Browning of white fat: does irisin play a role in humans?

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Abstract
The discovery of irisin as an exercise-regulated myokine inducing browning of WAT has gained interest as a potential new strategy to combat obesity and its associated disorders, such as type 2 diabetes. However, there are inconsistencies regarding the relevance of irisin in humans. The regulation of FNDC5 mRNA expression by exercise and contraction could not be reproduced by a number of human studies using several exercise protocols and in vitro approaches. Furthermore, the nature of FNDC5 fragments and the presence of irisin in humans are questionable and probably contribute to conflicting data obtained with commercially available ELISA kits. Most importantly, the information regarding the concentration of circulating irisin in humans is not clear, as different studies using different kits measure irisin levels in a wide range. Data about the role of irisin in states of human obesity and metabolic diseases are conflicting and, in some cases, changes in irisin levels have been observed; they were only moderate in 10–20%. Independent of the presence and regulation of FNDC5/irisin in humans, the application of recombinant irisin could still represent a therapeutic strategy to fight obesity. However, the current data obtained from human cell models reveal that FNDC5/irisin has no effect on browning of the major WAT depots in humans and is likely to selectively target a small subpopulation of adipocytes, which are located in classical BAT regions, such as the supraclavicular adipose tissue. Thus, other candidates, such as BMP7 or CNPs, seem to be more prominent candidates as inducers of browning in humans.

Key Words
- adipocyte
- diabetes
- exercise
- obesity
- muscle

Introduction
White adipose tissue (WAT) and brown adipose tissue (BAT) exert inverse functions in that WAT stores energy, whereas BAT, characterized by a large number of mitochondria containing uncoupling protein 1 (UCP1), mediates adaptive thermogenesis and contributes to the maintenance of body temperature. In 2009, five independent studies demonstrated the presence of BAT in adult humans (Cypess et al. 2009, van Marken Lichtenbelt et al. 2009, Saito et al. 2009, Virtanen et al. 2009, Zingaretti et al. 2009) and confirmed the existence of an inverse correlation between BMI and BAT activity. These observations have aroused considerable interest in the therapeutic potential of brown adipocytes for inducing weight loss, and recent data have confirmed that BAT oxidative metabolism contributes significantly to energy expenditure (Ouellet et al. 2012). The classical view of brown and white fat cells was modified in 2010 by a publication of the Cannon/Nedergaard group describing a third type of fat cells termed ‘brite’ (brown-in-white) adipocytes (Petrovic et al. 2010). These cells share biochemical features and the thermogenic potential with brown adipocytes, but are derived from different precursor cells (Seale et al. 2008).
A novel hormone-like myokine termed irisin has recently been described to activate such a white-to-brown shift in adipocytes (Boström et al. 2012a). As this molecule was originally reported to be released after physical activity, it gained huge interest as a potential mediator of the health-promoting effects of physical exercise. Irisin is a 112 amino acid peptide cleaved from fibronectin type III domain containing protein 5 (FNDC5), a type I membrane protein which was claimed to be the mediator of the health-promoting effects of physical activity, it gained huge interest as a potential molecule was originally reported to be released after exercise. Irisin is a 112 amino acid peptide cleaved from fibronectin type III domain containing protein 5 (FNDC5), a type I membrane protein which was claimed to be upregulated by exercise training in both mice and humans (Boström et al. 2012a). When writing this review, more than 100 papers dealing with irisin have been found in PubMed. However, the physiological role and the potential therapeutic value of irisin have remained highly controversial. In this review, we analyzed the current literature regarding exercise regulation and the functional impact of irisin in humans. We conclude that: i) the nature and concentration of circulating FNDC5 fragments remain unclear and ii) irisin has no effect on major WAT depots in humans and may only target a small subpopulation of adipocytes.

**Impact of exercise on browning of white adipose tissue**

As noticed before, the exercise-regulated myokine irisin was identified and described as a link between exercise and the promotion of WAT browning (Boström et al. 2012a). It is well known that the transcriptional coregulator PGC1α is induced in muscle in response to exercise in rodents and humans (Goto et al. 2000, Pilegaard et al. 2003). Therefore, transgenic mice overexpressing Pgc1α (Pparγc1α) in skeletal muscle can be used as an exercise model (Boström et al. 2012a). Interestingly, these transgensics exhibited increased expression of the brown marker genes Ucp1 and cell death-inducing DFFA-like effector A (Cidea) only in the inguinal depot, suggesting that muscle activity promotes remodeling of subcutaneous WAT. Indeed, UCP1 expression was also strongly upregulated in the subcutaneous inguinal depot of mice after 3 weeks of wheel running.

