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## Estrogen-Related Receptors Mediate the Adaptive Response of Brown Adipose Tissue to Adrenergic Stimulation

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### SUMMARY

Adrenergic stimulation of brown adipose tissue (BAT) induces acute and long-term responses. The acute adrenergic response activates thermogenesis by uncoupling oxidative phosphorylation and enabling increased substrate oxidation. Long-term, adrenergic signaling remodels BAT, inducing adaptive transcriptional changes that expand thermogenic capacity. Here, we show that the estrogen-related receptors alpha and gamma (ERR $\alpha$ , ERR $\gamma$ ) are collectively critical effectors of adrenergically stimulated transcriptional reprogramming of BAT. Mice lacking adipose ERRs (ERR $\alpha\gamma^{Ad-/-}$ ) have reduced oxidative and thermogenic capacity and rapidly become hypothermic when exposed to cold. ERR $\alpha\gamma^{Ad-/-}$  mice treated long term with a  $\beta_3$ -adrenergic agonist fail to expand oxidative or thermogenic capacity and do not increase energy expenditure in response to norepinephrine (NE). Furthermore, ERR $\alpha\gamma^{Ad-/-}$  mice fed a high-fat diet do not lose weight or show improved glucose tolerance when dosed with  $\beta_3$ -adrenergic agonists. The molecular basis of these defects is the finding that ERRs mediate the bulk of the transcriptional response to adrenergic stimulation.

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Conceptualization, E.L.B. and A.K.; Methodology, E.L.B. and A.K.; Investigation, E.L.B., B.C.H., E.E., J.-S.W., M.L.G., S.C., M.S.-A., and V.A.; Writing – Original draft, E.L.B. and A.K.; Writing – Review and Editing, E.L.B., B.C.H., E.E., J.-S.W., M.L.G., V.A., M.S.-A., B.C., and A.K.; Supervision, E.L.B. and A.K.; Funding Acquisition, E.L.B. and A.K.; Resources, B.C. and M.S.-A.

### DATA AND SOFTWARE AVAILABILITY

The RNA sequencing gene expression data can be found at GEO: GSE104285.

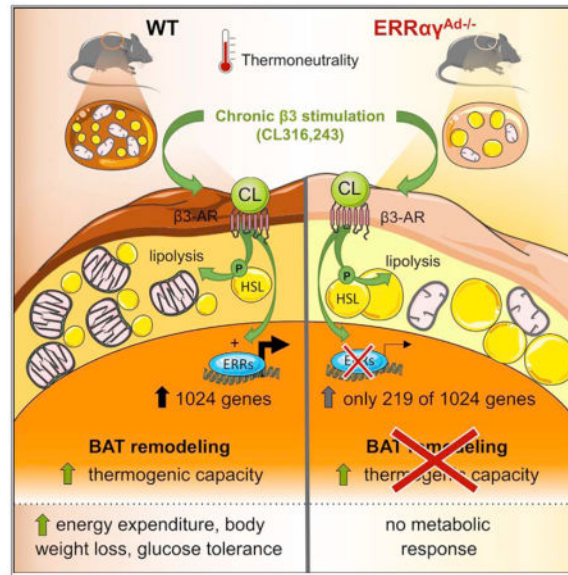
### SUPPLEMENTAL INFORMATION

Supplemental Information includes Transparent Methods, seven figures, and three tables and can be found with this article online at <https://doi.org/10.1016/j.isci.2018.03.005>.

### DECLARATION OF INTERESTS

The authors declare no competing interests.

## Graphical abstract



## INTRODUCTION

Brown adipose tissue (BAT) specializes in the regulated production of heat, also known as “adaptive” or “non-shivering” thermogenesis (Cannon and Nedergaard, 2004). To fulfill this function, BAT is rich in mitochondria and expresses the mitochondrial uncoupling protein 1 (Ucp1). Upon exposure to cold, local release of the adrenergic agonist norepinephrine (NE) induces thermogenesis by activating Ucp1, which generates heat by dissipating the mitochondrial proton gradient and allowing high rates of substrate oxidation. The capacity of BAT to convert stored chemical energy to heat is important for the defense of body temperature in cold environments and may also serve to expend energy at times of caloric excess, as in “diet-induced thermogenesis,” and thus counteract obesity (Cannon and Nedergaard, 2004). Importantly, the thermogenic capacity of BAT shows plasticity: it can be expanded in response to repeated or prolonged exposure to cold or attenuated and even lost in response to thermoneutral environments, aging, obesity, or denervation of the tissue (Dulloo and Miller, 1984; Ouellet et al., 2011; Saito et al., 2009; Yoneshiro et al., 2011). Although it is well recognized that adrenergic stimulation is the main signal that drives the expansion of thermogenic capacity, the transcription factors that transform this signal into long-term adaptive changes in energy expenditure remain poorly defined.

The estrogen-related receptors ( $ERR\alpha$ ,  $ERR\beta$ , and  $ERR\gamma$ ) are closely related members of the nuclear receptor family. They have no known endogenous ligands and, despite their name, do not bind estrogen (Giguere et al., 1988). Several features of ERRs suggest that these receptors could play important roles in the adrenergic-driven remodeling of BAT. First, they are highly and preferentially expressed in BAT vs. white adipose tissue (WAT), with  $ERR\alpha$  being the most abundant, followed by  $ERR\gamma$  and then  $ERR\beta$  (Bookout et al., 2006; Gantner et al., 2014). Second, ERRs are strongly activated by PGC-1 $\alpha$  and Gadd45 $\gamma$ , two regulators that are induced by cold in BAT (Gantner et al., 2014; Puigserver et al., 1998).

Finally, ERRs directly regulate the transcription of genes important for oxidative and thermogenic capacity. ERR $\alpha$  controls genes encoding factors of mitochondrial biogenesis, tricarboxylic acid (TCA) cycle, lipid oxidation, and angiogenesis (Arany et al., 2008; Huss et al., 2004, 2015; Mootha et al., 2004; Schreiber et al., 2004). ERR $\gamma$  shares many ERR $\alpha$  targets and when overexpressed can induce Ucp1 expression and oxidative capacity in brown adipocytes (Dixen et al., 2013; Dufour et al., 2007; Gantner et al., 2014). However, whole-body ERR $\alpha$  KO mice show no defects in the transcriptional response to adrenergic stimulation, even though they have decreased BAT mitochondrial content and are sensitive to cold (Luo et al., 2003; Villena et al., 2007). The physiological roles of ERR $\gamma$  or ERR $\beta$  in BAT are not known.

To address the role of adipose ERRs in BAT physiology and specifically the transcriptional remodeling that takes place in response to adrenergic stimulation, we have generated adipose tissue-specific knockout mice lacking different combinations of ERRs. Because young mice grown at regular vivarium temperatures need BAT function to effectively defend their body temperature, we have bred and raised WT and ERR<sup>Ad-/-</sup> at thermoneutrality. This approach minimizes confounding factors such as increased adrenergic tone in mice that have BAT defects (Schulz et al., 2013). Here, we show that mice lacking either ERR $\alpha$  alone or both ERR $\beta$  and ERR $\gamma$  in adipose tissue have minor changes in their BAT, whereas mice lacking ERR $\alpha$  and ERR $\gamma$  (the two main isoforms in BAT) have severe defects in oxidative and thermogenic capacity. Remarkably, when mice are treated with a  $\beta_3$ -adrenergic agonist, the transcriptional response of BAT is virtually entirely dependent on ERRs. Mice lacking adipose ERR $\alpha$  and ERR $\gamma$  also fail to enhance their oxidative and thermogenic capacity, even though adrenergic cytoplasmic signaling is preserved. Our findings show that ERRs are essential for BAT remodeling in response to adrenergic stimulation and suggest that ERR activation may sensitize BAT to adrenergic signals and lead to increased energy expenditure.

