Brown and beige adipose tissues can dissipate chemical energy as heat through thermogenic respiration, which requires uncoupling protein 1 (UCP1)\textsuperscript{1,2}. Thermogenesis from these adipocytes can combat obesity and diabetes\textsuperscript{3}, encouraging investigation of factors that control UCP1-dependent respiration \textit{in vivo}. Here we show that acutely activated thermogenesis in brown adipose tissue is defined by a substantial increase in levels of mitochondrial reactive oxygen species (ROS). Remarkably, this process supports \textit{in vivo} thermogenesis, as pharmacological depletion of mitochondrial ROS results in hypothermia upon cold exposure, and inhibits UCP1-dependent increases in whole-body energy expenditure. We further establish that thermogenic ROS alter the redox status of cysteine thiols in brown adipose tissue to drive increased respiration, and that Cys253 of UCP1 is a key target. UCP1 Cys253 is sulfenylated during thermogenesis, while mutation of this site desensitizes the purine-nucleotide-inhibited state of the carrier to adrenergic activation and uncoupling. These studies identify mitochondrial ROS induction in brown adipose tissue as a mechanism that supports UCP1-dependent thermogenesis and whole-body energy expenditure, which opens the way to improved therapeutic strategies for combating metabolic disorders.

The specialized capacity for thermogenic respiration in brown adipose tissue (BAT) and beige fat\textsuperscript{4} relies on UCP1. This mitochondrial inner membrane carrier dissipates protonmotive force ($\Delta p$) and increases the rate of substrate oxidation to generate heat\textsuperscript{5}. While UCP1 is critical for thermogenesis, mechanisms that support UCP1-dependent respiration \textit{in vivo} are not fully understood\textsuperscript{1,2}.

We examined features of mitochondrial function in mouse interscapular BAT after cold (4 °C) exposure. Unexpectedly, we found that acute activation of BAT thermogenesis \textit{in vivo} was associated with a substantial increase in mitochondrial ROS levels. Mitochondrial superoxide (Fig. 1a), lipid hydroperoxides (Fig. 1b), and mitochondrial hydrogen peroxide (Fig. 1c)\textsuperscript{9} levels increased significantly in BAT after acute cold exposure. Furthermore, primary brown adipocytes exposed to adrenergic stimulation of thermogenesis by noradrenaline exhibited acute dose-dependent increases in superoxide levels (Extended Data Fig. 1a). Having identified the induction of mitochondrial ROS as an early event during thermogenesis in BAT, we investigated whether it was a regulator of function \textit{in vivo}. We injected mice intraperitoneally (i.p.) with the mitochondria-targeted antioxidant MitoQ before cold exposure. MitoQ efficiently depletes mitochondrial free radical species \textit{in vivo} and achieves substantial localization in metabolically active tissues including adipose\textsuperscript{6}. Treatment with MitoQ before cold exposure inhibited the cold-dependent increases in BAT mitochondrial superoxide and lipid hydroperoxides (Fig. 1a, b). Notably, MitoQ administration resulted in a dose-dependent induction of hypothermia upon cold exposure (Fig. 1d). MitoQ had no effect on body temperature of mice without cold stress (Fig. 1d), and equimolar injections of decylTPP, the mitochondria-targeting moiety lacking the antioxidant group, had no significant effect on body temperature during cold exposure (Extended Data Fig. 1b).

While BAT and beige fat are the primary tissues for mediating adaptive thermogenesis, muscle shivering also plays an important role, particularly during the early thermogenic response\textsuperscript{2}. Shivering was monitored directly by electromyography (EMG) after the transition from thermoneutrality to cold. As expected, by 30 min cold exposure substantial shivering was observed (Extended Data Fig. 1c–e), which could be abrogated by the nicotinic acetylcholine receptor inhibitor curare (Extended Data Fig. 1c). Importantly, treatment of mice with MitoQ before cold exposure caused no detectable change in shivering activity (Extended Data Fig. 1c–e).

We next tested whether the hypothermic effects of depleting mitochondrial ROS depended on UCP1. Mice genetically lacking UCP1 (UCP1$^{-/-}$) are sensitive to acute cold challenge, but maintain thermal homeostasis after gradual acclimation to temperatures below thermoneutrality through compensatory upregulation of alternative thermogenic pathways\textsuperscript{8}. We injected wild-type (WT) and UCP1$^{-/-}$ mice with MitoQ after gradual cold acclimation. Notably, UCP1$^{-/-}$ mice were protected against the hypothermic effects of MitoQ (Fig. 1e).

It is increasingly appreciated that mitochondrial ROS act as signalling molecules in physiology, which involves oxidative modification of sensitive cysteine thiol residues on proximal protein targets\textsuperscript{10}. Strikingly, acute cold exposure drove substantial oxidation and depletion of the BAT glutathione pool (Extended Data Fig. 2a). Furthermore, by performing quantitative profiling of \textit{in vivo} protein thiol redox status in BAT using ratiometric labelling with iodoTMT (Extended Data Fig. 2b), we found that BAT protein thiols became substantially more oxidized upon cold exposure (Fig. 2a and Extended Data Fig. 2c). Pathway analysis of those protein cysteine targets most substantially oxidized during thermogenesis revealed pathways involved in mitochondrial metabolism to be the most enriched (Fig. 2b and Supplementary Table 1). Finally, we profiled levels of protein sulfenic acids\textsuperscript{12}—the proximal reversible cysteine modification generated by lipid hydroperoxides and hydrogen peroxide\textsuperscript{12}—and found them to be significantly elevated upon cold exposure (Fig. 2c). These findings suggested that oxidation of mitochondrial thiols might be important for signalling ROS-dependent thermogenesis in BAT.
We examined this possibility using N-acetylcysteine (NAC), a cell-permeable cysteine precursor shown to increase the intracellular pool of reduced thiols. Similar to what was observed with MitoQ, NAC treatment resulted in a significant decrease in core body temperature upon cold exposure (Fig. 2d). This hypothermic activity suggests an effect on BAT function, as shivering was unaffected (Extended Data Fig. 1c–e). Furthermore, acute elevation in oxygen consumption by CL administration was inhibited by NAC pretreatment (Fig. 2e and Extended Data Fig. 2d). Taken together, these findings suggest that increased mitochondrial ROS levels and consequent thiol oxidation play important roles in driving BAT thermogenesis in vivo.

