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Abstract: Brown adipose tissue (BAT), largely controlled by the sympathetic nervous system (SNS), has the ability to dissipate energy in the form of heat through the actions of uncoupling protein-1 (UCP-1), thereby critically influencing energy expenditure. Besides BAT, the SNS also strongly influences bone, and recent studies have demonstrated a positive correlation between BAT and bone mass, albeit the interactions between BAT and bone remain unclear. Here we show that UCP-1 is critical for protecting bone mass under conditions of permanent mild cold stress (22oC), since UCP-1-/- mice showed significantly reduced cancellous bone mass, with reduced trabecular number and thickness, a decreased bone formation rate and mineralising surface area, but unaltered osteoclast number, compared to wild type mice also exposed to mild cold stress. UCP-1-/- mice also displayed shorter femurs than wild types, with smaller cortical periosteal and endosteal perimeters. Importantly, these altered bone phenotypes where not observed when UCP-1-/- and wild type mice were housed in thermo-neutral conditions (29oC). When housed under conditions of mild cold stress, UCP-1-/- mice showed elevated hypothalamic expression of neuropeptide Y relative to cold-stressed wild types. This finding is consistent with the reduced bone formation and mass of UCP-1-/- versus wild type mice and the known bone catabolic effects of hypothalamic NPY induced by SNS modulation. The results from this study collectively suggest that UCP-1 exerts a protective effect on bone mass during cold mild stress, when BAT-dependent thermogenesis is required, possibly through alterations in central neuropeptide pathways known to regulate SNS activity.

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October 28th 2014

Dear Editor,

Please find enclosed our manuscript entitled '**Uncoupling protein-1 is protective of bone mass under mild cold stress conditions through stimulation of osteoblast activity by central neuropeptide Y pathways**' for consideration for publication in *Bone*.

Recently it has emerged that the regulation of whole body energy homeostasis is closely linked to the control of bone metabolism. Brown adipocytes together with white adipocytes, myocytes, chondrocytes and osteoblasts share the same precursor, mesenchymal stem cells, which raises the notion of shared regulatory influences. Brown adipose tissue (BAT) is a highly active area of research, related to the potential to reduce obesity through non-dietary means. Interestingly, a relationship between BAT and bone mass is emerging, however, much remains to be determined. Initial studies in rats undergoing cold exposure, when is highly activated, revealed reductions in plasma osteocalcin, suggesting that cold stress may reduce osteoblast function. More recently, the induction of BAT activity in adipose tissue has demonstrated a positive correlation between BAT and bone mass in transgenic mice exhibiting defects in brown adipocyte function. Thus the exact nature of the BAT/bone relationship remains to be defined.

Here we show that UCP-1, the main component in BAT responsible for heat production, is critical for protecting bone mass under mild cold stress conditions since UCP-1 KO mice reared under conditions of permanent mild cold stress (22^{o}C,RT) showed significantly reduced cancellous bone mass, with reduced trabecular number and thickness, and a decreased bone formation rate and mineralising surface. In addition, UCP-1$^{-/-}$ mice also displayed shorter femora, reduced cortical area, with smaller cortical periosteal and endosteal perimeters. Importantly, these altered bone phenotypes where no longer observed when UCP-1 KO mice were held at thermo-neutral condition (29^{o}C). The results from this study collectively suggest that UCP-1 exerts a critical protective role on bone mass during cold stress when BAT-dependent thermogenesis is required. Importantly, our data links UCP-1 activity to regulation of sympathetic nervous activity through control of neuropeptide Y expression in the hypothalamus, tying together observations made in previous studies outlining a role for SNS in BAT control of bone mass and the actions of NPY and stress on sympathetic tone. ***In this manner, we provide for the first time a unified mechanism whereby BAT activation stimulated osteoblast activity.***

We believe that the results presented in this manuscript, showing the specific and critical role played by BAT through UCP-1 action in the regulation of bone mass, will provide greater clarity for all future research into the complex regulation of bone homeostasis and would be of wide interest to readers of *Bone.*

Sincerely,

Herbert Herzog

Highlights:

- UCP-1 is critical for protecting bone mass under conditions of cold stress
- UCP-1 is not required for bone mass regulation under thermo-neutral conditions
- Lack of *Ucp-1* under cold stress conditions leads to increase expression of Arc NPY

Uncoupling protein-1 is protective of bone mass under mild cold stress conditions

Running title: UCP-1 is protective of bone mass

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Keywords: uncoupling protein-1, bone, energy homeostasis, cold stress, brown adipose tissue, neuropeptide Y

Abstract

Brown adipose tissue (BAT), largely controlled by the sympathetic nervous system (SNS), has the ability to dissipate energy in the form of heat through the actions of uncoupling protein-1 (UCP-1), thereby critically influencing energy expenditure. Besides BAT, the SNS also strongly influences bone, and recent studies have demonstrated a positive correlation between BAT and bone mass, albeit the interactions between BAT and bone remain unclear. Here we show that UCP-1 is critical for protecting bone mass under conditions of permanent mild cold stress (22^{o}C), since UCP-1$^{-/-}$ mice showed significantly reduced cancellous bone mass, with reduced trabecular number and thickness, a decreased bone formation rate and mineralising surface area, but unaltered osteoclast number, compared to wild type mice also exposed to mild cold stress. UCP-1$^{-/-}$ mice also displayed shorter femurs than wild types, with smaller cortical periosteal and endosteal perimeters. Importantly, these altered bone phenotypes where not observed when UCP-1$^{-/-}$ and wild type mice were housed in thermo-neutral conditions (29^{o}C). When housed under conditions of mild cold stress, UCP-1$^{-/-}$ mice showed elevated hypothalamic expression of neuropeptide Y relative to cold-stressed wild types. This finding is consistent with the reduced bone formation and mass of UCP-1$^{-/-}$ versus wild type mice and the known bone catabolic effects of hypothalamic NPY induced by SNS modulation. The results from this study collectively suggest that UCP-1 exerts a protective effect on bone mass during cold mild stress, when BAT-dependent thermogenesis is required, possibly through alterations in central neuropeptide pathways known to regulate SNS activity.

