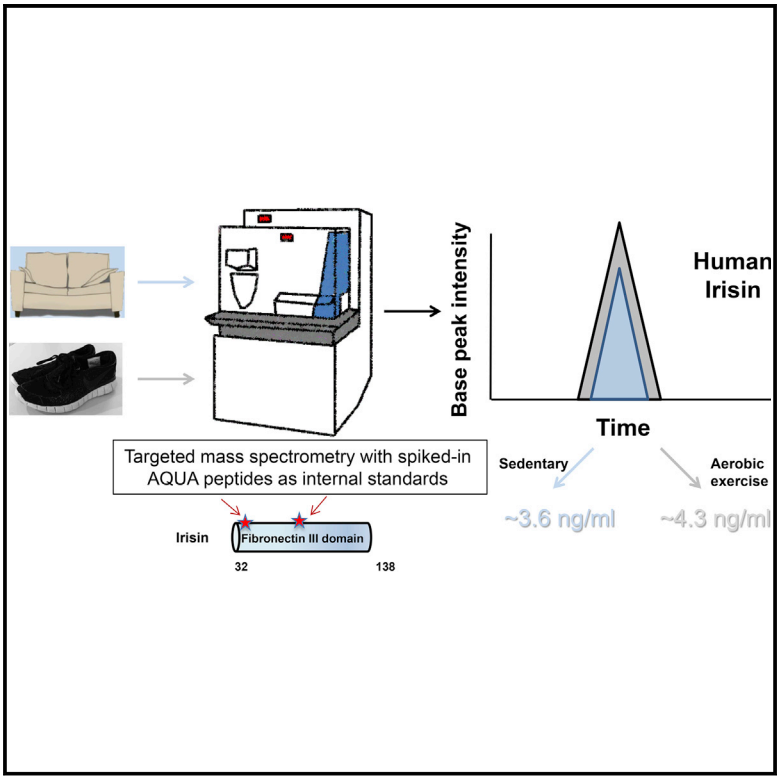
Short Article

Cell Metabolism

Detection and Quantitation of Circulating Human Irisin by Tandem Mass Spectrometry

Graphical Abstract



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In Brief

Irisin is an exercise-induced myokine with beneficial metabolic functions. Its detection in human plasma has, however, been problematic. Here, Jedrychowski et al. use state-of-the-art quantitative mass spectrometry to precisely detect and quantify circulating irisin and show that it goes up in individuals undergoing aerobic interval training.

Highlights

- Detection and quantitation of human plasma irisin by quantitative mass spectrometry
- Irisin is mainly translated from its non-canonical start codon
- Irisin circulates at ~3.6 ng/ml in sedentary individuals
- Irisin levels significantly increase in individuals undergoing aerobic training

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Detection and Quantitation of Circulating Human Irisin by Tandem Mass Spectrometry

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SUMMARY

Exercise provides many health benefits, including improved metabolism, cardiovascular health, and cognition. We have shown previously that FNDC5, a type I transmembrane protein, and its circulating form, irisin, convey some of these benefits in mice. However, recent reports questioned the existence of circulating human irisin both because human FNDC5 has a non-canonical ATA translation start and because of claims that many human irisin antibodies used in commercial ELISA kits lack required specificity. In this paper we have identified and quantitated human irisin in plasma using mass spectrometry with control peptides enriched with heavy stable isotopes as internal standards. This precise state-of-the-art method shows that human irisin is mainly translated from its non-canonical start codon and circulates at ~3.6 ng/ml in sedentary individuals; this level is increased to ~4.3 ng/ml in individuals undergoing aerobic interval training. These data unequivocally demonstrate that human irisin exists, circulates, and is regulated by exercise.

INTRODUCTION

The health benefits of physical activity and exercise are well recognized (Hawley et al., 2014; Mann and Rosenzweig, 2012; Voss et al., 2013). Exercise is the first line of therapy for various metabolic diseases like diabetes and obesity, but exercise also improves outcomes in diseases involving other tissues, such as the heart and brain. We recently described a novel polypeptide that is secreted from skeletal muscle and is increased with exercise. Irisin is the shed extracellular domain of a transmembrane protein called FNDC5. FNDC5, when expressed from adenoviral vectors in mice, causes an elevation of irisin in the blood and improved metabolic health in recipient animals (Bostrom et al., 2012). It also stimulates the expression of a potential neuroprotective gene program in the brain, particularly in the hippocampus (Wrann et al., 2013). Several papers have studied the effects of exercise on circulating irisin in humans; positive associations between irisin plasma level and exercise have been observed in some but not all cohorts and modes of exercise (Daskalopoulou et al., 2014; Hofmann et al., 2014; Huh et al., 2014; Kraemer et al., 2014; Lee et al., 2014; Norheim et al., 2014). Data suggest that early sampling after exercise and high-intensity training protocols are particularly effective at raising circulating irisin levels. Most of these studies have relied on commercial antibodies and ELISA assays.

