GADD45γ regulates the thermogenic capacity of brown adipose tissue

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The coactivator peroxisome proliferator-activated receptor-gamma coactivator 1 α (PGC-1α) is widely considered a central transcriptional regulator of adaptive thermogenesis in brown adipose tissue (BAT). However, mice lacking PGC-1α specifically in adipose tissue have only mild thermogenic defects, suggesting the presence of additional regulators. Using the activity of estrogen-related receptors (ERRs), downstream effectors of PGC-1α, as read-out in a high-throughput genome-wide cDNA screen, we identify here growth arrest and DNA-damage-inducible protein 45 γ (GADD45γ) as a cold-induced activator of uncoupling protein 1 (UCP1) and oxidative capacity in BAT. Mice lacking *Gadd45γ* have defects in *Ucp1* induction and the thermogenic response to cold. GADD45γ works by activating MAPK p38, which is a potent activator of ERRβ and ERRγ transcriptional function. GADD45γ activates ERRγ independently of PGC-1 coactivators, yet synergizes with PGC-1α to induce the thermogenic program. Our findings elucidate a previously unidentified GADD45γ/p38/ERRγ pathway that regulates BAT thermogenesis and may enable new approaches for the stimulation of energy expenditure. Our study also implicates GADD45 proteins as general metabolic regulators.

norepinephrine signaling | transcriptional regulation | adrenergic response | nuclear receptors

Through the regulated dissipation of energy as heat (thermogenesis), brown adipose tissue (BAT) can increase total body energy expenditure and impact adiposity (1, 2). In humans, activation of BAT by exposure to cold or pharmacologically leads to body-fat loss (3–5). Interestingly, the levels of BAT activity vary greatly among individuals, with a significant part of the population, in particular older and obese adults, having no detectable BAT activity (6, 7). Elucidating the regulatory mechanisms that control brown adipocyte thermogenesis is essential for understanding the wide variations in BAT activity and for enabling new approaches to activate BAT and counteract obesity and obesity-related diseases.

The thermogenic capacity of BAT depends on the tissue's high mitochondrial density and oxidative capacity, as well as the presence and activity of the uncoupling protein UCP1. UCP1 dissipates the mitochondrial proton gradient, thereby using respiration to generate heat instead of ATP (1). BAT oxidative capacity and the levels of UCP1 are regulated at the transcriptional level and are increased by exposure to cold, via the sympathetic nervous system-dependent release of norepinephrine. Norepinephrine, as well as other activators of thermogenesis, induces an intracellular signaling pathway that includes protein kinases A and G (PKA and PKG), the MAPK p38, and the transcriptional regulator peroxisome proliferator-activated receptor-gamma coactivator 1 α (PGC-1α) and leads to the enhanced expression of oxidative metabolism genes and *Ucp1* (1, 2, 7, 8).

PGC-1α is a regulator of mitochondrial biogenesis and oxidative capacity. It also enhances the expression of the thermogenic gene program, inducing oxidative capacity and *Ucp1*, when overexpressed (9, 10). Consequently, the induction of *Pgc-1α* is widely used as a marker of BAT activation and is thought to be critical for thermogenesis. However, mice lacking PGC-1α specifically in adipose tissue retain the transcriptional response to cold in BAT and show only a mild defect in thermogenesis (11). Furthermore, mice lacking estrogen-related receptor α (ERRα), a key downstream effector of PGC-1α, also maintain a wild-type transcriptional response to cold exposure (12). These findings suggest that, whereas PGC-1α is important for the basal oxidative capacity in BAT, there are additional regulators that induce the transcription of thermogenic genes in response to cold.

BAT also expresses the ERRα-related receptors ERRβ and ERRγ, which can activate similar genes as ERRα and have been implicated in the transcriptional control of oxidative genes and *Ucp1* (13–16). Interestingly, ERRβ and ERRγ can activate their target genes in the absence of PGC-1 regulators (15, 17), suggesting that they may provide an alternate pathway to induce thermogenic capacity in response to cold. Thus, to identify BAT thermogenic regulators, we used an unbiased, high-throughput cDNA screen for activators of ERRβ-mediated transcription. Here, we report the identification of growth arrest and DNA-damage-inducible protein 45 γ (GADD45γ) as an ERRβ and ERRγ activator that is rapidly induced by adrenergic signaling and show that GADD45γ regulates the thermogenic program (oxidative capacity and UCP1 levels) in BAT.

Results

Identification of GADD45γ as a Cold-Induced Activator of ERRβ and ERRγ. To find new regulators of ERR-controlled pathways, we set up a cell-based assay amenable to high-throughput screening and designed to identify modulators of ERR transcriptional activity (Fig. S1*A*). We focused on ERRβ and ERRγ because they are enriched in BAT relative to white adipose tissue (WAT) and are

Significance

Brown adipose tissue (BAT) specializes in converting stored chemical energy into heat. When activated in response to cold, BAT increases the body's metabolic rate and promotes fat loss. The recent identification of BAT in humans makes activation of this tissue an attractive target for counteracting obesity. However, our understanding of the intracellular pathways that regulate the response to cold is limited. Here, we identify a previously unknown regulator of BAT activation, growth arrest and DNA-damage-inducible protein 45 γ, which is induced in response to cold and works via the MAPK p38 and the transcription factor estrogen-related receptor γ to enhance the expression of genes important for thermogenesis. Our findings may provide new avenues for the stimulation of energy expenditure.