Introduction

Brown adipose tissue (BAT) has a primary function to generate heat by dissipating energy through the process of non-shivering thermogenesis. BAT is thus energy-utilising, and enhanced BAT activity reduces energy balance and may help to combat obesity. This is in contrast to white adipose tissue (WAT), which is primarily involved in the storage of energy as lipids, with its excess accumulation leading to obesity. In recent years, interest in the functions of BAT has increased markedly following the demonstration that the presence of BAT is not restricted to rodents or infants, but is also present in considerable amounts in adult humans (1-3). While BAT in mice is generally specific to the scapular region, in humans BAT is found in the neck, supraclavicular, paravertebral and suprarenal regions (1). Importantly, research has shown a certain plasticity of fat tissue leading to a phenomenon called 'browning' of white adipose tissue depots or skeletal muscle, resulting in 'beige' or 'brite' ('brown in white') adipocytes (4, 5). Browning occurs during events such as cold-exposure and strength training (4, 6). Interestingly, browning is also associated with skeletal-related events such as heterotopic ossification (7) or triggered by direct injection of bone morphogenetic protein 7 (BMP7) (8). This ability to induce brown adipocytes, or BAT-like depots, has sparked great research interest because the induction of brown adipocytes could be employed as a potential obesity treatment. Our understanding of the regulatory process surrounding BAT activity however is incomplete, and more research is required to determine the pathways involved in BAT thermogenesis and how altered BAT activity influences other tissues, including skeletal tissue.

Recently it has emerged that the regulation of whole body energy homeostasis is closely linked to the control of bone metabolism. Brown adipocytes together with white adipocytes,

myocytes, chondrocytes and osteoblasts share the same precursor, mesenchymal stem cells (9). Interestingly, rats undergoing cold exposure, when BAT is highly activated, exhibit reductions in circulating concentrations of bone formation markers, indicating that cold stress may reduce osteoblast function (10). Furthermore, increases in BAT activity have been demonstrated to positively correlate with bone mass (11, 12). In these studies, a model of impaired BAT function, the *Misty* mouse, was employed. This mouse model exhibited decreased cancellous and cortical bone mass, which resulted from elevated sympathetic tone (12). On the corollary, a mouse model of induced brown adipocyte growth exhibited anabolic effects on the skeleton via release of paracrine factors which affected bone remodelling (11). Human studies have also demonstrated a positive link between BAT and bone mass in children (13) and young, non-obese women (14), but these studies are correlative and cannot ascertain causality. These findings are of great interest given recent research showing that BAT function decreases in adult humans from approximately 50-60 years of age (15-17), an age where bone dysfunction is increasingly common (18). This research is in its relatively fundamental stages, however, and the BAT-bone relationship has not yet been studied in detail.

The ability of BAT to dissipate energy in the form of heat occurs specifically through the actions of uncoupling protein-1 (UCP-1). Various studies in rodents have looked at BAT contributions to energy homeostasis, either in models of BAT ablation (19), cold stress (20), at thermo-neutrality (21), and through transgenic overexpression (22-24) or disruption of BAT function (21, 25). However, the exact details of UCP-1's effect upon energy homeostasis have not been determined. These details are important as they may form part of an indirect regulatory pathway between BAT and bone. Several studies have corroborated that defects in BAT function or UCP-1 ablation resulted in obesity (19, 21, 26, 27). However,

other studies have demonstrated that BAT-defective or UCP-1 ablation mouse models, although having impaired thermogenesis, may be resistant to diet-induced obesity (25, 28-31). These conflicting data demonstrate that the metabolic as well as the skeletal phenotype of UCP-1$^{-/-}$ mice remains to be clearly elucidated. Thus several critical aspects of the BAT – bone relationship remain to be clarified: 1. What is the component of BAT that regulates bone? 2. What is the pathway by which BAT activity alters bone metabolism? Resolution of these issues will provide novel information regarding this emerging anabolic pathway to bone, but will also increase our understanding of the complex interactions between skeletal and energy homeostasis, an increasingly important issue in contemporary society.

In order to address these questions we analysed the skeletal phenotype of our novel UCP-1$^{-/-}$ mouse model, which unlike previously published models (21, 25) is based on a point mutation that renders the UCP-1 protein inactive. We specifically examined the role of UCP-1 in controlling bone mass and metabolic phenotypes at temperatures of thermo-neutrality (29°C), which does not require activation of BAT UCP-1, as well as under conditions of mild cold stress (22°C), when BAT UCP-1 activation is required for temperature control.

Results

A schematic showing the location of the introduced point mutation and the consequences for splicing and translation of the UCP-1 protein are shown in Figure 1A. This genetic ablation strategy avoids the deletion of large parts of surrounding genomic sequence and does not require the inclusion of a neo-cassette, both issues that might have affected the phenotypes of previous UCP-1 knockout models. Loss of UCP-1 protein in our knockout model was confirmed by Western Blot analysis (Fig. 1B).

UCP-1 deficiency decreases longitudinal and radial growth, as well as cancellous bone volume under cold stress conditions

In order to investigate whether UCP-1 is the causal factor responsible for BAT's reported anabolic effect on bone, we examined bones of UCP-$1^{-/-}$ mice reared under thermo-neutral (29°C) and mild cold stress conditions (22°C). When the requirement for UCP-1-mediated thermogenesis was reduced by housing mice at thermo-neutral temperatures, no differences in whole body bone mass, whole body bone mineral content or femur length were evident between UCP-$1^{-/-}$ and WT mice (Fig 2A,B,C). However, cold stress had significant effects upon skeletal parameters in WT mice, with decreased whole body bone mass and whole body bone mineral content and an increase in femur length (Fig 2A-C). On the other hand, UCP-$1^{-/-}$ mice displayed a reduced response to chronic cold stress, with no change in whole body bone mineral density or femur length relative to wild type mice in thermoneutral conditions (Fig. 2A,C) and the only significant change seen in a decrease in whole body bone mineral content relative to cold stressed wild type animals (Fig. 2B). Importantly, UCP-$1^{-/-}$ mice when compared to WT mice under cold stress conditions showed an even greater reduction in BMC and also a decreased femur length (Fig 2B,C). It appears that UCP-1 protects against bone loss under conditions of mild cold stress, because cancellous bone mass, trabecular thickness

and trabecular number were significantly reduced in cold stressed UCP-1$^{-/-}$ mice compared to WT animals under the same conditions, while they were similar between genotypes under thermo-neutral conditions (Fig. 2D-F). Representative images of cancellous bone of WT and UCP1$^{-/-}$ mice under cold stress conditions are shown in Figure 2G. Furthermore, UCP-1$^{-/-}$ compared to WT mice reared in cold stress conditions exhibited significant reductions in cortical periosteal (Fig. 2H) and endosteal (Fig. 2I) perimeters, as well as a reduction in cortical bone volume (Fig. 2J) but no change in cortical thickness (Fig. 2K). Representative images of cross sections of femurs of WT and UCP1$^{-/-}$ mice under cold stress conditions are shown in Figure 2L.

