NBR1 is a critical step in the repression of thermogenesis of p62-deficient adipocytes through PPARγ

Jianfeng Huang1, Juan F. Linares2,3, Angeles Duran2,3, Wenmin Xia4, Alan R. Saltiel4, Timo D. Müller5,6,7, María T. Diaz-Meco2,3 & Jorge Moscat2,3

Activation of non-shivering thermogenesis is considered a promising approach to lower body weight in obesity. p62 deficiency in adipocytes reduces systemic energy expenditure but its role in sustaining mitochondrial function and thermogenesis remains unresolved. NBR1 shares a remarkable structural similarity with p62 and can interact with p62 through their respective PB1 domains. However, the physiological relevance of NBR1 in metabolism, as compared to that of p62, was not clear. Here we show that whole-body and adipocyte-specific ablation of NBR1 reverts the obesity phenotype induced by p62 deficiency by restoring global energy expenditure and thermogenesis in brown adipose tissue. Impaired adrenergic-induced browning of p62-deficient adipocytes is rescued by NBR1 inactivation, unveiling a negative role of NBR1 in thermogenesis under conditions of p62 loss. We demonstrate that upon p62 inactivation, NBR1 represses the activity of PPARγ, establishing an unexplored p62/NBR1-mediated paradigm in adipocyte thermogenesis that is critical for the control of obesity.
there are at least three main morphologically and functionally different adipocyte types: white, brown, and beige. Unlike white adipocytes, which are specialized in the storage of chemical energy in the form of triglycerides, classical brown adipocytes (BAs) are found in the interscapular area (termed interscapular brown adipose tissue, IBAT) in rodents and generate heat during cold exposure by an adaptive mechanism called non-shivering thermogenesis. This process requires the expression of the uncoupling protein 1 (UCP1) in the inner mitochondrial membrane to uncouple oxidative phosphorylation from ATP regeneration, thereby dissipating the energy from electron transport as heat. Active BAT is detected in cervical, supraclavicular, paravertebral, and deep neck regions, and is acutely induced by cold exposure. Since the amount of metabolic active BAT inversely correlates with body mass index in adult humans, and BAT (Fig. 1b–d). Consistently, while p62-deficient mice exhibited increased lipid accumulation in BAT and enlarged adipocyte size in epididymal WAT (eWAT), this phenotype was completely rescued in Sgstm1 KO/Nbr1 KO mice (Fig. 1e–g). Moreover, the mRNA levels of two master thermogenic regulators (Ucp1 and Pgc1α), which were reduced in Sgstm1–/–/Nbr1 KO mice, were rescued in Sgstm1–/–/Nbr1 KO/BAT (Fig. 1h).

Furthermore, the expression of key lipogenic genes that were increased in the eWAT of Sgstm1–/– mice was partially reduced to WT levels in Sgstm1–/–/Nbr1–/– mice (Fig. 1i), likely reflecting the BAT-driven metabolic improvement in the host. These results demonstrate that NBR1 is an obligate step in the obesity phenotype unleashed by p62 deficiency, likely through the repression of the adipocyte’s thermogenic program.

Adipocyte’s NBR1 is required for increased adiposity driven by p62 deficiency. To determine whether the effect of inactivating global NBR1 on obesity could be accounted for by its potential role in adipocytes, we next generated a mouse line with the adipocyte-specific deletion of NBR1 either in WT mice (Nbr1 AKO) or in mice in which p62 has been selectively inactivated in adipocytes both in WAT and BAT (Sgstm1 AKO). These adipocyte-selective double KO mice (Sgstm1 AKO/Nbr1 AKO) demonstrated that the specific loss of NBR1 in p62-deficient adipocytes rescued the body weight gain of Sgstm1 AKO mice to levels close to those of the corresponding WT controls (Fig. 2a, b). That is, BAT and WAT masses and whole-body fat composition, which were increased in Sgstm1 AKO mice, were largely normalized in Sgstm1 AKO/Nbr1 AKO mice (Fig. 2c–e). Notably, the normalization of body weight and fat mass in Sgstm1 AKO/Nbr1 AKO mice is independent of age and sex (Supplementary Fig. 1a–c). Histological analyses showed that while Sgstm1 AKO mice have robustly increased adipocyte size and lipid content in BAT and eWAT, these alterations were completely abrogated in Sgstm1 AKO/Nbr1 AKO mice (Fig. 2f, g and Supplementary Fig. 1d). We next determined the development of fatty liver by Oil Red O (ORO) staining and quantification of hepatic lipid content. In accordance with changes in adiposity, the fatty liver phenotype of Sgstm1 AKO mice was normalized in Sgstm1 AKO/Nbr1 AKO mice (Fig. 2f, h). Chronic inflammation in WAT, characterized by severe macrophage infiltration, may result in systemic insulin resistance in obese diabetic animals. We found that the number of crown-like structures (a hallmark of macrophage infiltration) and the expression of macrophage marker F4/80 (encoded by Adgre1 gene), which were markedly increased in Sgstm1 AKO mice, were normalized in Sgstm1 AKO/Nbr1 AKO mice (Supplementary Fig. 1e, f). These findings suggest a functional dependence on NBR1 for p62 dysfunction in adipocytes as the mechanism underlying the whole-body obese phenotype of Sgstm1 AKO mice.