## RESULTS

### ERRs Act Collectively and in a Redundant Manner to Protect BAT Oxidative Capacity *In Vivo*

We recently showed that ERRs act redundantly to preserve mitochondrial function in primary brown adipocytes (Gantner et al., 2016), suggesting that ERRs may collectively control brown adipocyte oxidative and thermogenic function *in vivo*. To test this notion, we generated three lines of mice lacking different combinations of ERRs specifically in adipose tissue: mice lacking just ERR $\alpha$ , the most abundant ERR in adipose tissue (ERR $\alpha$ <sup>Ad-/-</sup>); mice lacking ERR $\beta$  and ERR $\gamma$ , the two ERRs that are highly enriched in BAT compared with WAT (ERR $\beta\gamma$ <sup>Ad-/-</sup>); and mice that lack ERR $\alpha$  and ERR $\gamma$ , the two most abundant ERRs in BAT and WAT (ERR $\alpha\gamma$ <sup>Ad-/-</sup>) (Dixen et al., 2013; Gantner et al., 2014). To minimize the effect of thermal stress at standard vivarium temperatures, all mice were born and raised at thermoneutrality (30°C). Loss of ERRs in BAT was confirmed at the mRNA level for all three isoforms (Figure 1A) and at the protein level for ERR $\alpha$  and ERR $\gamma$  (Figure 1B); ERR $\beta$  protein could not be detected reliably in WT BAT with currently available antibodies. Decreases in ERR mRNA were also observed in inguinal WAT, but not skeletal muscle, confirming that the adiponectin promoter-driven knockout was tissue specific

(Figures S1A and S1B). Notably,  $ERR\beta$  mRNA was nearly undetectable in the BAT of  $ERR\alpha\gamma^{Ad-/-}$  mice, suggesting that double knockout of  $ERR\alpha$  and  $ERR\gamma$  results in the practical loss of all three ERRs (Figure 1A). We next measured the mRNA levels in BAT of known ERR target genes involved in the TCA cycle and oxidative phosphorylation (OxPhos; Dufour et al., 2007; Schreiber et al., 2004; Villena et al., 2007). Loss of  $ERR\alpha$  alone ( $ERR\alpha^{Ad-/-}$ ) showed decreases in many but not all of these genes, whereas loss of  $ERR\beta$  and  $ERR\gamma$  ( $ERR\beta\gamma^{Ad-/-}$ ) had no significant effect (Figure 1C). However, the parallel loss of  $ERR\alpha$  and  $ERR\gamma$  ( $ERR\alpha\gamma^{Ad-/-}$ ) resulted in larger decreases and in all tested ERR targets, consistent with ERRs complementing each other in regulating gene expression in BAT (Figure 1C). Similarly,  $ERR\alpha^{Ad-/-}$  and  $ERR\beta\gamma^{Ad-/-}$  mice showed minor defects in BAT OxPhos protein and mtDNA content, but  $ERR\alpha\gamma^{Ad-/-}$  mice displayed a dramatic loss of BAT OxPhos complexes I–IV and a ~60% reduction in mtDNA content (Figures 1D–1F). These data show that ERRs display robust redundancy in protecting BAT oxidative capacity *in vivo*. Thus, to test the collective role of ERRs in adipose tissue function, we focused on studying BAT function in the  $ERR\alpha\gamma^{Ad-/-}$  mice, which showed pronounced defects in oxidative capacity.

### Loss of $ERR\alpha$ and $ERR\gamma$ Impairs BAT Thermogenic Capacity without Affecting Adipogenesis or BAT Identity

BAT is characterized by its brown color, the presence of small multilocular lipid droplets, a high density of mitochondria, and the expression of *Ucp1* (Cannon and Nedergaard, 2004). In mice lacking adipose  $ERR\alpha$  and  $ERR\gamma$ , the interscapular BAT depot was paler than WT BAT (Figure 2A) and showed notably increased lipid accumulation (Figure 2B). Electron microscopy imaging revealed sparse BAT mitochondria that were often malformed and with few visible cristae (Figure 2C). The abnormal mitochondrial structure was coupled with decreased expression of genes important for mitochondrial morphology and cristae structure (Figure 2D). Importantly, loss of adipose ERRs did not result in decreased expression of markers of BAT differentiation (*Prdm16*, *Zic1*, *Lhx8*, *Ebf2*) or adipogenesis (*Pparg*, *Fabp4*), indicating that the lipid accumulation and changes in mitochondria were not secondary to defects in the program of development or differentiation of BAT (Figure 2E). In support of the dramatic decrease in expression of TCA genes and OxPhos complexes I–IV shown in Figure 1, other genes of these pathways were also significantly reduced (*Cox7a1* and *Cs*) in  $ERR\alpha\gamma^{Ad-/-}$  BAT (Figure 2F).  $ERR\alpha\gamma^{Ad-/-}$  BAT also displayed decreased expression of genes important for fatty acid oxidation (Figure 2G) and increased expression of genes that regulate fatty acid biosynthesis and storage (Figure 2H), which may contribute to the increase in lipid accumulation. Additional lipid metabolism genes and other pathways, such as creatine metabolism, were also altered in  $ERR\alpha\gamma^{Ad-/-}$  mice (Figures S2A and S2B). Finally, the expression of two hallmark genes essential for thermogenesis, *Ucp1* and *Dio2*, was severely defective in BAT of  $ERR\alpha\gamma^{Ad-/-}$  mice (Figure 2I). *Ucp1* protein levels were also dramatically decreased (Figure 2J), in a manner consistent again with largely redundant roles of ERRs (Figure S2C). The decreased *Ucp1* expression suggested that thermogenic activity would be greatly reduced. Indeed, when exposed to 4°C,  $ERR\alpha\gamma^{Ad-/-}$  mice were very cold intolerant and became hypothermic in less than 2.5 hr (Figure 2K). These findings show that adipose ERRs are required to maintain proper oxidative and thermogenic capacity in BAT and to defend body temperature upon acute cold exposure.

To gain insights into the level at which ERRs control mitochondrial content and thermogenic function, we next assessed the expression levels of other transcriptional regulators of oxidative metabolism and thermogenesis genes. The two nuclear transcriptional co-regulators that drive mitochondrial biogenesis and *Ucp1* expression in BAT, *Pparg1a* and *Pparg1b* (Puigserver et al., 1998; Uldry et al., 2006), were moderately up- and down-regulated, respectively, at the mRNA level (Figure 2L); assessment of their protein expression showed a trend for both co-regulators to be at higher levels in  $ERR\alpha\gamma^{Ad-/-}$  than WT BAT (Figure S2D). The mRNA levels of other regulators of BAT oxidative capacity and *Ucp1* were not altered (*Nrf1* and *Gadd45γ*), moderately (320%) decreased (*Gabpa*), or increased (*Irf4*) (Figure 2L). The small changes in the expression of these regulators are thus unlikely to explain the dramatic loss of mitochondrial structure and proteins. On the other hand, we observed decreases in the expression of nuclear genes that encode regulators of mtDNA expression, such as *Tfam*, *Tfb2m*, *Polrmt*, and most notably the ERR targets *Sirt3* and *Endog* (Giralt et al., 2011; McDermott-Roe et al., 2011; Figure 2M), suggesting that the loss of ERRs is sufficient to compromise expression of genes important for mitochondrial biogenesis and thermogenesis even when other known nuclear transcriptional regulators of these programs are largely unaffected.

### Adipose ERRs Are Essential for the Remodeling of BAT Induced by $\beta_3$ -Adrenergic Agonists

Adrenergic stimulation of BAT promotes tissue remodeling by enhancing mitochondrial biogenesis and *Ucp1* expression, thereby increasing long-term oxidative and thermogenic capacity (Cannon and Nedergaard, 2004). To determine if ERRs are required for BAT remodeling in response to adrenergic stimulation, mice were injected with the  $\beta_3$ -specific adrenergic agonist CL316,243 or PBS as a control, for 10 days. As expected, WT mice treated with CL316,243 had darker BAT depots (Figure 3A; lower left panel) and decreased lipid accumulation, relative to WT mice treated with PBS (Figure 3B; lower left panel). These responses to CL316,243 treatment were completely dependent on ERRs, as PBS- and CL316,243-treated  $ERR\alpha\gamma^{Ad-/-}$  mice had similar pale color and high lipid content in their BAT depots (Figures 3A and 3B). Moreover, CL316,243 treatment resulted in mitochondrial changes, such as increased cristae density, in WT but not in  $ERR\alpha\gamma^{Ad-/-}$  mice (Figure 3C). Instead, mitochondria from  $ERR\alpha\gamma^{Ad-/-}$  mice treated with CL316,243 appeared to form abnormal circle-shaped cristae structures (Figure 3C; lower right panel). mtDNA content increased upon CL316,243 treatment in both WT and  $ERR\alpha\gamma^{Ad-/-}$  mice but remained very low in  $ERR\alpha\gamma^{Ad-/-}$  mice (Figure 3D). In WT BAT, CL316,243 treatment also elicited greater than 2-fold increases in protein expression of OxPhos complexes I and II and smaller but significant increases in complexes III and IV. These increases were considerably blunted in the BAT of  $ERR\alpha\gamma^{Ad-/-}$  mice (Figures 3E and 3F). Finally, the ability of CL316,243 to induce *Ucp1* protein (Figure 3G) and *Ucp1* mRNA (Figure 3H) in WT BAT was largely lost in the BAT of  $ERR\alpha\gamma^{Ad-/-}$  mice. Notably, the defects in the response to CL316,243 were not due to impaired adrenergic signaling. Primary brown adipocytes isolated from  $ERR\alpha\gamma^{Ad-/-}$  mice displayed full induction of hormone-sensitive lipase (HSL) phosphorylation and of lipolysis upon NE or CL316,243 stimulation (Figures S3A and S3B). Moreover, acute treatment of mice with CL316,243 induced the phosphorylation of HSL, the MAPK p38, and the transcription factor ATF2 in  $ERR\alpha\gamma^{Ad-/-}$  mice (Figures S3C

and S3D). Finally, CL316,243 induced lipolysis in  $ERR\alpha\gamma^{Ad-/-}$  BAT explants as efficiently, if not better than in WT BAT explants (Figure S3E).

