA) analysis revealed a distinct fat mass increment in IFT88 KO<sub>SF-1</sub>
mice (Figure 1D and Supplementary Figure 5B and 6B). In accordance with these findings, the subcutaneous white adipose tissue (scWAT) and gonadal white adipose tissue (gWAT) of the IFT88 KO\textsuperscript{SF-1} mice were significantly bigger than those of WT mice (Figure 1E). In addition, IFT88 KO\textsuperscript{SF-1} mice exhibited increases in the number of hypertrophic adipocytes, as revealed by histological analysis (Figure 1, F and G). Serum leptin levels were also increased in the IFT88 KO\textsuperscript{SF-1} mice (Figure 1H).

To provide further evidence in support of excluding potential peripheral effects and thus confirm that the obesity in IFT88 KO\textsuperscript{SF-1} mice came directly from the absence of primary cilia in the VMH, we bilaterally injected AAV2-GFP (control) or AAV2-Cre-GFP into the VMH of IFT88\textsuperscript{fl/fl} mice (Figure 1I). Bilateral Cre virus injection induced VMH-specific knockdown (KD) of primary cilia (Figure 1I). The KD mice exhibited the obese phenotype seen in IFT88 KO\textsuperscript{SF-1} mice (Figure 1, J and K), suggesting the primary cilia in the VMH are critical organelles for maintaining normal energy homeostasis.

Decreased sympathetic tone is the major cause of obesity in IFT88 KO\textsuperscript{SF-1} mice.

To determine potential factors responsible for inducing the obesity in IFT88 KO\textsuperscript{SF-1} mice, we monitored several metabolic parameters using indirect calorimetry (20, 22). Body weight-matched 12-week-old WT and IFT88 KO\textsuperscript{SF-1} littermates were maintained on a normal chow diet and subjected to metabolic cage studies (Figure 2A). Although food intake of IFT88 KO\textsuperscript{SF-1} mice was not markedly different from WT littermates, their VO\textsubscript{2}, VCO\textsubscript{2}, and energy expenditure (EE) were significantly lower, especially during the dark cycle (Figure 2, B-E). In the HFD feeding condition, the obesity phenotype of the IFT88 KO\textsuperscript{SF-1} mice was more apparent from early ages, with a significant increase in food
intake and lower VO₂, VCO₂, and EE (Supplementary Figure 6, C-J). The expression of hypothalamic orexigenic Agrp significantly increased and anorexigenic Pomc decreased without changes in Npy expression (Figure 2F and Supplementary Figure 6K). Physical activity was comparable between WT and IFT88 KO^SF-1_ regardless of the type of diet (Figure 2G and Supplementary Figure 6, L and M). Altogether, these results highlight that primary cilia in the VMH are required for maintaining normal energy balance.

The interscapular brown adipose tissue (iBAT) plays an important roles in controlling basal metabolic rate, including energy expenditure (EE), and the VMH is a brain region that regulates sympathetic tone to the iBAT (20, 22, 23). UCP1, a key regulator of thermogenesis in iBAT, is well known to be activated by sympathetic tone (24). Therefore, we examined if the decreased EE in IFT88 KO^SF-1_ mice was linked to iBAT dysfunction. We found that the VMH-primary cilia KO mice had hyperplasia in the iBAT, and H&E staining confirmed higher lipid infiltration in the IFT88 KO^SF-1_ mice with less mitochondria contents (Figure 2, H and I). In addition, immunohistochemistry and Western blot results showed markedly decreased UCP1 expression in IFT88 KO^SF-1_ mice (Figure 2, J-L). Furthermore, thermogenic or mitochondrial function-related genes such as Ucp1, Pgc1α, Dio2, and Tfam were down-regulated, whereas genes involved in de-novo lipogenesis including PPARγ and FASN were up-regulated (Figure 2M). These results highly suggest that VMH-primary cilia play an important role in maintaining normal iBAT function, potentially through sympathetic nerve activity. To directly confirm whether VMH-primary cilia are involved in sympathetic regulation, we evaluated serum norepinephrine (NE) levels of mice age from 8- to 14-weeks, when
the body weight of WT and IFT88 KO<sub>SF-1</sub> mice were comparable. The IFT88 KO<sub>SF-1</sub> mice showed a tendency of decreased NE levels from the first 8 weeks, with significantly different levels at 12 and 14 weeks (Figure 2N). In addition, correlation analysis between NE and body weight showed that the NE levels were inversely correlated with body weight only in IFT88 KO<sub>SF-1</sub> mice (Figure 2O), indicating that the decreased sympathetic activity would be one of the factors responsible for decreased EE and iBAT function.

If the decreased sympathetic activity is a major cause of the metabolic phenotypes of IFT88 KO<sub>SF-1</sub> mice, it is possible that the introduction of sympathomimetics would countervail the metabolic disorders. To this end, we administered mice daily with isoproterenol (ISO), a β-adrenoreceptor agonist, for 2 weeks with a concentration that does not disturb body weight for both WT and IFT88 KO<sub>SF-1</sub> mice (Supplementary Figure 7A). The marked difference of the VO<sub>2</sub>, VCO<sub>2</sub>, and EE levels induced by VMH-primary cilia KO as shown in Figure 2 was normalized by the ISO treatment without changing locomotor activity (Supplementary Figure 7, B-E). These results confirm that the metabolic phenotypes shown in IFT88 KO<sub>SF-1</sub> mice majorly come from the decreased sympathetic activity.

*Primary cilia are required for normal leptin sensitivity in the VMH.*

In the hypothalamus, the adipokine leptin suppresses food intake and increase sympathetic tone and EE (25, 26). The effects of Increased food intake and decreased sympathetic tone and EE in IFT88 KO<sub>SF-1</sub> mice led us to hypothesize that the VMH-primary cilia may be important factors for leptin action. To address this proposition, we
first monitored serum leptin levels of WT and IFT88 KO\textsuperscript{SF-1} mice from 8- to 14-weeks, a period when the WT and IFT88 KO\textsuperscript{SF-1} littermates show comparable body weight. Surprisingly, the IFT88 KO\textsuperscript{SF-1} mice showed markedly higher leptin levels from 12-week, along with a strong trend of increasing leptin levels even from 8- to 10-weeks, indicating that ciliary defects in the VMH may lead to primary leptin resistance (Table 1). Next, we examined leptin sensitivity either by measuring rebound food intake or by monitoring the feeding response to leptin injection after an 18-hour fast. Results portrayed that the IFT88 KO\textsuperscript{SF-1} mice showed significantly increased rebound food intake, and following leptin administration, exhibited blunted response to the injected leptin, as demonstrated by significantly increased food intake (Figure 3, A and B). In addition, leptin activates signal transducer and activator of transcription 3 (STAT3), and the phosphorylation of STAT3 (pSTAT3) in the brain is an indicator of leptin sensitivity. Thus, we examined the basal hypothalamic STAT3 level together with pSTAT3. Although there was no change in basal STAT3 expression, leptin-induced pSTAT3 activation was significantly impaired, specifically in the VMH where the primary cilia were removed (Figure 3, C-F). To further confirm the direct role of VMH-primary cilia in leptin action, we used metabolic chambers to monitor metabolic parameters in body weight-matched WT and IFT88 KO\textsuperscript{SF-1} littermates after leptin administration (Figure 3G). The IFT88 KO\textsuperscript{SF-1} mice exhibited resistance to the reduction in food intake and increase in energy expenditure induced by leptin (Figure 3, H-K). Altogether, these results suggest that the primary cilia in SF-1 neurons of the VMH are necessary for normal leptin action, and leptin resistance in IFT88 KO\textsuperscript{SF-1} mice is not a secondary consequence of obesity.
**VMH-specific deletion of primary cilia leads to glucose and insulin intolerance.**

We next examined whether the primary cilia expressed in SF-1 neurons of the VMH are involved in the regulation of glucose homeostasis and insulin sensitivity. The IFT88 KO<sup>SF-1</sup> mice exhibited significantly elevated serum glucose and insulin levels (Figure 4, A and B). Also, IFT88 KO<sup>SF-1</sup> mice showed impaired tolerance of glucose and insulin, without significant changes in glucose-induced insulin secretion (Figure 4, C-E). These results indicate that primary cilia in the VMH are required for the regulation of normal glucose and insulin homeostasis.

