Adipocyte HIF2α functions as a thermostat via PKA Cα regulation in beige adipocytes

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Thermogenic adipocytes generate heat to maintain body temperature against hypothermia in response to cold. Although tight regulation of thermogenesis is required to prevent energy sources depletion, the molecular details that tune thermogenesis are not thoroughly understood. Here, we demonstrate that adipocyte hypoxia-inducible factor α (HIFα) plays a key role in calibrating thermogenic function upon cold and re-warming. In beige adipocytes, HIFα attenuates protein kinase A (PKA) activity, leading to suppression of thermogenic activity. Mechanistically, HIF2α suppresses PKA activity by inducing miR-3085-3p expression to downregulate PKA catalytic subunit α (PKA Cα). Ablation of adipocyte HIF2α stimulates retention of beige adipocytes, accompanied by increased PKA Cα during re-warming after cold stimuli. Moreover, administration of miR-3085-3p promotes beige-to-white transition via downregulation of PKA Cα and mitochondrial abundance in adipocyte HIF2α deficient mice. Collectively, these findings suggest that HIF2α-dependent PKA regulation plays an important role as a thermostat through dynamic remodeling of beige adipocytes.
Adipose tissue is a central organ in the regulation of energy homeostasis. In mammals, adipose tissues are largely divided into white adipose tissue (WAT) and brown adipose tissue (BAT) based on their morphology and functional characteristics. While WAT is responsible for lipid storage and endocrine functions, BAT is specialized in the maintenance of body temperature by non-shivering thermogenesis to protect the body from hypothermia. Brown adipocytes in BAT contain multilocular lipid droplets (LDs) and numerous mitochondria, which are suitable for efficient heat generation by dissipating proton gradient via uncoupling protein 1 (UCP1). Recently, it has been reported that WAT is able to possess brown-like adipocytes, termed beige or brite adipocytes. As beige adipocytes exhibit morphological and functional plasticity upon stimuli, they are considered to be inducible types of thermogenic adipocytes.

Upon demands for long-term heat production such as chronic cold exposure or sustained β-adrenergic activation, enhanced beige adipocytes stimulate the thermogenic programs and increase UCP1 expression. In contrast, beige adipocytes lose their thermogenic characteristics and return to white adipocytes accompanied by unilocular LD and decreased mitochondrial abundance upon re-warming or alteration of external stimuli.

Upon sympathetic nerve activation during cold acclimation, activation of β-adrenergic receptor-dependent adenylyl cyclase produces cyclic AMP (cAMP), which in turn stimulates protein kinase A (PKA) signaling. In thermogenic adipocytes, PKA signaling promotes lipolysis through phosphorylation of lipases and related proteins to produce free fatty acids and glycerol. The fatty acids generated from PGA-induced lipolysis are not only used as a fuel but also directly activate UCP1 in mitochondria. Besides, PKA governs the activation of thermogenic programming including thermogenic gene expression and mitochondrial biogenesis. Although PKA activation largely depends on cellular cAMP levels, emerging evidence has proposed that the stoichiometric balance between PKA regulatory and catalytic subunits is also a key factor in modulating PKA activity, independent of cAMP levels.

Thermogenic adipocytes utilize large amounts of energy source and oxygen to fulfill their needs for sustaining their activities under cold conditions. As increased oxygen consumption leads to an oxygen deficit in adipose tissues, thermogenic adipose tissues become hypoxic upon cold exposure. Under oxygen deprivation, hypoxia-inducible factor α (HIF1α) escapes from prolyl hydroxylase (PHD)- and von Hippel-Lindau protein (VHL)-dependent proosomal degradation and modulates various cellular responses, including glucose and lipid metabolism. HIF1α has two major α subunits, HIF1α and HIF2α, which regulate common and specific target genes. During the catabolic process, HIF1α affects lipid metabolism by modulating lipolysis and mitochondrial fatty acid oxidation. In adipocytes, HIF1α attenuates PKA-induced lipolysis to prevent futile lipid breakdown. Moreover, HIF1α has been reported to suppress mitochondrial activities including β-oxidation and oxidative phosphorylation (OXPHOS) in hypoxic conditions and VHL deficient models. Recently, it has been reported that fat mass and adipocyte size are altered in adipocyte-specific HIF1α or HIF2α knockout mice upon high fat diet. However, the physiological roles of HIF1α in cold-induced hypoxia and, particularly, the key isofrom of HIF1α in thermogenic adipocytes have not been thoroughly studied.

In this study, we aimed to unravel the roles of HIF1α in thermogenic adipocytes. We comprehensively analyzed the thermoregulatory roles of HIF1α using various adipocyte-specific HIF1α knockout mouse models and pharmacological HIF1α modulation. In addition, bioinformatic approaches were adopted to identify the key mediator(s) of HIF2α-dependent pathways for PKA-dependent thermogenic regulation. Here, we elucidate the physiological roles of HIF2α in the activation of beige adipocytes and rewiring of beige-to-white adipocyte transition. Collectively, our data suggest that HIF2α is an important molecular brake that fine-tunes thermogenic activity in beige adipocytes to maintain whole-body energy homeostasis.