Human FNDC5 has an atypical translation start codon, ATA, in place of the more typical ATG. While it is now known that many eukaryotic mRNAs begin translation with non-ATG start codons (Ingolia et al., 2011; Ivanov et al., 2011; Peabody, 1989), two recent papers have claimed that this ATA codon in human FNDC5 represents a null mutation and therefore human irisin would not be produced (Albrecht et al., 2015; Raschke et al., 2013). These authors argue that if FNDC5 exists in humans, it is translated from a downstream ATG, and hence the irisin polypeptide is a "myth" and does not exist. In addition, these authors claim that the many papers measuring human irisin are all artifacts of poor antibody specificity (Albrecht et al., 2015; Erickson, 2013); this is despite the fact that Lee et al. had previously detected an irisin peptide in human plasma with mass spectrometry (Lee et al., 2014). In this paper we have investigated the presence of human irisin in blood using quantitative mass spectrometry. As internal standards, we synthesized irisin peptides and included a valine enriched in stable isotopes (six ^{13}C atoms). The peptides were used to develop a quantitative platform for the measurement of human irisin; these data should facilitate future studies of this molecule in both mice and humans.

RESULTS

Two peptides were chosen as standards for this mass spectrometric analysis. These were both chosen because they are unique to the irisin sequence (FNDC5 ectodomain) and not encoded in any other proteins in the annotated human genome. As shown in Figure 1A, one peptide represents the most extreme N-terminal 12 amino acids (DSPSAPVNVTVR^{12}) of the processed irisin molecule, coming immediately after the signal peptide (Figure 1A). Importantly, this peptide is downstream of the non-canonical ATA codon but upstream of the first ATG codon in the *FNDC5* mRNA. Therefore detection of this peptide would demonstrate use of the non-canonical start codon. A second tryptic peptide (^{48}FIQEVNTTTR^{57}) was chosen from the central