From a physiological point of view, the induction of brown-like fat in response to exercise appears to be surprising due to several factors. First, exercise itself is an energy-consuming process and skeletal muscle is supplied with energy sources from other organs, such as free fatty acids released from WAT during physical activity. In this context, Kelly (2012) raised the question as to why physical activity would induce a program that burns fat stores, which are needed for the exercising muscle. Secondly, it has been discussed by Cannon & Nedergaard (2004) that there should be a diminished demand for non-shivering thermogenesis during exercise, as heat is generated by skeletal muscle contraction. In accordance, several studies on rats have demonstrated that treadmill running has no effect on Ucp1 mRNA levels in BAT (Scarpace et al. 1994, Segawa et al. 1998, De Matteis et al. 2013) as well as on BAT mass (Segawa et al. 1998). The interscapular BAT of the Pgc1α transgenic mice used by Boström et al. (2012a) was also not altered when compared with WT mice. Furthermore, even a trend toward a decrease in Ucp1 mRNA expression in BAT has been observed in rats after 3 weeks of endurance exercise (Roca-Rivada et al. 2013). By contrast, only one study demonstrated that treadmill exercise training of mice leads to enhanced expression of certain brown adipocyte-specific genes in BAT (Xu et al. 2011). Thus, exercise intervention is likely to have no effect on classical BAT in rodents. Regarding the browning of WAT, an increase in the number of mitochondria and enhanced expression of brown-specific genes in the visceral epididymal WAT of mice have been demonstrated after exercise (Xu et al. 2011). Another study carried out on rats also observed induction of UCP1 expression in the visceral but not the subcutaneous WAT after 3 weeks of training (Roca-Rivada et al. 2013). Boström et al. (2012a) demonstrated the most prominent effect of exercise on the subcutaneous WAT and the data obtained were in contrast to the two above-mentioned studies.

In addition to these studies on rodents, a potential induction of WAT browning in response to exercise remains to be clarified in case of humans. A recent study performing a 12-week training intervention in humans could not detect any alterations in WAT. Thus, expression of several brown adipocyte-specific marker genes, such as UCP1 and PRDM16, were not significantly changed in subcutaneous adipose tissue after the intervention (Norheim et al. 2014). Hence, studies assessing the impact of exercise on WAT browning in rodents are conflicting, especially with regard to the WAT depot that undergoes remodeling in response to exercise. Furthermore, the browning of WAT in response to exercise has not yet been demonstrated in humans and, hence, further studies in humans are necessary.
Exercise regulation of FNDC5 mRNA in skeletal muscle: in vivo studies

In the initial report by Boström et al. (2012a), irisin is proposed to be a novel PGC1α-dependent and exercise-responsive myokine. This conclusion was based on the finding that skeletal muscle Fndc5 mRNA levels from mice after 3 weeks of free wheel running were enhanced compared with sedentary mice (about 2.8-fold). Moreover, enhanced Fndc5 mRNA expression was accompanied by increased Pgc1a mRNA levels (about 2.5-fold). Additionally, the expression levels of FNDC5 and PGC1A were examined in muscle biopsies from human subjects before and after 10 weeks of endurance training. Expression of both genes was enhanced after the training session (about twofold) (Boström et al. 2012a).

After this initial description of FNDC5 and irisin as exercise-regulated proteins in mice and humans, FNDC5 mRNA expression in skeletal muscle was analyzed in several human exercise cohorts (Table 1). Intriguingly, most of these studies performed in humans failed to reproduce enhanced FNDC5 mRNA levels after exercise. The upregulation of FNDC5 in skeletal muscle by exercise was only demonstrated in four out of 15 studies, including the study by Boström et al. In this context, they reported the most prominent increase in FNDC5 mRNA expression after exercise (twofold) (Boström et al. 2012a), whereas three other studies reported only a moderate increase of 1.3- to 1.4-fold (Timmons et al. 2012, Pekkala et al. 2013, Norheim et al. 2014).

Timmons et al. (2012) were the first unable to reproduce a substantial training-induced increase in FNDC5 expression, neither in young men after endurance training nor in a different cohort (20–80 years old subjects) after resistance training. However, it is possible that FNDC5 mRNA expression can be induced by exercise in a subset of individuals, as older active subjects had a 30% higher FNDC5 expression than sedentary control subjects, while no difference was observed in younger subjects (Timmons et al. 2012). As FNDC5 has been described to be PGC1α-dependent (Boström et al. 2012a), this early study by Timmons et al. (2012) has been criticized for including exercise intervention studies without reported induction of PGC1α expression in muscle (Boström et al. 2012b). Thus, a lack of PGC1α induction in some of the exercise cohorts may explain the conflicting results.

In line with this suggestion, exercise intervention studies reporting increased muscle PGC1α expression would potentially clarify the relation between FNDC5 and PGC1A mRNA expression in skeletal muscle. Recently, Norheim et al. (2014) have compared the effects of acute and chronic exercises and assessed the correlation between PGC1A and FNDC5 expression in these exercise types. Therefore, a cohort of 13 sedentary men, aged 40–65 years, underwent a 12-week intervention of combined endurance and strength training. Muscle biopsies were taken after an acute endurance workload, both before (acute) and after (chronic) the 12-week intervention period. Interestingly, after 12 weeks of exercise, PGC1α was slightly activated (1.2-fold) and FNDC5 mRNA levels were additionally increased (1.4-fold) (Norheim et al. 2014), being in line with the initial report by Boström et al. However, acute exercise intervention performed by the same subjects gave different results. Regardless of the prominent increase in PGC1A mRNA levels (7.4-fold), FNDC5 mRNA expression levels did not differ at all (Norheim et al. 2014). Prominent effects on PGC1A mRNA expression have also been described in other acute exercise cohorts (twofold and fourfold (Pekkala et al. 2013)), whereas chronic exercise had a moderate effect (1.2-fold (Norheim et al. 2014) and 1.5-fold (Boström et al. 2012a)).