**Results**

Cold acclimation increases HIF1α expression in thermogenic adipocytes. Given that HIF1α could be induced in thermogenic adipose tissues, we evaluated the extent of hypoxia in several fat depots at different temperatures. Compared to thermoneutral (TN) conditions, the overall intensity of pimonidazole staining was markedly increased in inguinal WAT (iWAT) and BAT during cold exposure (Fig. 1a, b), but not in epididymal WAT (eWAT) (Supplementary Fig. 1a). Simultaneously, the levels of HIF1α and HIF2α proteins gradually increased during cold exposure in iWAT and BAT (Fig. 1c, d), and HIF2α proteins were slightly induced in eWAT upon cold (Supplementary Fig. 1b). Moreover, in iWAT, cold-induced HIF1α and HIF2α protein levels were augmented in adipocytes with multilocular LDs (Fig. 1e, f). To examine whether HIF1α might be also promoted by β-adrenergic stimuli, we measured the levels of HIF1α and HIF2α protein upon treatment with a β3-adrenergic agonist, CL-316,243 (CI). In iWAT and BAT, chronic administration of CL elevated HIF1α and HIF2α as well as UCP1 (Fig. 1g, h). These data indicate that HIF1α protein expression would be augmented in thermogenic adipose tissues, accompanied by hypoxia.

HIF1α regulates thermogenic programming in adipocytes upon cold stimuli. To investigate the roles of HIF1α in thermogenic adipocytes, we generated adipocyte-specific HIF1α, HIF2α knockout and HIF1/2α double knockout (HIF1α AKO, HIF2α AKO, and HIF1/2α DKO, respectively) mice using Adiponectin-Cre. As expected, mRNA levels of the Hif1a and/or Hif2a genes were downregulated in adipose tissue (Supplementary Fig. 2a). Upon cold exposure, HIF1α AKO, HIF2α AKO, and HIF1/2α AKO mice were cold tolerant and exhibited higher body temperature than wild-type (WT) mice (Fig. 2a, b). Next, we subjected the thermogenic tissues to histological analyses. While BAT did not show significant differences in WT and adipocyte HIF1α deficient mice upon TN or cold (Supplementary Fig. 2b–d), multilocular LDs containing adipocytes were evidently elevated in iWAT of HIF1αAKO, HIF2α AKO, and HIF1/2α DKO mice upon cold (Fig. 2c–e). Thermogenic gene expression was significantly upregulated in iWAT of HIF2α AKO and HIF1/2α DKO mice and slightly increased in iWAT HIF1α AKO mice (Fig. 2f), implying that HIF2α might be a key player in the regulation of thermogenic programming. Consistently, the level of UCP1 protein was further promoted in cold exposed iWAT of HIF2α AKO mice than that of WT mice (Supplementary Fig. 2e, f). Accordingly, HIF1α deficient beige adipocytes potentiated thermogenic gene expression, while overexpression of HIF1α attenuated thermogenic gene expression (Supplementary Fig. 2g, h).

To explore whether HIF1α could modulate thermogenic function in classical brown adipocytes, we generated brown adipocyte-specific HIF1α and HIF2α knockout (HIF1α BKO and HIF2α BKO, respectively) mice using Ucp1-Cre. Similar to the pan-adipocyte HIF1α deletion mouse model, HIF1α BKO and HIF2α BKO mice were cold tolerant (Supplementary Fig. 3a, b). While the overall size of lipid droplets was relatively smaller in BAT of HIF1α BKO and HIF2α BKO than that of WT at 6 h of cold (Supplementary Fig. 3c), histological changes appeared to be comparable between the genotypes upon 3 days of cold (Supplementary Fig. 3d), implying that thermogenic function
might be maximized by 3 days of cold exposure. In addition, many thermogenic gene expressions were comparable in BAT and iWAT, except that Ucp1 and Elovl3 appeared to be upregulated in BAT of HIF2α BKO mice (Supplementary Fig. 3e, f).

In parallel, thermogenic properties were investigated by pharmacological interventions of HIFα with a general HIFα inhibitor (YC-1), HIF2α-specific inhibitor (PT2385), or HIFα activator (dimethyloxaloylglycine, DMOG) during cold acclimation (Supplementary Fig. 4a–f). Similar to the genetic ablation of HIFα, YC-1-treated WT mice were cold tolerant with increased thermogenic gene expression and beige adipocyte accumulation in iWAT upon cold (Fig. 2g–i). In addition, HIF2α-specific inhibitor PT2385-treated mice exhibited augmented thermogenic properties with elevated beige adipocytes in iWAT upon cold (Fig. 2j–l and Supplementary Fig. 4g). On the contrary, DMOG-administered WT mice were cold intolerant with attenuated thermogenic expression and beige adipocyte formation in iWAT upon cold (Fig. 2m–o). Therefore, these data suggest that adipocyte HIFα inhibition could stimulate thermogenic programming via beige adipocyte generation during cold exposure.

Loss of HIF2α in adipocytes increases energy expenditure upon β-adrenergic activation. As chronic β-adrenergic stimulation potentiates beige adipocyte formation in iWAT33, we examined the effects of chronic CL treatment on thermogenic activity in iWAT. As shown in Fig. 3a–c, iWAT of HIF2α AKO mice exhibited elevated thermogenic properties as well as increased UCP1 expression upon CL treatment. Similarly, CL boosted beige adipocyte generation and thermogenic gene expression in HIF1α AKO and HIF1/2α DKO iWAT (Supplementary Fig. 5a–d). To study whether increased thermogenic activity by adipocyte HIFα ablation might alter whole-body energy metabolism, metabolic rates were measured. As indicated in Fig. 3d–i and Supplementary Fig. 5e–g, the volume of oxygen consumption (VO2), the volume of carbon dioxide production (VCO2), and energy expenditure...
were increased in HIF2α KO and HIF1/2α DKO mice treated with CL. However, these parameters were comparable between WT and HIF1α KO mice treated with CL (Supplementary Fig. 5h–j), implying that adipocyte HIF2α deficiency would potentiate energy expenditure with β-adrenergic activation.

