Original Article
OBESITY BIOLOGY AND INTEGRATED PHYSIOLOGY

Obesity

Parkin-Mediated Mitophagy is Downregulated in Browning of White Adipose Tissue

David Taylor and Roberta A. Gottlieb

Objective: Browning of white adipose tissue (WAT) promotes increased energy expenditure through the action of uncoupling protein 1 (UCP1) and is an attractive target to promote weight loss in obesity. Lowering of mitochondrial membrane potential by UCP1 is uniquely beneficial in this context; in other tissues, reduced membrane potential promotes mitochondrial clearance via mitophagy. It is unknown how parkin-mediated mitophagy is regulated in beige adipocytes.
Methods: The relationship between parkin expression and WAT browning was investigated in 3T3-L1 adipocytes and parkin-deficient male C57BL/6 mice in response to pharmacological browning stimuli.
Results: Rosiglitazone treatment in 3T3-L1 adipocytes promoted mitochondrial biogenesis, UCP1 expression, and mitochondrial uncoupling. Parkin expression was decreased and reduced mitochondrial-associated parkin, and p62 indicated a reduction in mitophagy activity. Parkin overexpression prevented mitochondrial remodeling in response to rosiglitazone. In CL 316,243-treated wild-type mice, decreased parkin expression was observed in subcutaneous inguinal WAT, where UCP1 was strongly induced. CL 316,243 treatment weakly induced UCP1 expression in the gonadal depot, where parkin expression was unchanged. In contrast, parkin-deficient mice exhibited robust UCP1 expression in gonadal WAT following CL 316,243 treatment.
Conclusions: WAT browning was associated with a decrease in parkin-mediated mitophagy, and parkin expression antagonized browning of WAT.

Obesity (2017) **25**, 704-712. doi:10.1002/oby.21786

Introduction

It is now recognized that active brown fat is present in adult humans (1). Characterized by a high abundance of mitochondria, the presence of multiple lipid droplets within adipocytes, and an abundance of the thermogenic uncoupling protein 1 (UCP1), brown fat activation has been shown to increase total energy expenditure in adults (2). While brown fat activation is an attractive target to expend excess energy in the face of overnutrition, it is not considerably abundant and is scarce in people with obesity and diabetes (3).

Beige fat is a novel class of adipose tissue arising in the subcutaneous white adipose depots of rodents, bearing multiple brown fat-like characteristics, namely increased abundance of mitochondria, the presence of multilocular adipocytes, and expression of UCP1 (4). In mice, brown and beige adipocytes are derived from distinct embryonic precursors and may be differentially regulated (5). The presence of beige fat in humans has not yet been demonstrated; however, subcutaneous white adipose tissue (WAT)-derived stromal progenitor cells can be converted to a beige-like phenotype *in vitro* (6). Furthermore, human brown adipocytes have been shown to exhibit markers of classical brown and beige adipocytes in rodents (7). In recent years, a growing list of pharmacological, physiological, and environmental stimuli has been demonstrated to induce expression of beige fat in experimental models (8).

UCP1 was first purified in 1982, and its function in energy dissipation and heat generation has been well characterized over the past 50 years (9). This 32 kDa inner mitochondrial membrane protein is activated by long-chain fatty acids, resulting in increased proton conductance and, thus, dissipating the membrane potential. As such, brown adipose tissue mitochondria exhibit a modest membrane potential of -30 to -50 mV (10). In contrast, the membrane potential of brown fat mitochondria from UCP1-ablated mice is approximately -200 mV, similar to mitochondria of other tissues (11). Indeed, the less-negative membrane potential of brown (and beige) mitochondria is unique in that it is functionally beneficial. In other tissues, a sustained decrease in mitochondrial membrane potential is generally considered deleterious. Such instances arise as a result of various mechanisms

The Cedars-Sinai Heart Institute, Barbra Streisand Women's Heart Center, Cedars-Sinai Medical Center, Los Angeles, California, USA. Correspondence: Roberta A. Gottlieb (roberta.gottlieb@cshs.org)

Funding agencies: This work was funded in part by NIH P01 HL112730 (RAG).
Disclosure: The authors declared no conflict of interest.
Additional Supporting Information may be found in the online version of this article.
Received: 11 August 2016; **Accepted:** 11 January 2017; **Published online 27 February 2017. doi:10.1002/oby.21786**

such as apoptosis, mitochondrial permeability transition pore opening, mitochondrial membrane damage, or impaired electron transport (12).

Mitophagy describes the selective process by which damaged mitochondria are sequestered and subsequently removed via autophagic degradation. The importance of mitophagy in maintaining mitochondrial function is well established in cardiac and neuronal biology (13), while critical roles for mitophagy are emerging in other tissues and organ systems (14).

Parkin-mediated mitophagy is the best-characterized mitophagic pathway, wherein mitochondrial damage precipitates a fall in membrane potential, resulting in accumulation of PINK1 and recruitment of cytosolic parkin to the outer mitochondrial membrane. Activated parkin then mediates polyubiquitination of outer mitochondrial membrane proteins, targeting the organelle for subsequent autophagic degradation (15).

Our current understanding of the role of parkin-mediated mitophagy is to maintain a healthy mitochondrial population via culling of damaged or senescent organelles, which are characterized by a fall in membrane potential. The setting of WAT browning presents a unique mitochondrial environment wherein lowering of membrane potential is considered functionally beneficial. A rapid increase in UCP1 expression observed in white adipose browning elicits a fall in membrane potential, which we may predict would subsequently target the organelles for removal. Autophagic removal of mitochondria has been demonstrated in differentiating adipocytes (16); however, the potential role of parkin-mediated mitophagy has yet to be explored in adipose biology. Herein we explored the role of parkin in the browning of WAT.

Methods

Animals

All animal studies were performed in accordance with institutional guidelines and approved by the Institutional Animal Care and Use Committee of Cedars Sinai Medical Center. Male parkin$^{-/-}$ (C57BL/6 background) and wild-type mice were obtained from The Jackson Laboratory. At 9 to 10 weeks of age, rosiglitazone or CL 316,243 was administered via 10 daily intraperitoneal injections of 10 mg/kg/day and 1 mg/kg/day, respectively. Control animals received saline vehicle. Animals were housed at room temperature throughout. Nine-week-old db/db and control counterpart mice were obtained from The Jackson Laboratory.

Cell culture and differentiation

3T3-L1 (murine embryonic fibroblast) cells were purchased from Zen-Bio. All experiments were performed between cell passage 9-13. Undifferentiated preadipocytes were cultured in high-glucose DMEM supplemented with 10% bovine calf serum. For differentiation studies, cells were grown to confluence and growth arrested for 48 hours. Differentiation was initiated using DMEM/F12 supplemented with 10% FBS, 1 μg/mL insulin, 0.5 mM 3-isobutyl-1-methylxanthine, 0.25 μM dexamethasone, and 1 μM rosiglitazone. After 48 hours, the medium was refreshed with DMEM/F12 supplemented with 10% FBS and 1 μg/mL insulin. After a further 48 hours, cells were subsequently maintained in DMEM/F12 supplemented with 10% FBS. All treatments were initiated on day 5 of differentiation.