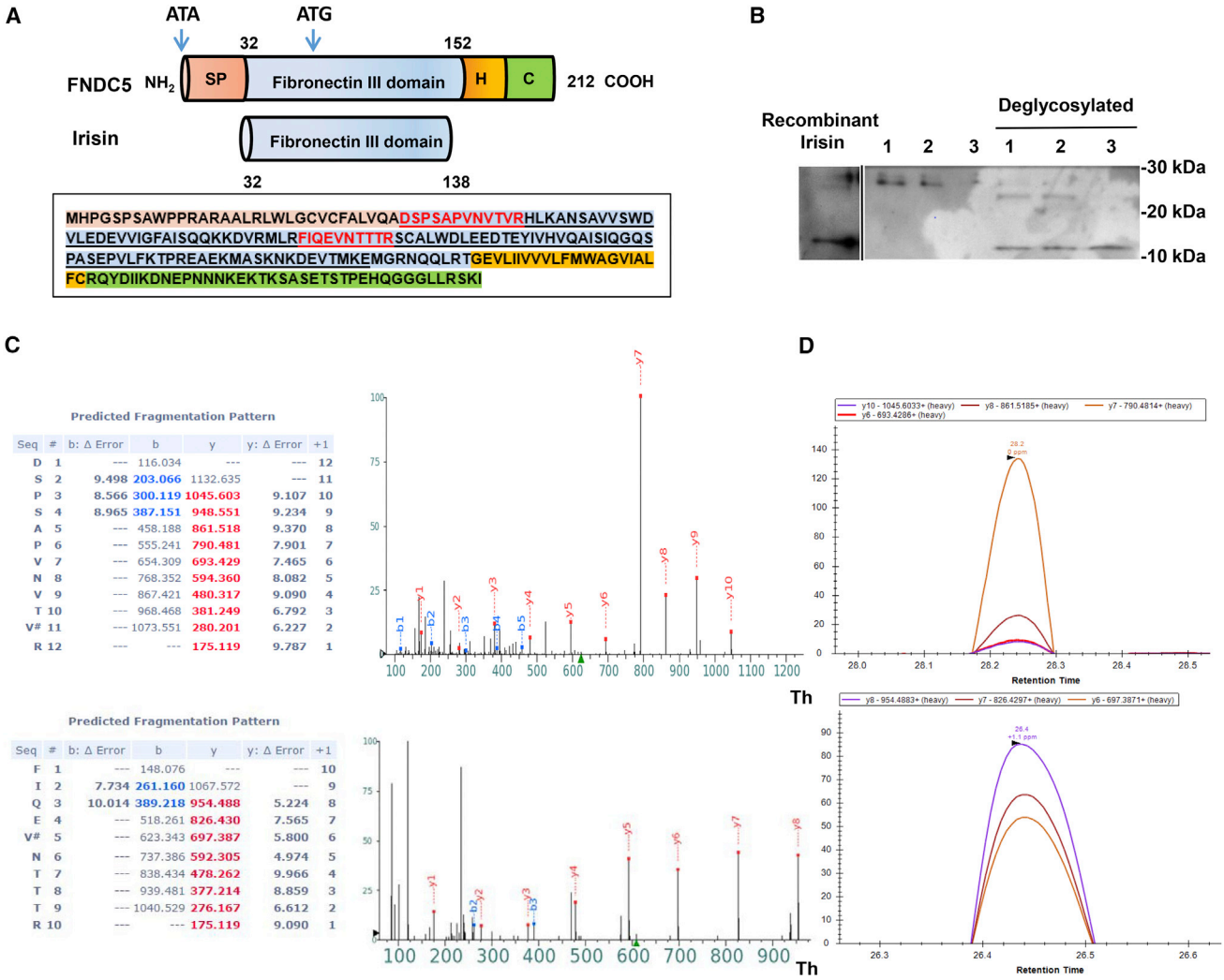

Figure 1. Analysis of Irisin Peptides by Mass Spectrometry

(A) Schematic representation of the FNDC5 protein structure (top) and irisin (bottom). SP, signal peptide; H, hydrophobic domain; C, C-terminal domain. Below is shown the human FNDC5 sequence with corresponding domains colored. Human irisin sequence is underlined as are synthetic AQUA peptides used in this study (red).

(B) Immunoblotting of irisin plasma samples from three subjects undergoing aerobic interval training with or without deglycosylation enzyme (Protein Deglycosylation Mix [NEB]) and deglycosylated recombinant irisin.

(C) MS^2 spectra acquired using a Q Exactive mass spectrometer for the two synthetic AQUA peptides and their b-, y-ion series *m/z* values. Mass accuracy values are given in PPMs and "#" denotes the heavy valine residue.

(D) PRM elution profile for the y-ions for the AQUA peptides using Skyline software. Retention times for each peptide are labeled on the x axis, and y axis represents the relative intensity for each y-ion peak. See also Figure S1.

portion of irisin, three amino acids downstream of the ATG. Plasma samples from human volunteers who had undergone aerobic interval training (see Experimental Procedures) were used to develop this assay. These plasma samples were first treated with a commercial affinity resin to remove the very abundant albumin and immunoglobulins, so that these proteins would not hinder analysis of less abundant proteins (see Experimental Procedures). Samples were then deglycosylated with the Protein Deglycosylation Mix from New England Biolabs (NEB), which contains PNGase F, O-glycosidase, neuraminidase, β1-4-galactosidase, and β-N-acetylglucosaminidase and results in complete deglycosylation. After electrophoresis, the anti-irisin antibody detected a band running at ~12 kDa, the predicted size of the irisin polypeptide (Figure 1B). To characterize the synthetic heavy irisin, peptides were subjected to liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis in both data-dependent and parallel reaction monitoring (PRM) acquisition modes. As shown in Figure 1C, the intensity of the y ions series from the MS^2 spectra for both peptides corresponds to the rank order elution profile in the PRM acquisition mode (Figure 1D), validating that these ions can be used for identification and quantification of irisin.

