The E3 ubiquitin ligase parkin is dispensable for metabolic homeostasis in murine pancreatic β cells and adipocytes

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Abstract
The E3 ubiquitin ligase parkin is a critical regulator of mitophagy and has been identified as a susceptibility gene for type 2 diabetes (T2D). However, its role in metabolically active tissues that precipitate T2D development is unknown. Specifically, pancreatic β cells and adipocytes both rely heavily on mitochondrial function in the regulation of optimal glycemic control to prevent T2D, but parkin’s role in preserving quality control of β-cell or adipocyte mitochondria is unclear. Although parkin has been previously reported to control mitophagy, here we show that parkin surprisingly is dispensable for glucose homeostasis in both β cells and adipocytes during diet-induced insulin resistance in mice. We observed that insulin secretion, β-cell formation, and islet architecture were preserved in parkin-deficient β cells and islets, suggesting that parkin is not necessary for control of β-cell function and islet compensation for diet-induced obesity. Although transient parkin deficiency mildly impaired mitochondrial turnover in β-cell lines, parkin deletion in primary β cells yielded no deficits in mitochondrial clearance. In adipocyte-specific deletion models, lipid uptake and β-oxidation were increased in cultured cells, whereas adipose tissue morphology, glucose homeostasis, and the beige-to-white adipocyte transition were unaffected in vivo. In key metabolic tissues where mitochondrial dysfunction has been implicated in T2D development, our experiments unexpectedly revealed that parkin is not an essential regulator of glucose tolerance, whole-body energy metabolism, or mitochondrial quality control. These findings highlight that parkin-independent processes maintain β-cell and adipocyte mitochondrial quality control in diet-induced obesity.

Introduction
Mitochondrial health is predicated on an intricate balance between biogenesis of new functional mitochondria and turnover of old, dysfunctional mitochondria. Selective turnover of dysfunctional mitochondria via the autophagy machinery, also known as mitophagy, is an essential quality control mechanism ensuring mitochondrial health. To date, the most comprehensively studied pathway of mitophagy is initiated by the E3 ubiquitin ligase parkin, encoded by the Prkn gene (1). Parkin targets damaged mitochondria for turnover following its recruitment to the mitochondrial surface by PTEN-induced kinase 1 (PINK1). Following ubiquitination of its substrate proteins, including Mitofusins 1 and 2 (MFN1/MFN2), and VDAC1, parkin initiates the recruitment of autophagy receptors necessary to ferry mitochondria within autophagosomes to the lysosome for degradation (2,3). Mutations or deficiency of parkin are implicated in T2D development, our experiments have prolonged maintenance of metabolically-beneficial beige/BRITE adipose tissue following withdrawal of β3-adrenergic activation (20-22). This beige-to-white adipocyte transition is highly dependent upon clearance of mitochondria via mitophagy and is impaired in global Prkn null mice (20). It is well-established that there are diverse roles for parkin in metabolic tissues, but precise tissue-specific functions of parkin-mediated mitophagy in the development of T2D have not been elucidated.

Here we report that parkin is not required for pancreatic development or β-cell function, that responses to DIO are unchanged following β-cell parkin deficiency, and that parkin is dispensable for mitochondrial turnover following damage in β-cells. We further demonstrate that loss of parkin in adipocytes does not modulate whole-body glucose...
metabolism, adipocyte morphology, or mitochondrial mass. We identify a role for parkin in β-oxidation of fatty acids within adipocytes, but not in preadipocyte differentiation. Finally, we determine that parkin is not required within adipocytes for the beige-to-white adipocyte transition following cessation of cold exposure or β3-adrenergic stimulation.

Results

Parkin deficiency in pancreatic endocrine cells does not affect glucose tolerance.

The importance of mitochondria and mitochondrial turnover (mitophagy) in pancreatic endocrine cells cannot be understated (15,16,23-26). However, the role of the key initiator of mitophagy, parkin, specifically in pancreatic endocrine cells has yet to be fully understood. Indeed, there are conflicting reports in the literature as to parkin’s contribution to β-cell function (7,17-19). To this end, we first investigated the role of parkin in pancreatic cells by utilizing the PrknFL/FL floxed mouse (hereafter known as Parkinflox) crossed to the Pdx1-Cre mouse (27) to elucidate the role for parkin in pancreatic islet function. Parkinflox, Pdx1-Cre; hereafter known as Panc-ParkinKO). Due to the expression of Pdx1 in pancreatic development, this model allows investigation into parkin’s involvement during development of the endocrine pancreas, as well as in mature islets after birth (28). We investigated the role of parkin both at baseline, as well as after high fat diet (HFD) to induce obesity as a diabetogenic stressor. Panc-ParkinKO islets exhibited loss of parkin protein expression when compared to Pdx1-Cre only controls (Fig. 1A), but maintained normal glucose tolerance at 10 weeks of age (Fig. 1B). We observed no fasting hypoglycemia (Fig. 1B), suggesting no defects in α-cell function. We also observed no steatorrhea, suggesting that parkin-deficient mice do not develop overt pancreatic exocrine function (data not shown).