To obtain an unbiased view of the contribution of ERRs to the  $\beta_3$ -adrenergic-induced remodeling of BAT, we next used RNA sequencing to compare the global transcriptional changes induced by 10 days of CL316,243 treatment in WT and  $ERR\alpha\gamma^{Ad-/-}$  BAT depots. CL316,243 enhanced the expression of 1,024 genes in WT mice but only 352 genes in  $ERR\alpha\gamma^{Ad-/-}$  mice (applying cut-offs of 1.5-fold increase and  $p < 0.05$ ; Figures 4A and 4B). Strikingly, only 219 of the 1,024 genes induced by CL316,243 in WT mice were significantly induced in  $ERR\alpha\gamma^{Ad-/-}$  BAT depots, showing that ERRs are required for the vast majority (380%) of the transcriptional adaptation to chronic  $\beta_3$ -adrenergic stimulation (Figures 4A and 4B). Moreover, the absolute levels of 109 of the 219 genes induced in both WT and KO mice were significantly lower in  $ERR\alpha\gamma^{Ad-/-}$  BAT relative to WT BAT (Figures 4Aii and 4Bii). Overall, 389% of the genes induced by CL316,243 in WT BAT were dependent on ERRs for their full induction. Pathway analyses of the differentially expressed genes identified “mitochondrial dysfunction” and “oxidative phosphorylation” as the top two canonical pathways regulated by CL316,243 in WT BAT; neither of these pathways was significantly regulated by CL316,243 in  $ERR\alpha\gamma^{Ad-/-}$  BAT (Table 1). Of the top pathways regulated by CL316,243 in WT BAT, “Fatty-acid  $\beta$ -oxidation” remained preserved and became the top regulated pathway in  $ERR\alpha\gamma^{Ad-/-}$  BAT (Tables 1 and S1). Thus the loss of ERRs selectively altered the nature of the CL316,243-induced response by affecting specific and highly regulated pathways, rather than just attenuating CL316,243-induced responses across all pathways. Notably, the role of ERRs in the CL316,243-induced transcriptional remodeling extended beyond the predicted regulation of mitochondrial, OxPhos, and TCA cycle genes. Among the 20 genes highest induced by CL316,243 in WT BAT, 14 genes rely on ERRs for their induction and these include genes of lipid, phospholipid, and glycosphingolipid synthesis, as well as signaling proteins, a  $K^+$  channel, a carbonic anhydrase, and proteins of unknown function (Figure S4A).

To gain insights into the global roles of BAT ERRs, we next analyzed the RNA-sequencing data to define genes and pathways whose expression is affected by ERRs, by comparing WT and  $ERR\alpha\gamma^{Ad-/-}$  BAT of PBS- or CL316,243-treated mice (Figures 4C and 4D). The top ERR-regulated pathways were “oxidative phosphorylation,” “mitochondrial dysfunction,” and “TCA cycle,” i.e., the same pathways that were induced by CL316,243 in WT BAT (Tables S2 and 1). Notably, a heatmap representation of ERR-dependent gene expression shows that the levels of many of these genes were enhanced by CL316,243 in WT BAT (Figure 4C). Furthermore, ERRs affected the expression of a broader set of genes in CL316,243-treated than in PBS-treated mice, suggesting that ERR signaling was enhanced by the  $\beta_3$ -adrenergic agonist (Figure 4D). In summary, there was a high correlation between ERR dependency and CL316,243 induction of gene expression, consistent with the notions that (1) the majority of the transcriptional changes induced by CL316,243 require ERRs and (2) ERR signaling is enhanced by CL316,243.

## ERRs Contribute to the CL316,243-Induced Browning of Inguinal WAT

In addition to its effects on BAT,  $\beta_3$ -adrenergic stimulation leads to the appearance of brown-like adipocytes in inguinal WAT (ingWAT), a process known as browning or beiging (Granneman et al., 2005; Guerra et al., 1998). Therefore we assessed the ability of CL316,243 to induce browning of ingWAT in WT and  $ERR\alpha\gamma^{Ad-/-}$  mice. CL316,243 treatment led to the prominent appearance of multilocular adipocytes in the ingWAT of WT mice but had minimal effects on the adipocytes of the ingWAT of  $ERR\alpha\gamma^{Ad-/-}$  mice (Figure 5A). CL316,243 also significantly increased the protein levels of all five OxPhos complexes in WT ingWAT (Figures 5B and 5C), a response that was blunted in  $ERR\alpha\gamma^{Ad-/-}$  mice. Similarly, CL316,243 treatment dramatically induced Ucp1 protein and mRNA in WT ingWAT (nearly 100-fold) but had notably more modest effects (310-fold) on *Ucp1* expression in  $ERR\alpha\gamma^{Ad-/-}$  ingWAT (Figures 5D and 5E). Similarly blunted responses to CL316,243 were seen for several other genes involved in the TCA and OxPhos (Figure 5F). The reduced response to CL316,243 was not due to impaired  $\beta_3$ -adrenergic signaling, as primary inguinal white adipocytes isolated from these mice displayed full induction of HSL phosphorylation and lipolysis upon NE or CL316,243 stimulation (Figure S5). In summary, the ingWAT of  $ERR\alpha\gamma^{Ad-/-}$  mice showed impaired “browning,” as assessed by histological analysis and measurements of mitochondrial protein and Ucp1 expression, suggesting that ERRs also contribute to the remodeling of ingWAT driven by  $\beta_3$ -adrenergic signaling.

## Adipose ERRs Are Important for the Acute Enhancement of Whole-Body Metabolism by $\beta$ -Adrenergic Stimulation

Adrenergic activation of BAT enhances whole-body energy expenditure (Xiao et al., 2015). To determine the relative significance of adipose ERRs to whole-body metabolism, we tested the acute metabolic response of WT and  $ERR\alpha\gamma^{Ad-/-}$  mice to adrenergic agonists, using indirect calorimetry. Notably,  $ERR\alpha\gamma^{Ad-/-}$  and WT littermates treated with PBS showed no differences in oxygen consumption, body temperature, or respiratory exchange ratio (RER), suggesting that the decreased oxidative and thermogenic capacity of  $ERR\alpha\gamma^{Ad-/-}$  mice is not important for whole-body metabolism in a thermoneutral, basal state (Figures 6A–6C and S6A). An injection of CL316,243 led to a dramatic increase in oxygen consumption in WT mice but had a minimal effect, similar to injection of PBS, in  $ERR\alpha\gamma^{Ad-/-}$  mice (Figure 6D), demonstrating that ERRs are required for the metabolic response to CL316,243. There were no differences in physical activity between the groups (Figures S6B and S6D). Parallel to the increase in oxygen consumption, CL316,243 caused a small but significant and sustained (over 36 hr) increase in basal body temperature of WT mice that was not seen in  $ERR\alpha\gamma^{Ad-/-}$  mice (Figure 6E). CL316,243 also led to a significant decrease in the RER of WT mice, consistent with increased lipid metabolism (Figures 6C and S6C). The RER of CL316,243-treated  $ERR\alpha\gamma^{Ad-/-}$  mice was not significantly different from that of WT and  $ERR\alpha\gamma^{Ad-/-}$  mice injected with PBS (Figure 6C, S6A, and S6C), although it is possible that a CL316,243-induced increase in adipose tissue lipolysis may have increased lipid utilization in other tissues, such as skeletal muscle. Interestingly, WT mice treated with CL316,243 for 10 days displayed a mild, but significant, increase in oxygen consumption even on the day after the injection (during the active phase, 32–44 hr after an injection [Figure 6F]), suggesting a prolonged effect of chronic  $\beta_3$ -adrenergic stimulation on whole-body energy expenditure. Again, this was not seen in

ERR $\alpha$  $\gamma^{Ad-/-}$  mice. Overall, our data show that ERRs are required for the metabolic response to  $\beta_3$ -adrenergic stimulation.

The  $\beta_3$ -adrenergic agonist CL316,243 is a pharmacologic agent that acts selectively and potently on adipose tissue. To test the relative significance of ERRs in a more physiological context, we next compared the metabolic response of WT and ERR $\alpha$  $\gamma^{Ad-/-}$  mice to the native agonist, NE, which activates a wider range of adrenergic receptors, including  $\beta_1$  and  $\alpha_1$  types (Nedergaard and Cannon, 2014). NE was administered to naive mice (treated with PBS), as well as to mice with “trained” BAT (treated with CL316,243 for 10 days). NE injection evoked significantly higher oxygen consumption rates in WT mice than in ERR $\alpha$  $\gamma^{Ad-/-}$  mice, in both PBS- and CL316,243-treated groups (Figures 6G and 6H), showing that adipose ERRs are important for whole-body oxidative and thermogenic capacity in a physiological context. Furthermore, the difference in maximal oxygen consumption rates between WT and ERR $\alpha$  $\gamma^{Ad-/-}$  mice treated with NE was more pronounced in mice that had received CL316,243 for 10 days relative to mice treated with PBS (Figure S6E), supporting the notion that CL316,243-dependent remodeling of adipose tissues increased the thermogenic capacity in WT but not in ERR $\alpha$  $\gamma^{Ad-/-}$  mice.