In conclusion, the regulation of FNDC5 expression by exercise could not be reproduced by the majority of studies performed in humans (Table 1). Furthermore, even a strong increase in gene expression of the transcriptional coactivator PGC1A occurring after acute exercise does not necessarily lead to an activation of FNDC5 expression (Pekkala et al. 2013, Norheim et al. 2014).

Exercise regulation of FNDC5 mRNA in skeletal muscle: in vitro models

To further investigate the effect of PGC1α on FNDC5 expression in human skeletal muscle cells, in vitro studies were performed on primary human myotubes (Besse-Patin et al. 2014, Raschke et al. 2013, Norheim et al. 2014).

Treatment of primary human myotubes with drugs mimicking the activation of exercise signaling pathways, namely caffeine, ionomycin, and forskolin, significantly increased PGC1A expression. However, this induction of PGC1A mRNA expression was not accompanied by enhanced FNDC5 expression. Indeed, FNDC5 mRNA was reduced after incubating the myotubes for 24 h with these exercise mimetics (Norheim et al. 2014). In line with this, a second study also demonstrated significantly decreased FNDC5 expression levels in primary human myotubes challenged with ionomycin and forskolin (Besse-Patin et al. 2014).
<table>
<thead>
<tr>
<th>Study (reference)</th>
<th>Patients/subjects</th>
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<th>Exercise mode</th>
<th>Intervention</th>
<th>Main results</th>
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</thead>
<tbody>
<tr>
<td>Bostrom et al. (2012a)</td>
<td>Healthy adults</td>
<td>8</td>
<td>A</td>
<td>10 weeks of supervised endurance training</td>
<td>Twofold increase in FNDC5 mRNA expression in muscle</td>
</tr>
<tr>
<td>Besse-Patin et al. (2014)</td>
<td>Obese, non-diabetic subjects</td>
<td>11</td>
<td>A</td>
<td>8 weeks of supervised endurance training</td>
<td>No change in FNDC5 mRNA expression in muscle</td>
</tr>
<tr>
<td>Kurdiova et al. (2014)</td>
<td>Sedentary, obese individuals</td>
<td>16</td>
<td>A</td>
<td>12 weeks strength/endurance training</td>
<td>No change in FNDC5 mRNA expression in muscle</td>
</tr>
<tr>
<td>Norheim et al. (2014)</td>
<td>Normoglycemic, sedentary men</td>
<td>13</td>
<td>A</td>
<td>12 weeks combined endurance and strength training</td>
<td>1.4-fold increase in FNDC5 mRNA expression in muscle</td>
</tr>
<tr>
<td>Pekkala et al. (2013)</td>
<td>Healthy, untrained male</td>
<td>17</td>
<td>B</td>
<td>1 h acute low-intensity aerobic exercise</td>
<td>No change in FNDC5 mRNA expression in muscle</td>
</tr>
<tr>
<td>Raschke et al. (2013)</td>
<td>Young, sedentary males</td>
<td>6</td>
<td>A</td>
<td>10 weeks of aerobic interval training</td>
<td>No change in FNDC5 mRNA expression in muscle</td>
</tr>
<tr>
<td>Timmons et al. (2012)</td>
<td>Young sedentary males</td>
<td>24</td>
<td>A</td>
<td>6 weeks intense endurance cycling</td>
<td>No change in FNDC5 mRNA expression in muscle</td>
</tr>
<tr>
<td>Young vs older sedentary and age-matched endurance trained</td>
<td>10 vs 10</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>1.3-fold increase in FNDC5 mRNA expression in muscle of older trained compared with older sedentary subjects</td>
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</table>
To circumvent exercise mimetics as an in vitro exercise model, electrical pulse stimulation (EPS) has been performed to induce muscle contraction in vitro. This model is well established and primary human myotubes subjected to EPS are characterized by enhanced PGC1A mRNA expression, enhanced mitochondrial biogenesis as well as enhanced secretion of the well-known myokines IL6 and VEGFA (Lambernd et al. 2012). Moreover, this EPS model reflects a training model rather than acute exercise as shown by the enhanced MYH1 mRNA level and enhanced mitochondrial content (Lambernd et al. 2012, Raschke et al. 2013). Similar to the results obtained with exercise mimetics, FNDC5 mRNA expression was not significantly enhanced in primary human myotubes after EPS, although PGC1A expression was significantly increased (Raschke et al. 2013).

To sum up, all of these in vitro studies performed with primary human skeletal muscle cells failed to demonstrate an increase in FNDC5 expression using several approaches to mimic exercise in vitro. The lack of PGC1α induction as a potential reason for the absence of augmented FNDC5 expression can be excluded, as PGC1A gene expression was significantly enhanced in all models.

Taken all described human in vivo and in vitro studies together, discussing all these discrepancies between PGC1A activation and FNDC5 mRNA expression, Norheim et al. (2014) speculated that FNDC5 is not a direct PGC1α target gene but is rather upregulated in skeletal muscle in vivo via secondary mechanisms.