Next, we sought to understand the role of parkin in response to obesity-related metabolic stress. Pdx1-Cre control and Panc-ParkinKO mice were fed HFD at weaning (Fig. 1C), and gained weight similarly overall, with the exception of a subtle but significant decrease in body weight in 19-week old Panc-ParkinKO mice (Fig. 1C). Glucose tolerance was again unchanged at this age (Fig. 1D), nor was any significant difference observed in glucose tolerance pre-HFD initiation, or at 4 and 8 weeks of HFD feeding (Fig. S1A-C). Histological analysis of pancreatic sections revealed normal islet architecture within Panc-ParkinKO mice, with no differences in β- or α-cell distribution by insulin and glucagon immunostaining, respectively (Fig. 1E). Altogether, these data suggest that parkin is dispensable for islet formation and glucose homeostasis at baseline and following DIO.

Parkin is dispensable for pancreatic β-cell function at baseline and following DIO.

Due to recent concerns regarding the study of pancreatic islet growth in Pdx1-Cre mice related to expression of the human growth hormone minigene (29), we wanted to further confirm the role of parkin specifically in pancreatic β-cells utilizing Parkinflox mice crossed to Ins1-Cre knock-in mice (30) (hereafter known as β-ParkinKO). β-ParkinKO mice also demonstrated loss of parkin protein in isolated islets (Fig. 2A). No significant differences were observed in body weight or glucose tolerance between Ins1-Cre alone or Parkinflox, Parkinflox/+, or Parkinflox+/- alone control mice (Fig. S2A-C), thus all studies utilized a mixture of control animals (Ctrl). Similar to findings following pancreas-specific parkin loss of function (Fig. 1), glucose tolerance in β-ParkinKO mice was indistinguishable from controls at 8 wk of age (Fig. 2B).

To further elucidate the role of parkin in β-cell function, GSIS was assessed in vivo to investigate whether β-cell secretory function is impacted by loss of parkin. Interestingly, 9 wk old β-ParkinKO mice exhibited higher insulin release 3 min after a glucose challenge compared to controls (Fig. 2C). However, this was not accompanied by changes in total pancreatic insulin content (Fig. 2D), glucose tolerance (Fig. 2B) or islet morphology (Fig. 2E). These data indicate that β-ParkinKO mice could have capacity for enhanced insulin secretion to potentially drive improved glucose clearance, yet no changes in glucose clearance were observed (Fig. 2B). Taken together, these data again suggest that loss of parkin is not detrimental to β-cell function or whole-body glucose homeostasis.

To determine a role for parkin during metabolic stress, β-ParkinKO mice and littermate controls were placed on HFD at weaning and monitored for 4 months. Both male and female Ctrl
and β-ParkinKO mice gained weight similarly throughout the study (Fig. 2F) and, as seen previously (Fig. 1D), no difference in glucose tolerance was observed between genotypes throughout the HFD study (Fig. 2G-H, Fig. S1D-E). Additionally, islet morphology was unchanged between Ctrl and β-ParkinKO mice. These data confirm that loss of parkin is dispensable for β-cell adaptation to DIO, and that β-cells deficient in parkin are fully capable of regulating whole-body glucose homeostasis.

**Parkin has mild effects on mitochondrial turnover in pancreatic β-cells.**

While it is evident that parkin is dispensable for β-cell function (Figs. 1-2), parkin is known to be a pivotal node in mitochondrial turnover in a number of other cell types (31-33). Therefore, we wanted to examine whether parkin deficiency affects mitochondrial turnover in β-cells. Utilizing parkin-specific siRNA, we transiently knocked down parkin in MIN6 β-cells. Following a ~40-50% reduction of parkin protein levels (Fig. 3A), we observed that expression of the outer mitochondrial membrane protein MFN2 was decreased, whilst another outer membrane protein, VDAC1, was not similarly affected. We next examined whether the rate of mitochondrial turnover was impacted by parkin loss following treatment with the mitochondrial uncoupler carbonyl cyanide m-chlorophenyl hydrazine (CCCP), which is known to dissipate mitochondrial membrane potential and initiate clearance via mitophagy (34). CCCP treatment caused a time dependent decrease in MFN2 and VDAC1 in control non-targeting (NT)-siRNA treated MIN6 β-cells (Fig. 3B-C); however, acute parkin deficiency significantly slowed the rate of turnover. These data suggest that acute loss of parkin does affect mitophagy in β-cells following robust mitochondrial damage (Fig. 3B-C), yet acute parkin deficiency does not appear to impact β-cell function as GSIS continues to be unaffected (Fig. 3D). Similarly, cellular stress responses are unaffected as reactive oxygen species (ROS) generation is not different following acute loss of parkin (Fig. S3A).

To further investigate the role of parkin in β-cell mitochondrial turnover, isolated islets from control or β-ParkinKO mice were treated ex vivo with the ionophore valinomycin to again dissipate mitochondrial membrane potential and induce mitochondrial clearance via mitophagy (34). Deletion of parkin in vivo had no effect on expression of outer mitochondrial membrane proteins at baseline (Fig. 3E). Surprisingly, we observed no overt effect of parkin on the rate of mitochondrial turnover after valinomycin treatment in primary islets (Fig. 3F). We also observed no significant differences in bulk autophagy machinery as measured by LC3 and p62 protein levels in Ctrl or β-ParkinKO islets ex vivo (Fig. S3B-C). Taken altogether, these results highlight that parkin is not required for mitochondrial turnover in β-cells in vivo, and has only a small effect on turnover after transient loss of function. These findings could suggest a novel and potentially important role for parkin-independent mitophagy (35,36), which may maintain appropriate β-cell mitochondrial quality control in the absence of parkin.

