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13. The 10 transcripts detected were those of early growth response protein 1 (EGR-1, GenBank accession number NM_001964), β-actin (NM_001101), γ-actin (NM_001614), c-myc (NM_002467), c-jun (NM_002228), cyclin D1 (NM_001758), interleukin-8 (IL-8, NM_000584), myeloid cell leukemia 1 (MCL1, NM_021960), transforming growth factor β immediate early gene (TIEG, NM_005655), and dual specificity kinase 1/MAP kinase phosphatase (DUSP, NM_004417). These genes were first detected singly, using Cy3 and Cy5 probes. Then they were barcoded to distinguishable combinations of fluorophores for multiplexed detection (coding scheme in Fig. 2C). This allows comparison of sensitivity of detection with different color codes to ensure reliability independent of color code. Full sequences of probes are provided in supporting online material.
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21. As in (*13*), but also including c-fos (GenBank accession number NM_005252), cysteine-rich angiogenic factor (Cyr61, NM_001554) and Fos-related antigen 1 (Fra-1, NM_005438) in the place of IL-8 and TIEG. Full sequences of probes are provided in supporting online material. Experiments were performed for logarithmically growing cells, starved (0 min), and 5, 10, 15, 20, 25, 35, 40, 45, 50, 60, 90, and 120 min after induction. Data were extrapolated for the 30-min time point and points between 60, 90, and 120 min.
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**Supporting Online Material**

www.sciencemag.org/cgi/content/full/297/5582/836/DC1
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# White Collar–1, a DNA Binding Transcription Factor and a Light Sensor

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**Blue light regulates many physiological processes in fungi, but their photoreceptors are not known. In *Neurospora crassa*, all light responses depend on the Per-Arnt-Sim (PAS) domain–containing transcription factor *white collar–1* (*wc-1*). By removing the WC-1 light, oxygen, or voltage domain, a specialized PAS domain that binds flavin mononucleotide in plant phototropins, we show that light responses are abolished, including light entrainment of the circadian clock. However, the WC-1–mediated dark activation of *frq* remains normal in this mutant, and the circadian clock can be entrained by temperature. Furthermore, we demonstrate that the purified *Neurospora* WC-1–WC-2 protein complex is associated with stoichiometric amounts of the chromophore flavin-adenine dinucleotide. Together, these observations suggest that WC-1 is the blue-light photoreceptor for the circadian clock and other light responses in *Neurospora*.**

Blue and near-ultraviolet light are known to regulate many physiological processes in a large number of organisms, including circadian clock functions from fungi to mammals. Currently, phototropins, cryptochromes, and a flavin-adenine dinucleotide (FAD)–containing adenylyl cyclase in *Euglena* are the three known types of eukaryotic blue-light photoreceptors, all of which are flavin-containing proteins (*1–3*). Most fungal photoresponses, ranging from growth responses to phototropism to carotenoid induction to circadian clock entrainment, are mediated by blue light (*4*). For *Neurospora crassa,* previous evidence has suggested that a flavin-containing blue-light photoreceptor is responsible for mediating all known light responses [reviewed in (*4*)].

The *white collar–1* (*wc-1*) and *wc-2* genes are required for all aspects of the known *Neurospora* blue-light responses, including induction of the circadian clock gene *frequency* (*frq*), which has been shown to mediate the light entrainment of the circadian clock (*4–10*). WC-1 and WC-2 are transcriptional activators that can bind to promoter elements upstream of light-inducible genes via their GATA-type zinc-finger DNA binding domains (*5*, *6*, *11*). Both proteins also contain Per-Arnt-Sim (PAS) domains, which are important for the in vivo formation of a nuclear complex between WC-1 and WC-2 (WCC) (*9*, *12*). In addition to their critical roles in light responses, WC-1 and WC-2 are also essential components of the *Neurospora frq-wc*–based circadian feedback loops because they activate the transcription of *frq* in the dark (*7*, *13–16*).

Sequence analysis of the WC-1 protein revealed that one of its three PAS domains (Fig. 1A) belongs to a specialized class of these domains known as a light, oxygen, or voltage (LOV) domain. These LOV domains have been best characterized in plant phototropins, in which each LOV domain binds a flavin mononucleotide (FMN) molecule and is able to undergo fully reversible photocycles (*1*, *17–19*). The crystal structure of a LOV-FMN complex revealed 11 residues in the vicinity of FMN that are highly conserved in LOV domains (*20*), including that of WC-1, suggesting that WC-1 may bind FMN and function as a blue-light photoreceptor.