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The authors declare no conflict of interest.

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transcriptionally active in 10T1/2 cells that do not express significant levels of PGC-1α or PGC-1β (Fig. S1 *B–D*), suggesting that they are capable of integrating signals in a PGC-1–independent manner. We thus screened the Mammalian Genome Collection cDNA expression library in 10T1/2 cells for activators of Gal4-ERRβ. Hits identified in the screen were subjected to secondary screening (see *SI Methods* for screen design and validation assays) to eliminate general activators that enhanced non–ERR-dependent luciferase activity and to select hits that activated full-length ERRβ (containing the native DNA binding domain) at ERRE-containing luciferase reporters and at endogenous ERR target genes (*Shp* and *Psca*) (Fig. S1 *E* and *F*). All validated hits activated ERRγ in addition to ERRβ and showed no activation of ERRα in 10T1/2 cells (Fig. 1*A*).

To identify potential regulators of thermogenesis, we measured the levels of the top eight validated hits in the BAT of mice acclimated to thermoneutrality (30 °C) for 10 d or exposed to acute cold (4 °C) for 6 h. Uniquely, *Gadd45γ* had high basal expression in BAT and was strongly induced upon cold exposure (Fig. 1*B*). GADD45γ belongs to a small family of proteins that include GADD45α and GADD45β. All three GADD45 members can modestly activate ERRβ and/or ERRγ (Fig. 1*A*) and are expressed in BAT, but only *Gadd45γ* was induced in vivo in response to cold (Fig. 1*B*).

To determine the signals that induce *Gadd45γ* in BAT in response to cold exposure, we examined *Gadd45γ* expression in primary brown adipocytes. *Gadd45γ* was rapidly and transiently induced by norepinephrine, the key signal of cold exposure in vivo, as well as cAMP, a downstream effector of norepinephrine (Fig. 1*C* and Fig. S1*G*). *Gadd45γ* induction preceded that of other norepinephrine-induced genes, such as *Pgc-1α* and *Ucp1* (Fig. 1*C*). Induction of *Gadd45γ* by norepinephrine was blocked by the PKA inhibitor H-89, but not the p38 MAPK inhibitor SB202190 (Fig. S1*H*). In summary, a cell-based unbiased cDNA screen for activators of ERRβ identified GADD45γ as an ERRβ and ERRγ activator that is induced by cold exposure in BAT and by adrenergic stimulation, via cAMP and PKA, in brown adipocytes.

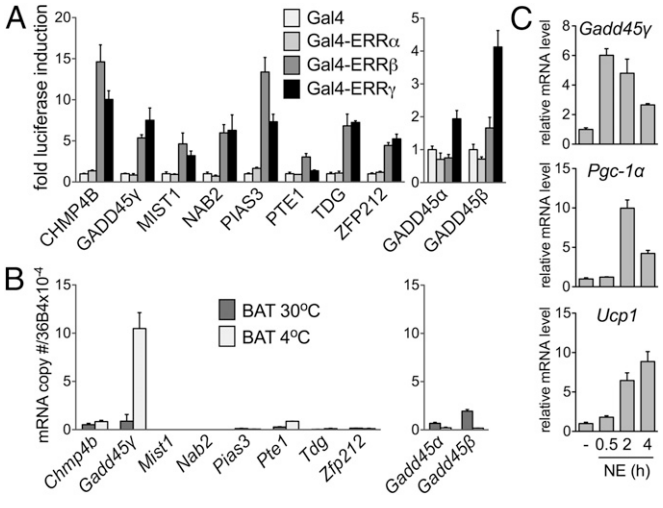

Fig. 1. GADD45γ is a cold-induced activator of ERRβ and ERRγ. (*A*) Gal4 luciferase activity assay in 10T1/2 cells expressing the indicated Gal4-ERR fusions and top eight ERRβ activators identified in the screen (*Left*) or Gadd45α and Gadd45β (*Right*). Data are expressed as fold-induction by each activator and are the mean ± SEM (n = 3–9). (*B*) Relative mRNA levels of top eight hits from the screen, Gadd45α and GADD45β in BAT of ∼12-wk-old male mice housed at thermoneutrality (30 °C) or exposed to cold (4 °C) for 6 h. Data are the mean ± SD (n = 3–4). (*C*) Relative mRNA levels in primary brown adipocytes treated with 1 μM norepinephrine (NE) for the indicated times. Data are the mean ± SD (n = 3).