The acute hypothermic effects of depleting mitochondrial ROS and altering thiol redox status occurred without any appreciable decrease in thermogenic gene expression in either BAT or inguinal white adipose tissue (Extended Data Fig. 3a, b). In fact, some thermogenic gene markers were further elevated following MitoQ or NAC treatment, possibly representing a compensatory increase in expression due to the functional compromise of thermogenic respiration (Extended Data Fig. 3a, b). Furthermore, stimulation of the lipolytic machinery in BAT was comparable upon cold challenge after MitoQ treatment, suggesting this upstream driver of thermogenesis was not compromised (Extended Data Fig. 3c, d). To test whether mitochondrial ROS might act as direct modulators of UCP1-dependent leak respiration, we examined primary brown adipocytes using cellular respirometry. As expected, adrenergic stimulation by noradrenaline triggered a substantial increase in oxygen consumption rate (OCR; Fig. 3a and Extended Data Fig. 3e); while inhibition of ATP synthase with oligomycin allowed for determination of leak respiration (Fig. 3a and Extended Data Fig. 3e). Depletion of mitochondrial ROS with MitoQ and reducing the thiol redox status with NAC both inhibited leak respiration (Fig. 3a, b and Extended Data Fig. 3e, f). Notably, these inhibitory effects were lost in adipocytes lacking UCP1 (Fig. 3c, d and Extended Data Fig. 3g, h).

Our findings indicated that ROS-supported thermogenesis relied on both UCP1 and cysteine thiol oxidation. So, we considered whether UCP1 cysteines were targets for oxidative modification during thermogenesis. We used a redox gel-shift method16 to determine whether BAT mitochondrial ROS drive cysteine-centred oxidative modifications on UCP1 in vivo. This approach labels reversibly oxidized protein cysteines with a polyethylene glycol maleimide, allowing for their determination by immunoblotting after SDS–polyacrylamide gel electrophoresis (Extended Data Fig. 3i). In BAT from mice housed at thermoneutrality, UCP1 existed predominantly in a cysteine non-oxidized form (Fig. 3e). However, upon cold challenge cysteine oxidation increased on UCP1 in a time-dependent manner (Fig. 3e). Calibration of UCP1 cysteine shifts indicated that a single cysteine site was redox modified during thermogenesis (Extended Data Fig. 4a–c), and this modification did not appear to participate in intermolecular disulfide bridging (Extended Data Fig. 4d). Notably, pharmacological reduction of BAT thiol status with NAC, which results in hypothermia (Fig. 2d), diminished UCP1 cysteine thiol oxidation upon cold exposure (Fig. 3f).

So, ROS-supported thermogenesis in BAT both depended on UCP1 (Fig. 1e) and acted directly on UCP1 cysteine(s) by oxidative modification (Fig. 3e, f). To examine the role of cysteine oxidation on UCP1 function, we first identified the site modified during thermogenesis. We profiled UCP1 cysteines for sulfenic acid modifications in vivo by selective dimedone labelling and mass spectrometry (Extended Data Fig. 4e) after acute cold exposure. We identified six of seven UCP1 cysteines, five of which were identified exclusively in the unmodified (N-ethyl maleimide (NEM)-labelled) form (Extended Data Fig. 4f and Supplementary Data File 1). However, UCP1 Cys253 was identified in the sulfenylated (dimedone-labelled) form (Fig. 4a, Extended Data Fig. 4f).
interactions could be destabilized owing to local electrostatic effects
acid modification on the Cys253 thiol suggested that these ‘c-state’
cardiolipin binding on UCP1 (Fig. 4d). Notably, modelling a sulfenic
binding is thought to occur23,24, and is proximal to a putative site of
opposing face of the cytosolic cavity where inhibitory nucleotide
bond21,22 (Fig. 4d and Extended Data Fig. 5b). This region forms the
contain residues highly conserved across the carrier family that stabilize
nucleotides1,20.

Since UCP1 Cys253 was identified as a redox-sensitive site for
activity on Cys253 increased significantly upon acute cold exposure (Fig. 4b).
Quantification of dimedone-labelled Cys253 provided an estimate of
diminished oxidation status during cold exposure (8.4%; Fig. 4b). However, owing
to the unstable nature of protein sulfenic acid23 this must be considered
a significant under-representation of in vivo levels of this modification.

Supplementary Data File 1). Moreover, sulfenic acid occupancy
Cys253 status should specifically modulate sensitivity to adrenergic activation
of the purine nucleotide inhibited ‘c-state’ of UCP11,20. To test this, in
UCP1−/− brown adipocytes we separately transduced WT UCP1 and seven mutants, each having one of the seven cysteine residues
in which modification correlated with thermogenesis, we investigated
the potential structural and functional importance of this residue. We
modelling UCP1 on the structure of the homologous mitochondrial
ATP/ADP carrier (AAC)19 (Fig. 4c and Extended Data Fig. 5a). This structure represents the matrix-closed ‘c-state’ of the carrier19, which for
UCP1 is maintained by inhibitory binding of cytosolic purine nucleotides1,20.

Examining the environment of the Cys253 thiol indicated that it
resides at an interface between two matrix-facing helices. These helices
contain residues highly conserved across the carrier family that stabilize
the ‘c-state’ by inter-helical hydrophobic interactions and a hydrogen
bond21,22 (Fig. 4d and Extended Data Fig. 5b). This region forms the
opposing face of the cytosolic cavity where inhibitory nucleotide
binding is thought to occur23,24, and is proximal to a putative site of
cardiolipin binding on UCP121 (Fig. 4d). Notably, modelling a sulfenic
acid modification on the Cys253 thiol suggested that these ‘c-state’
interactions could be destabilized owing to local electrostatic effects
(Extended Data Fig. 5b). Thus, on the basis of this structural model,
Cys253 oxidation status could play a role in determining stability of the
matrix closed ‘c-state’ of UCP1, and sensitivity to β-adrenergic activation and uncoupling.

and Supplementary Data File 1). Moreover, sulfenic acid occupancy
Cys253 increased significantly upon acute cold exposure (Fig. 4b).
Quantification of dimedone-labelled Cys253 provided an estimate of
sulfenylation status during cold exposure (8.4%; Fig. 4b). However, owing
to the unstable nature of protein sulfenic acid this must be considered
a significant under-representation of in vivo levels of this modification.