Gene expression analyses in liver showed increased expression of gluconeogenic glucose 6-phosphatase (\textit{G6Pase}) and phosphoenolpyruvate carboxykinase (\textit{PEPCK}), as well as decreased expression of glycolytic pyruvate kinase (\textit{Pklr}), implying that aberrant gluconeogenesis might contribute to the high glucose and insulin insensitivity in IFT88 KO<sup>SF-1</sup> mice (Figure 4F). In addition, liver from the IFT88 KO<sup>SF-1</sup> mice exhibited evident steatosis, along with a significant increment in hepatic lipogenic genes (Figure 4, G and H). The effects of both glucose and insulin intolerance were also distinct in AAV-Cre mediated VMH-specific IFT88 KD mice, confirming that the VMH-primary cilia play an important role for whole body glucose homeostasis and insulin sensitivity (Figure 4, I and J).

**Decreased sympathetic activity leads to the high bone density in IFT88 KO<sup>SF-1</sup> mice.**

Evidence from animal and human studies demonstrated that changes in sympathetic nervous system (SNS) is linked to alterations in bone density. Moreover, the VMH is
known to control bone homeostasis (27-30). Considering the decreased sympathetic tone and leptin resistance in IFT88 KO\textsuperscript{SF-1} mice, we thus examined whether the VMH-primary cilia are important factors in maintaining skeletal homeostasis.

We performed age-dependent DEXA analysis to examine bone parameters. Interestingly, the long bones of the male IFT88 KO\textsuperscript{SF-1} mice showed increased bone mineral density (BMD), bone mineral content (BMC), bone volume (BV), and bone area (BA) compared to WT littermates, independent of the effect of obesity (Figure 5, A-D). Similarly, the female IFT88 KO\textsuperscript{SF-1} mice also exhibited increased BMD, BMC, BV, and BA (Supplementary Figure 8, A-D). The bone parameters examined were inversely correlated with serum NE only for IFT88 KO\textsuperscript{SF-1} mice (Figure 5, E-H). In addition, femur NE levels significantly decreased in IFT88 KO\textsuperscript{SF-1} mice, indicating that the decreased sympathetic activity may contribute to the increased bone density in these mice (Figure 5I). Microcomputed tomography (\textmu CT) analysis recapitulated the DEXA data and showed increased BMD, BV, in addition to thicker trabeculae in the IFT88 KO\textsuperscript{SF-1} mice (Figure 5, J and K). To further confirm that the VMH-primary cilia are directly involved in the regulation of bone mass, we analyzed viral-mediated VMH-specific IFT88 KD mice. Bilateral injection of AAV-Cre into the VMH of the IFT88\textsuperscript{fl/fl} mice resulted in high bone density in the KD femur, indicating a critical role for VMH-primary cilia in the regulation of bone homeostasis (Table 2 and Figure 5, L and M). Overall, these results identify normal VMH-primary cilia as a requirement for bone mass homeostasis.

It has been shown that the effect of the SNS on bone homeostasis is mediated through \(\beta\)-adrenergic receptors (\(\beta\)-ARs), and several animal studies have demonstrated that \(\beta\)-blocker increased bone mass not only in normal, but also in ovariectomized conditions.
To investigate whether chronic β-AR agonist treatment could offset the high bone mass shown in IFT88 KO\textsuperscript{SF-1} mice, we administrated mice daily with isoproterenol (ISO) for 2 weeks and monitored bone parameters. The significantly increased basal BMD, BMC, BV, and BA shown in IFT88 KO\textsuperscript{SF-1} mice disappeared after the ISO treatment (Figure 6, A-D). In addition, using \(\mu\)CT analysis, we confirmed the countervailing effect of ISO, as ISO treatment revealed no differences in BMD, BV/TV, BS, Tb.Th., Tb.N., and Tb.Sp. between WT and IFT88 KO\textsuperscript{SF-1} mice (Figure 6, E and F). These results indicate that decreased sympathetic activity induced by primary cilia deletion in the VMH is a critical factor for the increased bone mass accrual in IFT88 KO\textsuperscript{SF-1} mice.

\textit{Increased osteoblastic and decreased osteoclastic activities in IFT88 KO\textsuperscript{SF-1} mice.}

SNS activities have previously been linked to greater osteoclastic bone resorption by increasing osteoblastic ligand for receptor activator of nuclear factor-κB (RANKL) expression (27). In addition, it has been shown that different hypothalamic nuclei influence SNS activity in distinct ways (32-34). To gain mechanistic insights into the observed bone density increment induced by primary cilia deletion in the VMH, we first examined genes involved in osteogenic- and osteoclastogenic-related signaling pathways. We found significantly decreased expression of \textit{Rankl}, a key factor of osteoclast differentiation, but no change in receptor activator of nuclear factor-κB (\textit{Rank}) expression (Figure 7, A and B). In addition, the osteoclastic markers tartrate-resistant acid phosphatase (\textit{Trap}) and dendrocyte-expressed seven transmembrane proteins (\textit{Dc-stamp}) were significantly decreased in IFT88 KO\textsuperscript{SF-1} mice (Figure 7, C and D).
Consistent with these results, TRAP-positive cells were significantly reduced in IFT88 KO<sup>SF-1</sup> femurs (Figure 7, E-G). In contrast, the osteogenic markers Sp7 transcription factor (Sp7) and alkaline phosphatase (Alp) were increased in femurs of the IFT88 KO<sup>SF-1</sup> mice (Figure 7, H and I). These results imply that the high bone mass phenotype in IFT88 KO<sup>SF-1</sup> mice might be due to a concomitant increase in bone formation factors and decrease in bone resorption factors. Next, we measured bone turnover markers, including serum C-terminal telopeptide of type 1 collagen (CTX-1) and procollagen type 1 N-terminal propeptide (P1NP). We found that serum P1NP was elevated in IFT88 KO<sup>SF-1</sup> mice, but serum CTX-1 did not change, indicating an increase in osteoblast differentiation (Figure 7, J and K). Finally, dynamic histomorphometry (calcein double-labeling) analysis confirmed the increased bone formation and mineral apposition rate in both cortical and trabecular bones of IFT88 KO<sup>SF-1</sup> mice (Figure 8, A-E). Altogether, these results demonstrate that the high bone density in IFT88 KO<sup>SF-1</sup> mice results from increased osteoblastic and decreased osteoclastic activities.
**Discussion**

Deletion of VMH-primary cilia by targeting the *Ift88* gene using either SF1-Cre or bilateral AAV-Cre injection resulted in metabolic dysregulations such as obesity, insulin/leptin resistance, impaired brown fat function, and increased fat mass. In addition, impaired primary cilia function in the VMH led to increased osteoblastic and decreased osteoclastic activities, resulting in high bone density. The VMH is known to regulate sympathetic activity, and decreased sympathetic activity is linked to reduction of metabolic rate and increase in bone density (6). Thus, we examined whether the VMH-primary cilia play a critical role in controlling sympathetic activity. The IFT88 KO^{SF-1} mice exhibited several indications of impaired sympathetic activity, including decreased energy expenditure (EE), blunted brown fat function, and leptin resistance. They also demonstrated significantly lower levels of serum NE independent of changes in body weight. These results indicate that the VMH-primary cilia are critical for the regulation of normal sympathetic activity. Therefore, our current study identifies primary cilia expressed in the VMH as a key component of normal energy and skeletal homeostasis through the regulation of sympathetic nerve activity.