GADD45γ and ERRγ Increase *Ucp1* Expression and Oxidative Capacity in Primary Brown Adipocytes. Next, we determined the consequences of increasing GADD45γ levels in brown adipocytes by exogenously expressing GADD45γ, alone or in combination with ERRγ. ERRβ and ERRγ behave similarly when overexpressed, and ERRγ was chosen for these assays based on its higher abundance in BAT (Fig. S1*B*). GADD45γ expression in primary brown adipocytes significantly increased *Ucp1* expression; it also enhanced the ability of ERRγ to induce *Ucp1* at the mRNA and protein level (Fig. 2 *A* and *B*). The induction of *Ucp1* by GADD45γ and ERRγ was comparable with the induction seen in response to norepinephrine (Fig. S2 *A* and *B*). GADD45γ also enhanced the ability of ERRγ to induce other known ERR gene targets, such as *Pdk4* and *Atp1b1* (18, 19), validating GADD45γ as an ERRγ activator in brown adipocytes (Fig. 2*A*). To determine the functional impact on oxidative capacity, we monitored cellular O_2 consumption rates. Both GADD45γ and ERRγ led to increases in basal and maximal respiration (Fig. 2*C*), suggesting that they remodel cellular metabolism beyond the observed increase in UCP1 expression. Furthermore, the combination of GADD45γ and ERRγ increased the percentage of uncoupled respiration from 61% (in control brown adipocytes) to 81% (Fig. 2*D*). Thus, GADD45γ and ERRγ enhance key cellular components important for thermogenesis: UCP1 levels, oxidative capacity, and mitochondrial uncoupling. Notably, they do so without increasing PGC-1α or PGC-1β expression (Fig. S2*C*).

GADD45γ and ERRγ Can Induce Thermogenic Gene Expression Independently of PGC-1α and PGC-1β. The identification of GADD45γ as an activator of ERRγ in cells that do not express detectable levels of PGC-1α or PGC-1β (Fig. 1*B* and Fig. S1*D*) suggests that GADD45γ activates ERRγ independently of PGC-1 coactivators. To test this hypothesis, we determined the ability of GADD45γ and ERRγ to induce ERR targets in cells genetically lacking PGC-1α and PGC-1β. Brown adipocytes were derived from mice with floxed *Pgc-1α* and *Pgc-1β* alleles and infected with GFP- or CRE-expressing lentiviruses to generate WT and Pgc-1α/β double KO cells, respectively. Both *Pgc-1* floxed alleles were recombined in ∼90–95% of adipocytes, leading to a dramatic loss of *Pgc-1α* and *Pgc-1β* mRNA (Fig. S2 *D* and *E*). The Pgc-1α/β double KO adipocytes had similar morphology to WT brown adipocytes and comparable expression of adipogenic markers *aP2* and *Pparγ* (Fig. S2 *E* and *F*). GADD45γ and ERRγ enhanced *Ucp1* and *Pdk4* expression as well (or even better) in Pgc-1α/β double KO as in WT adipocytes (Fig. 2*E*), indicating that GADD45γ/ERRγ-mediated regulation of ERR targets does not require PGC-1α or PGC-1β. Similar results were seen in adipocytes lacking just PGC-1α (Fig. S2 *G–I*).

Loss of GADD45γ Impairs Adaptive Thermogenesis in BAT. To test the function of endogenous GADD45γ in BAT adaptive thermogenesis, we challenged mice lacking Gadd45γ (20) and acclimated for 2 wk to thermoneutrality (30 °C), with exposure to cold. At thermoneutrality, Gadd45γ KO mice had similar BAT morphology, body temperature, and expression levels of adipogenic and thermogenic BAT genes as WT littermates (Fig. S3 *A–C*). After 6 h of exposure to 4 °C, Gadd45γ KO mice showed an ∼50% decrease in the induction of BAT *Ucp1* compared with WT littermates, indicating that GADD45γ is required for full induction of *Ucp1* in response to cold (Fig. 3*A*). To evaluate BAT-specific thermogenesis, we monitored the temperature of mice exposed acutely to mild cold, 21 °C (21), using transponders implanted above the interscapular BAT. Gadd45γ KO mice showed significant defects in their thermogenesis, having lower temperatures than their WT littermates at 3–4 h after transfer to 21 °C (Fig. 3*B*). The milder cold stress (21 °C) also induced *Ucp1* expression in BAT, and, as seen with the 4 °C challenge, Gadd45γ KO mice had significantly