We made a construct in which the LOV domain was deleted from the wild-type *wc-1* gene (Fig. 1A) and introduced it into a *wc-1* null strain (wc-1.lov) (*21*). The apparent molecular weight of mutant WC-1 was smaller than that of wild-type WC-1, and mutant WC-1 was present in a lower amount than wild-type WC-1 (Fig. 1B). The amount of FRQ in the wc-1.lov mutant was near normal in 22 hours of constant darkness (DD22) but did not increase in cultures grown in constant light (LL), indicating that the light induction of *frq* is lost in the mutant. This result was confirmed with Northern blot analyses of *frq* and *al-3* (a gene required for carotenoid biosynthesis) RNA amounts (Fig. 1C). As for FRQ in the dark, the amounts of *frq* mRNA were comparable in both strains (Fig. 1C, middle panel). These data indicate that the WC-1 LOV domain is essential for light-activated transcription of *frq* but not *frq* transcription activation in the dark.

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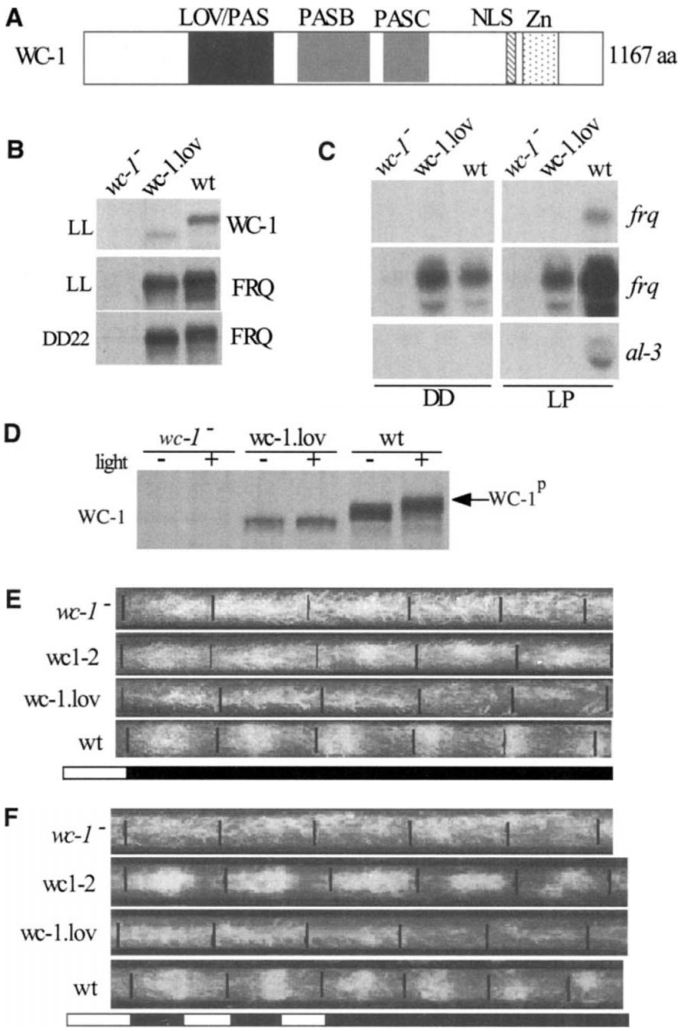



**Fig. 1.** The WC-1 LOV domain is required for light responses, including the light entrainment of the circadian clock. (**A**) The domain architecture of WC-1. NLS, nuclear localization signal; ZN, zinc-finger DNA binding domain; aa, amino acid. (**B**) Western blot analysis showing the amounts of WC-1 and FRQ. wt, wild-type; *wc-1*⁻, a *wc-1* null strain. (**C**) Northern blot analysis showing the amounts of *frq* and *al-3* mRNA. LP indicates that cultures were given 15 min of light treatment at the 24th hour of complete darkness. The middle panel is a longer exposure of the top panel. (**D**) Western blot analysis of WC-1 for cultures with (+) or without (–) 15 min of light pulse. WC-1ᵖ indicates the hyperphosphorylated WC-1 forms. (**E** and **F**) Race tube analysis in constant dark after a single LD transition (E) or LD cycles (F). wc1-2, the *wc-1*⁻ strain rescued by a wild-type *wc-1* gene.

