

Nuclear receptor corepressor and histone deacetylase 3 govern circadian metabolic physiology

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Rhythmic changes in histone acetylation at circadian clock genes suggest that temporal modulation of gene expression is regulated by chromatin modifications^{1–3}. Furthermore, recent studies demonstrate a critical relationship between circadian and metabolic physiology^{4–7}. The nuclear receptor corepressor 1 (Ncor1) functions as an activating subunit for the chromatin modifying enzyme histone deacetylase 3 (Hdac3)⁸. Lack of Ncor1 is incompatible with life, and hence it is unknown whether Ncor1, and particularly its regulation of Hdac3, is critical for adult mammalian physiology⁹. Here we show that specific, genetic disruption of the Ncor1–Hdac3 interaction in mice causes aberrant regulation of clock genes and results in abnormal circadian behaviour. These mice are also leaner and more insulin-sensitive owing to increased energy expenditure. Unexpectedly, loss of a functional Ncor1–Hdac3 complex *in vivo* does not lead to sustained increases in known catabolic genes, but instead significantly alters the oscillatory patterns of several metabolic genes, demonstrating that circadian regulation of metabolism is critical for normal energy balance. These findings indicate that activation of Hdac3 by Ncor1 is a nodal point in the epigenetic regulation of circadian and metabolic physiology.

Mammals display circadian rhythms in behavioural and physiological processes, such as sleep, feeding, blood pressure and metabolism^{10–12}, guided by external light–dark signals that are integrated through intrinsic central and peripheral molecular clocks^{13,14}. Several critical clock and clock output genes have daily cycling of histone acetylation, suggesting that epigenetic regulation of chromatin has a central role in circadian regulation^{1–3}. Nuclear receptors regulate circadian rhythm and metabolism by interacting with cofactor complexes that act at the level of chromatin^{15–17}.

Ncor1 is a large, multidomain protein that is recruited by nuclear receptors to mediate transcriptional repression. Ncor1 stably associates with Hdac3 through its deacetylase activation domain (DAD), which is conserved in the highly related Ncor2 (also known as SMRT)⁸. Hdac3 deacetylase activity requires association with the Ncor1 or SMRT DAD⁸, and DAD mutations significantly inhibit nuclear receptor-mediated repression¹⁸. Both *Hdac3* and *Ncor1* knockout mice die early in embryogenesis^{9,19}, indicating that these proteins are essential for normal development and postnatal survival.

The function of Ncor1 in the adult is unknown, as is the physiological significance of the Ncor1–Hdac3 association. We used a homologous recombination strategy to generate C57BL/6 mutant mice (referred to as DADm) with a single amino acid substitution (Y478A) in the Ncor1 DAD, creating a mutant protein that is stable but unable to associate with or activate Hdac3 (ref. 18; Supplementary Figs 1 and 2a, b). Levels of Ncor1 and SMRT were similar in DADm and wild-type littermates (Supplementary Fig. 2c, d), and the Hdac3 interaction with Ncor1 (but not SMRT) was lost in the DADm mice

(Supplementary Fig. 2e, f)¹⁸. DADm mice were viable, born at normal Mendelian frequencies (Supplementary Table 1) and morphologically indistinguishable from wild-type littermates at birth. Thus, Ncor1 binding to Hdac3 is not required for normal development, and the embryonic defects of mice lacking Ncor1 are due to factors other than, or in addition to, Hdac3 recruitment by Ncor1.