### ERRs Are Required for CL316,243-Induced Weight Loss and Improved Glucose Tolerance in HFD Mice

The mice studied earlier were fed a chow diet and were fairly lean. CL316,243 treatment induced a decrease in the adiposity of WT mice that was not seen in ERR $\alpha$  $\gamma^{Ad-/-}$  mice, but it did not result in significant changes in body weight (Figures S6F and S6G). CL316,243 has been shown to confer greater metabolic improvements in mice fed a high-fat diet (HFD) compared with chow diet (Xiao et al., 2015). Hence, we next put WT and ERR $\alpha$  $\gamma^{Ad-/-}$  mice on an HFD before evaluating the effects of CL316,243 treatment. WT and ERR $\alpha$  $\gamma^{Ad-/-}$  mice had a similar food intake (Figure S7A), and after 18 weeks on the HFD, similar body weights and glucose tolerance (Figures 7A and 7B). Subsequent treatment of the mice with CL316,243 (14 injections over 4 weeks, given every other day, since we observed sustained effects of CL316,243 on energy expenditure [Figure 6F]) led to significantly higher body weight loss in WT mice than in ERR $\alpha$  $\gamma^{Ad-/-}$  mice (Figures 7C and 7D), with WT mice losing on average 32.0% and ERR $\alpha$  $\gamma^{Ad-/-}$  mice losing just 3.7% of their body weight. CL316,243 treatment also improved glucose tolerance in WT mice (Figure 7E) but had no effect in ERR $\alpha$  $\gamma^{Ad-/-}$  mice (Figure 7F), revealing that adipose ERRs are required for the beneficial metabolic effects of  $\beta_3$ -agonists.

## DISCUSSION

Deciphering the transcriptional networks that regulate BAT development and function is important for developing therapeutic strategies to increase energy expenditure via non-shivering thermogenesis for the treatment of obesity. In the last several years, there has been much progress in understanding transcriptional mechanisms that control the commitment of stem cells to the brown/beige preadipocyte lineage and/or the differentiation of preadipocytes to mature brown or beige adipocytes (Lynes and Tseng, 2015; Wang and Seale, 2016). Once differentiated, however, brown adipocytes require additional signals and



factors to maintain and expand their thermogenic capacity (Cannon and Nedergaard, 2004; Peirce et al., 2014). Notably, exposure to cold and pharmacologic administration of  $\beta_3$ -adrenergic agonists can enhance the thermogenic capacity and/or energy expenditure in both rodents and humans (Cannon and Nedergaard, 2004; Cypess et al., 2015; van der Lans et al., 2013; Yoneshiro et al., 2013). We show here that ERRs perform a central and essential role in mature brown adipocytes in determining both the basal thermogenic capacity and the ability to expand this capacity. Although past studies revealed a role for ERR $\alpha$  in building the high basal mitochondrial content of BAT (Villena et al., 2007), the current study provides multiple lines of evidence that highlight that ERRs, collectively, play a substantially more important role than previously appreciated. First, double deletion of ERR $\alpha$  and ERR $\gamma$  in mature adipocytes resulted in an extreme loss of mitochondrial content, with the remaining mitochondria having abnormal cristae and overall morphology and almost no Ucp1 protein; in comparison, mice lacking just ERR $\alpha$  (the most abundant BAT ERR isoform) had a milder reduction in mitochondrial content, and their mitochondria had normal morphology, oxidative function, and Ucp1 expression (Villena et al., 2007). Second, mice lacking adipose ERR $\alpha$  and ERR $\gamma$  show remarkable defects in their adaptive response to adrenergic stimulation, failing to remodel their transcriptome, expand their thermogenic capacity, and attain the metabolic benefits of  $\beta_3$ -adrenergic agonists.

ERR $\alpha$  and ERR $\gamma$  have been shown to bind similar sites on the genome and to regulate overlapping sets of genes (Dufour et al., 2007; Huss et al., 2015), suggesting that a certain degree of redundancy in their action is to be expected. Recent studies have also highlighted the complimentary roles of ERRs in determining the oxidative capacity in primary brown adipocytes and in the heart (Gantner et al., 2016; Wang et al., 2015). Yet, the extent to which mice lacking either just ERR $\alpha$  (ERR $\alpha^{\text{Ad-/-}}$ ) or ERR $\beta$  and ERR $\gamma$  together (ERR $\beta\gamma^{\text{Ad-/-}}$ ) maintain their mitochondrial content and OxPhos levels, despite a substantial loss of total ERR protein, is surprising. This functional compensation is seen without an upregulation of the remaining ERR isoforms, suggesting that BAT, at least in young mice, expresses ERRs at much higher levels than necessary for maintaining basal oxidative function. It will be interesting to assess ERR expression in the BAT depots of mice and humans at different ages, to establish if this high ERR reserve diminishes with age, as BAT function declines.

A most striking finding in our study is the key role of ERRs in the adrenergic response of BAT. This was unexpected, based on studies of the ERR $\alpha$  KO mouse, which showed normal induction of gene expression in response to adrenergic stimulation (Villena et al., 2007). Importantly, adrenergic signaling, judged by phosphorylation of HSL, p38, and ATF2 and induction of lipolysis, is intact in BAT lacking ERRs, suggesting that ERRs act downstream of such signals to effect the transcriptional changes elicited by adrenergic stimulation. ERRs likely integrate adrenergic signaling in more than one way. PGC-1 $\alpha$  and PGC-1 $\beta$ , whose expression and/or activity are increased via cAMP, protein kinase A (PKA), and p38 signaling, are potent co-activators of all three ERRs (Gantner et al., 2014; Huss et al., 2002; Kamei et al., 2003; Schreiber et al., 2003). Moreover, Gadd45 $\gamma$ , whose expression is also induced by adrenergic signaling in a cAMP- and PKA-dependent manner, activates p38 and ERR $\beta/\gamma$ -dependent transcription in a PGC-1 independent manner (Gantner et al., 2014). Notably, although the absence of ERRs does not block adrenergic signaling, it may lead to a partial activation of the pathway in the basal state. We observed higher levels of phos-pho-

p38, phospho-ATF2, and PGC-1 $\alpha$  in the absence of adrenergic stimulation. The underlying mechanisms are not clear.

Once activated, ERRs are likely direct positive regulators of many adrenergically induced genes. Although some of the pathways induced by  $\beta_3$ -adrenergic agonism (e.g., mitochondrial function, oxidative phosphorylation, and TCA cycle) were known to be regulated by ERRs (Hock and Kralli, 2009; Villena and Kralli, 2008), our RNA sequencing data show a much broader set of adrenergic responses, extending beyond the control of mitochondrial biogenesis, that rely on ERRs. Some of the highly induced genes, such as *Ucp1*, *Dio2*, and *Fndc5* (Figure S4), have ERR response elements and are regulated by ERRs (Debevec et al., 2007; Dixen et al., 2013; Gantner et al., 2016; Wrann et al., 2013), suggesting that others are also direct targets. Notably, there are adrenergically induced genes that are not dependent on ERRs, consistent with adrenergic signaling being intact, and ERRs not required for all components of the adrenergic response. In particular, the induction of the FAO pathway is retained. Ingenuity Pathway Analysis for regulators of the genes in the FAO pathway suggests that this may be due to *Ppara*, whose expression is increased by CL316,243 in the BAT of  $ERR\alpha\gamma^{Ad-/-}$  mice, and *Klf15*, which is modestly increased in the BAT of  $ERR\alpha\gamma^{Ad-/-}$  (Figure S4). Even though the induction of FAO genes is retained, FAO is likely defective in  $ERR\alpha\gamma^{Ad-/-}$  BAT, owing to the mitochondrial impairment. Finally and importantly, we do not expect that all genes and proteins whose expression is reduced in the BAT of  $ERR\alpha\gamma^{Ad-/-}$  mice are direct ERR targets. Loss of mitochondria and mitochondria-derived signals, as well as of the further actions of direct ERR targets (e.g., a potential paracrine function of *Fndc5*), may contribute to the loss of adaptive responses to adrenergic stimulation. In addition, the decrease in mitochondrial content likely contributes to the post-translational loss of expression of many mitochondrial proteins whose stability requires proper localization, including *Ucp1*.