Next, for the quantification of irisin in human plasma by mass spectrometry, albumin- and immunoglobulin-depleted plasma

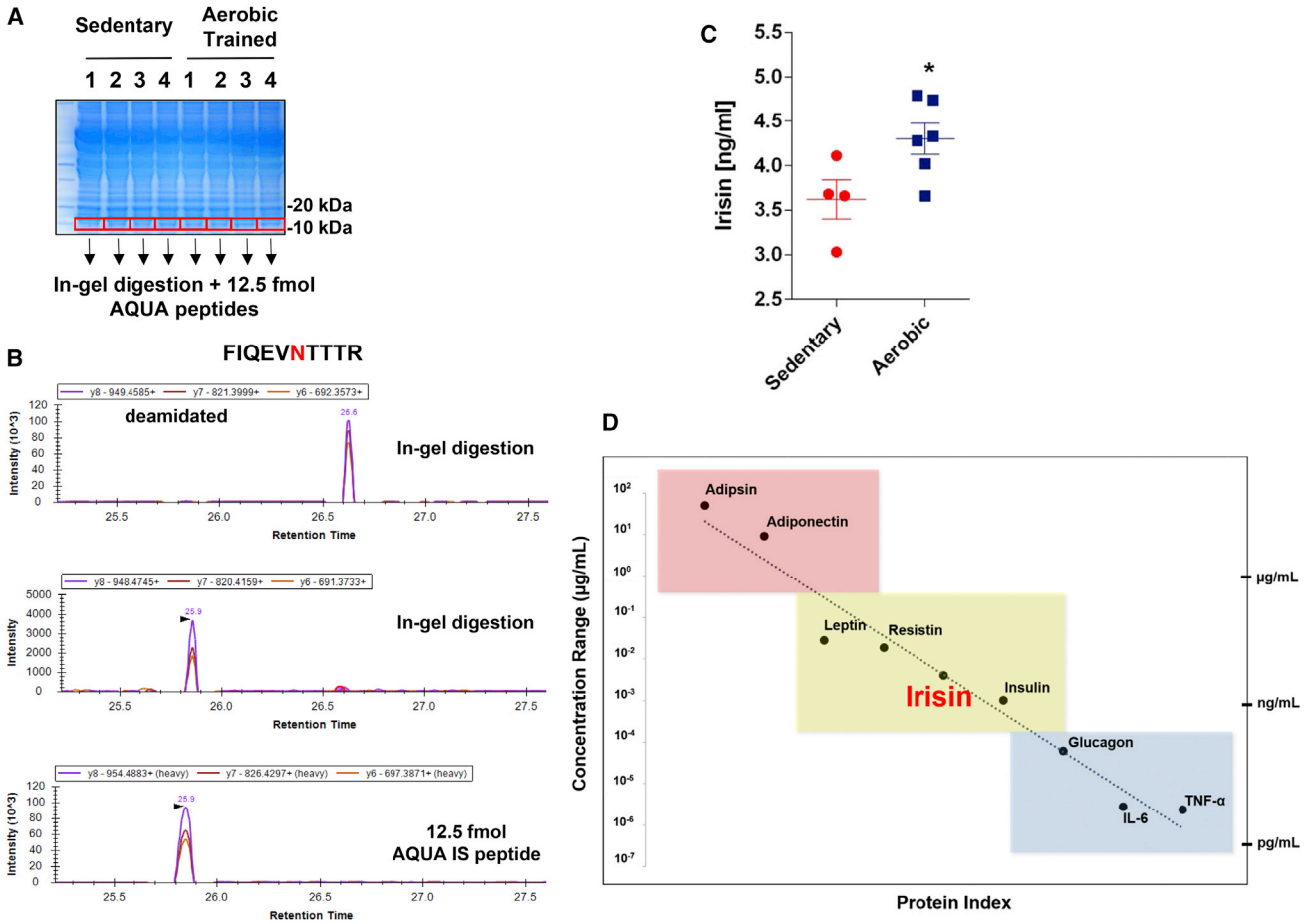

Figure 2. Detection of Irisin in Human Plasma

(A) SDS-PAGE separation of 50 μg of plasma from each subject and visualized by Coomassie staining. Molecular mass regions corresponding to completely deglycosylated irisin (10–15 kDa) were excised from six separate gels (300 μg from the original 100 μl plasma) for each subject and digested in-gel in the presence of 12.5 femtomoles of each internal standard AQUA peptide.