Ncor1 serves as corepressor for Rev-erb α (also known as Nr1d1), a nuclear receptor that represses the clock gene, *Bmal1* (also known as *Arntl*; refs 20 and 21). *Bmal1* is normally expressed at its lowest levels from zeitgeber time (ZT)7–9. Consistent with an *in vivo* role for repression by Ncor1–Hdac3, *Bmal1* messenger RNA was increased from ZT7–9 in DADm mice (Fig. 1a), although its levels continued to cycle (data not shown). Histone H4 acetylation was increased at the Rev-erb response element (RORE) of the gene during this period (Fig. 1b)²⁰. DADm mouse embryonic fibroblasts (MEFs) also displayed higher levels of *Bmal1* (Fig. 1c). HDAC inhibition increased *Bmal1* expression in wild-type but not DADm MEFs (Fig. 1d), suggesting that derepression of *Bmal1* in the DADm MEFs is epistatic with loss of Hdac3 activity. Moreover, depletion of *Rev-erba* or its haem ligand, derepressed *Bmal1* in wild-type but not DADm MEFs (Fig. 1e and Supplementary Fig. 3a), indicating that Rev-erb α is an important endogenous target of Ncor1–Hdac3. Although cyclic expression of a *Bmal1*-luciferase reporter requires Rev-erb α and Rev-erb β (ref. 22), *Bmal1* expression remained rhythmic in DADm MEFs (Fig. 1f), potentially due to compensation by SMRT–Hdac3 or by regions of the endogenous gene not contained in the reporter construct. Nevertheless, the rhythmic expression of *Bmal1* and *Rev-erba* was abnormal in the DADm MEFs, demonstrating a cell-autonomous role for the Ncor1–Hdac3 complex in maintaining normal circadian rhythm (Fig. 1f and Supplementary Fig. 3b). Cyclic histone acetylation was similarly altered in the DADm cells (Fig. 1g). Ncor1 did not oscillate and was recruited to the *Bmal1* RORE in the DADm as well as the wild-type MEFs (Supplementary Fig. 3c, d) whereas, as predicted, Hdac3 recruitment was markedly reduced at this site in the DADm cells (Supplementary Fig. 3d).

To evaluate whether the molecular dysregulation of the clock corresponds with circadian behavioural abnormalities, we monitored locomotor activity in constant darkness. DADm mice demonstrated a free-running period of ~23.2 h compared with 23.6 h in wild-type mice (Fig. 1h, i). This decrease in the average period length of the DADm mice is highly significant and coincides with the period change reported in *Rev-erba* knockout mice²¹. These findings identify a critical role for activation of Hdac3 by Ncor1 in regulating normal circadian rhythm and demonstrate the dependence of circadian behaviour on dynamic epigenetic modifications.

Owing to the strong links between circadian and metabolic physiology, as well as the role of nuclear receptors in both systems, we also examined metabolic parameters. The Ncor1 DADm mice weighed

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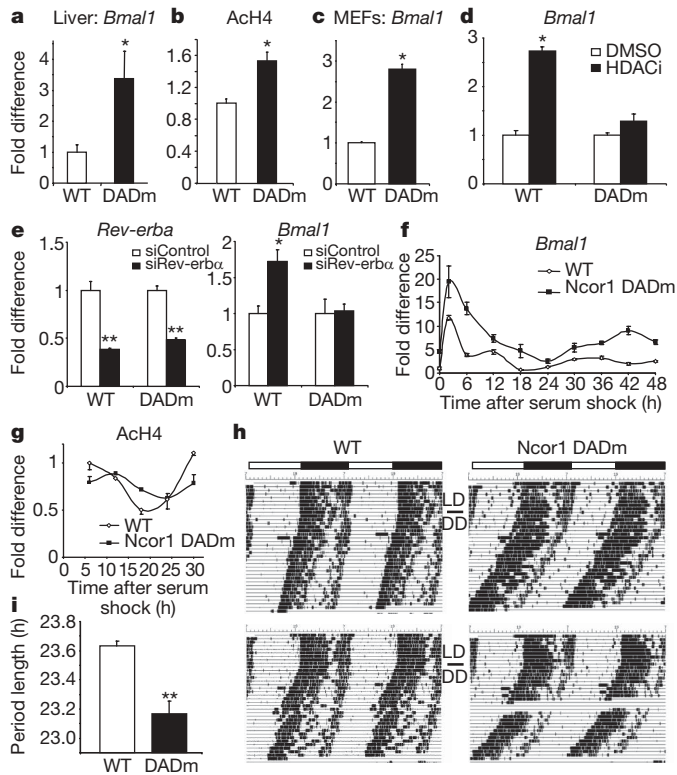