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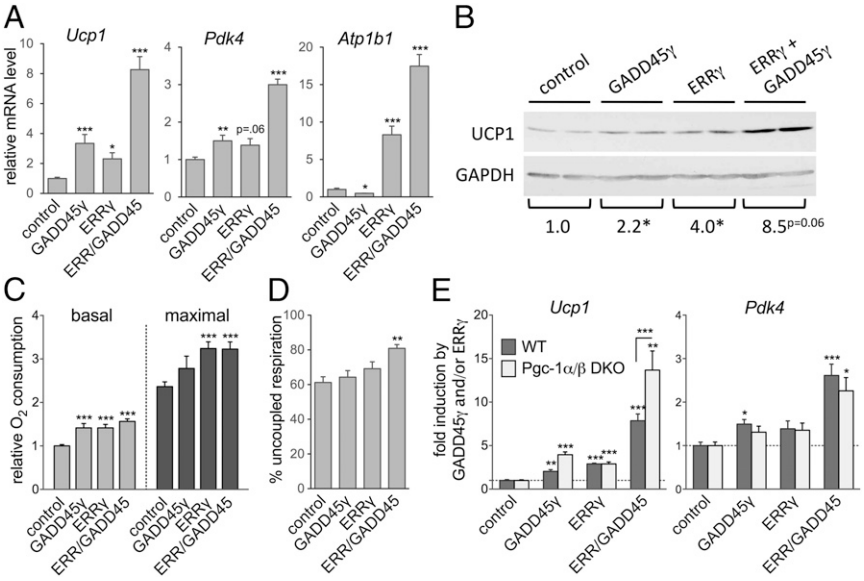

Fig. 2. GADD45γ and ERRγ enhance UCP1 expression and oxidative capacity in primary brown adipocytes, independently of PGC-1 coactivators. (*A*) Relative mRNA expression in adipocytes expressing GADD45γ and/or ERRγ for 24 h. Values represent the mean ± SEM ($n = 6$). (*B*) UCP1 protein levels in adipocytes expressing GADD45γ and/or ERRγ for 48 h; quantifications relative to control are indicated below ($n = 2$). (*C*) Oxygen consumption rates of adipocytes expressing GADD45γ and/or ERRγ for 48 h. Values represent the mean ± SEM ($n = 6$–9). (*D*) Uncoupled respiration (expressed as percentage of total respiration and determined in the presence of 3 μM oligomycin) in adipocytes expressing GADD45γ and/or ERRγ for 48 h. Values represent the mean ± SEM ($n = 4$). (*E*) Relative mRNA levels in WT and Pgc-1α/β double knockout (DKO) adipocytes expressing GADD45γ and/or ERRγ for 24 h. Data are expressed as fold-induction by GADD45γ/ERRγ and are the mean ± SD ($n = 3$). $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.005$, compared with control or as indicated.

reduced *Ucp1* levels at 21 °C (Fig. S3*D*). *Ucp1* levels were not significantly decreased in the inguinal or gonadal depots of these mice (Fig. S3*E*). In summary, mice lacking GADD45γ have impaired *Ucp1* induction in BAT and decreased BAT thermogenic activity when exposed to cold.

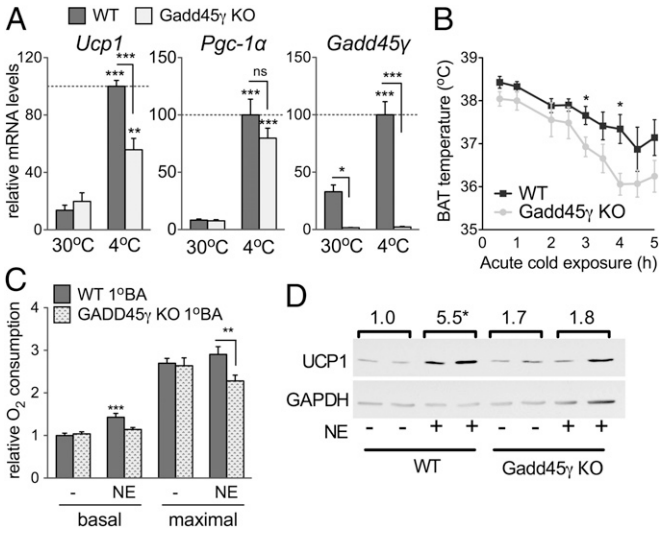

Fig. 3. Loss of GADD45γ impairs induction of *Ucp1* and thermogenesis by cold in BAT and norepinephrine responses in brown adipocytes in vitro. (*A*) Relative mRNA levels in the BAT of WT and Gadd45γ KO mice exposed to 4 °C for 6 h or kept at thermoneutrality (30 °C). Data are the mean ± SEM ($n = 5$–8). (*B*) Subcutaneous BAT temperature of WT and Gadd45γ KO littermates exposed to 21 °C for 5 h. Data are the mean ± SEM ($n = 7$). (*C*) Oxygen consumption rates of WT and Gadd45γ KO primary brown adipocytes treated with 1 μM NE for 20 h. Maximal rates were determined in the presence of 2.4 μM carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone (FCCP). Data are the mean ± SEM ($n = 3$). (*D*) UCP1 protein levels in WT and Gadd45γ KO adipocytes treated with 1 μM NE for 20 h. Protein quantification is indicated above the lanes ($n = 3$). ns, not significant; $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ compared with control of same genotype or as indicated.