(B) PRM elution profile for internal tryptic irisin peptide (FIQEVNTTR) using Skyline software found in sedentary subject 1. Top panel is the deamidated asparagine form of the peptide found in the plasma, middle panel is the unmodified peptide found in the plasma, and the bottom panel is 12.5 femtomoles of heavy internal standard (IS) AQUA peptide.

(C) Irisin levels in plasma from sedentary subjects (Sedentary) or subjects undergoing aerobic interval training (Aerobic). Values are shown as mean ± SEM; n = 4 (Sedentary) and n = 6 (Aerobic). *p = 0.0411 compared to sedentary subject group as determined by unpaired t test, two-tailed.

(D) Depicted are several plasma proteins and their circulating concentrations ranging from the μg/ml (red), ng/ml (yellow), and pg/ml (blue) levels. We quantify circulating plasma irisin at a 3–5 ng/ml. See also Figure S2.

from four sedentary and six aerobically interval-trained subjects was deglycosylated and resolved by SDS-PAGE prior to in-gel trypsin digestion. After this, 12.5 femtomoles of each heavy peptide were spiked into the sample prior to absolute quantification (AQUA) of irisin (Figure 2A) (Gerber et al., 2003). Of note, often with enzymatic deglycosylation of proteins there is a propensity for deamidation occurring on asparagine residues, increasing the mass of the residue by 0.984 Da and slightly delaying the reverse phase retention (Zielinska et al., 2010). Therefore, successful identification of human irisin peptides (as for other N-glycosylated plasma proteins) must take into account this mass shift. Deamidation modifications for both endogenous plasma irisin peptides are observed without dramatically changing the MS^2 spectra (Figure S1A) nor altering the PRM rank order elution profile (Figure 2B). Fragment ions for both peptides were quantified using Skyline version 3.1 (MacLean et al., 2010), and comparable levels of quantification for both peptides downstream of the ATA start codon and the later ATG, suggest irisin is mainly translated from its non-canonical start codon (Table 1, Figures S1B and S2). We found that irisin levels are present at ~3.6 ng/ml in sedentary individuals and are significantly increased to ~4.3 ng/ml in individuals undergoing aerobic interval training (Figure 2C, Table 1).

DISCUSSION

We have developed here a quantitative, precise, and unbiased assay for the detection of human irisin in plasma. This assay definitively shows that human irisin circulates and has a very similar or identical architecture to the mouse protein (Bostrom

Table 1. Quantification of Irisin in Plasma from Human Subjects

Sedentary	N-terminal peptide (femtomoles)	Internal (femtomoles)	Combined average (femtomoles)	ng/ml
Subject 1	16.66	12.78	14.72	3.68
Subject 2	11.98	12.3	12.14	3.03
Subject 3	13.73	15.59	14.66	3.66
Subject 4	16.21	16.7	16.455	4.11
Average	14.65	14.34	14.49	3.62
Aerobic	N-terminal peptide (femtomoles)	Internal (femtomoles)	Combined average (femtomoles)	ng/ml
Subject 1	13.98	14.78	14.38	3.66
Subject 2	18.22	19.74	18.98	4.74
Subject 3	16.11	22.22	19.165	4.79
Subject 4	16.28	17.38	16.83	4.28
Subject 5	16.68	19.2	17.94	4.48
Subject 6	15.1	17.11	16.105	4.02
Average	16.06	18.41	17.23	4.33

Skyline software was used to quantify absolute amounts of irisin peptides from the plasma of sedentary and aerobically trained subjects. The 25 kDa glycosylated bioactive form of irisin was used to calculate its ng/ml concentrations in plasma.

et al., 2012). Human irisin circulates at a level at or above the levels observed for many other important biological hormones, as shown in Figure 2D. It confirms earlier reports that have identified a unique peptide in human plasma by untargeted mass spectrometry (Albrecht et al., 2015; Lee et al., 2014), but provides quantitation of the circulating levels of human irisin in an unbiased and antibody-independent manner. Irisin concentrations are present at ~3.6 ng/ml in sedentary individuals and are significantly increased to ~4.3 ng/ml in individuals undergoing aerobic interval training. We therefore also confirm our earlier report of irisin being regulated by endurance exercise in humans (Bostrom et al., 2012).