Results
Loss of NBR1 inhibits increased adiposity of Sgstm1-deficient mice. To address the role of NBR1 in adipocyte biology, we generated a total body NBR1 KO (Nbr1–/–) mouse line and compared its phenotype with that of total body p62 KO (Sgstm1–/–). Interestingly, and as previously reported, Sgstm1–/– mice had more body weight than wild-type (WT) mice (Fig. 1a), which was associated with increased masses of both white adipose tissue (WAT) and BAT (Fig. 1b–d). In contrast, Nbr1–/– mice displayed no such metabolic phenotype (Fig. 1a–d), suggesting that NBR1 does not play a relevant role in whole-body metabolism. However, the analysis of mice with total body KO of both p62 and NBR1 (Sgstm1–/–/Nbr1–/–) revealed that the increased fat weight of Sgstm1–/– mice was restored to WT conditions in the absence of NBR1 (Fig. 1b–d). Consistently, while p62-deficient mice exhibited increased lipid accumulation in BAT and enlarged adipocyte size in epididymal WAT (eWAT), this phenotype was completely rescued in Sgstm1–/–/Nbr1–/– mice (Fig. 1e–g). Moreover, the mRNA levels of two master thermogenic regulators (Ucp1 and Pgc1α), which were reduced in Sgstm1–/–/Nbr1–/– mice, were rescued in Sgstm1–/–/Nbr1–/–/BAT (Fig. 1h).

NBR1 inactivation in adipocytes restores glucose intolerance and insulin resistance in Sgstm1 AKO mice. We next determined the impact of NBR1 deficiency in systemic glucose tolerance and insulin sensitivity. To this end, we measured glucose tolerance in mature mice of all genotypes. Interestingly, while glucose intolerance were evident characteristics of obese Sgstm1 AKO mice in GTT (Fig. 3a), this phenotype was not observed in Nbr1 AKO and Sgstm1 AKO/Nbr1 AKO mice (Fig. 3b, c). Next, insulin sensitivity was determined in these mice in ITT experiments. While insulin...
Fig. 1 Loss of NBR1 inhibits increased adiposity of Sqstm1-deficient mice. a–d Body mass (a) and fat tissue masses of eWAT (b), iWAT (c), and BAT (d) from WT and total body knockout mice at 10–12 weeks of age. WT (n = 18), Sqstm1+/− (n = 21), Nbr1−/− (n = 23), and Sqstm1+/−/Nbr1−/− (n = 26). p = 0.0422 WT vs Sqstm1+/− (a), p = 0.0281 vs Nbr1−/− (a), p < 0.0001 WT vs Sqstm1+/−; p = 0.0142 WT vs Sqstm1+/−/Nbr1−/−, p < 0.0001 Sqstm1+/− vs Sqstm1+/−/Nbr1−/− (b), p < 0.0001 WT vs Sqstm1+/− and Sqstm1+/− vs Sqstm1+/−/Nbr1−/− (c, d). e Representative H&E staining of BAT and eWAT (n = 5, per genotype). Scale bar: 100 μm (BAT) and 200 μm (eWAT). f, g Adipocyte size measurement from H&E staining of eWAT described above (n = 5 mice, per genotype). Distribution range and frequency (f) and mean diameter of adipocyte size (g) were shown. p = 0.045 Sqstm1+/− vs Sqstm1+/−/Nbr1−/− (<30), p = 0.0214 Sqstm1+/− vs WT, p = 0.0071 vs Sqstm1+/−/Nbr1−/− (30–40), p = 0.0136 Sqstm1+/− vs Sqstm1+/−/Nbr1−/− (40–50), p = 0.0083 Sqstm1+/− vs WT, p = 0.0008 vs Sqstm1+/−/Nbr1−/− (60–70), p = 0.0323 Sqstm1+/− vs WT, p = 0.0237 vs Sqstm1+/−/Nbr1−/− (70–80) (f), p = 0.0243 Sqstm1+/− vs WT, p = 0.0191 vs Sqstm1+/−/Nbr1−/− (g). h qPCR analysis of thermogenesis genes in BAT. WT (n = 10), Sqstm1+/− (n = 9), Nbr1−/− (n = 10), and Sqstm1+/−/Nbr1−/− (n = 11). 0.0011 Sqstm1+/− vs WT, p = 0.0003 vs Sqstm1+/−/Nbr1−/− (Pgc1a), p = 0.0073 Sqstm1+/− vs WT, p = 0.0461 vs Sqstm1+/−/Nbr1−/− (Ucp1). i qPCR analysis of lipogenesis-related genes in eWAT. WT (n = 8), Sqstm1+/− (n = 7), Nbr1−/− (n = 7), and Sqstm1+/−/Nbr1−/− (n = 8). p = 0.0066 Sqstm1+/− vs WT (Fasn), p = 0.0183 Sqstm1+/− vs WT (Srebf1), p = 0.016 Sqstm1+/− vs WT, p = 0.0364 vs Sqstm1+/−/Nbr1−/− (Hmgcr), p = 0.0115 Sqstm1+/− vs WT (Srebf2). Data are presented as mean ± SEM (a–d, f–i). *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001. Unpaired two-tailed Student’s t-test. Source data are provided as a Source Data file.
established that the loss of NBR1 in adipocytes protects mice from the dysfunctional glucose metabolism characteristic of adipocyte-specific p62 deficiency.