To determine whether the function of Gadd45γ specifically in the brown adipocytes underlies the thermogenic defects, we next tested the norepinephrine response of primary brown adipocytes derived from Gadd45γ KO and WT littermates. Gadd45γ KO cells differentiated into mature brown adipocytes with similar morphology as WT controls and comparable expression of most adipogenesis and brown adipocyte-specific markers (*aP2, Dio2, Pgc1-α*) (Fig. S3 *F* and *G*). WT and Gadd45γ KO adipocytes also had similar basal and maximal oxygen-consumption rates in the absence of norepinephrine (Fig. 3*C*). Treatment with norepinephrine enhanced basal oxygen consumption in WT cells but failed to do so in Gadd45γ KO cells (Fig. 3*C*). Furthermore, the norepinephrine-dependent induction of UCP1 protein levels (~5× in WT cells) was lost in the Gadd45γ KO adipocytes (Fig. 3*D*). In summary, Gadd45γ KO adipocytes showed diminished responses to norepinephrine, suggesting that intrinsic brown adipocyte defects in Gadd45γ KO mice underlie the impaired thermogenic response observed in vivo.

GADD45γ Functions via MAPK p38 to Enhance ERRβ and ERRγ Transcriptional Activity. The GADD45 family members have been reported to act as nuclear receptor coactivators (22). Although we detected interactions between the ERRs and GADD45γ in coimmunoprecipitations, we found no evidence of GADD45γ having "classic" coactivator function. We could not detect GADD45γ recruitment to chromatin or transcriptional activity of Gal4-GADD45γ chimeras. Thus, we explored other mechanisms whereby GADD45γ could activate ERRs.

GADD45γ is known to interact with and activate MTK1/MEKK4, which activates MKK3 and MKK6, which in turn phosphorylate and activate MAPK p38 (20, 23, 24), a kinase that has been implicated in the response of brown adipocytes to cold and to norepinephrine (25, 26). Indeed, we found that overexpression of GADD45γ in primary brown adipocytes led to the