We found that mice lacking the *Ift88* gene from SF-1 neurons of the VMH developed obesity with a calorimetric decrease in EE. The decreased EE in IFT88 KO^{SF-1} mice appeared to be a primary consequence of reduced sympathetic outflow rather than secondary to the obesity effect, considering that EE was already decreased when the mice were at comparable body weight (Figure 2, A and C-E). In addition to the decreased EE, the IFT88 KO^{SF-1} mice showed significantly increased *Agrp* and decreased *Pomc* expression in the hypothalamus, which are genes that potentially...
affect food intake (Figure 2F and Supplementary Figure 6K). Concomitantly, hyperphagia in the IFT88 KO SF-1 mice was evident in the HFD condition (Supplementary Figure 6C). As a contrast to the HFD setting, NC-fed IFT88 KO SF-1 mice showed comparable food intake with the WT littermates. Therefore, the decreased EE might play an important role in leading metabolic disorders of the IFT88 KO SF-1 mice, as the KO mice demonstrated an obese phenotype even in comparable NC consumption (Figure 1C and 2B).

In addition to metabolic disorders, impaired primary cilia function in the VMH resulted in high bone density in the present study. Previously, it was suggested that circulating leptin that acted on bone cells stimulated bone growth and formation (35, 36). On the other hand, it was believed that leptin action in the CNS inhibits bone formation through activation of the sympathetic nervous system (37). While these findings suggest that leptin may regulate bone density differently either in the periphery or the brain, experimental approaches using various doses of leptin or supra physiological ob/ob and db/db mice may potentially influence other hormones affecting skeletal homeostasis (38-41). Indeed, systemic injection of leptin in ob/ob mice concomitantly altered steroid and pancreatic hormones, both factors influencing bone homeostasis (40). Therefore, careful attention must be paid to understand leptin’s role in skeletal physiology in particular contexts.

The leptin receptor is widely expressed in several brain regions, including the hypothalamus and brainstem (42). Thus, it would be interesting to identify leptin’s region-specific roles in regulation of skeletal homeostasis. Unfortunately, however, early studies investigating brain region-specific function of leptin are majorly focused
on energy homeostasis (19, 43). As a result, only limited information is available for leptin’s brain site-specific role in regulation of bone homeostasis. Specific leptin receptor deletion in the ARC or VMH did not affect bone density (19, 43). However, it was suggested that the leptin receptor expressed in Tph2-expressing neurons plays an important role in regulation of cancellous bone homeostasis, although some discrepancies exist in energy homeostasis regulation (44, 45). Despite these findings, mice chemically lesioned in the VMH using gold thioglucose (GTG) exhibited loss of VMH neurons and showed high bone density phenotype, highly suggesting a possibility that bone homeostatic regulation through the VMH might require other components in addition to leptin, such as proper neuronal communication between the VMH and its targets (30). In the present study, the VMH-specific primary cilia KO mice showed primary leptin resistance, but this alone might not be enough to induce bone remodeling. Therefore, the high bone density observed in the IFT88 KO<sup>SF-1</sup> mice emphasizes combined results of both leptin resistance and SNS decrement. Although we showed strong evidence of decreased sympathetic tone in IFT88 KO<sup>SF-1</sup> mice, further examination of the direct mechanism mediating the primary cilia effects on skeletal homeostasis through neuronal elements would provide new insight into the function of neuronal primary cilia. In addition, it would also be interesting to investigate whether primary cilia located in extra VMH regions including the ARC, LH, or PVH are involved in regulation of skeletal homeostasis as well.

SF-1, a well-known marker of the VMH, is also expressed in pituitary gonadotrophs and the major steroidogenic tissues, such as the adrenal cortex and testicular Leydig cells (46). Surprisingly, primary cilia are not exhibited in SF1-expressing endocrine cells,
but are expressed in the pituitary somatotrophs and lactotrophs, adrenal capsule and medulla, and immatured testis cells, implying that the primary cilia either would not or would minimally be affected by SF1-Cre in the periphery organs (Supplementary Figure 3) (16-18). Intact structural integrities of the pituitary and adrenal glands, as well as the gonads, together with the functional HPA and HPG axes of IFT88 KO^SF-1^ mice, support this notion (Supplementary Figure 4 and Supplementary Table 1). Consequently, the metabolic and skeletal phenotypes of IFT88 KO^SF-1^ mice might be results of VMH-primary cilia deletion.

To further rule out potential influence from peripheral primary cilia and to verify that the IFT88 KO^SF-1^ phenotypes are indeed results from the VMH-primary cilia, we injected AAV-Cre virus directly into the VMH of IFT88fl/fl mice and established VMH-specific primary cilia KD models, with the purpose of investigating energy and bone homeostasis (Figure 1, I-K and Figure 4, I and J). Interestingly, the AAV-mediated KD mice phenocopied the metabolic and skeletal effects shown in IFT88 KO^SF-1^ mice (Figure 1 and Figure 5). These results confirm that the VMH-primary cilia, but not the periphery primary cilia exhibited in SF1-expressing cells, are responsible for the observed phenotypes, possibly through the regulation of sympathetic nervous system activity. One consideration is that the Cre virus will also be expressed in non-SF-1 neurons of the VMH. Therefore, it would be interesting to separate which neuronal populations in the VMH are mainly involved in the modulation of energy and skeletal homeostasis.

One interesting finding in this study is that the VMH-primary cilia are necessary for proper leptin action. The relationship between leptin action and the functional
involvement of primary cilia has long been questioned. Mechanistically, it was suggested that Bbs1, a component of the ciliary basal body, directly binds to the leptin receptor and is required for proper leptin receptor trafficking (47). Interestingly, leptin resistance developed independent of obesity only when Bbs1 was deleted in the leptin receptor-expressing neurons, suggesting that Bbs1 is required for proper leptin action (12). However, CAGG-CreER mediated IFT88 deletion or Bbs4 KO animals showed leptin resistance secondary to obesity. Based on these results, the authors suggested that leptin signaling is not directly affected by both cilia loss (IFT88 KO) and defective cilia signaling (Bbs4 KO) (48). Later, the development of minimal leptin resistance was reported when cilia were removed from leptin receptor-expressing neurons (12). Together with our current results, this data suggests that the impact of primary cilia on leptin action may differ from neuron to neuron (Figure 3). Therefore, a future goal may be to identify distinct neuronal populations involved in the maintenance of whole-body leptin homeostasis.

Another intriguing finding in our study is that the VMH-primary cilia are required for normal sympathetic nerve activity. It has been shown that VMH lesions affect the sympathetic nervous activity, and microinjection of glucose into the VMH increases the iBAT activity through the SNS (49, 50). In addition, genetic studies have also indicated that intact gene expression in the VMH is required for proper SNS regulation (13, 19, 22). These studies highlight that normal VMH function is necessary for intact SNS regulation. A key question regarding sympathetic activity regulation by the VMH-primary cilia is the role that primary cilia play in this regulation. Since we ablated signaling hub mediated by VMH-primary cilia, it is possible that gene expression might
be changed in the neurons with deleted primary cilia. To investigate this possibility, we examined gene expression levels of *SF-1*, *ObRb*, and *5-HT2c*, known factors involved in regulation of SNS activity (Supplementary Figure 9) (19, 22, 44). Although we could not detect a difference in expression levels of those genes, it is still possible to change transcriptomes in primary cilia-deleted neurons, which may be involved in the regulation of SNS activity. Approaches such as whole RNA sequencing after FACS-sorting of labeled SF-1 neurons could be used to investigate the mechanism behind SNS modulation. In addition to the changing of transcriptomes, another possible regulator of SNS activity is the changing character of SF-1 neurons, for they lead to impairment in neuronal communication to the sympathetic preganglionic neurons. Indeed, Guo et. al. reported that primary cilia are required for proper neuronal circuit formation, especially those that affect interneuron morphology, connectivity, and synaptic integration. This suggests that cilia deletion in SF-1 neurons may influence local neuronal circuits (51). Thus, future studies could also consider whether neuronal primary cilia are involved in the formation of neural circuits specific to the SNS in order to determine the functional role of primary cilia in the regulation of SNS activity.