Figure 1 | Ncor1-Hdac3 regulates peripheral clock and circadian physiology. **a**, *Bmal1* expression in wild type (WT) and DADm livers during ZT7–9 (WT: $n = 6$, DADm: $n = 4$). **b**, Chromatin immunoprecipitation (ChIP) for ACh4 from wild-type and DADm livers at the *Bmal1* RORE ($n = 3$). **c**, *Bmal1* expression in immortalized MEFs ($n = 3$). **d**, *Bmal1* expression in MEFs after treatment with the HDAC inhibitor MS-275 (HDACi, black bars) or dimethylsulphoxide control (white bars) ($n = 3$). **e**, Effect of *Rev-erba* knockdown on MEF *Rev-erba* and *Bmal1* expression, using small interfering RNA against green fluorescent protein (siControl) or against *Rev-erba* (siRev-erb α) ($n = 3$). **f**, *Bmal1* expression in MEFs after cell synchronization (fold difference relative to wild type, time 0; $n = 3$ per time point). **g**, ChIP of ACh4 at the *Bmal1* RORE following 50% serum shock. Mean \pm s.e.m. of duplicate samples. Independent experiments gave similar results. **h**, Voluntary locomotor wheel running activity, double plotted in each panel. LD, 12 h light/12 h dark; DD, complete darkness. **i**, Average free running period ($n = 3$). Three independent experiments gave similar results. Data are presented as mean and s.e.m. * $P < 0.05$, ** $P < 0.01$.

the same as wild-type littermates at birth, but began to weigh significantly less between 4–6 weeks of age and maintained this difference throughout adulthood (Fig. 2a). Perigonadal fat pad weight (Fig. 2b, c) and whole body fat measured by NMR (Fig. 2d) were decreased in DADm mice. Histological evaluation of Ncor1 DADm adipose tissue showed normal architecture, with a trend towards smaller adipocytes (Supplementary Fig. 4a). Consistent with previous reports that Ncor1 inhibits adipogenesis, adipocyte differentiation was modestly increased in DADm MEFs, indicating that the decrease in fat tissue is not due to impaired adipogenesis (Supplementary Fig. 4b, c)²³.

We next used metabolic cages to determine why the Ncor1 DADm mice are leaner. DADm mice demonstrated similar levels of locomotor activity to the wild-type mice and food intake was increased, indicating that their decreased weight did not result from increased activity or decreased feeding (Fig. 2e, f). Instead, DADm mice showed increased oxygen consumption and heat measured by indirect calorimetry, particularly during the wakeful dark cycle (Fig. 2g–i). Therefore, these mice probably ate more food to compensate for increased catabolism. The increase in heat production is unlikely to be a primary consequence of brown adipose UCP expression which was unaltered in DADm mice (Supplementary Fig. 4d).

Hyperinsulinemic-euglycemic clamp studies revealed increased insulin sensitivity of the DADm mice on a normal chow diet (Fig. 3a).

