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A PGC1-α-dependent myokine that drives brown-fat-like development of white fat and thermogenesis

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Exercise benefits a variety of organ systems in mammals, and some of the best-recognized effects of exercise on muscle are mediated by the transcriptional co-activator PPAR-γ co-activator-1 α (PGC1-α). Here we show in mouse that PGC1-α expression in muscle stimulates an increase in expression of FNDC5, a membrane protein that is cleaved and secreted as a newly identified hormone, irisin. Irisin acts on white adipose cells in culture and *in vivo* to stimulate UCP1 expression and a broad program of brown-fat-like development. Irisin is induced with exercise in mice and humans, and mildly increased irisin levels in the blood cause an increase in energy expenditure in mice with no changes in movement or food intake. This results in improvements in obesity and glucose homeostasis. Irisin could be therapeutic for human metabolic disease and other disorders that are improved with exercise.

PGC1-α is a transcriptional co-activator that mediates many biological programs related to energy metabolism. Originally described as a co-activator of PPAR-γ that modulated expression of uncoupling protein 1 (UCP1) and thermogenesis in brown fat[1], it has also been shown to control mitochondrial biogenesis and oxidative metabolism in many cell types. PGC1-α is induced in muscle by exercise and stimulates many of the best-known beneficial effects of exercise in muscle: mitochondrial biogenesis, angiogenesis and fibre-type switching[2]. It also provides resistance to muscular dystrophy and denervation-linked muscular atrophy[3]. The health benefits of elevated muscle expression of PGC1-α may go beyond the muscle tissue itself. Transgenic mice with mildly elevated muscle PGC1-α are resistant to age-related obesity and diabetes and have a prolonged lifespan[4]. This suggests that PGC1-α stimulates the secretion of factors from skeletal muscle that affect the function of other tissues. Here we show that PGC1-α stimulates the expression of several muscle gene products that are potentially secreted, including FNDC5. The *Fndc5* gene encodes a type I membrane protein that is processed proteolytically to form a newly identified hormone secreted into the blood, termed irisin. Irisin is induced in exercise and activates profound changes in the subcutaneous adipose tissue, stimulating browning and UCP1 expression. Importantly, this causes a significant increase in total body energy expenditure and resistance to obesity-linked insulin resistance. Thus, irisin action recapitulates some of the most important benefits of exercise and muscle activity.

Muscle PGC1-α transgenics

Mice with transgenically increased PGC1-α in muscle are resistant to age-related obesity and diabetes[4], suggesting that these animals have a fundamental alteration in systemic energy balance. We therefore analysed the adipose tissue of the PGC1-α transgenic mice for expression of genes related to a thermogenic gene program and genes characteristic of brown fat development. There were no significant alterations in the expression of brown-fat-selective genes in the interscapular brown adipose tissue or in the visceral (epididymal) white adipose tissue (Fig. 1a). However, the subcutaneous fat layer (inguinal), a white adipose tissue that is particularly prone to 'browning' (that is, formation of multilocular, UCP1-positive adipocytes), had significantly increased levels of *Ucp1* and *Cidea* messenger RNAs (Fig. 1b). We also observed increased UCP1 protein levels and more UCP1-positive stained multilocular cells in transgenic mice compared to controls (Fig. 1c, d). There are recent reports that exercise causes a mild increase in the expression of a thermogenic gene program in the visceral adipose tissue, a depot that has minimal expression of these genes[5]. As it is the subcutaneous white adipose depot that has the greatest tendency to turn on a thermogenic gene program and alter the systemic energy balance of mice[6], we re-investigated this with regard to browning of the white adipose tissues in two types of exercise. Similar to what has been reported[5], a twofold increase in *Ucp1* mRNA expression was observed in the visceral, epididymal fat with 3 weeks of wheel running (Supplementary Fig. 1). However, a much larger change (approximately 25 fold) was seen in the same mice in the subcutaeneous inguinal fat depot. Similarly, a small increase in *Ucp1* mRNA expression was seen in the epididymal fat with repeated bouts of swimming in warm (32 °C) water (Supplementary Fig. 1); however a very large increase (65 fold) was observed in the inguinal white depot (Supplementary Fig. 1). Thus, muscle-specific expression of PGC1α drives browning of subcutaneous white adipose tissue, possibly recapitulating part of the exercise program.

Media from PGC1-α-expressing myocytes

The effect on browning of the adipose tissues from PGC1-α-expressing muscle could be due to direct muscle–fat signalling or to another,

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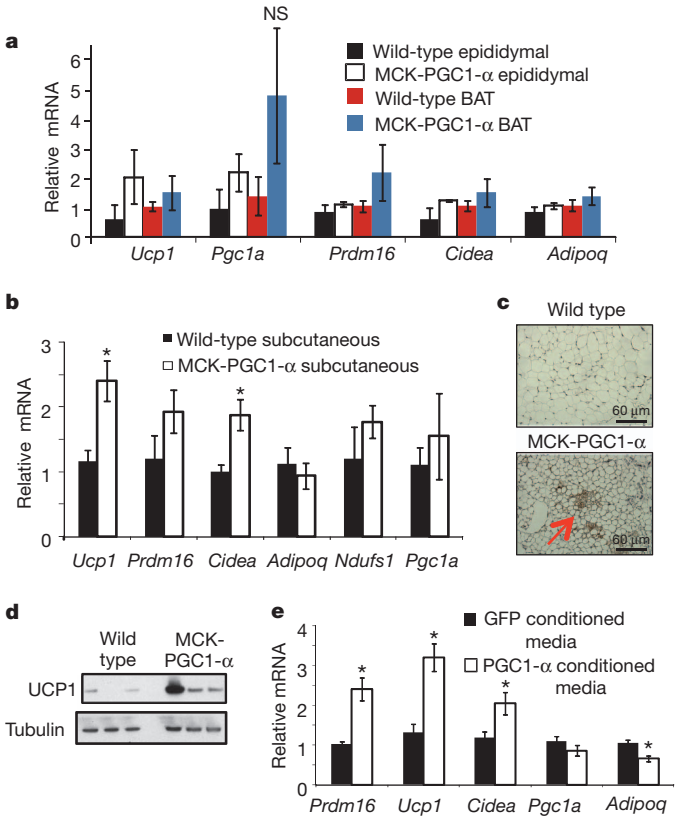