In summary, our study establishes the roles of VMH-primary cilia in the regulation of energy and skeletal homeostasis through sympathetic nerve activity. It provides new evidence that VMH-ciliary signaling directly modulates metabolic parameters such as leptin sensitivity, feeding behaviors, and brown fat function. We report that primary cilia are involved in bone homeostasis both locally and through neuronal signaling. Thus, proper VMH-primary cilia are required and indispensable for energy and skeletal homeostasis.
Methods

All experimental and surgical protocols were approved and conducted in accordance with Institutional Animal Care and Use Committee (IACUC) of Yonsei University Health System (YUHS). All animals were maintained in a specific pathogen free (SPF) facility accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care International (AAALAC). All mice procedures were maintained on an inbred C57BL/6J genetic background and were housed under controlled room temperature (22-24°C) with a 12-hour light/dark cycle (light on at 08:00), with free access to either normal chow (NC, PicoLab® Rodent Diet 20, #0007688; 13% from fat, 3.03 kcal·g⁻¹) or high-fat diet (HFD, Research Diets, #D12492; 60% from fat, 5.24 kcal·g⁻¹) and water.

Mouse models.

To generate experimental mice, IFT88 KO<sup>SF-1</sup>, male mice heterozygous for the SF1-Cre transgene and homozygous for the floxed Ift88 allele (Ift88<sup>fl/fl</sup>) were crossed with female mice (Jackson Laboratory Stock No. 022409) homozygous for the floxed Ift88 allele (Ift88<sup>fl/fl</sup>). The littermate mice homozygous for the floxed Ift88 allele (Ift88<sup>fl/fl</sup>) served as controls (WT).

The primers used for genotyping PCR. SF1-Cre: forward (F), 5’-CTGAGCTGCAGCGCAGGGACAT-3’, reverse (R), 5’-TGCGAACCTCATCACTCGTTGCAT-3’.

WT: F, 5’-GGTCAGCCTAATTAGCTCTGTCAT -3’, R, 5’-GATCTCCAGCTCCTCCTCCTGTCT-3’.

Ift88 floxed: F, 5’-GACCACCTTTTTAGCCTCCTG-3’, R, 5’-
To generate VMH-specific primary cilia knock-down (KD) mice, 10- to 12-week old Ift88^{fl/fl} male mice were used for stereotaxic surgery. Anesthetics were prepared by mixing Zoletil (30mg/kg) and Rompun (10mg/kg) in isotonic saline. Then, the mixture was intraperitoneally (i.p.) injected to the mice at 10 μL/g of body weight. Animals were placed into a stereotaxic apparatus (Kopf 1900, CA, USA) under sterile conditions. A small incision was made to expose the skull and drilled at the injection site. To target the injection site, three-dimensional MRI coordinate system was used as a reference. The coordinates for VMH were anterior-posterior: -4.80 mm, medio-lateral: ±0.50 mm, dorsal-ventral: -4.83 mm (500 nL injection). Viral constructs were injected at a rate of 50 nL/min for 10 minutes, and the injector remained in place for an additional 5 minutes before removal. After surgery, mice were placed on a heating pad for recovery. Adeno-associated virus (AAV) expressing Cre-GFP (#105545, AAV2-Cre-GFP) and GFP (#105530, AAV2-GFP) were purchased from Addgene (Watertown, Massachusetts, USA).

Validation of Mouse Models.

To validate the specific deletion or KD of primary cilia in the VMH, mice were perfused and their brains were collected. Mice were i.p. injected with Avertin (tribromoethanol, 25mg/kg of body weight) for anesthesia, then transcardially perfused with 20 mL of PBS (pH7.4) then fixed with 20 mL of 10% neutral buffered formalin. Brains were dissected out and post-fixed in 10% neutral buffered formalin in 2 hours at room temperature (RT), then were transferred to 20% of sucrose in PBS in overnight at 4°C
for cryoprotection. Brains were sliced into 20µm sections using a sliding microtome (Leica SM2010, Leica Biosystems, Wetzlar, Germany).

To visualize neuronal primary cilia, immunohistochemistry was performed on free-floating method. Briefly, brain sections were washed in PBS and permeated with 0.25% Triton X-100 in PBS (v/v) for 30 minutes. After blocking with 3% goat serum prepared in PBS containing 0.25% Triton X-100 (PBT) for 1 hour at RT, the brain sections were incubated for 36 hours at 4°C with primary antibodies in PBT-azide containing 3% (v/v) goat serum. Then, sections were rinsed with PBS 3 times and incubated for 2 hours in secondary antibodies diluted in PBT containing 3% (v/v) goat serum at RT. Then sections were rinsed and mounted on glass slides using mounting medium with DAPI (#H-1500, Vector Lab, CA, USA) and visualized by a confocal laser microscope (LSM700, Carl Zeiss AG, Oberkochen, Germany). Further analysis was done using the ZEN blue software (Zeiss) and ImageJ (National Institutes of Health).

To evaluate any effects of peripheral primary cilia by SF1-Cre, mice were perfused and the tissues known to express SF-1, including pituitary and adrenal glands, testis, and ovary, were collected. Post-fixed tissue samples were then paraffin-embedded and cut into 4-5µm slices. To visualize peripheral primary cilia, a regular immunohistochemistry method was performed using the paraffin-embedded tissue samples. After deparaffinization of the tissue samples, the slides were brought to a boil in 10mM sodium citrate buffer (pH6.0) for 10 minutes. Then, they were washed in PBS, permeated with PBT for 30 minutes, and blocked by incubating the tissue sections with 3% BSA in PBT for 1 hour at RT. Primary antibodies diluted in PBT-azide containing 3% BSA were added, then slides were incubated overnight at 4°C. The following day, tissue
samples were rinsed with PBS and incubated for 2 hours in secondary antibodies. Then, the sections were rinsed and a coverslip was placed on the tissue sections using mounting medium with DAPI. Slides were visualized using a confocal laser microscope (LSM700) and analysis was processed using the ZEN blue software (Zeiss).

The primary antibodies used were rabbit anti-ACIII (Adenylyl cyclase III, #sc-588, 1:2000, Santacruz, Dallas, Texas, USA), goat anti-SSTR3 (Somatostatin receptor 3, #sc-11617, 1:500, Santacruz), and mouse anti-Ac-Tub (Acetylated alpha tubulin, #T7451, 1:500, Sigma, Louis, MO, USA) for primary cilia and chicken anti-GFP (#GFP-1020, 1:10000, AvesLab, Tigard, OR, USA) for either viral infection or SF1-eGFP positive cells.

Secondary antibodies used were Alexa Fluor 488-conjugated goat anti-rabbit antibody (#A21206, 1:1000), Alexa Fluor 594-conjugated goat anti-rabbit antibody (#A11012, 1:1000), Alexa Fluor 594-conjugated goat anti-mouse antibody (#A11005, 1:1000), Alexa Fluor 594-conjugated donkey anti-goat antibody (#A11058, 1:1000) from Invitrogen (Waltham, Massachusetts, USA) and Alexa Fluor 488-conjugated donkey anti-chicken IgY†† (#703-545-155, 1:5000, Jackson immune Research, West Grove, PA, USA).

Cresyl violet staining (Nissl staining) was performed to examine overall neuron structure. Briefly, brain sections were embedded in Superfrost Plus microscope slides (Fisher Scientific, Waltham, MA, USA) and sections were left to dry at RT. After rinsing the sections in distilled water, the slides were dipped in 0.1% cresyl violet solution for about 10 minutes. Then, the slides were rinsed in distilled water and differentiated in 90% and 95% ethanol for 3 minutes each. Then, the slides were dehydrated in absolute
ethanol two times for 3 minutes each, and were dipped in the 100% xylene 3 times for 5 minutes each. Finally, slides were mounted with mounting media and checked under the microscope.