**NBR1 inactivation in adipocytes restores impaired systemic energy expenditure in Sqstm1KO mice.** To evaluate the role of adipocyte NBR1 in whole-body metabolic profile, especially in the context of p62 deficiency, we performed a full metabolic characterization of the adipocyte-selective KO mouse lines. In concordance with the previous study16, p62 ablation in adipocytes led to marked reduction in EE as determined by ANCOVA using body weight as covariate as previously described22 (Fig. 4a, b). Interestingly, the reduced EE of Sqstm1KO mice was restored in Sqstm1KO/Nbr1KO mice to WT levels (Fig. 4a, b). The respiratory exchange ratio (RER) denotes the preference for carbohydrates and lipids as fuels to fit the energy demand. The fact that Sqstm1AKO mice had lower RER during the dark (feeding) phase suggests that most of the dietary carbohydrates are stored rather than being metabolized (Fig. 4c, d). Notably, this parameter was largely restored in Sqstm1AKO/Nbr1AKO mice when comparing to Sqstm1AKO mice (Fig. 4c, d). This observation is consistent with an improvement in the whole-body metabolic rate, with no alterations in food intake and locomotor activity (Supplementary Fig. 3a, b). Furthermore, in agreement with their unaltered body weight and fat mass, Nbr1AKO mice showed no phenotypic changes in any of the metabolic parameters investigated, including EE, RER, food intake, and locomotor activity (Fig. 4a–d and Supplementary Fig. 3a, b). These data support the notion that the loss of NBR1 in adipocytes rescues the impaired systemic EE driven by p62 deficiency.
Adipocyte NBR1 is required for downregulation of adaptive thermogenesis in BAT and inguinal WAT of p62-deficient mice. We next tested in these mice the adaptive thermogenic capacity of BAT. To this end, mice of the different genotypes were exposed to cold (4 °C) for 7 h to stimulate their thermogenic program. In contrast to the hypothemic $S q s t m^ T m$ mice, the $S q s t m^ F f$ and $N b r t m$ mice were able to maintain their core temperature against acute cold exposure to levels similar to their respective WT controls, suggesting intact heat generation (Fig. 5a and Supplementary Fig. 4a). Interestingly, while a “whitening” histological feature was found in $S q s t m^ T m$ BAT upon cold exposure, indicative of insufficient lipid mobilization and metabolism, this abnormality was largely rescued in $S q s t m^ T m N b r t m$ mice (Fig. 5b), while $N b r t m$ mice showed no phenotype (Supplementary Fig. 4b). Consistently, the expression of thermogenic genes ($U c p 1, D i o 2$, $C i d e a$, and $C o x 7 a$) in response to cold-driven sympathetic stimulation was significantly decreased in $S q s t m^ T m$ BAT but was largely rescued in $S q s t m^ T m N b r t m$ BAT (Fig. 5c).

Beige adipocytes are the inducible form of thermogenic fat cells that emerge within inguinal WAT in rodents in response to a variety of external stimuli, such as chronic cold exposure and cancer cachexia$^{17,23}$. Injection of the $p 3$-adrenergic agonist CL316,243 in $S q s t m^ T m N b r t m$ and $N b r t m$ mice rapidly
switched adipocytes from the characteristic unilocular to the multicellular cell morphology, which is typical of beige/bright cells (Fig. 5d and Supplementary Fig. 4c). However, inguinal white adipocytes in Sqstm1f/f mice remained unilocular and enlarged in cell size (Fig. 5d), indicative of the absence of beige cells. The levels of UCP1 and mitochondrial complex proteins were upregulated by CL316,243 in differentiated mature BAs (Supplementary Fig. 5a). Treatment with ISO that transcriptionally upregulates UCP1 and drives mitochondrial OCR (basal uncoupling respiration) was decreased as that in WT controls whereas such an induction was largely preserved in Nbr1−/− mice. Two-tailed Student’s T-test. p = 0.0004 Sqstm1f/f vs Sqstm1−/−. BW body weight. c Respiratory exchange rate (RER) was recorded and plotted. Two-way ANOVA followed by Bonferroni’s post-test. p = 0.0421 (4 h), p = 0.0196 (5 h), p = 0.0308 (28 h) Sqstm1f/f vs Sqstm1−/−, p = 0.0126 (48 h) Nbr1f/f vs Nbr1−/−. d Quantification of respective AUC from (c) was analyzed by two-tailed Student’s T-test. p = 0.0012 Sqstm1f/f vs Sqstm1−/−. Data are presented as mean ± SEM (b-d). *p < 0.05, **p < 0.01, ***p < 0.001. Source data are provided as a Source Data file.

BAT takes up large amounts of glucose during cold exposure in mice and humans. The cold-induced expression of glycolytic enzymes could be mimicked in vitro by β-adrenergic stimulation21. We found that upon ISO stimulation, Sqstm1−/− Nbr1−/− iBAs exhibited higher ECAR relative to Sqstm1−/− iBAs (Fig. 6d). These results demonstrated that the loss of NBR1 in BAs largely rescued the thermogenic and mitochondrial defects in Sqstm1−/− BAT.

p62 and NBR1 interact with PPARγ in the nucleus of brown adipocytes. In search of the molecular crosstalk between p62 and NBR1 that might underly their role in adipocyte biology, we initially found that NBR1 expression was dramatically upregulated during brown adipogenesis, following a pattern similar to that of PPARγ expression (Supplementary Fig. 5a). Unlike NBR1, p62 showed just a marginal increase, detectable only in fully differentiated mature BAs (Supplementary Fig. 5a). Treatment with ISO that transcriptionally upregulates UCP1 and drives thermogenesis robustly induced the nuclear amounts of PPARγ and CREB, two master thermogenic regulators, but also triggered the nuclear translocation of p62 and NBR1 (Fig. 7a). We hypothesized that the impaired thermogenic and mitochondrial activity characteristic of p62 deficiency are due to a defective activation of the PPARγ transcriptional program. Since adrenergic stimulation augments the nuclear levels of p62, NBR1, and PPARγ (Fig. 7a), we speculated that they could be part of a multi-component protein complex. Consistent with this hypothesis, PPARγ and NBR1 were co-immunoprecipitated with p62 in iBAs treated with ISO and rosiglitazone (Fig. 7b and Supplementary Fig. 5b). These observations demonstrate that both p62 and NBR1 can form a complex with PPARγ. Because similar results...
Fig. 5 Role of NBR1 in adaptive thermogenesis in BAT and inguinal WAT. a–c Male mice at 25 weeks of age were subjected to acute cold exposure (4 °C) for 7 h to stimulate brown thermogenesis. a Rectal core temperature was measured for consecutive 7 h. Sqstm1f/f (n = 10), Sqstm1AKO (n = 6), Sqstm1f/fNbr1f/f (n = 9), and Sqstm1AKO Nbr1AKO (n = 10). Two-way ANOVA followed by Bonferroni’s post-test. b Representative H&E staining in BAT of indicated mice (n = 3, per genotype). Scale bar = 100 μm. c qPCR analysis of thermogenesis-related genes in BAT of mice. Results are presented as change fold related to individual controls. Sqstm1f/f (n = 7), Sqstm1AKO (n = 5), Sqstm1f/fNbr1f/f (n = 6), and Sqstm1AKO Nbr1AKO (n = 6). Two-tailed Student’s T-test.