UCP-1 deficiency decreases bone formation rate under cold stress conditions

To investigate the cellular basis for the anabolic effect of UCP-1 on bones, we also conducted histomorphometric analysis on bones from WT and UCP-1$^{-/-}$ mice under thermo-neutral as well as mild cold stress conditions. Consistent with the observed reduced cancellous bone parameters in UCP-1$^{-/-}$ versus WT mice, significant reductions in trabecular bone formation rate (Fig. 3A) and mineralising surface (Fig. 3B) can be seen in UCP-1$^{-/-}$ versus WT mice under cold stress conditions but not under thermo-neutral conditions. Interestingly, mineral apposition rate was significantly reduced in UCP-1$^{-/-}$ compared to WT mice under thermo-neutral conditions (Fig. 3C), but this effect of UCP-1 ablation was not seen under cold stress conditions (Fig 3C).

In terms of bone resorption, UCP-1$^{-/-}$ mice under thermo-neutral conditions exhibit a trend to increases in osteoclast number (Fig. 3D) and the surface area of bone that is covered by osteoclasts (Fig. 3E), which might in part explain the significantly reduced mineral apposition rate in these mice under these conditions (Fig. 3C). However, there are significant reductions

in osteoclast indices in both genotypes under cold stressed conditions compared to mice held under thermo-neutral conditions (Fig. 3D,E).

UCP-1 expression is absent from bone tissue

To investigate whether UCP-1 may exert a direct effect upon bone cells, immunohistochemistry against UCP-1 was conducted on femoral bone tissue. Staining of cells on both the bone surface and within the bone marrow was similar between WT (Supp. Fig. 1A) and UCP-1$^{-/-}$ mice (Supp Fig. 1B), indicating a lack of UCP-1 protein expression in bone from either genotype. As a positive immunohistochemistry control, BAT tissue from WT and UCP-1$^{-/-}$ mice was used (Supp. Fig. 1C), demonstrating that our antibody detects UCP-1. As a further confirmation of the functionality of our immunohistochemistry techniques, bone tissue from UCP-1$^{-/-}$ mice was immunostained for osteocalcin, an abundant protein found in bone, resulting in abundant staining (Supp. Fig. 1D).

UCP-1 regulates body composition under cold stress conditions

This study is the first to demonstrate the anabolic effect of UCP-1 activity upon bone; however, the mechanism involved seems to be indirect and thus may involve responses to altered metabolic activity. In order to establish the possible contribution of metabolic changes to this bone phenotype, we examined the metabolic consequences of UCP-1 deficiency, under thermo-neutrality and mild cold stress. Body weight and food intake were monitored from 7 weeks of age for a period of 10 weeks. Body weight was not significantly different between UCP-1$^{-/-}$ and WT at either temperature when analysed over the entire monitoring period (Fig. 4A,B). However at cull, cold stressed WT mice were significantly heavier than WT mice kept at a thermo-neutral temperature, with cold stressed WT mice also being significantly heavier than cold stressed UCP-1$^{-/-}$ mice (Fig. 4C). These differences in body weight may be due to

differences in lean body mass, which showed a similar pattern of change (Fig. 4D), and is consistent with the greater femur length in cold stressed WT mice (Fig. 2C). These lean mass changes were consistent with changes in food intake, with both genotypes showing significant increases under cold stress conditions, both under fasting induced conditions (Fig. 4E) and during spontaneous food intake (Fig. 4F). In contrast, cold stressed UCP-1$^{-/-}$ mice exhibited a significant gain in fat mass compared to thermo-neutral UCP-1$^{-/-}$ mice (Fig. 4G). As expected as a compensatory response to increased UCP-1 demand, BAT weight was significantly increased in cold stressed mice, but this was markedly greater in UCP-1$^{-/-}$ relative to WT mice (Fig. 4H).

Cold stress increases energy expenditure in both WT and UCP-1$^{-/-}$ mice

As demonstrated, experimental animals that are continuously held at temperatures below thermo-neutrality elicit a metabolic response to the mild cold stress mostly affecting body composition and food intake. In order to evaluate the metabolic response to such temperature shifts, as well as the role UCP-1 plays in this response, it is critical to assess energy expenditure, which we determined by indirect calorimetry. At thermo-neutrality, UCP-1$^{-/-}$ mice displayed no significant differences in energy expenditure, physical activity or respiratory exchange ratio compared to their WT counterparts (Fig. 4I-N). Importantly however, mild cold stress significantly and markedly increased energy expenditure in both genotypes of mice in both light and dark phases, reflecting the increased amount of energy required to maintain body temperature under these housing conditions (Fig. 4I,J). Similarly, cold stress had an inhibitory effect on the respiratory exchange ratio of both WT and UCP-1$^{-/-}$ mice, indicating a greater utilisation of fat oxidation as a source of energy (Fig. 4K,L). The increase in energy expenditure in cold stressed mice was not associated with significantly increased physical activity in mice of either genotype (Fig. 4M,N), albeit there was a

significant increase in activity in WT mice during the lesser active light phase. Thus, cold stress induces marked increases in energy expenditure that is not due to increased physical activity. These additional energy demands are supplied by the observed increases in food intake and fat oxidation.

ARC-specific NPY mRNA is altered in cold stressed UCP-1$^{-/-}$ mice

Mild cold stress and UCP-1 deletion produce shifts in food intake and energy homeostasis that are co-incident with changes in bone homeostasis. One of the major regulators of energy expenditure, food intake and bone mass is neuropeptide Y (NPY), which is produced widely within the hypothalamus of the brain. NPY mediates its effects on energy expenditure to a large extent by modulating sympathetic output, a known regulator of both BAT and bone mass. Particularly, NPY produced in the arcuate nucleus of the hypothalamus (ARC) is of critical importance in the regulation of energy expenditure, and alterations in the level of NPY in this location have been demonstrated to be responsible for changes in UCP-1 expression levels in BAT (32), as well as for changes in osteoblast activity in bone tissue (33), particularly under cold stress conditions (34). We therefore investigated how the absence of UCP-1 activity influences ARC NPY expression under cold stress conditions, because it has been demonstrated that UCP-1 ablation in mice induces effects on energy homeostasis that could be mediated by changes in hypothalamic orexigenic peptides. Importantly, compared to WT mice, NPY mRNA levels measured using *in situ* hybridisation were significantly reduced in the ARC of cold stressed UCP-1$^{-/-}$ mice (Fig. 5 A, B). On the other hand, ARC-specific POMC mRNA levels, a counter-regulatory component of this regulatory loop, was unchanged in UCP-1$^{-/-}$ mice when compared to WT counterparts (Fig. 5 C, D).