Transfections and gene silencing experiments were performed using TurboFect^{TM} Transfection Reagent (ThermoFisher) following manufacturer's guidelines. Parkin, optineurin, p62, and scrambled siRNA (Santa Cruz Biotechnology) were reconstituted following manufacturer instructions. mCherry-parkin was obtained from Dr. Richard Youle (17).

Western blotting

See Supporting Information for further details.

RNA extraction and quantitative RT-PCR

RNA was extracted from cells using NucleoSpin® RNA kits (Machery-Nagel) according to manufacturer's instructions. cDNA was synthesized from 1 μg of RNA using iScript^{TM} cDNA synthesis kit (Bio-Rad), and quantitative real-time PCR was performed with iTaq^{TM} SYBR® Green Supermix (Bio-Rad) on a CFX96 Thermal Cycler (Bio-Rad). A list of primer sequences used for RT-PCR analysis is shown in Supporting Information Methods S1 .

Mitochondrial isolation and functional measurements

See Supporting Information for further details.

Histology

Adipose tissues were excised, fixed in 4% PFA, and embedded in paraffin. General morphology was subsequently visualized by hematoxylin and eosin staining.

Oil Red O stain

Cells were fixed with 10% formalin and rinsed with PBS, then dehydrated with 60% isopropanol for 5 minutes. Cells were subsequently stained with filtered Oil Red O staining solution for 30 minutes (60% Oil Red O solution [0.5% Oil Red O in isopropanol]/40% water.) Cells were washed twice with PBS, then lipid staining was visualized via microscopy. Following imaging, the dye was extracted with 100% isopropanol and the absorbance was measured spectrophotometrically at 492 nm.

Statistical analysis

Data are expressed as means ± standard deviation (SD). Student *t* test was used for unpaired comparisons, while ANOVA followed by Tukey's multiple comparison test was used to determine differences among 3 or more groups. A P value of <0.05 was considered significant.

Results

Parkin expression is increased in differentiating adipocytes

3T3-L1 preadipocytes were selected as a cellular model for browning. 3T3-L1 cells are widely used as an *in vitro* model for white adipocytes; however, they have been reported to respond to browning

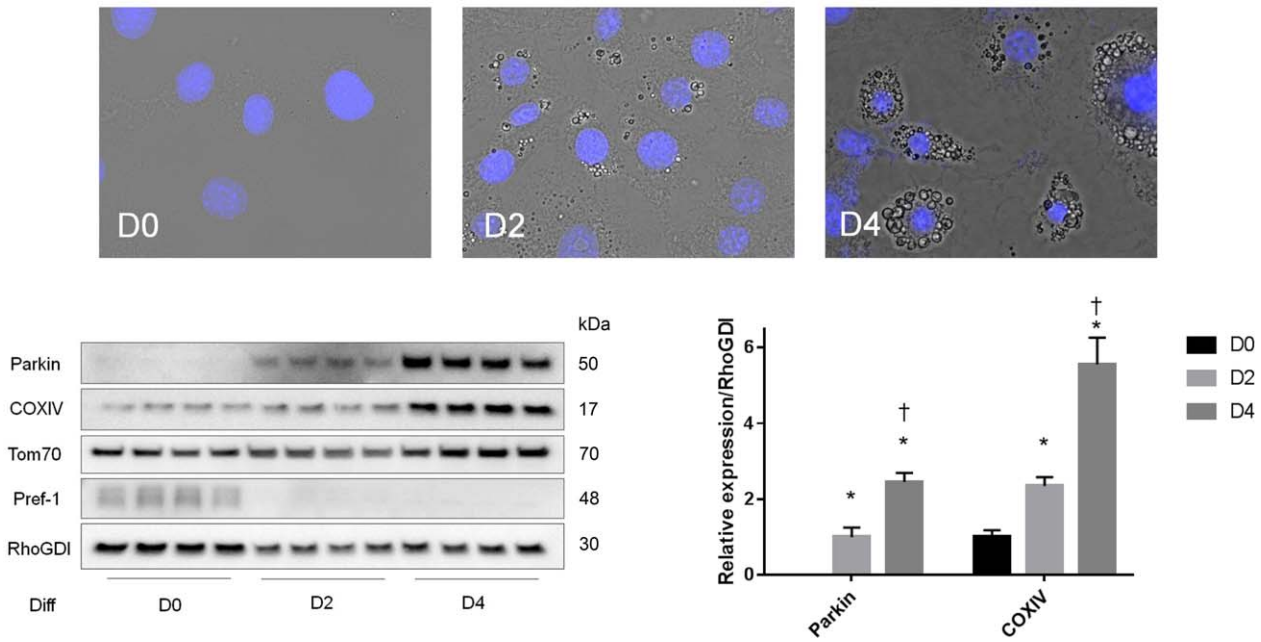

Figure 1 Parkin expression in differentiating 3T3-L1 adipocytes. Representative 40x magnification brightfield images (blue-DAPI) of 3T3-L1 adipocytes on D0, D2, and D4 of differentiation. Immunoblots and densitometric analysis of parkin and mitochondrial protein expression. Values are means ± SD ($n = 4$). *$P < 0.05$ vs. D0, †$P < 0.05$ vs. D2. [Color figure can be viewed at wileyonlinelibrary.com]

stimuli and upon induction can exhibit characteristics of multiple adipocyte lineages (18,19). We initially investigated the expression of parkin in differentiating 3T3-L1 preadipocytes. Parkin protein was undetectable in undifferentiated 3T3-L1 preadipocytes, rising 2-fold between days 2 and 4 of differentiation, in parallel with a 2- to 3-fold increase in COXIV expression (Figure 1). Initiation of differentiation was marked by the disappearance of the preadipocyte marker pref-1 after day 0 (20).

Rosiglitazone induces beiging of 3T3-L1 adipocytes

Differentiated 3T3-L1 preadipocytes (D5) were initially treated with several reported browning agonists in order to induce a beige phenotype. Seventy-two hours of treatment with sildenafil (1 μM) and CL 316,243 (100 nM) failed to stimulate mitochondrial biogenesis or induce UCP1 expression in our model (Supporting Information Figure S1). Following 72 hours of treatment with the PPARγ agonist rosiglitazone (1 μM), mitochondrial biogenesis and brown fat-associated gene expression was elevated, and UCP1 gene expression was significantly increased in rosiglitazone-treated cells (Figure 2C).

Respirometry revealed functional differences in rosiglitazone-treated cells, consistent with a beige phenotype (Figure 2B). Basal proton leak and respiratory reserve capacity were elevated in rosiglitazone-treated cells. Cells were acutely treated with the β3-adrenergic receptor agonist CL 316,243 to stimulate lipolysis, resulting in a 129% stimulation of basal respiration in rosiglitazone-treated cells (versus 7% in controls). Subsequent oligomycin treatment revealed that CL 316,243-stimulated respiration was attributable to an increased proton leak, indicating mitochondrial uncoupling. Mitochondrial membrane potential was lowered in rosiglitazone-treated cells, consistent with a more uncoupled phenotype (Figure 2D). Lipid accumulation was not significantly different in rosiglitazone-treated cells (Figure 2E.)