A *wc-2* mutant strain (234w) that lacks most of the COOH-terminal part of the protein, including the DNA binding domain, is defective in most aspects of the light response (*6*, *9*). However, the light-induced hyperphosphorylation of WC-1 remained unaffected in this mutant (*22*), showing that WC-1 hyperphosphorylation is independent of transcriptional activation, that WC-2 acts downstream of WC-1, and that WC-2 is not the photoreceptor that directly receives the light signal.

Could WC-1 be the photoreceptor? Several "blind" phenotypes of the wc-1.lov mutant are consistent with this hypothesis: (i) there was no WC-1 hyperphosphorylation observed in the wc-1.lov mutant after 15 min of light (Fig. 1D); (ii) the circadian conidiation rhythm could not be synchronized in the wc-1.lov mutant grown under light-dark (LD) cycles (Fig. 1F); (iii) the wc-1.lov mutant was arrhythmic in constant darkness after either a single LD transition or LD cycles (Fig. 1, E and F), suggesting that the clock was not running under these conditions; and (iv) in contrast to the wild-type strain, the amounts of both *frq* RNA and FRQ protein in the wc-1.lov mutant were unaffected by the LD transition, and the amounts of *frq*, FRQ, and *(ccg-1)* stayed relatively constant in LL and in constant darkness (DD) with no circadian fluctuations (fig. S1, A and B).

The PAS domain of WC-2 is involved in WC-1–WC-2 and WCC-FRQ protein-protein interactions and the maintenance of the steady-state level of WC-1 (*9*). However, both the wild-type and mutant WC-1 proteins could form complexes with WC-2 and FRQ under LL conditions, suggesting that the circadian oscillator is intact in the mutant (Fig. 2). Together, these data demonstrate that in the wc-1.lov mutant, light responses are abolished and the circadian clock cannot be entrained by light, despite the near-normal activation of *frq* in the dark and the maintenance of WC-2 and FRQ interactions by the mutant WC-1.

If the circadian clock in the wc-1.lov mutant was functional but just blind, then it should be entrained by other nonphotic cues. To test whether temperature could entrain the wc-1.lov mutant as it does for normal *Neurospora* (*23*), we grew cultures with temperature cycles (12 hours at 20°C, then 12 hours at 25°C) in DD for 3 days and then switched them to a constant temperature of 25°C (Fig. 3A). After transfer to constant temperature, the *wc-1*⁻ (the *WGl* null) strain became arrhythmic; and the wild-type strain, the *wc-1*⁻ strain rescued by a wild-type *wc-1* gene (wc1-2), and the wc-1.lov strain exhibited a circadian conidiation rhythm, indicating that the clock can be reset by the temperature treatment. The amplitudes of the conidiation rhythm in the wc-1.lov mutant were lower than those of the wild-type, and the conidiation rhythm usually damped out after 3 to 4 days in constant darkness, suggesting that the lack of the LOV domain partially affected its function in the dark. Similar experiments were performed in LL (Fig. 3B). After transfer to constant temperature, the wild-type strain immediately became arrhythmic be-

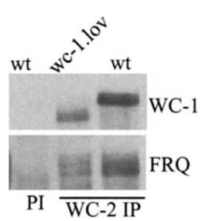
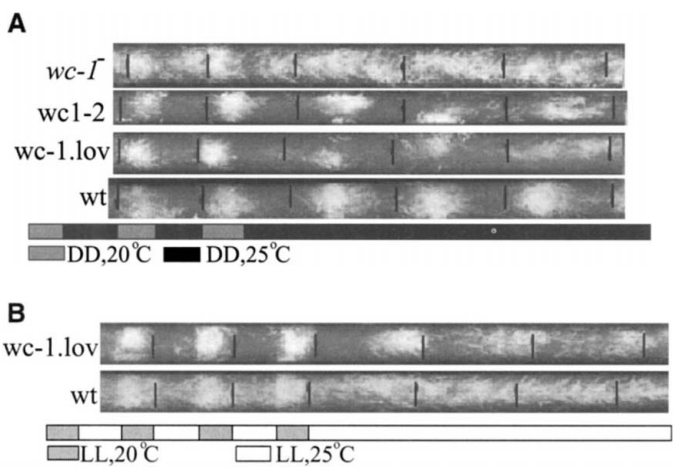



**Fig. 2.** Immunoprecipitation assay with WC-2 antiserum. PI, wild-type extract immunoprecipitated with the WC-2 preimmune serum; IP, immunoprecipitation.