The initial characterization of a candidate myokine is frequently limited to the detection of mRNA expression in skeletal muscle tissue, as it has also been done for FNDC5. Moreover, determination of gene expression or protein level in skeletal muscle biopsies is critical, as besides skeletal muscle fibers, skeletal muscle contains extended layers of connective tissues, capillaries, and nerve cells among others. Thus, gene expression studies must be followed by the detection of the encoded protein specifically in skeletal muscle fibers, e.g., by additional immunostaining of the skeletal muscle tissue sections. Finally, for full validation of a protein as a myokine, secretion from skeletal muscle cells has to be demonstrated.

Exercise regulation of circulating irisin in humans

Key points in the study by Bostrom et al. (2012a) were that the irisin fragment was present in the plasma of mouse and humans and that circulating levels were enhanced following exercise, as observed for skeletal muscle FNDC5 expression in this study. The presence of irisin protein in plasma was based on western blots using an antibody which detects the transmembrane segment of FNDC5 and thus would fail to detect the C-terminally cleaved, secreted irisin fragment (Erickson 2013, Raschke et al. 2013). However, later on, several ELISA assays to detect circulating irisin became commercially available and were used to quantify irisin concentrations in human exercise studies (Table 2).

Using these ELISA kits, some of these human studies reported moderately increased serum irisin levels after exercise intervention. Thus, Kraemer et al. (2014) reported transiently elevated circulating irisin levels in response to moderate aerobic exercise during the first hour after exercise (20% increase) (Kraemer et al. 2014). In line with this finding, Huh et al. (2012) observed a moderate increase in circulating irisin level 30 min after a sprint running session (18% increase) (Huh et al. 2012) and Norheim et al. (2014) demonstrated slightly increased irisin levels after 45 min cycling (20% increase) (Norheim et al. 2014). In contrast to FNDC5 mRNA data, enhanced circulating irisin levels were found in acute exercise studies rather than in long-term training studies.

Despite these three reports, 12 out of 15 studies failed to demonstrate that exercise affects circulating irisin levels in humans, neither after an acute bout of exercise nor after chronic exercise training (Huh et al. 2012, Aydin et al. 2013, Hecksteden et al. 2013, Moraes et al. 2013, Pekkala et al. 2013, Kurdiova et al. 2014, Norheim et al. 2014). Recently, Hecksteden et al. (2013) have published a randomized control trial and focused on the serum irisin concentrations. Subjects performed two guideline-conforming training interventions, either endurance or strength endurance training for 26 weeks. Once again, a training-induced increase in circulating irisin levels could not be confirmed (Hecksteden et al. 2013). Interestingly, although Norheim et al. (2014) observed an increased expression of PGC1A and FNDC5 after 12 weeks of exercise, this was not translated into chronically increased levels of irisin in plasma and training for 12 weeks even tended to reduce irisin levels.

Summarizing the above-mentioned ELISA results, the current data provide equivocal results for regulation of circulating irisin levels after exercise. Only some studies reported slightly increased irisin levels after acute exercise, while the majority of studies failed to reproduce the results obtained by Bostrom et al. (summarized in Table 2).
Impact of irisin on WAT browning

The idea of irisin as an exercise-regulated myokine in humans and the physiological role of exercise-mediated browning of WAT have been discussed in the previous section. Independent of the potential regulation of circulating irisin and skeletal muscle FNDC5 expression in response to acute or chronic exercise, the functionality of irisin in humans remains to be elucidated. Bostro¨m et al. (2012a) initially demonstrated that recombinant irisin and FNDC5 induce browning of WAT-derived murine preadipocytes in vitro. In the context of irisin as a therapeutic approach in the fight against obesity and its associated metabolic diseases, it is crucial to prove that irisin has an effect on white-to-brown transition in human cell models. Therefore, functional studies investigating the potency of irisin as an inducer of browning, performed in rodents and humans, will be discussed in this section.

After selection of secreted PGC1α- and exercise-regulated proteins/myokines in mice, Bostro¨m et al. (2012a) assessed their potential as inducers of browning and identified FNDC5 as a promising candidate. To study browning of WAT in vitro, murine preadipocytes were isolated from the inguinal fat depot and treated with commercially available recombinant FNDC5 (Abnova, Table 2