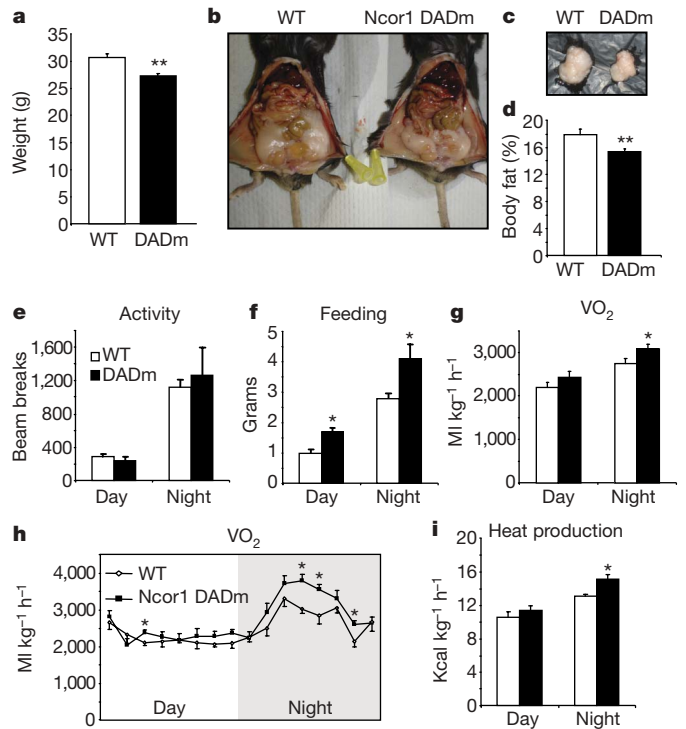


Figure 2 | DADm mice exhibit increased energy expenditure. **a**, Body weight of 24-week-old male wild type (WT) and DADm mice ($n = 10$). **b**, Representative abdominal images of wild type and DADm mice. **c**, Perigonadal fat pads obtained from mice in **b**. **d**, Percentage body fat composition measured by NMR for cohort presented in **a**. **e–i**, Effect of DADm on locomotor activity measured by photobeam breaks (**e**), on food intake (**f**), on oxygen consumption (VO_2) (**g, h**), and on heat production (**i**). Average VO_2 for three consecutive measurements taken every 27 min is shown in **h** ($n = 4$). Data are presented as mean and s.e.m. * $P < 0.05$, ** $P < 0.01$.

On a high-fat diet (HFD), DADm mice were resistant to diet-induced obesity and were protected from developing insulin resistance (Fig. 3b, c and Supplementary Fig. 5a), with reduced hepatic glucose production in the setting of the hyperinsulinemic clamp (Supplementary Fig. 5b). This was surprising because *Rev-erb α* recruits Ncor1–Hdac3 to gluconeogenic genes to repress hepatic glucose production¹⁷. However, on normal chow before the development of large differences in insulin sensitivity, hepatic glucose output was indeed upregulated in the DADm mice (Supplementary Fig. 5c). Also, expression of the gluconeogenic *Pepck* gene was increased in the DADm livers (Supplementary Fig. 5d), and the ability of the *Rev-erb α* ligand haem to repress *Pepck* gene expression was abrogated in DADm primary hepatocytes (Supplementary Fig. 5e). Apparently, in the HFD model, the gluconeogenic effects of reduced *Rev-erb α* function were more than counterbalanced by the markedly reduced insulin resistance of the DADm mice. Although this insulin sensitivity was consistent with the leaner phenotype of the mice, weight-matched DADm mice fed a HFD were also more insulin sensitive (Supplementary Fig. 6), suggesting that their improved insulin tolerance may not be entirely secondary to leanness.

To understand further the altered circadian and metabolic phenotypes of the DADm mice, ketone and free fatty acid levels were measured regularly during a 24-h cycle. Serum ketones and fatty acids were significantly increased in the DADm mice at specific times during the cycle (Supplementary Fig. 7a, b), suggesting temporal dysregulation of fat metabolism such that cumulative increases in hepatic beta oxidation drive increased lipolysis. Importantly, in contrast to mice lacking *Hdac3* in liver²⁴, there was no evidence of hepatic steatosis in the DADm mice (Supplementary Fig. 7c), suggesting a potential protective effect of residual SMRT-dependent *Hdac3* activity.

Microarray analysis at a single time point demonstrated modest changes in hepatic gene expression between the DADm and wild-type mice, although both circadian and metabolic pathways were enriched