Figure 1 | Muscle-specific PGC1-α transgenic mice have increased brown/beige fat cells in the subcutaneous depot. **a**, **b**, Quantitative polymerase chain reaction (qPCR) against brown fat and thermogenic genes in epididymal fat, BAT (**a**) and subcutaneous, inguinal (**b**) fat depots in muscle creatine kinase promoter (MCK)-PGC1-α transgenic mice or littermate controls. $n = 7$ for each group, repeated in a separate cohort with similar results. **c**, Representative immunohistochemistry against UCP1 in the inguinal depot from indicated mice. **d**, Western blot against UCP1 in the inguinal fat depot ($n = 3$ and repeated in an independent cohort with similar results). **e**, qPCR against indicated genes in adipocytes differentiated for 6 days from stromo vascular fraction (SVF) cells. This was done in the presence of conditioned media from primary myocytes with forced expression of GFP or PGC1-α (representative for three independent experiments). Data are presented as mean ± s.e.m., and $^*P < 0.05$ compared to control group. Student's *t*-test was used for single comparisons. NS, not significant.

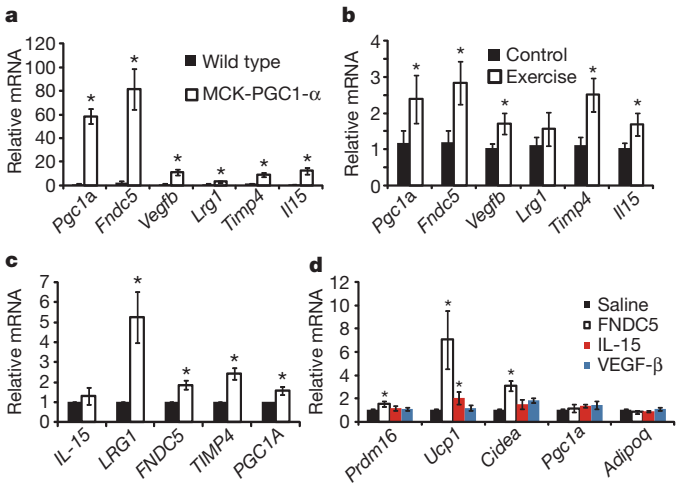

Figure 2 | FNDC5 is induced with forced PGC1-α expression or exercise, and turns on brown/beige fat gene expression. **a**, qPCR against indicated genes in skeletal muscle from MCK-PGC1-α transgenic mice or littermate controls ($n = 7$ from each group). **b**, qPCR against indicated genes in skeletal muscle from sedentary mice or mice given 3 weeks of free wheel running ($n = 10$ from each group). Mice were rested for 12 h before being killed. **c**, mRNA expression levels from human muscle biopsies before and after 10 weeks of endurance exercise training (8 subjects included). All data points are normalized to baseline levels. **d**, SVF from the inguinal fat depot, differentiated into adipocytes for 6 days in the presence of saline or recombinant FNDC5 (20 nM), IL-15 (10 μM) or VEGF-β (50 μM). The graph show normalized mRNA levels of indicated genes. This experiment was repeated three times with similar results. For **d**, we performed one-way ANOVA tests where $^*P < 0.05$ for the effect of FNDC5 on *Ucp1* and *Cidea* expression. All other statistics were performed using Student's *t*-test, and bar graphs are mean ± s.e.m.

indirect mechanism. To investigate this, we treated cultured primary subcutaneous adipocytes with serum-free media conditioned by myocytes expressing PGC1-α or cells expressing green fluorescent protein (GFP). As shown in Fig. 1e, the media from cells expressing ectopic PGC1-α increased the mRNA levels of several brown-fat-specific genes (Fig. 1e). This suggested that PGC1-α causes the muscle cells to secrete a molecule(s) that can induce a thermogenic gene program in the cells.

Candidate, secreted PGC1-α-dependent proteins

We used a combination of Affymetrix-based gene expression arrays and an algorithm that predicts protein secretion to search for proteins that could mediate the browning of adipose tissues under the control of muscle PGC1-α (Methods). Proteins with mitochondrial targeting sequences were excluded, and all candidates were validated in gain-of-function systems for PGC1-α *in vivo* (Methods). Five proteins were identified as PGC1-α target genes in muscle and as likely to be secreted: IL-15, FNDC5, VEGF-β, LRG1 and TIMP4 (Fig. 2a). Conversely, expression of these genes was reduced in mice with muscle-specific deletion of PGC1-α (Supplementary Fig. 2). Furthermore, they were also found to be increased at the mRNA level in muscle from exercised mice (Fig. 2b). The expression of this same set of genes was also examined in muscle biopsies from human subjects before and after a controlled period of endurance exercise[7] (Fig. 2c). *FNDC5*, *VEGFB* and *TIMP4* mRNAs were all significantly induced in humans with exercise. IL-15 has previously been reported as being secreted from muscle under the influence of exercise[8], while the regulation of FNDC5, VEGF-β, LRG1 and TIMP4 by exercise has not been described. *FNDC5* mRNA and brown fat markers in subcutaneous fat were not regulated by acute exercise, and *FNDC5* mRNA was not induced by exposure to cold (4 °C) for 6 h (Supplementary Fig. 2).

Fndc5 induces a browning *in vitro*

Several of the proteins encoded by these genes were commercially available, so they were applied directly to primary subcutaneous white adipocytes during differentiation. Factors such as IL-15 or VEGF-β had minimal effects on the expression of *Ucp1* and other brown fat genes at concentrations of 200 nM or higher. However, FNDC5 promoted a sevenfold induction of *Ucp1* mRNA at a concentration of 20 nM (Fig. 2d). The transcriptional changes induced by FNDC5 were addressed on a global scale using gene expression arrays (Supplementary Fig. 3). Notably, *Ucp1* and three other known brown fat genes, *Elovl3*, *Cox7a1* and *Otop1*, were among the eight most upregulated genes (Supplementary Fig. 4). Conversely, genes characteristic of white fat development were downregulated, such as leptin (Supplementary Fig. 3). These data indicate that the activation of browning and thermogenic genes by FNDC5 is a major part of the action of this polypeptide on these cells.