Supplementary Data File 1). Moreover, sulfenic acid occupancy
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In the cellular milieu, UCP1 is inhibited by endogenous purine nucleotides, and this inhibition can be overcome by β-adrenergic stimulation of lipolysis and activation of UCP1-dependent
respiration15. A prediction of the above model is that altering Cys253
status should specifically modulate sensitivity to adrenergic activation
of the purine nucleotide inhibited ‘c-state’ of UCP1. To test this, in
UCP1−/− brown adipocytes we separately transduced WT UCP1 and seven mutants, each having one of the seven cysteine residues
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ATP/ADP carrier (AAC)19 (Fig. 4c and Extended Data Fig. 5a). This structure represents the matrix-closed ‘c-state’ of the carrier19, which for
UCP1 is maintained by inhibitory binding of cytosolic purine nucleotides1,20.

The UCP1 Cys-mutant cells were subjected to noradrenaline and
oligomycin to examine the effect of adrenergic activation on leak
respiration. Noradrenaline stimulated rapid oxidation of protein
cysteine residues (Extended Data Fig. 6g) that was concomitant with increases in OCR (Extended Data Fig. 6h). As expected, adipocytes
lacking UCP1 exhibited a substantial reduction in respiration
compared with WT UCP1 cells after 100 nM noradrenaline
(Extended Data Fig. 6c, d), which exhibited compromise of UCP1-dependent
leak respiration (Fig. 4f). Interestingly, individual modification of Cys224
also inhibited UCP1 leak respiration (Extended Data Fig. 7f). We
additionally generated a C224A/C253A double mutant (Extended Data Fig. 6c, d), which exhibited compromise of UCP1-dependent
leak respiration (Extended Data Fig. 6c, d). This agreed with previous studies indicating that UCP1 cysteines are not critical for protein stability and
mitochondrial viability25.

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respiration comparable to the individual mutations (Extended Data Fig. 7g).

Figure 4 | UCP1 Cys253 is sulfonylated during thermogenesis and sensitizes UCP1 to adrenergic activation. a, MS<sup>2</sup> spectrum of dimedone-labelled UCP1 Cys253 peptide indicating sulfonylation of this site during thermogenesis. Fragment ions that span the dimedone-alkylated cysteine are highlighted in the peptide sequence. C#, dimedone-labelled cysteine; M<sup>+</sup>, oxidized methionine. b, Quantification of dimedone-labelled UCP1 Cys253 relative to the NEM-alkylated form (n = 5). c, d, Structure of Cys253 modelled on the AAC crystal structure and (d) Cys253 in a hydrophobic pocket between two matrix facing helices. IMS, intermembrane space. e, f, Basal, maximal, and UCP1-dependent OCR of UCP1<sup>−/−</sup> brown adipocytes ± transduction with (e) WT UCP1 (WT n = 7; UCP1<sup>−/−</sup> n = 6) or (f) UCP1 C253A (WT n = 17; C253A n = 19). g, UCP1-dependent leak respiration after stimulation by various concentrations of noradrenaline (50 nM noradrenaline n = 9; 100 nM noradrenaline n = 7; 500 nM noradrenaline n = 8; 2,000 nM noradrenaline n = 6). Comparison of UCP1 WT and C253A indicates that degree of UCP1 inhibition by C253A is inversely correlated with noradrenaline concentration (n = 19; 100 nM WT noradrenaline; n = 17,500 nM noradrenaline n = 18; 2,000 nM noradrenaline n = 10). h, A model of sensitization of UCP1-mediated uncoupling by mitochondrial ROS. Data are mean ± s.e.m. of at least five mouse replicates or cell replicates for respirometry experiments. *P < 0.05, **P < 0.01 (two-tailed Student’s t-test for pairwise comparisons).
thermogenic effector—is a target of thiol oxidation. We identify UCP1 Cys253 as a sulfenylation site that is increasingly modified during thermogenesis, and provide evidence that this residue can modulate sensitivity to activation of UCP1-dependent thermogenic respiration.

While UCP1 is identified here as a target of redox modification during thermogenesis, it is likely that other functional target proteins exist, which may act together to support thermogenic respiration. In addition to establishing a role for thiol redox signalling in thermogenesis in vivo, these findings provide insight into the long-standing investigations on the relationship between mitochondrial ROS and UCP1 (refs 1, 2, 26, 27). Interestingly, studies in this area have relied largely on the use of isolated mitochondria and reconstituted systems. Our data suggest that redox regulation of UCP1-dependent respiration is particularly relevant in adipocytes and in vivo where both redox homeostasis and free fatty-acid concentrations are subject to strict endogenous regulation; parameters that are necessarily divergent from in vitro conditions. Notably, a role for thiol redox status and UCP1-dependent uncoupling has been suggested in isolated BAT mitochondria28, while the importance of superoxide in particular for modulating UCP1 function is the subject of debate21,22,26,27. Previous investigation using a manganese superoxide dismutase transgenic mouse model suggested that matrix superoxide did not modify UCP1 function27. This previous work suggests that the role of thiol redox status in UCP1-dependent respiration detailed here is not directly mediated by matrix superoxide, but reliant on other ROS and related molecules the levels of which are not compromised by elevated manganese superoxide dismutase expression10.