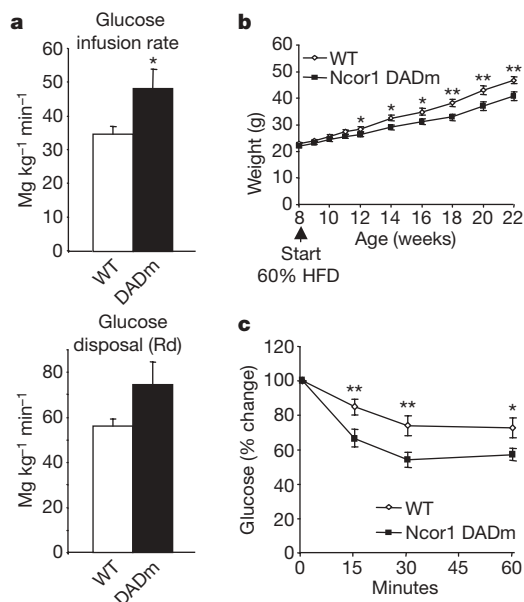


Figure 3 | DADm mice are resistant to diet-induced obesity. **a**, Hyperinsulinemic-euglycemic clamp measurements for 16-week-old male wild type (WT) and DADm mice fed a normal chow diet ($n = 4$). Top panel shows the glucose infusion rate, and the bottom panel shows the glucose disposal rate (Rd). **b**, Weight curve for age-matched wild type and DADm mice started on 60% HFD at 8 weeks. **c**, Insulin tolerance test for mice in **b** at ZT8 after 15 weeks on HFD (WT: $n = 13$, DADm: $n = 12$). Data are presented as mean \pm s.e.m. * $P < 0.05$, ** $P < 0.01$.

(data not shown). However, 24-h studies showed marked alterations in cyclic expression of several critical genes involved in lipid metabolism in the liver (Fig. 4a). DADm mice showed phase shifts in the expression of genes involved in fat breakdown, such as carnitine palmitotransferase 1a (*Cpt1a*), medium chain acyl-CoA dehydrogenase (*MCAD*, also known as *Acadm*), and their transcriptional regulator peroxisome proliferator activated receptor α (*Ppara*). There was a trend towards increased expression of *Cpt1a* and *MCAD* after treatment of DADm hepatocytes with a PPAR- α agonist (Supplementary Fig. 8). ATP citrate lyase (*Acly*), which produces acetyl coA, and acetyl coA carboxylase 2 (*Acc2*, also known as *Acacb*), the malonyl CoA product of which allosterically inhibits *Cpt1a*, were also dysregulated but antiphase with *Cpt1a* (Fig. 4a). Elongation of long-chain fatty acids family member 6 (*Elovl6*), the deficiency of which favours leanness and insulin sensitivity²⁵, showed fourfold lower expression at ZT10 in DADm liver. In white adipose tissue, modest circadian changes in *Cpt1a* were noted but, unlike in the liver, *Acc2* and *Ppar- γ* expression were not altered (Fig. 4a). Taken together, these results indicate that *Ncor1*-*Hdac3* is required for normal circadian regulation of genes involved in the breakdown, biogenesis and modification of lipids in the liver.

To determine the mechanism of altered beta oxidation cycling in DADm mice, we examined cofactor and histone acetylation at well-characterized nuclear receptor response elements in the *Cpt1a* and *MCAD* genes. As expected, *Ncor1* was similarly recruited to the nuclear receptor responsive elements in the *Cpt1a* and *MCAD* genes in wild-type and DADm cells (Supplementary Fig. 9a)^{26,27}. However, *Hdac3* recruitment was markedly reduced at these same genomic sites (Fig. 4b). Moreover, circadian oscillation at these response elements was disturbed in the DADm livers in a pattern that paralleled the alterations in gene expression (Fig. 4c, d), whereas *Ncor1* recruitment remained unchanged