**Body weight and compositions.**

The body weights of WT and IFT88 KO\textsuperscript{SF-1} mice were monitored weekly from the weaning time (4-week old). For HFD study, WT and IFT88 KO\textsuperscript{SF-1} littermates were maintained on the regular chow diet until 8-week old, then switched to HFD for an additional 12-16 weeks. Body weight measurement for AAV-injected KD mice started from 1 week after stereotaxic surgery. Body compositions of all mice were analyzed by nuclear magnetic resonance (NMR) (LF90 Minispec, Bruker Corp., TX, USA).

**Metabolic cage study.**

For metabolic cage studies, weight-matched 12-week WT (30.10±1.11) and IFT88 KO\textsuperscript{SF-1} (31.30±1.33) male littermates fed NC were used and 16-week WT (46.63.10±1.78) and IFT88 KO\textsuperscript{SF-1} (53.40±2.21) male littermates fed HFD were used. Metabolic rates were assessed with an indirect calorimetry system (CaloSys Calorimetry System, TSE Systems, Inc., Bad Homburg, Germany) as previously described (20, 24). For environmental acclimation, first, the experimental mice were housed 5 days in metabolic cages individually and maintained in the same room as where the metabolic analyses were performed. Then, the mice were individually housed in the metabolic chambers and acclimated for 48 hours. After the acclimation in the chamber, food intake, oxygen consumption (VO\textsubscript{2}), carbon dioxide production
(VCO₂), heat generation, and movement were measured and the relationship between metabolic rate and body mass was normalized using lean body mass. Diet (NC or HFD) and water were available ad libitum unless otherwise indicated.

To measure leptin sensitivity using metabolic cages, body weight matched (28.54±1.39 for WT and 29.33±1.10 for IFT88 KO⁹⁸⁻¹) 12-week old mice were used. After 3 days of regular cage studies, chow was removed at 6:00pm and the mice were fasted for 1 day. The following day, experimental mice were given leptin (i.p., 5mg/kg of body weight) with food, then the metabolic parameters including food intake, VO₂, VCO₂, heat generation, and movement were monitored during the experimental period.

To assess the metabolic effects of isoproterenol (ISO, #I6504, Sigma, Louis, MO, USA), an adrenoreceptor agonist, 16-week old WT and IFT88 KO⁹⁸⁻¹ mice were used and were given ISO (15mg/kg of body weight, daily i.p. injection) for 2 weeks.

Rebound food intake.

For the rebound feeding experiments, 10- to 12- week old mice were fasted overnight for 18 hours with water provided ad libitum. The following day, mice were given the same amount of food then food intake and body weight were recorded at the indicated time points. To assess the leptin sensitivity, 12-week old WT and IFT88 KO⁹⁸⁻¹ mice were given either saline or leptin (5mg/kg of body weight) via i.p. injection after an overnight fast. The refeeding food intake and body weight were recorded at the indicated time points.

Hypothalamic pSTAT3 staining.
For pSTAT3 immunohistochemistry, weight-matched 10-week old WT and IFT88 KO\textsuperscript{SF-1} mice fasted for 24 hours were given leptin (5mg/kg of body weight) or saline. After 1 hour, the animals were transcardially perfused with 10% neutral buffered formalin. The rabbit anti-pSTAT3 (#9131, 1:1000, Cell Signaling, Danvers, USA) were used and regular immunohistochemistry was followed, as described elsewhere.

**Serum analyses.**

To measure fed glucose levels, mice were acclimated for 2 hours in the experiment area before measurement. Then, a small drop of blood was obtained from the tail nick and measured with a glucometer (Contour TS, Ascensia Diabetes Care, NJ, USA). For insulin and leptin measurement, blood was collected from the tail-nicked blood drops after 2 hours of acclimation in the experiment area. The serum was analyzed with specific ELISA kits (Morinaga Institute of Biological Science, Yokohama Japan) in accordance with manufacturer’s instructions.

For corticosterone, aldosterone, and estradiol (E2) measurements, mice were either housed in a group or individually, and blood samples were collected at 2pm. An acclimation period of 3 days was given to the mice caged individually. Corticosterone and aldosterone levels were measured using ELISA kit obtained from Abcam (#ab108821 and #ab136933 respectively, Cambridge, UK). Plasma estradiol (E2) was measured using ELISA kits (#3830, BioVision Inc., CA, USA).

For serum P1NP and CTX-1 measurement, blood samples were collected from 12-week old WT and IFT88 KO\textsuperscript{SF-1} male littermates at 2pm. P1NP and CTX-1 were measured using commercial kits (#AC-33F1 for P1NP, and #AC-06F1 for CTX-1,
Immunodiagnostic Systems, East Boldon, UK) in accordance with the manufacturer’s instructions.

**Norepinephrine measurements.**

For serum norepinephrine (NE), blood was collected from the tail of 8- to 14-week old male mice with 2 week intervals. For femur NE, male mice were sacrificed and their right femur were dissected. Frozen femur powder was resuspended in glutathione tissue extraction buffer (0.01N HCl, 0.15mM EDTA, 0.1% reduced L-glutathione), and neutralized with 1.0M Tris pH8.0 (1/10 vol) prior to NE ELISA. Extracted serum and femur NE were acylated and then converted enzymatically before being quantitatively determined. The levels were determined by a competitive enzyme immunoassay method using commercial ELISA kits (#BA-E-5200, Labor Diagnostika Nord Gmbh & Co., Germany) following the manufacturer’s instructions. Aliquot of femur suspended in extraction buffer was used to quantify protein concentration, and used to normalize femur NE measurements.

**Glucose and insulin tolerance tests.**

For the glucose tolerance test (GTT), 12-week old mice were fasted overnight for 18 hours and provided with water *ad libitum*. The next day, mice were housed in individual cages and allowed to acclimate for 2 hours followed by i.p. injection of 1.0 g kg\(^{-1}\) glucose (#G8270, Sigma, Louis, MO, USA). For insulin tolerance test (ITT), mice were fasted for 2 hours in individual cages with free access to water. Insulin (0.8 U kg\(^{-1}\), Eli Lilly and Company, IN, USA) was administered by i.p. injection. Blood
samples were obtained from a tail nick and the blood glucose were measured at 0, 15, 30, 45, 60, 90, 120 and 150 minutes using a commercial glucometer (Contour TS, Ascensia Diabetes Care, NJ, USA).

**Bone densitometry (Dual-energy X-ray absorptiometry).**
WT and IFT88 KO^{SF-1} male littermates were examined for whole-body fat and lean masses, bone mineral density, bone mineral contents, bone volume, and bone area using DEXA (InAlyzer™, MEDIKORS, Seongnam, Republic of Korea) under light isoflurane anesthesia about 1 minute. The detection sensitivity of the DEXA instrument was 0.001 g/cm². A standard calibration block was used to calibrate the DEXA device before measurements in accordance with the operator’s manual. Software integrated to the scan was used for data analysis.

**Micro-computed tomography (μCT) analysis.**
The right femur from each mice was dissected and fixed overnight in 10% neutral buffered formalin and loaded into 12.3-mm-diameter scanning tubes, and imaged using a desk-top μCT scanner (SkyScan 1076, Bruker Micro CT, Belgium). The scans were integrated into three-dimensional (3D) voxel images and a region of interest (ROI) that consisted of 100 slices starting from about 0.5mm proximal to growth plate, constituting 1.6mm in length, was chosen for analysis. The following 3D indices in the defined ROI were analyzed including total tissue volume, BV; bone volume faction, BV/TV; trabecular number, Tb.N.; trabecular thickness, Tb.Th.; and trabecular separation, Tb.Sp.
Isoproterenol treatment.

For isoproterenol treatment, 14-week old WT and IFT88 KO^{SF-1} male littermates were used. Prior to treatment, mice were examined fat and lean masses, bone mineral density (BMD), bone mineral contents (BMC), bone volume (BV), and bone area (BA) using DEXA. After 2 days of recovery, mice were given isoproterenol (15mg/kg of body weight, daily i.p. injection) for 2 weeks. After 2 weeks, mice were measured again for fat and lean masses and bone parameters.