**Body weight, adiposity, and glucose tolerance are not affected by adipose-specific loss of parkin.**

Roles of parkin appear to be minimal in β-cell responses to excess metabolic demand, thus we next investigated if its role in adipose tissue, which also plays a causative role in T2D, elicited more of a phenotype. Parkin has been described as a regulator of fat uptake, as global parkin null mice are resistant to the weight gain, hepatic steatosis, and insulin resistance caused by feeding a HFD (6). To investigate cell-autonomous roles of parkin regulation of lipid metabolism in adipocytes, we generated mice lacking parkin in adipose tissue by crossing Parkinflox with Adiponectin-Cre mice to generate PrknlFL/FL;Adiponectin-Cre/+ mice (AD-ParkinKO). Adiponectin-Cre is a well-established model to delete floxed genes selectively and efficiently in adipocytes with minimal off-target effects (37). In contrast to the findings by Kim et al. (6) with global parkin deficiency, AD-ParkinKO and Parkinflox littermate controls did not have differences in weight gain over the course of 12 wk of feeding a high-fat (Fig. 4A) or normal chow diet (data not shown). Adipocyte-specific deletion of parkin was confirmed by genotyping of DNA (Fig. S4A, S4B), immunoblot of protein extracts from adipose tissue (Fig. S4C), and expression of Prknl mRNA (Fig. 4B). Body composition measured by NMR spectroscopy did not significantly differ
between Parkin\textsuperscript{flox} and AD-Parkin\textsuperscript{KO} mice (Fig. 4C). Individual tissue weights from Parkin\textsuperscript{flox} and AD-Parkin\textsuperscript{KO} mice were also relatively similar after 12 weeks of HFD feeding (Fig. S4D). Histological analysis of various tissues did not reveal gross abnormalities in cell size or morphology between AD-Parkin\textsuperscript{KO} mice and control littermates (Fig. 4D).

Next, we asked whether AD-Parkin\textsuperscript{KO} mice had metabolic alterations relative to the Parkin\textsuperscript{flox} controls, despite the lack of obvious changes in body weight, tissue weight, or tissue morphology. Neither glucose tolerance nor insulin sensitivity were significantly changed in AD-Parkin\textsuperscript{KO} mice compared to the Parkin\textsuperscript{flox} controls following HFD feeding (Figs. 4E and S4E). Fasting and random-fed blood glucose concentrations were also similar between experimental groups (Figs. 4F and S4F). We considered whether glucose intolerance might be masked by compensatory release of insulin; however, the concentration of insulin in the serum of Parkin\textsuperscript{flox} and AD-Parkin\textsuperscript{KO} mice was similar in both fasting and fed states (Fig. S4G). Circulating glycerol concentrations in the serum of Parkin\textsuperscript{flox} and AD-Parkin\textsuperscript{KO} mice were also unaffected by parkin deletion (Fig. S4H), suggesting that parkin does not affect global adiposity, glucose tolerance, or metabolic homeostasis in mice.

**Parkin is not required for normal adipocyte differentiation, but does play a role in adipocyte β-oxidation.**

To further investigate the molecular function of parkin in adipocytes, we isolated primary eMSCs from Parkin\textsuperscript{flox} mice and subjected the cells to a variety of molecular and metabolic analyses. The Parkin\textsuperscript{flox} eMSCs were infected with adenovirus expressing either GFP as a negative control (Ad-GFP), or Cre recombinase to induce parkin deletion (Ad-Cre). Recombination of the floxed allele was confirmed by PCR using primers flanking the loxP sites (Fig. S6A). These cells were then differentiated into mature adipocytes using standard adipogenic stimuli (insulin, dexamethasone, IBMX, and rosiglitazone). The ability of precursors to differentiate and the morphology of the mature adipocytes were not affected by parkin deletion, as observed by phase-contrast microscopy and Oil Red O staining (Fig. 5A).