<table>
<thead>
<tr>
<th>Study (reference)</th>
<th>Patients/subjects</th>
<th>n</th>
<th>Exercise mode</th>
<th>Intervention</th>
<th>Main result</th>
</tr>
</thead>
<tbody>
<tr>
<td>Boström et al. (2012a)</td>
<td>Healthy adults</td>
<td>8</td>
<td>A</td>
<td>10 weeks of supervised endurance training WB with Abnova antibody – does not detect irisin</td>
<td>No change in circulating irisin levels between groups</td>
</tr>
<tr>
<td>Hecksteden et al. (2013)</td>
<td>Healthy adults: Control</td>
<td>39</td>
<td>A</td>
<td>26 weeks of supervised aerobic endurance or strength endurance training</td>
<td>No change in circulating irisin levels</td>
</tr>
<tr>
<td></td>
<td>Aerobic training</td>
<td>23</td>
<td></td>
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<td></td>
<td>Strength training</td>
<td>40</td>
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<tr>
<td></td>
<td>Young, moderately trained, healthy males</td>
<td>15</td>
<td>B</td>
<td>1 week of exercise (2–3 sets of two 80-m sprints)</td>
<td>Circulating irisin levels were significantly induced (18%) 30 min after the exercise</td>
</tr>
<tr>
<td></td>
<td>Young, moderately trained, healthy males</td>
<td>15</td>
<td>A</td>
<td>8 weeks of exercise (three times a week, 2–3 sets of two 80-m sprints)</td>
<td>No change in circulating irisin levels</td>
</tr>
<tr>
<td>Kraemer et al. (2014)</td>
<td>Healthy, young male</td>
<td>7</td>
<td>B</td>
<td>90 min treadmill exercise</td>
<td>Circulating irisin levels were significantly induced (20%) by 54 min exercise</td>
</tr>
<tr>
<td>Kurdiova et al. (2014)</td>
<td>Sedentary vs trained</td>
<td>7 vs 8</td>
<td>B</td>
<td>1 h 75% of maximal capacity</td>
<td>No change in circulating irisin levels</td>
</tr>
<tr>
<td>Moraes et al. (2013)</td>
<td>Hemodialysis patients</td>
<td>26</td>
<td>A</td>
<td>6 months supervised resistance exercise</td>
<td>No change in circulating irisin levels</td>
</tr>
<tr>
<td>Norheim et al. (2014)</td>
<td>Normoglycemic, sedentary men</td>
<td>13</td>
<td>A</td>
<td>12 weeks combined endurance and strength training</td>
<td>No change in circulating irisin levels</td>
</tr>
<tr>
<td></td>
<td>Normoglycemic, sedentary men</td>
<td>13</td>
<td>B</td>
<td>45 min cycling at 70% VO2 max</td>
<td>1.2-fold increase in circulating irisin levels directly after exercise</td>
</tr>
<tr>
<td>Pekkala et al. (2013)</td>
<td>Healthy, untrained male</td>
<td>17</td>
<td>B</td>
<td>1 h acute low-intensity aerobic exercise</td>
<td>No change in circulating irisin levels</td>
</tr>
<tr>
<td></td>
<td>Healthy, young male</td>
<td>14</td>
<td>B</td>
<td>Single resistance exercise bout</td>
<td>No change in circulating irisin levels</td>
</tr>
<tr>
<td></td>
<td>Healthy, young male</td>
<td>10</td>
<td>B</td>
<td>Single resistance exercise bout</td>
<td>No change in circulating irisin levels</td>
</tr>
<tr>
<td></td>
<td>Healthy, untrained middle-aged male</td>
<td>9</td>
<td>A</td>
<td>21 weeks high-intensity endurance exercise</td>
<td>No change in circulating irisin levels</td>
</tr>
<tr>
<td></td>
<td>Healthy, untrained middle-aged male</td>
<td>9</td>
<td>A</td>
<td>21 weeks combined endurance and resistance exercise</td>
<td>No change in circulating irisin levels</td>
</tr>
<tr>
<td>Aydin et al. (2013)</td>
<td>Obese males vs healthy males</td>
<td>7 vs 7</td>
<td>B</td>
<td>45 min of moderate outdoor running (5.5 km/45 min)</td>
<td>No change in circulating irisin levels</td>
</tr>
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</table>

A, long-term exercise intervention; B, acute exercise.
Taiwan, China) during adipogenic differentiation. The inguinal depot is regarded as a subcutaneous and generally white fat depot, but has a high ability to undergo browning in response to a cold environment (Walden et al. 2012) and hormonal stimuli such as BMP7 (Schulz et al. 2011). Treatment of primary murine subcutaneous preadipocytes with 20 nM recombinant FNDC5 increased the expression of the BAT marker genes Ucp1, Cidea, and Pgc1a as well as oxygen consumption as a functional readout (Boström et al. 2012a). By contrast, FNDC5 failed to enhance brown marker genes in classical brown adipocytes isolated from the interscapular depot, suggesting tissue- and/or lineage-specific effects for FNDC5. The beneficial effect of irisin has also been demonstrated in vivo, as high-fat diet-induced obesity was reduced by adenoviral-mediated overexpression of FNDC5 in mice (Boström et al. 2012a).

The idea of a tissue-dependent impact of FNDC5 on browning was further elucidated by a second study from the same group. They proposed that brite or beige adipocytes display a distinct subpopulation within white adipose tissue and are highly responsive to β-adrenergic stimulation similar to classical brown adipocytes (Wu et al. 2012). In this context, CD137 (TNFRSF9) and TMEM26 were identified as selective cell surface marker genes highly expressed in brite adipocytes, but with a low expression in classical brown and white adipocytes. Interestingly, CD137-sorted cells from the stromal vascular fraction isolated from the inguinal depot of mice display a strong browning response toward 20 nM FNDC5 (Abnova) or 100 nM of the fusion protein irisin-Fc. On the other hand, cells expressing CD137 to a lesser extent did not show any significant response toward FNDC5 or irisin-Fc, indicating that only a subpopulation of preadipocytes isolated from the subcutaneous depot, which highly express CD137, is responsible for the browning effect of irisin (Wu et al. 2012).

