Autophagy-mediated NCOR1 degradation is required for brown fat maturation and thermogenesis

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Abstract

Brown adipose tissue (BAT) thermogenesis affects energy balance, and thereby it has the potential to induce weight loss and to prevent obesity. Here we document a macroautophagic/autophagic-dependent mechanism of PPARG activity regulation that induces brown adipose differentiation and thermogenesis, and that is mediated by TP53INP2. Disruption of TP53INP2-dependent autophagy reduced brown adipogenesis in cultured cells. In vivo specific-tp53inp2 ablation in brown precursor cells or in adult mice decreased the expression of thermogenic and mature adipocytes genes in BAT. As a result, TP53INP2-deficient mice had reduced UCP1 content in BAT and impaired maximal thermogenic capacity, leading to lipid accumulation and to positive energy balance. Mechanistically, TP53INP2 stimulates PPARG activity and adipogenesis in brown adipose cells by promoting the autophagic degradation of NCOR1, a PPARG co-repressor. Moreover, the modulation of TP53INP2 expression in BAT and in human brown adipocytes suggest that this protein increases PPARG activity during metabolic activation of brown fat. In all, we have identified a novel molecular explanation to the contribution of autophagy to BAT energy metabolism that could facilitate the design of therapeutic strategies against obesity and its metabolic complications.

Keywords

Autophagy; brown adipose tissue; metabolism; obesity; thermogenesis
**Abbreviations**

BAT: brown adipose tissue; GTT: glucose tolerance test; HFD: high fat diet; ITT: insulin tolerance test; MAP1LC3/LC3: microtubule-associated protein 1 light chain 3; NCOR1: nuclear receptor co-repressor 1; PPARG/PPARγ: peroxisome proliferator-activated receptor gamma; TRP53INP2/TP53INP2: transformation related protein 53 inducible nuclear protein 2; UCP1: uncoupling protein 1 (mitochondrial, proton carrier); WAT: white adipose tissue.
Introduction

Brown adipose tissue (BAT) uses nutrients to produce heat in order to maintain body temperature through non-shivering adaptive thermogenesis, and plays a relevant role in energy expenditure [1, 2]. Brown adipocytes contain many lipid droplets and mitochondria that express UCP1 (uncoupling protein 1 (mitochondrial, proton carrier)), the final effector of heat dissipation. Functional BAT depots have been detected in lean adult humans that can be activated by cold exposure [3-5]. Of relevance, human BAT activity correlates with lower body mass index (BMI) and improved glycemia [3, 6], and associates with cardiometabolic health [7] indicating that strategies that could increase BAT mass and/or its activation could become promising targets to combat obesity and its metabolic complications.

Macroautophagy/autophagy has been reported to modulate the differentiation and the metabolic activity of BAT. Initial studies showed that concurrent depletion of the autophagic protein ATG7 in white and brown adipocytes promoted a decrease in the mass of interscapular BAT, as well as lower deposition of lipid droplets, consequence of increased beta-oxidation [8]. Later, the selective depletion of ATG7 in MYF5+ (myogenic factor 5) progenitors (including brown preadipocytes) showed that autophagy is required for BAT differentiation and function. Thus, BAT from MYF5-specific atg7 KO mice had diminished levels of UCP1 and mitochondrial markers, which limited its thermogenic capacity and resulted in the enlargement of its lipid droplets [9]. The contribution of autophagy receptor proteins such as SQSTM1/p62 or NBR1 in brown adipocytes has also been evaluated, which revealed that actually they affect thermogenesis through non-autophagic dependent mechanisms, but rather through the modulation of nuclear functions [10, 11]. Specifically, SQSTM1 has been reported to activate the transcription factor ATF2 [12], and to promote the formation of PPARG/PPARγ (peroxisome
proliferator-activated receptor gamma)-RXR (retinoid X receptor) heterodimers, whereas NBR1 inhibits PPARG-RXR association in brown adipocytes [11]. These observations are relevant since PPARG is an essential transcription factor in brown adipogenesis [13-15], and genetic mutations that impair its activity are associated with decreased BAT development and thermogenic capacity [16]. Yet, the reasons why autophagy activity per se is essential for brown adipogenesis and thermogenesis remain to be elucidated.

In this work, we have focused on the modulator protein TRP53INP2/TP53INP2 (for the mouse or human transformation related protein 53 inducible nuclear protein 2 respectively, and thereafter referred as TP53INP2 for both proteins for simplicity), which induces autophagy in different in vitro models and also in mice [17-19]. We provide evidence supporting the concept that TP53INP2-dependent autophagy promotes brown adipose maturation and maintains the metabolic properties of brown adipocytes. Mechanistically, TP53INP2 sustains PPARG activity and brown fat differentiation through the autophagy-mediated degradation of NCOR1 (nuclear receptor co-repressor 1) in brown adipose cells.
Results

Autophagy is required for the differentiation and maturation of brown adipocytes.

Initially, we analyzed the role of autophagy on the capacity of brown preadipocytes to undergo differentiation into adipocytes. To this end, we focused into the autophagy protein TP53INP2, which accelerates autophagy in different cell types, and its loss-of function \textit{in vitro} and \textit{in vivo} reduces but does not cancel autophagy flux \cite{17, 19-21}. Brown preadipocytes were isolated from \textit{Tp53inp2 \textit{LoxP}+/-} mice and they were immortalized. The \textit{tp53inp2} ablation (KO) was conducted by adenoviral-mediated Cre-recombinase expression. \textit{Tp53inp2} mRNA and protein expression was undetectable in KO cells compared to controls (Figures S1A and S1B). In keeping with the autophagy function of TP53INP2 documented in other cell types \cite{17, 19, 20}, autophagy flux, measured by the accumulation of MAP1LC3/LC3B-II upon bafilomycin A\textsubscript{1} treatment, was markedly impaired in \textit{tp53inp2} KO preadipocytes (Figures 1A and 1B). To further validate these results, we also measured autophagy flux in brown preadipocytes upon acute repression of TP53INP2 induced by siRNA transfection. LC3B-II accumulation upon bafilomycin A\textsubscript{1} was reduced when using \textit{Tp53inp2} siRNA compared to siScramble (siSCR) transfected cells (Figures 1C and 1D). Importantly, the repression of TP53INP2 also reduced its autophagic degradation as detected by a low accumulation upon autophagy inhibition (Figures 1C and S1C). This suggests that TP53INP2 is also degraded through autophagy, and that this process is altered when the protein is downregulated.

Once validated the autophagic function of TP53INP2 in brown preadipocytes, these cells were induced to differentiate for 9 days, as reported \cite{22}. Morphological analysis of differentiated brown adipocytes revealed that KO preadipocytes lost their adipogenic
capacity compared to respective control cells (Figure 1E). Protein expression analysis showed that KO preadipocytes presented reduced PPARG1 and PPARG2 induction in response to differentiation (Figures 1F, S1D and S1E). Moreover, mitochondrial biogenesis, assessed by the increase in mitochondrial proteins TIMM44 and MFN2, and the thermogenic protein UCP1, was blunted in tp53inp2 KO adipocytes (Figures 1F, S1F-H). KO adipocytes also exhibited downregulation of genes involved in brown adipogenesis, such as *Prdm16* and *Pparg1*, and reduced mRNA levels of genes encoding mitochondrial proteins, such as *Ucp1*, *Cox7a1* and *Cox8b* (Figure 1G). In parallel studies, tp53inp2 downregulation (KD) was induced by lentiviral *Tp53inp2* siRNA stable expression, which achieved a 70% repression (Figures S1I and S1J). tp53inp2 KD cells also showed impaired adipogenic potential and reduced expression of protein markers (Figures S1K-M). In order to provide an independent evidence for a role of autophagy on brown adipogenesis, we differentiated brown preadipocytes with a submaximal concentration of chloroquine during different times. Chloroquine treatment for the last 3 or 7 days of the differentiation protocol induced a time-dependent accumulation of LC3B-II (Figure 1H and S1N), along with reduced expression of adipogenic markers PPARG2, PPARG1 and UCP1 (Figure 1H and S1O-Q). These results further support a role of autophagy in brown adipocyte differentiation.

As we found that TP53INP2 is required for brown adipocyte differentiation, we next assessed whether this process is involved in the acquisition of metabolic properties of BAT. With that aim, we generated a *Myf5*-specific *tp53inp2* KO (KO*Myf5*) mice by crossing *Tp53inp2* LoxP+/+ mice with Cre-recombinase-expressing animals under the control of the BAT and skeletal muscle precursor cell-specific promoter *Myf5* [23]. We selected this specific ablation because our interest was to determine the role of TP53INP2 on brown differentiation *in vivo*, and we had previously reported that the specific *tp53inp2*
Ablation in muscle does not alter body weight, adiposity, glucose metabolism or mitochondrial content and functionality in muscle [19]. Gene expression analysis showed that \( Tp53inp2 \) mRNA levels were specifically reduced in iBAT and muscle (Figure S2A), and unchanged compared with control (LoxP) littermates in all other tissues evaluated. In addition, TP53INP2 protein levels were also ablated in BAT from KO\(^{\text{Mryf5}}\) mice (Figure S2B). The resulting KO\(^{\text{Mryf5}}\) mice were born in normal Mendelian ratios and were indistinguishable from their LoxP littermates. At 3 months of age, no changes were found in body weight, composition or tissue weight in either male or female \( tp53inp2 \) KO mice (Figures S2C-G). However, iBAT weight was significantly increased in male KO\(^{\text{Mryf5}}\) mice (Figure 1I), thereby suggesting an early alteration in this tissue. Histological examination of iBAT showed a loss of multilocularity in \( tp53inp2 \) KO iBAT compared to controls in both sexes (Figures 1J and S2H), characterized by a marked reduction in lipid droplet (LD) number per area unit and an increase in LD size (Figures S2I and S2J). These morphological alterations are associated to thermogenically incompetent BAT [24-26]. In contrast, two major white adipose depots, namely inguinal WAT (ingWAT) and perigonadal WAT (pgWAT), showed similar weights in both sexes and genotypes (Figures S2K and S2L). The fact that KO\(^{\text{Mryf5}}\) mice do not show reduced brown fat mass indicates that TP53INP2 is dispensable for embryonic brown adipose development, or that its function \textit{in vivo} is compensated by other factors. To further characterize the alterations occurring in BAT we analyzed total DNA in the whole iBAT depot and we also performed histological analyses. Total DNA per iBAT depot was unchanged between genotypes (Figure S2M), indicating that the total number of cells was not altered upon TP53INP2 depletion. Additional histological analyses using wheat germ agglutinin (WGA, to label the cell surface), DAPI (to label the nuclei) and anti-UCP1 antibody (to measure the differentiation state of brown adipocytes) were performed in iBAT sections.
Triple staining showed a reduced abundance of UCP1 in brown adipocytes from KO^Myf5^ mice, again validating a reduced adipose differentiation (Figure 1K and S2N). In addition, data showed an altered cell size distribution of adipocytes in KO^Myf5^ mice, with a greater abundance of larger adipocytes and with less smaller cells (Figure S2O), which occurred in the absence of changes in the number of nuclei or in the number of adipocytes per surface unit (Figures 1K, S2P and S2Q). These data clearly indicate the existence of hypertrophy of brown adipocytes in BAT from KO^Myf5^ mice. In all, these evidences also indicate that TP53INP2 probably functions in brown adipose maturation or in brown adipocyte cell fate maintenance in adult mice, thus its ablation reduces BAT thermogenic capability leading to lipid accumulation and brown adipocyte enlargement.

To test this hypothesis, we evaluated the expression of adipogenic and thermogenic genes in iBAT. The results revealed a reduced expression of the adipogenic genes Prdm16, Cebpb, Pparg1 and Pparg2 in tp53inp2 KO iBAT, as well as of that involved in BAT thermogenesis, such as Ucp1, Ppargc1a/Pgc1a, Cox8b, Cox7a1, Dio2 and Elovl3 (Figure 1L). The mRNA levels of the Dlk1/Pref1 (delta like non-canonical Notch ligand 1), known to inhibit adipogenesis in preadipocytes, was also significantly enhanced in KO^Myf5^ mice compared to control animals (Figure S2R). Taken together, our data indicate that TP53INP2 is required for the proper metabolic maturation of brown adipocytes in vivo.