Our data are consistent with a model whereby redox modification of Cys253 sensitizes purified nucleotide inhibited UCP1 to uncoupling due to acute adrenergic stimulus (Fig. 4h), which is compatible with fatty-acid-mediated activation and shuttling26,28. This model reconciles the apparent sensitizing role of both redox status and UCP1 Cys253 with the sufficient role of fatty acids for UCP1-dependent uncoupling29, as well as previous observations that cysteines are not essential for UCP1 activity in reconstituted systems25. However, previous study of UCP1 cysteines used yeast expression systems that are subject to protein misfolding and artefactual uncoupling29. Although Cys224 was not identified here as a target for reversible redox modification it appears to affect UCP1-dependent respiration, albeit distinctly from the sensitizing role of Cys253. It could be that this site is modified by other ROS-dependent species that are known to interact with UCP1 cysteines28. Additionally, these sites may also be important for UCP1 structure/function independent of redox-modification. Finally, it is worth noting that UCP1 Cys253 is conserved to a high degree across several mitochondrial carriers, including UCP2, UCP3, and AAC. This suggests that redox modification of this site may be a pervasive signalling modality for mitochondrial carriers in physiology and pathophysiology.

Online Content Methods, along with any additional Extended Data display items and Source Data, are available in the online version of the paper; references unique to these sections appear only in the online paper.

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Author Contributions E.T.C. designed research, performed biochemical, cellular, and in vivo experiments, analysed data, and co-wrote the paper. L.K. designed and performed cellular and mutagenesis experiments. M.P.J. and K.A.P. performed and analysed data from mass spectrometric experiments. G.Z.L. performed cellular experiments. C.B.C. and S.P.G. oversaw mass spectrometric experiments. A.J.R. designed the research and co-wrote the paper, with assistance from all other authors.

Author Information Reprints and permissions information is available at www.nature.com/reprints. The authors declare no competing financial interests. Readers are welcome to comment on the online version of the paper. Correspondence and requests for materials should be addressed to B.M.S. (bruce_spiegelman@dfci.harvard.edu).
METHODS

Animal procedures and ethics statement. Animal experiments were performed according to procedures approved by the Institutional Animal Care and Use Committee of the Beth Israel Deaconess Medical Center. Unless otherwise stated, mice used were male C57BL/6 (8–12 weeks of age; Jackson Laboratories), and housed in a temperature-controlled (20–22°C) room on a 12 h light/dark cycle. In vivo compound administration. All compounds administered to mice in vivo were injected at the stated dose i.p. 10 min before subsequent interventions unless otherwise stated.

Body temperature and cold exposure. Body temperature and cold exposure experiments were assessed using a mouse rectal probe (World Precision Instruments). When studying acute activation of thermogenesis, mice were housed from birth at 20–22°C to allow for recruitment of thermogenic adipose tissue. Before individual housing at 4°C, mice were placed at thermoneutrality (30°C) for 3 days which allows both for maintenance BAT UCP1 protein content and for measurement of acute induction of BAT thermogenesis upon cold exposure. Upon exposure to 4°C, temperature was measured every 30 min. When studying body temperature after 4°C acclimation, WT and Ucp1−/− mice (equal numbers of male and female mice in each group) were acclimated using established protocols: mice were individually housed for 1 week at 15°C, 1 week at 10°C, and 24 h at 4°C before the experiment.

EMG determination of muscle shivering. Mice were individually restrained to limit non-shivering muscle activity and two EMG needle electrodes were inserted subcutaneously above the nuchal muscles in the back of the neck. EMG leads were connected to a computerized data acquisition system via a communicator. EMG was recorded at thermoneutrality to determine non-shivering basal nuchal muscle activity, before placement of mice at 4°C. EMG data were collected and burst activity was determined as described previously. Briefly, EMG data were collected from the implanted electrodes at a sampling rate of 2 kHz using LabChart 8 Pro Software (ADInstruments). The raw signal was converted to root mean square activity. Root mean square activity was analysed for shivering bursts in 10 s windows.

Metabolic phenotyping. Whole-body energy metabolism was evaluated using a Comprehensive Lab Animal Monitoring System (CLAMS, Columbia Instruments). For 6h measurements, mice were acclimated in the metabolic chambers for 48 h before experiments to minimize stress from the housing change. CO2 and O2 levels were collected every 12 or 32 min for each mouse over the period of the experiment. For acute measurements, CO2 and O2 levels were collected every 10 s. CL 316,243 (Sigma-Aldrich; 1 mg kg−1) was injected i.p. into mice at the indicated times.

Assessment of mitochondrial aconitase inactivation in mouse BAT. Aconitase activity was measured as described previously. In brief, after the relevant in vivo intervention mouse BAT was rapidly excised and homogenized in mitochondrial isolation buffer (250 mM sucrose, 2 mM EDTA, 10 mM sodium citrate, 0.6 mM MnCl2, 100 mM Tris-HCl, pH 7.4) followed by mitochondrial isolation by differential centrifugation. Samples (1–2 mg mitochondrial protein) were added to a 96-well plate and 190 nl assay buffer (50 mM Tris-HCl (pH 7.4), 0.6 mM MnCl2, 5 mM sodium citrate, 0.2 mM NADP, 0.1% (v/v) Triton X-100, 0.4 µM iodoTMT6 (ECDFI)). Absorbance was measured at 340 nm for 7 min at 37°C. To control for mitochondrial content aconitase activity was normalized to citrate synthase activity and expressed the result as a percentage of control levels.

Assessment of lipid hydroperoxides in mouse BAT. Lipid hydroperoxide content in mouse BAT was estimated by rapid snap freezing of BAT tissue followed by lipid extraction and assessment using a modified ferric thiocyanate assay (Cyaman Chemical Lipid Hydroperoxide Assay Kit) according to the manufacturer’s instructions.

Assessment of cysteine redox status by gel shift. Cysteine redox status of Pnx3 and Ucp1−/− tissue was measured as described previously. After the relevant in vivo intervention, mouse BAT was rapidly excised and homogenized in 100 mM NEM, 1 mM EGTA, 50 mM Tris-HCl, pH 7.4. Samples were incubated at 37°C for 5 min before the addition of SDS (2% final) and further incubation at 37°C for 10 min. Incubations at 37°C proceeded in a thermostatin at 1,300 r.p.m. Samples were then precipitated in five volumes of ice-cold acetone to remove excess NEM before resuspension in 1 mM EGTA, 2% SDS, 10 mM TCEP, 50 mM Tris-HCl, pH 7.4 containing a polyethylene glycol polymer conjugated to maleimide (50 mM PEG-Mal). Resuspended samples were incubated for 30 min at 37°C before a second acetone precipitation to remove excess PEG-Mal before sample resuspension and immunoblot detection. For standard methods described below for UCP1 experiments, to ensure gel shift signals were specific to reversible cysteine oxidation, oxidized samples were separately treated with TCEP before differential labelling as described above. Calibrating the number of UCP1 cysteines oxidized was achieved by treating TCEP-reduced samples with increasing proportions of PEG-Mal:NEM to generate a cysteine-dependent ladder. In addition, to ensure higher molecular mass signals were specific to UCP1, UCP1 antibody specificity was tested in BAT. It should be noted that while the UCP1 antibody used here is highly specific for UCP1 in BAT (Extended Data Fig. 4c), the same antibody applied to cultured brown adipocyte samples can generate non-specific signals at molecular mass >35 kDa. So, the UCP1 gel shift assay as described here is only compatible with in vivo tissue experiments.