Discussion

This study demonstrates for the first time that UCP-1 presence during mild cold stress is protective of bone mass, thus identifying a critical mediator of BAT's anabolic actions on bone. This is demonstrated by the significant reduction in linear and radial growth of bone in mice lacking *Ucp-1* under mild cold conditions, and the fact that this change is absent when UCP-$1^{-/-}$ mice are kept under thermo-neutral conditions. In addition to these growth effects, the skeletal action of UCP-1 activation is also evident in cancellous bone of mature mice, where it stimulates bone formation. This study also highlights the importance of standard housing conditions for experimental mice. Mice reared under conditions of mild cold stress (22°C) exhibited markedly increased food intake and energy expenditure. This is likely an adaptive response to cold, because in addition to providing greater fuel for heat generation via shivering or non-shivering thermogenesis, increasing food intake *per se* generate heat, as digestion increases metabolic rate (35). It is interesting to note that in UCP-$1^{-/-}$ but not in WT mice, this increased energy intake led to an increase in adiposity, perhaps due to the inability of UCP-$1^{-/-}$ mice to convert their increased food energy into heat due to a lack of brown adipocyte-driven thermogenesis. In WT, cold stress increased lean mass, in line with greater food intake, and consistent with increased linear bone growth, and may relate in part to regulation of IGF-1 signalling with cold exposure (36). The decreased bone mass of cold-exposed UCP-$1^{-/-}$ mice was associated with reduced hypothalamic expression of NPY, and this change could conceivably contribute to changes in bone mass in the KO mice, via changes in SNS activity for example, which is regulated by NPY.

In recent years it has become increasingly apparent that the interaction between skeletal and energy homeostasis is vastly more complex than was appreciated, with both systems releasing factors that can concurrently affect either system (37). In cold stress conditions and when compared to WT values, UCP-1$^{-/-}$ mice display reductions in both cancellous and cortical bone parameters, likely via modulation of osteoblast activity as evidenced by reductions in bone formation with no differences in body weight or bone resorption. The process by which this regulation occurs is yet to be completely determined. A recent publication reported the presence of UCP-1 protein in bone marrow using immunohistochemistry in a mouse model with induced overexpression of brown adipocytes (11). Therefore, it is possible that UCP-1 may exert a local effect upon bone metabolism. However, using immunohistochemistry we were unable to conclusively demonstrate the presence of UCP-1 on the bone surface or within the bone matrix of cold stressed WT mice. It should be noted that if UCP-1 were to be present on the bone surface or within the bone marrow, it would most likely be present in very low abundance in WT animals, and thus, perhaps at levels below the detectable sensitivities of our current immunohistochemical methodologies.

Given the apparent lack of local UCP-1 protein in bone, UCP-1 is likely to influence bone mass via an indirect pathway, and the downstream effects of central regulators remains a plausible possibility (Fig. 6). The hypothalamus is the most critical mediator of energy homeostasis and is also involved in maintenance of body temperature and bone mass. NPY, a highly abundant neurotransmitter, is known to regulate bone formation via Y1 and Y2 receptor signalling (38-42). NPY also has known roles in modulating thermogenesis (12). For instance, a recent study showed that NPY signalling in the arcuate nucleus of the hypothalamus inhibits sympathetic activity and subsequent BAT-mediated thermogenesis through Y1 receptor signalling (32). Specifically, arcuate NPY signals within the

paraventricular nucleus (PVN) to decrease local expression of tyrosine hydroxylase (TH), a modulator of sympathetic outflow, thus reducing activity of the sympathetic nervous system. In addition to this, it has been recently shown that NPY plays a pivotal role in protecting bone mass during chronic stress, such as cold stress (34). Using a mouse model in which NPY is only expressed in the sympathetic nervous system, we showed that stress-induced increases in NPY act via Y2 receptors to inhibit TH neurons in the PVN. This in turn down-regulates noradrenaline release, subsequently preserving osteoblast activity, since noradrenaline inhibits osteoblasts (43). Thus, NPY exerts a protective role against sympathetically-driven bone loss resulting from chronic exposure to cold stress. These recent studies and the current findings from UCP-1$^{-/-}$ mice suggest an integrated system mediating the BAT protection of bone mass during chronic cold exposure (Fig. 6). In this system, UCP-1 responses in BAT are a result of cold exposure triggering elevations in arcuate NPY expression, which in turn stimulate food intake, increasing digestion-induced thermogenesis and supply of substrate for BAT-induced thermogenesis. In addition, arcuate NPY also inhibits TH expression and consequently sympathetic tone (4, 41). This reduction in SNS activity is permissive for greater bone formation (43) and greater BAT activity (12). Moreover, the reduction in arcuate NPY evident in UCP-1$^{-/-}$ mice would indicate an increase in sympathetic tone, which in turn increases noradrenaline release, and dis-inhibition of NPY's protective effects on osteoblast activity, resulting in bone loss. While this central pathway is consistent with our current data, it may not be the sole pathway involved. IGF-1 signalling has been strongly implicated in BAT/bone signalling (36). Interestingly, recent data has linked several bone-active molecules to BAT activation, including PTHrP (44) and IL-6 (45). Thus it appears that the integration of BAT with skeletal metabolism displays a level of complexity akin to the integration of WAT with bone (38,40).

As expected, energy expenditure and physical activity were not different at thermo-neutrality between WT animals and those deficient in UCP-1. Interestingly, these parameters also remained unchanged when the UCP-$1^{-/-}$ mice were tested under conditions of mild cold stress, apart from a modest reduction in physical activity. However, energy expenditure, representative of heat generation, was noticeably increased during cold stress in both genotypes when compared to counterparts housed under thermo-neutral conditions, similar to findings of other studies (29). These marked increases demonstrate the extent to which mice must accommodate to maintain core temperature under standard housing conditions (typically 20^{o}C to 24^{o}C in published studies), and the systemic effects that result from this mild thermal stress. This result was not due to corresponding increases in physical activity. However, it has been consistently reported that increased adiposity, such as that seen in our UCP-$1^{-/-}$ cold-stressed mice compared to those in thermo-neutral conditions, is associated with increased thermogenesis and accompanied by elevated resting metabolic heat production (46-48). Additionally, it should be noted that during cold stress, a switch to fat oxidation in both WT and UCP-1 knockout mice was seen, as evidenced by a reduction in respiratory exchange ratio. Even though no differences in fat oxidation levels were detected between UCP-$1^{-/-}$ and WT mice, this switch to fat oxidation indicates that UCP-1 ablation still allows these mice to successfully employ other forms of thermogenesis that are utilised by WT mice. Given this, and that UCP-$1^{-/-}$ mice undergoing gradual reductions in ambient temperature develop cold tolerance (49, 50), it is likely that animals deficient in UCP-1 are able to utilise alternative mechanisms besides BAT-dependent thermogenesis to generate heat to maintain thermoregulation, to an extent that is comparable to that of WT mice with intact UCP-1 (29).