The effects of FNDC5 treatment were remarkably robust; *Ucp1* mRNA was increased 7–500 fold in more than ten experiments using FNDC5 at a concentration of 20 nM (Fig. 3a and Supplementary Fig. 5). Moreover, we could demonstrate a clear dose-dependence, with an effective range between 20–200 ng ml^{-1} (1.5–15 nM) (Fig. 3b). In contrast, BMP7, reported as a potent inducer of browning[9], had a much smaller effect (maximum of twofold) on the same cells at 3.3 μM (Fig. 3a).

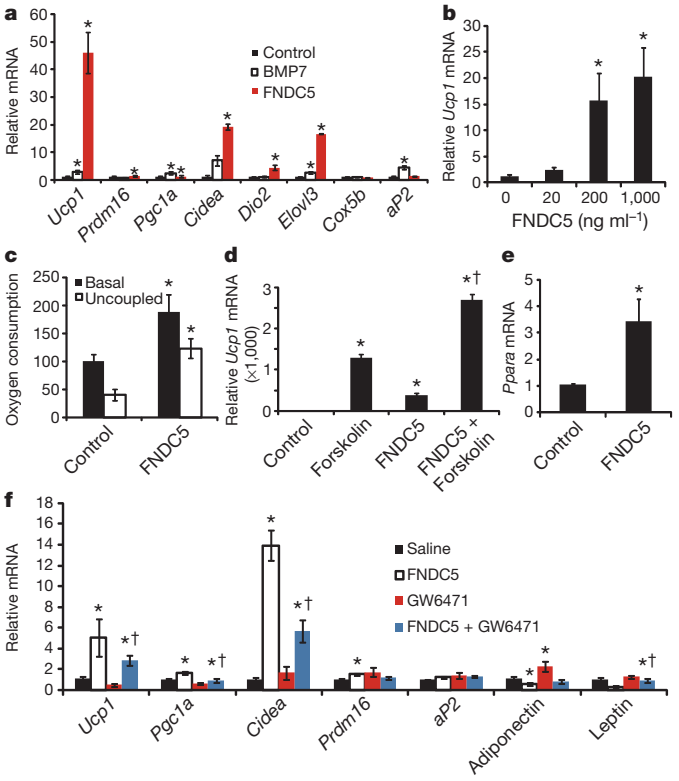

Figure 3 | FNDC5 is a potent inducer of the brown/beige fat gene program **a**, SVF from the inguinal fat depot was differentiated into adipocytes for 6 days in the presence of saline, recombinant FNDC5 (20 nM) or BMP7 (3.3 μM). The graph show normalized mRNA levels for indicated genes. Similar results were obtained in more than 10 experiments with the fold induction of *Ucp1* between 7–500 fold. **b**, mRNA levels of *Ucp1* from inguinal-derived SVF treated with FNDC5 for 6 days at indicated doses. **c**, Clark electrode measurements of oxygen consumption in SVF from the inguinal fat depot, differentiated into adipocytes for 6 days in the presence of saline or recombinant FNDC5 (20 nM). Data are representative for three independent experiments and normalized to total cellular protein. **d**, qPCR of *Ucp1* mRNA from SVF, differentiated into adipocytes, and treated with FNDC5 or saline for 6 days followed by addition of forskolin for 8 h. †$P < 0.05$ compared to forskolin treatment. **e**, qPCR of *Ppara* after 6 days of FNDC5 treatment (20 nM) during differentiation of primary SVF. **f**, SVF differentiated into adipocytes and treated with FNDC5 and/or GW6471 for 6 days. The graph shows qPCR of indicated genes. †$P < 0.05$ compared to FNDC5 treatment. For **d** and **f**, combined one- and two-way ANOVA was used. *$P < 0.05$. All other statistics were performed using Student's *t*-test, and bar graphs are mean ± s.e.m.

We also used immunohistochemistry to study cells treated with FNDC5 and observed a robust increase in UCP1-positive adipocytes with multilocular lipid droplets (Supplementary Fig. 4). Electron microscopic analysis of FNDC5-treated cells showed a higher density of mitochondria compared to control cells, consistent with a brown-fat-like phenotype and elevated mitochondrial gene expression (Supplementary Fig. 5). The sizes of mitochondria, however, were similar between groups (Supplementary Fig. 4). Lastly, measurements of oxygen consumption provided functional evidence of increased energy expenditure with FNDC5 exposure. Total oxygen consumption was greatly increased (100%) by 20 nM of FNDC5, and the majority of this respiration was uncoupled (Fig. 3c). Thus, FNDC5 potently induces thermogenesis and a brown-fat-like gene program in cultured adipocytes. In marked contrast, FNDC5 showed little or no effects on the classical brown fat cells isolated from the interscapular depot (Supplementary Fig. 4).

We sought to define the timeframe during the differentiation process when FNDC5 was effective. FNDC5 was applied to cells in 2-day windows from day 0–6, and this was compared to cells to which the protein was added during the entire 6-day differentiation process. As shown in Supplementary Fig. 5, treatment during days 3–6 was effective at inducing *Ucp1* mRNA, although not as effective as when FNDC5 was present throughout the differentiation process. Furthermore, treatment during the initial 2 days had no effect on *UCP1* levels, suggesting that FNDC5 acts mainly during the differentiation process of cells committed to the adipocyte lineage. Cyclic AMP (cAMP) is an important signalling pathway in thermogenesis, promoting the brown-fat gene program downstream of β-adrenergic stimulation. We therefore asked whether FNDC5 effects were additive or redundant with cAMP signalling. As shown in Fig. 3d, FNDC5-exposed cells increase UCP1 expression in an additive manner when exposed to forskolin, an adenyl cyclase activator. Two-way ANOVA tests demonstrated that there was a significant ($P < 0.01$) interaction between FNDC5 and forskolin treatment, indicating synergistic effects.