Next, we analyzed the ability of eMSC adipocytes to metabolize fatty acids and found that β-oxidation of [\textsuperscript{3}H]-labeled palmitic acid (Fig. 5B) or [\textsuperscript{3}H]-oleic acid was significantly increased in Parkin\textsuperscript{flox}-AdCre adipocytes compared to Parkin\textsuperscript{flox}-AdGFP controls (Fig. S6B). Etomoxir, a selective inhibitor of the mitochondrial fatty acid transporter, carnitine palmitoyltransferase 1 alpha (CPT1α), blocked β-oxidation in both Parkin\textsuperscript{flox}-AdGFP and Parkin\textsuperscript{flox}-AdCre adipocytes to a similar extent (Fig. 5B and S6B). Fatty acid uptake into eMSC adipocytes was also significantly increased in the absence of parkin (Fig. S6C). However, we did not observe differential β-oxidation when adipocytes were incubated with medium-chain [\textsuperscript{3}H]-labeled octanoic acid (Fig. S6D), either in the presence or absence of etomoxir. These data indicate that the increase in β-oxidation is specific to long-chain fatty acids and is dependent on the activity of CPT1α, which facilitates transport of long-chain fatty acids across the outer mitochondrial membrane. Indeed, we observed higher CPT1α protein and mRNA in the Parkin\textsuperscript{flox} eMSCs treated with Ad-Cre (Fig. 5C-D). Interestingly, adiponectin protein was reduced in parkin-deficient cultured adipocytes (Fig. 5C), and expression of mRNA for
adipocyte markers AdipoQ and Fabp4 was significantly reduced in Parkin^{flox-AdCre} adipocytes (Fig. 5D) despite the lack of morphological changes in the cells (Fig. 5A). Expression of oxidative phosphorylation complex proteins was also slightly reduced in Parkin^{flox-AdCre} adipocytes (Fig. 5C); however, parkin deletion did not significantly alter expression of the mitochondrial or regulatory genes Cpt1α, Cox1, or Pgc1α (Fig. 5D), nor the total number of mitochondria (Fig. 5E). Protein expression of FABP4, MFN2, or VDAC1 were also unchanged (data not shown). In mature adipocytes, parkin deletion did not affect lipolytic activity, either in the basal state or when induced with forskolin, nor insulin-stimulated glucose uptake (Fig. S6E-F). We observed no differences the generation of cellular ROS in Parkin^{flox-AdGFP} and Parkin^{flox-AdCre} adipocytes (Fig. S6G). We also measured the levels of the autophagic proteins LC3 and p62 in Parkin^{flox-AdGFP} and Parkin^{flox-AdCre} adipocytes and found no change in LC3 expression but an increase in p62 levels following parkin deletion (Fig. S6H). Together these data demonstrate that parkin may influence specific aspects of lipid metabolism in cultured eMSC adipocytes; however, these changes are not significant enough to induce phenotypic changes in mice lacking parkin expression in adipose tissue.

**Formation and reversion of beige adipose tissue is not dependent on parkin expression.**

Autophagy and mitophagy are essential for the maintenance of beige adipocytes as induced by cold exposure or β3-adrenergic stimulation (20,21). Lu et al. recently reported that whereas parkin is not required for development of beige adipose tissue, the beige-to-white adipocyte transition of inguinal white adipose tissue (iWAT) upon withdrawal of beige-inducing stimuli is impaired in global Prkna null mice (20). Thus, we investigated whether parkin deletion in adipocytes impaired either the ability of iWAT to acquire beige characteristics, or the ability of beige fat to revert back to WAT after cessation of cold exposure or β3-adrenergic stimulation.

To address these questions, we treated Parkin^{flox} and AD-Parkin^{KO} mice with the β3-adrenergic agonist CL-316,243 for 1 wk to induce beige fat formation, then let the animals recover without drug administration for 15 days. The body weights and tissue weights did not differ between the Parkin^{flox} and AD-Parkin^{KO} mice following CL-316,243 administration and recovery (Fig. 6A-B). In contrast to the reported findings with the global parkin null mice (20), differences were not observed in the beige-to-white adipocyte transition upon cessation of CL-316,243 treatment, as evidenced by histological analysis (Fig. 6C), and expression of UCP1 and oxidative phosphorylation complex proteins (Fig. 6D). These changes were not dependent on diet, as we observed similar phenotypes in Parkin^{flox} and AD-Parkin^{KO} mice fed a HFD (Fig. S7A-C). Furthermore, we did not observe any differences in the circulating levels of leptin or adiponectin in the serum from Parkin^{flox} and AD-Parkin^{KO} mice (Fig. S7D-E).

To determine whether these phenotypes were dependent on type of beige-inducing stimuli, we also subjected a group of female mice to cold exposure (6°C for 7 days), followed by 15 days of recovery at room temperature. Again, no significant differences in the capacity of iWAT to beige or transition back to white adipocytes were observed with parkin deletion (Fig. 7A-E). These findings demonstrate that adipocyte-specific deletion of parkin is insufficient to affect the formation or reversion of beige adipose tissue and suggest that other cell types contribute to inhibition of the beige-to-white adipocyte transition observed in global parkin null mice. These data indicate that loss of parkin in adipocytes does not affect adipose tissue morphology, expression of metabolic proteins, or maintenance of beige adipocytes following cold exposure or β3-adrenergic stimulation.

**Discussion**

Mitochondrial function and homeostasis are critical to maintain normal cellular activities. Disruption of mitochondrial quality control is implicated in numerous disease states including obesity and β-cell dysfunction in T2D (12-14). Despite a wide body of evidence identifying parkin as a critical regulator of mitophagy, we did not observe mitochondrial dysfunction in mice with pancreatic-, β-cell-, or adipocyte-specific parkin deletion, nor any phenotypes affecting glucose homeostasis or metabolic health. Our findings suggest that parkin is largely dispensable for adipose and pancreatic islet/β-cell function and whole-body glucose
Parkin function in β-cells and adipocytes

Parkin is considered to be a master regulator of mitophagy, and mitochondria and mitochondrial turnover are essential for proper cellular function, especially in pancreatic β-cells (12,15,16,24). As such, it was surprising that loss of a key mitochondrial quality control protein (i.e. parkin) elicited little to no phenotype. The role of parkin in β-cells has been inconclusive to date, with studies showing that loss of parkin results in impaired insulin release and production, as well as increased susceptibility to streptozotocin-induced diabetes (7,19), but also that overexpression or activation of parkin-dependent pathways results in aberrant β-cell function (17,18). While studies in other systems have described the importance of parkin in the initiation of mitophagy, these studies were primarily performed in ex vivo cell-based systems, following parkin overexpression and severe mitochondrial damage (34,36). The role of parkin in physiologically relevant contexts of mitophagy in vivo is still not well-developed. Our study importantly demonstrates that in the context of obesity caused by HFD consumption, parkin deficiency does not lead to β-cell failure. This could indicate that the stress of overnutrition does not exceed the capacity of β-cells to adapt to increased mitochondrial metabolic demand, or that parkin may have a redundant role in mitochondrial turnover with other pathways. Indeed, we identify here that mitochondrial turnover remains largely intact following loss of parkin, indicating the likelihood of compensatory parkin-independent mechanisms.