Taken together, the results from this study suggest that UCP-1-dependent thermogenesis initiated during cold stress plays a role to promote bone mass, and corroborates the link

between sympathetically driven BAT function and bone metabolism. The discoveries in this work provide possible avenues for the treatment of metabolic diseases, as well as bone loss diseases such as osteoporosis.

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Figure Legends

Figure 1. UCP-1 gene inactivation

A.) A cytosine (C) to adenosine (A) point mutation generates an alternative splice-acceptor side within exon V of the *Ucp1* gene that then leads to the deletion of 13 amino acids. B.) Western Blot analysis revealed that UCP-1 protein was absent in the brown adipose tissue (BAT) of germline UCP-1deficient mice (UCP-$1^{-/-}$), but present in wildtype littermate controls (UCP-$1^{+/+}$) and dose-dependently reduced in BAT of mice heterozygous for UCP-1 (UCP-$1^{+/}$). All tissue samples were also blotted for the housekeeping marker, glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

Figure 2. Protective role of UCP-1 in bone during cold exposure

Analysis of whole body DXA scan of (A) bone mineral density and (B) bone mineral content of 16 week old wild-type (WT) and knockout (KO) mice under thermo-neutral (TN) and cold stress (CS) conditions. (C) Femur lengths in UCP-$1^{-/-}$ and WT mice. Micro computed tomography (micro-CT) analysis of (D) cancellous bone volume (BV/TV), (E) trabecular thickness and (F) trabecular number. (G) Representative 3-dimensional reconstructions of cancellous bone of the distal femoral metaphysis isolated from WT and UCP-$1^{-/-}$ mice.

Cortical parameters evaluated by micro-cT include: (H) cortical periosteal and (I) endosteal perimeters, (J) cortical bone volume (BV) and (K) cortical thickness in UCP-$1^{-/-}$ and WT mice. (L) Representative picture of mid-femoral cortical bone cross sections of UCP-$1^{-/-}$ and WT mice. Abbreviations: BV, bone volume; TV, tissue volume; WB, whole body. Plotted values are means ± SEM of more than 5 mice per group. *P<0.05, **P<0.01 or ***P<0.001 as compared to WT of same ambient temperature condition, or as indicated.

Figure 3. UCP-1 mediates its anabolic effects through osteoblast action

(A) Bone formation rate, (B) mineralising surface, (C) mineral apposition rate (MAR), (D) osteoclast number and (E) osteoclast surface of trabecular femoral bone was assessed using histomorphometry. Abbreviations: CS, cold stress; TN, thermo-neutral; Plotted values are means ± SEM of more than 5 mice per group. *P<0.05, **P<0.01 or ***P<0.001 as compared to WT of same ambient temperature condition, or as indicated.

Figure 4. UCP-1 regulates body composition during cold stress.

Body weight curve of UCP-$1^{-/-}$ and WT mice recorded from the age of 7 to 17 weeks at (A) thermo-neutral and (B) cold stress environments. (C) Final body weight of mice at cull. (D) Lean mass measured using dual energy x-ray absorptiometry in UCP-$1^{-/-}$ and WT mice at 15-16 weeks of age. (E) Accumulated 24-hour fasting-induced and (F) spontaneous food intake in UCP-$1^{-/-}$ and WT mice. (G) Fat mass measured using dual energy x-ray absorptiometry in UCP-$1^{-/-}$ and WT mice at 15-16 weeks of age. (H) Weight of dissected brown adipose tissue (BAT) depots of UCP-$1^{-/-}$ and WT mice. 24-hour time course and phase averages of energy expenditure (I,J), respiratory exchange ratio (K,L) and physical activity (M,N) in UCP-$1^{-/-}$ and WT mice at thermo-neutrality and mild cold stress conditions. Open and filled horizontal bars indicate light and dark phases, respectively. Abbreviations: CS, cold stress; TN, thermo-neutral; Plotted values are means ± SEM of 5-7 mice per group. *P<0.05, **P<0.01 or ***P<0.001 as compared to WT of same ambient temperature condition, or as indicated.

Figure 5. Lack of functional UCP-1 leads to decreased NPY levels

Bright field photomicrographs of coronal brain sections from cold-stressed WT and UCP-$1^{-/-}$ mice showing (A) NPY and (C) POMC mRNA expression determined by *in situ* hybridisation. Quantification of relative optical density of radioactive labelled sections after *in*

situ hybridisation for (B) NPY and (D) POMC mRNA. Abbreviations: NPY, neuropeptide Y; POMC, proopiomelanocortin. Plotted values are means ± SEM of 10-11 standard chow-fed samples per group. ***P<0.001 as compared to WT, or as indicated.

Figure 6. Cold stress regulates both BAT and bone mass through a NPY/TH circuit. A). Cold stress acting upon BAT induces UCP-1 activity, which subsequently stimulates NPY expression within the arcuate nucleus. B). Arcuate NPY signals through Y2 receptors to stimulate Y1 receptors within the PVN. C). During chronic cold stress, NPY signalling in the PVN inhibits Tyrosine hydroxylase (TH) expression in the PVN. D). Reduced central TH via hind-brain circuits reduces circulating noradrenaline and sympathetic nervous system (SNS) activity. E). Reduced SNS activity stimulates bone formation and increases BAT thermogenesis.