d–g Male mice at 25 weeks of age were injected with CL316,243 or saline as control for consecutive 5 days. d Representative H&E staining in iWAT of indicated mice. Sqstm1f/f (n = 4), Sqstm1AKO (n = 4), Sqstm1f/fNbr1f/f (n = 3), and Sqstm1AKO Nbr1AKO (n = 3). Scale bar = 100 μm. e, f Immunoblot analysis of mitochondrial OXPHOS genes and UCP1 in BAT of Sqstm1AKO (e) and Sqstm1AKO Nbr1AKO (f) and their respective controls (n = 5, per genotype). g Densitometric quantification of gene intensity from western blot (e, f). Results are presented as change fold related to individual controls. Two-tailed Student’s T-test. Data are presented as mean ± SEM (a, c, g). *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001. Source data are provided as a Source Data file.
were obtained when these precipitations were performed with purified recombinant proteins, we concluded that these interactions were direct (Fig. 7d, e). Furthermore, we found in co-transfection experiments that p62 and NBR1 influence each other’s interaction with PPARγ (Supplementary Fig. 5d). Consistently, mutations of p62 (K7A) and NBR1 (D50R) within their respective PB1 domains that abolished p62-NBR1 interaction11,19,20 also clearly abrogated the synergistic binding of p62 and NBR1 to PPARγ (Fig. 7f and Supplementary Fig. 5e). These results demonstrate a nuclear interaction among p62, NBR1, and PPARγ, which might influence PPARγ’s activities in BATs.

**p62 and NBR1 regulate PPARγ-RXRα heterodimerization to control thermogenesis in brown adipocytes.** PPARγ functions as an obligate heterodimer with RXRα, which together bind to PPAR-responsive regulatory elements (PPRE) to activate the expression of target genes25. Our data also showed that NBR1 co-immunoprecipitated with RXRα in PPARγ immunoprecipitates (Fig. 7c and Supplementary Fig. 5c). Given the functional importance of the PPARγ-RXRα interaction, we hypothesized that p62 and NBR1 might play a critical role on its formation. Interestingly, the expression of NBR1 impaired the PPARγ-RXRα complex, which was restored by p62 expression (Fig. 8a). However, such a restoration was abolished by mutations that disrupt the interaction between p62 and NBR1 (Fig. 8a). Consistent with this model, the levels of PPARγ-RXRα heterodimer in cold-exposed BAT were reduced in Sqstml−/− mice but were normal in Sqstml−/−Nbr1−/− mice (Fig. 8b). We next reconstituted Sqstml1 and Nbr1 double KO iBAs with either p62 or NBR1 or both together and determined the PPARγ-RXRα heterodimerization. While p62 was able to enhance interaction between PPARγ and RXRα, NBR1 blunted p62 effects (Fig. 8c). Of great functional relevance, the luciferase assay using a reporter construct revealed an enhanced PPARγ transcriptional activity by p62, but this was reverted by NBR1 co-reconstitution (Fig. 8d). Furthermore, the expression of Ucp1, whose transcription is driven by PPARγ26, was increased upon p62 reconstitution, but the expression of NBR1 severely inhibited p62 effect (Fig. 8e). Our data support the role of p62 and NBR1 in the regulation of PPARγ-RXRα heterodimerization and PPARγ-mediated thermogenic program in BAs.

**Discussion**

We reported previously that the obese and insulin-resistant phenotypes observed in the whole-body p62 deficient mice resulted from reduced systemic EE14 that underscores the critical role of adipocyte’s p62 in sustaining β3-adrenergic signaling-induced mitochondrial function and thermogenesis in BAT16, as well as cancer-associated browning of subcutaneous WAT17. Importantly, the impaired thermogenesis is not secondary to the obese phenotype because impaired EE has previously been demonstrated in newborn p62 mutant pups and in young p62 mutant mice that yet do not differ in body weight or body composition18. Further evidence has been shown in isolated and in vitro cultured brown and inguinal white adipocytes harvested from either young lean non-obese p62 mutant mice16,18 or neonates from this study.

The severe adiposity and gain of fat mass characteristic of mature p62 mutant mice raises an important question whether glucose tolerance test should be assessed by dosing glucose by lean body mass but not by total body weight. Although dosing according to lean mass has been suggested by some studies especially when body composition is relatively similar, a larger body of literature argues against that because non-lean tissue mass like the white and brown fat and the brain can significantly contribute to whole-body glucose uptake27–29. This is particularly
important in obese animals, in which the non-lean mass can make up to 50% of the body weight, and relative to the muscles, adipose glucose uptake is as high as 30% in obese mice. Thus, total body weight has been adapted for glucose/insulin tolerance tests and normalization of EE data in this study.

