



OPEN Nardilysin in adipocyte regulates insulin sensitivity via HIF1 α and PPAR γ

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Obesity is a critical global health issue and a major risk factor for type 2 diabetes mellitus (T2DM). However, molecular mechanism by which obesity leads to T2DM has not been fully elucidated. Here, we investigated the role of adipocyte nardilysin (NRDC) in insulin sensitivity using adipocyte-specific NRDC-knockout mice (Adipo-KO). Despite no difference in body weight, Adipo-KO demonstrated enhanced glucose tolerance and insulin sensitivity exclusively under high fat diet (HFD) conditions. Epididymal white adipose tissue (eWAT) in Adipo-KO showed the decreased crown-like structures and F4/80-positive cells. Consistently, insulin-stimulated Akt phosphorylation was elevated in the eWAT of HFD-fed Adipo-KO and 3T3-L1 adipocytes with NRDC knockdown. In addition, NRDC knockdown led to a decrease in cellular oxygen consumption rate and hypoxia-induced HIF1 α expression in 3T3-L1 adipocytes. The augmented Akt phosphorylation by NRDC knockdown was reversed by daprodustat, which stabilizes HIF1 α , suggesting that the effect of NRDC on insulin sensitivity is mediated by HIF1 α . Upstream analysis of the differentially expressed genes (DEGs) in NRDC knockdown 3T3-L1 adipocytes revealed that PPAR γ is enriched in the promoters of the DEGs. We also confirmed the complex formation of NRDC and PPAR γ . In conclusion, NRDC in adipocyte regulates insulin sensitivity through HIF1 α and PPAR γ .

Keywords Nardilysin (NRDC), Adipocyte, Insulin sensitivity, Oxygen consumption, HIF1 α , PPAR γ

Type 2 diabetes mellitus (T2DM) is a global health issue, primarily driven by obesity due to overeating and lack of exercise^{1,2}. Adipose tissue plays a central role in the causal relationship of obesity and T2DM, which, beyond serving as an energy storage depot, functions as an endocrine organ that secretes various adipokines. In individuals with obesity, excess adipose tissue undergoes pathological changes including hypertrophy, hypoxia, and macrophage and lymphocyte infiltration, leading to a state of chronic inflammation^{3,4}. This inflammatory state certainly contributes to adipose tissue dysfunction and insulin resistance^{5,6}, whereas detailed molecular mechanisms of the pathogenesis have not been fully clarified.

Numerous studies have explored how obesity leads to chronic inflammation and decreased insulin sensitivity in white adipose tissue, and one of the leading working hypotheses is that hypoxia in adipose tissue is responsible^{7–9}. The expansion of adipose tissue in obesity is often coupled with hypoxic conditions due to the limitations in local oxygen supply relative to tissue demand¹⁰. Intriguingly, recent studies have also shown that obese adipocytes exhibit increased cellular oxygen consumption, which cause the relative hypoxia¹¹. Hypoxia in adipose tissue triggers various cellular responses, including the upregulation of hypoxia-inducible factor 1 α (HIF1 α), which mediates metabolic adaptations and inflammatory responses in adipocytes^{12–15}. The transcription factor peroxisome proliferator-activated receptor gamma (PPAR γ) is another critical regulator in adipose tissue, playing a central role in adipocyte differentiation, lipid storage, and insulin sensitivity¹⁶. Importantly, PPAR γ agonists, such as thiazolidinediones, have been used in diabetes treatment to improve insulin sensitivity. There is some evidence to suggest that HIF1 α and PPAR γ may functionally interplay and influence adipose tissue metabolism and other diseases^{15,17,18}.

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Nardilysin, also known as N-arginine dibasic convertase (NRDC), is a metalloendopeptidase of the M16 family that plays a complex role in energy and glucose metabolism. Mice globally lacking NRDC (*Nrd1*^{-/-}) exhibited enhanced brown adipose tissue (BAT) thermogenesis and improved systemic insulin sensitivity despite of severe impairment in glucose-stimulated insulin secretion (GSIS)¹⁹. Mice with β -cell specific deletion of NRDC showed severe diabetic phenotype with marked decrease in GSIS, indicating that NRDC in β cells are indispensable for the maintenance of GSIS. However, how NRDC regulates insulin sensitivity has not been elucidated. To determine which tissue-expressed NRDC is important in regulating insulin sensitivity, we first analyzed adipocyte-specific deficient (Adipo-KO) mice. Under normal chow diet (NCD), Adipo-KO mice showed enhanced BAT thermogenesis and hyperthermia through two mechanisms. First, NRDC and PPAR γ co-activator-1 α (PGC-1 α) interact and co-localize at the UCP1 enhancer, where NRDC represses PGC-1 α activity²⁰. Second, NRDC negatively regulates the protein stability of UCP1 via the ubiquitin-proteasome pathway²¹.

In the present study, we show that Adipo-KO mice exhibit enhanced glucose tolerance and insulin sensitivity exclusively under HFD feeding. Interestingly, under HFD, Adipo-KO did not show hyperthermia due to BAT thermogenesis, but showed reduced chronic inflammation in the eWAT. Moreover, NRDC knockdown in 3T3-L1 adipocytes led to a decrease in cellular oxygen consumption rate and hypoxia-induced HIF1 α expression. We also confirmed the association of NRDC and PPAR γ , and that mRNA levels of several key targets of PPAR γ are elevated in Adipo-KO eWAT. These results demonstrate that NRDC in adipocyte regulates insulin sensitivity through functional interaction with HIF1 α and PPAR γ .

Results

Improvement in glucose tolerance and insulin sensitivity in adipocyte-specific NRDC knockout mice on a high-fat diet

To clarify the role of NRDC in adipocyte on glucose metabolism and insulin sensitivity, glucose and insulin tolerance tests were performed in adipocyte-specific NRDC knockout mice (Adipo-KO). Adipo-KO were generated by crossing Adipoq-Cre^{+/-} with *Nrd1*^{fllox/fllox} mice (Floxed) on a C57BL/6 background as previously described²¹, and Floxed mice without Adipoq-Cre expression were used as control mice. These mice were fed a normal chow diet (NCD) or a high-fat diet (HFD) from 6 weeks of age for 12 weeks. The general appearance and growth, including body weight, epididymal white adipose tissue (eWAT) and liver weight, remained similar in Floxed and Adipo-KO, either in NCD nor in HFD groups (Fig. 1A-C). In spite of no differences in glucose tolerance and insulin sensitivity after NCD between Floxed and Adipo-KO mice, Adipo-KO showed significantly improved glucose tolerance and insulin sensitivity after HFD for 12 weeks compared to Floxed mice (Fig. 1D-G). There were no significant differences in plasma insulin levels between Floxed and Adipo-KO mice during fasting or after glucose loading on NCD or HFD (Fig. 1H). Additionally, body temperature, VO₂, locomotor activity, and the respiratory exchange ratio (RER) did not significantly differ between Floxed and Adipo-KO mice after HFD (Supplementary Fig. 1A-D). These findings suggest that NRDC deletion in adipocytes enhances systemic glucose tolerance and insulin sensitivity specifically under HFD conditions.

Reduction of crown-like structures and F4/80-positive cells in adipo-KO on an HFD

To investigate the involvement of NRDC in HFD-induced chronic inflammation of eWAT, we performed H&E staining which revealed that Adipo-KO tissue showed significantly less inflammatory cell infiltration and crown-like structure (CLS) development compared to Floxed mice (Fig. 2AB). The size measurement of white adipocytes demonstrated that the composition of adipocytes in Adipo-KO WAT consisted of fewer small-sized cells and more large-sized cells compared to Floxed WAT (Fig. 2CD). This suggests a reduction in the number of adipocytes impacted by CLS. Consistently, F4/80 positive cells and the expression of F4/80 mRNA in eWAT, which are upregulated by HFD, were significantly decreased in the Adipo-KO group (Fig. 2EF). Residual expression of *Nrd1* in Adipo-KO eWAT likely reflects NRDC expression in non-adipocyte cell types, including stromal or inflammatory cells.