Figure 1
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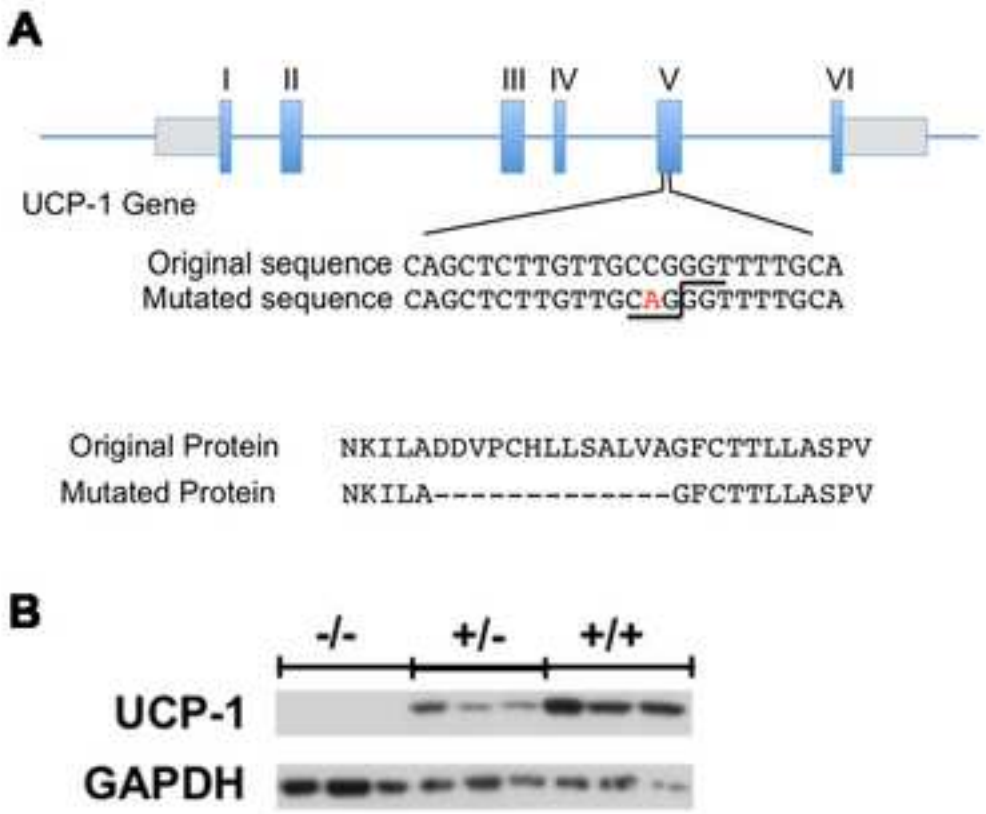

Figure 1

Figure 2

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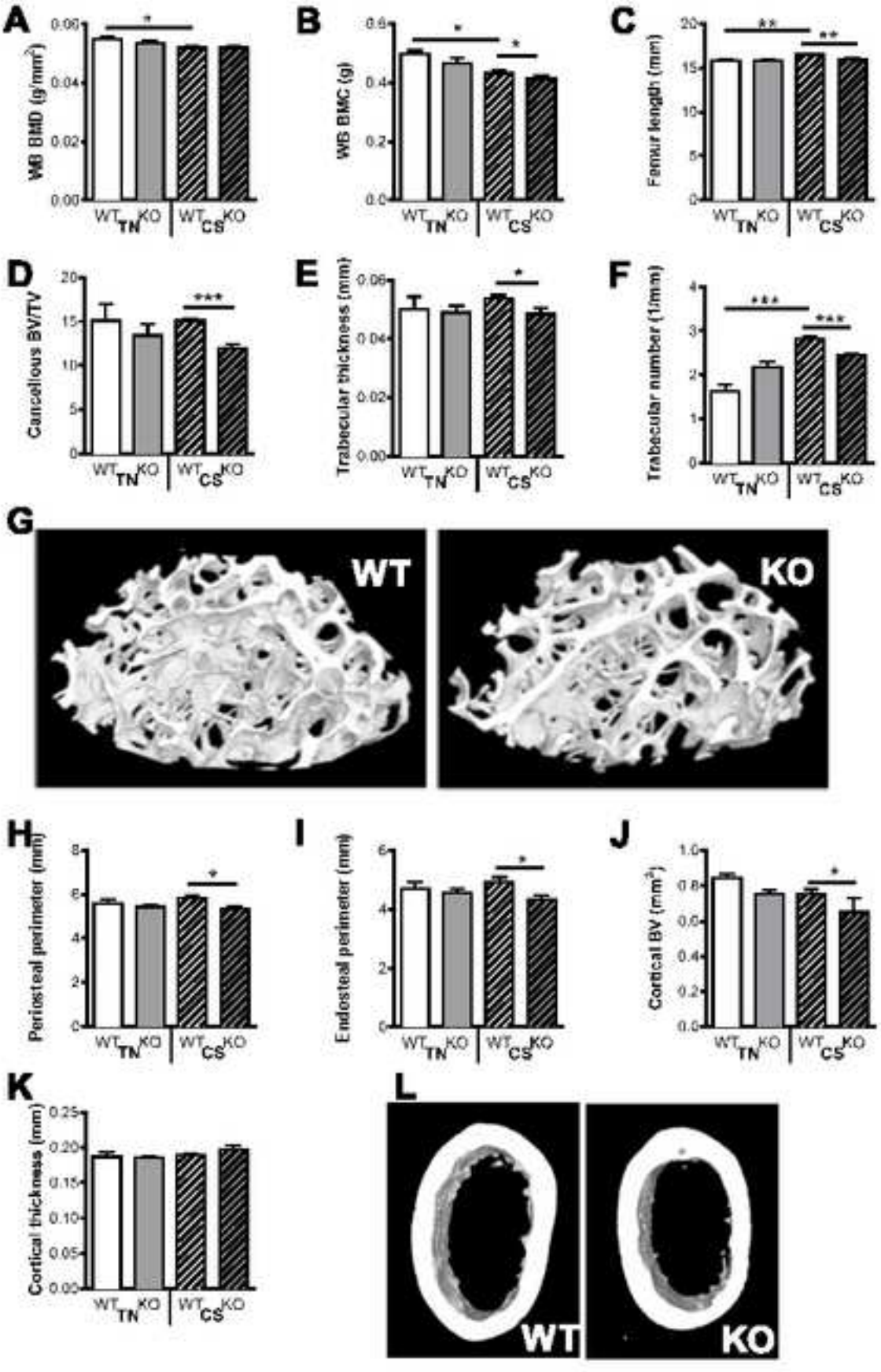