Assessment of BAT-reduced and -oxidized glutathione content by mass spectrometry. Reduced and oxidized glutathione were profiled in negative ionization mode by liquid chromatography tandem mass spectrometry (LC–MS) methods as described previously. Data were acquired using an ACQUITY UPLC (Waters) coupled to a 5500 QTRAP triple quadrupole mass spectrometer (AB SCIEX). Tissue homogenates (30 µl) were extracted using 120 µl of 80% methanol containing 0.05 ng µl−1 insulin−15N6, 0.05 ng µl−1 thymidine−d4, and 0.1 ng µl−1 glycodeoxycholate−d4 as internal standards (Cambridge Isotope Laboratories). The samples were centrifuged (10 min, 9,000g, 4°C) and the supernatants (10 µl) were injected directly onto a 150 mm × 2.0 mm Luna NH2 column (Phenomenex). The column was eluted at a flow rate of 400 μl min−1 with initial conditions of 10% mobile phase A (20 mM ammonium acetate and 20 mM ammonium hydroxide (Sigma-Aldrich) in water (VWR)) and 90% mobile phase B (10 mM ammonium hydroxide in 75:25 v/v acetonitrile/methanol (VWR)) followed by a 10 min linear gradient to 100% mobile phase A. The ion spray voltage was −4.5 kV and the source temperature was 500°C. Raw data were processed using MultiQuant 2.1 software (AB SCIEX) for automated peak integration. LC–MS data were processed and visually inspected using TraceFinder 3.1 software (Thermo Fisher Scientific).

Assessment of BAT protein thiol redox status using iodoTMT. After the relevant in vivo intervention, mouse BAT was rapidly excised and homogenized in 20% (v/v) TCA to stabilize thiols. Tissue homogenates were incubated on ice for 30 min and then pelleted for 30 min at 16,000g at 4°C. The pellet was washed with 10% and 5% (v/v) TCA and then resuspended in 80 µl denaturing alkylating buffer (DAB; 6 M urea, 2% (v/v) SDS, 200 mM Tris- HCl, 10 mM EDTA, 100 µM DTPA, 10 µM neocupeine). The contents of one vial of iodoTMT6 reagent (Thermo Scientific) was added to each of three biological replicate samples to label reduced cysteine residues at 37°C and 1,300 r.p.m. for 1 h. Sample protein was precipitated with five volumes of ice-cold acetone, incubated at −20°C for 2 h, and pelleted at 4°C and 16,000g for 30 min. The amount of protein to be processed was optimized to ensure saturation of thiol labeling by the iodoTMT reagent as per the manufacturer’s instructions. The pellet was washed twice with ice-cold acetone and then re-solubilized in 80 µl DAB containing 1 mM tris(2-carboxyethyl)phosphine (TCEP), reducing previously reversibly oxidised cysteine residues in the presence of a second, distinct iodoTMT6 reagent. Proteins were incubated at 37°C and 1,400 r.p.m. for 1 h, precipitated and resuspended for protease digestion. After digestion, iodoTMT-labelled cysteine-containing peptides were enriched using the anti-TMT resin as per the manufacturer’s instructions.

Profiling of redox-sensitive protein targets. Proteins with cysteine thiols exhibiting differential redox status (defined as >10% shift in cysteine oxidation status upon cold exposure) were assessed for Gene Ontology (GO) enrichment and functional enrichment. The total identified population of cysteine thiols containing proteins was used as the reference background. Enriched GO terms were filtered after benjamini-hochberg correction at an adjusted P value <0.1. All data analysis used R (R Core Team, Vienna, Austria, http://www.R-project.org).

Assessment of protein thiol sulfenic acids. Tissue or cellular samples were prepared adapting a protocol used previously to stabilize endogenous protein sulfenic acids. Briefly, samples were homogenized in 50 mM Tris base, containing 100 mM NaCl, 100 mM DTPA, 0.1% SDS, 0.5% sodium deoxycholate, 0.5% Triton X-100, 5 mM dithiothreitol. To minimize lysis-dependent oxidation, buffers were bubbled with argon before use. Samples were incubated for 15 min at room temperature, at which point SDS was added to a final concentration of 1% and samples were incubated for a further 15 min. After didecomposition treatment, 10 mM TCEP and 50 mM NEM were added and samples were incubated for a further 15 min at 37°C to reduce and alkylate all non-sulfenic acid protein cysteine residues. Protein sulfenic acids were then assessed by immunoblotting against didecomposition (1:1,000 antibody dilution).

Targeted assessment of UCP1 cysteine sulfenylation status using dimedone-MS. After didecomposition and NEM labelling of samples as described above, samples were resolved by SDS–PAGE and bands in the UCP1 containing region of the gel were excised, destained with acetonitrile and subjected to dehydration by a speed vacuum concentrator. Gel bands were rehydrated with digestion buffer (75 µl of 50 mM HEPES and 500 ng of trypsin (Promega) and subjected to 12 h of digestion at 37°C. Peptides were extracted and labelled with TMT 10 reagents (Thermo Fisher) as previously described.
Protein digestion. Protein pellets were resuspended and re-suspended in 8 M urea containing 50 mM HEPES (pH 8.5). Protein concentrations were measured by BCA assay (Thermo Scientific) before protease digestion. Protein lysates were diluted to 4 M urea and digested with LysC (Wako, Japan) in a 1/100 enzyme/protein ratio overnight. Protein extracts were diluted further to a 1.0 M urea concentration, and trypsin (Promega) was added to a final 1/200 enzyme/protein ratio for 6 h at 37 °C. Digests were acidified with 20 μl of 20% formic acid (FA) to a pH ~2, and submitted to C18 solid-phase extraction (Sep-Pak, Waters).