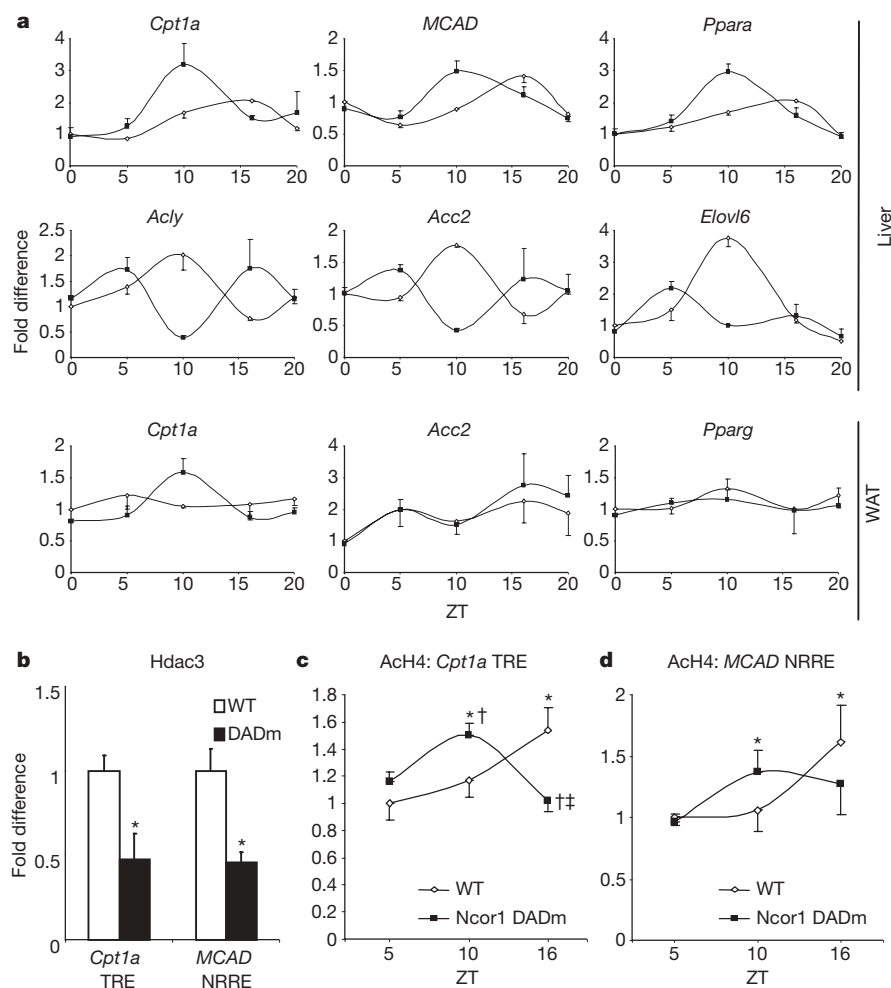


Figure 4 | Activation of Hdac3 by Ncor1 regulates circadian metabolic gene expression in the liver. **a**, Diurnal expression of lipid metabolic genes (wild type, open diamonds; DADm, black squares relative to wild type, ZT0). Lights on and off at ZT0 and ZT12, respectively ($n = 2-3$ per genotype per ZT). WAT, white adipose tissue. **b**, ChIP for Hdac3 from wild type (WT) and DADm MEFs ($n = 3$). **c**, **d**, ChIP for AcH4 from livers at *Cpt1a* TRE (**c**) and *MCAD* NRRE (**d**) ($n = 3$ per genotype per ZT). Quantitative PCR results are relative to wild type, ZT5. Data are presented as mean \pm s.e.m. * $P < 0.05$ relative to ZT5, † $P < 0.05$ relative to wild type, ‡ $P < 0.05$ relative to ZT10.

(Supplementary Fig. 9b). An intronic region of the *Cpt1a* gene did not exhibit oscillation of histone acetylation, demonstrating site specificity of circadian histone acetylation (Supplementary Fig. 9c).

We have demonstrated that the association between Hdac3 and the transcriptional corepressor Ncor1 regulates circadian behaviour and metabolism. A transcriptional coactivator recently identified as a regulator of circadian and metabolic pathways, PGC-1 α (also known as PPARGC1 α), does not possess intrinsic histone acetylase (HAT) activity, but interacts with HAT-containing coactivators⁷, and the Clock protein was recently shown to possess HAT activity². These findings emphasize that circadian metabolic transcriptional regulation is regulated at the level of histone acetylation and that the cycling of epigenetic modifications is critical to maintaining normal energy homeostasis. Loss of Ncor1-dependent Hdac3 activity did not lead to constant increases in gene expression, as might have been predicted, probably owing to complex interaction with other histone-modifying circadian factors. Indeed, dysregulation of the molecular clock and metabolic gene expression in the DADm mice undoubtedly reflects alterations in several interconnected pathways including the molecular clock as well as signalling by several nuclear receptors.