Figure 2

Figure 3

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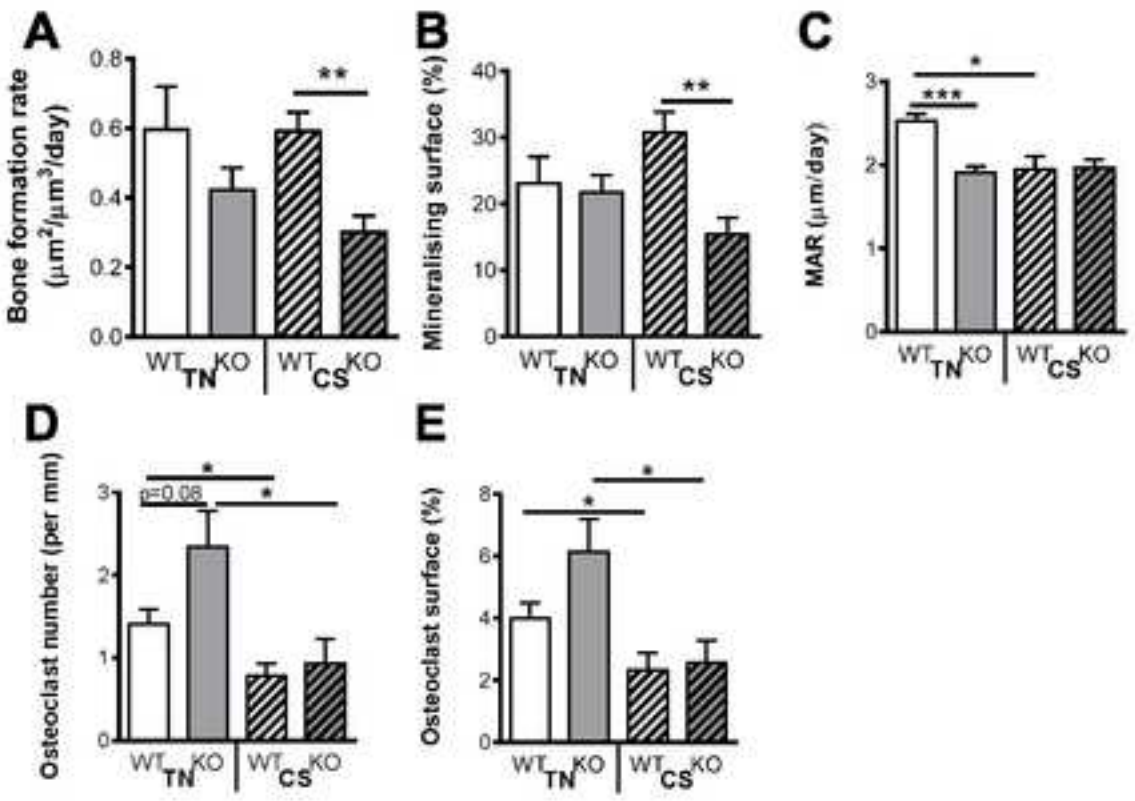

Figure 3

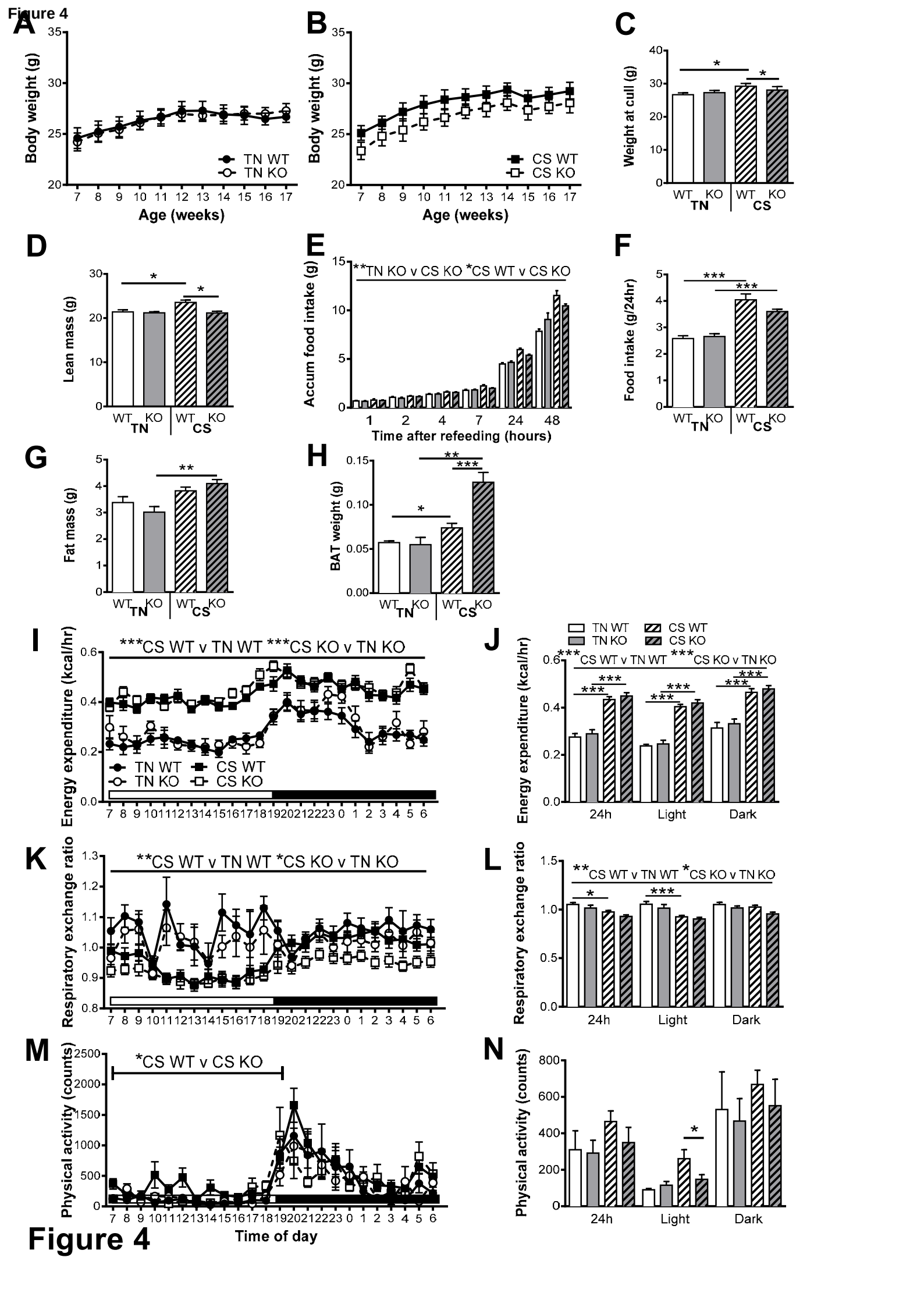
Figure 4
A
Body weight (g)
Age (weeks)
TN WT
TN KO
B
Body weight (g)
Age (weeks)
CS WT
CS KO
C
Weight at cull (g)
WT KO WT KO
TN CS
D
Lean mass (g)
WT KO WT KO
TN CS
E
**TN KO v CS KO *CS WT v CS KO
Accum food intake (g)
Time after refeeding (hours)
F
Food intake (g/24hr)
WT KO WT KO
TN CS
G
Fat mass (g)
WT KO WT KO
TN CS
H
BAT weight (g)
WT KO WT KO
TN CS
I
***CS WT v TN WT ***CS KO v TN KO
Energy expenditure (kcal/hr)
TN WT
TN KO
CS WT
CS KO
J
TN WT
TN KO
CS WT
CS KO
***CS WT v TN WT ***CS KO v TN KO
Energy expenditure (kcal/hr)
24h
Light
Dark
K
**CS WT v TN WT *CS KO v TN KO
Respiratory exchange ratio
L
**CS WT v TN WT *CS KO v TN KO
Respiratory exchange ratio
24h
Light
Dark
M
*CS WT v CS KO
Physical activity (counts)
Time of day
N
Physical activity (counts)
24h
Light
Dark