Enhanced insulin-induced phosphorylation of AKT in eWAT of Adipo-KO mice and NRDC-knockdown 3T3-L1 adipocytes

We further investigated insulin sensitivity in adipose tissues of Floxed and Adipo-KO mice following NCD or HFD feeding. As shown in Fig. 3A-C, insulin-induced phosphorylation of AKT was elevated in Adipo-KO compared to that of Floxed mice in both NCD and HFD group. While the global level of AKT phosphorylation was lower in HFD group, the difference between Floxed and Adipo-KO eWAT was more obvious in HFD group compared to NCD group (Fig. 3A-C). Next, we examined 3T3-L1 adipocytes with NRDC knockdown to investigate whether NRDC directly influences insulin signaling in a cell-autonomous manner. Insulin stimulation of the differentiated 3T3-L1 adipocytes with siRNA (siNRDC1 and 2) knockdown of NRDC showed higher levels of AKT phosphorylation than in cells treated with control siRNA (Fig. 3DE). These in vitro results suggest that NRDC plays a cell-autonomous role in modulating insulin signaling.

NRDC knockdown impacts cellular metabolism in 3T3-L1 adipocytes

NRDC knockdown did not visibly impact the differentiation of 3T3-L1 cells into mature adipocytes (Fig. 4A). However, despite similar cell densities, medium acidification occurred more slowly in NRDC-knockdown cells than in the control cells (Fig. 4B). These findings led us to propose that NRDC might affect cellular respiration in 3T3-L1 adipocytes. Subsequent analysis of the respiratory state of these adipocytes using an extracellular flux analyzer revealed that the oxygen consumption rate (OCR) was reduced in NRDC-knockdown-adipocytes at baseline and during maximal respiration following oligomycin and FCCP addition, compared to control adipocytes (Fig. 4C, D). Notably, the reduction in OCR by NRDC-knockdown was not clearly observed in

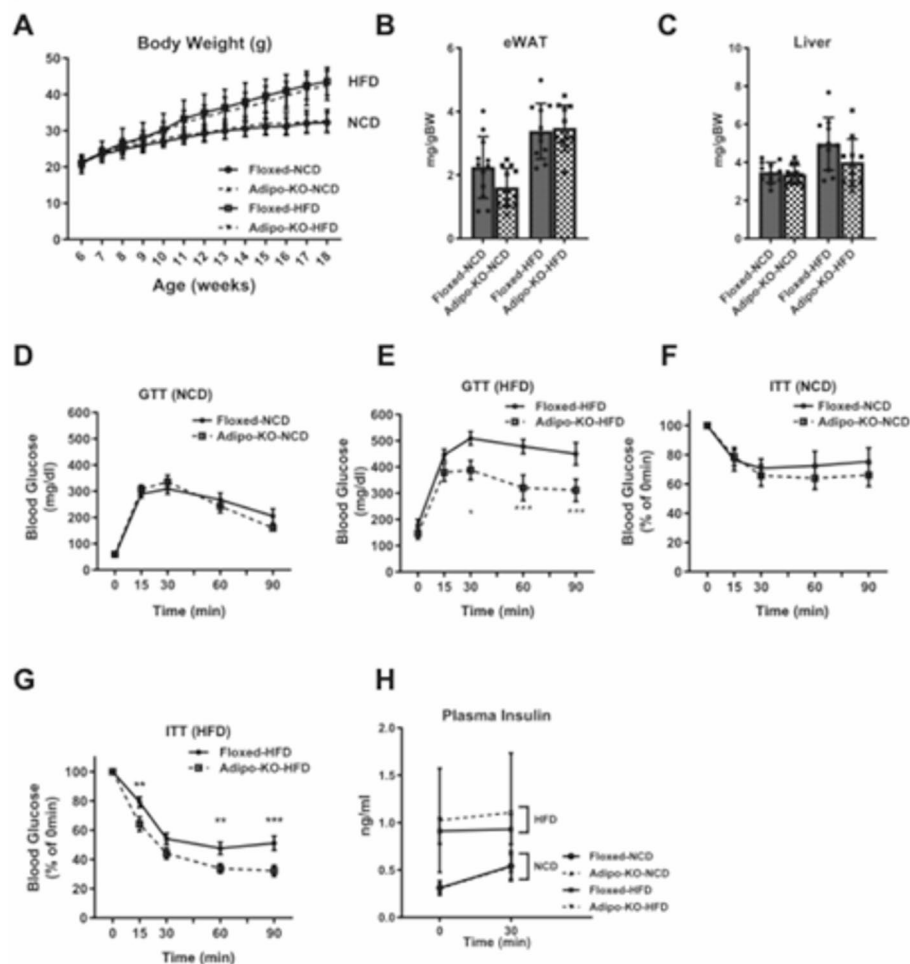


Fig. 1. Improvement in glucose tolerance and insulin sensitivity in adipocyte-specific NRDC knockout mice on a high-fat diet. **(A)** Body weight trend of Floxed and Adipo-KO mice on a normal chow diet (NCD) or high-fat diet (HFD) ($N=10-11$ /group). **(B,C)** Weights of epididymal WAT **(B)** and liver **(C)** normalized to body weight for Floxed and Adipo-KO mice (at 18 weeks of age, after 12 weeks of NCD or HFD feeding) ($N=11-12$ /group) **(D,E)** Glucose tolerance test (GTT) results for mice on NCD ($N=6/8$) **(D)** and HFD ($N=9/7$) **(E)**. * $P<0.05$, ** $P<0.001$ **(F,G)** Insulin tolerance test (ITT) results for mice on NCD ($N=9/11$) **(F)** and HFD ($N=15/15$) **(G)**. ** $P<0.01$, *** $P<0.001$ **(H)** Plasma insulin levels of Floxed and Adipo-KO mice before and 30 min after glucose loading on NCD ($N=7/6$) and HFD ($N=5/7$).

undifferentiated 3T3-L1 preadipocytes (Fig. 4E, F), suggesting that the effect of NRDC on oxygen consumption is dependent on the differentiation state of adipocytes.

NRDC in adipocytes regulates insulin signaling via HIF1 α

NRDC knockdown reduced oxygen consumption in 3T3-L1 adipocytes, which likely alleviates relative intracellular hypoxia and thereby suppresses HIF1 α stabilization under hypoxic conditions. Under this hypothesis, we assessed whether NRDC deletion could affect HIF1 α expression induced by low oxygen environment (3% O_2 condition). As expected, only control adipocytes exhibited an induction of HIF1 α , whereas NRDC-knockdown adipocytes displayed no significant induction of HIF1 α expression (Figure 5A). We also examined 3T3-L1 preadipocytes, which did not show HIF1 α induction both in control and NRDC-knockdown cells (Figure 5A). This led us to hypothesize that mature 3T3-L1 adipocytes might experience relative hypoxia compared to preadipocytes and that NRDC knockdown could protect adipocytes from hypoxia. NRDC deficiency attenuates HIF1 α induction and simultaneously enhances insulin sensitivity. To assess whether NRDC deficiency enhances insulin sensitivity via attenuation of HIF1 α induction, we examined the effect of daprostustat, an inhibitor of HIF1 α degradation. Daprostustat treatment markedly upregulated the expression of HIF1 α , which reversed the increased phosphorylation of AKT by NRDC knockdown, suggesting that the effect of NRDC is mediated by HIF1 α (Fig. 5BC).