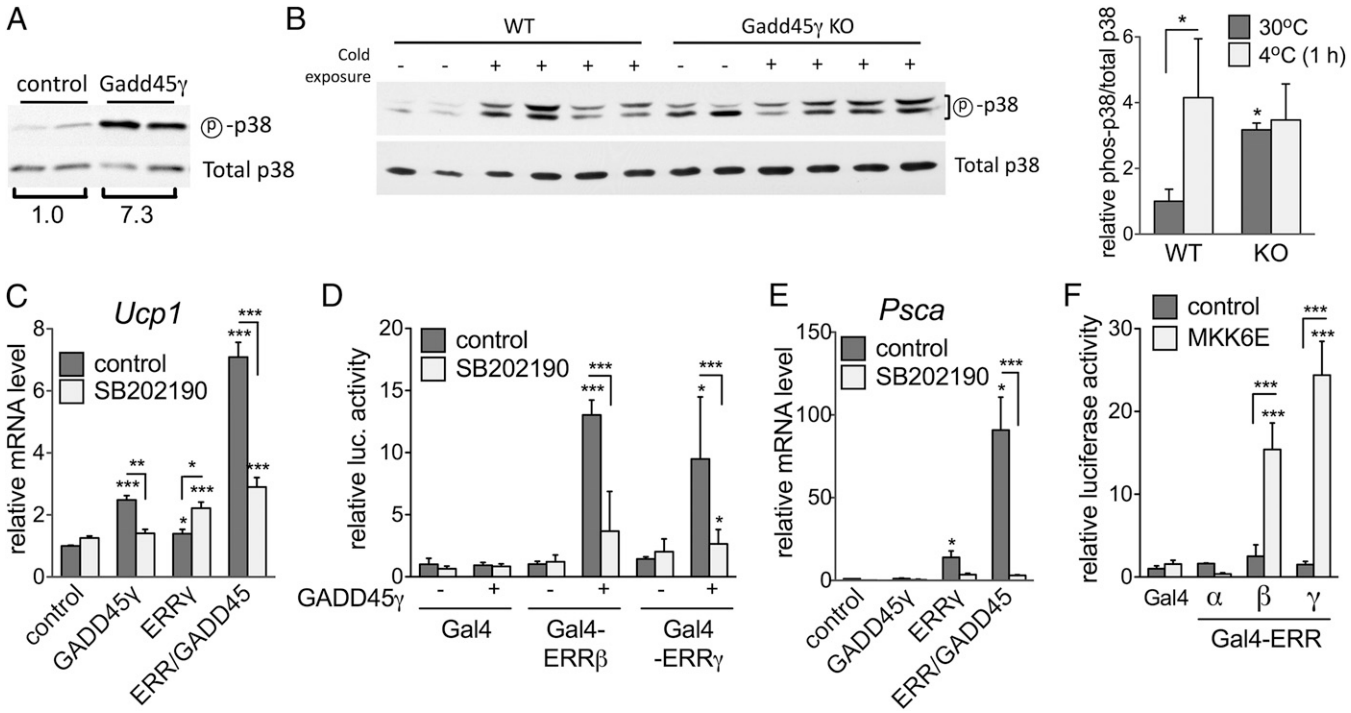

Fig. 4. GADD45γ activates the MAPK p38, which enhances the transcriptional activity of ERRβ and ERRγ. (*A*) Phospho-p38 and total p38 protein levels in adipocytes expressing control (LacZ) or GADD45γ for 13 h, with relative quantification of phospho-p38/total p38 indicated. (*B*, *Left*) Phospho-p38 protein levels in BAT isolated from WT or Gadd45γ KO mice kept at 30 °C (–) or exposed for 1 h to 4 °C (+). The doublet reflects different BAT p38 isoforms recognized by the antibody. Quantification of phospho-p38 levels (*Right*) normalized to total p38 protein. Data are the mean ± SD ($n = 2$–4). (*C*) Relative *Ucp1* expression in adipocytes expressing GADD45γ and/or ERRγ for 24 h and treated with the p38 inhibitor (SB202190, 10 μM). Data are the mean ± SEM ($n = 6$). (*D* and *F*) Activation of Gal4-ERRs in 10T1/2 cells by (*D*) Gadd45γ in the absence or presence of SB202190 (0.5 μM) or (*F*) MKK6E, an activator of p38. Data are the mean ± SD ($n = 4$). (*E*) Relative *Psca* levels (an ERR target gene) in 10T1/2 cells expressing GADD45γ and/or ERRγ, treated with SB202190 (10 μM) for 24 h. Data are the mean ± SD ($n = 3$). $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, compared with control of the same colored bar or as indicated.

activation (increased phosphorylation) of p38 (Fig. 4*A*). Furthermore, phosphorylation of p38 was induced by cold exposure in the BAT of WT but not of Gadd45γ KO mice (Fig. 4*B*). Gadd45γ KO BAT showed elevated basal levels of phosphorylated p38, compared with WT mice, possibly due to activation of compensatory mechanisms or feedback regulatory loops (Fig. 4*B*). These data indicate that GADD45γ activates p38 in adipocytes and mediates the activation of p38 in response to cold in BAT.

We next tested whether p38 is necessary for the ability of GADD45γ to activate ERRβ and ERRγ and transcription of ERRβ/γ targets. In primary brown adipocytes, inhibition of p38 (using SB202190) blocked the ability of GADD45γ to induce *Ucp1* and *Pdk4*, as well as to enhance ERRγ activity at these targets (Fig. 4*C* and Fig. S4*A*), showing that GADD45γ depends on p38 to activate gene expression. Furthermore, in 10T1/2 cells, where we identified GADD45γ as an ERRβ/γ activator, inhibition of p38 activity blocked the ability of GADD45γ to activate Gal4-ERRβ and Gal4-ERRγ and to enhance ERRβ- and ERRγ-dependent induction of endogenous ERR target genes (Fig. 4 *D* and *E* and Fig. S4*B*). In 10T1/2 cells, inhibition of p38 also impaired the ability of ERRβ and ERRγ to induce endogenous targets in the absence of cotransfected GADD45γ, suggesting that the basal transcriptional activity of ERRβ and ERRγ in these cells relies on p38 signaling (Fig. 4*E* and Fig. S4*B*). Finally, a constitutively active version of MKK6 (MKK6E), which phosphorylates and activates p38 (27), strongly activated Gal4-ERRβ and Gal4-ERRγ but not Gal4-ERRα (Fig. 4*F*). MKK6E also dramatically enhanced the ability of ERRβ and ERRγ to induce endogenous ERR target genes in a p38-dependent manner (inhibited by SB202190) (Fig. S4*C*). In summary, the MAPK p38 is a potent activator of ERRβ and ERRγ activity.

GADD45γ Acts Synergistically with PGC-1α to Enhance the Thermogenic Program. Our results suggest that the newly identified GADD45γ/p38/ERRγ pathway can act independently of PGC-1α. However, GADD45γ and PGC-1α are coinduced by adrenergic stimulation, and both enhance the expression of genes important for thermogenesis. To test for possible interactions between the two regulators, we coexpressed PGC-1α and GADD45γ in primary brown adipocytes. Unlike GADD45γ, PGC-1α alone did not induce *Ucp1* expression (Fig. 5*A*). However, the combined expression of PGC-1α with GADD45γ led to a synergistic activation of *Ucp1* and other genes, including other ERR targets important for oxidative capacity in BAT (e.g., *Pdk4*, *Cpt1b*, *Shp*, *Tfam*, and *Pparα)* (Fig. 5*A* and Fig. S5). UCP1 protein levels were also greatly enhanced when GADD45γ and PGC-1α were coexpressed (Fig. 5*B*). Furthermore, combined expression of GADD45γ and PGC-1α potently enhanced both basal and maximal O_2 consumption rates, better than either regulator by itself (Fig. 5*C*). PGC-1α is phosphorylated and activated by p38 (28, 29); therefore, the observed synergism may, in part, be due to GADD45γ activating p38. Consistent with this notion, GADD45γ enhanced the activity of Gal4-PGC-1α in a p38-dependent manner (Fig. 5*D*). These findings indicate that GADD45γ, via p38 activation, enhances both PGC-1α–independent and PGC-1α–dependent transcriptional pathways.