In beige adipocytes, HIF2α ablation potentiates PKA signaling via PKA Ca upregulation. To decipher the underlying mechanism(s) by which HIF2α modulates thermogenesis, transcriptome profiles of iWAT were scrutinized. Compared to WT iWAT, HIF2α KO iWAT showed numerous differentially
expressed genes (DEGs) (Supplementary Fig. 6a). Among the upregulated DEGs, “thermogenesis” and “mitochondrion” gene ontology signatures were ranked as the top biological pathways in cold exposed iWAT of HIF2α KO mice (Fig. 4a, b). To investigate the key mediator(s) influencing DEGs in thermogenesis and related pathways, in silico analyses were conducted. Network propagation (NP) scores of genes were calculated to measure their relevance to HIF2α (Supplementary Fig. 6b and Supplementary Data 1) and the centrality of genes was calculated to measure their importance in the network (Supplementary Fig. 6c). Among several candidates, it was of interest to note that Prkaca, which encodes PKA catalytic subunit α (PKA-Cα), was one of the highly ranked genes which could influence numerous catabolic pathways including PKA-related, TCA cycle, fatty acid β-oxidation, OXPHOS, and thermogenesis in iWAT of HIF2α AKO mice upon cold (Fig. 4c, d).

Next, we examined whether PKA-Cα expression and PKA signaling might be indeed altered by HIF2α. As shown in Fig. 4e–g, PKA-Cα was elevated in iWAT of HIF2α AKO mice upon cold or CL treatment. Also, the levels of phosphorylated PKA target proteins were further augmented in iWAT and beige adipocytes of HIF2α AKO mice than those of WT mice upon cold or β-adrenergic activation (Fig. 4h–j). Additionally, the levels of serum glycerol and glycerol released from beige adipocytes were enhanced in HIF2α deficient adipocytes upon cold or ISO treatment (Supplementary Fig. 6d and Fig. 4k). Nevertheless, there was no significant difference in the cAMP level between WT and HIF2α deficient beige adipocytes (Fig. 4l), implying that HIF2α could regulate PKA activity independent of cAMP. To ascertain whether PKA-Cα would be regulated by HIF2α in beige adipocytes, the levels of PKA-Cα expression were examined with HIF2α modulation. PKA-Cα protein was upregulated in HIF2α deficient beige adipocytes (Fig. 4m, n), whereas overexpression of HIF2α attenuated PKA-Cα expression (Fig. 4o, p). Similar to HIF2α AKO iWAT, HIF1α AKO and HIF1/2α DKO iWAT also exhibited enhanced PKA signaling and PKA-induced lipolysis (Supplementary Fig. 6e–g). However, Prkaca expression was not altered by HIF1α modulation (Fig. 4e and Supplementary Fig. 6h–k). Together, these results suggest that HIF2α suppresses PKA signaling in beige adipocytes, and PKA-Cα expression is selectively regulated by HIF2α.

In beige adipocytes, HIF2α ablation stimulates mitochondrial activity. The finding that “mitochondrion” was top-ranked in iWAT of HIF2α AKO mice upon cold (Fig. 4b) led us to test whether mitochondrial activity might be influenced by HIF2α. As shown in Fig. 5a–c, mtDNA and OXPHOS complexes were increased in HIF2α AKO iWAT upon cold. Similarly, chronic CL administration augmented mitochondrial quantity in HIF2α AKO iWAT (Fig. 5d–f). In contrast, in beige adipocytes, HIF2α overexpression attenuated mtDNA content, whereas HIF2α deficiency increased mtDNA content (Supplementary Fig. 7a, b). Similar to iWAT in HIF2α AKO, mitochondrial OXPHOS complexes were also upregulated in HIF1α AKO iWAT upon cold (Supplementary Fig. 7c, d). Next, to study whether the altered mitochondrial content in HIF2α AKO iWAT might affect mitochondrial activity, mitochondrial oxygen consumption rates (OCRs) were determined. OCRs in basal, UC1P1-dependent, and maximal states were repressed by overexpression of HIF2α (Fig. 5g). In contrast, HIF2α deficient beige adipocytes exhibited increased basal, UC1P1-dependent, and maximal respiration (Fig. 5h), with an increase in mitochondrial membrane potential (Supplementary Fig. 7e). Further, we found that enhanced mitochondrial respiration, as well as upregulated thermogenic gene expression in HIF2α deficient beige adipocytes, was impaired by PKA inhibitor H89 treatment or knockdown of Prkaca (Fig. 5i and Supplementary Fig. 7f). To elucidate in vivo roles of PKA-Cα in iWAT of HIF2α AKO mice, siPrkaca was delivered to iWAT (Fig. 5j). Suppression of PKA-Cα via siRNA downregulated UC1P1 expression, mitochondrial OXPHOS complexes, accompanied with attenuated beige adipocytes formation in iWAT of HIF2α AKO mice upon cold (Fig. 5k, l). These data propose that HIF2α deficiency could result in elevated mitochondrial content and mitochondrial activity in beige adipocytes.