The thermogenic capacity of BAT can be harnessed to increase energy expenditure and counteract obesity and obesity-related metabolic dysfunction (Lee et al., 2014). Consistent with this notion, when treated with the  $\beta_3$ -adrenergic agonist CL316,243, WT mice increased their energy expenditure, lost weight, and showed improvements in glucose homeostasis.  $ERR\alpha\gamma^{Ad-/-}$  littermates were defective in all three responses, demonstrating the importance of ERR-controlled pathways in adipose tissue for the effects of  $\beta_3$ -adrenergic stimulation on energy balance. Nonetheless,  $ERR\alpha\gamma^{Ad-/-}$  mice were not prone to obesity in the absence of CL316,243 administration, whether on chow or HFD diet. This was irrespective of gender, as similar findings were seen in female and male mice fed a HFD (Figure S7). On the one hand, this finding is not surprising, because our studies were performed at thermoneutrality, when BAT receives minimal adrenergic stimulation (Xiao et al., 2015). Other mutant mice with BAT thermogenic defects are also not prone to obesity (Emmett et al., 2017; Lee et al., 2016). On the other hand, BAT thermogenic capacity has been proposed to be important for diet-induced thermogenesis, i.e., the ability to increase energy expenditure in response to caloric excess, as in HFD (Feldmann et al., 2009; Kazak et al., 2017). There can be many explanations as to why  $ERR\alpha\gamma^{Ad-/-}$  mice do not show increased obesity. First, diet-induced thermogenesis, the basis of which is less well understood than cold-induced thermogenesis, may involve signals and pathways that are sufficiently retained in  $ERR\alpha\gamma^{Ad-/-}$  mice. Second, minor differences in vivarium

temperatures or in the length of time spent at thermoneutrality may explain the different outcomes seen with different models. In our study, mice were born, raised, and studied at thermoneutrality, to minimize the impact that standard vivarium temperatures have on mice with defective BAT (Schulz et al., 2013). Finally, the adrenergic system can be activated by stress, so additional differences in vivarium conditions housing may affect findings.

Even though loss of BAT may not always give rise to obesity, it is clear that increased BAT capacity, coupled to activation, as seen at standard vivarium temperatures for mice, can lead to protection from obesity (Cohen and Spiegelman, 2015). Indeed, mice with increased adipose PGC-1 $\alpha$ /ERR $\alpha$  signaling, as seen in mice lacking the tumor suppressor folliculin, show elevated oxidative metabolism, resistance to hypothermia, and protection from HFD-induced obesity (Yan et al., 2016). Although ERRs are nuclear receptors with no known endogenous ligand, crystallography studies show that they are amenable to drug design, with hydrophobic pockets that can accommodate synthetic ligands (Coward et al., 2001; Kallen et al., 2007; Wang et al., 2006). Synthetic agonists, such as GSK4716, can enhance the activity of ERR $\beta$  and ERR $\gamma$  (Wang et al., 2006; Zuercher et al., 2005). Newer GSK4716-derived molecules can activate ERR $\gamma$  and promote WAT browning (Xu et al., 2015), although the specificity of these ligands has not been tested yet *in vivo*. Our findings suggest that pharmacological activation of ERRs may be beneficial by increasing brown (and beige) adipose tissue thermogenic capacity. Moreover, increased ERR activity may sensitize adipose tissues to the action of adrenergic agonists. The concept of using  $\beta_3$ -adrenergic agonists to pharmacologically activate BAT in humans was floated long ago but has been difficult to apply (Lee and Greenfield, 2015). Recently, mirabregon, a  $\beta_3$ -adrenergic agonist used in the treatment of overactive bladder, was shown to activate BAT in humans, albeit at doses four times higher than that of the current US Food and Drug Administration–approved dose and with adverse effects on the cardiovascular system (Cypess et al., 2015). Administration of ERR agonists could prime the adipose tissue for a more effective response to  $\beta_3$ -adrenergic agonism, thereby enabling the use of lower doses. Future studies will be needed to evaluate the potential of ERR agonists to enhance BAT thermogenesis and energy expenditure, alone or in combination with  $\beta_3$ -adrenergic stimulation.

In conclusion, we have demonstrated that ERRs are critical transcriptional regulators that control adipose tissue plasticity and orchestrate the adaptive remodeling induced by adrenergic stimuli. Our findings underscore the potential of targeting ERRs to expand energy expenditure capacity, which may be harnessed for the treatment of metabolic disease.

## METHODS

All methods can be found in the accompanying Transparent Methods supplemental file.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

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**HIGHLIGHTS**

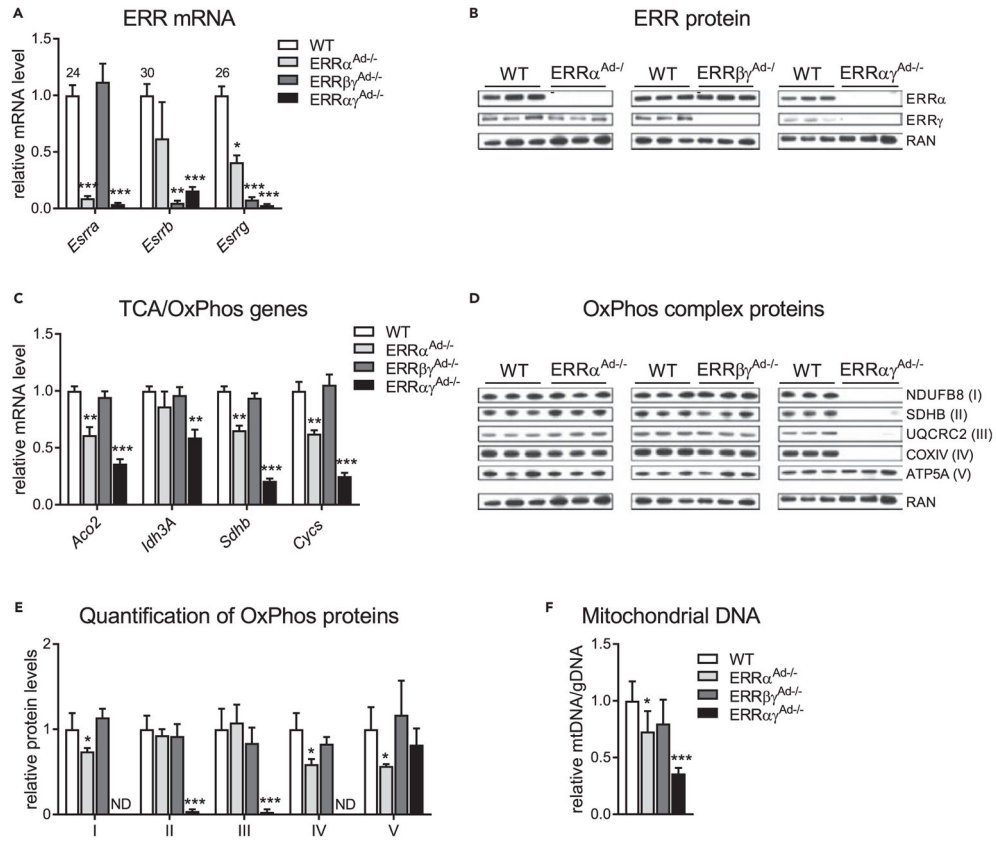
Adipose ERRs collectively control brown fat oxidative and thermogenic capacity

Adipose ERRs are essential for BAT remodeling induced by  $\beta$ -adrenergic agonism

ERRs control the bulk of the transcriptional response to adrenergic stimulation

Mice that lack adipose ERRs show no metabolic benefits of  $\beta$ -adrenergic agonism





**Figure 1. ERRs Act Collectively and in a Redundant Manner to Promote BAT Mitochondrial Oxidative Capacity**

(A) Relative ERR mRNA levels in BAT from WT or littermate mice with adipose-specific deletions of ERR $\alpha$  (ERR $\alpha^{Ad-/-}$ ), ERR $\beta$  and ERR $\gamma$  (ERR $\beta\gamma^{Ad-/-}$ ), or ERR $\alpha$  and ERR $\gamma$  (ERR $\alpha\gamma^{Ad-/-}$ ), expressed relative to the levels of each gene in WT BAT and normalized to *Ppia*. Numbers above bars represent Ct (threshold cycle) values for each ERR isoform in WT BAT.

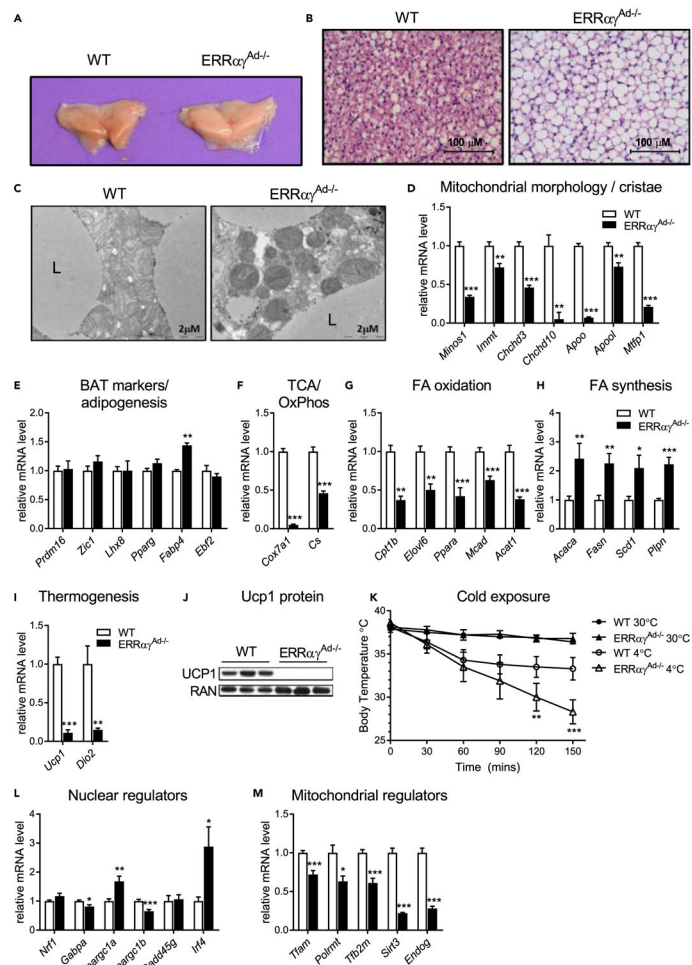
(B) Western blot of ERR $\alpha$ , ERR $\gamma$ , and RAN (ras-related nuclear protein; loading control).