Parkin is downregulated in beiged 3T3-L1 adipocytes

Following 72 hours of rosiglitazone treatment, parkin expression was suppressed at the protein and mRNA levels (Figure 3A-B). No differences in expression of the negative regulators of parkin, deubiquitinating enzymes USP30 and USP35, were observed in rosiglitazone-treated adipocytes (Figure 3B). Mitochondria-associated p62 and parkin, markers of mitochondria targeted for degradation, were significantly decreased in rosiglitazone-treated adipocytes (Figure 3C). These findings indicate that the induction of mitochondrial biogenesis in browning is associated with decreased activity of parkin-mediated mitophagy.

Parkin downregulation *in vivo* is specific to the subcutaneous WAT

In rodent models, it has previously been shown that the induction of UCP1 expression in response to pharmacological or environmental stimuli is specific to subcutaneous WAT depots (21). We investigated tissue expression of beige fat-associated mitochondrial proteins in response to rosiglitazone or the selective beta 3-adrenoreceptor agonist CL 316,243 in the subcutaneous inguinal (sWAT) and epididymal (gonadal) (gWAT) depots in male C57BL/6 mice. Following 10 days of treatment, lipid droplet size was diminished in response to both agents in sWAT (Figure 4A). Both rosiglitazone and CL 316,243 induced UCP1 expression in sWAT; however, it was more potently induced with the latter. In contrast, UCP1

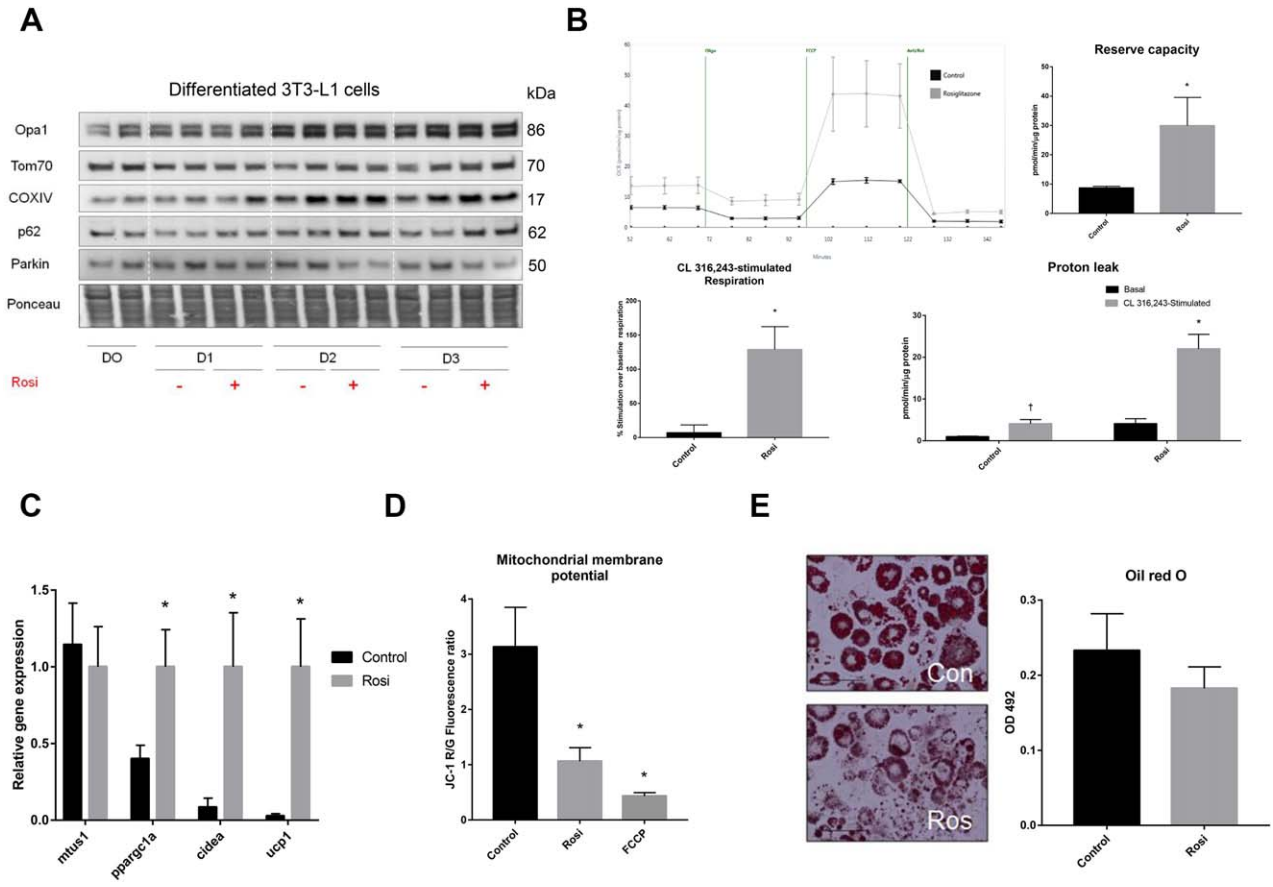

Figure 2 Characterization of browning model. (**A**) Representative immunoblot illustrating mitochondrial biogenesis in differentiated 3T3-L1 cells in response to rosiglitazone administration (1μM) for 72 h. (**B**) Respirometry trace and respiratory rates from rosiglitazone-treated 3T3-L1 cells subjected to mitochondrial stress test and acute CL 316,243-stimulated oxygen consumption; values are means ± SD ($n = 3$). *$P < 0.05$ vs. untreated control, †$P < 0.05$ vs. CL 316,243-stimulated control. (**C**) Relative gene expression in rosiglitazone-treated 3T3-L1 cells, normalized to GAPDH; values are means ± SD ($n = 3$). *$P < 0.05$ vs. control. (**D**) Relative mitochondrial membrane potential in rosiglitazone-treated 3T3-L1 cells; values are means ± SD ($n = 6$). *$P < 0.05$ vs. control. (**E**) Representative 40x magnification lipid stains in rosiglitazone-treated 3T3-L1 adipocytes (scale bars 100 μm) and quantified extracted lipid stain intensity; values are means ± SD ($n = 4$). [Color figure can be viewed at wileyonlinelibrary.com]

expression was not detected in gWAT in response to rosiglitazone and was only weakly expressed in CL 316,243-treated animals. Interestingly, the outer mitochondrial membrane proteins Tom70, Mfn2, and VDAC were comparably upregulated in both depots, suggesting distinct mechanisms controlling induction of mitochondrial biogenesis per se and UCP1 expression (Figure 4A). In CL 316,243-treated mice (Figure 4B), in which the magnitude of UCP1 induction was greatest, the increase in UCP1 in sWAT was coupled with a decrease in parkin expression, while parkin expression was unchanged in gWAT. We also investigated parkin expression in sWAT of untreated db/db mice (Supporting Information Figure S2), a model reported to exhibit an impaired induction of UCP1 following cold exposure (22). While mitochondrial protein expression was similar to controls, parkin expression was elevated threefold in db/db mice, implying increased mitophagy activity. These findings suggest that parkin must be downregulated in order for UCP1-containing mitochondria to persist.