PPAR-α acts downstream of Fndc5

A key question is how FNDC5 is able to stimulate a thermogenic gene program. One potentially important transcription factor induced by FNDC5, identified using gene expression arrays, was PPAR-α. This nuclear receptor has been shown to drive *Ucp1* expression and several other genes involved in browning of adipose cells[10]. *Ppara* is increased threefold at the mRNA level by FNDC5 treatment (Fig. 3e). Importantly, the FNDC5-mediated increase in UCP1 was significantly reduced when cells were simultaneously subjected to the selective PPAR-α antagonist GW6471 (Fig. 3f). The functional interaction between the FNDC5 and GW6471 treatments on *UCP1* expression was confirmed using two-way ANOVA ($P < 0.05$). Conversely, the PPAR-α antagonist normalized the reduction seen in white adipose genes leptin and adiponectin after FNDC5 treatment. Together, these data indicate that FNDC5 acts to induce *Ucp1* gene expression, at least in part, via PPAR-α.

Irisin is a cleaved and secreted fragment of Fndc5

FNDC5 (also known as FRCP2 and PeP), was previously shown to have a signal peptide, two fibronectin domains and one hydrophobic domain that is likely to be membrane inserted[11,12] (Fig. 4a). Previous studies did not investigate whether part of this protein might be secreted[11,12]. With this structure in mind, we considered the possibility that FNDC5 might be synthesized as a type I membrane protein, followed by proteolytic cleavage and release of the amino (N)-terminal part of the protein into the extracellular space. Thus, any carboxy (C)-terminal or N-terminal tags would be lost during processing of the mature protein or interfere with localization. Indeed, expression of a C-terminally Flag-tagged FNDC5 (Fig. 4a) did not result in any Flag immunoreactivity in the medium from cells expressing this construct (Fig. 4b). However, when we immunoblotted the same samples with an antibody that recognizes the endogenous FNDC5 protein, we could easily detect substantial amounts of FNDC5 in the media at approximately 32 kilodalton (kDa): this is slightly larger than the cellular FNDC5 (Fig. 4b). These data indicate that FNDC5 is C-terminally cleaved, secreted and possibly further modified.

Western blot of media fractions with antibodies against wild-type FNDC5 showed multiple bands, suggestive of glycosylation, a common feature of secreted proteins. Treatment of supernatants from FNDC5-expressing cells with peptide *N*-glycosidase F (PNGase F) resulted in a significant size decrease as detected by SDS–polyacrylamide gel electrophoresis (SDS–PAGE), from 32 kDa to 20 kDa (Fig. 4c).

Mass spectrometry was used to determine the sequence of the FNDC5-derived polypeptide found in the media (Methods). To do this, we fused the N terminus of FNDC5 (without the signal peptide) to the C terminus of the crystallisable fragment (Fc) domain of immunoglobulin G (IgG). After purification and enzymatic deglycosylation of the secreted material, mass spectrometry analyses indicated that secreted FNDC5 was truncated at glutamic acid 112 (not including the signal sequence), as shown in Fig. 4e. The secreted portion of FNDC5 has remarkable conservation between species, with 100% identity between

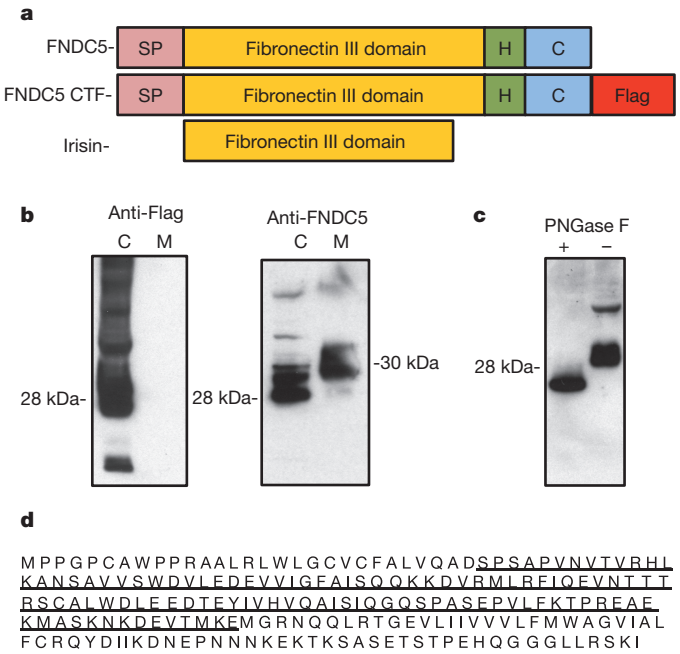

Figure 4 | FNDC5 is proteolytically cleaved and secreted from cells. **a**, Schematic representation of the FNDC5 protein structure (top), Flag-tagged FNDC5 protein (middle) and irisin (bottom). C, C-terminal domain; H, hydrophobic domain; SP, signal peptide. **b**, HEK 293 cells transfected with a vector expressing the C-terminal Flag-tagged FNDC5 (CTF-F5, third panel from **a**), followed by isolation of cell and culture media protein. Samples were adjusted for protein content and western blots were performed against the Flag antigen (left) or FNDC5 (right). This was repeated in several experiments with similar results. Adjusting for volume (instead of protein content) also gave similar results. C, cell fraction; M, media fraction. Arrows indicate molecular weight. **c**, HEK 293 cells transfected with a vector expressing FNDC5–CTF, followed by isolation of cell and media protein. Respective protein fraction was then treated with PNGase F followed by western blot against FNDC5 after SDS–PAGE. **d**, Representation of the full-length FNDC5 and the irisin fragment mapped by mass spectrometry (underlined).