Adipocyte HIF2α suppresses thermogenesis via miR-3085-3p-dependent PKA-Cα repression. To delineate how HIF2α modulates Prkaca, we assessed DEGs between WT and HIF2α AKO mice. Although several DEGs in HIF2α AKO iWAT were identified, it appeared that there were no candidate genes which could directly repress transcription of Prkaca (Supplementary Data 2). Since HIFα could suppress expression of certain genes via induction of miRNA34, we hypothesized that HIF2α-dependent miRNA expression might mediate the downregulation of Prkaca. Using in silico analysis, we identified miR-3085-3p as one of the highest-scoring miRNAs targeting the evolutionarily conserved region of 3′UTR of Prkaca. In a reporter assay, miR-3085-3p attenuated luciferase activity of WT Prkaca 3′UTR, but not that of the miR-3085-3p binding defective mutant of Prkaca 3′UTR (Fig. 6a, b). To evaluate whether HIF2α would be recruited to stimulate miR-3085-3p expression, ChIP-qPCR analysis was performed. As shown in Fig. 6c, d and Supplementary Fig. 8a, overexpressed HIF2α interacted with several hypoxia-response elements in the upstream region of miR-3085. Moreover, the level of miR-3085-3p was regulated in a HIF2α-dependent manner in
iWAT, BAT, and thermogenic adipocytes (Fig. 6e, f and Supplementary Fig. 8b–i). Consistent with these, HIF2α-specific inhibitor PT2385 decreased miR-3085-3p expression, accompanied by increased Prkaca expression in iWAT upon cold exposure (Fig. 6g, h). These data indicate that HIF2α could selectively activate miR-3085-3p expression in thermogenic adipocytes.

Next, we investigated whether miR-3085-3p might function as a downstream mediator of HIF2α in the regulation of PKA Cα and thermogenesis. Increased PKA Cα expression in HIF2α deficient beige adipocytes was potently attenuated by miR-3085-3p mimic, leading to repression of PKA signaling and thermogenic gene expression (Fig. 6i and Supplementary Fig. 8j). Further, miR-3085-3p mimic suppressed the effect of HIF2α deletion on elevated mitochondrial respiration (Fig. 6j). Conversely, miR-3085-3p inhibitor restored decreased PKA Cα expression, PKA signaling, thermogenic gene expression, and OCRs in HIF2α overexpressing beige adipocytes (Fig. 6k, l and Supplementary Fig. 8k), indicating that miR-3085-3p would be a key mediator of HIF2α-dependent thermogenic regulation. To further examine the in vivo effects of miR-3085-3p in iWAT of HIF2α AKO mice, miR-3085-3p mimic was administered into iWAT (Fig. 6m). As shown in Fig. 6n, o, miR-3085-3p mimic downregulated beige adipocyte generation and UCP1 expression in cold exposed HIF2α AKO iWAT. Similar to beige adipocytes, miR-3085-3p mimic attenuated PKA signaling and thermogenic gene in brown adipocytes (Supplementary Fig. 8l, m). Moreover, administration of miR-3085-3p mimic in BAT suppressed PKA...
Ca, UCP1, and thermogenic genes (Supplementary Fig. 8n–p). Together, these data suggest that adipocyte HIF2α could promote miR-3085-3p expression, eventually leading to suppression of PKA Ca-mediated thermogenic properties.

HIF2α confers whitening of beige adipocytes upon re-warming stimuli. It has been reported that beige adipocytes could regain key features of white adipocytes upon re-warming or removal of β-adrenergic activation.4,7 During re-warming, mitochondria in beige adipocytes undergo dynamic remodeling, including down-regulation of mitochondrial function–related gene expression and up-regulation of mitochondrial clearance.5,35 The findings that HIF2α could act as a negative regulator of thermogenesis prompted us to evaluate that HIF2α might be involved in the whitening process of beige adipocytes. As shown in Fig. 7a, HIF2α was sustainably expressed in iWAT upon re-warming. To
determine the roles of HIF2α during re-warming. WT and HIF2α AKO mice were placed in a cold environment for 2 weeks, at which time the number of beige adipocytes appeared to reach a maximum level and to be comparable between the genotypes (Fig. 7b, c), and were then exposed to TN for 1 week. Intriguingly, beige-to-white transition upon re-warming was delayed in iWAT of HIF2α AKO mice (Fig. 7c). As miR-3085-3p could mediate the thermoregulatory function of HIF2α, the level of miR-3085-3p was examined. As shown in Fig. 7d, e, miR-3085-3p was downregulated, concurrently with an increase of Prkaca upon re-warming. Although the expression of thermogenic genes in iWAT was attenuated upon re-warming (Fig. 7e), the levels of OXPHOS complexes, PKA Ca, and UCP1 in iWAT of HIF2α AKO mice were higher than those in WT mice (Fig. 7f, g), implying that increased PKA Ca in adipocyte HIF2α deletion might maintain mitochondrial abundance during re-warming.