Despite the marked phenotypes, the mechanisms whereby p62 deficiency in adipocytes impaired these processes was unclear from those previous studies. The data shown now demonstrate that whole-body and adipocyte-specific NBR1 ablation reverts the obese phenotype induced by p62 deficiency by restoring global EE and thermogenesis in BAT. We also establish here that these obese phenotype induced by p62 deficiency by restoring global EE and thermogenesis in BAT. We also establish here that these

their respective PB1 domains. However, the physiological relevance of that interaction was not clear until now.

The PPARs are members of the nuclear receptor (NR) superfamily of ligand-inducible transcription factors. PPARγ is a critical transcriptional regulator of both WAT and BAT development as well as browning of WAT. Chronic treatment with synthetic ligands of PPARγ strongly induces beige adipocyte differentiation in subcutaneous WAT. PPARγ coordinates with several key co-regulators (PRDM16, PGC1α), controlling brown adipogenesis. Ligand binding induces a conformational change in PPARγ, promoting dissociation of transcriptional repressors and recruitment of co-activator, leading to activation of downstream gene expression. Collectively, our studies show that upon adrenergic stimulation, PPARγ together with p62 and NBR1 translocates into the nucleus of BAs and establish a multi-protein
complex. In this way, p62 and NBR1 emerge as co-regulators of PPARγ with opposite activities. That is, whereas p62 favors PPARγ:RXRα heterodimerization to drive thermogenic gene expression, NBR1 impairs PPARγ:RXRα complex formation, decreasing PPARγ activity. Interestingly, our results are consistent with a model whereby p62 contributes to PPARγ activation by restraining NBR1 from its inhibitory binding to PPARγ (Fig. 9). In support of this model, we show here that mutations that disrupt the p62-NBR1 interaction, or deletion of p62, allow the unleashed NBR1 to dampen PPARγ:RXRα heterodimerization and subsequent function.

This proposed mechanism is reminiscent of the role that p62 plays in hepatic stellate cells, in which p62 facilitates the formation of a VDR:RXRα heterodimeric complex through its binding to these NRs.32 Thus, it is conceivable that p62 could bridge different NRs, potentially increasing their proximity to respective co-regulators for an optimal transcriptional activation. The ability of p62 to interact with NRs should be considered in the context of different NRs, potentially increasing their proximity to respective co-regulators for an optimal transcriptional activation.

Fig. 8 p62 and NBR1 regulate PPARγ:RXRα heterodimerization to control thermogenesis in brown adipocytes. a FLAG-RXRα, GST-PPARγ, WT/ mutants of HA-p62, and HA-NBR1 were overexpressed in HEK293T cells and the interaction of RXRα with PPARγ was analyzed by immunoblotting in pull-downs using glutathione-beads against GST-PPARγ, in the present of NBR1 and/or p62. Representative immunoblotting and densitometric quantification were shown (n = 3 independent experiments). EV empty vector, wt wild-type, mu mutant. b Endogenous interaction of PPARγ with RXRα in BAT of mice exposed to cold for 7 h. PPARγ immunoprecipitates were analyzed by immunoblotting. Densitometric quantification was shown (n = 6, per Sqtstm1Δ/Δ, Sqtstm1Δ/Δ:Nbr1Δ/Δ, and Sqtstm1Δ/Δ:Nbr1Δ/Δ). c Endogenous interaction of PPARγ with RXRα in double KO iBAs (sgSqtstm1sgNbr1) reconstituting p62, NBR1, or both. PPARγ immunoprecipitates were analyzed by immunoblotting. Densitometric quantification was shown (n = 3 independent experiments). d Luciferase assay determining transcriptional activity of PPARγ in iBAs transfected with indicated cDNA vectors, cells were treated with ISO (1 μM) and rosiglitazone (1 μM) for 48 h (n = 6 biological replicates). e qPCR analysis of Ucp1 expression in Sqtstm1Δ/Δ:Nbr1Δ/Δ iBAs reconstituting p62 or NBR1 or both with/without overexpression of PPARγ. Cells were treated with ISO (1 μM) for 48 h. EV (n = 3 biological replicates), PPARγ (n = 5 biological replicates). Data are presented as mean ± SEM (a–e), *p < 0.05, **p < 0.01. Two-tailed Student’s T-test (a–e). Source data are provided as a Source Data file.
fibroblasts to control tumorigenesis. Direct interaction of p62 with AT2F is required for genomic binding of AT2F and AT2F-mediated transcription of thermogenic target genes during β-adrenergic stimulation in BAs. Whether NBR1 also impacts the VDR, ATF4, and/or the AT2F systems still needs to be addressed. Since autophagy is suppressed by β-adrenergic signaling during fat browning, it is conceivable that the accumulation of p62 and NBR1 that we describe in this paper could be the consequence of autophagy inhibition. Therefore, autophagy in this context would be a mechanism for tuning the signaling capabilities of p62/NBR1 in thermogenesis and adiposity. Although the role of p62 and NBR1 in mitophagy has been questioned, at least in some systems, it is still possible that mitophagy inhibition might play a role in the regulation of mitochondrial levels during thermogenesis, whereas the accumulated p62/NBR1 tandem described here will insure that mitochondrial biogenesis and UCP1 expression are efficiently activated transcriptionally. Therefore, the p62-NBR1 complex emerges as a central hub organizing two branches of the thermogenic program converging into mitochondrial function, acting both as autophagy adaptors and as signaling mediators through PPARγ:RXRα signaling.

Methods

Animal. WT and Sqtmt1−/− mice were previously described. Ap2 cre Sqtmt1floxed (Sqtmt1 FX) mice were previously described. Nbr1floxed mice were available from the previous study. Sqtmt1 and Nbr1 dual flox mice were generated by cross-bred with individual flox mice than to breed to aP2 cre to generate adipocyte-specific KO mice (Sqtmt1FX Nbr1 KOX mice). All mouse strains were generated in a C57BL/6 background. All mice were born and maintained under pathogen-free conditions. Mice were fed a normal chow diet and kept on a 12-h light/12-h dark cycle with free access to food and water in a temperature (22 ± 1 °C) and humidity (50 ± 5%) controlled room. All genotyping was done by PCR. Mice were sacrificed, and adipose tissues and liver sections were dissected. Animal handling and experimental procedures conformed to institutional guidelines and were approved by the Sanford Burnham Prebys Medical Discovery Institute Institutional Animal Care and Use Committee.