Parkin is not required for induction of browning of adipocytes

We next silenced parkin expression in differentiated 3T3-L1 cells to investigate whether suppression promotes the browning response. Forty-eight hours following siRNA treatment, parkin expression was decreased by 60% (Figure 5A). In untreated cells, expression of the outer mitochondrial membrane protein and parkin target Tom70 was moderately increased, implying a role for parkin in basal turnover of mitochondria (left panel). Basal expression of other mitochondrial proteins did not differ following parkin silencing and basal oxygen consumption and proton leak were similar to control cells (Figure 5C). Following 48 hours of rosiglitazone treatment, mitochondrial protein expression was comparable (Figure 5A), and parkin-silenced cells exhibited comparable upregulation of UCP1 expression (Figure 5C). Similarly, basal respiration was increased in parkin-silenced cells following rosiglitazone treatment (Figure 5D), indicating that suppression of parkin does not affect the magnitude of the browning response. We also assessed UCP1

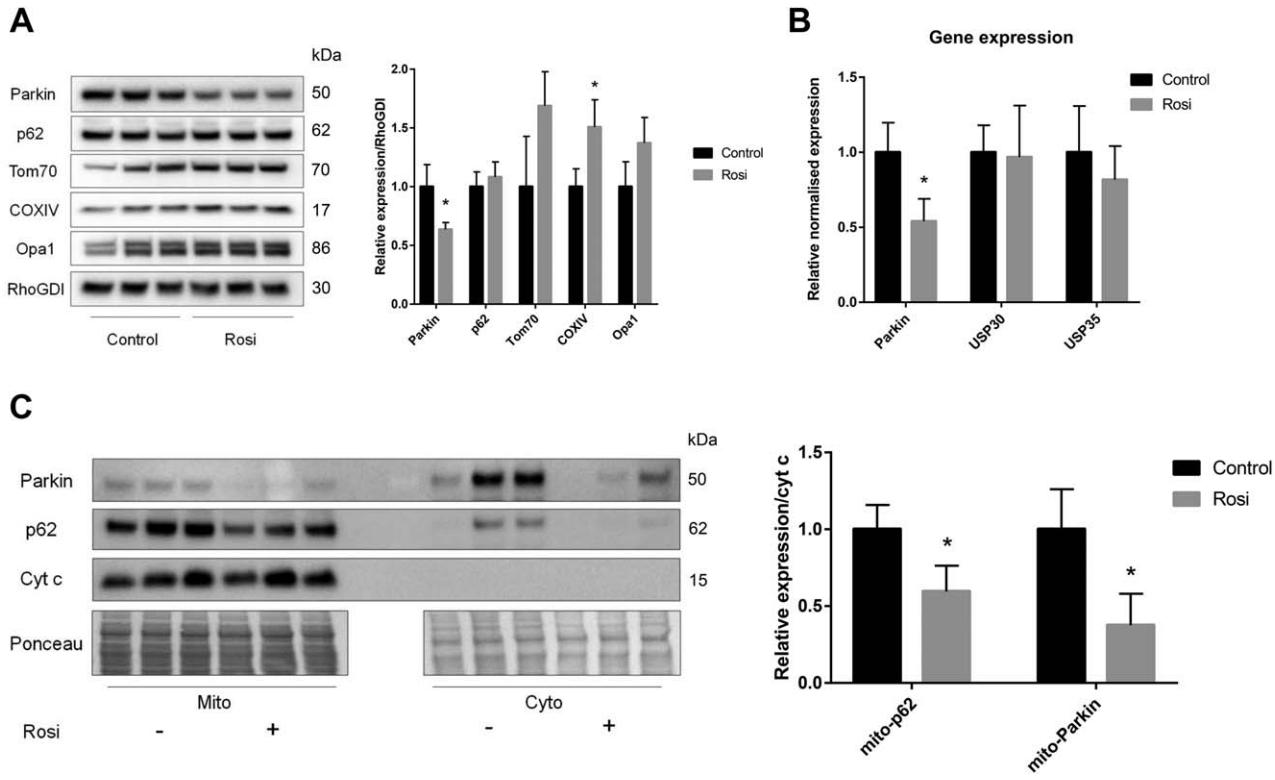

Figure 3 Parkin is downregulated following rosiglitazone treatment. (**A**) Immunoblots and densitometric analysis of mitophagy and mitochondrial protein expression in differentiated 3T3-L1 adipocytes treated with rosiglitazone for 72 h (1 μM); values are means ± SD ($n = 3$). *$P < 0.05$ vs. control. (**B**) Relative gene expression in rosiglitazone-treated 3T3-L1 cells; values are means ± SD relative to expression of actin and hydroxymethylbilane synthase ($n = 3$). *$P < 0.05$. (**C**) Immunoblots and densitometric analysis of mitochondrial and cytosolic fractions from rosiglitazone-treated 3T3-L1 cells; values are means ± SD ($n = 3$). *$P < 0.05$ vs. control.

mRNA expression in p62 and optineurin siRNA-treated 3T3-L1 cells following rosiglitazone treatment (Supporting Information Figure S4). UCP1 expression was significantly increased in both cases following rosiglitazone treatment, indicating that suppression of these proteins does not inhibit browning in this context.

Parkin overexpression prevents browning of adipocytes

We then transfected differentiated 3T3-L1 adipocytes with mCherry-parkin to observe whether parkin overexpression impacts the browning response (Figure 5B). Under basal conditions, Tom70 abundance was drastically decreased in transfected cells, while abundance of other inner and outer mitochondrial membrane proteins did not change. Following 48 hours of rosiglitazone treatment, an approximate 2-fold increase in expression of mitochondrial proteins was observed in control cells, while parkin-overexpressing cells exhibited no change in expression of mitochondrial proteins. Consistent with these findings, UCP1 expression was significantly lower in rosiglitazone-treated parkin-overexpressing cells, compared to rosiglitazone-treated controls (Figure 5C). Moreover, rosiglitazone had no stimulatory effect on basal respiration and proton leak in mCherry-parkin-expressing cells (Figure 5D). These results demonstrate that parkin antagonizes the browning response to rosiglitazone in adipocytes.

Parkin expression underlies the differential response of subcutaneous and visceral adipose tissues to browning stimuli

We next explored the *in vivo* response of sWAT and gWAT of parkin-deficient (PKO) mice to CL 316,243 administration (Figure 6). In sWAT, PKO mice exhibited similar induction of UCP1 and other mitochondrial proteins to wild-types (Figure 6A). In isolated mitochondria, both strains exhibited similar increases in state 3 and state 4 respiration following CL 316,243 administration (Supporting Information Figure S3). Wild-type mice exhibited a similar though nonsignificant degree of parkin downregulation, in response to CL 316,243 treatment (Figure 4). In mitochondria isolated from sWAT (Figure 6B), mitochondrial-associated p62 was decreased in both wild-type and PKO mice, suggesting that the browning treatment reduced the number of mitochondria targeted for mitophagy.

In gWAT, wild-type parkin expression did not change in response to the CL 316,243 treatment, while UCP1 expression was only mildly induced, consistent with the apparent resistance of the gonadal depot to browning stimuli (Figure 6C). Unexpectedly, PKO mice exhibited robust induction of UCP1 expression in the gonadal depot, indicating that parkin may contribute to the blunted browning response in gWAT compared with sWAT. Collectively, these findings indicate that the browning response is associated with a decrease in mitophagy and that parkin expression antagonizes browning of WAT.