TP53INP2-dependent autophagy is also required for the maintenance of the differentiation state of brown adipocytes.

Next, we evaluated whether TP53INP2-dependent autophagy, in addition to regulating brown adipose differentiation, also maintains the maturation state of brown adipocytes.
To do so, we treated *in vitro* differentiated brown adipocytes isolated from Cre-recombinase-inducible *Tp53inp2 LoxP*/*+* mice with tamoxifen for 3 days, to induce TP53INP2 repression. Tamoxifen treatment efficiently reduced *Tp53inp2* mRNA levels by 60%, but also significantly downregulated the expression of *Prdm16, Ucp1* and *Ppargc1a*, which are brown adipogenic and thermogenic genes, compared to vehicle (DMSO)-treated adipocytes (Figure 2A). Under these conditions, the expression of *Cox7a1, Cox8b* or *Pparg2* was unchanged (Figure 2A). No difference in gene expression was found when using *in vitro* differentiated brown adipocytes without Cre-recombinase expression under the same experimental conditions (Figure 2B). To determine the role of TP53INP2 in mature brown adipocytes *in vivo*, we eliminated it from adult mice by tamoxifen administration to Cre-recombinase-inducible *Tp53inp2 LoxP*/*+* mice [20] under the control of the *Ubc* (ubiquitin C) promoter. A tamoxifen diet was administered to both Cre-positive (KO*Ubc*) and Cre-negative (LoxP) mice for one month, leading to TP53INP2 loss-of-function in iBAT (Figures S3A and S3B). Six months after the onset of tamoxifen treatment, KO*Ubc* mice showed an increased iBAT weight compared with control littermates (Figure 2C). Under these conditions, body weight was also enhanced in KO*Ubc* mice (Figure S3C), in keeping with reported data [20]. Under these conditions, all adipose depots analyzed in KO*Ubc* mice were enhanced (ingWAT and pgWAT), liver weight was also increased, and no changes were detected in quadriceps, gastrocnemius and tibialis anterior muscles (Figures 2C and S3D). Histological staining of iBAT sections again revealed a thermogenically inactive morphology (Figure 2D), as reflected by an increase in LD size and a decrease in LD number per surface (Figures 2E and 2F). Moreover, the number of adipocytes or nuclei per surface unit and UCP1 protein abundance was decreased in KO*Ubc* mice (Figures 2G, 2H, S3E and S3F), and adipocyte area was enhanced (Figure 2I). Gene expression analysis of iBAT samples showed that
KO^Ubc^ mice had a similar expression profile to that of KO^Myf5^ mice, characterized by reduced expression of BAT adipogenic genes, including Pparg2 and Cebpb, accompanied by downregulation in genes related to thermogenesis (Figure 2J). Altogether, our data indicate that TP53INP2 plays a key role in the maintenance of the differentiation state of mature brown adipocytes under in vivo conditions.

**TP53INP2 induces BAT PPARG activity.**

As we found that TP53INP2-dependent autophagy is required for brown adipocyte differentiation and for the maintenance of mature BAT properties, we next analyzed the mechanisms involved. To this end, we performed microarray gene expression profiling using iBAT samples from KO^Myf5^ and from LoxP mice as controls. More than 700 genes were significantly upregulated and 464 were downregulated by tp53inp2 ablation (Figure 3A). In addition, to examine the impact of TP53INP2 downregulation on BAT gene modulation, we compared our microarray results with published RNAseq data evaluating the transcriptomic changes associated to thermoneutrality [27]. In this study, the authors compared gene expression from iBAT samples of mice housed at 30°C with mice housed under standard conditions (22°C). Interestingly, around 40% of the genes modulated by the lack of TP53INP2 in iBAT were also naturally modified by the physiological inhibition of thermogenesis (Figure 3A). Moreover, we defined two gene sets with the top 100 up- and top 100 downregulated genes by tp53inp2 ablation in iBAT, and analyzed them in the 22°C vs 30°C RNAseq. Our results showed that the top hits in the KO^Myf5^/LoxP experiment were over-represented in the extremes of the 30°C/22°C ranked list, being modulated with a common direction (Figures 3B and 3C), which indicates that
the gene variation showed a similar pattern in both conditions (thermoneutrality and

*tp53inp2* ablation).

The transcriptomic profiling of iBAT samples from KO^Ubc^ and from LoxP mice also
identified a substantial number of dysregulated genes (291 genes were upregulated and
250 genes were downregulated) by *tp53inp2* ablation. Gene set enrichment analysis
revealed several dysregulated pathways upon *tp53inp2* ablation, both in KO^Myf5^ (Figure
3D) and in KO^Ubc^ mice (Figure 3E). Importantly, one of the most significantly
downregulated gene sets by the absence of TP53INP2 in the two mouse models was
adipogenesis (Figures 3D, 3E and S4A), further validating the view that TP53INP2
induces brown fat differentiation. Fatty acid metabolism, oxidative phosphorylation and
peroxisome gene sets were also downregulated in both mouse models (Figures 3D, 3E,
S4B and S4C), revealing that BAT oxidative metabolism is compromised when
TP53INP2 is depleted. These data indicate the existence of common defective processes
operating in BAT from KO^Myf5^ and from KO^Ubc^ mice upon *tp53inp2* ablation.

Further exploration of additional gene sets affected revealed a significant downregulation
of the PPAR signaling pathway upon loss of TP53INP2 in BAT in KO^Myf5^ mice (Figure
3F), and in KO^Ubc^ mice (Figure S4D). These findings are of interest since it has been
documented that TP53INP2 overexpression co-regulates the transcriptional activity of
various nuclear receptors, including PPARs [21], and, in addition, PPARG is an essential
protein for brown adipogenesis [13-15]. Thus, we next focused on the potential role of
TP53INP2 in the modulation of PPAR transcriptional activity in the context of mouse
brown preadipocytes. PPAR response element (PPRE) transcriptional activity was
measured upon the addition of rosiglitazone, a PPARG-specific ligand. Preadipocytes
displayed low PPARG activity even in the presence of rosiglitazone, consistent with a
low endogenous expression of PPARG protein (Figure 3G). Thus, scramble and *tp53inp2*
KD cells were co-transfected with PPARG and treated with vehicle (-) or with rosiglitazone (+) to enhance PPRE activity. Under these conditions, tp53inp2 KD cells showed a reduced response, thereby implying a disruption in PPAR transcriptional activity (Figure 3G). tp53inp2 KO preadipocytes also displayed a similar profile of changes (Figure S4E). In addition, control and tp53inp2 KO preadipocytes stably expressing PPARG (+) or empty vector (-) were incubated in the presence or absence of rosiglitazone. Ligand-induced PPARG transcriptional activity was completely blunted in TP53INP2-deficient cells stably expressing HA-PPARG (Figure 3H). Taken together, our evidence indicates that the lack of TP53INP2 in brown preadipocytes results in defective PPARG activity, which is independent of PPARG protein levels.

**TP53INP2-dependent autophagy promotes the degradation of the co-repressor NCOR1.**

The presence of co-repressors and co-activators modulates PPARG activity. Under inactive conditions, PPARG is physically bound to a co-repressor complex containing NCOR1. However, when PPARG binds to its ligand, a conformational change results in the release of the co-repressor complex and the binding with the co-activator complex [28]. NCOR1 has recently reported to be a substrate of autophagy [29, 30], and TP53INP2 participates in this process [17, 19]. Thus, we analyzed whether TP53INP2 regulates NCOR1 through this degradative pathway. TP53INP2-deficient brown preadipocytes showed a marked upregulation of NCOR1 protein levels under basal conditions (Figure 4A), which is in concordance with a reduced PPARG activity as documented. We also analyzed the rate of degradation of NCOR1 through the autophagy pathway. To this end, control and tp53inp2 KD cells were incubated in the absence or presence of bafilomycin.
A1, and we measured the build-up of autophagy markers and NCOR1. Results revealed that TP53INP2-deficient cells exhibited reduced autophagy flux and decreased autophagic degradation of NCOR1 (Figures 4A-C). Alterations in NCOR1 protein abundance were not associated to changes in mRNA levels (Figure S5A). Acute repression of *Tp53inp2* mediated by siRNA transfection also reduced autophagic degradation of NCOR1 (Figures S5B and S5C). As TP53INP2 overexpression has been reported to enhance autophagy activity [17], we tested whether it could also mediate enhanced NCOR1 degradation through this pathway. Thus, TP53INP2 stable overexpressing brown preadipocytes were generated. Coherent with reported data[17], TP53INP2 overexpression enhanced LC3B-II accumulation upon bacilomycin A1 treatment, and also increased autophagic degradation of NCOR1 in brown preadipocytes (Figures 4D-F). However, overexpression of a form of TP53INP2 with a mutation on its LIR motif (TP53INP2-LIR) that lacks its capacity to interact with Atg8-family proteins and to induce autophagy, did not alter LC3B-II or NCOR1 autophagy fluxes (Figures 4D-F). TP53INP2-LIR expression was higher than TP53INP2 WT (Figure S5D), which may be a consequence of a reduced degradation through autophagy (Figure S5E).

Next, we studied whether TP53INP2 also mediates NCOR1 degradation during differentiation of brown preadipocytes. PPARG expression is highly induced from day 2 to 4 of differentiation (Figures S1D and S1E). Thus, we reasoned that under these conditions, autophagic degradation of NCOR1 should increase in order to propitiate PPARG activation. With that idea in mind, we induced TP53INP2 downregulation from day 2 to 4 of differentiation by siRNA transfection, and then evaluated autophagy flux at day 4. TP53INP2 repression reduced LC3B-II autophagic flux and also TP53INP2 degradation through autophagy (Figures 4G, S5F and S5G), similarly to preadipocytes (Figures 4A-C, S5B and S5C). Importantly, NCOR1 levels in si*Tp53inp2* adipocytes were
also increased compared to siSCR cells, and its degradation rate through autophagy was lower (Figures 4G and S5H). Enhanced NCOR1 protein abundance was also detected in iBAT from KO\textsuperscript{Myf5} compared to LoxP mice (Figures 4H and 4I), supporting the idea that TP53INP2-mediated autophagic degradation of NCOR1 also occurs \textit{in vivo}.

Considering that autophagy-mediated NCOR1 degradation occurs in the cytosol upon translocation of the co-repressor from the nucleus \cite{29, 30} and that TP53INP2 recycles between nucleus and cytosol \cite{31}, we analyzed the subcellular distribution of NCOR1 by imaging and by subcellular fractionation. Coherent with its co-repressor function, NCOR1 mainly localized in the nucleus (Figures 5A and 5B). However, confocal microscopy showed that TP53INP2-deficient preadipocytes displayed greater levels of nuclear NCOR1 (Figures 5A and 5B). Enhanced abundance of NCOR1 was also detected in nuclear fractions from \textit{tp53inp2} KD preadipocytes (Figures 5C and 5D). In contrast, TP53INP2 overexpression reduced the localization of NCOR1 in the nuclei, an effect that was not induced by TP53INP2-LIR (Figures S5I-M). These data support the view that the autophagic activity of TP53INP2 is instrumental for the proper cytosolic shuttling of NCOR1 and its regulation by autophagy, thus, TP53INP2 repression impedes NCOR1 cytosolic transport and in turn, its degradation.

Next, we analyzed whether the function of TP53INP2 on NCOR1 involves the formation of a complex between the two proteins. With that aim, we investigated whether TP53INP2 interacts with NCOR1 in HEK cells stably expressing empty vector (-) or HA-TP53INP2 (+). The affinity isolation of HA-tagged TP53INP2 protein with anti-HA beads revealed co-immunoprecipitation of endogenous NCOR1 (Figure 5E). Furthermore, the endogenous immunoprecipitation with an anti-NCOR1 antibody in HEK cells specifically detected NCOR1 protein, which co-immunoprecipitated with HA-TP53INP2 (Figure 5F). We also validated the interaction of NCOR1 with TP53INP2 in brown
preadipocytes by proximity ligation assays (PLA) (Figures 5G and 5H). Moreover, we
detected that the interaction of the two proteins was maintained by TP53INP2-LIR
(Figures 5G and 5H). These data suggest that NCOR1 and TP53INP2 participate in a
complex, and that TP53INP2 could facilitate the translocation of NCOR1 to the cytosol
for its autophagic degradation.