Recent studies have demonstrated critical links between circadian regulation and normal health, longevity and diet^{4,28,29}. The DADm mice showed markedly altered metabolic oscillatory expression, with a net effect of increased lipid consumption and a lean, insulin sensitive, obesity-resistant metabolic phenotype. Thus, although shift work and other disruptions of normal rhythms can be metabolically deleterious³⁰, alteration of normal circadian physiology can also be associated with a favourable metabolic profile. Targeting the Ncor1–Hdac3 deacetylase enzyme would be a highly specific means of combating diseases of nutritional excess including obesity, diabetes and the metabolic syndrome.

METHODS SUMMARY

Generation of DADm mice. The Y478A mutation was introduced into exon 13 of the *Ncor1* gene using a targeting vector containing a loxP-flanked neomycin resistance (*neo^R*) gene downstream from exon 13. The targeting vector was electroporated into C57BL/6 embryonic stem cells and a clone confirmed to contain the homologous targeted mutation was injected into C57BL/6 blastocysts. Blastocysts were implanted in pseudopregnant female mice and germline transmission led to the generation of C57BL/6 F₁ Ncor1^{Y478A/Neo^R+} mice. These mice were mated to C57BL/6 Tg^{EIIa-Cre} mice to remove the floxed *neo^R* cassette, generating C57BL/6 Ncor1^{Y478A/+} mice, which were bred to wild-type mice to obtain (Cre-)Ncor1^{Y478A/+} mice. Experimental cohorts were generated from (Cre-)Ncor1^{Y478A/+} × (Cre-)Ncor1^{Y478A/+} breedings.

Mice. Age-matched male mice were used for all experiments. Animals were housed at up to five per cage in a ventilated isolator cage system in a 12 h light/dark cycle, with free access to water and chow or a HFD (60 kcal% fat). Mice used for circadian gene expression experiments were housed under strict light/dark regulation and minimal disturbance for 3 weeks. For metabolic experiments, mice were placed on a HFD at 8 weeks and insulin tolerance tests were performed after 16 weeks on the diet using 0.75 U kg⁻¹ insulin. Mice underwent body composition analysis by NMR and monitoring of feeding, locomotor activity and indirect calorimetry. For hyperinsulinemic-euglycemic clamp studies, 2.5 or 5 mU kg⁻¹ min⁻¹ insulin was administered in chow-fed mice and HFD mice, respectively.

Full Methods and any associated references are available in the online version of the paper at www.nature.com/nature.

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

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Author Contributions T.A., M.B., K.H.K., R.S.A. and M.A.L. designed the research, T.A., K.M., K.L., A.A.-A., S.E.M. and J.A. acquired the data, T.A., A.A.-A., S.E.M., M.B., R.S.A. and M.A.L. analysed and interpreted the data, and T.A. and M.A.L. drafted the manuscript.

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METHODS

Generation of DADm mice. The targeting vector containing 3.5-kb arms was generated using RP23 Ncor1-containing BAC clone templates (Invitrogen) followed by subcloning of PCR products into a pBluescript KS backbone (Stratagene). The Y478A mutation was introduced into exon 13 of the *Ncor1* gene using site directed mutagenesis (Stratagene). A cassette containing a loxP-flanked neomycin resistance (*neo^R*) gene expressed from the phosphoglycerate kinase promoter was cloned within an intron approximately 500 base pairs (bp) downstream from exon 13. The negative selection diphtheria toxin gene was cloned downstream of the 3' arm. The targeting vector was linearized with XhoI and SacII (New England Biolabs) and electroporated into C57BL/6 embryonic stem cells (Chemicon). A clone confirmed to contain the homologous targeted mutation was injected into C57BL/6 blastocysts and these blastocysts were implanted in pseudopregnant female mice. Germline transmission led to the generation of C57BL/6 F₁ Ncor1^{Y478A^{Neo}/+} mice. These mice were mated to C57BL/6 Tg^{Elia-Cre} mice (Jackson Laboratory) to remove the floxed *neo^R* cassette, generating C57BL/6 Ncor1^{Y478A/+} mice which were bred to wild-type mice to obtain (Cre-)Ncor1^{Y478A/+} mice. Experimental cohorts were generated from (Cre-)Ncor1^{Y478A/+} × (Cre-)Ncor1^{Y478A/+} breedings.