Metabolic phenotyping. EE, O2 consumption rate, CO2 production rate, RER, food intake, and locomotor activity were assessed in male mice at 50–55 weeks of age using an automated indirect calorimetry Oxymax system of the Comprehensive Lab Animal Monitoring System (CLAMS; Prometheus System) at UCSD. After 48 h of adaptation, O2 consumption and CO2 production were measured to determine the respiratory quotient and EE. EE was measured using ANCOVA with body weight as covariate as previously described. Whole-body composition (fat and lean mass) was measured using Dual-Energy X-ray Absorptiometry at UCSD Animal Care Program. For glucose tolerance test, mice at 25–28 weeks of age were fasted overnight and then challenged with 1.5 g glucose per kg body weight. For insulin tolerance test, mice at 23–28 weeks of age were fasted 4 h and administrated with 0.5 U insulin per kg body weight. Glucose concentrations of tail blood were then measured by using an ACCU-CHEK Aviva (Roche) glucometer at indicated time points. For acute cold exposure, all ap2 Cre mice lines at 23–27 weeks of age were singly housed at 4 °C in a non-bedded cage with access to water but not food for 7 h. Core body temperature was measured using a rectal probe (BAT-10, Physitemp). At the end of the experiment, BAT was resected for histological and gene expression analyses. To induce browning in WAT, mice at 25–27 weeks of age were i.p. administered with β3-adrenergic agonist CL316,243 at 0.5 mg/kg BW for 4 days and at 1 mg/kg for the last day. After injection for 5 days, inguinal WAT was dissected for histological and gene expression analyses.

Histological analysis. Tissues from indicated mice were isolated, rinsed in ice-cold PBS, fixed in 10% neutral buffered formalin for 24 h, dehydrated, and embedded in paraffin. Livers were embedded in Tissue Tek O.C.T. compound and snap frozen in dry ice, then kept in −80°C. Tissue sections (5 μm) were stained with hematoxylin and eosin (H&E). Histological sections of fat pads were stained with periodic acid-Schiff (PAS) and captured under 20-fold magnification by the AxioVision LE software to determine adipocyte size. At least seven fields per section from four different mice of each genotype were randomly selected to determine the adipocyte size and number according to morphological feature using “ImageJ”-based software “Adiposoft.” Frozen liver sections (5 μm) were stained with ORO (Sigma-Aldrich) to detect lipid accumulation. Sections were fixed in paraformaldehyde and stained for 3 h in 0.5% ORO in propylene glycol, followed by 1 min incubations in 85% aqueous propylene glycol. After the slides were washed in distilled water, they were counterstained with Harris’s hematoxylin for 10 s.

Lipid analysis. For determination of lipids mass, liver sample was washed with PBS and frozen. Total lipids were isolated from homogenates by Folch extraction. Briefly, around 50 mg tissue samples were homogenized in 1 ml methanol, homogenates were further mixed with 2 ml chloroform and rotated mildly for 2 h to extract lipid. Samples were then mixed roughly with 1 ml H2O for 30 s to separate phases. The lipid-containing organic phase (bottom) was collected and dried under N2. Total lipids were dissolved in PBS containing 1% Triton X 100, followed by quantification by kits. The tissue lipid concentrations were determined spectrophotometrically (Wako Diagnostics, USA) and normalized to tissue weight.

Cell culture. HEK293T/HEK293FT cells were purchased from ATCC. Primary and iBAs were generated in house. HEK293T/HEK293FT cells and iBAs were cultured in Dulbecco’s Modified Eagles Medium (DMEM, Corning) supplemented with 10% fetal bovine serum (FBS), 2 mM glutamine, and primary cells were cultured in DMEM/F-12 supplemented with GlutaMAX ( Gibco) and 10% FBS. All cells were maintained in an atmosphere of 95% air and 5% CO2. Only cells that were tested negative for mycoplasma were used for experiments.

Generation of primary and immortalized brown adipocytes. For preparation of primary BA, BAT was excised from neonates of WT, Sqtmt1−/−, Nbr1−/−, and Sqtmt1−/−Nbr1−/− mice regardless of gender, and minced in 2 ml PBS, then added with collagenase D (1.5 U/ml), dispase II (2.4 U/ml), and CaCl2 (10 mM) and cultured under 20-fold magnification by the AxioVision LE software to determine adipocyte size. At least seven fields per section from four different mice of each genotype were randomly selected to determine the adipocyte size and number according to morphological feature using “ImageJ”-based software “Adiposoft.” Frozen liver sections (5 μm) were stained with ORO (Sigma-Aldrich) to detect lipid accumulation. Sections were fixed in paraformaldehyde and stained for 3 h in 0.5% ORO in propylene glycol, followed by 1 min incubations in 85% aqueous propylene glycol. After the slides were washed in distilled water, they were counterstained with Harris’s hematoxylin for 10 s.