To evaluate if the decreased PPARG activity detected in TP53INP2-deficient
preadipocytes is a consequence of increased NCOR1 abundance, we repressed NCOR1
in control and TP53INP2-deficient cells, and monitored PPARG activity. Interestingly,
the downregulation of the co-repressor NCOR1 rescued the transcriptional defect
observed in *tp53inp2* KD cells (Figure 5I). In the absence of PPARG ligand, NCOR1
knockdown significantly stimulated PPRE activity both in SCR and in *tp53inp2* KD
compared with siCtr transfected cells (Figure 5I). In contrast, in the presence of
rosiglitazone, which induces PPARG activity, the increase in PPARG transcriptional
activity induced by the downregulation of NCOR1 was only significant in *tp53inp2* KD
cells (Figure 5I) demonstrating that NCOR1 exerts stronger PPARG repression when
TP53INP2 is deficient.

Overall, our data indicate that TP53INP2 activates the autophagic degradation of NCOR1
in brown adipose cells, through a process that involves the formation of a complex and
the movement of NCOR1 from the nucleus to the cytosol. This leads to the activation of
PPARG, which in turn promotes the normal induction of brown adipogenesis.

*Loss of BAT-specific TP53INP2 results in thermogenic dysfunction and altered energy
balance.*
Based on the processes triggered by TP53INP2-dependent autophagy we next addressed the metabolic implications induced by the lack of TP53INP2 in BAT. Indirect calorimetry assays revealed that KOMyf5 mice significantly reduced O2 consumption, CO2 production and energy expenditure (Figures 6A-C and S6A-C). The decrease in energy expenditure was detected mainly in the light phase, pointing to a thermogenic defect (Figure S6D). These alterations were observed in the absence of changes in locomotor activity (Figure S6E) or in food and water intake (Figures S6F and S6G). The respiratory exchange ratio (RER) and nutrient oxidation were unchanged in KOMyf5 mice (Figures S6H-J). To determine whether the decreased energy expenditure was indeed a defect in BAT-specific non-shivering thermogenesis, calculated as the differential increase in oxygen consumption caused in response to adrenergic stimuli when analyzed under different thermogenic conditions [32], we measured whole body norepinephrine (NE)-induced VO2 (ΔVO2) in anesthetized mice housed at a range of temperatures. Under low thermogenic conditions (30°C), both LoxP and KOMyf5 mice showed a similar capacity to induce oxygen consumption in response to NE (Figures 6D and 6E). However, when mice were housed under standard conditions (22°C), which induces significant BAT thermogenic activity, KOMyf5 mice presented blunted NE-induced VO2 compared with control littermates (Figures 6D and 6E). Our results demonstrate a defective maximal thermogenic capacity caused by the loss of TP53INP2 (Figures 6D and 6E). To further confirm that BAT dysfunction contributed to the decrease in whole-body energy expenditure, we directly measured mitochondrial respiration using high-resolution respirometry. Indeed, mitochondrial complex II activity decreased in iBAT mitochondrial-enriched fractions from KOMyf5 mice (Figure 6F). Under these conditions, no alterations in mitochondrial respiration were detected in tibialis anterior muscles (Figure S6K). Furthermore, the skeletal muscle-specific tp53inp2 knockout mice
displayed normal metabolic parameters, showing similar energy expenditure, locomotor activity and substrate utilization than control mice (Figures S6L-R), supporting the view that the metabolic defects documented in KO\textsuperscript{Myf5} mice are associated with a deficient BAT thermogenesis. In addition to these findings, cultured \textit{tp53inp2} KO brown adipocytes showed a reduced capacity to induce \textit{Ucp1} or \textit{Prdm16} in response to the β3-adrenergic agonist CL-316,243 (CL) (Figures S6S and S6T), and a blunted ability to increase oxygen consumption when treated with NE \textit{in vitro} (Figure S6U), proving that the metabolic alterations are cell autonomous.

The energy imbalance observed in 3-month-old \textit{tp53inp2} KO mice occurred in the presence of unaltered body weight, and with a non-significant trend for an increased fat mass. Based on this, we analyzed the gain of body weight and of fat mass in a group of mice studied at either 3 or 6 months, which were maintained at 22° C and subjected to a control chow diet. Data clearly indicated that at 6 months of age, male KO\textsuperscript{Myf5} mice were heavier, had more fat mass than controls, and similar lean mass (Figures 6G, S7A and S7B). iBAT from male KO\textsuperscript{Myf5} mice weighed twice as much as that of LoxP animals (Figure 6H). Expansion of both the ingWAT and the pgWAT depots was markedly more significant in \textit{tp53inp2} KO mice (Figures S7C and S7D). As a result, male KO\textsuperscript{Myf5} mice showed a greater body weight gain and fat mass gain between 3 and 6 months of age (Figures 6I and 6J). Our data clearly indicate that the reduced BAT metabolism of TP53INP2 depleted mice leads to energy imbalance and greater fat mass gain.

Histological analysis of iBAT confirmed that tissue morphology was severely impaired and the loss in multilocularity caused by \textit{tp53inp2} ablation was exacerbated at 6 months compared to 3 months both in male and in female mice (Figures S7E-G). Again, brown adipocytes from KO\textsuperscript{Myf5} displayed hypertrophy, monitored by an increased adipocyte area (Figures 6K and S7H), and by reduced number of nuclei or adipocytes per surface
Coherent with the decreased maximal thermogenic capacity documented in KO^Myf5, iBAT displayed a marked reduction in UCP1 protein expression (Figures 6K, 6L, S7K and S7L).

In addition to the disruption of BAT metabolism, KO^Myf5 mice showed systemic alterations. Thus, both males and females KO^Myf5 mice showed impaired glucose use in glucose tolerance test (GTT) assays (Figures S7M and S7N), which occurred in the presence of fasting hyperinsulinemia in male mice (Figures S7O and S7P). In addition, a reduced hypoglycemic effect of insulin was detected in male KO^Myf5 mice upon insulin tolerance test (ITT) assays (Figures S7Q and S7R).

Overall, these results indicate that tp53inp2 ablation in brown adipocyte precursor cells results in the thermogenic dysfunction of iBAT, which impacts energy balance and contributes to weight gain. In addition, tp53inp2 ablation causes alterations in glucose tolerance as a consequence of a reduced capacity to respond to insulin.

TP53INP2 is essential for diet-induced thermogenesis.

Next, we studied the impact of tp53inp2-deficient BAT in the absence of cold stress. Under increased environmental temperature conditions (30°C), cold-induced thermogenesis is abolished, but there is still a thermogenic component induced by diet, a process that is specific to brown adipocytes. Thus, we housed two groups of mice at 30°C for 5 months, starting just after weaning. One group was fed a standard chow diet (CD), while the other received a high-fat diet (HFD), potentially inducing an increase in thermogenic capacity due to the high consumption of fat [33, 34].

Exposure of mice to 30°C and to a CD diet resulted in no differences in body weight or in iBAT weight between wild-type and KO^Myf5 groups, but a slight increase in the total
fat mass of the KO\textsuperscript{Myf5} mice was detected (Figures 7A-C). When animals were challenged with a HFD at 30ºC, KO\textsuperscript{Myf5} mice again showed a greater body weight (Figure 7A), together with an increased total fat mass, iBAT mass, and size of white adipose depots (Figures 7B-E) compared to control littermates. No changes in lean mass were detected between genotypes (Figure 7F).

These results further support the view that TP53INP2 deficiency in BAT decreases non-shivering adaptive thermogenesis and, consequently, induces fat accumulation. Morphological analysis of iBAT showed again that brown adipocytes were unilocular and undistinguishable between groups under 30ºC conditions and with a standard diet (Figures 7G-I). HFD administration increased the appearance of multilocular adipocytes in control mice in keeping with active thermogenesis. This effect was blunted in KO\textsuperscript{Myf5} mice, as reflected by an increase in the average LD size compared to control animals (Figures 7G-I). Increased environmental temperature blocks the effect of \textit{tp53inp2} ablation, and argues strongly that this is a BAT specific effect only present under conditions of thermogenic activation, as it is prolonged feeding with HFD. Thus, 30ºC acclimated mice were fed either with CD or HFD and then were challenged with NE to measure whole body ΔVO\textsubscript{2}. Interestingly, the results obtained clearly demonstrate that diet-induced maximal thermogenic capacity is also impaired in \textit{tp53inp2} KO mice (Figures 7J and 7K).

\textit{Thermogenesis modulates TP53INP2 expression in brown adipose tissue.}

Based on the data indicating that TP53INP2-dependent autophagy modulates BAT metabolism and brown adipose differentiation, we analyzed whether TP53INP2 expression is modulated upon enhanced BAT function. Mice subjected to a HFD showed
enhanced expression of $Tp53inp2$ (2.3-fold) and $Ucp1$ (Figure 8A) in iBAT, suggestive of active diet-induced thermogenesis, as previously described [33, 34]. A HFD also induced the expression of TP53INP2 and UCP1 proteins (Figures 8B and 8C). In order to evaluate whether HFD-induced increase in TP53INP2 expression is a mechanism that prevents the excessive accumulation of fat in vivo, we fed LoxP and KO$^{Myf5}$ mice with HFD for 16 weeks. Under these conditions, $tp53inp2$ KO mice gained more body weight and fat mass compared with control littermates already at 8 weeks after HFD, and without alterations in lean mass (Figures 8D-F and S8A). 16 weeks after, the iBAT and ingWAT adipose depots were significantly expanded in KO$^{Myf5}$ mice, but pgWAT showed similar weight between genotypes (Figures S8B-D). As a result, fat mass gain between 8 and 16 weeks of HFD was 3-fold greater in KO$^{Myf5}$ mice compared to controls (Figure 8G). Under these conditions, liver weight was also increased in $tp53inp2$ KO animals (Figure S8E), while muscles weight was unchanged (Figure S8F). All these data indicate that TP53INP2 expression in iBAT prevents the development of obesity in mice and its metabolic complications.

Low environment temperature is another situation characterized by enhanced brown adipose thermogenesis. Exposure of mice to cold (4°C for 10 h) also acutely upregulated the expression of $Tp53inp2$ (2.2-fold), followed by the expected increase in $Ucp1$ and $Ppargc1a$ mRNA (Figure 8H) [35-37]. The converse situation, BAT thermogenesis inactivation in C57/BL6 mice induced by increasing environmental temperature to 30°C versus standard housing conditions (22°C) showed marked downregulation of $Tp53inp2$ in BAT (Figure 8I), in parallel with the expected low expression of thermogenic genes such as $Ucp1$, $Ppargc1a$ and $Prdm16$ [24, 25, 38] under these conditions (Figure 8I).

To further confirm that $Tp53inp2$ expression was modulated specifically in brown adipocytes, we studied the mRNA levels of this gene in cultured brown adipocytes.
Differentiation of human or mouse brown preadipocytes markedly induced TP53INP2 mRNA and protein levels (Figures 8J and S8G-K). In addition, brown adipocytes were stimulated or not with the β3-adrenergic agonist CL for 4 h. Indeed, Tp53inp2 expression was markedly increased (56%), alongside the induction of Ucp1 and Ppargc1a (Figure S8L). Intracellular stimuli such as the cAMP analogues 8-bromo-cAMP (8Br) and dibutyryl-cAMP (dcAMP) or the adenylyl cyclase activator forskolin (FSK) were also capable to dramatically induce Tp53inp2 mRNA and protein levels in mature human PAZ6 brown adipocytes (Figures 8K, 8L and S8M). Our results suggest that the modulation of TP53INP2 expression is part of a mechanism aimed to activate PPARG and mitochondrial biogenesis upon BAT recruitment both in human and in murine cells.
In this work, we document that TP53INP2-dependent autophagy plays a relevant role in brown adipose cell metabolism and differentiation. Disruption of TP53INP2-dependent autophagy reduced brown adipogenesis in cultured cells, and in vivo \textit{tp53inp2} ablation in BAT caused dysregulated gene expression and enhanced lipid accumulation in brown adipocytes. This was the result of decreased UCP1 protein and reduced BAT non-shivering thermogenesis, leading to a positive energy balance. Furthermore, TP53INP2 also maintains the differentiation state of brown adipocytes in adult mice. Mechanistically, TP53INP2 promotes the autophagic degradation of the PPAR\textsubscript{gamma} co-repressor NCOR1, which stimulates PPAR\textsubscript{gamma} activity and adipogenesis in brown preadipocytes. Our data manifest the relevance of autophagy activity in the control of BAT metabolism, and emphasize the potential use of autophagy-directed drugs to treat obesity and related metabolic diseases.