Figure 4

Figure 5

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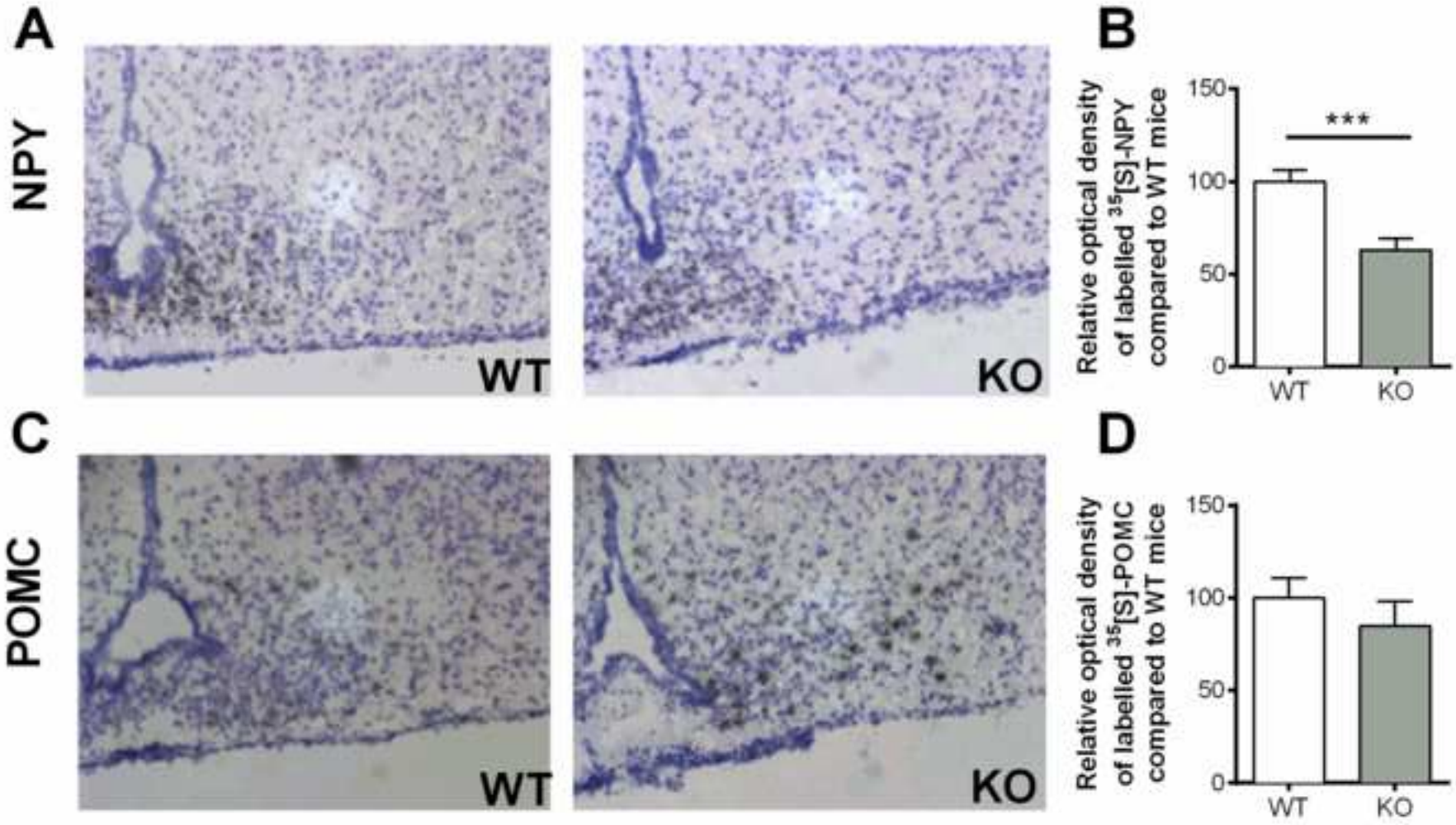

Figure 5

Figure 6
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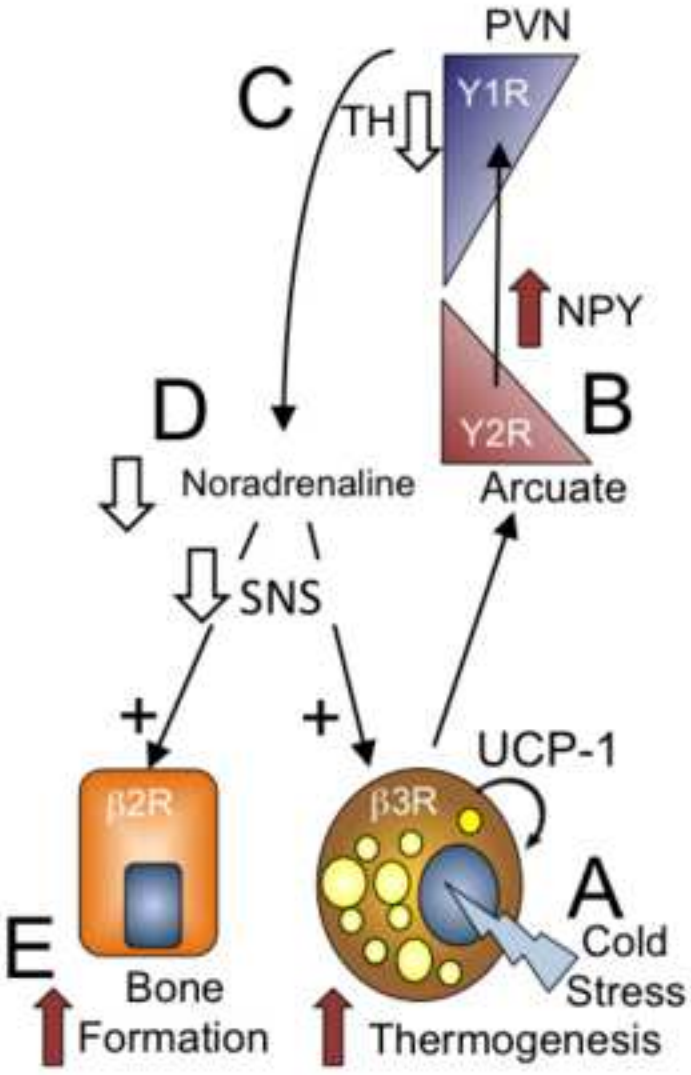

Figure 6

Supplementary Material

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