Fig. 9 Model for the role of p62 and NBR1 in the regulation of PPARγ:RXRα heterodimerization and PPARγ-mediated thermogenic program in brown adipocytes. p62 and NBR1 translocate into the nucleus of brown adipocytes upon adrenergic stimulation and form a multi-protein complex with the nuclear receptor PPARγ and its obligate co-activator RXRα. p62 separately interacts with PPARγ and RXRα, increasing their nuclear proximity and facilitates their heterodimerization to drive thermogenic gene expression. NBR1 emerges as an opposite player that impairs PPARγ:RXRα complex formation and decreases PPARγ activity through direct interaction with PPARγ. The fact that p62 and NBR1 synergistically bind to PPARγ allows p62 to restrain NBR1 from its inhibitory binding to PPARγ and RXRα and facilitates their heterodimerization to drive thermogenic gene expression. To induce browning in WAT, mice at 25–27 weeks of age were i.p. administered with β3-adrenergic agonist CL316,243 at 0.5 mg/kg BW for 4 days and at 1 mg/kg for the last day. After injection for 5 days, inguinal WAT was dissected for histological and gene expression analyses.

Cell culture. HEK293T/HEK293FT cells were purchased from ATCC. Primary and iBAs were generated in house. HEK293T/HEK293FT cells and iBAs were cultured in Dulbecco’s Modified Eagles Medium (DMEM, Corning) supplemented with 10% fetal bovine serum (FBS), 2 mM glutamine, and primary cells were cultured in DMEM/F-12 supplemented with GlutaMAX (Gibco) and 10% FBS. All cells were maintained in an atmosphere of 95% air and 5% CO2. Only cells that were tested negative for mycoplasma were used for experiments.

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induction cocktail consisting of dexamethasone (2 µg/ml), isobutylmethylxanthine (0.5 mM), indomethacin (125 µM), rosiglitazone (0.5 µM), T3 (1 nM), and insulin (5 µg/ml) in growth medium. After 2 days, cells were maintained in growth medium supplemented only with rosiglitazone, T3, and insulin till day 7 for experiments. For transfection assay, differentiating cells at day 5 were transfected with indicated vectors using X-tremeGENE HP transfection reagent (Roche). To stimulate thermogenesis, cells were treated with ISO (Sigma-Aldrich) for indicated times when cells were fully differentiated.

**Generation of knockout cell by CRISPR/Cas9.** To knockout p62 and NBR1 in immortalized SVF cells, 20-nucleotide single-guide RNA sequences targeting the mouse genes (GACUCCCCUGCCAGAGA Aspntm1 and UCACAGACGCA for Nbr1) were purchased from Synthego and transduced into reprogrammed mouse genes (GACUCUCCCUGCCAGAGA for Sqstm1 and CUACAGA for Nbr1) were purchased from Synthego and transduced into reprogrammed mouse genes. To eliminate off-targets, several clones were expanded and screened for p62 and NBR1 expression by protein immunoblotting.

**Cytoplasmic and nuclear fractionation.** Adipocytes were differentiated in P100 dishes. At day 7, mature adipocytes were lysed on ice with Buffer A (20 mM Tris-HCl at pH 7.9, 1.5 mM MgCl2, 10 mM KCl) containing protease and phosphatase inhibitors. Lysates were centrifuged at 750 g for 5 min at 4 °C. The supernatant was collected and centrifuged at 9000 g for 10 min (cytoplasmic fraction). The pellets from previous centrifugation were washed with Buffer A to remove cytoplasmic contamination, then resuspended in Buffer C (20 mM Tris-HCl at pH 7.9, 1.5 mM MgCl2, 0.42 M NaCl) followed by sonication. Lysates were centrifuged at 16,800 g for 15 min and the resulting supernatant was collected as nuclear fraction.

**Immunoprecipitation.** For total cell lysis, cells were rinsed once with ice-cold PBS and lysed on mice in e3 lysis buffer (25 mM Tris-HCl at pH 8.0, 100 mM NaCl, 1% Triton X-100, 10% glycerol) with phosphatase and protease inhibitors. Lysates were centrifuged at 13,000 g for 15 min at 4 °C to remove cell debris. For tissue extracts, BAT was homogenized in 0.5 ml ice-cold lysis buffer (50 mM HEPES at pH 7.5, 0.42 M NaCl, 1% IGEPAL CA-630, 1% NP-40, 0.1% SDS, 1 mM PMSF, 10 µg/ml aprotinin, 10 µg/ml leupeptin). Homogenates were centrifuged at 13,000 g for 15 min at 4 °C to remove fat layer and insoluble material. Protein content of lysates was quantified by DC Protein Assay Kit (Bio-Rad). For immunoprecipitation, 1 mg proteins of tissue or total cell lysates, 3–4 mg proteins of nuclear extracts from cells (for immunoprecipitating endogenous protein), or 0.5 mg of total cell lysates (for co-transfection immunoprecipitation) were cleared with 30 μl 50% slurry of protein G-agarose (Genesee Scientific) for 30 min. Then 2 µg of primary antibodies (anti-p62, Progen, #GP62-C; anti-PPARγ, Santa Cruz Biotechnology, #sc-7273; anti-FLAG, Sigma-Aldrich, #P2983) or control immunoglobulins were added to the lysates and incubated with rotation overnight at 4 °C. The next day, 30 μl 50% slurry protein G-agarose (Genesee Scientific) was added with 20 µl slurry of Anti-FLAG M2 affinity gel (Sigma-Aldrich) for 2 h. Then, immunoprecipitates were centrifuged at 2000 g at 4 °C for 5 min, followed by washing several times with BC300 buffer (50 mM Tris-HCl at pH 7.9, 300 mM KCl, 2 mM EDTA, 10% glycerol, 0.1% NP-40) for nuclear extract, or the same lysis buffers for total cell lysates and tissue lysates. Immunoprecipitated proteins were denatured by adding 10 µl of sample buffer and boiled for 10 min before subject to immunoblotting.