Studies using mouse brown preadipocytes and cells undergoing differentiation have revealed that TP53INP2 is required for the autophagy-mediated degradation of NCOR1 to maintain PPAR\textsubscript{gamma} activity. Thus, TP53INP2 repression is linked to impaired autophagy flux and increased NCOR1 protein levels in the nuclear compartment. In addition, TP53INP2 overexpression enhanced autophagic flux, NCOR1 degradation through autophagy, and in parallel, reduced NCOR1 nuclear abundance. The effects of TP53INP2 overexpression were linked to its ability to trigger autophagy and mutations on its LIR motif cancelled them. These results are coherent with prior data indicating that both NCOR1 and TP53INP2 proteins localize on the nucleus under basal conditions and they are shuttled to the cytosol upon autophagy activation \cite{17, 29, 30, 39, 40}. Moreover, cells with altered autophagy exhibit either retention of NCOR1 in the nucleus or aberrant NCOR1 accumulation on the cytosol \cite{29, 30, 41}. Notably, the defective PPAR\textsubscript{gamma} activity
detected in TP53INP2-deficient cells was ameliorated upon NCOR1 down-regulation. In all, our data reveal a causal connection between the modulation of NCOR1 protein levels by TP53INP2 and the observed PPARG transcriptional activity.

Based on the results documenting a physical interaction between TP53INP2 and NCOR1, we propose that TP53INP2 facilitates NCOR1 translocation to autophagosomes for its degradation. As to the potential mechanisms by which TP53INP2 sense a thermogenic stimulus and promotes the nuclear exit of NCOR1, they remain unknown. Perhaps relevant to this question, are the following observations: i) TP53INP2 shuttles between the nucleus and the cytosol, and rapamycin or amino acid starvation causes the rapid recruitment of nuclear TP53INP2 to the cytosol in cells [17, 42]; ii) TP53INP2 binds to Atg8-family proteins such as GABARAPL2 or LC3, and this is key for the binding of LC3 to ATG7 proteins to initiate autophagy [17, 42, 43]; and iii) SIRT1-dependent deacetylation of LC3 enables its interaction with TP53INP2, promoting its recruitment to the cytosol [44]. Based on these observations, we propose that similarly to what occurs for LC3, NCOR1 requires TP53INP2 for its exit from the nucleus, and its subsequent engagement into autophagic degradation upon a thermogenic stimulus.

Depletion of the autophagic protein ATG7 in white and brown adipocytes has been reported to reduce interscapular BAT mass, lipid droplet deposition, and to increase beta-oxidation [8]. Ablation of *atg7* in MYF5+ precursor cells, revealed a role of autophagy on the differentiation of brown preadipocytes [9], and BAT from KO mice had bigger lipid droplets and diminished levels of UCP1 and mitochondrial markers [9]. Further mechanistic studies nicely documented that depletion of the autophagy receptor protein SQSTM1 in brown adipocytes attenuated the expression of PPARGC1A and UCP1 in BAT and decreased energy expenditure in mice [10, 11]. These effects were not directly associated to impaired autophagic flux, but consequence of the inhibition of the
transcription factor ATF2 or the reduction in the formation of PPARG-RXR heterodimers [11, 12]. Thus, the direct mechanisms that link autophagic activity with brown fat differentiation remained unknown. The results obtained in the present study support the requirement of autophagy for the optimal differentiation of brown adipocytes, and autophagy inhibition with chloroquine treatment or by TP53INP2 deficiency reduced adipogenic markers such as UCP1, PPARG2 and PPARG1. In this work we expand our view on the mechanistic role of autophagy on BAT metabolism, and we document that TP53INP2-dependent autophagy controls NCOR1 abundance and PPARG activity in brown adipose cells. In this connection, we support the view that TP53INP2 is involved in constitutive autophagy, as TP53INP2 depleted cells displayed reduced autophagic flux determined by LC3B-II accumulation upon bafilomycin A1 treatment, and TP53INP2 overexpressing cells showed the opposite phenotype. However, this does not rule out the possibility that TP53INP2 is involved in the selective autophagic degradation of NCOR1, which will require further analysis.

Our data have revealed that the TP53INP2 protein is a positive regulator of brown adipogenesis and that it maintains brown adipocyte identity in adult mice through PPARG activation. Ablation of tp53inp2 in adult mice cause BAT dysfunction, altered expression of metabolic genes, and obesity. In keeping with these data, we have also documented that TP53INP2 deficiency in brown preadipocytes reduces the expression of crucial brown adipose differentiation genes such as Prdm16, Ucp1 or Ppargc1a, and UCP1 protein levels in iBAT. Thus, TP53INP2 induces BAT-specific maximal thermogenic capacity and prevents the development of obesity in mice. However, TP53INP2 seems to be dispensable for the embryonic development of BAT. This phenotype is similar to what detected when genes that are essential in BAT differentiation where specifically ablated, such as Prdm16, Tyk2 or Stat3 [26, 45], and suggests that
under conditions of TP53INP2 deficiency, alternative pathways that maintain adipogenesis may be triggered in brown preadipocytes in vivo. In this connection, we have clearly documented that *tp53inp2* ablation reduces the differentiation state of brown adipocytes without altering the total number of adipocytes in BAT, and adipocytes displayed hypertrophy as a consequence of reduced UCP1-mediated uncoupling activity and enlargement of lipid droplets. This is in agreement with the described role of PPARG in BAT, which is not essential for its commitment but rather for its maturation [15].

Of note, TP53INP2 has opposite effects on adipose differentiation in white and brown preadipocytes. In this regard, this protein has been reported to block white adipose differentiation by enhancing TCF activity through a process that depends on the sequestration of GSK3B in an endosomal compartment and the accumulation of CTNNB1/β-catenin [20]. Another study using bovine white preadipocytes also documented an adipogenic role of TP53INP2 [46]. In this study, we document that TP53INP2 has a stimulatory effect in brown preadipocytes through the activation of PPARG transcriptional activity. Based on the current evidence, we propose that the maintenance of high TP53INP2 activity in white and brown adipose depots prevents the development of obesity in mice.

TP53INP2 promotes BAT-specific diet-induced thermogenesis. In this connection, *tp53inp2* ablation reduced BAT thermogenesis, and this was followed by enhanced adiposity in mice. The repression of TP53INP2 expression in conditions of increased environmental temperature, when combined with maintained or increased food intake, may contribute to the development of obesity in mice. Furthermore, HFD-induced TP53INP2 expression in BAT represents an adaptive mechanism to prevent an excessive accumulation of body fat under obesogenic conditions. Thus, the induction or activation of TP53INP2 in the absence of cold-stress through a HFD-independent stimulus could be
an efficient tool through which to increase thermogenic activity at the whole-body level, thus preventing obesity.

Interestingly, our findings indicate that the expression of TP53INP2 in interscapular BAT is upregulated by conditions characterized by enhanced BAT thermogenic activity, namely exposure to cold or a HFD. In contrast, the expression of TP53INP2 is repressed under 30°C environment, i.e., a condition with no need to generate heat. Sympathetic nerves directly control BAT thermogenesis through the action of NE. In this regard, cold exposure or a chronic HFD cause the sympathetic nervous system to release NE and to activate the β-adrenergic receptors present on the surface of brown adipocytes [3, 6, 47-49]. Given these findings and the observations that a β3-adrenergic agonist enhances TP53INP2 expression in mouse brown adipocytes, and that cAMP analogs increase TP53INP2 expression in human PAZ6 adipocytes, we propose that the adrenergic-mediated PKA signaling pathway is involved in the upregulation of TP53INP2 in BAT under in vivo conditions. Overall, our results unravel a novel protein target that, if selectively activated, could increase BAT thermogenic metabolism and prevent obesity and metabolic disorders associated to this condition.
Materials and Methods

Mice strains

The KO\textsuperscript{Myf5} mouse line was generated by crossing homozygous \textit{Tp53inp2} \textit{LoxP}+/+ mice [19] with a Cre-recombinase-expressing mouse strain under the control of the \textit{Myf5} promoter (The Jackson Laboratory, 007893). Experimental groups contained \textit{LoxP}+/+ Cre negative (LoxP) and \textit{LoxP}+/+ Cre positive mice (KO\textsuperscript{Myf5}).

The total \textit{tp53inp2} knockout (KO\textsuperscript{Ubc}) mouse line was obtained by crossing homozygous \textit{Tp53inp2} \textit{LoxP}+/+ mice with a mouse strain expressing the Cre-recombinase under the control of the \textit{Ubc} promoter (UBC-Cre-ERT2), as previously described [20].

Skeletal muscle specific \textit{tp53inp2} knockout mouse line (KO\textsuperscript{Mlc1}) was generated by crossing homozygous \textit{Tp53inp2} \textit{LoxP}+/+ mice with a mouse strain with Cre-recombinase expression under the control of the \textit{Myl1} (myosin, light polypeptide 1) promoter [19].

Mice were bred in a C57BL/6J genetic background, kept under a 12-h dark-light period, and provided with a standard chow-diet and water \textit{ad libitum}. When indicated, animal cages were placed inside a thermostatic enclosure at 30°C for 5 months, and/or were fed a high-fat diet (HFD) for the indicated period (60 kcal\% Fat, Research Diets Inc.).

Body composition analysis

Mouse body composition was measured using magnetic resonance with the EchoMRI\textsuperscript{TM} Body Composition Analyzer.

Histology

Interscapular BAT (iBAT) samples were fixed overnight in 4% PBS-buffered formalin (PanReac, 252931). Fixed samples were dehydrated and embedded in paraffin. iBAT
sections were stained with hematoxylin and eosin (H&E), and lipid droplet (LD) area and LD number were quantified with Ilastik software. For immunohistochemistry studies, iBAT sections were stained with the rabbit polyclonal anti-UCP1 (Abcam, ab10983) followed with the Alexa Fluor Plus 647 Goat anti-Rabbit IgG (H+L) (Invitrogen, A32733) as a marker of brown adipocytes. Wheat germ agglutinin (WGA), Alexa Fluor® 488 conjugate (Invitrogen, W11261) was used to label plasma membranes. Nucleus were stained with DAPI. Specificity of staining was confirmed by staining with a rabbit IgG isotype control (Abcam, ab37415). Fluorescent images were acquired with a NanoZoomer-2.0 HT C9600 digital scanner (Hamamatsu, Photonics, France) equipped with a 20X objective and coupled to a mercury lamp unit L11600-05 and using NDP.scan2.5 software U10074-03 (Hamamatsu, Photonics, France). DAPI signal of all the samples has been acquired with the filter DAPI350 with an exposure time of 13 ms and a gain of 6. WGA-488 signal of all the samples has been acquired with the FITC filter with an exposure time of 28 ms and a gain of 4. UCP1-647 signal of all the samples has been acquired with the Cy5 filter with an exposure time of 57 ms and a gain of 8. All images were visualized with the NDP.view 2 U123888-01 software (Hamamatsu, Photonics, France) with a gamma correction set at 1.0 in the image control panel of the software. Slides were evaluated in blind manner, using QuPAth software[50] for the UCP1-647 intensity with the “Add intensity features”, HALO® imaging analysis software (Indica Labs) software using the “Highplex IF v4.1.3” analysis for the nuclei count and the “DenseNet AI V2 (plugin)” classifier followed by the “Highplex IF v4.1.3” analysis independently trained for the detection of the adipocytes following the WAG labeling. The classifier algorithm was trained to segment the image between “adipocytes” and “rest of the tissue + background”. Selection of tissue was performed manually, and
tissue artefacts such as broken areas were excluded. Finally, the number of adipocytes was determined with a 25-1000 µm² cell size filter.