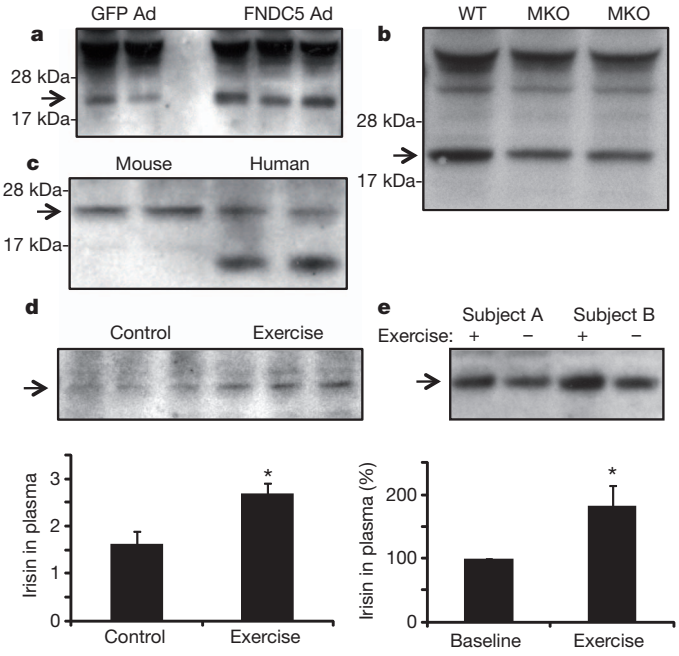

Figure 5 | Detection of irisin in mouse and human plasma. **a**, Plasma from mice injected intravenously with adenoviral vectors (Ad) expressing FNDC5 or GFP was subjected to western blot against FNDC5. **b**, Western blot against irisin in plasma from muscle-specific PGC1-α knockout (MKO) mice or control littermates (flox/flox). WT, wild type. **c**, Western blot against irisin in plasma from wild-type mice or two healthy human subjects (representative for 8 subjects analysed identically). **d**, Western blot against irisin in serum from control or 3 weeks exercised mice, followed by 12 h rest. Bottom panel shows quantification of the bands. **e**, Western blot analysis of irisin in plasma from human subjects before and after 10 weeks of endurance exercise. Eight subjects in total were analysed; quantification after internal normalization is displayed in bottom panel. For all plasma analyses, samples were depleted for albumin/IgG, and deglycosylated using PNGase F. Arrow indicated irisin band. Data are presented as mean ± s.e.m., and $*P < 0.05$ compared to control group. Student's *t*-test was used for single comparisons.

mice and humans (Supplementary Fig. 6). Because this distinct, secreted polypeptide has not been previously described and signals from muscle to other tissues we named it irisin, after Iris, the Greek messenger goddess.

The ability of the anti-FNDC5 antibodies to react with irisin allowed us to investigate the contribution made by irisin to the browning activity caused by muscle cells expressing PGC1-α. Media conditioned by muscle cells that had forced expression of PGC1-α were incubated with control or anti-FNDC5 antibodies before they were applied to the fat cell cultures. As shown in Supplementary Fig. 6, the FNDC5 antibody caused a marked reduction in the ability of the PGC1-α conditioned media to induce *Ucp1* and *Cidea* mRNA in the primary inguinal cells. This suggests that irisin accounts for a significant fraction of this activity found in secreted media from muscle cells with forced PGC1-α expression. We cannot, however, exclude the possibility that other factors might also contribute to this response.

Irisin is present in mouse and human plasma

We next analysed levels of irisin in plasma from wild-type mice, using intravenous adenoviral delivery of full-length FNDC5 as a positive control. This method results in strong forced expression from the liver and potential secretion to the plasma, where we detected irisin using western blot after albumin/IgG-depletion and deglycosylation. As seen in Fig. 5a, FNDC5-expressing virus resulted in a clear increase in an immunoreactive band at 22 kDa. Importantly, this was the only band altered on these blots. Using western blots of purified FNDC5 protein as a quantitative standard, irisin is present in the plasma of control mice at a concentration of approximately 40 nM. We also analysed plasma of PGC1-α muscle-specific knockout mice as a negative control, and the irisin band at 22 kDa was decreased by 72% in these animals (Fig. 5b). Furthermore, an immunoreactive band of identical electrophoretic mobility was found in plasma from healthy human subjects (Fig. 5c). This band was greatly diminished when the anti-FNDC5 antibody was neutralized with an excess of antigen (Supplementary Fig. 7).

We examined blood levels of irisin after exercise in mice and human subjects. Mice had significantly elevated (65%) plasma concentrations of irisin after they were subjected to 3 weeks of free wheel running (Fig. 5d). Similar analyses in healthy adult humans subjected to supervised endurance exercise training for 10 weeks revealed a twofold increase in the circulating irisin levels compared to the non-exercised state (Fig. 5e). Thus, irisin is present in mouse and human plasma, and is increased with exercise. The increase in circulating protein in both species is roughly proportional to the increases observed at the mRNA level in muscle (Fig. 2c).

Irisin reduces obesity and insulin resistance

We used adenoviral vectors to express full-length FNDC5 (or a control GFP) and examined its biological and therapeutic effects. This method resulted in a 15-fold increase in liver *Fndc5* mRNA, although the liver expresses very low endogenous levels of this mRNA. Plasma levels of irisin were increased 3–4 fold (Fig. 5a). The mice did not display any adverse reaction, and upon gross pathological examination, there was no apparent toxicity in any major organ system. There was also no increase in plasma AST levels, and inflammatory genes were not significantly altered in the liver when the two groups were compared

(Supplementary Fig. 8). Ten days after injection, *Ucp1* mRNA was increased by 13-fold in the subcutaneous depot relative to the same depot in mice receiving the virus expressing GFP (Fig. 6a, b); *Cidea* was also significantly upregulated (Fig. 6a). There were no changes in expression of UCP1 in the interscapular classical brown fat (BAT), but we did observe a minor elevation in *Prdm16* and *Pgc1a* mRNA (Supplementary Fig. 8). The changes in gene expression in the subcutaneous adipose tissues were accompanied by a clear increase in the number of UCP1-positive, multilocular adipocytes (Fig. 6c). We did not, however, detect any change in body weight in the GFP versus FNDC5 groups of animals. We observed similar results in young C57BL/6 mice (Supplementary Fig. 9). Thus, moderate increases in circulating irisin can induce browning of white adipose tissues *in vivo,* including increased expression of UCP1.

As activation of the classical brown fat or browning of white fat has been shown to improve obesity and glucose homeostasis *in vivo*[6,13], we delivered FNDC5-expressing adenovirus to mice rendered obese and insulin-resistance by feeding a high fat diet. We chose C57BL/6 mice for these experiments because they are highly prone to diet-induced obesity and diabetes. The expression of irisin increased *Ucp1* gene expression to the same degree as in lean mice (Supplementary Fig. 9).