Several papers have called the start codon of the human *FNDC5* gene, which is an ATA, rather than the more common ATG, a mutation. Indeed, these authors concluded that human *FNDC5* is a non-coding "pseudogene" or that "the human species has an effective gene knockout of *FNDC5*" (Albrecht et al., 2015; Raschke et al., 2013). This claim was based on a transfection assay expressing human FNDC5 from a CMV-promoter-driven plasmid, which yielded protein levels lower than human FNDC5 expressed with an ATG instead of an ATA from the same plasmid. However, several lines of reasoning stand against that claim. First, the high degree of conservation of the irisin amino acid sequence across most mammalian species (including humans) strongly argues against *FNDC5* in humans being a pseudogene. Second, the simple fact that Raschke et al. detect human FNDC5 protein made from the ATA-FNDC5 sequence proves that human *FNDC5* is not a pseudogene; these are generally defined as genes that have lost their protein-coding ability (Vanin, 1985). Third, their conclusion that low protein production from CMV-promoter-driven plasmid expressed in HEK293 cells translates to inefficient FNDC5 translation in vivo is completely speculative, since this experiment did not consider endogenous regulation of human FNDC5 in its native state. Indeed, non-canonical starts of translation are often indicative of complex regulation of translation (Chang and Wang, 2004; Starck et al., 2012). Fourth, as mentioned above, our detection here of equal amounts of peptide 1 and 2 in human plasma demonstrates that human irisin is, in fact, mainly translated from its non-canonical start codon and not the further downstream ATG.

The earlier report (Albrecht et al., 2015) had several serious methodological deficiencies. First, their failure to detect irisin in human serum at 12 kDa by western blotting relied on deglycosylation by only one enzyme, namely PNGase F; however, this leads to only incomplete deglycosylation. PNGase F is an effective enzymatic method for removing almost all N-linked oligosaccharides, but not other oligosaccharides. Hence, with PNGase F, no visible band will appear at 12 kDa and the irisin signal will be diluted across the lane, leading to apparent lower levels. In our previously published method (Wrann et al., 2013), we used the Protein Deglycosylation Mix from NEB, which contains, in addition to PNGase F, O-glycosidase, neuraminidase, β1-4-galactosidase, and β-N-acetylglucosaminidase; this leads to complete deglycosylation and the appearance of 12 kDa bands in recombinant mammalian irisin and human plasma by immunoblot (Figure 1).

Second, these authors (Albrecht et al., 2015) used a method of protein mass spectrometry called "shotgun proteomics," which randomly samples peptides for detection from all the peptides contained in the sample. While the method has the potential to detect irisin, it would be suboptimal for detection because the peptides of interest can be missed in complex samples due to their low abundance. In these cases targeted proteomics is required. This allows the mass spectrometer to focus on the targeted peptides and ignore signal from co-eluting peptides. AQUA-based quantification concomitantly with PRM produces spectra that are highly specific because all potential product ions of a peptide and elution profile confirm the identity of the peptide.

Third, and perhaps most importantly, the authors report their own detection limits for irisin at about 100 ng/ml. However, many reports of human irisin fall below this level (Kraemer et al., 2014; Kurdiova et al., 2014; Moraes et al., 2013; Wang et al., 2015; Zhang et al., 2014). Hence it is rather surprising that these authors concluded that human irisin did not exist or was a "myth."

It is worth noting that limitations of own study include that the AQUA heavy peptides were added to the irisin preparations after the extraction of the proteins from the SDS-PAGE gel; we therefore cannot account for how much irisin protein was lost during the sample preparation (albumin/IgG removal, deglycosylation, and retrieval from the gel band, etc.); the numbers reported here must therefore be considered a slight underestimation of the irisin levels. In our experience, typical losses during sample preparation range between 10% and 30%. In addition, this assay is relatively costly and relies on available mass spectrometry instrumentation and capabilities. However, while this assay is relatively low throughput, it should prove useful for benchmarking more high-throughput assays as they are developed. Taken together, targeted mass spectrometry with the use of heavy irisin AQUA peptides settles the existence, the overall architecture of human irisin in the plasma, and its regulation by exercise.

EXPERIMENTAL PROCEDURES

Aerobic Interval Training

Plasma samples were collected from young healthy participants (n = 6 males, 25 ± 5 years, BMI = 24.3 ± 2.5 kg/m^2) following 12 weeks of high-intensity aerobic training. Training consisted of 3 days/week of intervals on a cycle ergometer (4 × 4 min > 90% peak aerobic capacity + 3 min rest) separated by 2 days/week of walking on a treadmill (45 min at 70% peak aerobic capacity). All training was supervised at the Dan Abraham Healthy Living Center at the Mayo Clinic, Rochester. A separate sedentary group served as no-treatment control (n = 4 males, 26 ± 3 years, BMI = 26.1 ± 3.4 kg/m^2). Written informed consent approved by the Mayo Clinic Institutional Review Board was given by all participants before entering the study.