(C) Relative mRNA levels of genes acting in the tricarboxylic acid (TCA) cycle and/or oxidative phosphorylation (OxPhos) and normalized to *Ppia*.

(D) Representative western blot of OxPhos complexes I–V and RAN (loading control) protein.

(E) Quantification of OxPhos complexes, based on blots of panel D (expressed relative to levels in WT BAT and normalized to RAN protein). (F) Relative mitochondrial DNA (mtDNA) copy number, normalized to gDNA in BAT of the same mice.

(A–F) Mice were born and raised at thermoneutrality (30°C) and euthanized at 12 weeks of age. Data are mean  $\pm$  SD of 14 WT, 4 ERR $\alpha^{Ad-/-}$ , 6 ERR $\beta\gamma^{Ad-/-}$ , and 5 ERR $\alpha\gamma^{Ad-/-}$  mice. \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$  vs. WT BAT. WT data in panels A, C, E, and F are the accrued data of all WT littermates of ERR $\alpha$  floxed, ERR $\beta\gamma$  floxed, and ERR $\alpha\gamma$  floxed mice. There were no differences between WT mice of the different cohorts. The same loading control (RAN) is presented in panels B and D. See also Figure S1, Table S3.



**Figure 2. Loss of ERR $\alpha$  and ERR $\gamma$  Impairs BAT Thermogenic Capacity without Affecting Adipogenesis or BAT Identity**

(A) Gross morphology of interscapular BAT depots from WT and ERR $\alpha$  $\gamma$ <sup>Ad-/-</sup> mice.

(B) H&E staining of BAT at 400 $\times$  magnification.

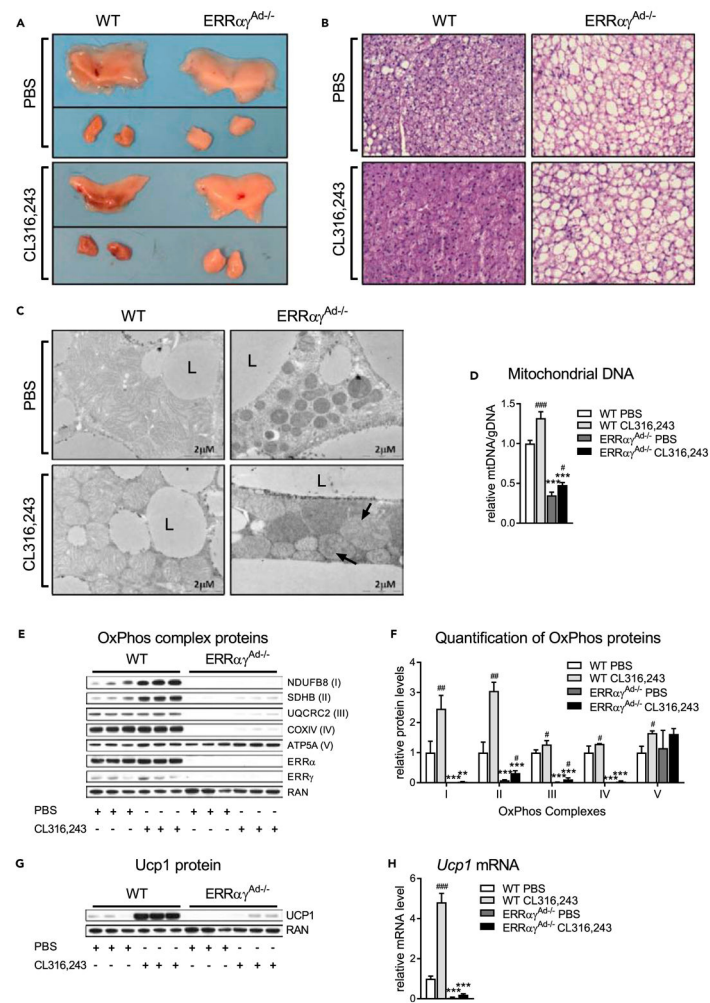
(C) Electron microscopy of mitochondria and surrounding lipid droplets in BAT. L, lipid droplet.

(D–I, L, and M) Relative mRNA levels of genes involved in mitochondrial morphology and cristae structure (D), adipogenesis and BAT identity (E), OxPhos and TCA cycle (F), fatty acid (FA) oxidation (G), FA biosynthesis and storage (H), thermogenesis (I), nuclear regulation of the mitochondrial oxidative metabolism program (L), and mitochondrial regulation of mitochondrial biogenesis (M).

(J) Western blot of Ucp1 protein and RAN (loading control).

(K) Body temperature of WT and ERR $\alpha$  $\gamma$ <sup>Ad-/-</sup> female mice either at 30 $^{\circ}$ C or moved from 30 $^{\circ}$ C to 4 $^{\circ}$ C for 2.5 hr (n = 5). \*\*p < 0.01, \*\*\*p < 0.001 vs. WT 4 $^{\circ}$ C.

Mice were born and raised at thermoneutrality (30 $^{\circ}$ C) and euthanized at 12 weeks of age. mRNA data are from male and female mice combined and normalized to *Ppia* (n = 7–18). Protein data shown here are from male mice. Data are mean  $\pm$  SEM. \*p < 0.05, \*\*p < 0.01, \*\*\*p < 0.001 vs. WT BAT. See also Figure S2 and Table S3.



**Figure 3. Effect of 10 Days' Treatment with CL316,243 on BAT of WT and ERRαγ<sup>Ad-/-</sup> Mice**  
 (A) Gross morphology of interscapular BAT from WT and ERRαγ<sup>Ad-/-</sup> mice. Upper panel: PBS (before and after dissection from surrounding WAT). Lower panel: CL316,243 (before and after dissection from surrounding WAT).  
 (B) H&E staining of BAT at 400× magnification.  
 (C) Electron microscopy of mitochondria and surrounding lipid droplets in BAT. L, lipid droplet; arrows point to abnormal cristae formations.  
 (D) Relative mtDNA copy number, normalized to gDNA (n = 9–19).  
 (E) Representative western blot of OxPhos complexes I–V, ERRα, ERRγ, and RAN (loading control).  
 (F) Quantification of OxPhos western blots, normalized to RAN protein levels (n = 6–9).  
 (G) Representative western blot of Ucp1 protein and RAN (loading control).  
 (H) *Ucp1* mRNA, normalized to *Ppia* mRNA (n = 6–7).

Male mice were born and raised at thermoneutrality (30°C), treated with PBS or CL316,243 for 10 days, and euthanized at 12 weeks of age. Data are mean ± SEM \*\*p < 0.01, \*\*\*p < 0.001 vs. WT in the same treatment; #p < 0.05, ##p < 0.01, ###p < 0.001 vs. PBS in the same

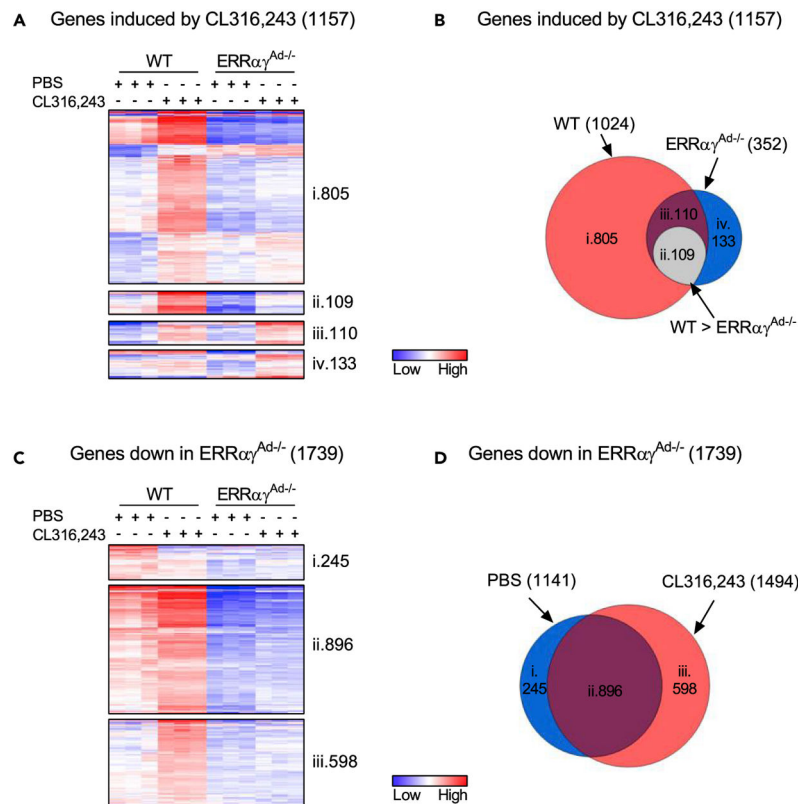
genotype. The same loading control (RAN) is presented in panels E and G. See also Figure S3 and Table S3.