**Indirect calorimetry and thermogenesis assessment**

Oxygen consumption (VO₂), carbon dioxide production (VCO₂) and locomotor activity were measured using an indirect calorimetry system (Oxymax, Columbus Instrument). Energy expenditure (3.815*VO₂+1.232*VCO₂), VO₂ and VCO₂ were calculated with an adjusted body weight of 29.4714 g (KO Myf5) or 27.0625 g (KO Mlc1), determined using ANCOVA. The respiratory exchange ratio (RER, VCO₂/VO₂), glucose oxidation (4.545*VCO₂-3.205*VO₂), and lipid oxidation (1.672*(VO₂-VCO₂)) were calculated using the respective formulas.

Norepinephrine (NE)-induced maximal thermogenic capacity was assessed in mice that had been acclimated either to 22°C or to 30°C. The animals were anesthetized by an intraperitoneal injection of pentobarbital (80 mg/kg), maintained inside the 30°C enclosure, and placed inside the metabolic chamber to measure basal respiration. After 20 min, the cage was opened and mice were injected with a subcutaneous dose of NE (Sigma-Aldrich, A0937; 1 mg/kg) to measure NE-induced respiration. Data are presented as increase in oxygen consumption (ΔVO₂) from basal respiration. For statistical comparison of the curves (Figures 6D and 7J), we fitted a random slope mixed effects model separately for measures taken at 22°C and 30°C that take into consideration the longitudinal structure of the data. We considered the ΔVO₂ as the dependent variable. The time, genotype (LoxP or KO Myf5) and interaction of the two were taken as independent variables. The difference in time linear effect between KO Myf5 and LoxP was estimated by a REML procedure using the lm function from the nlme R package [51]. Although only time points between 56 min to 100 min were considered for this analysis,
the results obtained were consistent for other starting times. Diet-induced maximal thermogenic capacity was measured with the same methodology, using mice that were acclimated to 30°C and fed either a CD or a HFD.

**Glucose and insulin tolerance tests**

Intraperitoneal GTT or ITT were performed in male and female mice at 3 months of age housed at 22°C. For the GTT, mice were fasted 16 h before the administration of 2 g/kg glucose dose. Blood glucose levels were measured from time 0 to 150 min after glucose injection. At determined points, blood was also collected with Microvette® tubes (Starstedt, 16444), for the posterior determination of plasma insulin levels using an Ultra Sensitive Mouse Insulin ELISA kit (Cristal Chem, 90080) following manufacturer instructions. ITT was performed after 4 h of fasting with an insulin (Lilly, Humalog® 100 U/ml) dose of 0.7 U/kg. Blood glucose levels were monitored as for the GTT.

**Food and water intake**

Mice were placed individually in metabolic cages and allowed to acclimate to the new environment for 48 h. Food and water intake measurements per mouse were collected every 24 h over two consecutive days.

**High-resolution respirometry**

The respiration of permeabilized muscle fibers and BAT mitochondria was measured at 37°C in MiR05 buffer by high-resolution respirometry with the Oxygraph-2k (Oroboros Instruments, Innsbruck, Austria). iBAT was homogenized in a sucrose buffer (250 mM sucrose [Sigma-Aldrich, 84100], 50 mM KCl, 5 mM EDTA, 5 mM MgCl₂, 1 mM Na₄P₂O₇, pH 7.4) and centrifuged at 740g for 5 min. The supernatant was centrifuged at 9,000g for 15 min and the mitochondrial pellet was washed and resuspended in sucrose
buffer. Tibialis anterior fibers were prepared and permeabilized as previously described [52]. All respiration measurements were made on fresh tissues immediately after dissection, and each sample was assayed in duplicate. Oxygen flux (shown as “Leak” in figures) was measured with 2 mM malate (Sigma-Aldrich, M1000) and 10 mM glutamate (Santa Cruz Biotechnology, sc-211703), in the absence of ADP (Sigma-Aldrich, A5285). Complex I-derived mitochondrial respiration (denoted as “C I” in figures) was measured with the addition of ADP (1 mM for iBAT mitochondria and 2.5 mM for permeabilized fibers). Finally, succinate (Sigma-Aldrich, S2378) was added to a final concentration of 10 mM to measure electron flow through both complex I and II (denoted as “C I+II” in the figures). Non-mitochondrial respiration levels were subtracted and determined by the addition of 2.5 μM antimycin A (Sigma-Aldrich, A8674). In order to ensure the integrity of the outer mitochondrial membrane, 10 μM CYCS/cytochrome c (Sigma-Aldrich, C2037) was added, and no stimulation of respiration was observed.

**DNA and RNA extraction and qPCR**

Total DNA was extracted from mouse iBAT. Tissue was digested with proteinase K (Merck Life Science S.L.U., 03115801001) and total DNA was purified using DNeasy Blood & Tissue Kit (Qiagen, 69504).

Total RNA was extracted from mouse tissues or mouse cell cultures by homogenization with TRIzol reagent (Thermo Fisher Scientific, 15596018) and purification with PureLink™ RNA Mini Kit (Thermo Fisher Scientific, 12183018A). An amount of 2 μg of RNA was reverse-transcribed with the SuperScript™ II RT kit (Thermo Fisher Scientific, 18064014). Gene expression was analyzed by quantitative real-time PCR performed using Power SYBR™ Green PCR Master Mix (Thermo Fisher Scientific, 4367659) and ABI Prism 7900 HT real-time PCR system (Applied Biosystems).
*Arpc/Arp* was used as internal control for normalization. The primers used for qPCR are listed in Table S1.

Human Paz6 cells were lysed in RLT buffer containing 1% β-ME. RNA extraction was carried out using RNeasy Qiagen Kit (Qiagen, 74004) following manufacturer’s instructions. RNA concentration was quantified using an Epoch™ 2 microplate reader. cDNA was generated from 1 µg of isolated RNA using M-MLV reverse transcriptase (Promega, M1701) and diluted 1:20 for use in 15 µL qPCR reactions using SYBR® Green PCR master mix (Applied Bioscience, 4344463) and run on the Applied Biosystems StepOnePlus™ system. The geometrical average of 4 different genes (*GAPDH, ACTB/β-actin, HPRT* and *TBP*) was used as an internal control, following an already described normalization method [53].

**Transcriptomic analysis**

Microarray services were provided by the IRB Barcelona Functional Genomics and Biostatistics/Bioinformatics Core Facilities, as described [20], including quality control tests of total RNA using an Agilent Bioanalyzer and nanodrop spectrophotometry. Briefly, the complementary DNA library was prepared and amplified from 25 ng total RNA using the TransPlex® Complete Whole Transcriptome Amplification Kit (Sigma-Aldrich, WTA2) with 17 cycles of amplification. cDNA (8 µg) was subsequently fragmented by DNaseI and biotinylated by terminal transferase obtained from the GeneChip Mapping 250K Nsp Assay Kit (Affymetrix, 900766). The hybridization mixture was prepared following Affymetrix’s protocol. Each sample was hybridized to a Mouse Genome 430 PM strip (Affymetrix, 901570). Arrays were washed and stained in a Fluidics Station 450 (Fluidics protocol FS450_002) and scanned in a GeneChip Scanner
3000 (both Affymetrix), following the manufacturer’s recommendations. CEL files were generated from DAT files using GCOS software (Affymetrix).

Microarray samples were processed using the affy [54] and affyplm packages [55] from Bioconductor [54]. Raw CEL files were normalized using RMA background correction and summarization [56]. Technical metrics PM median, PM interquartile range (IQR), RMA IQR and RNA degradation described in [57] were computed and recorded as additional features for each sample. Differential expression between tp53inp2 KO vs. LoxP conditions was determined using the moderated t-statistics by empirical Bayes shrinkage method [58]. Batches representing the strip and Eklund metric RNA degradation were included as adjusting variables in the model to correct for technical variability. The moderated t-statistic information (positive change when tp53inp2 KO was higher than LoxP and negative change when tp53inp2 KO was lower than LoxP) was considered to rank all genes in the genome, and Gene set enrichment analysis (GSEA) was performed using the Broad Institute's implementation [59] on the KEGG (Kyoto Encyclopedia of Genes and Genomes) collection [60].

We downloaded paired-end RNA-seq data from the GSE86338 study [27]. Only samples GSM 2300503 (22°C 1), GSM 2300504 (22°C 2), GSM 2300505 (30°C 1), and GSM 2300506 (30°C 2) were considered for normalization and data analysis. Files were aligned against the mm10 genome with STAR 2.3.0e in strand-specific paired-end mode with default parameters [61]. Alignments were sorted and indexed with sambamba v0.5.1 [62]. Counts per genomic feature were computed with the R package casper [63] function wrapKnown. A quantile normalization was applied to the resulting rpkm expression matrix. Differential expression between 30°C vs. 22°C conditions was analyzed on the normalized data using the moderated t-statistics by the empirical Bayes shrinkage method...
A size factor that measured the total number of reads per sample was included in the model as an adjusting variable.

We compared the differential expression results obtained from the two transcriptomic analyses, the 30°C vs 22°C and \textit{tp53inp2} KO vs LoxP contrasts, by performing a GSEA (33). We used as background ranked list the moderated t-statistics of the 30°C/22°C contrast and as testing gene-sets two signatures of 100 genes containing the most KO/LoxP differentially expressed genes (ordered by the t-statistic), distinguishing between up- and downregulated genes.

**Cell culture**

Primary brown preadipocytes were isolated from iBAT as previously described [22]. Briefly, 6-8 iBAT depots from 1-month-old \textit{Tp53inp2} \textit{LoxP}+/+ or \textit{Tp53inp2} \textit{LoxP}+/+ \textit{UBC-Cre-ERT2} mice were minced and digested in collagenase buffer at 37°C for 30-40 min (1 mg/ml collagenase A [Sigma-Aldrich, 10103578001], 4% BSA fraction V [Sigma-Aldrich, A9647] and penicillin-streptomycin 100 U/ml in HBSS medium [Thermo Fisher Scientific, 14025050]). Stromal vascular fraction cells were seeded in primary culture medium (DMEM: Hams F12 (1:1) [Thermo Fisher Scientific, 11320074], 10% FCS, 20 mM HEPES, pH 7.4 and penicillin-streptomycin 100 U/ml) and immortalized through retroviral expression of SV40 large-T antigen (Addgene, 13970; deposited by Thomas Roberts). Control (C) and \textit{tp53inp2} KO preadipocytes were generated by GFP or Cre-GFP adenoviral infection, respectively, and sorted by flow cytometry against GFP fluorescence. Parallel cultures of immortalized brown preadipocytes were also used to generate TP53INP2 deficiency model. Lentivirus encoding for a siRNA scramble or against \textit{Tp53inp2}, as control (SCR) or knockdown (KD), were produced in HEK 293T cells (ATCC, CRL-11268) as reported [18]. Transduced cells were selected based on GFP
fluorescence and sorted with FACS Aria Fusion flow cytometer. Acute TP53INP2 repression was induced by transfection with siRNAs using Lipofectamine 3000 (Thermo Fisher Scientific, L3000015). Transfections included 15 nM of MISSION siRNA Universal Negative Control #1 (Sigma-Aldrich, SIC001), siTp53inp2 #1 (Sigma-Aldrich, SASI_Mm01_00032790) or siTp53inp2 #2 (Sigma-Aldrich, SASI_Mm02_00348576).

HA-PPARG2-overexpressing brown preadipocytes were generated by retroviral infection with pBABE-empty-Puro (Addgene, 1764; deposited by Hartmut Land, Jay Morgenstern and Bob Weinberg) or pBABE-PPARG2-Puro (Addgene, 1764; deposited by Bruce Spiegelman) and puromycin selection (Santa Cruz Biotechnology, sc-108071; 3 μg/ml). TP53INP2 was ablated in these cells following the same methodology described above.

TP53INP2 or TP53INP2W35,I38A mutant (TP53INP2-LIR) [17, 18] were subcloned into a lentiviral Gateway destination vector, and empty vector was used as control (Addgene; 19067 and 17478, deposited by Eric Campeau and Paul Kaufman). Lentiviral particles were produced in HEK 293T cells and used to infect brown preadipocytes. Infected cells were selected with neomycin (Thermo Fisher Scientific, 10131-027; 400 μg/ml).