LC-MS/MS analysis. Total MS spectra were acquired using an Orbitrap Fusion mass spectrometer (Thermo Fisher) in line with an Easy-nLC 1000 (Thermo Fisher Scientific) ultra-high pressure liquid chromatography pump. Peptides were separated onto a 100 μm inner diameter column containing 1 cm of Magic C4 resin (5 μm, 100 Å, Michrom Bioresources) followed by 30 cm of Sepax Technologies GP-C18 resin (1.8 μm, 120 Å) with a gradient consisting of 9–30% (ACN, 0.125% FA) over 180 min at ~250 nA min⁻¹. For all LC-MS/MS experiments, the mass spectrometer was operated in the data-dependent mode. We collected MS₁ spectra at a resolution of 120,000, with an AGC target of 150,000 and a maximum injection time of 100 ms. The ten most intense ions were selected for MS². MS² precursor ions were excluded using a dynamic window (75 ± 10 ppm). The MS³ precursors were isolated with a quadrupole mass filter set to a width of 0.57 FWHM. For the MS³ based TMT quantitation, MS² spectra were collected at an AGC of 4,000, maximum injection time of 150 ms, and CID collision energy of 35%. MS² spectra were acquired with the same Orbitrap parameters as the MS² method except HCD collision energy was increased to 55%. Synchronous precursor-selection was enabled to include up to six MS² fragment ions for the MS³ spectrum.

Data processing and MS² spectra assignment. A compilation of in-house software was used to convert raw files to MXF format, as well as to adjust monoisotopic m/z measurements and erroneous peptide charge state assignments. Assignment of MS² spectra was performed using the SEQUEST algorithm. All experiments used the Mouse UniProt database (downloaded 10 April 2014) where reversed protein sequences and known contaminants such as human keratins were appended. SEQUEST searches were performed using a 20 ppm precursor ion tolerance, while requiring each peptide’s amino/carboxy (N/C) terminus to have trypsin protease specificity and allowing up to two missed cleavages. IodoTMT tags on cysteine residues (±329.226959 Da) was set as static modifications, while methionine oxidation (±15.999492 Da) was set as variable modifications. For targeted assessment of UCP1 cysteine sulhydration, TMT tags on lysine residues and peptide N termini (±229.16293 Da), NEM on cysteine residues (±125.047679 Da) were set as static modifications and oxidation of methionine residues (±15.999492 Da) and dimered on cysteine residues (±13.020401 Da versus NEM) as variable modifications. Determination of sulhydration status of the Cys253 peptide was determined by comparing TMT reporter ion abundance of the dimered-alkylated and NEM-alkylated peptides as a proportion of total precursor ion intensity. An MS² spectra assignment false discovery rate of less than 1% was achieved by applying the target-decoy database search strategy. Protein filtering was performed by using an in-house linear discrimination analysis algorithm to create one combined spectrum for each TMT reporter ion. The following peptide ion and MS² spectra metrics: XCorr, ΔCn score, peptide ion mass accuracy, peptide length and missed-cleavages were used to assign probabilities to each MS² spectrum for being assigned correctly, and these probabilities were further used to filter the data set to a 1% protein-level false discovery rate.

Determination of iodoTMT reporter ion intensities and quantitative data analysis. For quantification, a 0.03 m/z window centred on the theoretical Th value of each reporter ion was used for the nearest signal intensity. Reporter ion intensities were adjusted to correct for the isotopic impurities from the different TMT reagents (manufacturer specifications). The signal to noise values for all peptides were normalized for each TMT reporter ion. Following peptide ion and MS² spectra metrics: XCorr, ΔCn score, peptide ion mass accuracy, peptide length and missed-cleavages were used to assign probabilities to each MS² spectrum for being assigned correctly, and these probabilities were further used to filter the data set to a 1% protein-level false discovery rate.

Production of adenovirus and transduction of cysteine-null UCP1 mutants. Production of adenovirus and transduction of cysteine-null UCP1 mutants. Production of adenovirus and transduction of cysteine-null UCP1 mutants. Production of adenovirus and transduction of cysteine-null UCP1 mutants. Production of adenovirus and transduction of cysteine-null UCP1 mutants. Production of adenovirus and transduction of cysteine-null UCP1 mutants. Production of adenovirus and transduction of cysteine-null UCP1 mutants. Production of adenovirus and transduction of cysteine-null UCP1 mutants. Production of adenovirus and transduction of cysteine-null UCP1 mutants.
MODELLER energy score was taken as the best representative structure. The cardiolipin molecules of the AAC were added to the modelled UCP1 structure by aligning the two structures, and copying the lipid molecules. This structure was examined and figures produced by using the PyMOL molecular visualization system (PyMOL Molecular Graphics System, version 1.4.1, Schrödinger).

Assessment of superoxide levels in primary brown adipocytes. ROS production was estimated by oxidation of DHE and ratiometric assessment as described previously. Cells were plated and differentiated onto 96-well plates suitable for fluorescence analysis. Before imaging, cell media was removed and replaced with imaging buffer (156 mM NaCl, 1.25 mM KH2PO4, 3 mM KCl, 2 mM MgCl2, 10 mM HEPES, pH 7.4) supplemented with 1 mM sodium pyruvate. Cells were loaded with 5 μM DHE (Invitrogen), which remained present throughout the time course. DHE was excited at 535 nm and the emitted signal was acquired at 560 nm. Oxidized DHE was excited at 544 nm and emission was acquired at 590 nm.

Assessment of mitochondrial membrane potential in permeabilized primary brown adipocytes. Mitochondrial membrane potential was measured in permeabilized cells using TMRM (Life Technologies) in dequench mode. In this mode, mitochondrial depolarization causes redistribution of a high concentration of signal quenched TMRM from mitochondria to the cytosol, such that the lower concentration results in dequenching and an increase in fluorescence. Cells were pre-loaded at room temperature with imaging buffer containing 1 μM TMRM. TMRM fluorescence was excited at 544 nm and emission was collected at 590 nm.