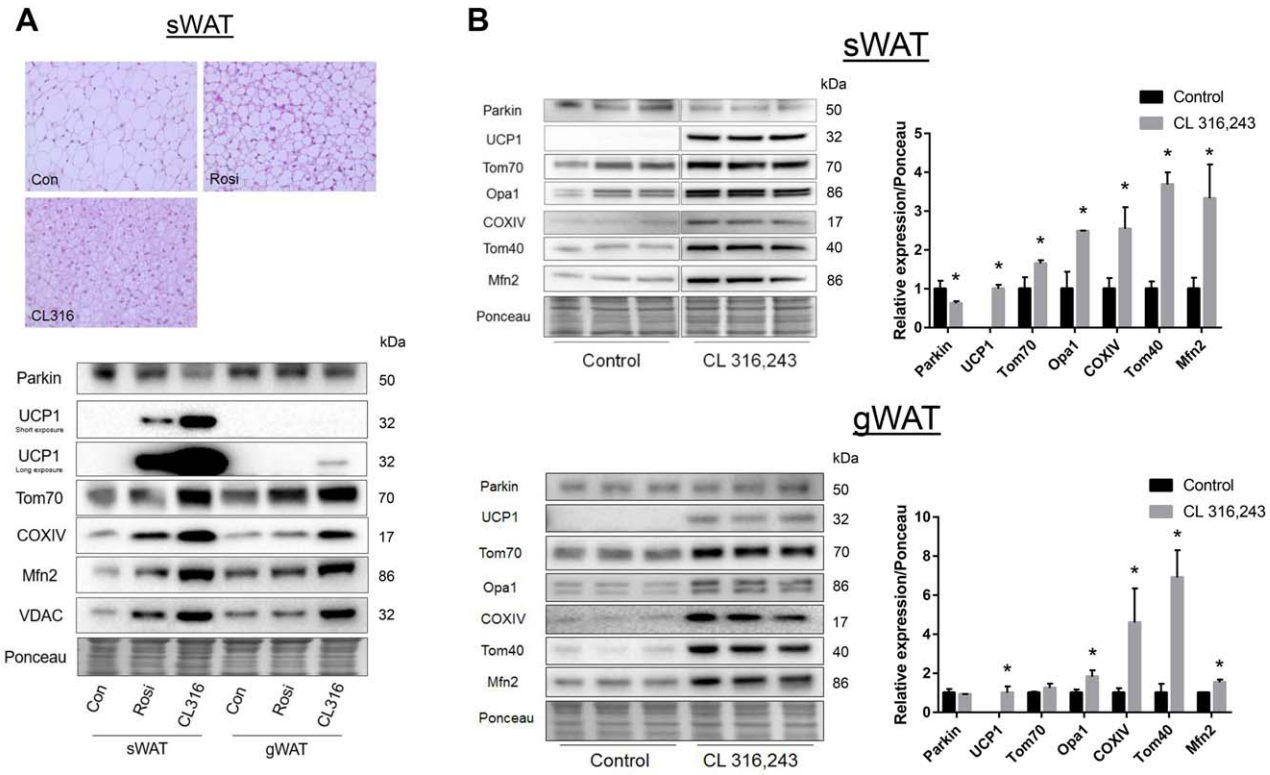

Figure 4 *In vivo* browning of subcutaneous (sWAT) and gonadal (gWAT) white adipose tissue. (**A**) Representative hematoxylin and eosin stains of sWAT from male wild-type mice following 10 d of vehicle, rosiglitazone (10 mg/kg/d), and CL 316,243 (1 mg/kg/d) administration; and representative immunoblots illustrating comparative induction of mitochondrial protein expression in sWAT and gWAT depots following each treatment. (**B**) Immunoblots and densitometric analyses of sWAT (upper panel) and gWAT (lower panel) following 10 d of CL 316,243 administration. Panels acquired from same blot and exposure; values are means ± SD ($n = 3$). *$P < 0.05$ vs. control. [Color figure can be viewed at wileyonlinelibrary.com]

Discussion

Our current paradigm of mitochondrial quality control dictates that when mitochondria are dysfunctional, their membrane potential decreases, providing a signal to the cell to initiate their clearance. Beige adipocytes perform a specialized function that is critically dependent upon the regulated lowering of mitochondrial membrane potential, yet their mitochondria persist. Here we provide the first evidence that decreased activity of parkin-mediated mitophagy underlies the transition from white to beige adipocytes. Furthermore, results from our knockout experiments suggest that differential activity of parkin-mediated mitophagy may contribute to the distinct responses of subcutaneous and visceral WATs to browning stimuli.

While the central components of parkin-mediated mitophagy have been well characterized, relatively little is known about regulation of parkin expression. Parkin expression and mitophagic activity increase in response to oxidative stress (23), promoting clearance of damaged mitochondria. Parkin is transcriptionally regulated in part by ATF4, a transcription factor of the unfolded protein response (24). ATF4 has also been shown to regulate adipogenic differentiation, which is consistent with our observation of robust parkin upregulation in differentiating adipocytes (25). Interestingly, ATF4 null mice have a lean phenotype and are resistant to diet-induced obesity, though parkin expression has yet to be evaluated in these mice (26).

Parkin knockout mice have previously been characterized (27), exhibiting a leaner phenotype with resistance to high-fat-diet-induced weight gain with enhanced oxygen consumption, despite increased food intake. These findings were attributed in part to an alteration in systemic lipid metabolism due to a role for parkin in regulating hepatic CD36 protein stability. It is well established that in the acute setting, a high-fat diet promotes expression of UCP1 in brown and white adipose depots (28). Our findings suggest the observed resistance to weight gain in PKO mice may also manifest as a result of unbridled UCP1 induction, in particular in the visceral WAT as we have observed.

Our finding that UCP1 expression can be robustly induced in parkin-ablated gonadal WAT suggests that differential activity of the mitophagy machinery may contribute to the contrasting responses of subcutaneous and visceral adipose tissues to browning stimuli. The transcriptional regulatory protein PRDM16 has been identified as a mediator of browning of WAT, and its higher expression in subcutaneous than visceral adipose tissue correlates with the responsiveness of those tissues to browning agents (21). Similarly, PRDM16 ablation promotes metabolic dysfunction and a propensity for increased visceral adiposity (29). However, when transgenically expressed in epididymal adipose tissue, PRDM16 expression failed to induce expression of brown like adipocytes (30).