Cells were maintained in proliferation medium (DMEM, 10% FBS, 20 mM HEPES, pH 7.4 and penicillin-streptomycin 100 U/ml). Differentiation was achieved by growing cells to confluence for 3 days in differentiation medium (proliferation medium supplemented with 20 nM insulin and 1 nM T3 [Sigma-Aldrich, I1882 and T6397]). Subsequently, differentiation was triggered by induction medium (day 0) (differentiation medium supplemented with 0.5 μM dexamethasone, 0.125 mM indomethacin and 0.5 mM IBMX [Sigma-Aldrich, D2915, I8280 and I7018]) for 48 h. Cells were then switched again to differentiation medium for 7 days. When indicated, adipocytes were treated with 1 μM CL-316,243 (Sigma-Aldrich, C5976), 1 μM 4-hydroxy-tamoxifen (Sigma-Aldrich,
Autophagy flux was assessed upon 200 nM bafilomycin A1 (Santa Cruz Biotechnology, sc-201550) treatment for 4 or 6 h.

The human immortalized brown cell line PaZ6 was provided by A Vidal-Puig and was cultured and differentiated as previously described [64]. When incubated with different compounds i.e 8-Br cAMP, Forskolin and dcAMP (Sigma-Aldrich, B7880, F3917 and D0260), the mature adipocytes were kept overnight in differentiation medium without serum and then treated with the different agents at the concentration of $10^{-5}$ M for 4 h. Then the cells were harvested for RNA or protein extraction.

**Monoclonal antibody production**

BALB/c mice were immunized, three times, by intraperitoneal injection with the purified peptide (40 μg) SPPAPSLMDESWFVTPPAC covalently linked to KLH, corresponding to human TP53INP2 (residues 60-78). Hybridomas were produced by fusing spleen cells with myeloma cells using polyethylene glycol in mHAT+HFCS-RPMI medium and following standard hybridoma techniques. Supernatants of the hybridomas were screened by indirect ELISA on polystyrene plates coated with the peptide. Bound antibodies were detected using horseradish peroxidase-labelled goat anti-mouse antibody (Dako, P 0447). The positive hybridomas were subcloned by limiting dilution, and the specificity of antibodies was validated by western blot assays.

**Protein extraction and western blot**

Total homogenates were extracted with RIPA buffer (50 mM Tris-HCl pH 8.0, 150 mM NaCl, 1% NP-40 [Sigma-Aldrich, I3021], 0.1% SDS and 1 mM EDTA) or lysis buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1% Triton X-100 and 1 mM EDTA) from cell
cultures or iBAT respectively, with freshly added phosphatase inhibitors 1 mM Na$_3$VO$_4$, 5 mM Na$_4$P$_2$O$_7$ and 50 mM NaF, and protease inhibitor cocktail (Complete-Mini; Roche, 11836153001). After centrifugation at 16,000g for 20 min at 4°C, supernatants were quantified by the BCA protein Assay (Thermo Fisher Scientific, 23225). The same amount of proteins was resolved in acrylamide gels for SDS-PAGE and transferred to PVDF membranes (Millipore, IPFL00010). The following primary antibodies were used: PPARG (H-100) and LMNA/lamin A/C (N-18) (Santa Cruz Biotechnology, sc-7196 and sc-6215); UCP1 and MFN2 (Abcam, ab10983 and ab56889); TIMM44 (BD Biosciences, 612582); and NCOR1, LC3B and HA-tag (Cell Signaling Technology, 5448, 2775 and 3724). The TP53INP2 antibody was generated in our laboratory as described. TUBA4A/α-tubulin and ACTB/β-actin (Sigma-Aldrich, T5168 and A1978), and TBP or VCL/vinculin (Abcam, ab818 and ab18058) antibodies or Revert™ 700 Total Protein Stain (LI-COR, 926-11011) were used as loading controls. Proteins were detected by the ECL method and quantified by densitometry using ImageJ, or by NIR-fluorescence detected with LI-COR Odyssey System and measured with Image Studio software.

**NE-induced respiration in cultured brown adipocytes**

Control and tp53inp2 KO brown preadipocytes were plated in SeaHorse Bioscience XF24 plates (3,000 cells/well) and induced to differentiate as described above. Mitochondrial respiration was evaluated at day 9 of differentiation. Cells were switched to respirometry medium containing DMEM (Sigma-Aldrich, D5030) supplemented with 1 mM glutamine, 2 mM pyruvate and 5 mM glucose. Oxygen consumption rate was detected under basal and NE-stimulated conditions (1 μM). Non-mitochondrial oxygen consumption was subtracted and measured by adding 2.5 μM rotenone (Sigma-Aldrich, R8875) and 2.5 μM antimycin A. Data are shown as NE-induced mitochondrial respiration.
**PPAR transcriptional activity**

For transcriptional activity assays brown preadipocytes were transfected with PEI MAX 40K (Polysciences Inc., 24765) or with Lipofectamine 3000 when the reactions contained siRNA. Transfections included 1 μg of the reporter plasmid PPREx3-TK-Luc (kindly provided by Dr. D. Haro, Institute of Biomedicine of the University of Barcelona [IBUB], Barcelona, Spain) and, when indicated, 500 ng of PPARG. siRNAs used, MISSION siRNA Universal Negative Control #1 (Sigma-Aldrich, SIC001) or siNcor1 (Sigma-Aldrich, SASI_Mm01_00098740), were transfected with a final concentration of 15 nM. To normalize for transfection efficiency, 100 ng of TK-Renila plasmid (Promega, E2261) was used. Twenty-four hours after transfection, cells were treated, when indicated, with 10 μM rosiglitazone (Sigma-Aldrich, R2408) for an additional 24 h. Extracts were obtained and assays of dual-luciferase reporter were performed (Promega, E2920).

**Immunofluorescence**

Cells were fixed in 2% paraformaldehyde (PFA) for 3 min and then switched to 4% PFA for a total of 30 min. Permeabilization was performed in 0.1% Triton X-100 in PBS for 15 min and then autofluorescence was blocked using TrueBlack (Biotium, 23007). Coverslips were washed, blocked with 5% FBS for 1 h and then incubated in NCOR1 (dilution 1:200 in blocking buffer; Cell Signaling Technology, 5448) and/or TP53INP2 (dilution 1:250 in blocking buffer) primary antibodies overnight at 4°C. Cells were then washed with PBS and incubated in Alexa Fluor 568 anti-rabbit (dilution 1:400 in blocking buffer; Invitrogen, A11036) or Alexa Fluor 488 anti-mouse (1:500 in blocking buffer; Invitrogen, A11029) secondary antibodies for 1 h. Cells were washed with PBS and nucleus were stained with Hoechst 33342 (Invitrogen, H3570). Coverslips were washed once again and mounted on microscope slides with Fluoromount (Sigma-Aldrich, F4680).
Confocal images were obtained using Leica TCS SP5 confocal scanning microscope. Z-stacks were acquired with a constant thickness of 0.5 µm. Nuclear to cytosol (N:C) ratio was determined as described [65] using ImageJ software. Briefly, the central section of each cell was used for quantification. Hoechst 33342 staining was used to determine the nucleus perimeter, and GFP fluorescence of the siRNA lentiviral construct to determine the cell perimeter. N:C ratio was calculated dividing the mean intensity of each compartment.

Subcellular fractionation

Nuclear and cytosolic enriched fractions from brown preadipocytes were obtained using NE-PER kit (Thermo Fisher Scientific, 78835) following the manufacturer’s recommendations.

Immunoprecipitation

Protein extracts from Flp-In T-REx 293 cells (Thermo Fisher Scientific, R78007) stably expressing empty vector or HA-TP53INP2 were used for immunoprecipitation experiments. Cells were lysed in IP Buffer (20 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.5% NP-40, 1 mM EDTA, 1mM DTT) and centrifuged at 16,000 g at 4°C for 20 min. HA affinity isolation was performed incubating protein supernatants with HA-conjugated agarose beads (Sigma-Aldrich, A2095) overnight at 4°C with rotation. Then, beads were washed five times with one volume each of IP Buffer and finally eluted in Laemmli sample buffer at 95°C for 5 min. Endogenous NCOR1 immunoprecipitation was carried out with 10 µg of mouse NCOR1 (F-1) antibody (Santa Cruz Biotechnology, sc-515934), or normal mouse IgM (Santa Cruz Biotechnology, sc-3881) as a negative control. Corresponding antibodies were conjugated to Protein L magnetic beads (Thermo Fisher Scientific, 88849) for 2 h at room temperature. Five mg of protein extracts were
precleared with IgM-conjugated Protein L magnetic beads overnight prior to
immunoprecipitation. Beads were incubated with precleared lysates, washed and eluted
in the same way as HA-beads. Input fractions and immunoprecipitated proteins were run
in 4–15% gradient SDS-PAGE gels (Bio-Rad, 4568084), and immunodetection was
performed as described.

Proximity ligation assay

Proximity ligation assays were performed in brown preadipocytes stably expressing
TP53INP2, TP53INP2-LIR or empty vector cultured in 96-well imaging plates (Zell-
Kontakt, 5242-20) according to the manufacturer’s instructions (Duolink® In Situ
Detection Reagents FarRed; Sigma-Aldrich, DUO92013). Briefly, cells were fixed with
4% PFA, permeabilized with 0.2% Triton X-100 in PBS for 20 min at room temperature
and blocked for 30 min at 37°C. Then, cells were incubated with NCOR1 (dilution 1:200;
Cell Signaling Technology, 5448) and TP53INP2 (dilution 1:250) primary antibodies
overnight at 4°C. After that, cells were washed and incubated with PLA probes anti-rabbit
PLUS (Sigma-Aldrich, DUO092002) and anti-mouse MINUS (Sigma-Aldrich,
DUO092004) for 1 h at 37°C. Ligation and amplification were performed for 30 and 100
min respectively at 37°C. Finally, cells were stained with Hoechst 33342 (Invitrogen,
H3570) for 10 min and kept in citrate buffer (150 mM NaCl, 15 mM sodium citrate, pH
7.4). NIKON LIPSI, a high content and high-speed screening platform, equipped with an
Eclipse Ti2 inverted microscope, a Yokogawa W1 confocal spinning disk unit, a Prior
stage, and two Prime BSI Photometrics sCMOS cameras was used to scan PLA
experiments. In every well, at least at 20 fields of view 4 µm deep z-stacks were taken
with the Apo LWD 40x water lens of 1.15 NA. The two cameras were used to scan
simultaneously Hoechst and Far Red with the spinning disk unit and the 405 and 638
lasers. To analyze the number of PLA dots, NIS Elements AR 5.30.05 software was used.
First, z-stacks were maximum intensity projected and the total PLA dots were identified using a bright spot detection method and counted. Nuclei images were processed with Rolling ball background subtraction, thresholded and watersheded.

**Study approval**

The animal studies followed established guidelines. This project was approved by the Institutional Animal Care and Use Committee of the Parc Científic de Barcelona (IACUC-PCB), which considered that it complied with standard ethical regulations and met the requirements of current applicable legislation (RD 53/2013 Council Directive; 2010/63/UE; Order 214/1997/GC).

**Statistical analysis**

Data are presented as means ± SEM. Statistical analysis of the data presented was performed using the Student t-test. Statistical analysis was conducted only to data sets with an n ≥ 3 independent experiments. Calorimetry data was analyzed using analysis of covariance (ANCOVA).
Acknowledgments

We thank Jorge Manuel Seco, Vanesa Hernández and Laura Alcaide for technological assistance, and Neus Prats, Mònica Aguilera and the Histopathology Core Facility (IRB Barcelona). We also thank the Mouse Mutant and the Functional Genomics Core Facilities (IRB Barcelona). We also want to acknowledge Adrià Caballe and the Biostatistics/Bioinformatics Core Facility (IRB Barcelona) for the analysis of transcriptomic data. We are grateful to Nikolaos Giakoumakis, Anna Lladó and the Advanced Digital Microscopy Core Facility (IRB Barcelona) for help with confocal microscopy and in particular for PLA imaging. A.S. was recipient of a pre-doctoral fellowship from the University of Barcelona. This study was supported by grants from the MINECO (SAF2016-75246R), the Generalitat de Catalunya (Grants 2017 SGR 1015, ICREA Acadèmia), INFLAMES (PIE-14/00045, ISCIII), CIBERDEM, ISCIII, Fundación Ramón Areces (CIVP18A3942), the Fundación BBVA and the Fundació la Marató TV3 (201634-30; 20132330). We gratefully acknowledge institutional funding from the MINECO through the Centres of Excellence Severo Ochoa Award, and from the CERCA Programme of the Generalitat de Catalunya.
References


peroxisome proliferator-activated receptor (P465L PPARgamma) function.