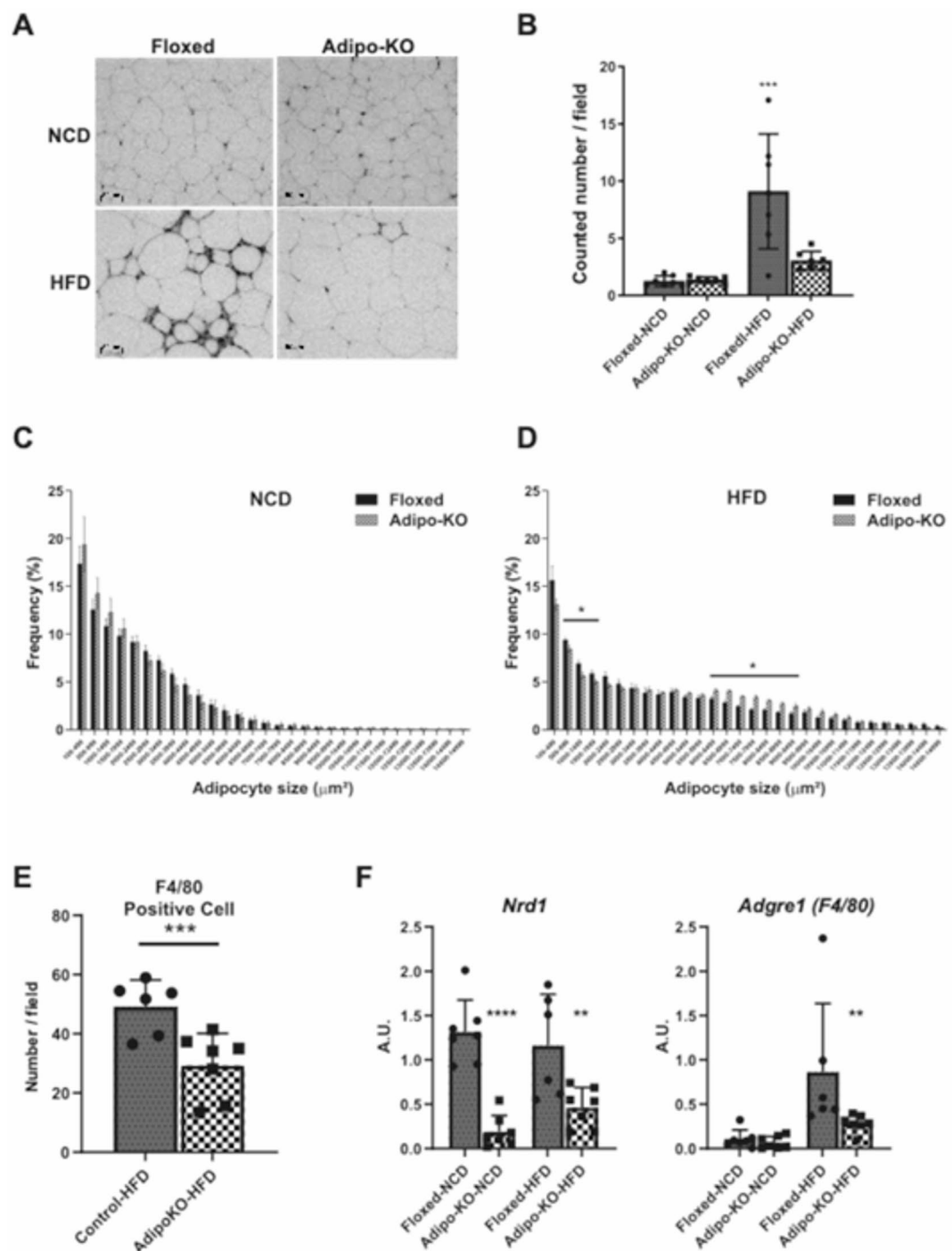


Fig. 2. Reduction of crown-like structures (CLS) and F4/80-positive cells in Adipo-KO on HFD. **(A)** Representative H&E-stained sections of eWAT from Floxed and Adipo-KO mice on NCD or HFD. Scale bars, 50 μm . **(B)** Quantification of CLS per field in eWAT from Floxed and Adipo-KO mice on NCD or HFD. $N=7$ /group. *** $P<0.001$ **(C, D)** Histogram showing adipocyte size distribution in eWAT from Floxed and Adipo-KO mice on NCD or HFD. In panel **(D)**, the asterisk indicates that each pair of bars (Floxed vs. Adipo-KO) within the marked size bins are significantly different. $N=7$ /group. * $P<0.05$ **(E)** Quantification of F4/80-positive cells per field in eWAT from Floxed and Adipo-KO mice on HFD, based on immunostaining ($N=6/7$ per group). *** $P<0.001$ **(F)** qPCR analysis of *Nrdc* (*Nrd1*) and F4/80 (*Adgre1*) in eWAT from Floxed and Adipo-KO mice (mean $\pm$ SE, $N=7/7/6/7$ per genotype). **** $P<0.0001$, ** $P<0.01$.

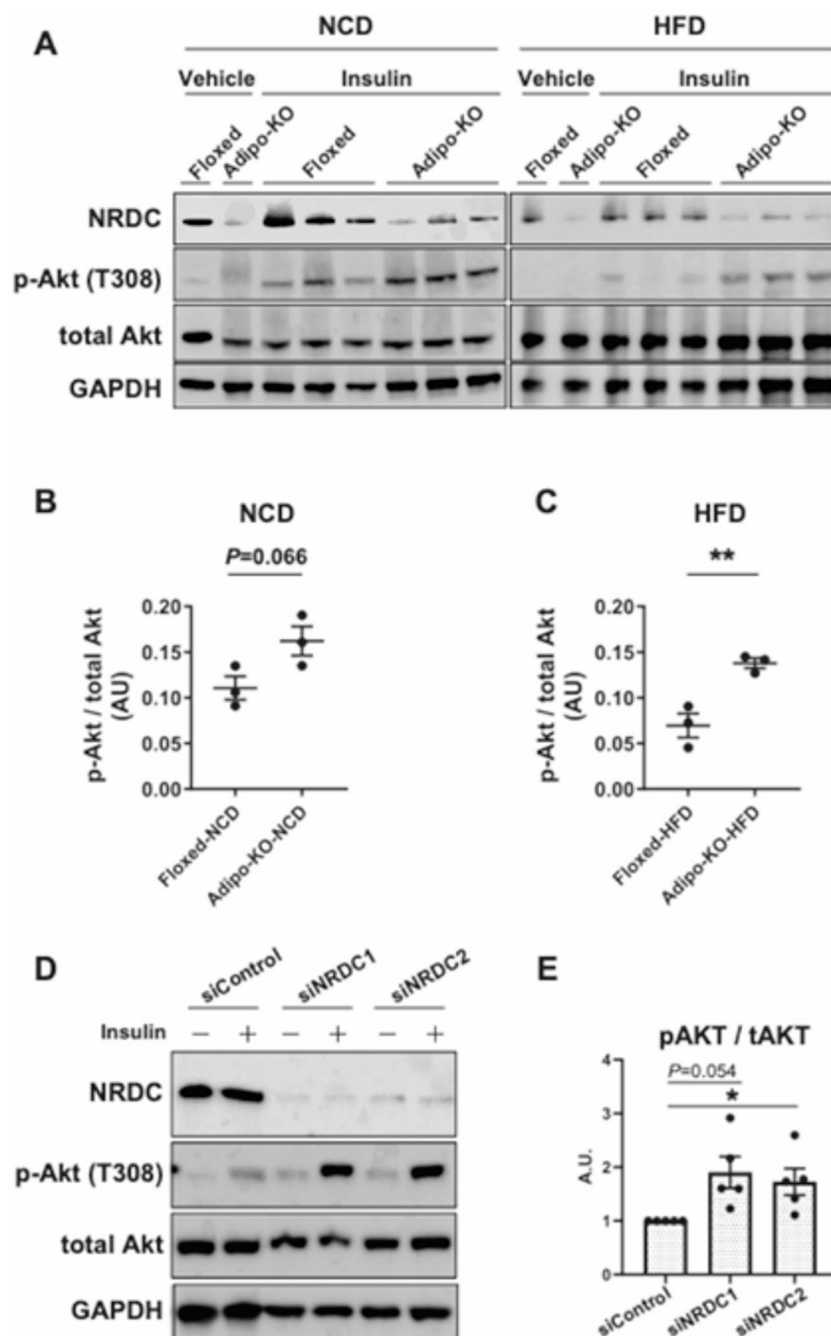


Fig. 3. Enhanced insulin-induced phosphorylation of AKT in eWAT of Adipo-KO mice and NRDC-knockdown 3T3-L1 adipocytes. **(A)** Western blot analysis of phospho-AKT (T308) and total AKT in eWAT from Floxed and Adipo-KO mice with or without insulin stimulation for 30 min on either NCD or HFD. **(B, C)** Quantification of the intensity of p-AKT signal normalized by that of total AKT signal in **(A)** using ImageJ software (NIH). $N=3/\text{group}$. $^{**}P<0.01$. **(D, E)** Western blot analysis for phospho-AKT and total AKT in 3T3-L1 adipocytes in which NRDC is knocked down by siRNA (siNRDC1, siNRDC2), with or without insulin treatment for 15 min **(D)**. Quantitative analysis of the intensity of p-AKT signal normalized by that of total AKT for siNRDC1, for siNRDC2 vs. siControl **(E)**. $N=5$. $^{*}P<0.05$.