Expression of parkin and its upstream activating kinase, PINK1, increases during adipocyte differentiation, and is also increased in white adipose tissue of mice fed a HFD relative to normal chow-fed controls (38,39). This suggests a role for mitophagy during the mitochondrial remodeling that occurs in WAT of obese mice (39). Further, our studies suggest that previously described roles for parkin in prevention of DIO and maintenance of beige adipocytes (6,20,21) occur in an adipocyte-independent manner. These findings, in addition to those in β-cells above, place previous findings in whole-body knockouts in appropriate cellular context and suggest a need to refine interpretations of genetic links between parkin and T2D in humans. In general, it is still not understood whether mitophagy is beneficial or detrimental in the progression of diseases such as cancer or metabolic syndrome (34). Our data to date suggest that parkin deficiency is dispensable for adipocytes and pancreatic β-cells in the regulation of whole-body metabolism.

Our findings agree with recent publications describing mild phenotypes when parkin or PINK1 are depleted in vivo (34-36). The emergence of these studies places the importance of parkin in physiologically relevant contexts into question and suggests the potential for parkin-independent mitophagy pathways to compensate for maintenance of mitochondrial quality control. Mitophagy still occurs in mice lacking PINK1 or in Drosophila with either PINK1 or parkin deficiency, suggesting that other pathways maintain mitochondrial homeostasis despite their absence (34-36). These parkin-independent pathways may include receptor-mediated mitophagy (including BNIP3, NIX/BNIP3L, or FUNDC1 among others), lipid-mediated mitophagy (via cardiolipin on the inner mitochondrial membrane), E3 ubiquitin ligases (such as MUL1), or ubiquitin-binding protein (34). Further work is needed to better understand mechanisms by which these pathways compensate for the absence of parkin. For instance, how and under what conditions are specific mitophagy pathways activated to maintain healthy mitochondrial function (34)? Parkin may also have broader functions, as recent reports suggest roles in cellular processes unrelated to mitophagy (40). These pathways remain poorly understood, but will likely be a major focus of future studies.

This study offers crucial contributions to the study of metabolic diseases by highlighting that parkin, a T2D-associated gene and crucial regulator of mitophagy, is not necessary during overnutrition to control metabolic phenotypes in pancreatic β-cells or adipose tissue. Loss of parkin subtly alters lipid uptake and β-oxidation in cultured adipocytes, and mildly impairs mitochondrial turnover in β-cell lines; however, this is not sufficient to disrupt whole-body glucose metabolism. Further study will be essential to dissect alternative regulators of mitophagy in pancreatic β-cells and adipocytes, and their importance to the development of T2D.

**Experimental Procedures**
**Animals**

Prkn<sup>FL/FL</sup> (Parkin<sup>lox/lox</sup>) mice were a generous gift from Ted Dawson (Johns Hopkins University) and Lexicon Genetics, and were generated with loxP sites flanking exon 7 of the Prkn allele (41). Pdx1-Cre mice were a generous gift from Doris Stoffers (University of Pennsylvania) (27). Ins1-Cre mice (#026801) and Adiponectin-Cre mice (#028020) were obtained from The Jackson Laboratory (Ellsworth, ME) (30,37). For DIO studies, mice were fed high-fat diet (HFD) containing 60% calories from fat (Research Diets #12492, New Brunswick, NJ). For beigeing studies, male mice were administered with 1 mg/kg CL-316,243 intraperitoneally (Cayman Chemical, Ann Arbor, MI), once daily for 7 days, followed by a 15 day rest period without drug treatment. Female mice were placed in thermal chambers at 6°C (with normal 12 hr light cycle and free access to chow and water) for 3 wk to induce beigeing, followed by 15 days at room temperature. All animal studies were performed in compliance with policies of the University of Michigan Institutional Animal Care and Use Committee.

**Glucose tolerance tests and in vivo glucose stimulated insulin secretion**

For adipose tissue studies, animals were fasted for 16 h then administered 1 mg/kg glucose intraperitoneally (IPGTT). For pancreatic tissue studies, animals were fasted for 6 h, then administered 2 mg/kg glucose intraperitoneally. Blood glucose concentrations were monitored at 0, 15, 30, 60, and 120 min post-injection using Contour® next blood glucose strips (Bayer AG, Leverkusen, Germany). For glucose-stimulated insulin secretion, animals were fasted for 6 h, then 3 mg/kg glucose was administered intraperitoneally. Glucose concentrations were measured, and plasma samples were collected at 0 and 3 min post-injection. Plasma insulin concentrations were measured by ELISA (Alpco, Salem, NH).