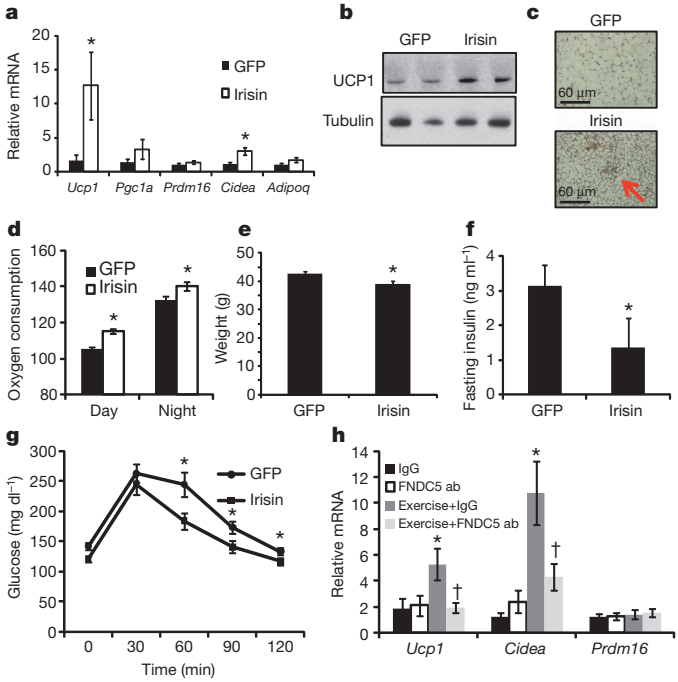

Figure 6 | Irisin induces browning of white adipose tissues *in vivo* and protects against diet-induced obesity and diabetes. **a**–**c**, Wild-type BALB/c mice were injected with 10^{10} GFP- or FNDC5-expressing adenoviral particles intravenously ($n = 7$ for each group). **a**, **b**, Animals were killed after 10 days and inguinal/subcutaneous fat pads were collected and analysed using qPCR analysis of indicated mRNAs (**a**) and western blot against UCP1 (**b**). **c**, Representative images from immunohistochemistry against UCP1 in these mice. All results in **a**–**c** were repeated two times with similar results. **d**–**g**, C57BL/6 mice fed a 60% kcal high-fat diet for 20 weeks were intravenously injected with GFP- or FNDC5-expressing adenovirus and all analyses were done 10 days thereafter ($n = 7$ for both groups). **d**, Oxygen consumption at day and night. **e**, Body weights of mice 10 days after injection with indicated adenovirus. **f**, Fasting plasma insulin measured using enzyme-linked immunosorbent assay (ELISA). **g**, Intraperitoneal glucose tolerance test. **h**, Mice were injected intraperitoneally with 50 μg of rabbit IgG or a rabbit anti-FNDC5 antibody (ab) and were either subjected to swimming for 7 days or kept sedentary ($n = 10$ for all groups). Data show mRNA expression levels from inguinal white adipose tissue. All data in **d**–**j** were performed at least twice in a separate mouse cohort with similar results. †$P < 0.05$ compared to exercise and IgG. One-way ANOVA was used for statistics in **h**. All other statistics were performed using Student's *t*-test, and bar graphs are mean ± s.e.m.

There was also an elevation in expression of several mitochondrial genes (Supplementary Fig. 8). Notably, these changes occurred with only moderately increased irisin blood levels, threefold compared to the GFP-expressing mice. This effect was accompanied with a large increase in oxygen consumption (Fig. 6d), consistent with the gene expression data, and body weights of the irisin-expressing mice were slightly reduced after 10 days compared to GFP-expressing controls (Fig. 6e). These effects of irisin on mitochondrial gene expression in the fat were not seen in skeletal muscle *in vivo* or in cultured myocytes (Supplementary Fig. 10). Irisin expression in the mice fed a high fat diet caused a significant improvement in glucose tolerance when compared to the control mice expressing GFP. In addition, fasting insulin was also reduced (Fig. 6f, g). These data illustrate that even moderately increased levels of circulating irisin potently increase energy expenditure, reduce body weight and improve diet-induced insulin resistance.

Finally, we asked whether irisin is required for the exercise-induced effects on the subcutaneous white fat. Injection of anti-FNDC5 antibodies into mice before 10 days of swim training dramatically reduced the effect of this exercise on *Ucp1* and *Cidea* gene expression, compared to injection of control antibodies (Fig. 6h). In contrast, *Prdm16* mRNA levels were not increased with exercise and were also not affected by the anti-FNDC5 antibodies. Thus, irisin is required for a substantial part of the effect of exercise on these gene expression events in the browning of white fat.

Discussion

Exercise has the capacity to improve metabolic status in obesity and type 2 diabetes, but the mechanisms are poorly understood. Importantly, exercise increases whole body energy expenditure beyond the calories used in the actual work performed[14]. However, the relative contribution of the adipose tissues to this phenomenon has not been clarified. Because transgenic mice expressing PGC1-α selectively in muscle showed a remarkable resistance to age-related obesity and diabetes[4], we sought factors secreted from muscle under the control of this co-activator that might increase whole body energy expenditure. We describe a new polypeptide hormone, irisin, which is regulated by PGC1-α, secreted from muscle into blood and activates thermogenic function in adipose tissues. Irisin is remarkable in several respects. First, it has powerful effects on the browning of certain white adipose tissues, both in culture and *in vivo*. Nanomolar levels of this protein increase UCP1 in cultures of primary white fat cells by 50 fold or more, resulting in increased respiration. Perhaps more remarkable, viral delivery of irisin that causes only a moderate increase (~3 fold) in circulating levels stimulates a 10–20 fold increase in UCP1, increased energy expenditure and an improvement in the glucose tolerance of mice fed a high fat diet. As this is in the range of increases seen with exercise in mouse and man, it is likely that irisin is responsible for at least some of the beneficial effects of exercise on the browning of adipose tissues and increases in energy expenditure. It is important to note that the evidence provided here does not exclude a role for other tissues besides muscle in the biological regulation and secretion of irisin.