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Figure legends

Figure 1. TP53INP2-dependent autophagy induces brown fat differentiation and maturation. (A) LC3B protein abundance and (B) LC3B-II fold accumulation in control (C) and *tp53inp2* knockout (KO) mouse brown preadipocytes treated with vehicle (-) or with bafilomycin A1 (BAF) (+) 200 nM for 6 h (n=3). (C) LC3B protein abundance and (D) LC3B-II fold accumulation in mouse brown preadipocytes transfected with siRNA control (siSCR) or with different siRNAs targeting *Tp53inp2* (si#1 and si#2) and treated with vehicle (-) or with bafilomycin A1 (BAF) (+) 200 nM for 4 h (n=5). Panels (E) to (G): C and KO mouse brown preadipocytes. (E) Optical microscopy images at day 9 of differentiation. (F) PPARG, UCP1, TIMM44 and MFN2 protein abundance during differentiation (n=3–6). (G) Relative mRNA levels of adipogenic and thermogenic genes at day 9 of differentiation (n=4–5). (H) LC3B, PPARG and UCP1 protein abundance in adipocytes treated for the last 3 or 7 days of differentiation with 15 μM chloroquine (CQ). Panels (I) to (L): control (LoxP) and KO*Myf5* male or female mice at 3 months of age housed at 22°C and subjected to a chow diet (n=7–9 LoxP or KO*Myf5* mice). (I) Weight of iBAT. (J) Hematoxylin-eosin (H&E) staining of iBAT sections. (K) iBAT sections stained with DAPI (blue), wheat germ agglutinin (WGA, green) and UCP1 (red). (L) Expression of adipogenic and thermogenic genes in iBAT. Data are mean ± SEM. *p<0.05 vs. control group. Scale bar: 100 μm (H&E images) or 50 μm (DAPI, WGA, UCP1 images).

Figure 2. TP53INP2 maintains the differentiation state of brown adipocytes. Expression of genes in brown adipocytes from (A) *tp53inp2 LoxP*+/ Ubc-Cre-ERT2 mice or from (B) *Tp53inp2 LoxP*+/ Cre-negative mice treated with vehicle or with 4-hydroxy-
tamoxifen for 3 days (n=3). Panels (C) to (I): control (LoxP) and KO\textsuperscript{Ubc} male mice at 8 months of age housed at 22°C and subjected to a chow diet (n=4–8 LoxP or KO\textsuperscript{Ubc} mice).

(C) Weight of iBAT. (D) Hematoxylin-eosin (H&E) staining of iBAT sections, (E) lipid droplet (LD) number and (F) LD average area measurements. (G) iBAT sections stained with DAPI (blue), wheat germ agglutinin (WGA, green) and UCP1 (red), (H) number of adipocytes per surface unit and (I) adipocyte size distribution measurements. (J) Expression of adipogenic and thermogenic genes in iBAT from 4-month-old LoxP and KO\textsuperscript{Ubc} male mice housed at 22°C and subjected to a chow diet (n=4–7 LoxP or KO\textsuperscript{Ubc} mice). Data are mean ± SEM. *p< 0.05 vs. control group. Scale bar: 100 μm (H&E images) or 50 μm (DAPI, WGA, UCP1 images).

**Figure 3.** TP53INP2 stimulates PPARG activity. Panels (A) to (C): transcriptomic analysis performed in iBAT from LoxP (n=4) or KO\textsuperscript{Myf5} mice (n=4) and compared to transcriptomic analysis from WT mice housed at 22°C (n=2) or 30°C (n=2) in iBAT samples. (A) Number of genes upregulated or downregulated (hypergeometric test p-val<0.001). Enrichment plot (GSEA) of the gene sets composed by the (B) 100 top up- and (C) downregulated genes in iBAT from KO\textsuperscript{Myf5} mice compared to the LoxP group using as background ranked list the 30°C /22°C test statistics (p-val<0.001). GSEA of the most significantly downregulated pathways from Broad Hallmarks by tp53inp2 ablation in iBAT from (D) KO\textsuperscript{Myf5} or (E) KO\textsuperscript{Ubc} mice. (F) GSEA of PPAR signaling pathway from Kyoto Encyclopedia of Genes and Genomes (KEGG) in the LoxP vs KO\textsuperscript{Myf5} transcriptomic analysis (p-val<0.001). (G) PPRE reporter activity in control (SCR) or tp53inp2 knockdown (KD) brown preadipocytes transfected with empty vector (-) or PPARG (+) and treated with vehicle (-) or with rosiglitazone (+) 1 μM for 24 h (n=5). (H) PPRE reporter activity in brown preadipocytes stably expressing empty vector (-) or
HA-PPARG (+), with TP53INP2 endogenous levels (C) or tp53inp2 knockout (KO) treated with vehicle (-) or with rosiglitazone (+) 1 μM for 24 h (n=3). Data are mean ± SEM. *p<0.05 vs. control group.

**Figure 4.** TP53INP2 promotes NCOR1 degradation through autophagy. (A) NCOR1, LC3B and TP53INP2 protein abundance, (B) NCOR1 and (C) LC3B-II fold accumulation in control (SCR) or tp53inp2 knockdown (KD) brown preadipocytes in basal or upon treatment with 200 nM bafilomycin A1 (BAF) for 4 h (n=9, 7 and 9 respectively). (D) NCOR1, LC3B and TP53INP2 protein abundance, (E) NCOR1 and (F) LC3B-II fold accumulation in control (Empty), TP53INP2 or TP53INP2-LIR stably overexpressing brown preadipocytes in basal (-) or upon treatment with BAF (+) for 4 h (n=4 and 6 respectively). (G) NCOR1, LC3B and TP53INP2 protein abundance in day 4 adipocytes transfected at day 2 with siRNA control (SCR) or against Tp53inp2 in basal (-) or upon treatment with BAF (+) for the last 6 h (n=5, 6 and 6 respectively). (H) NCOR1 protein abundance and (I) quantification in iBAT from LoxP or KOMyf5 male mice at 3 months of age housed at 22ºC and subjected to a chow diet (n=4–7 LoxP or KOMyf5 mice).

**Figure 5.** TP53INP2 promotes the cytosolic recruitment of NCOR1 in an autophagy-dependent manner. Panels (A) to (D): Control (SCR) or tp53inp2 knockdown (KD) brown preadipocytes. (A) NCOR1 immunostaining and (B) nuclear:cytosolic ratio quantification (n=3, each experiment is the average of 17–65 cells). (C) NCOR1 protein abundance in total, cytosolic and nuclear homogenates, and (D) nuclear NCOR1 quantification (n=5). Panels (E) and (F): HEK cells stably expressing empty vector (-) or...
HA-TP53INP2 (+). (E) HA affinity isolation and NCOR1, TP53INP2 and VCL immunoblot in input and pull-down fractions (n=7). (F) NCOR1 immunoprecipitation and NCOR1, TP53INP2 and VCL immunoblot in input and immunoprecipitated fractions (n=3). (G) Proximity ligation assay (PLA) and nuclei staining (DAPI), and (H) PLA dots by nucleus quantification in control (Empty), TP53INP2 or TP53INP2-LIR stably overexpressing brown preadipocytes (representative experiment of n=3 independent experiments). (I) PPRE reporter activity in cells transfected with control (siCtr) or Ncor1 (siNcor1) siRNA and with empty vector (-) or PPARG (+), and treated with vehicle (-) or with rosiglitazone (+) 1 μM for 24 h (n=4). Data are mean ± SEM. *p<0.05 vs. control group. #p<0.05 vs. siCtr. Scale bar: 10 μm.

Figure 6. TP53INP2 induces non-shivering adaptive thermogenesis. Panels (A) to (C): control (LoxP) and KO^Myf5^ male mice at 3 months of age housed at 22°C and subjected to a chow diet (n=7 LoxP or KO^Myf5^ mice). (A) Energy expenditure, (B) oxygen consumption (VO\(_2\)) and (C) carbon dioxide production (VCO\(_2\)) plotted against body weight. (D) Oxygen consumption increase (ΔVO\(_2\)) upon norepinephrine injection (NE) and (E) area under the curve quantification in anesthetized mice at 30°C. LoxP or KO^Myf5^ mice were acclimatized to the indicated temperatures for 2 months before performing the experiment (n=7–11 LoxP or KO^Myf5^ mice at 22°C and n=5–9 LoxP or KO^Myf5^ mice at 30°C). (F) High-resolution respirometry in iBAT isolated mitochondria (n=5–6 LoxP or KO^Myf5^ mice). Panels (G) to (L): LoxP and KO^Myf5^ male or female mice at 6 months of age housed at 22°C and subjected to a chow diet (n=4–9 LoxP or KO^Myf5^ mice). (G) Body weight. (H) Weight of iBAT. (I) Body weight gain and (J) fat mass gain from 3 to 6 months of age. (K) iBAT sections stained with DAPI (blue), wheat germ agglutinin
(WGA, green) and UCP1 (red). (L) UCP1 protein abundance in iBAT. Data are mean ± SEM. *p<0.05 vs. control group. Scale bar: 50 μm.

**Figure 7.** BAT-specific diet induced thermogenesis is impaired by *tp53inp2* ablation.
Panels (A) to (I): control (LoxP) and KO*Myf5* male mice at 6 months of age housed at 30°C for 5 months and subjected to a chow diet (CD) (n=8–12 LoxP or KO*Myf5* mice) or to a high-fat diet (HFD) (n=8–10 LoxP or KO*Myf5* mice). (A) Body weight. (B) Total fat mass. (C) Weight of iBAT. (D) Weight of ingWAT. (E) Weight of pgWAT. (F) Total lean mass. (G) Hematoxylin-eosin staining of iBAT sections, (H) lipid droplet (LD) number and (I) LD average area measurements. (J) Oxygen consumption increase (ΔVO2) upon norepinephrine injection (NE) and (K) area under the curve quantification in anesthetized mice at 30°C. LoxP or KO*Myf5* mice were acclimated to 30°C and to the indicated diet for 2 months before performing the experiment (n=5–9 LoxP or KO*Myf5* mice subjected to a CD and n=7–9 LoxP or KO*Myf5* mice subjected to a HFD). Data are mean ± SEM. *p<0.05 vs. LoxP control group. Scale bar: 100 μm.

**Figure 8.** TP53INP2 expression in brown adipose tissue is modulated by thermogenesis.
(A) *Tp53inp2*, *Ucp1*, *Ppargc1a* and *Prdm16* mRNA levels (n=4–6), (B) TP53INP2 and UCP1 protein abundance and (C) quantification (n=5–6) in iBAT from control mice subjected to a chow diet (CD) or a high-fat diet (HFD) for 16 weeks. Panels (D) to (G): control (LoxP) and KO*Myf5* male mice housed at 22°C and subjected to a HFD for a total of 16 weeks (n=6–10 LoxP or KO*Myf5* mice). (D) Body weight. (E) Body weight gain. (F) Total fat mass. (G) Fat mass gain from 8 to 16 weeks of HFD. (H) *Tp53inp2*, *Ucp1*, *Ppargc1a* and *Prdm16* mRNA levels in iBAT from control mice housed at 22°C or 4°C.
for 10 h (n=4–5). (I) Tp53inp2, Ucp1, Ppargc1a and Prdm16 mRNA levels in iBAT from control mice housed at 22°C or at 30°C for 5 months (n=5–6). (J) TP53INP2 protein abundance in human PAZ6 preadipocytes (Pre) or differentiated adipocytes (Ad) (n=3).