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**Figure 4. Analyses of RNA Sequencing Data of BAT of WT and ERRαγ<sup>Ad-/-</sup> Mice Treated with CL316,243**

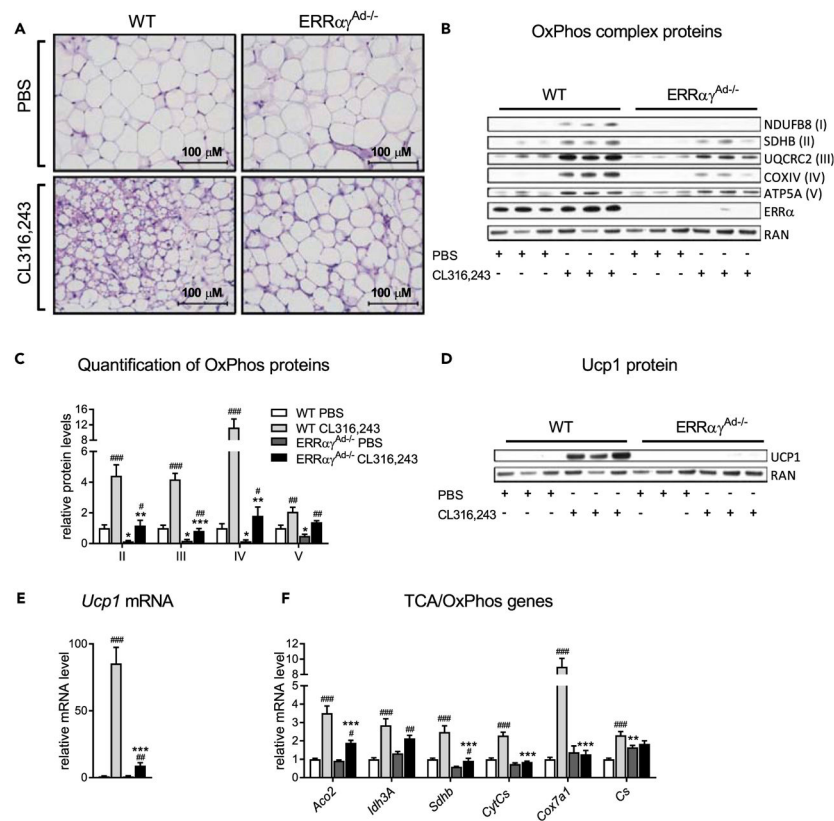
(A) Heat maps of genes significantly induced ( $p < 0.05$ ) by CL316,243 in WT and ERRαγ<sup>Ad-/-</sup> BAT, using a 1.5-fold cut-off. Minimum and maximum log transformed values are 39 and 6, respectively, with visual contrasts set to 2. (i) Genes induced significantly by CL316,243 in WT BAT only. (ii) Genes induced by CL316,243 in both WT and ERRαγ<sup>Ad-/-</sup> BAT, but expression is significantly higher in WT CL316,243 vs. ERRαγ<sup>Ad-/-</sup> CL316,243. (iii) Genes induced by CL316,243 in both WT and ERRαγ<sup>Ad-/-</sup> mice. (iv) Genes induced by CL316,243 in ERRαγ<sup>Ad-/-</sup> BAT only.

(B) Venn diagram of genes significantly induced ( $p < 0.05$ ) by CL316,243 in BAT. Sections i–iv correspond with genes displayed in heat maps (A).

(C) Heat maps of genes significantly reduced ( $p < 0.05$ ) in ERRαγ<sup>Ad-/-</sup> vs. WT BAT, in PBS- and/or CL316,243-treated mice, using a 1.5-fold cut-off. Minimum and maximum log transformed values were 35 and 6, respectively, with visual contrasts set to 2. (i) Genes reduced in PBS-treated mice only. (ii) Genes reduced in both PBS- and CL316,243-treated ERRαγ<sup>Ad-/-</sup> mice. (iii) Genes reduced in CL316,243-treated ERRαγ<sup>Ad-/-</sup> mice only.

(D) Venn diagrams of genes significantly reduced ( $p < 0.05$ ) in ERRαγ<sup>Ad-/-</sup> BAT. Sections i–iii correspond with genes displayed in heat maps (C).

Male mice were born and raised at thermoneutrality (30°C), treated with PBS or CL316,243 for 10 days, and euthanized at 12 weeks of age ( $n = 3$ ). See also Figure S4 and Tables 1, S1, and S2.



**Figure 5. Effect of 10 Days' Treatment with CL316,243 on ingWAT of WT and  $ERR\alpha\gamma^{Ad-/-}$  Mice**

(A) H&E staining of inguinal white adipose tissue (ingWAT) at 400 $\times$  magnification.

(B) Representative western blot of OxPhos complexes I–V,  $ERR\alpha$ , and RAN (loading control).

(C) Quantification of OxPhos complex levels, normalized to RAN protein levels.

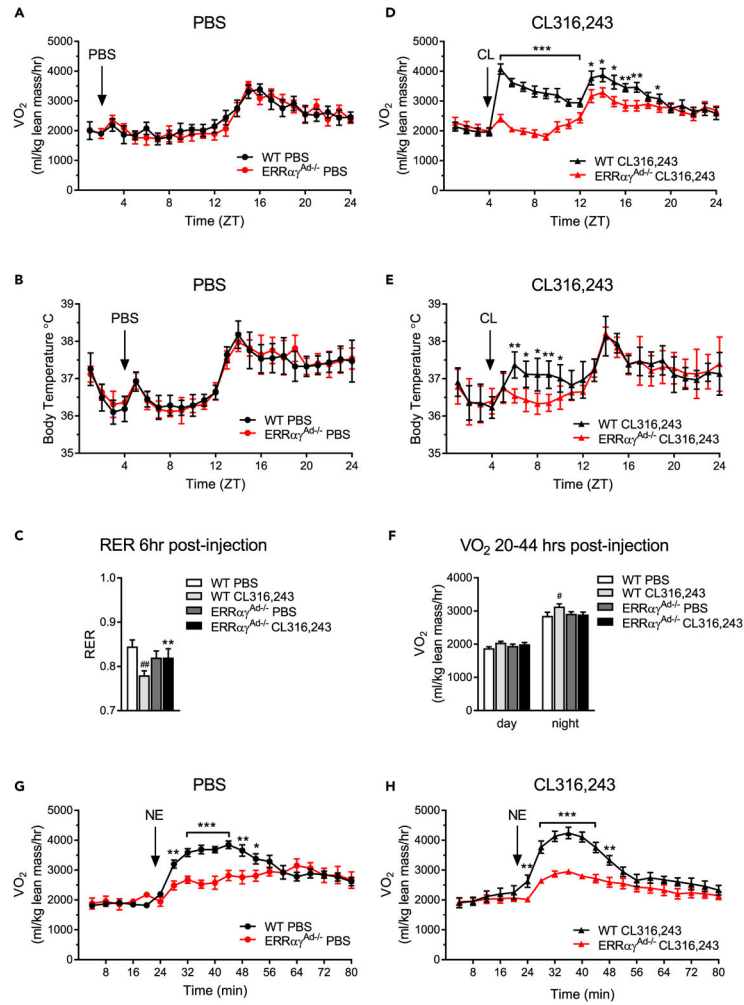
(D) Western blot of Ucp1 protein and RAN (loading control).

(E and F) mRNA levels of *Ucp1* and TCA/OxPhos genes, normalized to *Ppia* and expressed relative to levels in WT mice treated with PBS (n = 8–14).

Mice are from the same experiment as in Figure 3. Data are mean  $\pm$  SEM \*p < 0.05, \*\*p < 0.01, \*\*\*p < 0.001 vs. WT in the same treatment; #p < 0.05, ##p < 0.01, ###p < 0.001 vs.

PBS in the same genotype. The same loading control (RAN) is presented in panels B and D.

See also Figure S5 and Table S3.