Immunohistochemistry and H&E staining.

For SF-1 staining, brain sections were mounted on the Superfrost Plus microscope slides (Fisher Scientific, MA, USA) then dried overnight. The following day, samples were pretreated with 0.3% hydrogen peroxide in PBS (pH7.4) for 30 minutes at RT, then antigen retrieval was done for 5 minutes. Samples were blocked in 3% normal donkey serum, then incubated with a rabbit anti-SF-1 antibody (22, 52) for overnight at 4°C. After washing in PBS, sections were incubated in biotinylated donkey anti-rabbit IgG (#065-152, 1:1000, Jackson ImmunoResearch) for 2 hours at RT, followed by incubation for 1 hour in a solution of avidin-biotin complex (Vectastain Universal ABC kit, #PK-6200, Vector Laboratories, Burlingame, California, USA). The sections next were washed in PBS and stained with 3,3'-diaminobenzidine (DAB)-peroxidase substrate solution (0.04% DAB and 0.01% H_{2}O_{2}) in PBS. The stained slides were visualized by a Nikon Digital Camera DXM1200 microscope system (Nikon Corporation, Tokyo, Japan).
For UCP1 staining, iBAT was sectioned and paraffin-embedded. The sections were incubated with primary UCP1 antibody (#ab10983, 1:1000, Abcam, Cambridge, UK) at 4°C for 24 hours. The slides were washed 5 times with PBS and then incubated with secondary antibody for additional 2 hours using Vectastain Universal ABC kit. After washing 3 times in PBS for 10 minutes each, the slides were then stained with DAB-peroxidase substrate solution. The stained slides were visualized by a Nikon Digital Camera DXM1200 microscope system (Nikon Corporation, Tokyo, Japan). Peripheral tissues including white adipose tissue (WAT), brown adipose tissue (BAT), liver, pituitary, adrenal gland, testis, ovary and femurs dissected and post-fixed overnight at 4°C. The tissue samples were then paraffin embedded and cut into 4μm slices. The slices were stained with hematoxylin and eosin (H&E) following the standard H&E procedure.

**Dynamic bone histomorphometry.**

The left femur from each animal was dissected and fixed in 10% of neutral buffered formalin. Samples were decalcified in ChelatorCal™ (BBC Biochemical, Washington, USA) and embedded in paraffin, then cut into 4μm slices. Femur sections were stained by either H&E or tartrate-resistant acid phosphatase (TRAP) for osteoclast analysis. TRAP staining was carried out with the TRAP Stain kit (TRAP/ALP stain kit, Wako Pure Chemical Industries, Ltd., Tokyo, Japan). Deparaffinized and rehydrated sections were incubated at 37°C for 30 minutes in TRAP substrate buffer and then slides were mounted with Vectamount (Vector Laboratories, Inc., Burlingame, CA, USA). Images
were visualized with an Olympus IX71/F22PH microscope (Tokyo, Japan) and measurements performed with Bio-quant software. The TRAP-positive osteoclast number (N. Oc) and surface (Oc. S) were normalized with bone surface (BS).

To determine bone formation rate and mineralization, 6-week old male mice were injected with 10mg/kg of calcein (Sigma, Louis, MO, USA) dissolved in 2.0% sodium bicarbonate (pH7.0) 2 times a day for 7 days. Bones were fixed in 4% formalin. Femurs were embedded in paraffin blocks and sectioned to be 4µm thick. Before histomorphometric analysis, femur metaphyseal sections were viewed with a fluorescent Nikon Digital Camera DXM1200 microscope system (Nikon Corporation, Tokyo, Japan) and five digital images per section were taken. The distance between the calcein lines (mineral apposition rate; MAR) and the length of the calcein lines (mineralized surface) along the bone surface (BS) were measured to calculate bone formation rate (BFR) using ImageJ software.

**Western blot analysis.**

Tissues samples were lysed in radio-immunoprecipitation assay buffer (RIPA buffer; 150mM NaCl, 50mM Tris pH8.0, 1% Triton-X-100, 0.5% sodium deoxycholate and 0.1% SDS) containing protease and phosphatase inhibitors (Roche, Basel, Switzerland). Protein extracts were obtained by centrifugation at 13,000× g for 15 minutes at 4°C.

The protein concentration was measured using Bio-Rad Protein Assay reagent (#500006, Bio-Rad Laboratories, Hercules, California, USA). Equal amounts of protein were loaded and separated on sodium dodecyl sulfate-acrylamide (SDS/PAGE) gels and transferred to PVDF membranes (Millipore, Burlington, Massachusetts, USA).
Then, membranes were incubated with specific antibodies in Tris-buffered saline with 0.1% Tween-20 (TBST) overnight at 4°C. Primary antibodies used were rabbit anti-UCP1 (#ab10983, 1:10000, Abcam, Cambridge, UK), STAT3 (#9139, 1:5000, Cell Signaling Technology, Danvers, MA), and rabbit anti-beta Actin (#GTX109639, 1:5000, GeneTex, California, USA). Secondary antibodies conjugated to horseradish peroxidase (HRP) were added for 1 hour at RT. Secondary antibody used was goat anti-rabbit IgG-HRP (#31460, 1:5000, Thermo Fisher Scientific, MA, USA). The protein levels were detected using Pierce ECL Western blotting substrate (Waltham, MA, USA) following the standard Western blot procedure and the blots were visualized using the chemiluminescent image analyzer LAS 4000 (GE Healthcare Life Science, USA).

Mitochondria contents in the iBAT.

For mitochondrial DNA content analysis, total DNA from iBAT was extracted using DNAzol Reagent (Invitrogen, CA, USA) according to the manufacturer’s instructions. Mitochondrial DNA was amplified using primers specific for the mitochondrial cytochrome C oxidase subunit 2 (COX2) gene and normalized to genomic DNA by amplification of the 40S ribosomal protein s18 (Rps18) nuclear gene (53). Primers used were as follows. Mouse COX2: F, 5’-ATAACCGAGTCTTCTGCCAAT-3’, R, 5’-TTTCAGAGCATTGGCCATAGAA-3’. Rps18: F, 5’-TGTGTTAGGGGACTGGTGACA-3’, R, 5’-CATCACCCTGTTACCCCCAAA-3’.

RNA isolation and real-time quantitative PCR (qPCR).

Total RNA was extracted from tissue samples (hypothalamus, liver, iBAT, and femur)
using TRIzol reagent (Life Technologies, Carlsbad, CA, USA) following the manufacturer’s instructions. One μg of total RNA was used to synthesize cDNA using the ReverTra Ace® qPCR RT Master Mix with gDNA Remover (TOYOBO, Kita-ku, Osaka, Japan). An aliquot (1/100 vol) of the cDNA was then subjected to Real-time quantitative PCR using the iQ SYBR® Green Supermix (Bio-rad, Hercules, California, USA) in a 96 well Real-Time PCR machine (CFX96, Bio-Rad Laboratories, Hercules, California, USA). Fold changes were calculated and determined using the 2^{-ΔΔCt} method and expression levels normalized to the average of the housekeeping genes either 18S or HRPT.

F, 5'-TGGGCAAAATGGCAAGGA-3', R, 5'-TCTGCCCCAGGAATCAAAAAT-3'.