(K) Tp53inp2, Ucp1, Ppargc1a and Prdm16 mRNA levels in mature human PAZ6 brown adipocytes treated with vehicle (PBS), with 8-bromo-cAMP (8Br), with forskolin (FSK) or with dibutyryl-cAMP (dcAMP) for 4 h (n=3). (L) TP53INP2 protein abundance in mature human PAZ6 brown adipocytes treated with PBS, 8Br or FSK for 4 h (n=3). Data are mean ± SEM. *p<0.05 vs. control group in each case.
SUPPLEMENTARY DATA

Autophagy-mediated NCOR1 degradation is required for brown fat maturation and thermogenesis

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Table S1. List of primers used for qPCR.

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Figure S1

A. Relative mRNA levels of Tp53inp2 in Brown preadipocytes. C: Control, KO: Knockout.

B. Western blot analysis showing the expression of TP53INP2 and TUBA4A in Brown preadipocytes.

C. BAF-induced TP53INP2 (fold).

D. Relative protein levels of PPARG2 to control day 9.

E. Relative protein levels of PPARG1 to control day 9.

F. Relative protein levels of UCP1 to control day 9.

G. Relative protein levels of TIMM44 to control day 9.

H. Relative protein levels of MFN2 to control day 9.

I. Relative mRNA levels of Tp53inp2 in Brown preadipocytes.

J. Western blot analysis showing the expression of TP53INP2 and VCL in Brown preadipocytes.

K. Adipocytes - Day 9: SCR and KD.

L. Western blot analysis showing the expression of PPARG, UCP1, TP53INP2, and TUBA4A in Adipocytes - Day 9.

M. Relative protein levels of Adipocytes - Day 9 (% of control).

N. Relative protein levels of LC3B-II.

O. Relative protein levels of PPARG2.

P. Relative protein levels of PPARG1.

Q. Relative protein levels of UCP1.
**Figure S1.** TP53INP2 is a positive regulator of brown adipogenesis. (A) \( Tp53inp2 \) mRNA levels (n=4) and (B) TP53INP2 protein abundance (n=3) in control (C) or \( tp53inp2 \) knockout (KO) brown preadipocytes. (C) TP53INP2 fold accumulation in mouse brown preadipocytes transfected with siRNA control (siSCR) or with different siRNAs targeting \( Tp53inp2 \) (si#1 and si#2) treated with vehicle or with 200 nM bafilomycin A1 (BAF) for 4 h (n=4). (D) PPARG2, (E) PPARG1, (F) UCP1, (G) TIMM44 and (H) MFN2 protein quantification during differentiation of C or KO brown preadipocytes (n=3–6). Panels (I) to (M): control (SCR) or \( tp53inp2 \) knockdown (KD) brown preadipocytes. (I) \( Tp53inp2 \) mRNA levels (n=4). (J) TP53INP2 protein abundance (n=8). (K) Optical microscopy images at day 9 of differentiation. (L) PPARG, UCP1 and TP53INP2 protein abundance and (M) quantification at day 9 of differentiation (n=5). (N) LC3B-II, (O) PPARG2, (P) PPARG1 and (Q) UCP1 quantification in adipocytes treated for the last 3 or 7 days of differentiation with 15 \( \mu M \) chloroquine (CQ). Data are mean ± SEM. *p<0.05 vs. control group. Scale bar: 100 \( \mu m \).
Figure S2

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Figure S2. TP53INP2 loss of function alters BAT identity. Panels (A) to (R): control (LoxP) and KO\(^{Myf5}\) male or female mice at 3 months of age housed at 22°C and subjected to a chow diet (n=7–9 LoxP or KO\(^{Myf5}\) mice). (A) \(Tp53inp2\) mRNA levels in iBAT, quadriceps muscle (Quad), ingWAT and pgWAT. (B) TP53INP2 protein abundance in iBAT. (C) Body weight. (D) Total fat mass. (E) Total lean mass. Weight of Quad, gastrocnemius muscle (Gast), tibialis muscle (Tib) and liver in (F) female and (G) male mice. (H) Hematoxylin-eosin staining of iBAT sections, (I) lipid droplet (LD) number and (J) LD average area measurements. (K) Weight of ingWAT. (L) Weight of pgWAT. (M) Total DNA amount per BAT depot. (N) UCP1 mean intensity, (O) adipocyte size distribution, (P) number of nuclei per surface unit and (Q) number of adipocytes per surface unit measurements in iBAT sections. (R) \(Dlk1\) mRNA levels in iBAT. Data are mean ± SEM. *p<0.05 vs. LoxP control group. Scale bar: 100 \(\mu\)m.
Figure S3. Ablation of $tp53inp2$ in adult mice enhances body weight and adiposity.

Panels (A) to (F): Control (LoxP) and KO$^{Ubc}$ male mice at 8 months of age housed at 22°C and subjected to a chow diet ($n=4$–8 LoxP or KO$^{Ubc}$ mice). (A) $Tp53inp2$ mRNA levels in iBAT. (B) T53INP2 protein abundance in iBAT. (C) Body weight. (D) Relative weight of ingWAT, pgWAT, liver, quadriceps (Quad), gastrocnemius (Gast) and tibialis muscles (Tib). (E) Number of nuclei per surface unit and (F) UCP1 mean intensity measurements in iBAT sections. Data are mean ± SEM. *p<0.05 vs. LoxP control group.
Figure S4. TP53INP2 induces PPARG activity. Panels (A) to (C): transcriptomic analysis performed in iBAT from LoxP (n=4) or KO^{Myf5} mice (n=4). Heat maps showing gene expression modulation of (A) adipogenesis, (B) oxidative phosphorylation and (C) fatty acid metabolism gene sets from Broad Hallmarks. (D) Transcriptomic analysis performed in iBAT samples from LoxP (n=4) and KO^{Ubc} (n=4) mice. Enrichment plot (GSEA) of PPAR signaling pathway from Kyoto Encyclopedia of Genes and Genomes (KEGG) (p-val<0.001). (E) PPRE reporter activity in control (C) or tp53inp2 knockout (KO) brown preadipocytes transfected with empty vector (-) or PPARG (+) and treated with vehicle (-) or with rosiglitazone (+) 1 μM for 24 h (n=3). Data are mean ± SEM. *p<0.05 vs. control group.
Figure S5. TP53INP2 induces the autophagic degradation of the co-repressor NCOR1. 

(A) Tp53inp2, Ncor1 and Map1lc3b mRNA levels in control (SCR) or tp53inp2 knockdown (KD) brown preadipocytes (n=7–8). (B) NCOR1 protein abundance and (C) NCOR1 fold accumulation in mouse brown preadipocytes transfected with siRNA control (siSCR) or with different siRNAs targeting Tp53inp2 (si#1 and si#2) and treated with vehicle (-) or with 200 nM bafilomycin A1 (BAF) (+) for 4 h (n=3). (D) TP53INP2 protein abundance and (E) TP53INP2 fold accumulation in mouse brown preadipocytes stably overexpressing TP53INP2 or TP53INP2-LIR treated with vehicle (-) or with BAF (+) for 4 h (n=5). (F) LC3B-II and (G) TP53INP2 fold accumulation and (H) NCOR1 quantification in day 4 adipocytes transfected at day 2 with siRNA control (SCR) or against Tp53inp2 in basal (-) or upon treatment with BAF (+) for the last 6 h (n=6, 6 and 5 respectively). Panels (I) to (M): control (Empty), TP53INP2 or TP53INP2-LIR stably overexpressing brown preadipocytes. (I) NCOR1 and TP53INP2 immunostaining, (J) NCOR1 and (K) TP53INP2 nuclear:cytosolic ratio quantification (representative experiment of n=3 independent experiments). (L) NCOR1 protein abundance in total, cytosolic and nuclear homogenates, and (M) nuclear NCOR1 quantification (n=4). Data are mean ± SEM. *p<0.05 vs. control group. #p<0.05 vs. non treated group. Scale bar: 10 μm.
Figure S6. TP53INP2 enhances BAT specific thermogenesis. Panels (A) to (K): control (LoxP) and KO^{Myf5} male mice at 3 months of age housed at 22°C and subjected to a chow diet (n=7 LoxP or KO^{Myf5} mice). (A) Energy expenditure. (B) Oxygen consumption (VO2). (C) Carbon dioxide production (VCO2). (D) Energy expenditure during light (inactive) and dark (active) phase. (E) Locomotor activity during light and dark phase. (F) Food intake. (G) Water intake. (H) Respiratory exchange ratio (RER). (I) Glucose oxidation. (J) Lipid oxidation. (K) High-resolution respirometry in tibialis anterior muscle. Energy expenditure, VO2, VCO2, glucose oxidation and lipid oxidation are shown as adjusted means based on a normalized mouse weight of 29.4714 g determined using ANCOVA. Panels (L) to (R): control (LoxP) and KO^{Micl} male mice at 4 months of age housed at 22°C and subjected to a chow diet (n=4 LoxP or KO^{Micl} mice). (L) Energy expenditure. (M) VO2. (N) VCO2. (O) Locomotor activity. (P) RER. (Q) Glucose oxidation. (R) Lipid oxidation. Energy expenditure, VO2, VCO2, glucose oxidation and lipid oxidation are shown as adjusted means based on a normalized mouse weight of 27.0625 g determined using ANCOVA. Panels (S) to (U): control (C) and tp53inp2 knockout (KO) mouse brown adipocytes. (S) Ucp1 and (T) Prdm16 mRNA levels in adipocytes treated with CL-316,243 (CL) for different times (n=4). (U) Norepinephrine (NE)-induced (1 μM) mitochondrial respiration (ΔOCR) (n=4). Data are mean ± SEM. *p<0.05 vs. control group.
Figure S7. TP53INP2 prevents fat mass expansion through BAT thermogenesis. Panels (A) to (L): control (LoxP) and KO^Myf5^ male or female mice at 6 months of age housed at 22°C and subjected to a chow diet (n=4–9 LoxP or KO^Myf5^ mice). (A) Total fat mass. (B) Total lean mass. (C) Weight of ingWAT. (D) Weight of pgWAT. (E) Hematoxylin-eosin staining of iBAT sections, (F) lipid droplet (LD) number and (G) LD average area measurements. (H) Adipocyte size distribution, (I) number of adipocytes per surface unit, (J) number of nuclei per surface unit and (K) UCP1 mean intensity measurements in iBAT sections. (L) UCP1 protein quantification in iBAT. Panels (M) to (R): LoxP and KO^Myf5^ male or female mice at 3 months of age housed at 22°C and subjected to a chow diet (n=7–10 LoxP or KO^Myf5^ mice). Blood glucose levels during glucose tolerance test (GTT, 2 g/kg) in (M) males or (N) females. Plasma insulin levels during GTT in (O) males or (P) females. Blood glucose levels during insulin tolerance test (ITT, 0.7 U/kg) in (Q) males or (R) females. Data are mean ± SEM. *p<0.05 vs. LoxP control group. Scale bar: 100 μm.
Figure S8. Thermogenesis activity and TP53INP2 expression are modulated in parallel. Panels (A) to (F): control (LoxP) and KOMyf5 male mice housed at 22°C and subjected to a high-fat diet (HFD) for a total of 16 weeks (n=6–10 LoxP or KOMyf5 mice). (A) Total lean mass. (B) Weight of iBAT. (C) Weight of ingWAT. (D) Weight of pgWAT. (E) Weight of liver. (F) Weight of quadriceps (Quad), gastrocnemius (Gast) or tibialis (Tib) muscles. (G) Tp53inp2 mRNA levels and (H) TP53INP2 protein quantification in human PAZ6 preadipocytes (Pre) or differentiated adipocytes (Ad) (n=3). (I) Tp53inp2 mRNA levels (n=4), (J) TP53INP2 protein abundance and (K) quantification (n=3) in mouse brown preadipocytes (Pre) or differentiated brown adipocytes (Ad). (L) Tp53inp2, Ucp1, Ppargc1a and Prdm16 mRNA levels in mature mouse brown adipocytes treated with vehicle (PBS) or with the β3-adrenergic agonist CL-316,243 (CL) for 4 h (n=4). (M) TP53INP2 protein quantification in mature human PAZ6 brown adipocytes treated with vehicle (PBS), with 8-bromo-cAMP (8Br) or with forskolin (FSK) for 4 h (n=3). Data are mean ± SEM. *p<0.05 vs. control group in each case.