**Figure 6. Metabolic Responses to Adrenergic Stimulation in WT and  $ERR\alpha\gamma^{Ad-/-}$  Mice**  
 (A) Oxygen consumption ( $VO_2$ ) in WT and  $ERR\alpha\gamma^{Ad-/-}$  mice injected with PBS for 10 days, on the last day of injections.  
 (B) Core body temperature of WT and  $ERR\alpha\gamma^{Ad-/-}$  mice injected with PBS for 10 days, on the last day of injections.  
 (C) Average respiratory exchange ratio (RER) over 6 hr following a PBS or CL316,243 injection.  
 (D)  $VO_2$  in WT and  $ERR\alpha\gamma^{Ad-/-}$  mice injected with CL316,243 for 10 days, on the last day of injections.  
 (E) Core body temperature of WT and  $ERR\alpha\gamma^{Ad-/-}$  mice injected with CL316,243 for 10 days, on the last day of injections.  
 (F) Average  $VO_2$  over the day and night on the day after the last PBS or CL316,243 injection (day, 20–32 hr after last injection; night, 32–44 hr after the last injection).  
 (G) Acute  $VO_2$  response to norepinephrine (NE) in WT and  $ERR\alpha\gamma^{Ad-/-}$  mice that had been treated with PBS for 10 days.  
 (H) Acute  $VO_2$  response to NE in WT and  $ERR\alpha\gamma^{Ad-/-}$  mice that had been treated with CL316,243 for 10 days.

Male mice were born and raised at thermoneutrality (30°C), treated with PBS or CL316,243 for 10 days, and euthanized at 12–14 weeks of age. NE responses were tested 2 days after the last PBS or CL316,243 injection. Data are mean  $\pm$  SEM (n = 5–10). \*p < 0.05, \*\*p < 0.01, \*\*\*p < 0.001, WT vs. ERR $\alpha$  $\gamma^{Ad-/-}$  for each condition; #p < 0.05, ##p < 0.01 vs. WT PBS. ZT = zeitgeber time. Different mice were used for calorimetry and telemetry experiments. See also Figure S6.

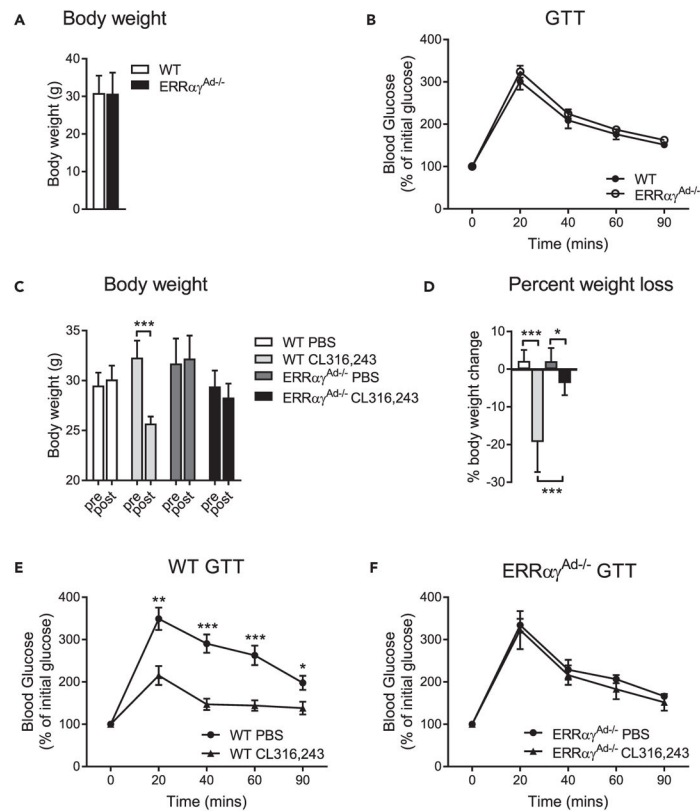
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**Figure 7. Effect of Long-term Adrenergic Stimulation on Body Weight and Glucose Tolerance of HFD-fed WT and  $ERR\alpha\gamma^{Ad-/-}$  Mice**

(A) Body weights of WT and  $ERR\alpha\gamma^{Ad-/-}$  mice fed a 60% high fat diet (HFD) for 18 weeks (n = 12–18).

(B) Glucose tolerance test (GTT) of WT and  $ERR\alpha\gamma^{Ad-/-}$  mice fed a 60% HFD and before treatment. Values are presented as percentage of starting blood glucose (WT,  $111.7 \pm 6.1$  mg/dL;  $ERR\alpha\gamma^{Ad-/-}$ ,  $107.1 \pm 3.7$  mg/dL; p = 0.52), (n = 12–18).

(C) Body weights of WT and  $ERR\alpha\gamma^{Ad-/-}$  mice before and after treatment with PBS or CL316,243 (n = 6–9). \*\*\*p < 0.001.

(D) Body weight loss of WT and  $ERR\alpha\gamma^{Ad-/-}$  mice, expressed as percentage of body weight before treatment (n = 6–9). \*p < 0.01; \*\*\*p < 0.001.

(E) GTT of WT mice treated with PBS or CL316,243. Values are presented as percentage of starting blood glucose (WT PBS,  $74.3 \pm 9.9$  mg/dL; WT CL316,243,  $99.4 \pm 8.2$  mg/dL; p = 0.074). (n = 6–9). \*p < 0.05, \*\*p < 0.01, \*\*\*p < 0.001 vs. WT CL316,243.

(F) GTT of  $ERR\alpha\gamma^{Ad-/-}$  mice treated with PBS or CL316,243. Values are presented as percentage of starting blood glucose ( $ERR\alpha\gamma^{Ad-/-}$  PBS,  $79.9 \pm 4.9$  mg/dL;  $ERR\alpha\gamma^{Ad-/-}$  CL316,243,  $75.2 \pm 6.2$  mg/dL; p = 0.56) (n = 6–9).

Female mice were born and raised at thermoneutrality (30°C) and fed a 60% HFD starting at weaning. At 22 weeks of age, mice were given an intraperitoneal injection of PBS or CL316,243 every other day for 4 weeks. Mice were euthanized at 26 weeks of age. Data are presented as mean  $\pm$  SEM.

Table 1

Top Canonical Pathways Regulated by CL316,243 in WT BAT and Their Ranking in ERR $\alpha$ . $\gamma$ Ad $^{-/-}$  BAT

Canonical Pathway	WT Mice				ERR $\alpha$ . $\gamma$ Ad $^{-/-}$ Mice				-log(p Value)
	Rank	Downregulated	Upregulated	-log(p Value)	Rank	Downregulated	Upregulated		
Mitochondrial dysfunction	1	8/142 (6%)	79/142 (56%)	9.29E+00	NA				
Oxidative phosphorylation	2	2/85 (2%)	62/85 (73%)	8.92E+00	NA				
TCA cycle II (eukaryotic)	3	0/19 (0%)	17/19 (89%)	7.22E+00	27	0/19 (0%)	13/19 (68%)	2.03E+00	
Fatty acid $\beta$ -oxidation I	4	0/26 (0%)	16/26 (62%)	6.80E+00	1	0/26 (0%)	21/26 (81%)	8.28E+00	
Glutaryl-CoA degradation	5	1/15 (7%)	11/15 (73%)	6.66E+00	39	1/15 (7%)	11/15 (73%)	1.72E+00	
Tryptophan degradation III (eukaryotic)	6	1/15 (7%)	11/15 (73%)	6.66E+00	40	1/15 (7%)	11/15 (73%)	1.72E+00	
Valine degradation I	7	0/17 (0%)	13/17 (76%)	5.81E+00	8	0/17 (0%)	15/17 (88%)	4.01E+00	
Acetyl-CoA biosynthesis I (pyruvate dehydrogenase complex)	8	0/5 (0%)	5/5 (100%)	4.34E+00	215	0/5 (0%)	3/5 (60%)	5.08E-01	
Allograft rejection signaling	9	11/19 (58%)	0/19 (0%)	4.23E+00	5	12/19 (63%)	0/19 (0%)	4.60E+00	
LXR/RXR activation	10	13/64 (20%)	16/64 (25%)	4.20E+00	15	17/64 (27%)	8/64 (13%)	2.76E+00	
Autoimmune thyroid disease signaling	11	9/13 (69%)	0/13 (0%)	4.12E+00	30	8/13 (62%)	1/13 (8%)	1.95E+00	
Isoleucine degradation I	12	1/13 (8%)	9/13 (69%)	4.12E+00	69	1/13 (8%)	11/13 (85%)	1.21E+00	
Complement system	13	13/23 (57%)	1/23 (4%)	4.11E+00	3	14/23 (61%)	2/23 (9%)	6.83E+00	
Antigen presentation pathway	14	11/24 (46%)	1/24 (4%)	3.90E+00	4	17/24 (71%)	1/24 (4%)	5.58E+00	
Gluconeogenesis I	15	2/17 (12%)	10/17 (59%)	3.88E+00	104	6/17 (35%)	3/17 (18%)	9.28E-01	

NA, non-applicable; LXR, liver X receptor; RXR, retinoid X receptor; these pathways were not detected as regulated by CL316,243 in ERR $\alpha$ . $\gamma$ Ad $^{-/-}$  BAT. See also Figures 4, S4 and Tables S1 and S2.