**Fig. 3.** Circadian rhythms in the wc-1.lov strain could be entrained by temperature. (**A** and **B**) The race tube cultures were entrained by three 20°–25°C temperature cycles before they were kept at a constant temperature of 25°C in either DD (A) or LL (B).



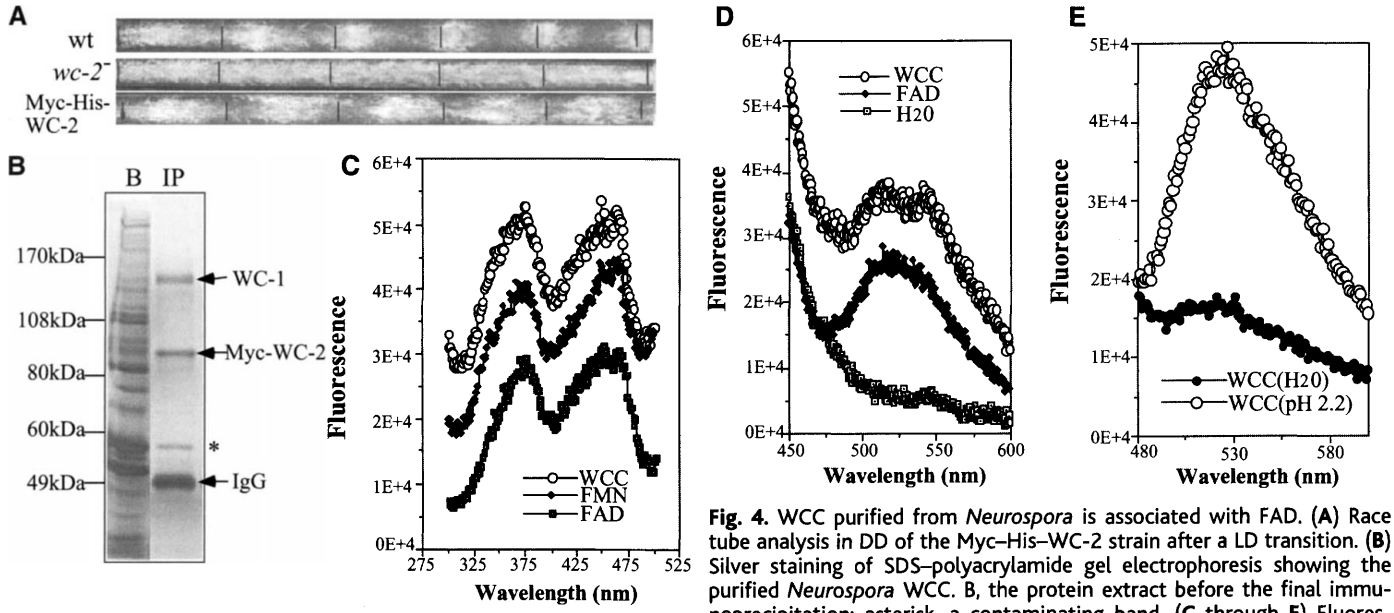



**Fig. 4.** WCC purified from *Neurospora* is associated with FAD. (**A**) Race tube analysis in DD of the Myc–His–WC-2 strain after a LD transition. (**B**) Silver staining of SDS–polyacrylamide gel electrophoresis showing the purified *Neurospora* WCC. B, the protein extract before the final immunoprecipitation; asterisk, a contaminating band. (**C** through **E**) Fluorescence excitation (C) and emission [(D) and (E)] spectra analyses.

cause of the suppression of the clock function by light, whereas the rhythm of the wc-1.lov strain persisted. These results, plus experiments on the circadian fluctuations of FRQ protein and *ccg-1* mRNA after the temperature treatment (fig. S2, A and B), demonstrate that the functions of WC-1 in light responses and in circadian feedback loops can be effectively separated by removing the LOV domain of WC-1. Furthermore, although the resetting of the clock requires changes in FRQ level, the signaling mechanisms of light and temperature entrainment are different, and the temperature entrainment pathway does not require the function of the WC proteins, as compared with the temperature and light entrainment pathways in *Drosophila* (*24*).