**Animal phenotyping**

Body composition was measured by NMR spectroscopy using the LF90 II Minispec (Bruker, Billerica, MA). Food intake, activity, energy expenditure, and oxygen consumption were monitored for 3 d using the Comprehensive Lab Animal Monitoring Systems (Columbus Instruments, Columbus, OH). All animal phenotyping was performed by the University of Michigan Mouse Metabolic Phenotyping core.

**Cell culture**

Primary mesenchymal stem cells were obtained from the ears of Parkin<sup>lox/lox</sup> mice as previously described (42) and maintained in DMEM:F12 media (Thermofisher Scientific, Waltham, MA) +10% fetal bovine serum (FBS; Sigma-Aldrich, St. Louis, MO), 100 U/mL penicillin (Thermofisher Scientific, Waltham, MA), 100 µg/mL streptomycin (Thermofisher Scientific, Waltham, MA), and 10 ng/ml recombinant bFGF (PeproTech INC, Rocky Hill, NJ). For adipogenesis, eMSCs were grown to confluency and two days later recombinant bFGF was removed, and DMEM:F12 media containing 10% FBS, 0.5 mM IBMX, 1 µM dexamethasone, 5 µg/ml insulin, and 5 µM rosiglitazone was added. Two days later, cells were fed with DMEM:F12 media +10% FBS, 5 µg/ml insulin, and 5 µM rosiglitazone. Every 2 days thereafter, cells were fed with DMEM:F12 +15% FBS + pen/strep.

MIN6 β-cells were maintained as previously described (15). siRNA studies were carried out as previously described (43). Briefly, MIN6 β-cells were seeded on 6-well plates, 24 h later they were treated with 2 µM non-targeting (NT) or Parkin-specific siRNA (Dharmacon, Lafayette, CO) using Dharmafect 3 transfection reagents (Dharmacon, Lafayette, CO). Cells were cultured for 48 h before protein isolation or glucose-stimulated insulin secretion (GSIS) assays that were performed as previously described (43).

**Islet isolation and culture**

Primary mouse islets were isolated as previously described (43), briefly pancreata were digested with 1 mg/ml Collagenase P (Roche, Basel, Switzerland) for 13 min at 37°C, filtered, and subjected to density gradient centrifugation with Histopaque (Sigma-Aldrich, St. Louis, MO) for 30 min. Islets were then maintained in RPMI 1640 (Thermofisher Scientific, Waltham, MA) supplemented with 10% FBS (Gemini BioProducts, West Sacramento, CA), 100 U/mL penicillin (Thermofisher Scientific, Waltham, MA), 100 µg/mL streptomycin (Thermofisher Scientific, Waltham, MA), 10 mM HEPES (Thermofisher Scientific, Waltham, MA), 100 U/mL penicillin (Thermofisher Scientific, Waltham, MA), and 10 µM heparin (Thermofisher Scientific, Waltham, MA).
Scientific, Waltham, MA) and 0.2 mM glutamine (Thermofisher Scientific, Waltham, MA).

**Measurement of cellular ROS**

Total cellular ROS was measured in MIN6 β-cells +/- parkin-specific siRNA, or in Parkin\textsuperscript{flox} AdGFP and Parkin\textsuperscript{flox} AdCre adipocytes, using the Cellular ROS Assay Kit per the manufacturer’s instructions (Abcam, Cambridge, UK). For MIN6 assays; cells were seeded on black, clear bottom 96-well plates (Grenier Bio-One, Kremsmünster, Austria), and cellular ROS assessed using the red fluorescence kit (#ab186027, Abcam, Cambridge, UK). For adipocyte assays; cells were seeded on 24-well black, clear bottom plates (Perkin Elmer, Turku, Finland) and cellular ROS assessed using the ROS only portion of the ROS/superoxide detection kit (#ab139476, Abcam, Cambridge).

**Adipocyte and stromal vascular cell fractionation**

Using a protocol modified from Rodbell (44), white adipose tissue (inguinal and gonadal combined) was isolated from Parkin\textsuperscript{flox} and AD-Parkin\textsuperscript{KO} mice, minced with scissors, and digested with 1 mg/ml collagenase (type I; Worthington Biochemical, Lakewood, NJ) in Krebs-Ringer-HEPES buffer +3% fatty acid-free BSA (Gold Biotechnology, St. Louis, NJ). After 1 h digestion at 37°C, the cell suspension was filtered through 100 µm cell strainers. Adipocytes and stromal vascular fraction were separated by differential centrifugation (100xg for 8 min) and washed with Krebs-Ringer-HEPES buffer containing 3% BSA.

**Histology**

Tissues were fixed in 10% neutral buffered formalin for 24 h and processed for paraffin embedding by the University of Michigan Microscopy and Imaging Analysis core. Sections (5 µm) were stained with hematoxylin and eosin as previously described (45). Pancreata were harvested and fixed in 4% paraformaldehyde overnight, and either processed for paraffin embedding as above, or incubated in 50% sucrose overnight and processed in OCT for cryosections. Immunostaining for insulin (Dako (Agilent), Santa Clara, CA) and glucagon (Santa Cruz Biotechnology INC, Dallas, TX) was performed as described (15).

**Immunoblot analysis**

Immunoblots were performed as previously described (15,46). In brief, 5-20 µg of cell or tissue protein extract was separated by SDS-PAGE, transferred onto PVDF or nitrocellulose membranes, and immunoblotted with primary antibodies listed in Supplementary Table 1.