Plasma Collection and Purification

Participants consumed a weight-maintaining diet for 3 days and then were admitted to the Clinical Research Unit at Mayo Clinic Hospital. The aerobic training group did not exercise during the 3-day meal period. Participants consumed a standardized evening meal and then remained fasted overnight. Arterialized blood was collected at 7:00 a.m. into sodium heparin tubes from a retrograde catheter in a dorsal hand vein (heated to 49°C). Plasma was separated by centrifugation and stored at −80°C until analysis. Human plasma specimens (100 μl) were depleted of albumin and IgG using the ProteoExtract kit (Millipore) and subsequently concentrated using 3 kDa molecular weight cut-off spin-filter columns (Millipore). Deglycosylation of plasma was performed using Protein Deglycosylation Mix (NEB) as per the manufacturer's denaturing protocol. Deglycosylated plasma samples were reduced with 10 mM DTT and alkylated with 50 mM iodoacetamide prior to being resolved by SDS-PAGE using 4%–12% NuPAGE Bis-Tris precast gels (Life Technologies).

Western Blotting

For western blotting analyses, 50 μg of protein was resolved for each plasma sample by SDS-PAGE. Gels were transferred to 0.2-μm pore size Protran (BA83) nitrocellulose membranes (Whatman) in 25 mM Tris, 200 mM glycine. Membranes were blocked with 10% nonfat dry milk in PBS containing 0.1% Tween (PBST) for 1 hr at room temperature. Membranes were then probed with anti-FNDC5 (Irisin) rabbit polyclonal antibody (1:1,000) (IN102, Adipogen) overnight at 4°C, washed with PBST, and incubated with horseradish peroxidase-conjugated secondary antibodies (1:3,000) (Sigma) followed by chemiluminescent (Perkin Elmer Life Sciences) detection. Recombinant mammalian irisin was obtained from Ember Therapeutics.

In-Gel Digestion

For in-gel digestions, deglycosylated plasma samples (300 μg) were reduced with 10 mM DTT and alkylated with 50 mM iodoacetamide prior to being resolved by SDS-PAGE using 4%–12% NuPAGE Bis-Tris precast gels (Life Technologies). Gels were Coomassie stained and fragments were excised from the 10–15 kDa region. Gel pieces were destained and dehydrated with 100% acetonitrile, vacuumed dried, and resuspended with 50 mM ammonium bicarbonate (Sigma-Aldrich), and 500 ng sequencing-grade trypsin (Promega) was added for an overnight incubation at 37°C (Shevchenko et al., 1996). Digests were quenched after 12 hr with 5% formic acid and de-salted using homemade C18 StageTips as previously described (Rappsilber et al., 2007). Peptides were eluted with 70% acetonitrile and 1% formic acid, then dried using a speedvac and resuspended in 10 μl of 5% formic acid and 4% acetonitrile containing the heavy valine-synthesized irisin peptides (Table S1).

LC-MS/MS

Mass spectrometry data were collected using a Q Exactive or LTQ Orbitrap Elite mass spectrometer (Thermo Fisher Scientific) coupled with a Famos Autosampler (LC Packings) and an Accela 600 liquid chromatography (LC) pump (Thermo Fisher Scientific). Peptides were separated onto a 100-μm inner diameter microcapillary column packed with ~0.5 cm of Magic C4 resin (5 μm, 100 Å, Michrom Bioresources) followed by ~20 cm of Accucore C18 resin (1.6 μm, 150 Å, Thermo Fisher Scientific). For each analysis, we loaded ~4 μl onto the column. Peptides were separated using a 50-min gradient of 8%–30% acetonitrile in 0.125% formic acid with a flow rate of ~250 nl/min.