Given that HIF2α-dependent mitochondrial regulation was mediated by miR-3085-3p in beige adipocytes (Fig. 6), mRNA levels of OXPHOS genes were examined. MiR-3085-3p mimic attenuated OXPHOS gene expression in HIF2α deficient beige adipocytes (Supplementary Fig. 9a), while miR-3085-3p inhibitor slightly but substantially rescued the expression of OXPHOS genes under HIF2α overexpression (Supplementary Fig. 9b). To assess the in vivo roles of miR-3085-3p in mitochondrial regulation, cold exposed mice were administered miR-3085-3p mimic at iWAT and subjected to re-warming (Fig. 7h). As indicated in Fig. 7i, the number of unilocular adipocytes in iWAT was increased by miR-3085-3p mimic. Further, the increase in OXPHOS complexes in iWAT of HIF2α AKO mice was suppressed by miR-3085-3p mimic, accompanied by downregulation of PKA Ca and UCP1 (Fig. 7j). Meanwhile, overall autophagy-related genes and proteins were comparable between the genotypes (Supplementary Fig. 9c, d), implying that general autophagy might not be involved in the HIF2α-dependent whitening of beige adipocytes. Therefore, these data suggest that adipocyte HIF2α could induce beige-to-white transition via miR-3085-3p-dependent mitochondrial regulation during re-warming.

Discussion
Thermogenesis has to be tightly fine-tuned to avoid the exhaustion of stored energy sources and cytotoxicity. Here, we demonstrated that cold-induced HIFα functions as a molecular brake on hyperactive thermogenesis in adipocytes. Mechanistically, HIF2α suppresses PKA Ca through miR-3085-3p expression, thereby mitigating thermogenic execution in beige adipocytes. These findings suggest that HIF2α would play a key regulatory role in the thermogenic programming of adipocytes via HIF2α-miR-3085-3p-PKA Ca axis (Fig. 8).

To maintain whole-body energy homeostasis, it is important to sense environmental alterations. Consistent with a previous report19, we found a cold-induced hypoxic environment in BAT and iWAT. HIFα protein expression was increased in the thermogenic adipose tissue upon cold or chronic CL treatment, probably, due to elevated UCP1-dependent oxygen consumption. In addition, recent studies have provided clues that HIFα could be induced in thermogenic adipose tissues independent of oxygen deprivation19,36. For example, increased mitochondrial ROS generation during OXPHOS could prevent PHD-dependent hydroxylation, leading to stabilization of HIFα in thermogenic adipocytes37. Also, accumulation of succinate in thermogenic adipose tissues might limit PHD activity upon cold38. Under various physiological and pathological conditions, including certain types of tumors, HIFα is activated by metabolic or microenvironmental alterations and relieves metabolic stress through modulating cellular pathways39–41. Thus, it is plausible to speculate that HIFα is a key sensor and responder for adaptation to the cellular metabolic status in thermogenic adipocytes.

While many studies have revealed various pro- and anti-thermogenic factors, their fine-tuning roles and underlying mechanisms are rather unclear. To prevent overheating which could be potentially triggered by hyperactive thermogenesis, the homeothermic function should be properly accomplished by sensing tissue-specific microenvironments and regulating cellular metabolic pathways to coordinate systemic homeostasis. In this study, we propose the roles of cold-induced HIF2α as a thermostat in adaptive thermogenesis. Several lines of evidence suggest that HIF2α would control thermogenic execution in beige adipocytes. First, HIF2α AKO mice exhibited cold-tolerant phenotypes with increased beige adipocytes upon cold exposure. Moreover, pharmacological modulation of HIFα and several adipocyte HIFα deficient mouse models including HIF1α AKO, HIF1/2α DKO, HIF1α BKO, and HIF2α BKO mice clearly showed that HIFα could suppress thermogenic function upon cold. Second, HIF2α attenuated PKA activity in beige adipocytes. Mechanistically, we elucidated that HIF2α suppressed PKA Cα expression via miR-3085-3p, resulting in downregulation of the PKA signaling cascade and thermogenic gene expression. Third, mitochondrial contents and activities were augmented in HIF2α deficient adipocytes, thereby increasing energy expenditure upon CL treatment. Conversely, HIF2α overexpression suppressed mitochondrial functions in beige adipocytes. Lastly, the retention of beige adipocytes was extended in HIF2α AKO iWAT during re-warming. In contrast, administration of miR-3085-3p promoted beige-to-white transition via PKA Ca suppression in iWAT of HIF2α AKO mice. Together, the present study proposes that HIFα acts as a key safeguard to maintain physiologically appropriate thermogenesis.

Homeothermic animals control thermogenic activity during seasonal changes. In rodents and humans, the expression of thermogenic genes and beige marker genes is activated in adipose
tissue during winter as compared to summer. Including Prkaca, the expression levels of PKA-related genes are also elevated during hibernation in ground squirrels. These findings raise the possibility that PKA subunit regulation might affect functional plasticity in beige adipocytes for adaptation to seasonal temperature changes. Here, we showed that PKA Cα would be a critical node for the maintenance of beige characteristics as well as the generation of beige adipocytes. In particular, increased PKA Cα in HIF2α deficient beige adipocytes promoted PKA activity and thermogenic function. In contrast, PKA Cα suppression via miR-3085-3p boosted the whitening of beige adipocytes. As cAMP is a factor to mediate immediate feedback...
loops, it appears that quantitative regulation of PKA subunits might be another effective way to sustain long-term thermogenic properties during chronic cold exposure. In this regard, it has been reported that chronic PKA activation via overexpression of Prkaca or deletion of Prkaca increases UCP1 and contributes to the induction of beige adipocytes in iWAT, independent of cAMP changes16,18. Thus, it is feasible to propose that PKA subunit regulation might be important for the modulation of thermogenic programming in adipocytes upon metabolic and environmental stimuli.