**Quantitative RT-PCR**

Total RNA was isolated from frozen tissue or isolated cells using RNA STAT-60 (AMS Biotechnology, Cambridge, MA) according to the manufacturer’s instructions. Reverse transcription and qRT-PCR were performed as previously described (46). To assess mitochondrial number, total RNA was treated with DNase and reverse transcribed, and the expression of mitochondrial genes relative to nuclear genes was measured by qRT-PCR. A list of qRT-PCR primers is listed in Supplementary Table 2.

**Transmission electron microscopy**

Brown adipose tissue from Parkin\textsuperscript{flox} and AD-Parkin\textsuperscript{KO} mice was minced into small fragments and fixed in 2.5% glutaraldehyde in Sorensons' phosphate buffer (pH 7.4) overnight at 4°C. Samples were washed in Sorenson’s buffer, post-fixed in 2% osmium tetroxide in Sorenson’s buffer for 1 hr at room temperature, then washed again with Sorenson’s buffer and dehydrated through ascending concentrations of acetone before embedding in epoxy resin. Semi-thin sections (500 nm) were stained with toluidine blue for tissue identification. Selected regions of interest were sectioned at 70 nm in thickness and post stained with uranyl acetate and Reynolds lead citrate. The sections were examined using a JEOL JEM-1400 Plus transmission electron microscope at 80 kV with support from the University of Michigan Microscopy and Imaging Analysis core.

**Statistics**

All data are presented as mean ± SD and analyzed by 2-tailed Student’s t-test or ANOVA, unless otherwise indicated. Differences were considered significant for $P < 0.05$ or 0.01, as indicated in the figure legends.
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Figure 1. Parkin is dispensable for endocrine cell function in the pancreas. A, Representative immunoblot of parkin protein expression in isolated pancreatic islets from either Pdx1-Cre or Panc-ParkinKO mice. B, Blood glucose concentrations during an IPGTT of Pdx1-Cre (blue circles) or Panc-ParkinKO (orange squares) mice on a normal chow diet at 10 weeks of age (n = 5/group). C, Weights over duration of HFD feeding in Pdx1-Cre or Panc-ParkinKO mice (n = 4-5/group). D, Blood glucose concentrations during an IPGTT of Pdx1-Cre (blue circles) or Panc-ParkinKO (orange squares) mice after being fed HFD for 12 wk (n = 4-5/group). E, Representative immunofluorescence images of pancreatic sections from Pdx1-Cre or Panc-ParkinKO mice stained for insulin (green), glucagon (red) and DAPI (blue). *p<0.05 two-way ANOVA with Sidak’s multiple comparison post-test.
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Figure 2. Parkin is not required for pancreatic β-cell function, either at baseline or after DIO. 
A, Representative immunoblot of parkin protein expression in isolated islets from Ins1-Cre or β-ParkinKO. B, Blood glucose concentrations during an IPGTT of Ins1-Cre (blue diamonds) or β-ParkinKO (orange triangles) mice at 8 wk of age. (n = 4/Ins1-Cre, 7/β-Parkin KO). C, Plasma insulin concentrations at baseline (0 min) and 3 min after a 3 mg/kg glucose bolus in Ins1-Cre (blue diamonds) and β-ParkinKO (orange triangles) at 9 wk of age (n = 4/Ins1-Cre, 7/β-Parkin KO). D, Total pancreatic insulin content from Ins1-Cre (blue bars) and β-ParkinKO (orange bars) pancreas at 9 wk of age. (n = 4/Ins1-Cre, 7/β-ParkinKO). E, Representative immunofluorescence images of pancreatic sections from Ins1-Cre or β-ParkinKO mice stained for insulin (green), glucagon (red), and DAPI (blue). F, Weights of male (left panel) and female (right panel) Ins1-Cre or β-ParkinKO mice following HFD feeding (Males: n = 7/Ins1-Cre, 10/β-ParkinKO. Females: n = 7/Ins1-Cre, 12/β-ParkinKO). G-H, Blood glucose concentrations during an IPGTT of Ins1-Cre (blue diamonds) and β-ParkinKO (orange triangles) mice after 4 wk (G) or 16 wk (H) HFD feeding (n = 15/Ins1-Cre, n = 23/β-ParkinKO). I, Representative immunofluorescence images of pancreatic sections from 20 wk HFD fed Ins1-Cre or β-ParkinKO mice stained for insulin (green), glucagon (red), and DAPI (blue).