Quantitative RT–PCR. Total RNA was extracted from frozen tissue using TRIzol (Invitrogen), purified with RNeasy Mini spin columns (QIAGEN) and pre-loaded at room temperature with imaging buffer containing 1 μM TMRM. TMRM fluorescence was excited at 544 nm and emission was collected at 590 nm.

Statistical analyses. Data were expressed as mean ± s.e.m. and P values were calculated using two-tailed Student’s t-test for pairwise comparisons, one-way ANOVA for multiple comparisons, and two-way ANOVA for multiple comparisons involving two independent variables. ANOVAs were subjected to post-hoc Bonferroni's multiple comparison tests. Sample sizes were determined on the basis of previous experiments using similar methodologies. For in vivo studies, mice were randomly assigned to treatment groups. Mass spectrometric analyses were blinded to experimental conditions.

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Extended Data Figure 1 | Assessing superoxide in brown adipocytes and effect of mitochondria-targeted compounds on shivering, body temperature, and movement. a, Noradrenaline stimulates superoxide-dependent oxidation of DHE in primary brown adipocytes (n = 5). b, Effect of i.p. decyl-TPP on core body temperature after acute cold exposure (n = 10). c, d, Representative (c) raw and (d) root mean square mouse EMG traces at thermoneutrality and after acute cold exposure ± MitoQ, NAC, or curare (0.3 mg kg\(^{-1}\)). e, Quantification of muscle burst frequency as determined by EMG at thermoneutrality and after acute cold exposure ± MitoQ or NAC (n = 3). f, Absolute oxygen consumed immediately before acute CL treatment described in Fig. 1f (n = 5). g, Effect of i.p. CL ± MitoQ on movement as assessed by number of beam breaks (n = 8). NS, not significant. Data are mean ± s.e.m. of at least three replicates. *P < 0.05, **P < 0.01, ***P < 0.001 (two-tailed Student’s t-test for pairwise comparisons, one-way ANOVA for multiple comparisons, two-way ANOVA for multiple comparisons involving two independent variables).
Extended Data Figure 2 | Assessing thiol redox status in vivo and the effect of NAC on movement. a, Mass spectrometric quantification of BAT-reduced and -oxidized glutathione at thermoneutrality and after acute cold exposure (n = 5). b, Scheme for quantitative assessment of protein thiol redox status by ratiometric labelling of BAT protein cysteine thiols. In vivo BAT thiol status is stabilized by protein precipitation in 20% TCA. Unmodified cysteine thiols are labelled with ‘light’ iodoTMT tags (126, 127, 128). After removal of light iodoTMT, reversibly modified protein thiols are reduced with TCEP in the presence of ‘heavy’ iodoTMT (129, 130, 131). Samples are combined and subjected to trypsin digestion. Ratiometric assessment of iodoTMT labelled peptides provides a quantitative profile of overall protein cysteine thiol redox status. c, Average percentage oxidation status of total BAT and BAT mitochondrial protein thiols at thermoneutrality and after acute cold exposure (n = 3). d, Effect of i.p. NAC on movement as assessed by number of beam breaks (vehicle, n = 11; NAC, n = 7). Data are mean ± s.e.m. of at least three replicates. *P < 0.05, ***P < 0.001 (two-tailed Student’s t-test for pairwise comparisons, one-way ANOVA for multiple comparisons, two-way ANOVA for multiple comparisons involving two independent variables).
Extended Data Figure 3 | Assessing thermogenic gene expression, adrenergic response parameters, and strategy for determination of UCP1 cysteine thiol redox status. **a, b**, Quantitative polymerase chain reaction (qPCR) analysis of mRNA expression of selected (**a**) BAT and (**b**) inguinal white adipose tissue (iWAT) genes ± cold exposure, ± MitoQ or NAC (n = 5). **c, d**, Immunoblot analysis of (**c**) PPAR-γ protein expression levels and (**d**) lipolytic phosphorylation cascade activation in BAT after cold exposure ± MitoQ. **e, f**, Raw OCR of primary brown adipocytes under basal conditions and after noradrenaline stimulation ± oligomycin ± (**e**) MitoQ (n = 10) or (**f**) NAC (vehicle and 1 mM NAC, n = 8; 0.1 mM NAC, n = 7). **g, h**, OCR of primary brown adipocytes lacking UCP1 under basal conditions and after noradrenaline stimulation ± oligomycin ± (**g**) MitoQ (n = 10) and (**h**) NAC (n = 10). **i**, Cys-redox mass shift strategy. After in vivo interventions, mouse BAT is excised and unmodified protein thiols are labelled with NEM, after which reversibly oxidized thiols are chemically reduced and labelled with polyethyleneglycol maleimide (PEG-Mal) allowing for determination of cysteine oxidation status on UCP1. Data are mean ± s.e.m. of at least five replicates. *P < 0.05, **P < 0.01 (two-tailed Student’s t-test for pairwise comparisons, one-way ANOVA for multiple comparisons, two-way ANOVA for multiple comparisons involving two independent variables).
Extended Data Figure 4 Assessing UCP1 reversible cysteine oxidation status in vivo by immunoblot and mass spectrometry. a, Calibration of UCP1 cysteine gel shift immunoblot. Calibration of cysteine-dependent shifts by incubation of BAT protein with TCEP and different ratios of NEM and PEG-Mal indicates that a single PEG-mal labelling event shifts UCP1 by ~10 kDa above the native molecular mass. b, Calibration of UCP1 cysteine oxidation status indicates that the gel shift observed upon cold exposure (lane 1) is cysteine dependent, as TCEP pretreatment results in a loss of the shift (lane 2). In addition, the cysteine-dependent mass shift is due to a single oxidation event as determined by including the calibrating markers (lanes 4–6). c, Calibration of specificity of UCP1 antibody in BAT. d, Reducing and non-reducing SDS–PAGE analysis of UCP1 to monitor cysteine-dependent and -independent inter-protein interactions during acute cold exposure. e, Scheme for identification of sulfenylated cysteines on UCP1 by dimedone labelling and mass spectrometry. After acute cold exposure, BAT protein thiols are differentially alkylated with dimedone to selectively label sulfenylated thiol and NEM to label non-sulfenylated thiols. Samples are subjected to trypsin digestion, followed by Lys-TMT labelling, and MS quantification of UCP1 cysteine containing peptides in their dimedone and NEM alkylated forms. Two technical points should be noted in this strategy when interpreting relative quantitation of NEM and dimedone-alkylated peptides. First, these differently alkylated peptides may not necessarily ionize with the same efficiency. Second, NEM is reported to react with sulfenic acids albeit less efficiently than with free thiols, which should be factored in when considering the order of addition of dimedone/PEG-Mal and potential underestimation of sulfenylation status. f, Top: amino-acid sequence alignment of UCP1 proteins highlighting the level of conservation across various species. Bottom: summary of MS determination of UCP1 cysteine sulfenylation status. Six of seven UCP1 cysteines were identified, with all but one being identified exclusively in the unmodified (NEM-alkylated state). Cys253 is identified as dimedone labelled, indicating that it is a site for sulfenylation.
Extended Data Figure 5 | Structure of human UCP1 modelled on the AAC crystal structure including bound cardiolipin and sulfenylation of Cys253. a, Entire UCP1 modelled structure including bound cardiolipin (green), and Cys253 in the oxidized sulfenic acid form. b, UCP1 region containing Cys253 in the oxidized sulfenic acid form. Cys253 localizes to a hydrophobic pocket between two matrix facing helices. Hydrophobic residues (pink) surround the Cys253 thiol, and a hydrogen bond between Arg238 and Glu261 (aqua) is proximal. These residues that stabilize interaction between the matrix facing helices are probably important for stabilization of the purine bound 'c-state' of the carrier. Two separate cardiolipin (green) binding domains are localized proximal to Cys253 within the UCP1 modelled structure.
Extended Data Figure 6 | Assessing transduced UCP1 constructs, OCR, and sulfenylation in brown adipocytes. a, Quantitative PCR analysis of UCP1 mRNA in WT, and UCP1−/− brown adipocytes transduced with WT and cysteine null UCP1 mutants (n = 4). b, Immunoblot of UCP1 protein in WT and UCP1−/− brown adipocytes transduced with WT and cysteine null UCP1 mutants. c, Immunoblot analysis of UCP1 protein in UCP1−/− brown adipocytes transduced with C224A/C253A double mutant compared with WT. d, Densitometry analysis of transduced UCP1 forms relative to WT across separate transduction experiments (n = 4; C224A/C253A n = 3). e, f, Summary of basal (e) and maximal (f) OCR of primary brown adipocytes containing cysteine-null UCP1 mutants. Raw OCR values provided in Extended Data Fig. 7. g, Immunodetection of protein sulfenic acid levels in primary brown adipocytes in the seconds after treatment with 100 nM noradrenaline. h, Time course of brown adipocyte OCR after stimulation with 100 nM noradrenaline (n = 12). Data are mean ± s.e.m. of at least three replicates.
Extended Data Figure 7 | Assessing OCR under basal and FCCP-stimulated maximal respiration, and after noradrenaline stimulation + oligomycin in UCP1−/− primary brown adipocytes transduced with UCP1 cysteine null mutants. a–g. Raw basal, maximal, and UCP1-dependent OCR from representative experiments of WT and UCP1−/− brown adipocytes transduced with (a) UCP1 C24A (WT n = 11; C24A n = 12), (b) C188A (n = 7), (c) UCP1 C213A (n = 19), (d) UCP1 C287A (WT n = 9; C287A n = 10), (e) UCP1 C304A (WT n = 7; C304A n = 10), (f) UCP1 C224A (WT n = 9; C224A n = 10), (g) UCP1 C224A/C253A (n = 8). Data are mean ± s.e.m. of at least seven replicates. **P < 0.01 (two-tailed Student's t-test for pairwise comparisons).
Extended Data Figure 8 | Assessing UCP1-dependent respiration and uncoupling following increasing degrees of adrenergic stimulus.