To further test whether WC-1 is the photoreceptor that directly receives the light signal, we sought in vivo evidence to show that WC-1 is associated with a flavin chromophore. Using *Neurospora* protein extracts from a strain containing epitope-tagged WC-2 (Myc–His–WC-2) (Fig. 4A), the WCC was purified (*21*). The final purification product (immunoprecipitation with c-Myc monoclonal antibody–coupled beads) contained four protein bands, including the c-Myc monoclonal immunoglobulin G band (Fig. 4B); the top two bands are WC-1 and the tagged WC-2, respectively, and the minor band at 60 kD is a contaminating band because it could also be immunoprecipitated with hemagglutinin antibody–coupled beads (*25*). No other protein was found to tightly bind WCC. The relative molar concentration of WC-1 and WC-2 was about 1:1, consistent with the 200-kD dimeric WCC (*16*).

To detect the existence of chromophore in WCC, fluorescence spectroscopic analyses were performed. As shown in Fig. 4, C and D, WCC contained a noncovalently bound fluorescent cofactor, with excitation and emission spectra very similar to those of free FMN and FAD standards. Like both of these, the WCC cofactor exhibited two excitation peaks at 370 and 450 nm and an emission peak at 520 nm, all of which are characteristic of flavins. The emission spectra of the WCC cofactor showed a substantial fluorescence increase at an acidic pH, indicating that it is FAD (Fig. 4E) (*26*). This identification was confirmed by thin-layer chromatography: The WCC fluorescent cofactor and FAD standard had identical retention factor values (0.08). In addition, comparison of the concentrations of WC-1 and FAD showed that FAD bound WC-1 stoichiometrically, with a molar ratio of about 1:1. Together, these observations suggest that FAD is the chromophore that binds to WC-1 and confirm previous physiological evidence suggesting that the *Neurospora* phototransduction mechanism is mediated by a flavin species.

A notable difference between the LOV domains of WC-1 and phototropins is a large extension in the loop connecting the αA and αC helices in the WC-1 LOV domain (fig. S3A). This extension may be important for accommodating the larger FAD molecule as compared with FMN (*21*). Similar extensions were also found in the LOV domains of several other proteins, including the *Neurospora* protein VIVID (VVD), a repressor of light-regulated processes (*27*), and ZTL and FKF1, the two closely related *Arabidopsis* proteins that regulate the timing of floral development and circadian periods (*28*, *29*). Phylogenetic analysis showed that the *ztl* and *fkf1* LOV domains are more closely related to those of WC-1 and VVD than to those of the plant phototropins (fig. S3B). Therefore, we suggest that these three proteins may also bind FAD to accomplish their roles in the light responses and circadian clock.

In this study, we have presented physiological, molecular, and biochemical data which strongly suggest that WC-1 is the blue-light photoreceptor for the circadian clock and other light responses in *Neurospora*. Thus, this fungal photoreceptor functions as both a DNA binding transcription factor and a light sensor. The nuclear localization of WC-1 and its ability to sense light and (together with WC-2) to bind to the promoters of light-inducible genes (*11*) ensure that the light signal is directly targeted to gene promoters to regulate transcription.

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Figs. S1 to S3

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# βAR Signaling Required for Diet-Induced Thermogenesis and Obesity Resistance

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**Excessive caloric intake is thought to be sensed by the brain, which then activates thermogenesis as a means of preventing obesity. The sympathetic nervous system, through β-adrenergic receptor (βAR) action on target tissues, is likely the efferent arm of this homeostatic mechanism. To test this hypothesis, we created mice that lack the three known βARs (β-less mice). β-less mice on a Chow diet had a reduced metabolic rate and were slightly obese. On a high-fat diet, β-less mice, in contrast to wild-type mice, developed massive obesity that was due entirely to a failure of diet-induced thermogenesis. These findings establish that βARs are necessary for diet-induced thermogenesis and that this efferent pathway plays a critical role in the body's defense against diet-induced obesity.**