NRDC associates with PPAR γ and may regulate its target genes

We performed RNA-sequence to comprehensively analyze gene expression changes caused by NRDC knockdown in 3T3-L1 adipocytes. Upstream analysis of the differentially expressed genes (DEGs: Supple Table 1) using public database of ChIP-seq results in white adipose tissue revealed that PPAR γ and Tle3 were enriched in the promoters of the DEGs (Fig. 6A). As we have previously reported that NRDC makes a complex with PPAR γ co-activator-1 α (ppargc1 α and PGC-1 α) and repress its transcriptional activity²⁰, we examined the triad complex of NRDC, PPAR γ and PGC-1 α . Immunoprecipitation and Western blotting demonstrated that these three factors

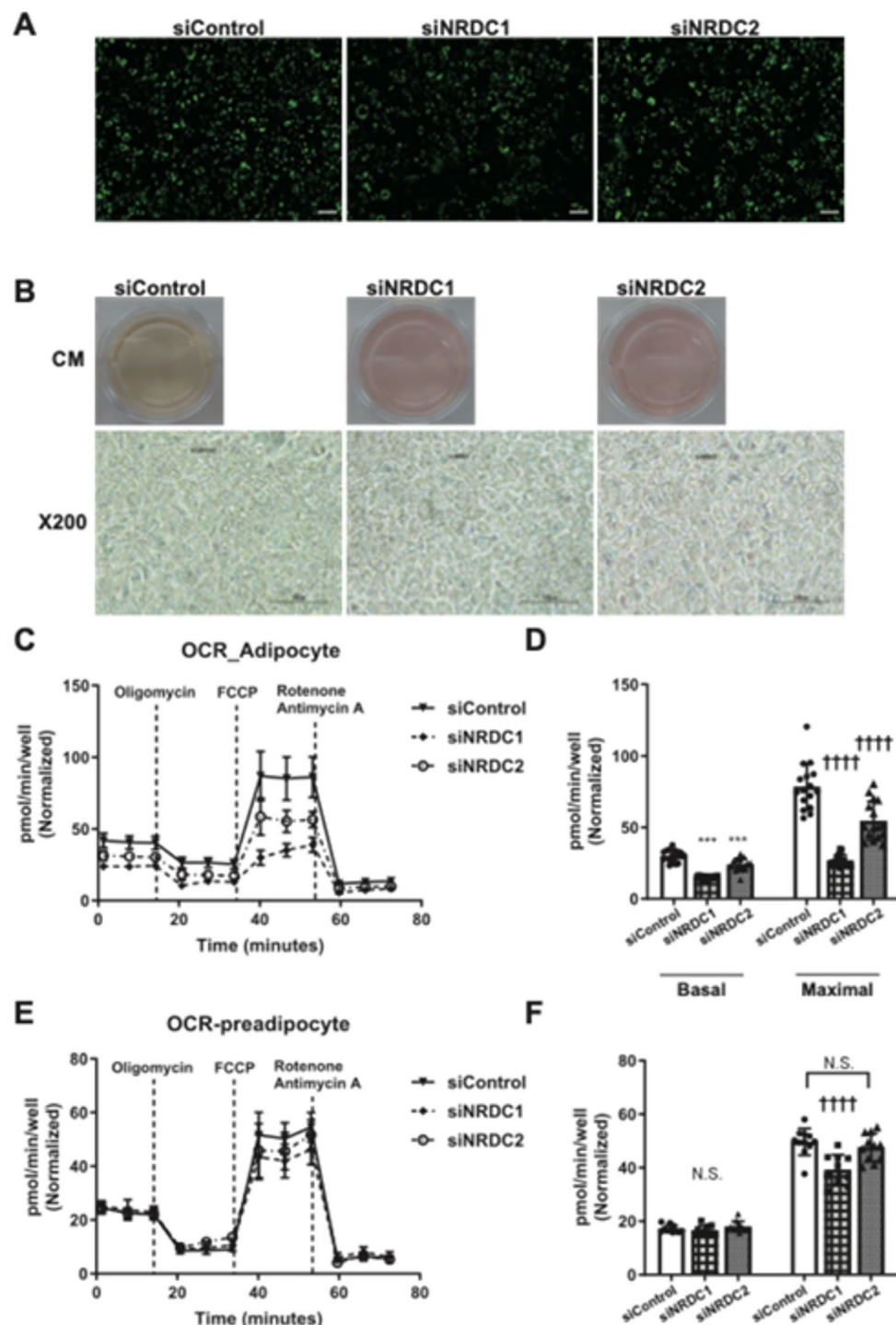


Fig. 4. NRDC knockdown impacts cellular metabolism in 3T3-L1 adipocytes. **(A)** Representative images of NRDC-knockdown 3T3-L1 adipocytes in which lipid droplets were stained with Lipidye 2 fluorescent dye. Scale bar: 500 μ m. **(B)** Representative images of the culture medium and bright-field images of control or anti-NRDC siRNA-transfected 3T3-L1 adipocytes. **(C)** Representative results of oxygen consumption rate (OCR) in NRDC-knockdown 3T3-L1 adipocytes measured by a Seahorse XF analyzer. **(D)** Basal and maximal OCR in NRDC-knockdown 3T3-L1 adipocytes. Basal OCR represents baseline OCR minus rotenone/antimycin A-insensitive OCR; maximal OCR represents FCCP-induced OCR minus rotenone/antimycin A-insensitive OCR. $N = 16$ /group. Data presented as mean $\pm$ SD; **** and + + + + for maximal OCR vs. siControl. $P < 0.0001$ for basal $P < 0.0001$. **(E)** Representative results of OCR in NRDC-knockdown 3T3-L1 pre-adipocytes measured by a Seahorse XF analyzer. **(F)** Basal and maximal OCR in 3T3-L1 pre-adipocytes. $N = 10-12$ /group. Data presented as mean $\pm$ SD; N.S. denotes not significant. + + + + siControl. $P < 0.0001$ for maximal OCR vs. siControl.