**Ehhadh**: F, 5’-CTTGGAATTCTGGATGTAG-3', R, 5’-GGGGTTTACCTATAACCG-3'.

**PPARy**: F, 5’-CAAGAATACCAAAGTGCGATCAA-3', R, 5’-GAGCTGGGTCTTTTCGAATAAATAAG-3'.

**Srebpl**: F, 5’-GGAGCCATGGATTGCACATT-3', R, 5’-GGCCCCGGAAGTCACTGT-3'.

**FASN**: F, 5’-GGTGTGGGAGGGTTTGGGAATTGT-3', R, 5’-TCACGAGGAGCGCATGCTTTTAGCACC-3'.

**HSL**: F, 5’-GCGCTGGAGGAGTGTTTTT-3', R, 5’-CCGCTCTCCAGTTGAAACC-3'.

**Rank**: F, 5’-CCAGGAGGAGGCATTATGAGCA-3', R, 5’-ACTGTCGGGAGGTAGGAGTGC-3'.

**Rankl**: F, 5’-CAGCATCGCTCTGTCTTCTGTGA-3', R, 5’-CTGCGGCTTTTCATGGAGTCTCA-3'.

**Trap**: F, 5’-GCAACATCCCCTGGTATGTG-3', R, 5’-GCAAACGGTAGTAAGGGCTG-3'.

**Dc-Stamp**: F, 5’-GGGGACTTATGTGTTTCCACG-3', R, 5’-ACAAGCAACAGACTCCCAAAT-3'.

**Sp7**: F, 5’-TCCCTGGGATATGACTCATCCCT-3', R, 5’-CCAAGGAGTAGGTGGTGGTGCC-3'.

**Alp**: F, 5’-CTTGAAAAATGCCCTGAAA-3', R, 5’-TTACTGTTGGAGAGCACCATA-3'.

**18S**: F, 5’-AACCCTGTTGAAACCCATT-3', R, 5’-CCATCCAATCGGTAGTAGC-3'.

**HRPT**: F, 5’-CTCATGGACTATTGAGGACAGGAC-3', R, 5’-GCAGGTCAGCAAAGAATTATAGGCC-3'.

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**Data and software availability.**

All data generated or analyzed during this study are available from the corresponding author on reasonable request.

**Statistics.**
All statistical tests for data values were conducted using GraphPad Prism (version 5) and R software (version 3.6.0). Full details of each statistical test used is described in each figure legend. The $P$ values less than 0.05 were defined as a statistically significant difference. Statistical significance was determined by unpaired 2-tailed Student’s t-test to compare two groups and two-way analysis of variance (ANOVA) with the Bonferroni post-test to compare multiple groups. For correlation testing, linear regression models in R was utilized, and $R^2$ values were reported. The R package ggplot2 was used to plot the result. The various asterisk abbreviations denote *: $P < 0.05$, **: $P < 0.01$, ***: $P < 0.001$, ns: not significant.

**Study approval.**

All animal studies were approved by the Institutional Animal Care and Use Committee of Yonsei University Health System.
Author contributions

J.S.S. and D.J.Y. performed the experiments and analyzed data.

K.W.K. wrote the Abstract, Introduction, Results, and Discussion sections.

D.J.Y. and J.S.S. wrote Methods and Figure Legends sections.

A.W.K. performed preliminary experiment.

S.G.Y. and J.K.S. performed metabolic cage studies.

S.J.M., J.K., D.M.S., Y.-H.C., and K.W.K. conceptualized the research, analyzed data, edited and finalized the manuscript. The order of co-first authorship was determined by effort in data acquisition and drafting manuscript. J.S.S. and D.J.Y. share first authorship, and the order in which they are listed was determined by workload.
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References


Figure 1. Deletion of primary cilia in the VMH leads to obesity.

(A) Primary cilia were visualized by ACIII (green) in the VMH of the WT and IFT88 KO<sup>SF-1</sup> mice. Scale bar = 10μm.

(B) Length and percentage of the VMH cilia shown in (A). **P<0.01 and ***P<0.001 for Student’s t-test.

(C) Left, weekly body weight of WT and IFT88 KO<sup>SF-1</sup> mice fed on normal chow. Right, representative picture of 18-week old male mice on normal chow diet. *P<0.05 for two-way ANOVA.

(D) Left, fat and lean mass were measured by NMR. Right, representative DEXA image. **P<0.01 for Student’s t-test.

(E) Representative subcutaneous white adipose tissue (scWAT) and gonadal WAT (gWAT) from WT and IFT88 KO<sup>SF-1</sup> mice. Scale bar = 0.5mm.

(F) and (G) H&E staining (F) and adipocyte cell surface (G) of gWAT. Adipocyte cell surface measured by ImageJ software. Scale bar = 50μm. ***P<0.001 for two-way ANOVA.

(H) Serum leptin levels in WT and IFT88 KO<sup>SF-1</sup> mice at 14-week old. **P<0.01 for Student’s t-test.

(I) Left, schematics showing the bilateral viral injection into the VMH. Right, schematics for AAV2-GFP and AAV2-Cre-GFP constructs used for viral injection (upper) and the validation images of primary cilia KD in the VMH (lower). Scale bar, 0.1mm for yellow and 10μm for white.

(J) Weekly body weight after injection either AAV2-GFP or AAV2-Cre-GFP into the VMH. ***P<0.001 for two-way ANOVA.

(K) Left, body compositions after 10 weeks post-injection of AAV2-GFP or AAV2-Cre-GFP. Right, representative DEXA image. ***P<0.001 for Student’s t-test.
Number of animals examined is expressed parentheses in each graph. The results are expressed as mean ± SD.
Figure 2. The VMH-primary cilia are important for regulation of EE and BAT function.

(A) Comparable body weight of WT and IFT88 KO$^{SF-1}$ littermates at 12-week old.

(B) Cumulative daily food intake under the normal chow diet.

(C)-(E) Temporal changes of VO$_2$ (C), VCO$_2$ (D), and EE (E) in WT and IFT88 KO$^{SF-1}$ littermates. *P<0.05 with red color for two-way ANOVA, **P<0.01 for Student’s t-test.

(F) Hypothalamic Pomc, Agrp, and Npy expression. *P<0.05 for Student’s t-test.

(G) Temporal changes of movement in WT and IFT88 KO$^{SF-1}$ littermates.

(H) Morphology and H&E staining of the iBAT from WT and IFT88 KO$^{SF-1}$ littermates. Scale bar, 0.5mm for yellow and 50μm for black.

(I) Mitochondria DNA contents in the iBAT. **P<0.01 for Student’s t-test.

(J) Immunohistochemistry for UCP1 in the iBAT of WT and IFT88 KO$^{SF-1}$ mice. Scale bar = 100μm.

(K) and (L) Western blot (K) and relative fold changes of protein levels (L) for UCP1. ***P<0.001 for Student’s t-test.

(M) Relative gene expression levels in the iBAT. *P<0.05, **P<0.01, and ***P<0.001 for Student’s t-test.

(N) Age-dependent serum norepinephrine (NE) level in WT and IFT88 KO$^{SF-1}$ mice. *P<0.05 and **P<0.01 for two-way ANOVA.

(O) Correlation analysis between serum NE and body weight of WT and IFT88 KO$^{SF-1}$ mice.

EE, energy expenditure; Ucp1, uncoupling protein 1; Pgc1α, Peroxisome proliferator-activated receptor gamma coactivator 1; Dio2, Type II iodothyronine deiodinase; Tfam, mitochondrial transcription factor A; PPARγ, peroxisome proliferator-activated
receptor gamma; *FASN*, fatty acid synthase. Number of animals examined is expressed in parentheses in each graph. The results are expressed as mean ± SD.
Figure 3. The VMH-primary cilia is required for leptin action.

(A) Cumulative rebound food intake of 10-week old littermates after overnight fasting for 18 hours. *P<0.05 for two-way ANOVA.

(B) Rebound food intake of 12-week old littermates after leptin administration. **P<0.01 for two-way ANOVA.

(C) and (D) Western blot (C) and relative fold changes of protein levels (D) for hypothalamic STAT3 in the WT and IFT88 KO^SF-1 mice.

(E) Immunohistochemical analysis of pSTAT3 activation after leptin injection. Ten-week old WT and IFT88 KO^SF-1 mice were used for analysis. Note that pSTAT3 positive cells are specifically decreased in the VMH. Scale bar = 100μm.

(F) Relative pSTAT3 expression in the VMH or ARC. ***P<0.001 for Student’s t-test.

(G) Body weight for 12-week old WT and IFT88 KO^SF-1 littermates used for metabolic cage study shown in (H) to (K).

(H) Cumulative food intake after leptin administration measured in metabolic chamber. **P<0.01, two-way ANOVA.