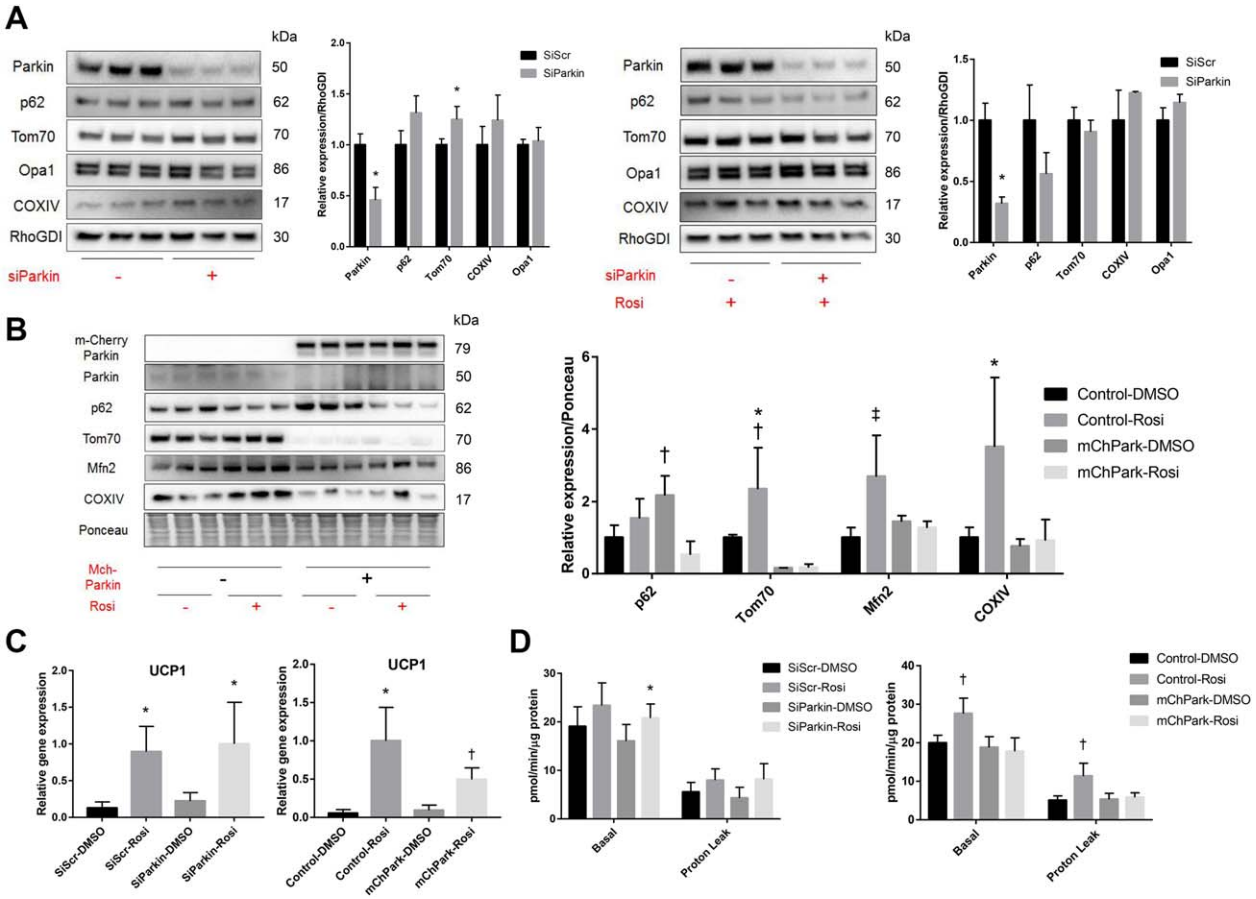

Figure 5 Impact of manipulating parkin expression upon browning response of differentiated 3T3-L1 cells. (**A**) Immunoblot and densitometric analysis of vehicle-treated (left panel) and rosiglitazone-treated (right panel) parkin-silenced 3T3-L1 cells; values are means ± SD ($n = 3$). *$P < 0.05$ vs. control. (**B**) Immunoblot and densitometric analysis of mCherry-parkin-expressing 3T3-L1 cells following rosiglitazone treatment; values are means ± SD ($n = 3$). †$P < 0.05$ vs. mCherry-parkin-rosi, *$P < 0.05$ vs. mCherry-parkin-DMSO, ‡$P < 0.05$ vs. control-DMSO. (**C**) UCP1 mRNA expression in parkin-silenced (left panel) and mCherry-parkin-expressing 3T3-L1 cells (right panel) following rosiglitazone treatment; values are means ± SD relative to expression of hydroxymethylbilane synthase and actin ($n = 6$). *$P < 0.05$ vs. DMSO-treated, †$P < 0.05$ vs. control-rosi. (**D**) Respiratory parameters from parkin-silenced (left panel) and mCherry-parkin-expressing (right panel) 3T3-L1 cells following rosiglitazone treatment; values are means ± SD ($n = 6$). *$P < 0.05$ vs. si-parkin-DMSO, †$P < 0.05$ vs. all groups. [Color figure can be viewed at wileyonlinelibrary.com]

Our studies have shown that browning stimuli promote increased expression of mitochondrial proteins and a functional increase in proton leak; however, overexpression of parkin prevents this remodeling. Aside from our hypothesized role in preventing clearance of UCP1-expressing mitochondria, decreased mitophagy may also be necessary in white adipose browning to permit the robust increase in mitochondrial mass that accompanies this remodeling. Loss of mitophagy adaptor BNip3 in the liver reportedly gives rise to an increased mitochondrial mass (31). When we silenced p62 and optineurin, rosiglitazone still promoted UCP1 expression, supporting the notion that a reduction in mitophagy machinery is permissive of beige remodeling. It was recently demonstrated, in an opposite scenario to the present context, namely, the reversion from beige-to-white fat, that autophagy-mediated mitochondrial clearance is required for this transformation (32). When autophagy was inhibited, the authors observed that the beige phenotype persisted. This report provides indirect evidence supporting our findings that mitochondrial autophagy must be reduced in order to permit beige transformation, though they did not investigate parkin in their model.

We observed elevated parkin expression in subcutaneous WAT of db/db mice that we hypothesize may contribute to the resistance of this tissue to browning stimuli (22). High-fat-diet-fed mice have also been shown to have increased WAT parkin expression in concert with mitochondrial impairments and a decrease in p62 and LC3-I (33). Because p62 functions as an adaptor protein responsible for recruiting mitochondria to autophagosomes, while LC3-I serves as a substrate that is recruited to autophagosomal membranes, these findings are suggestive of increased activity of parkin-mediated mitophagy. In such a setting, rampant mitophagy may promote rapid turnover and degradation of mitochondria and, thus, prevent browning from occurring. Similarly, the "whitening" of brown adipose tissues observed in obese mice occurs in concert with a rise in parkin expression (34).

It is unclear what factors drive increased parkin expression of adipose tissues in obesity. Inflammation is one such possibility in which macrophage infiltration, proinflammatory cytokine secretion, and oxidative stress may lead to oxidative damage to adipose tissue