Another study using murine 3T3-L1 adipocytes and primary rat adipocytes also observed a white-to-brown shift after exposure to 20 nM recombinant irisin, which was produced in yeast using the human irisin cDNA sequence (Zhang et al. 2014). Notably, the irisin-mediated increase in Ucp1 mRNA expression was higher in 3T3-L1 adipocytes compared with primary rat adipocytes (sevenfold vs fourfold), indicating potential differences in the action of irisin between species. Unfortunately, CD137 expression was not assessed in this study. In line with these results in murine and rat cell systems, a third study demonstrated the impact of irisin on browning of murine WAT. Treatment of primary murine preadipocytes isolated from the inguinal depot with 20 nM of a commercially available FNDC5 peptide (Abcam, ab117436) enhanced the expression of Ucp1 and other brown marker genes (Shan et al. 2013). However, the synthetic peptide from Abcam used for this study corresponds to the C-terminal region of human FNDC5 and comprises the amino acids 149–178. This peptide is a part of the transmembrane domain and does not share any sequences with the irisin fragment, which comprises amino acids 32–143 (UniProt entry Q8NAU1). Therefore, the results observed by Shan et al. (2013) with this peptide are surprising and not related to the irisin fragment.

Summarizing the current data assessing the role of irisin in rodents, there is evidence for an impact of irisin on the browning of WAT. However, the function of irisin has not yet been validated in a human cell system and remains unclear. In addition to the previously described lineage-selective effect of FNDC5, potential differences in the molecular signature of adipose tissue depots between mice and humans have been proposed. Thus, Wu et al. (2012) observed that human adipose tissue from the supraclavicular region, and proven to be BAT positive by [18F]FDG-PET/CT, shares the molecular signature of murine beige adipose tissue rather than murine classical BAT with a myogenic origin (Wu et al. 2012). However, three recent studies have provided evidence for the existence of classical brown adipocytes in humans, suggesting that the human neck adipose tissue depot is composed of both classical and brite adipocytes (Cypess et al. 2013, Jespersen et al. 2013, Lidell et al. 2013). In conclusion, these studies raise the question of whether results about browning obtained in murine models can be extrapolated to the human situation.

Relevance of irisin as an inducer of browning in humans

Studies investigating the functionality of FNDC5 and/or irisin in humans are rare and criticism has been raised regarding the relevance of irisin in humans. In this context, primary human preadipocytes isolated from the subcutaneous depot of different donors did not exhibit browning in response to irisin or FNDC5 (Raschke et al. 2013). The effects of three different recombinant proteins obtained from several sources (Phoenix, Abnova, and Caymen Chemicals) were compared in this study to exclude any potential differences between the suppliers. However, none of the proteins were able to induce browning, even when applied at high concentrations (1000 ng/ml for FNDC5 and 600 ng/ml for irisin).
Moreover, preadipocytes isolated from donors highly expressing CD137 strongly induced the browning program in response to BMP7, while no response toward irisin or FNDC5 was observed (Raschke et al. 2013).

Until recently, this was the sole study assessing the direct effect of FNDC5/irisin on browning in primary human adipocytes. More recently, a second study was published investigating the potency of FNDC5 to induce browning in primary human adipocytes from different depots. Lee et al. (2014) demonstrated a strong induction of several brown marker genes in primary human adipocytes isolated from neck biopsies after FNDC5 treatment for 6 days. Moreover, FNDC5 treatment of neck adipocytes increased basal and uncoupled oxygen consumption rates and FNDC5-treated cells were able to respond to β-adrenergic stimulation, a crucial feature of brown adipocyte functionality. As the myogenic marker ZIC1 was not detectable, these neck adipocytes represent brite adipocytes rather than the classical brown ones. Interestingly, the effect of FNDC5 was only marginal or completely absent in primary adipocytes isolated from the subcutaneous and omental depots (Lee et al. 2014). In line with the study by Raschke et al. (2013), adipocytes from the subcutaneous depot do not appear to be able to undergo browning in response to FNDC5/irisin. One explanation could be the low expression of the brite-specific marker genes TMEM26 and CD137 in the subcutaneous depot compared with deep neck adipose tissue depots (Cypess et al. 2013, Lee et al. 2014). However, high CD137 expression in human subcutaneous adipose tissue was not associated with a browning effect of FNDC5/irisin (Raschke et al. 2013).

The current data dealing with the effect of FNDC5/irisin on browning in humans lead to the suggestion that the sensitivity toward FNDC5/irisin is potentially dependent on the adipocyte lineage and in consequence the adipose tissue depot and the species (Fig. 1). Thus, FNDC5/irisin does not activate classical brown adipocytes (Boström et al. 2012a) and has no effect on pure white adipocytes (Wu et al. 2012, Raschke et al. 2013, Lee et al. 2014). It is likely that only a small subpopulation of adipocytes, which are highly expressing brite-specific markers, is responsible for the irisin effect (Wu et al. 2012). In this case, the relevance of irisin in humans and its potency as a pharmacological agent to treat obesity is questionable, as the major human adipose tissue depots (subcutaneous and omental) are pure white depots with a low expression of brite-specific markers and do not undergo browning upon FNDC5/irisin treatment. Probably, a subpopulation of cells present in deep neck adipose tissue from humans, which is suggested to be composed of classical brown and brite adipocytes, responds to FNDC5/irisin. However, the contribution of these cells to whole-body energy expenditure is not clearly determined. Moreover, other candidates seem to be more promising and have been shown to induce browning in human subcutaneous adipocytes, such as CNPs (Bordicchia et al. 2012) or BMP7 (Schulz et al. 2011, Elsen et al. 2014).