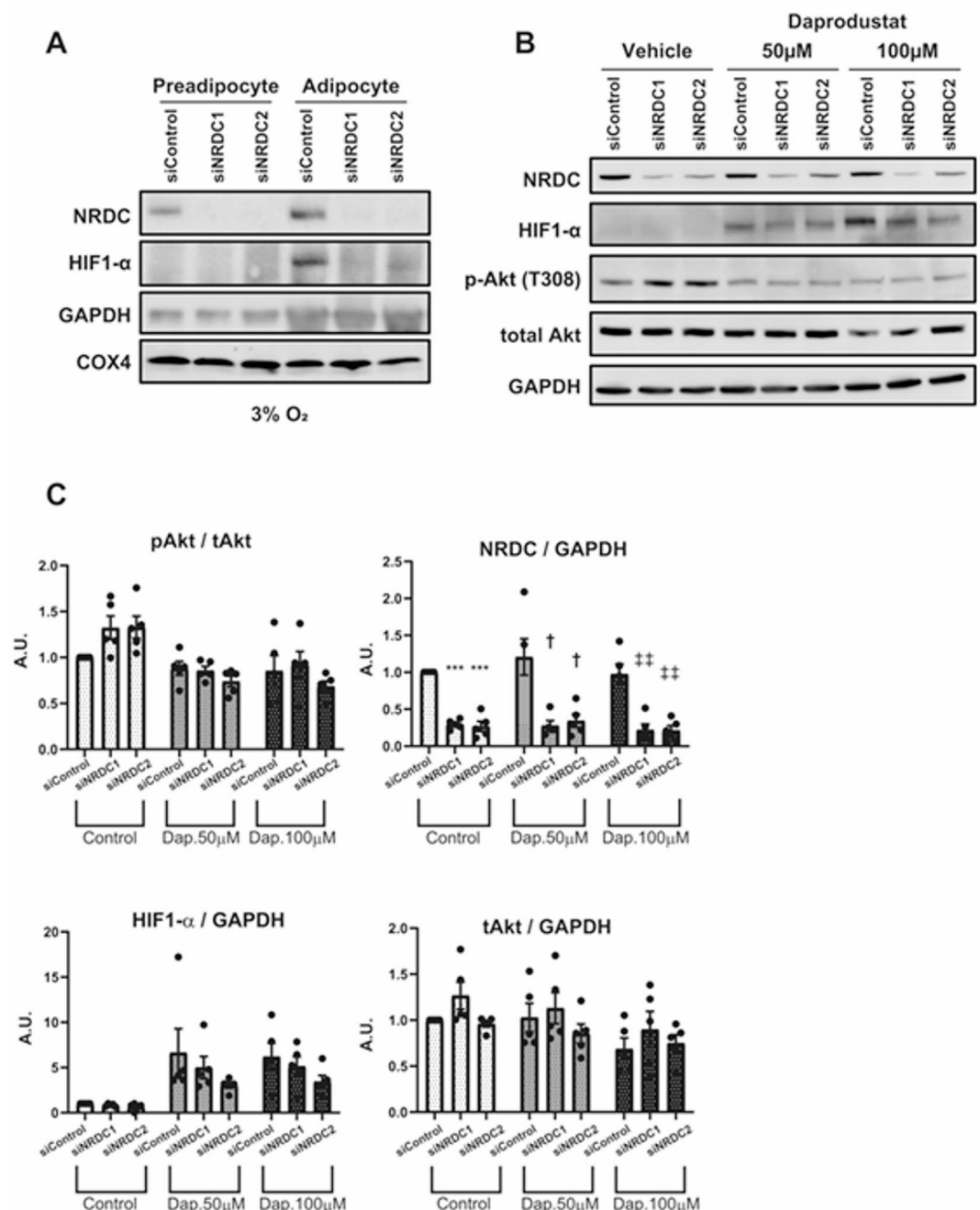


Fig. 5. NRDC in adipocytes regulates insulin signaling via HIF1 α . **(A)** Western blot analysis showing the induction of HIF1 α under hypoxic condition (3% of O₂ for – hours) in 3T3-L1 preadipocytes or differentiated adipocytes transfected with control or anti-NRDC siRNA. **(B)** Western blot analysis of NRDC-knockdown 3T3-L1 adipocytes treated with or without Daprodustat under normoxic conditions. **(C)** Quantitative analysis of the intensity of protein signals described in **(B)**. $N=5$, Data presented as mean $\pm$ SE; pAkt/tAkt ($p=0.13$ for siNRDC1, $p=0.11$ for siNRDC2 vs. siControl), NRDC/GAPDH ($***P<0.001$ vs. siControl under Control conditions, $\dagger P<0.05$ vs. siControl under 50 μ M Daprodustat, $\ddagger P<0.01$ vs. siControl under 100 μ M Daprodustat).

form a complex and pioglitazone, an activator of PPAR γ , tended to reduce the association of NRDC with PPAR γ (Fig. 6B). We next examined mRNA levels of several major PPAR γ target genes in eWAT of HFD-fed mice, and found that Glut4 (*Slc2a4*) is significantly, while adiponectin (*Adipoq*) and lipoprotein lipase (*Lpl*) tend to be elevated in Adipo-KO eWAT. Notably, a major target of HIF1 α , VEGF (*Vegfa*) also tended to be higher in Adipo-KO eWAT (Fig. 6C).

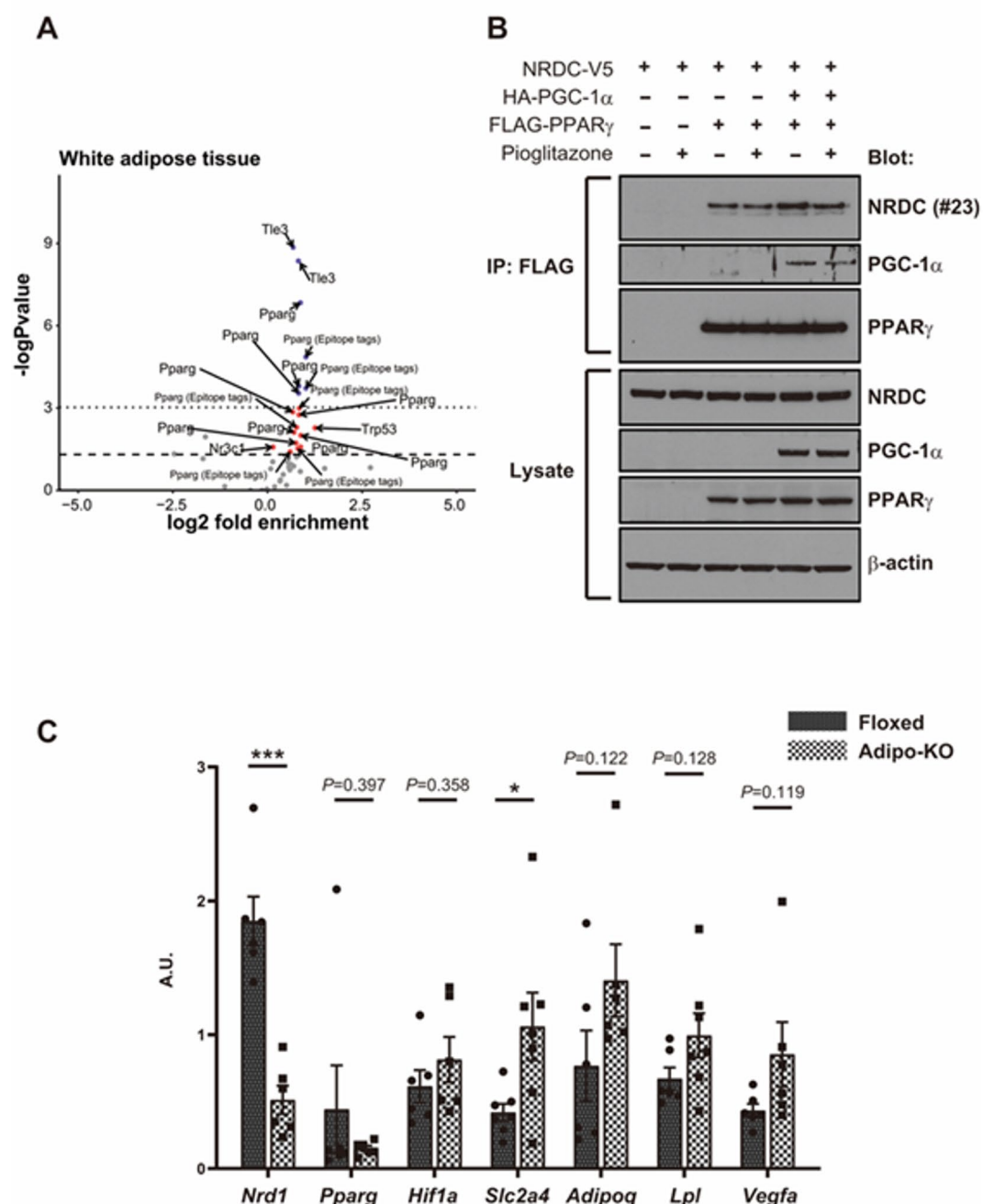


Fig. 6. NRDC associates with PPAR γ and may regulate its target genes. **(A)** Volcano plot showing transcription factor enrichment analysis of differentially expressed genes (DEGs) based on public ChIP-seq datasets from murine WAT, analyzed using the ChIP-Atlas platform. The DEGs were identified by RNA-seq in NRDC-knockdown 3T3-L1 adipocytes. Each point represents a transcription factor: blue indicates enriched factors, and red indicates those with $P < 0.05$. P values were calculated using a two-tailed Fisher's exact probability test. The x-axis shows $\log_2$ -transformed fold enrichment of transcription factors in the DEGs relative to all coding genes. The y-axis shows the $-\log_{10}$ -transformed P values for enrichment. The dashed line indicates a significance threshold of $P = 0.05$; the dotted line indicates the Bonferroni-adjusted threshold ($P = 0.05/53$). **(B)** Immunoprecipitation and Western blot analysis showing the formation of a complex of NRDC, PPAR γ , and PGC-1 α in COS7 cells transfected with expression vectors for NRDC-V5, HA-PGC1 α and FLAG-PPAR γ . **(C)** Relative mRNA expression levels of *Nrd1*, *Pparg*, *Hif1a*, and key *Pparg* and *Hif1a* target genes in eWAT from HFD-fed Floxed and Adipo-KO mice, which were normalized to β -actin mRNA levels. $N = 6/\text{group}$. Data presented as mean $\pm$ SE; * $P < 0.05$, *** $P < 0.0001$.

Discussion

We demonstrate here that the adipocyte-specific deletion of NRDC suppresses HFD-induced insulin resistance and chronic inflammation in adipose tissue. We also show that NRDC knockdown in 3T3-L1 adipocytes enhanced insulin-induced AKT phosphorylation, suggesting a cell-autonomous effect of NRDC on insulin sensitivity in adipocyte. Our previous study showed that global deletion of NRDC enhanced systemic insulin sensitivity, but adipose tissue-specific NRDC deficiency had an insulin sensitizing effect only under HFD, suggesting that insulin sensitivity, at least under NCD, is mediated by NRDC expressed in non-adipose tissue, possibly liver or skeletal muscle. On the other hand, glucose-stimulated insulin secretion is markedly suppressed by either global or β -cell-specific deletion of NRDC¹⁹, suggesting that NRDC expressed in each tissue regulates systemic insulin metabolism through different functions.