Enhanced mitochondrial function is one of the key features of beige adipocytes. PKA signaling not only induces mitochondrial biogenesis but also stimulates mitochondrial respiration via phosphorylation of mitochondrial proteins14,46. Given that increased OXPHOS complexes and mitochondrial respiration in HIF2α-deficient beige adipocytes were inhibited by miR-3085-3p, HIF2α-dependent PKA Ca suppression would be critical for mitochondrial regulation in beige adipocytes. Along with mitochondrial activation and biogenesis, PKA has been proposed to attenuate autophagy and mitophagy in beige adipocytes, leading to the preservation of mitochondrial contents35,47. Further, it has been reported that HIF1α induces mitophagy via Bnip3 and Bnip3l expression, and hypoxia activates Fundc1-mediated mitophagy38,49. However, the expression of general autophagy-related proteins and lysosomal genes did not differ between WT and HIF2α AKO iWAT upon re-warming. Although we cannot exclude the possibility that HIF2α might affect mitophagy and mitochondrial proteolytic pathways, our findings suggest that HIF2α would control the balance of mitochondrial functions, contributing to the regulation of beige adipocyte plasticity.

HIF1α and HIF2α share many target genes and participate in common cellular pathways. However, they also exert distinct roles and even opposing functions via their unique target gene expression. In this study, we found that HIF1α AKO and HIF2α AKO mice exhibited concordant phenotypes upon cold exposure. In accordance with our previous report25, both HIF1α and HIF2α suppressed PKA activity in beige adipocytes. In line with this, HIF1α overexpression in adipose tissue suppressed whole-body energy expenditure and oxygen consumption in BAT90. Furthermore, it has been reported that HIF1α inhibitor PX-478 and HIF2α inhibitors PT2385 and PT2399 prevent diet-induced obesity and liver steatosis, accompanied by an induction of thermogenic gene expression in adipose tissues31–32. Especially, intestinal HIF2α could provoke insulin resistance via induction of ceramide salvage pathway and regulate thermogenesis via lactate-dependent remodeling of gut microbiota32,54. Here, we found that HIF2α seemed to have more potent effects than HIF1α in the regulation of thermogenic gene expression and energy expenditure. Notably, inhibition of PKA Ca by miR-3085-3p was selective to HIF2α, as no such regulation was observed upon HIF1α modulation. Although further studies for the detailed roles of HIF1α and HIF2α remain to be elucidated, it is plausible to postulate that there may exist another HIF1α- and/or HIF2α-dependent thermogenic regulation besides PKA Ca inhibition.

In conclusion, the present study proposes that HIF2α-dependent PKA regulation exerts a crucial role in the delicate control of thermogenesis in beige adipocytes. It is likely that HIF2α coordinates the plasticity of beige adipocytes in order to strengthen adaptation to a temperature shift as well as fine-tune thermogenesis, to eventually avoid futile energy wastage. Given that thermogenic adipocytes play pivotal roles in whole-body energy expenditure, it seems that these findings may provide clues for the development of promising interventions to counteract obesity and related complications by promoting energy consumption.

Methods

Animals. All experiments with mice were approved by the Seoul National University Institutional Animal Care and Use Committee. HIF1α AKO, HIF2α AKO, and HIF2α/1α DKO mice were generated by crossing mice with Hif1afl/flloxox, Hif2afl/flloxox, and Hif1afl/flloxox/Hif2afl/flloxox mice (C57BL/6-Hif1atm1Mcs/J and C57BL/6-Hif2atm1Mcs/J). HIF1α BKO and HIF2α BKO mice were generated by crossing Ucp1-Cre mice with Hif1atm1Cre and Hif2atm1Cre mice. Male mice were housed in groups of 3–5 mice per cage at 22°C and 55% relative humidity conditions and maintained under 12-h/12-h light/dark cycles with free access to water and a normal chow diet (Ziegler Feed; DDL; 22.4% protein, 4.88% lipid of total calories). For TN and cold exposure experiments, 10–12-week-old male mice (1–2 mice per cage) were placed in an environmental cabinet (Environmental Cabinet, DDL Co.) at 30°C or 4–6°C, respectively. Rectal temperature was measured using a thermal probe (Testo925, Testo Inc.). Infrared thermography was conducted using an infrared camera (CX320, COX Co.). For in vivo PKA modulation, male mice received daily intraperitoneal injections of YC-1 (30 mg/kg) to inhibit PKA activation. For pharmacological inhibition of HIF2α, male mice were given PT2385 (10 mg/kg) orally twice a day from 1 day before cold exposure to 3 days after cold exposure. PT2385 was dissolved in a mixture of 10% ethanol, 40% PEG300, 5% Tween-80, and 45% saline. For beige adipocytes induction via β3-adrenergic signaling, CL-316,243 (0.5 mg/kg; C5367, Sigma) was intraperitoneally injected into male mice for 4 consecutive days. For siRNA or miRNA mimic delivery, 4 μg of siPrkaca, miR-3085-3p, or control mimic was directly injected into iWAT of male mice using in vivo-jetPEI™ (201-10 G, Polypus) according to the manufacturer’s protocol.