In a prevalent view of body weight homeostasis, it is proposed that dietary excess is sensed by the brain, which, to avoid excessive weight gain, then triggers a reduction in food intake and an increase in energy expenditure (*1*). The latter phenomenon is termed "diet-induced thermogenesis" and is thought to be mediated by the sympathetic nervous system (SNS) and stimulation of βARs on thermogenically active target tissues (*2*, *3*). Brown adipose tissue (BAT), with its uncoupled mitochondrial respiration, is one such target tissue and has been suggested to be an important mediator of diet-induced thermogenesis (*4–6*). While this model (diet → brain → SNS → βARs → thermogenesis → protection from obesity) has great appeal and is widely cited, there has been no direct demonstration that such a pathway operates and is important in preventing diet-induced obesity. Previous attempts have included ablation of sympathetic nerves (*7–9*) and the generation of genetically altered mice that are unable to synthesize catecholamines (*10*). However, neither these perturbations, nor gene knockouts of individual βARs (*11–13*), have resulted in obesity, possibly because of nonspecific complications caused by loss of all adrenergic signaling (*10*) and functional redundancy between the three known βARs, which are coexpressed on brown adipocytes (*11*, *14*). To test this model, we created mice that lack the three known βARs (β-less).

Two lines of mice were derived for both wild-type (wt) and β-less genotypes from existing strains (*11*, *13*, *15*) (fig. S1). This was done to confirm that the phenotype of β-less mice is due to absence of βARs and to rule out genetic background effects. Experiments were performed with both lines of male and female β-less and wt mice, unless otherwise indicated, and representative data from males are shown. β-less mice were viable and fertile. On a Chow diet, β-less mice developed mild obesity by 20 weeks compared with wt mice (Fig. 1A). β-less females similarly developed mild obesity (*16*). The increased body weight was attributable mostly to increased body fat in β-less mice (table S1). Leptin levels were also increased in β-less mice (7.63 ± 0.9 ng/ml) versus wt mice (3.9 ± 1.03 ng/ml, $P < 0.05$, $n = 3$), consistent with increased fat mass. Food intake (Fig. 1 B) and body temperature (*15*) in β-less mice were similar to those of wt controls. Metabolic rate, however, as indicated by oxygen consumption, was on average 16% lower in 8-week-old β-less mice (56 ± 1.46 ml $kg^{-1}$ $min^{-1}$) compared with weight-matched wt controls (67.34 ± 1.57 ml $kg^{-1}$ $min^{-1}$; $n = 8$, $P < 0.05$). This decrement in metabolic rate persists when oxygen consumption is expressed per gram of lean body mass (Fig. 1C) and per mouse (1.38 ± 0.05 versus 1.59 ± 0.09 ml $min^{-1}$ per mouse in wt; $P < 0.05$, $n = 6$ in each group). The lower metabolic rate in β-less mice was not due to measurable differences in thyroid hormone levels or physical activity (*15*).

We analyzed BAT in β-less versus wt mice because βARs have been shown to stimulate the development and function of this thermogenic adipose tissue (*17*). The interscapular BAT in β-less mice housed at room temperature (22°C) was markedly enlarged and pale in comparison with BAT from wt controls (*16*). The BAT from β-less mice contained large cells with unilocular triglyceride deposits (line 1 mice in Fig. 2A, line 2 mice in fig. S2), similar to BAT from denervated or catecholamine-deficient mice (*10*, *18*). Because $\beta_1$ and $\beta_3$ ARs stimulate proliferation and differentiation of BAT in vitro (*17*), we also derived mice lacking only these two receptors. Unexpectedly, $\beta_{1,3}$-less mice had normal BAT weight (*16*), and normal BAT appearance by histology (Fig. 2A). Thus, the presence of $\beta_2$AR alone is sufficient for normal BAT morphology. The BAT-specific thermogenic molecule, uncoupling protein–1 (UCP-1), was abundantly expressed in wt mice, whereas β-less mice expressed lower levels that were apparent as a cytoplasmic rim around unilocular triglyceride deposits (Fig. 2B). Leptin expression, which is normally restricted to white adipose tissue (WAT), was expressed in BAT of β-less mice, but not wt mice (Fig. 2C). Thus,

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