In the present study, adiponectin Cre was used for adipocyte-specific gene deletion, thus NRDC is deleted in both white and brown adipose tissue. We previously showed that NRDC expressed in BAT regulates adaptive thermogenesis via modulation of UCP1 expression and is involved in body temperature regulation under NCD²¹. Namely, on NCD, Adipo-KO mice exhibited hyperthermia and lower physical activity compared to control mice. Under HFD, however, there were no significant differences in body temperature and physical activity between Adipo-KO and control mice. Although it is not clear why HFD stress abolished the effect of NRDC deficiency on BAT activity, it is unlikely that NRDC in BAT had an effect on the insulin sensitivity observed in this study.

We show here that the oxygen consumption of NRDC-knockdown 3T3-L1 adipocytes is reduced compared to control cells. Furthermore, we show that HIF1 α induction by hypoxia is suppressed in NRDC-knockdown cells and that enhancement of HIF1 α expression by daprodustat, an inhibitor of HIF1 α degradation, abolishes the insulin-sensitizing effect of NRDC knockdown. These results suggest that NRDC regulates insulin sensitivity via HIF1 α . Evidence that HIF1 α in adipocytes plays an important role in insulin sensitivity includes the fact that adipocyte-specific loss of HIF1 α improves insulin sensitivity^{15,22} and inhibits atherosclerosis progression²³. If the reduced oxygen consumption in adipocyte due to NRDC deficiency also occurs in vivo, it is possible that the hypoxic situation predicted in eWAT with obesity is less likely to develop, which may lead to the improved insulin sensitivity. Intriguingly, a recent study by Feng et al. showed that lactate binds directly to PHD2 and inhibits its hydroxylase activity²⁴. Our results show that NRDC knockdown decreases lactate secretion into the medium, thus suggesting that the decrease in lactate may suppress HIF1 α expression via enhanced PHD2 activity.

Since our data suggested that NRDC regulates HIF1 α activity, the next question was whether HIF1 α accumulated upstream of the gene that was altered by knockdown of NRDC. To this end, we analyzed the upstream regulators of DEGs using public databases of ChIP-seq analysis. Unfortunately, HIF1 α ChIP-seq data in adipocyte were not publicly available. However, the analysis demonstrated that PPAR γ was significantly accumulated in the upstream regions of the DEGs. Since we had previously reported that PPAR γ coactivator, PGC-1 α and NRDC regulate UCP1 expression in BAT via mutual interaction²⁰, we examined the interaction between NRDC and PPAR γ and confirmed the triad complex of NRDC, PPAR γ and PGC-1 α . NRDC inhibits the transcriptional activity of PGC-1 α , and pioglitazone, the activating ligand of PPAR γ , weakened the NRDC-PPAR γ interaction. Given the several evidence showing mutual interplay of HIF1 α and PPAR γ ^{17,18}, our data suggest that NRDC may mediate the mutual repression of PPAR γ and HIF1 α .

One of the striking findings in this study is that inflammatory cell infiltration is markedly suppressed in visceral adipose tissue. We identified NRDC as a binding protein for HB-EGF²⁵, and the subsequent studies showed that NRDC enhances ectodomain shedding of membrane precursors such as HB-EGF and TNF α ^{26–28}. Furthermore, we showed that NRDC deficiency leads to suppression of inflammatory diseases such as gastritis, non-alcoholic steatohepatitis, and rheumatoid arthritis via attenuation of TNF α activation^{29–31}. Although this study demonstrated that the nuclear actions of NRDC are important in adipocyte metabolism and that they contribute to the regulation of insulin sensitivity, it is possible that the extracellular functions of NRDC, especially in inflammatory cytokine regulation, are partially involved, which should be clarified in the future. Taken together, we demonstrate here that NRDC in adipocyte regulates insulin sensitivity possibly through the functional interaction with two critical transcription factors, HIF1 α and PPAR γ .

Methods

Animals

All animal experiments were performed according to procedures approved by the Institute of Laboratory Animals in Kyoto University, and Research Center for Animal Life Science in Shiga University of Medical Science under the Institutes' guidelines for animal and recombinant DNA experiments. Animal protocols were approved by the Animal Research Committee, Graduate School of Medicine, Kyoto University and the Animal Care and Use Committee of the Shiga University of Medical Science. Generation of *Nrd1*^{flox/flox} mice (Floxed) were previously described (accession no. CDB1019K; <http://www.clst.riken.jp/arg/mutant%20mice%20list.html>)¹⁹. Adipocyte-specific *Nrd1* knockout mice (Adipo-KO) were generated by crossing *Nrd1*^{flox/flox} mice with *Adipoq*-Cre mice²¹. *Adipoq*-Cre mice were obtained from the Jackson Laboratory. Male mice were used for all animal experiments. Floxed and Adipo-KO mice were fed a normal chow diet (NCD) or high-fat diet (HFD) for 12 weeks starting at 6 weeks of age, and then were subjected to each experiment. For HFD, high-fat and high-sucrose diet (F2HFHSD, Oriental Yeast CO.), in which 54.5% of the total caloric intake was derived from fat, was utilized. To obtain mouse tissue for the biochemical analysis, mice were deeply anesthetized by isoflurane and euthanized by cervical dislocation, and epididymal white adipose tissue (eWAT) were then removed and immediately frozen in liquid nitrogen. The study is reported in accordance with ARRIVE guidelines (<https://arriveguidelines.org>).

Respiratory gas analysis

Oxygen consumption (VO_2), respiratory exchange ratio (RER), and ambulatory physical activity were measured using the Oxymax indirect calorimetry system (Comprehensive Lab Animal Monitoring System: Oxymax/CLAMS, Columbus Instruments, TX, US). The light period in the chamber was set for 12 h (8:00–20:00) and the dark period for 12 h (20:00–8:00). Room temperature was set at 23 °C. Mice were placed in the chamber and VO_2 , RER, and physical activity were measured continuously for 24 h at 10-minute intervals starting at 8:00. Food and water were taken freely during the measurement.

Telemetric body temperature monitoring was performed on conscious mice using a wireless ETA-F10 Mouse Implantable Transmitter System by Data Science International (New Brighton) as previously described²¹.

Glucose and insulin tolerance tests

For glucose tolerance tests, mice were fasted overnight and injected intraperitoneally with 10 ml/kg body weight of 20% D-glucose solution (2 g/kg body weight of D-glucose) (Otsuka Pharmaceutical). For insulin tolerance test, mice were fasted for 4 h, and then injected intraperitoneally with 1 (under NCD) or 1.5 (under HFD) unit/kg body weight of insulin (Humulin R, Eli Lilly). Blood samples were collected from the tails of mice before and at the indicated periods after the injection. A self-test glucometer was used to measure blood glucose levels. An ELISA kit (MS303; Morinaga Institute of Biological Science, Japan) was used to measure plasma insulin levels.

For analysis of insulin-induced AKT phosphorylation in eWAT, mice were fasted for overnight, and then injected intraperitoneally with 1 unit/kg body weight of insulin (under NCD and HFD). Epididymal WAT was harvested 30 min after insulin injection.

Histological procedures and immunohistochemistry

Mice were deeply anesthetized by isoflurane and transcardially perfused with 4% paraformaldehyde [volume %] in 0.1 M phosphate-buffered saline. Epididymal white adipose tissue (eWAT) was removed and fixed in 4% paraformaldehyde solution for paraffin block preparation. Paraffin sections of 4 μm thickness were obtained from four distinct layers, with each layer separated by more than 150 μm . A Keyence BZ-700 microscope was used for imaging. To calculate the number of crown-like structures (CLS), images of 3×3 consecutive fields at 40x magnification from three distinct layers were captured and jointed. The number of CLS was then normalized to the total adipose tissue area in the jointed image. Immunofluorescent staining for mouse tissues was conducted as previously described²⁸, using an anti-F4/80 antibody (#70076, Cell Signaling Technology) to identify F4/80 positive cells. Images of 3×3 consecutive fields at 200x magnification from two distinct layers were captured and jointed, and the number of F4/80 positive cells was determined by identifying nuclei stained with 4',6-diamidino-2-phenylindole (DAPI). LipiDyeII staining for the detection of lipid droplets in mature adipocytes was performed according to the manufacturer's protocol (Funakoshi), followed by observation using a Keyence BZ-700 microscope.