*p<0.05 two-way ANOVA with Sidak’s multiple comparison post-test.
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Figure 3. Mitochondrial protein turnover in β-cells is affected by transient loss of parkin, but not by constitutive deficiency. A, Representative immunoblot showing parkin protein expression after non-targeting (NT) or parkin-siRNA in MIN6 β-cells. Parkin protein was quantified by ImageJ software from immunoblot results, using cyclophilin B as a loading control, after NT- (black bars) or Parkin-siRNA (striped bars) in MIN6 β-cells (n = 4/group). B, Representative immunoblot images of MFN2 and VDAC1 proteins in NT- or parkin siRNA treated MIN6 β-cells, after a time course of CCCP (10 µM) treatment. Quantification of basal (0 hr) protein levels below. C, Quantification of MFN2 (top panel) and VDAC1 (bottom panel) protein expression from immunoblots as shown in Fig.3B, normalized to cyclophilin B as a loading control, in NT- (black circles) or parkin-siRNA (white circles) treated for 0, 2, 4, or 6 h with CCCP (n = 4 experiments). Expression is presented as fold change over time compared to respective basal (0 h) levels for each condition. D, Insulin release from MIN6 β-cells treated with either NT- (black bars) or parkin-siRNA (striped bars) after 2 mM or 20 mM glucose stimulation for 30 min (n = 3 experiments). E, Representative immunoblot images of MFN2 and VDAC1 proteins from Ins1-Cre or β-ParkinKO islets following treatment with valinomycin (250 nM) for 0, 3 or 6 h. Quantification of basal (0 hr) protein levels below. F, Quantification of MFN2 (top panel) and VDAC1 (bottom panel) from immunoblots as shown in Fig.3E, normalized to vinculin as a loading control, in Ins1-Cre (black diamonds) and β-ParkinKO (white triangles) following 250 nM valinomycin treatment (n = 3/group). Expression is presented as fold change over time compared to respective basal (0 h) levels for each condition. ***p<0.001 student’s unpaired t-test, $p<0.05$ two-way ANOVA Sidak’s multiple comparison test.
Figure 4. Body weight, adiposity, and glucose tolerance are not affected by adipose-specific loss of parkin. A, Body weights of Parkin<sup>lox</sup> and AD-Parkin<sup>KO</sup> mice over the course of 12 wk of HFD feeding (n = 7-9 animals per group). B, Relative expression of Prkn mRNA in adipocytes (AD) and stromal vascular fraction (SVF) isolated from the iWAT of Parkin<sup>lox</sup> and AD-Parkin<sup>KO</sup> mice after 12 wk of HFD feeding (n = 2 animals per group). C, Body composition measured by NMR spectroscopy after 12 wk of HFD feeding (n = 5 animals per group). D, Representative histological images of the iWAT, gonadal WAT (gWAT), brown adipose tissue (BAT), and liver after 12 wk of HFD feeding. E, Glucose tolerance after 12 wk of HFD feeding. Mice were fasted 16 h, then injected with 1 mg/kg glucose intraperitoneally. Blood glucose concentrations during an IPGTT in Parkin<sup>lox</sup> and AD-Parkin<sup>KO</sup> mice at the indicated timepoints (n = 5 animals per group). F, Blood glucose concentrations during random ad libitum feeding or after fasting (16-h food restriction) following 12 wk of HFD (n = 5 animals per group). *p<0.05, Student’s unpaired t-test.
Figure 5. Parkin deletion enhances β-oxidation but does not affect adipogenesis in cultured adipocytes. A, Representative images of Parkin<sup>flox</sup> eMSCs infected with Ad-GFP or Ad-Cre, before and after 12 days of adipogenesis (n = 6 wells per group). B, β-oxidation of [3H]-palmitic acid in mature Parkin<sup>flox</sup> adipocytes infected with Ad-GFP or Ad-Cre. CPM normalized to total protein per well (n = 4 wells per group). C, Immunoblot for Parkin<sup>flox</sup> adipocytes infected with Ad-GFP or Ad-Cre. D, qRT-PCR analysis for Parkin<sup>flox</sup> adipocytes infected with Ad-GFP or Ad-Cre. E, Quantification of the mitochondrial DNA to nuclear DNA ratio in Parkin<sup>flox</sup> adipocytes infected with Ad-GFP or Ad-Cre. *p < 0.01, Student’s unpaired t-test.
Figure 6. Adipocyte-specific parkin deletion does not affect beige-to-white adipocyte transition following β3-adrenergic activation. A, Schematic representation of experimental design. B, Body weights of Parkin^flox^ and AD-Parkin^KO^ male mice 15 days after CL-316,243 withdrawal (n = 7-8 animals per group). C, Tissue weights from Parkin^flox^ and AD-Parkin^KO^ male mice 15 days after CL-316,243 withdrawal (n = 7-8 animals per group). D, Representative histological images from Parkin^flox^ and AD-Parkin^KO^ male mice prior to CL-316,243 treatment and 0 and 15 days after CL-316,243 withdrawal. E, Immunoblot for protein isolated from the iWAT of Parkin^flox^ and AD-Parkin^KO^ male mice 0 and 15 days after CL-316,243 withdrawal.
Figure 7. Adipocyte-specific parkin deletion does not affect beige-to-white adipocyte transition following cold exposure. A, Schematic representation of experimental setup. B, Body weights of Parkin\textsuperscript{flox} and AD-Parkin\textsuperscript{KO} female mice 15 days after cessation of cold exposure (n = 5 animals per group). C, Tissue weights from Parkin\textsuperscript{flox} and AD-Parkin\textsuperscript{KO} female mice 15 days after cessation of cold exposure (n = 5 animals per group). D, Representative histological images from Parkin\textsuperscript{flox} and AD-Parkin\textsuperscript{KO} female mice before and after cold exposure. E, immunoblot for protein isolated from the iWAT of Parkin\textsuperscript{flox} and AD-Parkin\textsuperscript{KO} female mice 0 and 15 days after cessation of cold exposure.
The E3 ubiquitin ligase parkin is dispensable for metabolic homeostasis in murine pancreatic β cells and adipocytes
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