Role of irisin in metabolic diseases

In addition to the discovery of irisin as an exercise-regulated myokine, which induced browning of WAT, Boström et al. (2012a) demonstrated the beneficial effects of irisin on whole-body metabolism. Thus, adenoviral-mediated FNDC5 overexpression led to browning of WAT in lean mice as well as diet-induced obese mice. In addition, a moderate increase in circulating irisin levels by threefold augmented energy expenditure, reduced the body weight gain under high-fat diet, and improved diet-induced insulin resistance (Boström et al. 2012a). These results suggested a potential protective role of irisin in the development of type 2 diabetes, one of the major obesity-associated metabolic diseases. In order to investigate the relevance of irisin in humans, many clinical studies have focused on the relation between circulating irisin levels and metabolic parameters and diseases. The following section will give a comprehensive overview about the correlation of circulating irisin with metabolic parameters in humans and highlight existing discrepancies (Table 3).

As irisin has initially been described to protect against diet-induced weight gain, mediated by browning of WAT and thus increased energy expenditure, many studies have investigated the correlation of circulating irisin with obesity in humans. In line with the suggested protective role of the myokine irisin in the development of obesity, negative correlations of circulating irisin levels with the BMI have been reported in humans (Aydin et al. 2013, Choi et al. 2013, Moreno-Navarrete et al. 2013, Polyzos et al. 2014). However, controversy exists regarding the relation between irisin levels and the BMI. Several studies reported a positive correlation of serum irisin levels with BMI (Liu et al. 2013, Stengel et al. 2013, Crujeiras et al. 2014a, Liu et al. 2014, Park et al. 2014) or could not detect a change in circulating irisin in obesity (Huh et al. 2012, Gouni-Berthold et al. 2013, Kurdiya et al. 2014). This could be related to different populations analyzed in the different studies, as some include obese subjects without...
metabolic disorders whereas others enclose obese patients with metabolic diseases such as type 2 diabetes.

However, recent intervention studies provide evidence for a positive correlation between BMI and circulating irisin levels, which is in conflict with the proposed anti-obesity effect of irisin. The earliest intervention study by Huh et al. (2012) assessed morbidly obese subjects undergoing gastric banding or gastric bypass and blood samples were collected at baseline and 6 months after surgery. Bariatric surgery led to a significant weight loss after 6 months, which was accompanied by decreased circulating irisin levels. These lower irisin levels were attributed to a lower fat-free mass and decreased FNDC5 mRNA expression in skeletal muscle (Huh et al. 2012). However, the reduction of fat mass was not analyzed as a factor causing lower circulating irisin levels. The recent observation that irisin is not only a myokine but also an adipokine (Roca-Rivada et al. 2013) raises the question if circulating irisin can be solely attributed to skeletal muscle. Moreover, secretion of irisin is higher from WAT of diet-induced obese rats compared with lean controls (Roca-Rivada et al. 2013), suggesting that adipose tissue, especially in states of obesity, represents an important source of irisin besides skeletal muscle. Two other interventional studies, both with 8 weeks of hypocaloric diet, observed decreased irisin levels after weight loss (de la Iglesia et al. 2013, Crujeiras et al. 2014b). In addition to a positive association between irisin and BMI, irisin has been described to be positively associated with waist circumference and fat mass. (Crujeiras et al. 2014a). In summary, there is stronger evidence for a positive correlation of circulating irisin with adiposity markers. Nevertheless, data are still conflicting and it is not clear which organ is the major source of irisin.

Besides the association of irisin with obesity, many studies assessed the regulation of irisin in type 2 diabetes
and other metabolic diseases. Similar to the conflicting observations regarding BMI, which have been discussed above, controversy exists. While some studies reported decreased irisin levels in type 2 diabetic subjects compared with lean subjects (Choi et al. 2013, Liu et al. 2013, Kurdiova et al. 2014) and a negative correlation of HOMA-IR with circulating irisin levels in girls (Al-Daghri et al. 2013), other studies demonstrated a positive correlation of circulating irisin with HOMA-IR (Park et al. 2013, Ebert et al. 2014a). In line, increased irisin levels were found in subjects with metabolic syndrome (Park et al. 2013) and higher circulating irisin concentrations have been reported in mothers with prior gestational diabetes (GDM) compared with those without prior GDM (Ebert et al. 2014b). Besides, reduced circulating irisin levels have been demonstrated in patients with chronic kidney disease (CKD) (Zhang et al. 2013) and non-alcoholic fatty liver disease (NAFLD) (Wen et al. 2013, Ebert et al. 2014a).

In addition to the different study populations, the different assays used to detect irisin may be a reason for these controversial results. Criticism regarding the reliability of commercially available irisin/FNDC5 antibodies and non-validated ELISA kits has already been raised (Erickson 2013). In this context, we summarized the cohort, the irisin concentrations measured, and the assay used in each study to gain an overview about the range of detected irisin concentrations (Supplementary Table 1, see section on supplementary data given at the end of this article). Interestingly, there is a wide range of irisin concentrations measured, from very low concentrations of 38.86±2.48 pg/ml (Choi et al. 2013) to relatively high concentrations of 2157.9±600.7 ng/ml (Moreno-Navarrete et al. 2013) in lean individuals. The concentrations measured in these different studies differ strongly, with about 50 000 times higher concentrations measured in the study by Moreno-Navarrete et al. compared with those values originating from Choi et al. This tremendous difference between the circulating irisin concentrations indicates that at least some of the available ELISAs are unspecific and may have a high cross-reactivity to other proteins present in serum and plasma. Moreover, striking differences are observed even when using the same kit (Supplementary Table 1).