Discussion

BAT thermogenesis defends body temperature in cold environments and can dissipate excess energy in states of high caloric

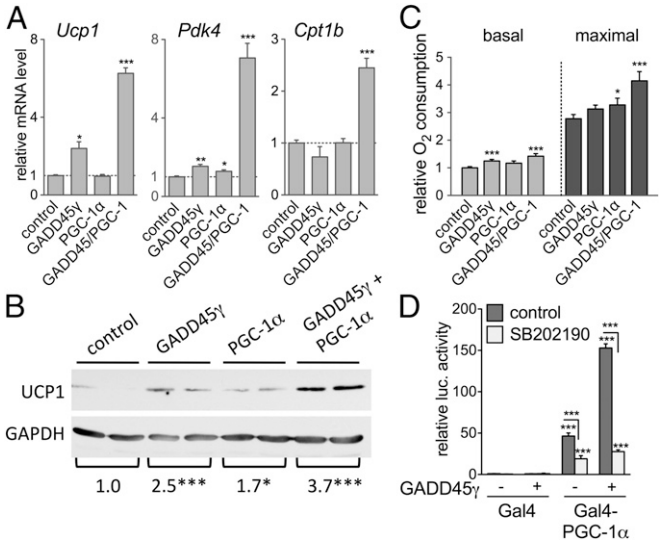

Fig. 5. GADD45γ acts synergistically with PGC-1α to enhance gene expression and oxidative capacity in primary brown adipocytes. (*A*) Relative mRNA levels in adipocytes expressing GADD45γ and/or PGC-1α for 24 h. Data are the mean ± SEM (n = 5–9). (*B*) UCP1 protein levels in adipocytes expressing GADD45γ and/or PGC-1α for 48 h, with quantification relative to control indicated (n = 6). (*C*) Relative oxygen consumption rates of adipocytes expressing GADD45γ and/or PGC-1α for 48 h. Maximal rates were determined in the presence of 2.4 μM FCCP. Data are the mean ± SEM (n = 6–8). (*D*) Activation of Gal4-PGC-1α in 10T1/2 cells by coexpression of GADD45γ, in the absence or presence of the p38 inhibitor (SB202190, 2 μM). Data are the mean ± SEM ($n \geq 4$). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

intake (1, 2, 7). Greater understanding of the transcriptional networks that regulate BAT thermogenic capacity is important to design strategies that enhance energy expenditure, which may serve to counteract obesity. Here, we identify GADD45γ as a regulator of thermogenic capacity in brown adipocytes. *Gadd45γ* is rapidly and transiently induced by norepinephrine in brown adipocytes, in a PKA-dependent manner, and by cold exposure in BAT of mice. GADD45γ mediates the norepinephrine-dependent activation of MAPK p38, which activates ERRγ transcriptional activity, leading to the induction of UCP1 and enhancement of oxidative capacity (Fig. S6*A*). Our study establishes GADD45γ/p38/ERRγ as a BAT signaling pathway that links adrenergic stimulation to the induction of the thermogenic program. It also suggests that Gadd45 proteins may have more general metabolic roles.

GADD45γ and PGC-1α are coinduced in response to cold and synergistically induce genes important for BAT thermogenesis. Part of the observed synergy may reflect the ability of GADD45γ, via p38, to enhance the transcriptional activity of PGC-1α (Fig. S6*A*) (28, 29). In addition, GADD45γ and PGC-1α may selectively activate different DNA-binding transcription factors that synergize with each other (e.g., ERRβ/γ and ERRα, respectively). The existence of multiple inducible transcriptional regulators likely enables broad and robust thermogenic responses to signals like cold. It may also enable more specific activation of a narrower target gene set in response to signals, yet to be identified, that selectively induce just GADD45γ or just PGC-1α.

Our study shows that activation of p38 is the major mechanism by which GADD45γ induces *Ucp1* and other ERR targets. In brown adipocytes, activation of p38 by norepinephrine requires PKA and is mediated by MKK3 and MKK6; the mechanism by which PKA activates the MKK3/6-p38 kinase cascade has so far been unknown (1, 25). Our findings suggest that activated PKA drives the induction of *Gadd45γ*, which activates MTK1, the upstream activator of MKK3/6, which then phosphorylate p38 (Fig. S6*A*) (23, 24). GADD45γ action via p38 signaling is also seen in other physiological contexts (20, 30, 31). The two other GADD45 family members, GADD45α and GADD45β, can also activate p38 in a signal-dependent manner (23). Consistent with this conserved GADD45 function, overexpression of GADD45α in primary adipocytes mimicked GADD45γ and enhanced ERRγ-dependent transcription (Fig. S6*B*). Although GADD45α and GADD45β are not induced in BAT by cold, they may be induced by other signals that activate the thermogenic program.