(I)-(K) Temporal change of VO₂ and its average (I), VCO₂ and its average (J), and EE and its average (K). *P<0.05 for Student’s t-test.

Number of animals examined is expressed parentheses in each graph. Data are expressed as mean ± SD.
Figure 4. Impaired VMH-primary cilia result in glucose and insulin intolerance.

(A) and (B) Blood glucose (A) and insulin (B) levels in WT and IFT88 KO SF-1 littermates at 12-week old. *P<0.05 for Student’s t-test.

(C) Left, glucose tolerance test (GTT) between WT and IFT88 KO SF-1. Right, area under the curve (AUC) from the GTT. *P<0.05 and **P<0.01 for two-way ANOVA.

(D) Left, insulin tolerance test (ITT) in indicated genotypes. Right, AUC for the ITT. *P<0.05, **P<0.01, and ***P<0.001 for two-way ANOVA.

(E) Glucose-stimulated insulin secretion (GSIS) during the GTT experiments.

(F) Relative expression of gluconeogenic genes in the liver. **P<0.01 and ***P<0.001 for Student’s t-test.

(G) H&E staining of the liver from WT and IFT88 KO SF-1 mice. Scale bar = 50μm.

(H) Relative expression of lipogenesis-related genes in the liver. *P<0.05, **P<0.01, and ***P<0.001 for Student’s t-test.

(I) The GTT (left) and its AUC (right) in viral-mediated VMH-specific IFT88 KD models. *P<0.05 for two-way ANOVA.

(J) The ITT (left) and its AUC (right) in viral-mediated VMH-specific IFT88 KD models. *P<0.05 and **P<0.01 for two-way ANOVA.

Number of animals examined is expressed parentheses in each graph. Data are expressed as mean ± SD.
Figure 5. Deletion of VMH-primary cilia leads to bone mass increment.

(A)-(D) DEXA analyses were performed at indicated ages and measured bone parameters. BMD (A), BMC (B), BV (C), and BA (D) levels of WT and IFT88 KO SF-1 mice. *P<0.05 and **P<0.01 for two-way ANOVA.

(E)-(H) Correlation between serum NE and BMD (E), BMC (F), BV (G), and BA (H) respectively. For correlation testing, linear regression models in R was utilized and R² values were reported.

(I) Femur NE level of WT and IFT88 KO SF-1 littermates at 16-week old. ***P<0.001 for Student’s t-test.

(J) Representative μCT images of the femurs. The 12-week old WT and IFT88 KO SF-1 littermates were used for analyses.

(K) The BMD, BV/TV, BS, Tb.Th., Tb.N., and Tb.Sp. were analyzed using μCT from (J). *P<0.05 and **P<0.01 for Student’s t-test.

(L) Representative μCT images of the femurs in the control (AAV2-GFP) and viral-mediated VMH-specific primary cilia KD (AAV2-Cre-GFP) mice.

(M) The BMD, BV/TV, BS, Tb.Th., Tb.N., and Tb.Sp. were analyzed using μCT from (M). *P<0.05 for Student’s t-test.

BMD, bone mineral density; BMC, bone mineral contents; BV, bone volume; BA, bone area; BV/TV, bone volume/trabecular volume; BS, bone space; Tb.Th., trabecular thickness; Tb.N., trabecular number; Tb.Sp., trabecular separation.

Number of animals examined is expressed parentheses in each graph. Data were expressed as mean ± SD.
Figure 6. VMH-primary cilia regulate bone homeostasis through sympathetic nervous system.

(A)-(D) Changes of bone parameters; BMD (A), BMC (B), BV (C), and BA (D) in both WT and IFT88 KO SF-1 littermates before and after i.p. injection of isoproterenol (ISO, 15mg/kg) for 2 weeks. Bone parameters measured using DEXA analysis. *P<0.05, **P<0.01 for two-way ANOVA. The ns indicates no significance.

(E) Representative femur images of WT and IFT88 KO SF-1 littermates after ISO administration.

(F) The BMD, BV/TV, BS, Tb.Th., Tb.N., and Tb.Sp. were analyzed using μCT in WT and IFT88 KO SF-1 mice given ISO.

Number of animals examined is expressed parentheses in each graph. The results are expressed as mean ± SD.
Figure 7. Bone parameter changes in VMH-specific primary cilia KO mice.

(A)-(D) Expression patterns of genes related to either osteoblast- or osteoclast-differentiation.

(E) H&E (left) and TRAP chromogenic staining (right) were done in trabecular bone of WT and IFT88 KO^SF-1^ littermates. Scale bar = 100μm.

(F) The number of osteoclasts per bone surface from (E).

(G) The amount of osteoclasts surface per bone surface from (E).

(H) and (I) Relative expression of osteoblastogenic genes.

(J) Serum P1NP levels of 12-week old WT and IFT88 KO^SF-1^ mice.

(K) Serum CTX-1 levels of 12-week old WT and IFT88 KO^SF-1^ mice.

N. Oc/BS, number of osteoclast/bone surface; Oc.S/BS, osteoclast surface/bone surface. Number of animals examined is expressed parentheses in each graph. Data are expressed as mean ± SD (*P<0.05, **P<0.01, and ***P<0.001 for Student’s t-test).
Figure 8. Increment of bone growth in VMH-specific primary cilia KO mice.

(A) Calcein double-labeling in cortical bone (left) and trabecular bone (right). Scale bar = 50μm.

(B) and (C), Bone formation rate (B) and mineral apposition rate (C) of cortical bone of WT and IFT88 KO^SF-1^ mice.

(D) and (E), Bone formation rate (D) and mineral apposition rate (E) of trabecular bone of WT and IFT88 KO^SF-1^ mice.

Ct, cortical bone; Tb, trabecular bone; BFR, bone formation rate; MAR, mineral apposition rate. Number of animals examined is expressed parentheses in each graph. Data are expressed as mean ± SD (**P<0.001 for Student’s t-test).
Table 1. Serum leptin level in 8 to 14-week old of WT and IFT88 KO^{SF-1} mice

<table>
<thead>
<tr>
<th>Week</th>
<th>WT (n=6)</th>
<th>IFT88 KO^{SF-1} (n=6)</th>
</tr>
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<tbody>
<tr>
<td>8</td>
<td>2.932 ± 1.173</td>
<td>3.198 ± 2.466</td>
</tr>
<tr>
<td>10</td>
<td>3.616 ± 1.496</td>
<td>5.954 ± 3.336</td>
</tr>
<tr>
<td>12</td>
<td>4.097 ± 2.125</td>
<td>10.400 ± 2.569*</td>
</tr>
<tr>
<td>14</td>
<td>4.790 ± 2.658</td>
<td>10.480 ± 4.699*</td>
</tr>
</tbody>
</table>

Age-dependent serum leptin levels. Note that the significant difference in leptin levels from 12-week when the WT and IFT88 KO^{SF-1} have comparable body weight. Data are expressed as mean ± SD (*P<0.05, two-way ANOVA).
Table 2. Bone parameters measured by DEXA analysis in KD mice

<table>
<thead>
<tr>
<th>Parameter</th>
<th>AAV2-GFP (n=8)</th>
<th>AAV2-Cre-GFP (n=6)</th>
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<tbody>
<tr>
<td>BMC (g)</td>
<td>0.770 ± 0.042</td>
<td>0.855 ± 0.043**</td>
</tr>
<tr>
<td>BMD (g/cm²)</td>
<td>0.093 ± 0.003</td>
<td>0.109 ± 0.013**</td>
</tr>
<tr>
<td>BV (cm³)</td>
<td>0.466 ± 0.025</td>
<td>0.517 ± 0.026**</td>
</tr>
<tr>
<td>BA (cm²)</td>
<td>8.312 ± 0.319</td>
<td>7.869 ± 0.622</td>
</tr>
</tbody>
</table>

Data are expressed as mean ± SD. **P<0.01 for Student’s t-test compared to AAV2-GFP control.