Second, the cleaved and secreted portion of FNDC5, the hormone irisin, is highly conserved in all mammalian species sequenced. Mouse and human irisin are 100% identical, compared to 85% identity for insulin, 90% for glucagon and 83% identity for leptin. This certainly implies a highly conserved function that is likely to be mediated by a cell surface receptor. The identity of such a receptor is not yet known.

On the basis of the gene structure of *Fndc5*, with a signal peptide that was evidently missed in previous studies[12], we considered that FNDC5 might be a secreted protein. Indeed, we have observed that the signal peptide is removed, and the mature protein is further proteolytically cleaved and glycosylated, to release the 112-amino-acid polypeptide irisin. The cleavage and secretion of irisin is similar to the release/shedding of other transmembrane polypeptide hormones and hormone-like molecules such as epidermal growth factor (EGF) and transforming growth factor-α (TGF-α).

As the conservation of calories would probably provide an overall survival advantage for mammals, it seems paradoxical that exercise would stimulate the secretion of a polypeptide hormone that increases thermogenesis and energy expenditure. One explanation for increased irisin expression with exercise in mouse and man may be that it evolved as a consequence of muscle contraction during shivering. Muscle secretion of a hormone that activates adipose thermogenesis during this process might provide a broader, more robust defence against hypothermia.

The therapeutic potential of irisin is obvious. Exogenously administered irisin induces the browning of subcutaneous fat and thermogenesis, and it presumably could be prepared and delivered as an injectable polypeptide. Increased formation of brown or beige/brite fat has been shown to have anti-obesity, antidiabetic effects in multiple murine models[6], and adult humans have significant deposits of UCP1-positive brown fat[15]. Data presented here show that even relatively short treatments of obese mice with irisin improves glucose homeostasis and causes a small weight loss. Whether longer treatments with irisin and/or higher doses would cause more weight loss remains to be determined. The worldwide, explosive increase in obesity and diabetes renders attractive the therapeutic potential of irisin in these and related disorders.

Another potentially important aspect of this work relates to other beneficial effects of exercise, especially in some diseases for which no effective treatments exist. The clinical data linking exercise with health benefits in many other diseases suggests that irisin could also have significant effects in these disorders.

METHODS SUMMARY

Primary mouse stromal vascular fractions from adipose tissues were differentiated as described[6]. FNDC5/irisin were purchased from ABNOVA (GST fused), or produced from Syd Laboratories (GST fused) or LakePharma (Fc fusions). Hydrodynamic injections[16], electron microscopy[17] and treadmil running[18] were performed as previously described. Unless otherwise stated, bar graph data are presented as mean $\pm$ s.e.m., and $^{*}P < 0.05$ compared to control group. Student's *t*-test was used for single comparisons and one-way ANOVA for multiple.

Full Methods and any associated references are available in the online version of the paper at www.nature.com/nature.

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Author Contributions P.B. and B.M.S. planned the majority of experiments and wrote the paper, and P.B. executed most of the experiments. J.W. performed a subset of cultured cell experiments and contributed valuable materials. M.P.J. and S.P.G. performed the peptide fingerprinting identification of irisin cleavage. A.K. contributed with technical assistance and L.Y. and S.K. performed the CLARK electrode experiments. E.A.B. assisted with the hydrodynamic injections. J.C.L. assisted with intravenous injections and K.A.R. with bioinformatics. J.Z.L. and J.H.C. performed *in vitro* experiments. P.B. and H.T. and LakePharma designed and provided Fc fusion proteins. K.H. and B.F.V. performed the human cohort study, and M.C.Z. and S.C. performed the electron microscopy studies.

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 this article at www.nature.com/nature. Correspondence and requests for materials should be addressed to B.M.S. (bruce_spiegelman@dfci.harvard.edu).

METHODS

Materials. Antibodies against UCP1, tubulin and FNDC5 were from Abcam. Forskolin, insulin, dexamethasone, rosliglitazone, GW6471 and antibody against Flag were from Sigma. Primers for all qPCR experiments are listed in Supplementary Table 1. Recombinant FNDC5, LRG1, IL-15, VEGF-β and TIMP4 were from ABNOVA (Taiwan). Coomassie staining kit and Lipofectamine 2000 were from Invitrogen.

Identification of PGC1-α-dependent secreted proteins. All PGC1-α-induced genes as judged from gene expression analysis in MCK-PGC1-α muscle with a fold change of at least 2 and $P < 0.05$ were subjected to the following analysis. The protein sequence of the longest transcript were analysed in the SignalP-software[19] (http://www.cbs.dtu.dk/services/SignalP/). Sequences with positive *S*, *C*, *Y* and *D* scores were considered positive for a signal sequence. All positive proteins were then screened for mitochondrial target sequences (http://www.cbs.dtu.dk/services/TargetP/) whereas positive sequences were removed. All remaining hit proteins were then analysed using qPCR in muscle from MCK-PGC1-α mice and myocytes overexpressing PGC1-α.

Primary cell cultures and recombinant protein treatments. The SVF from inguinal fat depots of 8–12-week-old BALB/C mice were prepared and differentiated for 6 days as previously described[20]. Rosiglitazone was used for the two first days of differentiation. For all experiments, unless otherwise indicated, recombinant FNDC5 was added to the culture media at a concentration of 1 μg ml^{-1} for the last 4 days of differentiation. Primary myoblasts were cultured and differentiated as described previously[21].

Preparation of protein fractions from cells and media. 293HEK or primary myocytes were transfected by standard methods or transduced with adenovirus at a multiplicity of infection (m.o.i.) of 20 as indicated. Twenty-four hours after transfection, media was removed, and cells were washed in large volumes of PBS five times, followed by incubation in Freestyle serum-free media (GIBCO) for 24 h. The cells and media were then collected separately, and media were centrifuged three times at 2,000*g* to pellet debris. Thereafter, a quarter volume of ice-cold TCA was added and precipitated protein was pelleted at 6,000*g* and washed three times in acetone. Pellet was then dried and resuspended in SDS-containing lysis buffer. Protein concentration was measured in both cell and media fraction and adjusted either by protein or volume as indicated.