**GST pull-down assay.** For the purification of Flag-p62 and Flag-NBR1 proteins, HEK293T cells in P150 dishes were transfected with 20 µg cDNA vectors of each genes. After 48 h, cells were lysed in lysis buffer (50 mM Tris-HCl at pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100) with phosphatase and protease inhibitors on ice for 30 min. After centrifugation at 13,000 g at 4 °C for 15 min, cell lysates were added with 20 µl slurry of anti-FLAG M2 affinity gel (Sigma-Aldrich) for 2 h. Then, immunoprecipitates were centrifuged at 2000 g at 4 °C for 5 min and washed with TBS buffer (50 mM Tris-HCl at pH 7.4, 150 mM NaCl) three times to remove unspecific binding. Flag-tagged proteins were eluted by 5XFLAG peptide (Sigma-Aldrich) at 100 µg/ml for 30 min at 4 °C in gentle shaking. Eluted proteins were added and stored in −80 °C immediately. To generate GST, GST fused PPARγ, PPARα, Flag-p62 or Flag-NBR1 in 500 µl of glutathione agarose-bound GST-PAPRγ, GST were mixed with 1 µl of eluted Flag-p62 or Flag-NBR1 in 500 µl of NELTE-N binding buffer (50 mM Tris-HCl at pH 8.0, 100 mM NaCl, 6 mM EDTA, 0.5% NP-40) with phosphatase and protease inhibitors for 1 h. After that, samples were washed three times with 1 ml of binding buffer and proteins were denatured by adding 20 µl of sample buffer followed by boiling for 10 min, subjected to immunoblotting. For the co-transfection pull-down assay, HEK293T cells in P60 dishes were transfected with 4 µg cDNA vectors of HA-tagged p62 or NBR1, Flag-tagged RXRa, and GST-tagged PPARγ. After 48 h, cells were washed on ice for 30 min with 50 mM HEPES at pH 7.5, 150 mM NaCl, 2.5 mM EDTA, 2.5 mM EGTA, 1% NP-40, 10% glycerol) with phosphatase and protease inhibitors. After centrifugation at 13,000 g at 4 °C for 15 min, cell lysates were added with 30 µl 50% slurry of glutathione agarose beads overnight followed by washing with lysis buffer for four times. Proteins pulled down by GST-PPARγ were denatured by adding 30 µl of sample buffer followed by boiling for 10 min, subjected to immunoblotting.

**Luciferase assay.** PPAR transcriptional activity was monitored in vitro using reporter construct consisting of three PPRE copies upstream of a luciferase reporter. At day 5 of differentiation, BAs differentiated from immortalized SVF were transiently transfected with the following plasmids using X-tremeGENE PPRE3-TK-luc (Addgene#1015), pRL-TK (control Renilla), HA-p62, HA-NBR1 or Flag-PPARγ1 (Addgene#78769). The level of promoter activity was evaluated by determining the firefly luciferase activity relative to renilla luciferase activity using the Dual Luciferase Assay System (Promega) according to the manufacturer’s instruction.

**RNA analysis.** Total RNA from mouse tissues and cultured cells was isolated using the TRIZOL reagent (Invitrogen) and the RNeasy Mini Kit (QIAGEN), followed by DNase treatment. After quantification using a Nanodrop 1000 spectrophotometer (Thermo Scientific), RNA was reverse transcribed using random primers and MultiScrIte Reverse Transcriptase (Applied Biosystems). Gene expression was analyzed by amplifying 20 ng of the complementary DNA using the CFX96 Real Time PCR Detection System with SYBR Green Master Mix (Bio-Rad). The amplification parameters were set at 95 °C for 30 s, 58 °C for 30 s, and 72 °C for 30 s (40 cycles total). Gene expression values for each sample were normalized to the 18S RNA. A complete list of all primers used is listed in Supplementary Table 1.

**Measurement of respiration in adipocytes.** The cellular OC of BAs was determined using an XFP Extracellular Flux Analyzer and analyzed by Agridata Seahorse Wave Software (Seahorse Bioscience). Prior to assay, 10,000 immortalized SVF cells were seeded into XFP microplates. One day later, adipogenic differentiation was initiated using a protocol mentioned above. Seven days post differentiation, adipocyte culture medium was changed to XF basal medium containing 5 mM glucose, 1 mM pyruvate, and 2 mM GlutaMAX. The basal uncoupled OCR was determined using 1 µM oligomycin. To determine the impact of ISO stimulation in uncoupled OCR. Then, 5 µM ISO was injected three cycles after oligomyacin injection. Oxygen consumption values were normalized to protein content.

**Statistical analysis.** GraphPad Prism software (v. 8.3.0) was used for graphing and statistical analysis. For comparison between two groups, datasets were analyzed by Unpaired Student’s two-tailed t-test. Multiple comparisons were analyzed by two-way ANOVA to determine the statistical significance between groups on basis of the variance of one variable. Differences in EE were calculated using ANCOVA with body weight as covariate using SPSS (version 24). Values of p < 0.05 were considered as significantly different.

**Reporting summary.** Further information on research design is available in the Nature Research Reporting Summary linked to this article.

**Data availability** The support that the findings of this study are provided in the data source file and available from the corresponding author upon reasonable request. The statistical p value
from GraphPad Prism or SPSS reports is provided in the individual figure legends. Source Data are provided with this paper.

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Author contributions

J. H., M. T. D.-M., and J. M. designed the experiments. J. H., J. F. L., and W. X. performed the experiments and analyzed the data. A. D. provided p62CAFpc2-Cre mice and expertise in mouse metabolism and analyzed data. A. R. S. offered access to the CLAMS study. All authors discussed the results and commented on the manuscript. M. T. D.-M. and J. M. conceived and supervised the project. J. H., M. T. D.-M., and J. M. wrote the manuscript.

Competing interests

The authors declare no competing interests.

Additional information

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Correspondence and requests for materials should be addressed to J.M.

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