Although multiple kinases have been shown to regulate ERRα activity (32), our observation that p38 activates ERRβ and ERRγ provides, to our knowledge, the first insight into how these other ERRs integrate extracellular signals. Notably, p38 was able to strongly activate ERRβ and ERRγ in 10T1/2 cells that lack endogenous PGC-1α and PGC-1β expression, indicating that its effects were not mediated by phosphorylation and activation of PGC-1 coactivators. It remains to be determined whether MAPK p38 activates ERRβ and ERRγ via direct phosphorylation or through regulation of alternative coregulators. Interestingly, additional ERRβ/γ activators identified in the cDNA screen are known p38 activators, suggesting that multiple hits in our screen activated Gal4-ERRβ by increasing p38 signaling (e.g., the scaffold protein p62/SQSTM1 scored in the top 12 hits and has been shown to regulate p38 activity) (33).

The ability of p38 to activate ERRβ and ERRγ may have implications for physiological responses other than BAT thermogenesis where ERRs play roles, such as exercise-induced adaptations in skeletal muscle. The MKK3/6 and p38 signaling cascade is activated in skeletal muscle by exercise, and p38γ is necessary for aspects of the adaptive response to exercise (34, 35). Muscle ERRγ expression levels correlate with exercise performance in humans (36). Moreover, mice lacking ERRβ and ERRγ in skeletal muscle have decreased running capacity whereas mice overexpressing ERRγ in muscle show increased exercise capacity (36–38). We speculate that p38-mediated activation of ERRβ and ERRγ may link exercise to adaptive metabolic changes in skeletal muscle.

Our work underscores the complexity of the transcriptional network that controls BAT thermogenesis. Multiple members of the PGC-1 and GADD45 families are expressed in BAT and have the potential to activate the thermogenic program. The existence of multiple interacting pathways likely explains why single knock-out mouse models (e.g., Pgc-1α or Gadd45γ) display relatively mild thermogenic phenotypes and highlights the challenge of defining the roles of these regulators in whole-body energy homeostasis. On the upside, the discovery of more molecular players that stimulate BAT activity may provide further avenues to increase energy expenditure for potential therapeutic purposes.

Methods

Constructs and Viruses. For constructs and viruses, see *SI Methods*.

Cell Culture. Mouse mesenchymal C3H-10T1/2 cells (ATCC CCL-226) were grown in DMEM (Gibco), supplemented with 10% (vol/vol) FCS (Gemini Bio), and maintained at 37 °C with 5% CO_2. Primary brown adipocytes were isolated and differentiated as described (*SI Methods*) (39). Cells were analyzed 7 d after the initiation of differentiation, unless indicated otherwise. Drugs (norepinephrine, 8-CPT-cAMP, SB202190, or H-89) were added on day 6 for overnight treatment or day 7 for 1- to 4-h treatments.

Transfection, Luciferase Assays, and Cell-Based cDNA Library Screen. For the ERR activity assays, C3H 10T1/2 cells were reverse transfected using Fugene6 (Roche). Twenty-four hours after the transfection, BriteLite reagent (Perkin-Elmer) was added to each well, and luminescence was measured on a Pherastar Spectrophotometer (BMG Labtech). Relative luciferase values have been normalized to Gal4-DBD activity. For the screen, the cDNA library (Open Biosystems Mammalian Gene Collection, containing ~18,000 cDNAs) was arrayed in 384-well plates, in duplicates. For screening procedures, see *SI Methods*.

Infection of Primary Brown Adipocytes with Adenoviruses and Lentiviruses. For overexpression assays, mature adipocytes were infected on day 5 or 6 of differentiation with the indicated adenoviruses in serum-free DMEM with 2 mg/mL poly-L-lysine, at a multiplicity of infection (MOI) of 20. For the generation of Pgc-1α/Pgc-1β knockout cells, preadipocytes isolated from mice carrying floxed *Ppargc1a* and *Ppargc1b* alleles (40, 41) were infected at 70% confluency with GFP (control) or CRE-expressing lentivirus, resuspended in 4 μg/mL polybrene.

DNA, RNA, and Protein Analyses. For DNA, RNA, and protein analyses, see *SI Methods*.

O_2 Consumption. Basal and maximal O_2 consumption rates were determined using a Clark-type oxygen electrode. Percentage mitochondrial uncoupling was determined using a Seahorse XFe96 flux analyzer. See *SI Methods* for assay conditions.

Animal Studies. For full description of mouse strains and care, see *SI Methods*. Gadd45γ KO mice show complete male-to-female sex reversal (30, 31); therefore, female (XX) mice [8–9 wk old and acclimated to thermoneutrality (30 °C) for 2–3 wk] were used for experiments, unless noted otherwise. For the transcriptional response to cold exposure, mice were transferred to 4 °C for 6 h, housed singly and with full access to water and food. Control animals remained at 30 °C. At 6 h, mice were euthanized, and the interscapular BAT was isolated. Mice that developed hypothermia (more than a 6 °C drop in body temperature) before 6 h (one of nine WT and one of seven Gadd45γ KO mice) were removed from the cohort and the analysis. For BAT thermogenesis measurements, mice that carried injectable transponders in the vicinity of BAT depots for the report of temperature (IPTT-300; Bio Medic Data Systems) were transferred to 21 °C. Temperatures were recorded hourly after transfer, for 5 h.

Statistical Analysis. For statistical analysis, see *SI Methods*.

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