RT–PCR. qPCR was carried out after Trizol-based RNA extraction using RNAeasy (Invitrogen) and thereafter SYBR green. All data were normalized to TBP, 18S or indicated in-house genes and quantitative measures were obtained using the $\Delta\Delta C_T$ method.

Western blot and quantification. Protein amounts from all samples were assessed using the BCA-kit (Thermo Scientific) followed by protein concentration normalization before all western blot experiments. Western blot was carried out following standard procedure and final band intensity (QL-BG) was quantified using BioPix iQ[22]. All data were normalized to background and loading controls.

Additional methods. CLARK electrode measurements, energy expenditure *in vivo*, interaperitoneal glucose tolerance test (IGTT) and immunohistochemistry against UCP1 were performed as described previously[6], with the exception that CLARK output was normalized to total cell protein. Fc-fusion construction and protein purification was performed by LakePharma.

Comprehensive laboratory animal monitoring system. C57/Bl6J mice were fed a high fat (60% kcal) diet (D12492, Research Diets) for 20 weeks, starting at 6 weeks of age. Mice were then injected with indicated doses of adenovirus expressing GFP or FNDC5, and comprehensive laboratory animal monitoring system (CLAMS; Columbia Instruments)-cage analysis was performed as described previously[6]. Briefly, mice were acclimated in metabolic chambers for 2 days before analysis in order to minimize stress. CO_2 and O_2 levels were then collected every 36 min for a period of 3 days. Data on activity, heat generation and food intake were measured at more frequent intervals. Circadian rhythm was observed for most parameters. Data were not normalized to body weights unless otherwise stated.

Mass spectrometry and peptide fingerprinting. Gel bands were digested with sequencing grade trypsin (Promega) or ASP-N (Sigma-Aldrich) as per manufacturer's instructions. Extracted in-gel protein digests were resuspended in 8 μl 5% formic acid/5% acetonitrile, and 4 μl were analysed by microcapillary liquid chromatography electrospray ionization tandem mass spectrometry (LC-MS/MS). Analyses were done on a LTQ Orbitrap Velos mass spectrometer (Thermo Fisher Scientific) equipped with a Thermo Fisher Scientific nanospray source, an Agilent 1100 Series binary HPLC pump and a Famos autosampler. Peptides were separated on a 100 m × 16 cm fused silica microcapillary column with an in-house made needle tip. The column was packed with MagicC18AQ C_{18} reversed-phase resin (particle size, 5 μm; pore size, 200 Å; Michrom Bioresources). Separation was achieved through applying a 30 min gradient from 0–28% acetonitrile in 0.125% formic acid. The mass spectrometer was operated in a data-dependent mode essentially as described previously[23] with a full MS scan acquired with the Orbitrap, followed by up to ten LTQ MS/MS spectra on the most abundant ions detected in the MS scan. Mass spectrometer settings were: full MS (automated gain control, 1×10^6; resolution, 6×10^4; m/z range, 375–1,500; maximum ion time, 1,000 ms); MS/MS (AGC, 5×10^3; maximum ion time, 120 ms; minimum signal threshold, 4×10^3; isolation width, 2 Da; dynamic exclusion time setting, 30 s). After MS data acquisition, RAW files were converted into mzXML format and processed using a suite of software tools developed in-house for analysis. All precursors selected for MS/MS fragmentation were confirmed using algorithms to detect and correct errors in monoisotopic peak assignment and refine precursor ion mass measurements. All MS/MS spectra were then exported as individual DTA files and searched with no enzyme using the Sequest algorithm. These spectra were then searched against a database containing sequence of mouse FNDC5 in both forward and reversed orientations. The following parameters were selected to identify FNDC5: 10 p.p.m. precursor mass tolerance, 0.8 Da product ion mass tolerance, fully tryptic or ASP-N digestion, and up to two missed cleavages. Variable modifications were set for methionine (+15.994915). In addition, a fixed modification for the carbamidomethylation for cysteine (+57.021464) was used as well. The C-terminal fragment for FNDC5 was identified (KDEVTMKE) by trypsin digestion and reconfirmed by a separate ASP-N digestion.

Preparation of plasma samples for western blot. Thirty-five microlitres of mouse or human plasma were precleared for albumin/IgG using the ProteoExtract-kit (CalBiochem) as recommended by the manufacturer. Samples were then concentrated to approximately 100 μl and >8 μg μl^{-1}, followed by deglycosylation of 150 μg using PNGase F (New England Biolabs). Totally, 80 μl were then prepared containing 1× sample buffer with reducing agent and 1.7 μg μl^{-1} protein, sonicated, boiled and analysed using western blot against FNDC5 or indicated antibody.

Construction of adenoviral vectors. The FNDC5 expression vector was purchased with a C-terminal Flag-tag from OriGene. The QuickChange Multi Site XL Directed Mutagenesis Kit (Aligent Technologies) was used to introduce a Flag tag downstream of the signal sequence and to mutate the C-terminal Flag tag, thus resulting in the N-terminal Flag (NTF)–FNDC5 construct (Fig. 5a). The NTF and CTF FNDC5 constructs were then subcloned into the pENTR1a vector (Invitrogen) and recombined into the pAd-CMV-DEST-V5 vector (Invitrogen) and adenovirus was produced using the virapower system (Invitrogen), including three rounds of amplification. Thereafter, virus was concentrated using the Vivapure adenopack 100 (Sartorius Stedim Biotech) and buffer exchanges to saline reaching a concentration of 9–10 i.f.u. μl^{-1}. A GFP-containing adenovirus previously used was prepared in parallel.

Transgenic mice. The MCK-PGC1-α transgenic and muscle-specific PGC1-α knockout mice have been described previously[24].

Exercise protocols. Twelve-week-old B6 mice were exercised either using swimming[25] or using free wheel running, as described previously[26].

Human material and exercise training program. Blood samples and skeletal muscle biopsies were obtained from eight male non-diabetic individuals before and after 10 weeks of aerobic training as described previously[7]. In brief, the exercise-training program consisted of cycling on stationary bikes with 4–5 sessions of 20–35 min per week at an average intensity of ~65% of maximal oxygen consumption. Informed consent was obtained from all volunteers before participation. The study was approved by the Local Ethics Committee and was performed in accordance with the Helsinki Declaration.

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