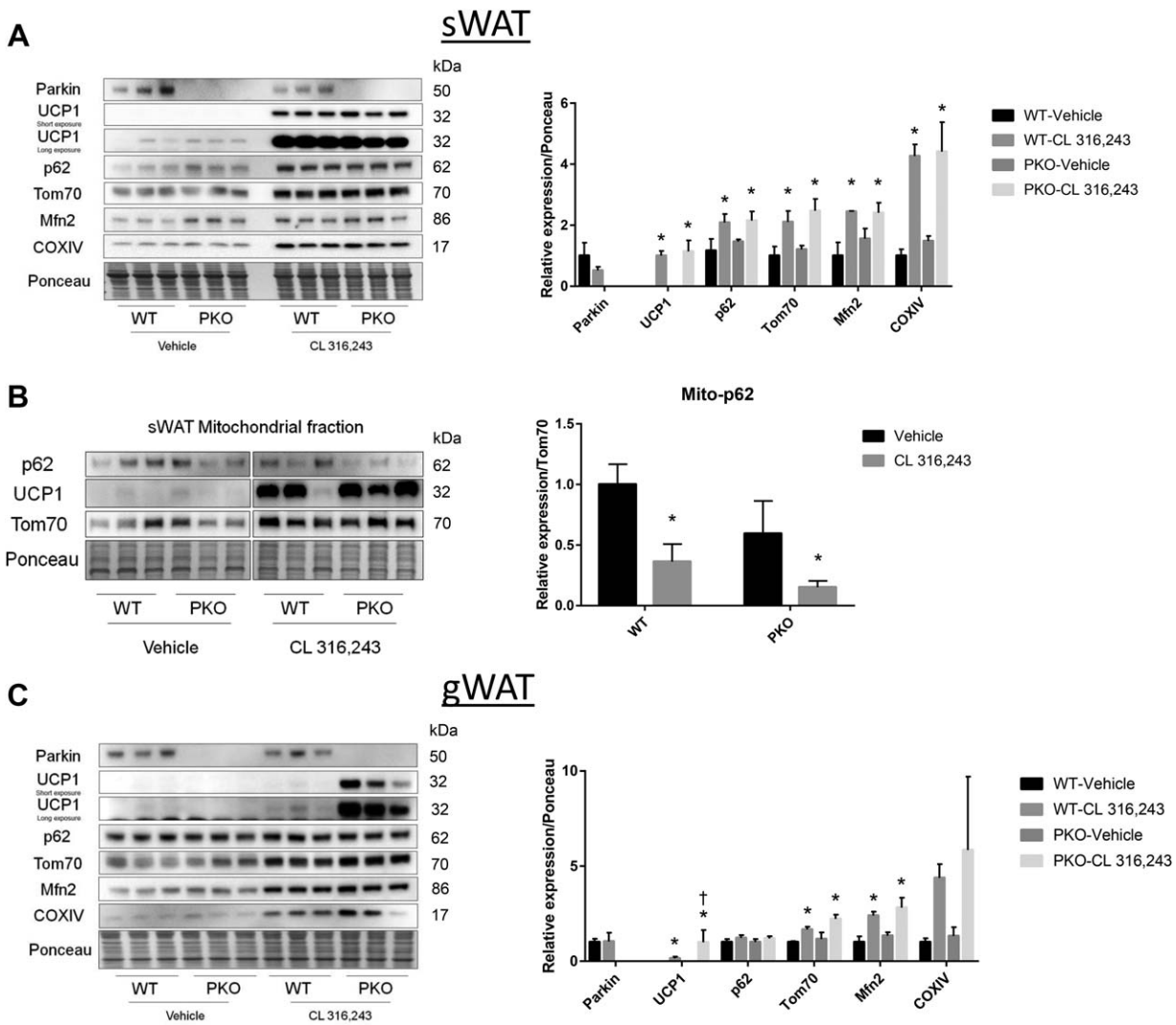

Figure 6 Differential browning response in subcutaneous (sWAT) and gonadal (gWAT) white adipose tissue of parkin-deficient mice. (**A**) Immunoblot and densitometric analysis of mitophagy and mitochondrial proteins in subcutaneous sWAT following 10 d of treatment with CL 316,243. (**B**) Isolated mitochondrial protein immunoblots and densitometric analysis of mitochondria-associated p62. Panels were acquired from same blot and exposure. (**C**) Immunoblot and densitometric analysis in gWAT. Values are means ± SD ($n = 3$). *$P < 0.05$ vs. wild-type (WT) vehicle, †$P < 0.05$ vs. WT CL 316,243.

mitochondria, thus triggering activation of mitophagy. Indeed, parkin plays an essential role in mitigating against neuroinflammation (35).

While parkin may be best characterized in the context of mitophagy, several other proteins appear to function in a somewhat similar manner, with their recruitment to mitochondria mediating degradation of the organelle. The role of one such protein, BNip3, was recently investigated in a similar context to the present manuscript (36). The authors observed an increase in BNip3 expression in 3T3-L1 adipocytes following rosiglitazone treatment, while the adipose tissues of high-fat-diet-fed and db/db mice exhibited decreased BNip3 expression. This apparent inverse response of BNip3 expression to parkin expression in the same setting is intriguing; however, the consequence of increased BNip3 expression in these contexts is unclear. When the authors silenced BNip3 expression, UCP1 mRNA induction was blunted, as was rosiglitazone-mediated stimulation of basal respiration and proton leak, mirroring our results from overexpressing parkin. It is likely that additional adaptor proteins will be identified in the future that regulate selective mitochondrial clearance, as mitochondrial remodeling is likely driven by more intracellular cues than a simple membrane-potential dependent trigger. For example, the adaptor pair NDP52 and optineurin were recently shown to be involved in activating mitophagy (37).

These findings highlight the central importance of mitochondrial turnover and mitophagy in the remodeling of WAT; however, some questions remain. Namely, if the robust browning response observed in gWAT of PKO mice is solely consequent to the absence of parkin, why was the degree of browning observed in subcutaneous WAT comparable to wild-types? It is feasible that the degree of browning observed in subcutaneous WAT represents a ceiling of maximal induction, in a manner analogous to the resistance of

interscapular brown adipose tissue to further browning stimuli in mice housed at room temperature (38). If correct, we may anticipate an exaggerated browning response in subcutaneous WAT of PKO mice if the experiment was repeated in thermoneutral conditions. It is also likely that other mechanisms contribute to the distinct browning responses observed between the depots. For example, subcutaneous adipose tissue reportedly exhibits decreased activation of JNK signaling compared with visceral fat (39). Inhibition of JNK signaling has been shown to reduce parkin recruitment to mitochondria in response to stress (40); thus, it is feasible that the sensitivity of parkin recruitment may be modified by such stress pathways.

Another unexplained observation is the apparent similar degree of induction of numerous mitochondrial proteins between the subcutaneous and visceral depots. Our results suggest that CL 316,243 treatment provokes a similar degree of mitochondrial biogenesis between depots. These low UCP1-expressing mitochondria have a very limited thermogenic capacity; therefore, it will be interesting to explore what role they play in a tissue that typically has a low complement of mitochondria and low respiratory activity.

The results of the present study have identified parkin downregulation as a prerequisite for browning of white adipocytes. Along with recent data from others, our findings highlight the importance of regulation of mitophagy and mitochondrial turnover in white adipose browning. Furthermore, our results provide novel evidence that parkin activity may contribute to the blunted response of visceral adipose tissue to browning stimuli.O

Acknowledgments

The authors acknowledge the Cedars-Sinai Metabolism and Mitochondrial Research Core for respirometry studies.