Cell culture

3T3-L1 cells obtained from ATCC were cultured in Dulbecco's Modified Eagle Medium (DMEM) with 4.5 g/L glucose (Nacalai Tesque) supplemented with 10% adult bovine serum (BS)(B9433) and 1% penicillin/streptomycin solution (P/S) (Gibco) at 37 °C in a 5% CO_2 atmosphere. For adipocyte differentiation, cells were initially cultured in DMEM with 10% fetal bovine serum (FBS) (N4637) and 1% P/S. Differentiation was induced with a medium containing 1 mM 3-isobutyl-1-methylxanthine (IBMX) (I5879, Sigma-Aldrich), 1 μM dexamethasone (DEX) (D1756, Sigma-Aldrich), and 10 $\mu\text{g}/\text{ml}$ insulin (I0516, Sigma-Aldrich) for 2 days, followed by a medium with 10 $\mu\text{g}/\text{ml}$ insulin alone for another 2 days, and finally, cells were cultured in insulin-free medium for 2 more days. For serum-free cultures, DMEM with 0.5% fatty acid-free bovine serum albumin was used (FUJIFILM Wako Pure Chemical).

For analysis of insulin-induced AKT phosphorylation, differentiated 3T3-L1 adipocytes were treated with 17 nM of insulin for 15 min. To evaluate the expression of HIF1 α in cells under hypoxic conditions, differentiated cells were incubated in a 3% O_2 -regulated incubator for 24 h, and then immediately lysed using the procedure described below. To assess the effect of Daprodustat on HIF1 α expression and AKT phosphorylation, siRNA-transfected and differentiated 3T3-L1 adipocytes were treated with or without Daprodustat (50 μM , 100 μM) for 48 h.

SiRNA transfection

Sub-confluent 3T3-L1 cells were transfected with Stealth RNAi[™] siRNA targeting *Nrd1* (MSS214013 (siNRDC1: F: 5'–3' AAU CCU AGA AUU CCA GAU GUG UUC C, R: 5'–3': GGA ACA CAU CUG GAA UUC UAG GAU U) and MSS214014 (siNRDC2: F: 5'–3' GGCUCACAGAGGACCUGGAAAGUAAA, R: 5'–3': UUUACUUC AGGUCCUCUGUGAGCC), Thermo Fisher Scientific) using Lipofectamine[™] RNAiMAX Transfection Reagent (Thermo Fisher Scientific). Differentiation of 3T3-L1 cells into adipocytes were then induced the day after the transfection, as described above.

Western blotting

Cultured cells or tissues were lysed in RIPA buffer containing 50 mM Tris-HCl, pH 8.0, 150 mM sodium chloride, 0.5 w/v% sodium deoxycholate, 0.1 w/v% sodium dodecyl sulfate, 1.0 w/v% NP-40 substitute, protease inhibitor cOmplete[™] (Roche), and phosphatase inhibitor cocktail 3 (P0044, sigma). The lysed samples were sonicated and centrifuged at 4 °C, 15,000 rpm for 5 min to remove impurities and fat, and then used for SDS-PAGE and western blotting. Anti-NRDC antibody (clone #1, #135, and #23) was raised in our laboratory. The clone #135 are commercially available at Millipore (MABS2057). Antibodies against anti phosphorylated (p)-AKT (T308), total AKT, GAPDH, HIF1 α , and COX4 were obtained from Cell signaling technology. Antibody against

PGC1 α (#16) was developed in our laboratory as previously described²⁰, and anti-PPAR γ antibody was obtained from the Santa Cruz Biotechnology. Goat anti-mouse or anti rat-peroxidase conjugated IgG (1:5000, GE), or anti rabbit-peroxidase conjugated IgG (1:3000, CST) were used as secondary antibody. Intensity of signals on western blots was quantified using ImageJ software (<https://imagej.net/ij/index.html>; version 1.54 g). Each signal intensity was normalized with that of GAPDH. For p-AKT, the intensity was normalized with that of total AKT. Full-length images of the Western blots shown in the main figures are provided in Supplementary Fig. 2.

Real-time PCR

RNA from cultured cells or tissues was extracted using TriPure Isolation Reagent (Roche), and reverse transcribed to synthesize cDNA. Expression levels of individual genes were quantitated by real-time PCR as described previously¹⁹. The primers are listed in the Supplementary Table.

Measurement of oxygen consumption rate in 3T3-L1 adipocytes

The XF96 Extracellular Flux Analyzer (Agilent Technologies, USA) was used to measure oxygen consumption rate (OCR) of 3T3-L1 adipocytes. The day before the assay, confluent cells were suspended in trypsin and re-seeded onto XF96-well microplates at 20% confluency. Measurements of OCR was performed according to the instruction manual of Agilent Seahorse XF Cell Mito Stress Test (Part Number 103016-400). Briefly, OCR was measured at baseline and after sequential addition of (1) oligomycin (ATP synthase inhibitor), (2) carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (mitochondrial oxidative phosphorylation uncoupler; FCCP), and (3) rotenone and antimycin A (complex1 inhibitor). To normalize the OCR value by cell numbers, the number of cells per well was counted using ArrayScan VTI (ThermoFisher Scientific) after the staining with DAPI.

RNA sequence analysis

Total RNA from 3T3-L1 adipocytes was extracted using TriPure Isolation Reagent (Roche). RNA quality was assessed using a Bioanalyzer 2100 (Agilent Technologies). Poly(A) + RNA was purified and libraries were prepared using the NEBNext Ultra II RNA Library Prep Kit for Illumina (New England Biolabs) with multiplexed barcode adaptors, following the manufacturer's protocol. Paired-end sequencing was performed on an Illumina NovaSeq platform. Read quality was assessed by FastQC v0.11.9. Adapter sequences and reads shorter than 50 bp were trimmed using Cutadapt v4.5 with Python v3.10.13. Trimmed reads were aligned to the mouse reference genome (GRCm39) using STAR Aligner v2.7.8a, and transcript abundance was quantified using RSEM v1.3.3 against the Gencode M33 annotation. The DEGs were identified using edgeR v4.2.1 with the estimateGLMCommonDisp function (method = "deviance", robust = TRUE, subset = NULL). Genes with a log₂ fold change > 1 or < -1 and a false discovery rate (FDR) < 0.1 were considered DEGs. To identify potential upstream transcriptional regulators of these DEGs, we conducted an in silico enrichment analysis using ChIP-Atlas³² (Threshold for Significance = 100), a public database that integrates published ChIP-seq datasets, including those from murine WAT.

Statistical analysis

Data are presented as mean values $\pm$ the standard deviation (SD) or standard error (SE). Differences between treatments, groups, and genotypes were analyzed using an unpaired 2-tailed Student's t-test for 2 groups or a one-way ANOVA (the post hoc Tukey–Kramer HSD test) for more than 3 groups.

Data availability

All data generated or analyzed during this study are included in this published article and its supplementary information files.

Received: 9 December 2024; Accepted: 19 September 2025

Published online: 27 October 2025

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Acknowledgements

Expression vectors for PPAR γ and PGC1 α are generous gifts from Bruce Spiegelman via Addgene. We are grateful to M. Yoshida, R. Morinaga and H. Iwai for their technical assistance, and T. Kita for continuous encouragement.

Author contributions

S.M and M.O conceived and performed experiments, data analysis and preliminary manuscript writing. E.N conceived experiments and wrote final manuscript. K.N, S.I, H.I, Y.H and T.E performed experiments and analyzed data. K.O and T.K supervised the work. All authors read and edited the manuscript.

Declarations

Competing interests

The authors declare no competing interests.

Additional information

Supplementary Information The online version contains supplementary material available at <https://doi.org/10.1038/s41598-025-21276-z>.