Cell culture and transfection. Stromal vascular fraction (SVF) was isolated from iWAT. For beige adipogenesis, SVF was grown to confluence in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 units/ml penicillin, and 100 μg/ml streptomycin. To induce beige adipocyte differentiation, cells were incubated with DMEM containing 10% FBS, 20 nM insulin, 1 mM 3-isobutyl-1-methylxanthine (IBMX), 0.5 mM 3-isobutyl-1-methylxanthine (IBMX), 2 μM rosiglitazone, 125 nM indomethacin, 1 nM flutamide, and 2 μM dexamethasone ( Dex), 0.5 μM 3-isobutyl-1-methylxanthine (IBMX), 2 μM rosiglitazone, 125 nM indomethacin, 1 nM 3-isobutyl-1-methylxanthine (IBMX), and 2 μM dexamethasone ( Dex) for 2 days. The culture medium was then replaced with DMEM containing 10% FBS, 20 nM insulin, and 1 nM T3 for 2 additional days. The culture medium
was changed every other day with DMEM containing 10% FBS. HEK293FT cells were grown in DMEM supplemented with 10% FBS, 100 units/mL penicillin, and 100 μg/mL streptomycin.

**RNA-sequencing analysis.** Raw sequence reads were trimmed and quality-controlled using TrimGalore (version 0.4.4). The trimmed reads were mapped to the mouse genome GRCm38/mm10 using the STAR aligner (version 2.6.1d)\textsuperscript{55}. Raw read counts per gene were computed using HTSeq-count (version 0.13.5)\textsuperscript{56}. Significant DEGs were identified using DESeq2 (version 1.30.0)\textsuperscript{57}. The significance thresholds were |log2FC| > 0.2 and \( P < 0.15 \) for cold HIF2α AKO samples versus cold WT samples. Gene ontology enrichment analysis of the upregulated DEGs in cold HIF2α AKO samples was conducted using EnrichR\textsuperscript{58}.

**Network analysis.** Network analysis was performed to prioritize candidate genes at the network level that would mediate thermogenesis and mitochondrial biogenesis in HIF2α AKO iWAT. A *Mus musculus* protein-protein interaction (PPI) network was constructed using STRING\textsuperscript{59}.
network from the STRING database (version 10.5) was used to construct a condition-specific DEG interaction network. Network nodes consisted of Hif2α with DEGs between WT and Hif2α AKO iWAT upon cold exposure, and network edges consisted of high-confidence STRING PPI edges (confidence score >0.55). Network propagation was used to rank genes based on their relevance to Hif2α in the network by the Walker module using Hif2α as a seed gene. For centrality analysis, betweenness centrality was used to rank genes based on centrality in the network topology using the NetworkX package (version 2.2). To analyze therogenic genes, Wikipathway gene annotation was used, and gene ontology annotation related to therogenic genes was assessed using mouse genome informatics. Network nodes were clustered using the Markov Cluster Algorithm in clusterMaker2 (version 1.3.1) in Cytoscape. The network was visualized using the Cytoscape software.

**Indirect calorimetry.** Indirect calorimetry was performed using PhenoMaster (TSE Systems) according to the manufacturer’s protocol. Twelve-week-old male mice were placed in a calorimetric chamber for 48 h prior to measurements of VO₂, VCO₂, and energy expenditure. To activate β3-adrenergic signaling, mice were intraperitoneally injected with CL-316,243 (0.5 mg/kg). The experiments were performed at the Korea Mouse Phenotyping Center (K MPC), SNU, Seoul, Korea.

**Cellular oxygen consumption assay.** The cellular OCR was analyzed using a Seahorse XF296 extracellular flux analyzer (Agilent) according to the manufacturer’s instructions. Differentiated beige adipocytes were incubated in assay medium (25 mM glucose, 1 mM sodium pyruvate, 2 mM l-glutamine, and 1% fatty acid-free BSA in Seahorse XF base medium at pH 7.4). To evaluate mitochondrial activity, the OCR was measured following treatment with 5 μM oligomycin (75371, Sigma), 1 μM ISO, 5 μM carbonyl cyanide-p-trifluoromethoxyphenylhydrazone (FCCP, C2920, Sigma), 5 μM antimycin A (A8874, Sigma), and 5 μM rotenone (R8875, Sigma). For PKA inhibition, cells were pre-treated with 50 μM H89 (B1427, Sigma) for 1 h. Cell numbers were determined through Hoechst staining and used to normalize the OCRs. Parameter values were calculated using previously reported equations. Basal respiration: value prior to the injection of oligomycin minus non-mitochondrial respiration. OCR-dependent respiration: maximal value after the injection of ISO minus minimal value after injection of oligomycin. Maximal respiration: maximal value after the injection of FCCP minus non-mitochondrial respiration. Non-mitochondrial respiration: minimal value after the injection of rotenone and antimycin A.

**Chromatin immunoprecipitation qPCR.** BA-Gs were crosslinked with 1% formaldehyde for 20 min and lysed with lysis buffer (1% SDS, 10 mM EDTA, 50 mM TRIS- pH 8.1, 100 mM NaCl, and protein inhibitor cocktail). The samples were mixed with a dilution buffer (0.01% SDS, 0.1% Triton X-100, 1.2 mM EDTA, 16.7 mM TRIS-HCl [pH 8.1], 167 mM NaCl, and protease inhibitor cocktail) and sonicated for 15 min. After being precleared with protein A agarose (17-0780-01, GE Healthcare) and salmon sperm DNA, the samples were immunoprecipitated with antibodies overnight. The immunoprecipitated samples were collected by adding protein A-sepharose beads and sequentially washed with low salt buffer (0.1% SDS, 1% Triton X-100, 1 mM NaCl, 0.1% protease inhibitor cocktail) and sodium saline, followed by washing with 1% SDS, 1% Triton X-100, 10 mM EDTA, 50 mM TRIS-HCl [pH 8.1], 167 mM NaCl, LiCl buffer (0.25 M LiCl, 1 mM NaF, 1 mM Na3VO4, and 1 mM NaH2PO4, pH 8.1), and lysis buffer (10 mM Tris, 1 mM EDTA). The supernatant was used for reverse transcription-qPCR. The qPCR was performed in triplicate using SYBR Green on a 7900HT Fast Real-Time PCR System (Applied Biosystems). The relative expression of each gene was calculated using the delta Ct method. The expression levels of GAPDH were used as an internal control.
Adenovirus infection. HIF1α and HIF2α adenoviruses were generously provided by Dr. Jang-Soo Chun (Gwangju Institute of Science and Technology, Gwangju, South Korea). Green fluorescent protein-containing adenovirus was used as a negative control (mock). Four days after differentiation, beige adipocytes or BACs were incubated with DMEM containing 10% FBS and adenovirus (at a multiplicity of infection of 500) for 16 h. The culture medium was replaced with fresh medium for 2 days.