References

1. Sacks H, Symonds ME. Anatomical locations of human brown adipose tissue: functional relevance and implications in obesity and type 2 diabetes. *Diabetes* 2013; 62:1783-1790.
2. Ouellet V, Labbé S, Blondin DP, et al. Brown adipose tissue oxidative metabolism contributes to energy expenditure during acute cold exposure in humans. *J Clin Invest* 2012;122:545-552.
3. Shimizu I, Walsh K. The whitening of brown fat and its implications for weight management in obesity. *Curr Obes Reps* 2015;4:224-229.
4. Harms M, Seale P. Brown and beige fat: development, function and therapeutic potential. *Nat Med* 2013;19:1252-1263.
5. Seale P, Bjork B, Yang W, et al. PRDM16 controls a brown fat/skeletal muscle switch. *Nature* 2008;454:961-967.
6. Bartesaghi S, Hallen S, Huang L, et al. Thermogenic activity of UCP1 in human white fat-derived beige adipocytes. *Mol Endocrinol* 2015;29:130-139.
7. Jespersen NZ, Larsen TJ, Peijs L, et al. A classical brown adipose tissue mRNA signature partly overlaps with brite in the supraclavicular region of adult humans. *Cell Metab* 2013;17:798-805.
8. Sidossis L, Kajimura S. Brown and beige fat in humans: thermogenic adipocytes that control energy and glucose homeostasis. *J Clin Invest* 2015;125:478-486.
9. Nicholls DG, Rial E. A history of the first uncoupling protein, UCP1. *J Bioenerg Biomembr* 1999;31:399-406.
10. Girardier L, Seydoux J, Clausen T. Membrane potential of brown adipose tissue: A suggested mechanism for the regulation of thermogenesis. *J Gen Physiol* 1968;52: 925-940.
11. Matthias A, Jacobsson A, Cannon B, Nedergaard J. The bioenergetics of brown fat mitochondria from UCP1-ablated mice: UCP1 is not involved in fatty acid-induced de-energization ("uncoupling"). *J Biol Chem* 1999;274:28150-28160.
12. Petronilli V, Cola C, Bernardi P. Modulation of the mitochondrial cyclosporin A-sensitive permeability transition pore. II. The minimal requirements for pore induction underscore a key role for transmembrane electrical potential, matrix pH, and matrix Ca2+. *J Biol Chem* 1993;268:1011-1016.
13. Hamacher-Brady A, Brady NR. Mitophagy programs: mechanisms and physiological implications of mitochondrial targeting by autophagy. *Cell Mol Life Sci* 2016;73:775-795.
14. Lazarou M. Keeping the immune system in check: a role for mitophagy. *Immunol Cell Biol* 2015;93:3-10.
15. Eiyama A, Okamoto K. PINK1/Parkin-mediated mitophagy in mammalian cells. *Curr Opin Cell Biol* 2015;33:95-101.
16. Goldman SJ, Zhang Y, Jin S. Autophagic degradation of mitochondria in white adipose tissue differentiation. *Antioxid Redox Signal* 2011;14:1971-1978.
17. Narendra D, Tanaka A, Suen DF, Youle RJ. Parkin is recruited selectively to impaired mitochondria and promotes their autophagy. *J Cell Biol* 2008;183:795-803.
18. Morrison S, McGee SL. 3T3-L1 adipocytes display phenotypic characteristics of multiple adipocyte lineages. *Adipocyte* 2015;4:295-302.
19. Zhang Y, Li R, Meng Y, et al. Irisin stimulates browning of white adipocytes through mitogen-activated protein kinase p38 MAP kinase and ERK MAP kinase signaling. *Diabetes* 2014;63:514-525.
20. Hudak CS, Sul HS. Pref-1, a gatekeeper of adipogenesis. *Front Endocrinol* 2013;4: 79. doi:10.3389/fendo.2013.00079.
21. Ohno H, Shinoda K, Spiegelman BM, Kajimura S. PPAR agonists induce a white-to-brown fat conversion through stabilization of PRDM16 protein. *Cell Metab* 2012; 15:395-404.
22. Masaki T, Yoshimatsu H, Chiba S, Sakata T. Impaired response of UCP family to cold exposure in diabetic (db/db) mice. *Am J Physiol Regul Integr Comp Physiol* 2000;279:R1305-R1309.
23. Yang YX, Muqit MM, Latchman DS. Induction of parkin expression in the presence of oxidative stress. *Eur J Neurosci* 2006;24:1366-1372.
24. Bouman L, Schlierf A, Lutz AK, et al. Parkin is transcriptionally regulated by ATF4: evidence for an interconnection between mitochondrial stress and ER stress. *Cell Death Diff* 2011;18:769-782.
25. Yu K, Mo D, Wu M, et al. Activating transcription factor 4 regulates adipocyte differentiation via altering the coordinate expression of CCATT/enhancer binding protein beta and peroxisome proliferator-activated receptor gamma. *FEBS J* 2014; 281:2399-2409.
26. Seo J, Fortuno ES, Suh JM, et al. Atf4 regulates obesity, glucose homeostasis, and energy expenditure. *Diabetes* 2009;58:2565-2573.
27. Kim KY, Stevens MV, Akter MH, et al. Parkin is a lipid-responsive regulator of fat uptake in mice and mutant human cells. *J Clin Invest* 2011;121:3701-3712.
28. Fromme T, Klingenspor M. Uncoupling protein 1 expression and high-fat diets. *Am J Physiol Regul Integr Comp Physiol* 2011;300:R1-R8.
29. Cohen P, Levy JD, Zhang Y, et al. Ablation of PRDM16 and beige adipose causes metabolic dysfunction and a subcutaneous to visceral fat switch. *Cell* 2014;156:304-316.
30. Seale P, Conroe HM, Estall J, et al. Prdm16 determines the thermogenic program of subcutaneous white adipose tissue in mice. *J Clin Invest* 2011;121:96-105.
31. Glick D, Zhang W, Beaton M, et al. BNip3 regulates mitochondrial function and lipid metabolism in the liver. *Mol Cell Biol* 2012;32:2570-2584.
32. Altshuler-Keylin S, Shinoda K, Hasegawa Y, et al. Beige adipocyte maintenance is regulated by autophagy-induced mitochondrial clearance. *Cell Metab* 2016;24:402-419.
33. Cummins TD, Holden CR, Sansbury BE, et al. Metabolic remodeling of white adipose tissue in obesity. *Am J Physiol Endocrinol Metab* 2014;307:E262-E277.
34. Shimizu I, Aprahamian T, Kikuchi R, et al. Vascular rarefaction mediates whitening of brown fat in obesity. *J Clin Invest* 2014;124:2099-2112.
35. Frank-Cannon TC, Tran T, Ruhn KA, et al. Parkin deficiency increases vulnerability to inflammation-related nigral degeneration. *J Neurosci* 2008;28: 10825-10834.
36. Choi JW, Jo A, Kim M, et al. BNIP3 is essential for mitochondrial bioenergetics during adipocyte remodelling in mice. *Diabetologia* 2016;59:571-581.
37. Lazarou M, Sliter DA, Kane LA, et al. The ubiquitin kinase PINK1 recruits autophagy receptors to induce mitophagy. *Nature* 2015;524:309-314.
38. Virtue S, Vidal-Puig A. Assessment of brown adipose tissue function. *Front Physiol* 2013;4:128. doi:10.3389/fphys.2013.00128.
39. Liu R, Pulliam DA, Liu Y, Salmon AB. Dynamic differences in oxidative stress and the regulation of metabolism with age in visceral versus subcutaneous adipose. *Redox Biol* 2015;6:401-408.
40. Park JH, Ko J, Park YS, Park J, Hwang J, Koh HC. Clearance of damaged mitochondria through PINK1 stabilization by JNK and ERK MAPK signaling in chlorpyrifos-treated neuroblastoma cells [published online February 18, 2016]. *Mol Neurobiol* doi:10.1007/s12035-016-9753-1.