a, Glycerol release from brown adipocytes as an index of adrenergic stimulus and lipolysis in response to increasing concentrations of noradrenaline (n = 4; 0 and 2,000 nM noradrenaline n = 6).

b, Representative raw noradrenaline + oligomycin leak OCR values in WT and UCP1−/− brown adipocytes after stimulation with a range of noradrenaline concentrations indicates that UCP1-dependent leak respiration is consistently ~25–35% of total leak OCR (50 nM n = 9; 100 nM n = 7; 500 nM n = 8; 2,000 nM n = 6). c, Assessment of UCP1-dependent leak respiration after stimulation by various concentrations of noradrenaline + oligomycin. Comparison of WT and UCP1−/− OCR (replotted data from Fig. 4 for comparison) indicates that UCP1-dependent respiration is consistently ~25–35% of leak respiration.

d, Plasma-membrane-permeabilized OCR of brown adipocytes ± endogenous fatty-acid depletion with BSA (WT n = 30; KO n = 20; C224A n = 9; C253A n = 8; C224A/C253A n = 10). e, Comparison of UCP1-dependent uncoupling absent purine nucleotide inhibition in plasma-membrane-permeabilized brown adipocytes (n = 6; C224A/C253A, KO n = 4). f, Comparison of UCP1-dependent uncoupling after titration of GDP in permeabilized adipocytes containing WT UCP1, UCP1 C224A, C253A, and C224A/C253A (n = 6; C224A/C253A n = 12). Data are mean ± s.e.m. of at least four replicates. *P < 0.05, **P < 0.01, ***P < 0.001 (two-tailed Student’s t-test for pairwise comparisons, one-way ANOVA for multiple comparisons).
CORRIGENDUM

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Corrigendum: Mitochondrial ROS regulate thermogenic energy expenditure and sulfenylation of UCP1

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In this Letter, owing to a typographical error, Fig. 4h was erroneously referred to as Fig. 4j in the text and in the Fig. 4 legend. This error has been corrected online.