**Prkaca 3′ UTR target miRNA prediction and validation.** TargetScan mouse 7.264 and miRDB Version 6.065 were used to predict miRNA candidates targeting Prkaca 3′ UTR. For luciferase assays, pGL3UC-Prkaca 3′ UTR WT or pGL3UC-Prkaca 3′ UTR Mut. construct was transfected with control or miR-3085-3p mimic into HEK293FT cells using Lipofectamine 3000 (L3000001, Thermo Fisher Scientific). After 24 h of transfection, cell lysates were analyzed for luciferase activity. A pCMV-β-galactosidase plasmid was used as an internal control for transfection.

**Fig. 7** During the re-warming process, adipocyte HIF2α deficiency fails to mitigate the mitochondrial activity. a Experimental scheme (top) and western blot analysis of HIFα and UCP1 in iWAT of upon cold exposure or re-warming (bottom). b Experimental scheme of re-warming. c Representative images of H&E staining of iWAT from WT and HIF2α AKO mice upon cold exposure or re-warming (cold 2 weeks + TN 1 week). Scale bars, 100 μm. d miR-3085-3p level of iWAT from WT (n = 6) and HIF2α AKO (n = 5) mice upon cold and re-warming (cold 2 week + TN 1 week). e, f mRNA levels in iWAT from WT (n = 6) and HIF2α AKO (n = 5) mice upon cold exposure and re-warming (cold 2 weeks + TN 1 week). g Western blot analysis of OXPHOS complexes, PKA Cα, and UCP1 in iWAT from WT and HIF2α AKO mice upon cold exposure and re-warming (cold 2 weeks + TN 1 week). h Experimental scheme of re-warming with con or miR-3085-3p mimic injection. i Representative images of H&E staining of iWAT from WT and HIF2α AKO mice upon re-warming (cold 2 weeks + TN 1 week) with con or miR-3085-3p mimic injection. Scale bars, 50 μm. j Western blot analysis of OXPHOS complexes, PKA Cα, and UCP1 in iWAT from WT and HIF2α AKO mice upon re-warming (cold 2 weeks + TN 1 week) with con or miR-3085-3p mimic injection. Data were expressed as the mean ± SEM by two-tailed unpaired Student t-tests in (d) or two-way ANOVA in (e, f) followed by Holm–Sidak’s multiple comparisons test.
Fig. 8 Proposed model. Upon cold exposure, an activated thermogenic program in beige adipocytes stimulates oxygen consumption, thereby resulting in the stabilization of HIFα. Among the HIFα isoforms, upregulated HIF2α fine-tunes thermogenic execution via miR-3085-3p-dependent Prkaca regulation. However, adipocyte HIF2α deficiency augments PKA signaling and potentiates thermogenic functions, leading to the retention of beige adipocytes. Our findings suggest that the HIF2α-miR-3085-3p-PKA Cα axis forms negative feedback for appropriate regulation of thermogenesis.

RT-qPCR. Total RNA was isolated from tissues or cells using TRIzol Reagent (RiboEx, GeneAll) and subjected to cDNA synthesis using the ReverTra Ace qPCR RT Kit (Toyobo). RT-qPCRs were run using SYBR Green Master Mix (DQ384-40h, Biofact). Target gene expression levels were normalized to cyclophilin gene expression levels. The primers used for RT-qPCR are listed in Supplementary Table 1. For miRNA extraction, Direct-zol™ RNA MiniPrep (Zymo Research) was used. The miRNA was reverse-transcribed using the MicroRNA Reverse Transcription Kit (4366596, Thermo Fisher Scientific) and TaqMan MicroRNA Assay (4440886, 4427975, Thermo Fisher Scientific). RT-qPCRs were run using TaqMan Master Mix (RT600S, Enzynomics). The miR-3085-3p level was normalized to that of snoRNA202.

Statistics and reproducibility. Data were presented as the mean ± standard error of the mean (SEM). In the figures, sample numbers and sizes are indicated by dots. In immunoblotting and DNA gel blotting, representative result from three independent experiments is shown. Representative images of H&E staining and immunohistochemistry were obtained from 4–5 replicates in each group. Comparisons between two groups were performed using a two-tailed unpaired Student t-test. Multiple comparisons were performed using one-way analysis of variance (ANOVA) or two-way ANOVA when two conditions were involved. Statistical analyses were conducted using GraphPad Prism 7. p < 0.05 was considered significant.
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Competing interests
The authors declare no competing interests.

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