**Is the irisin fragment present in humans?**

Based on the huge variation in the current studies assessing circulating irisin concentrations in humans,
the physiological circulating irisin levels in humans remain unclear. Moreover, the term irisin is often incorrectly and indistinctly used with FNDC5. The secretion mechanism of irisin by extracellular cleavage of FNDC5 proposed by Bostrom et al. (2012a) and the presence of a 12.6 kDa irisin fragment (theoretical molecular weight of the irisin chain amino acids 32–142 according to UniProt entry Q8NAU1) in the circulation has not been proven. As mentioned previously, for the initial description of irisin, an antibody raised against a peptide corresponding to amino acids 149–178 and thus mainly located in the transmembrane domain was used (Erickson 2013, Raschke et al. 2013). This antibody (ab117436, Abcam) is not predicted to detect irisin and is no longer available at Abcam. Nevertheless, a band of 22 kDa was designated to irisin, which was detected in western blot of deglycosylated cell media from HEK293 cells transfected with FNDC5 (Bostrom et al. 2012a). This molecular weight of 22 kDa is more likely to reflect full-length FNDC5 (23.7 kDa, according to Q8NAU1) than irisin (12.6 kDa, see above), which would not be in conflict with the used antibody. A later study by Roca-Rivada et al. (2013) used two different antibodies, one solely against full-length FNDC5 (Abcam, raised against amino acids 149–178) and the other also detecting the irisin fragment (Phoenix Pharmaceuticals, raised against amino acids 42–142), to discriminate between these two proteins. With both antibodies, a band of 25 kDa was observed in conditioned media from rat skeletal muscle corresponding to full-length FNDC5. Interestingly, no additional band at the predicted lower molecular weight of irisin was observed with the anti-irisin antibody (Roca-Rivada et al. 2013), questioning the release of the initially described irisin fragment. Recently, a mass spectrometry approach has been used to clarify the identity of FNDC5-immunoreactive bands in human serum samples, which underwent deglycosylation. Several bands were observed with an anti-FNDC5 antibody from Abcam (ab131390) detecting both FNDC5 and the irisin fragment; but exclusively in a band of 24 kDa, a unique peptide located in the irisin sequence was identified by mass spectrometry, confirming that this band represents FNDC5 or fragments of FNDC5 (Lee et al. 2014). Unfortunately, this band was also designated as the irisin fragment, although the molecular weight is 24 kDa and the band could also represent other forms of FNDC5. Summarizing these studies, it remains unclear which fragments of FNDC5 are present in the circulation of humans.

Additionally, sequence differences between species have been reported. FNDC5 has already been described in 2011 to contain a non-canonical ATG start codon in humans (Ivanov et al. 2011). In line, multi-species alignment of the FNDC5 exon 1 revealed that the canonical ATG start site is conserved in mouse, rat, gorilla, and chimp, but displays a mutation in the human sequence to ATA (Raschke et al. 2013). In vitro studies demonstrated that the translational efficiency of full-length FNDC5 is strikingly reduced in this case and in consequence also the release of FNDC5 fragments. Usage of the next in-frame ATG as an alternative start site would lead to a truncated form of FNDC5 lacking the first 76 amino acids (Raschke et al. 2013). Interestingly, the specific fragment in the FNDC5-immunoreactive band with a molecular weight of 24 kDa identified by mass spectrometry (Lee et al. 2014) is a part of this truncated form (represented by NP_715637) described by Raschke et al. Taken together, FNDC5 fragments are present in humans, but the nature and the secretion mode have to be clarified in future studies and the presence of the initially described irisin peptide in humans is unclear.

### Summary and conclusion

The discovery of irisin as an exercise-regulated myokine inducing browning of WAT has gained interest as a potential new strategy to combat obesity and its associated disorders, such as type 2 diabetes. However, there are inconsistencies regarding the relevance of irisin in humans. The regulation of FNDC5 mRNA expression by exercise and contraction could not be reproduced by a number of human studies using several exercise protocols and in vitro approaches. Furthermore, the nature of FNDC5 fragments and the presence of irisin in humans are questionable and probably contribute to conflicting data obtained with commercially available ELISA kits. Most importantly, the information regarding the concentration of circulating irisin in humans is not clear, as different studies using different kits measure irisin levels in a wide range. Data about the role of irisin in states of human obesity and metabolic diseases are conflicting and, in some cases, changes in irisin levels have been observed; they were only moderate with 10–20%. Independent of the presence and regulation of FNDC5/irisin in humans, the application of recombinant irisin could still represent a therapeutic strategy to fight obesity. However, the current data obtained from human cell models reveal that FNDC5/irisin has no effect on browning of the major WAT depots in humans and is likely to selectively target a small subpopulation of adipocytes, located in classical BAT.
regions, such as the supraclavicular adipose tissue. Thus, other candidates, such as BMP7 or CNPs, seem to be more prominent candidates as inducers of browning in humans.

Supplementary data
This is linked to the online version of the paper at http://dx.doi.org/10.1530/JOE-14-0189.

Declaration of interest
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