PRM Acquisition

PRM analyses were performed using a Q Exactive mass spectrometer (Thermo Fisher Scientific) using the following parameters: a full MS scan from 400 to 700 Thomson (Th) at an orbitrap resolution of 70,000 (at *m/z* 200), AGC target 5 × 10^6, and a 500 ms maximum injection time. Full MS scans were followed by 25–50 PRM scans at 35,000 resolution (at *m/z* 200) (AGC target 5 × 10^6, 500 ms maximum injection time) as triggered by a scheduled inclusion list (Table S2). The PRM method employed an isolation of target ions by a 2 Th isolation window, fragmented with normalized collision energy (NCE) of 25. MS/MS scans were acquired with a starting mass range of 100 *m/z* and acquired as a profile spectrum data type. Precursor and fragment ions were quantified using Skyline version 3.1 (MacLean et al., 2010).

Data-Dependent Acquisition

For data-dependent acquisitions using Q Exactive, the scan sequence began with an Orbitrap MS1 spectrum with the following parameters: resolution 70,000, scan range 400–1,400 Th, automatic gain control (AGC) target of 5 × 10^6, maximum injection time of 250 ms, and centroid spectrum data type. We selected the top 20 precursors for MS^2 analysis, which consisted of high-energy collision dissociation (HCD) with the following parameters: resolution 17,500, AGC 1 × 10^5, maximum injection time 60 ms, isolation window 2 Th, NCE 25, and acquired as a centroid spectrum data type. The underfill ratio was set at 9%, which corresponds to a 1.5 × 10^5 intensity threshold. In addition, unassigned and singly charged species were excluded from MS^2 analysis and dynamic exclusion was set to automatic.

For data-dependent acquisitions using an LTQ Orbitrap Elite, the MS1 survey scan was performed in the orbitrap in the range of 300–1,500 Th at a resolution of 3 × 10^4, followed by the selection of the ten most intense ions (TOP10) for CID-MS^2 fragmentation using a precursor isolation width window of 2 Th. The AGC settings were 3 × 10^6 and 2.5 × 10^5 ions for survey and MS^2 scans, respectively. Ions were selected for MS^2 when their intensity reached a threshold of 500 counts and an isotopic envelope was assigned. Maximum ion accumulation times were set to 1,000 ms for survey MS scans and to 250 ms for MS^2 scans. Singly charged ion species and ions for which a charge state could not be determined were not subjected to MS^2. Ions within a 10 ppm *m/z* window around ions selected for MS^2 were excluded from further selection for fragmentation for 120 s.

Peptide and Protein Identification

Following mass spectrometry data acquisition, Thermo Fisher RAW files were converted into mzXML format and processed using a suite of software tools developed in-house for analysis of proteomics datasets. 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 using the Sequest algorithm (Eng et al., 1994). These spectra were searched against a database containing sequences of all human proteins reported by Uniprot (Magrane and Consortium, 2011) in both forward and reversed orientations. Common contaminating protein sequences (e.g., human keratins, porcine trypsin) were included as well. The following parameters were selected to identify peptides from unenriched peptide samples: 25 ppm precursor mass tolerance, 0.02 Da product ion mass tolerance, no enzyme digestion, up to two tryptic missed cleavages, variable modifications: oxidation of methionine (+15.994915) and deamidation of asparagine (0.984016), fixed modifications: carbamidomethylation of cysteine (+57.021464). The AScore algorithm was implemented to quantify the confidence with which each deamidation modification could be assigned to a particular residue in each peptide (Beausoleil et al., 2006). Peptides with AScores above 13 were considered to be localized to a particular residue ($p < 0.05$).

Statistical Analysis

Data analysis was performed using GraphPad Prism 6 software. Where appropriate, an unpaired, two-tailed Student's t test was used. Significance was

assigned to differences with a p value < 0.05. Pooled data are presented as mean ± SEM.

SUPPLEMENTAL INFORMATION

Supplemental Information includes two figures and two tables and can be found with this article online at http://dx.doi.org/10.1016/j.cmet.2015.08.001.

AUTHOR CONTRIBUTIONS

M.P.J., C.D.W., S.P.G., and B.M.S. planned the majority of experiments and wrote the paper. M.P.J. developed the mass spectrometry assay for the irisin identification with heavy peptides. C.D.W. developed the serum clean-up and deglycosylation protocol and performed the statistical analysis. M.P.J. and S.P.G. performed the peptide fingerprinting identification of irisin. J.A.P., J.S., and K.K.G. contributed with technical assistance for the serum preparation, western blotting, or mass spectrometry. M.M.R. and K.S.N. performed the human cohort study.

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