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Tissue and cell collection. Tissue samples were collected after euthanasia and immediately frozen in liquid nitrogen. MEFs were collected from day 12.5 embryos and immortalized by repeated passages. Primary hepatocytes were obtained as described previously²⁰. Cells were grown at 37 °C in 5% CO₂. Primary MEFs were treated with 0.6 μM dexamethasone, 10 μg ml⁻¹ insulin, and 500 μM 3-isobutyl-1-methylxanthine (no thiazolidinedione) to induce adipocyte differentiation. Cells were collected and stained with oil red O on day 5 after the addition of the differentiation cocktail. Immortalized MEF lines were treated with MS-275 for 24 h at 1 μM before collection. For cell synchronization studies, MEFs were grown in serum free media for 24 h before synchronization

with 50% horse serum for 2 h, after which cells were switched back to serum-free media³³. Cells were treated with 5 μM succinylacetone for 12 h, 6 μM haem for 6 h, or 50 μM Wy-14,643 for 48 h. One micromolar of the siControl or siRev-erbβ (Dharmacon) double-stranded oligonucleotides were prepared with Nucleofector Solution V (Amaxa biosystems) and 1 × 10⁶ cells were electroporated according to the manufacturer's protocol. After 48 h, media was changed and cells were collected 24 h later.

Immunoprecipitation and immunoblotting. Samples for immunoprecipitation experiments were homogenized in a modified RIPA buffer containing protease inhibitor cocktail (Roche). Lysates were precleared with Protein G agarose beads and then incubated with mouse Hdac3 (Upstate) or mouse IgG (Santa Cruz) antibodies at 4 °C overnight followed by 1 h incubation with protein G agarose beads. Immunoprecipitates were washed five times with modified RIPA, eluted and subjected to immunoblot analysis. Blots were probed with the following primary antibodies: rabbit Ncor1³⁴, rabbit SMRT (Bethyl), rabbit Hdac3 (Abcam). For liver chromatin immunoprecipitations, 50 mg of tissue from each mouse was minced and crosslinked in 1% formaldehyde for 15 min. After two washes, cells were manually lysed using a dounce and then subjected to nuclear lysis and sonication. ChIP assays were performed according to the protocol of Upstate Biotechnology with minor modifications and the following antibodies: rabbit Hdac3 (Santa Cruz/Abcam), rabbit acetylated histone H4 (Upstate) and rabbit Ncor1 (Abcam). The following primer pairs were used for ChIP experiments: *Bmal1*, 5'-AGCCTAACGCAGAGCAGAAC-3', 5'-GCCAATCAGAGAGAGCGAAC-3'; *MCAD*, 5'-CACTGGGCACACAGTCTTCTTC-3', 5'-CCTTGCCCGAGCCTAAACT-3'; *Cpt1a* TRE, 5'-GGTGACGTTGGCTGAGCAA-3', 5'-TGAGCCCTGTACACGTTTTG-3'; *Cpt1a* intron, 5'-CAGCGCCTTGAACTTGCA-3', 5'-CAAACGGTCAAAGTACAGGAAAGTC-3'.

RNA isolation and real-time PCR analysis. Tissue samples were homogenized using a TissueLyser (Qiagen). RNA was isolated using a RNeasy lipid tissue kit (Qiagen) then subjected to reverse transcription (Applied Biosystems). mRNA transcripts were quantified by real-time PCR analysis using TaqMan (Applied Biosystems) or Sybr (Applied Biosystems). Samples were analysed using a Prism 7900 thermal cycler and sequence detector (Perkin Elmer/ABI). Sybr dissociation curves always indicated the formation of a single PCR product. Data was analysed with a threshold set in the linear range of amplification and processed on the basis of a standard curve of serial tenfold dilutions for each primer set. The gene of interest was normalized to an unaffected endogenous control gene (*GAPDH* or *36B4*) and plotted as mean fold change (± s.e.m.). Statistical analysis was performed using a Student's *t*-test.

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