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Supplementary Information

Nardilysin in adipocyte regulates insulin sensitivity via HIF1 α and PPAR γ

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Taiki Eifuku, Yoshinori Hiraoka, Takeshi Kimura, Koh Ono & Eiichiro Nishi^{*}

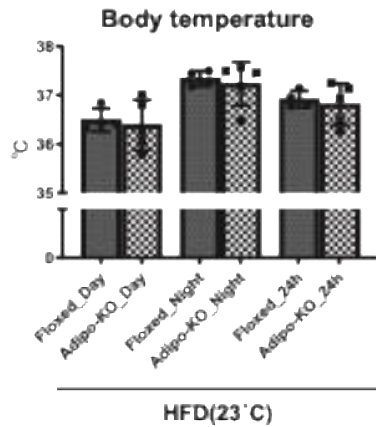
Supplementary Figure 1, 2

Supplementary Table 1

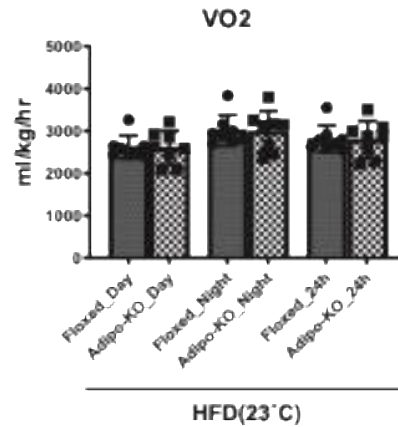
Supplementary File 1 (Excel file of DEGs)

Supplementary figure 1

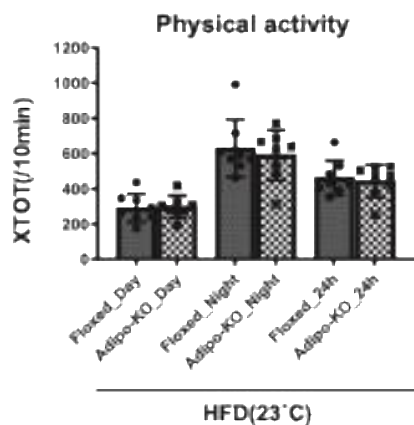
A



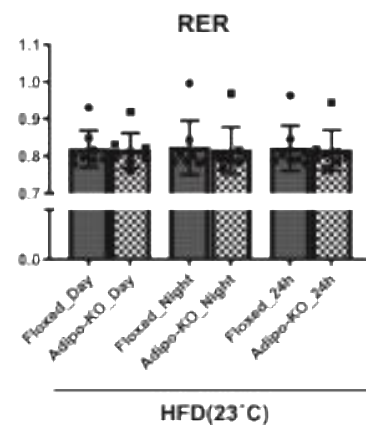
B



C



D



Supplementary Figure 1

A-D. Body temperature (A), Oxygen consumption (VO₂) (B), locomotor activity (C), and the respiratory exchange ratio (RER) (D) were measured using the Oxymax indirect calorimetry system. There were no significant differences in all measurements between Floxed and Adipo-KO mice after HFD.

Supplementary figure 2 Full length blotts

Figure 3A

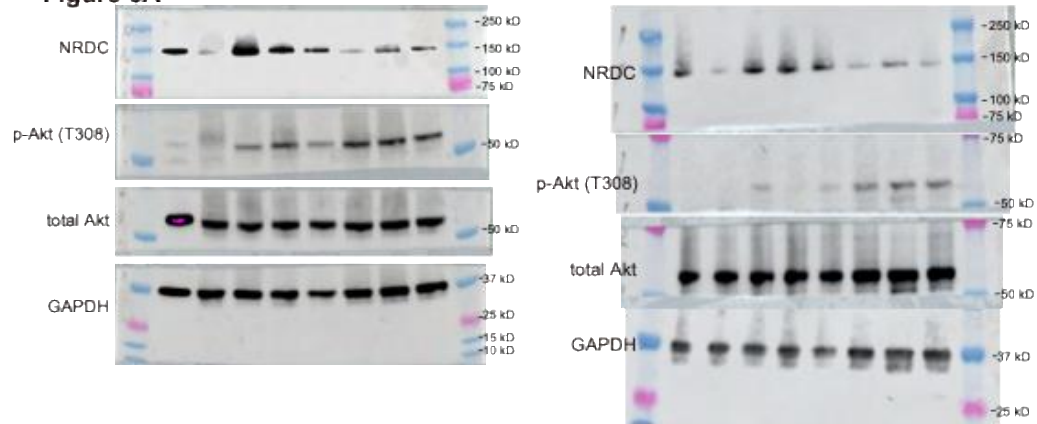


Figure 3D

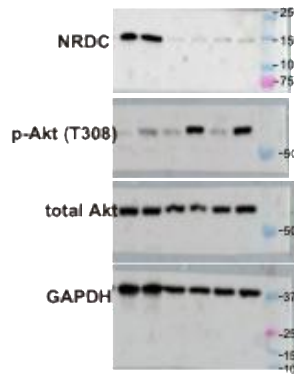


Figure 5A

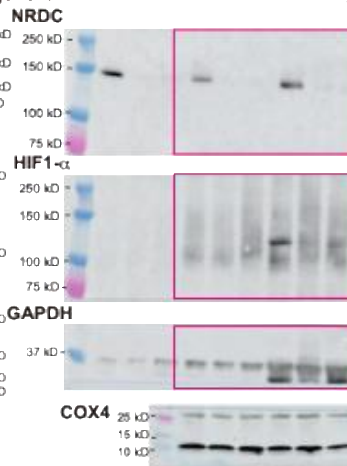


Figure 5B

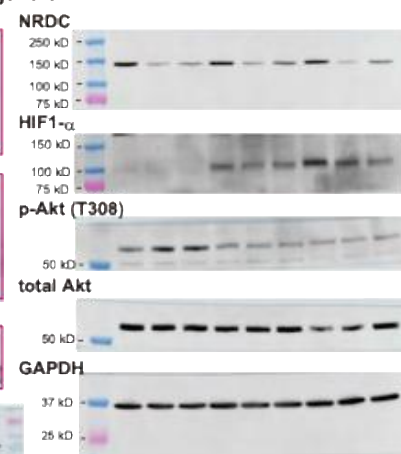
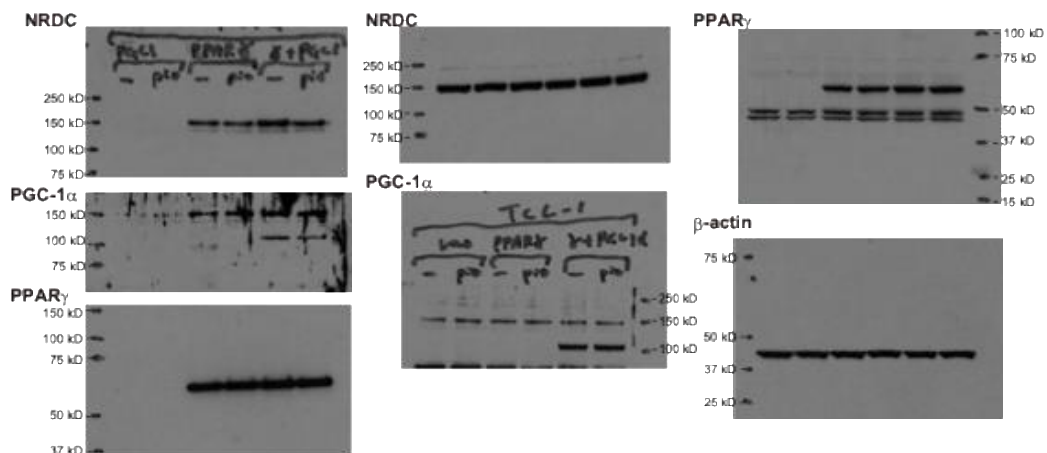


Figure 6B



Supplementary Figure 2.

Full length blots of Figures presented in the main manuscript.

Primer	Fw	Rev
<i>Nrd1</i>	ATG GAT GGC CTT TCC CTT G	CGC CGA AGT TCA GCT TGT CAA
<i>Actb</i>	CTG ACT GAC TAC CTC ATG AAG ATC CT	CTT AAT GTC ACG CAC GAT TTC C
<i>Pparg</i>	GTA CTG TCG GTT TCA GAA GTG CC	ATC TCC GCC AAC AGC TTC TCC T
<i>Hif1a</i>	CAA GAT CTC GGC GAA GCA A	GGT GAG CCT CAT AAC AGA AGC TTT
<i>Adgre1</i>	GCT GTG AGA TTG TGG AAG CA	CTG TAC CCA CAT GGC TGA TG
<i>Slc2a4</i>	CAA TGG T TG GGA AGG AAA AGG GCT A	GTA GGC GCC AAT GAG GAA CCG TC
<i>Adipoq</i>	TCT CCT GTT CCT CTT CTT AAT CCT GCC	CAT CTC CTT TCT CTC CCT TCT CTC C
<i>Lpl</i>	TGT TTC TCC TGC CTG ATG TCT TC	GGC TAA CCC AGG GTG AGG AA
<i>Vegfa</i>	TAC CTC CAC CAT GCC AAG TGG T	AGG ACG GCT TGA AGA TGT AC

Supplementary Table 1.

Primer sequences used for RT-PCR analysis.