Cell Metabolism, Volume *36*

Supplemental information

Fndc5 is translated from an upstream ATG start codon and cleaved to produce irisin myokine precursor protein in humans and mice

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Supplementary Figure 1

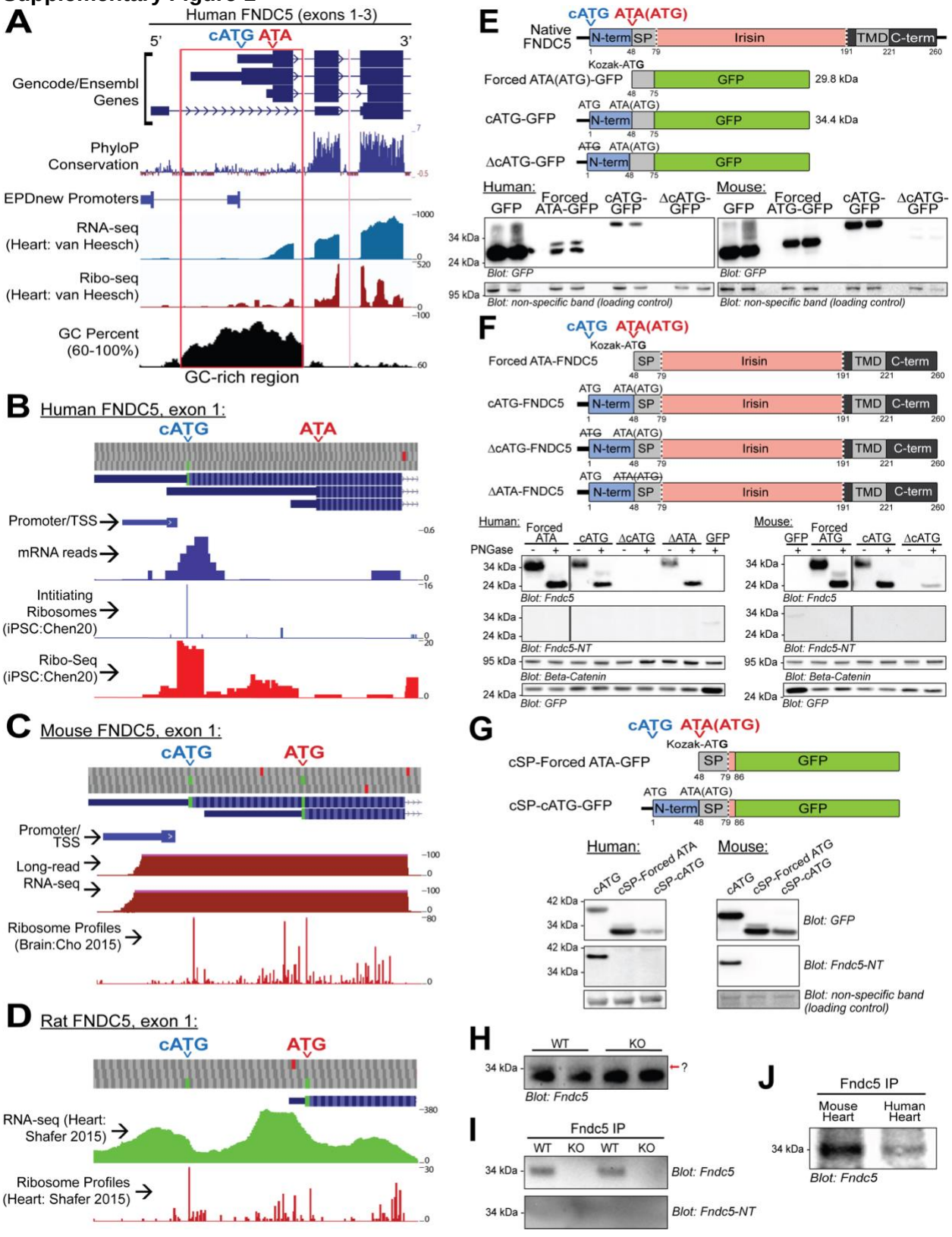

Figure S1: Fndc5 is translated from an upstream ATG start codon and cleaved to produce irisin myokine precursor protein in humans and mice

A) Schematic of the human FNDC5 genomic architecture (exons 1-3, introns removed) based on GENCODE/Ensembl (v44) annotations. Screenshots of PhyloP conservation track, and experimentally validated promoter track (EPDnew) visualized on the UCSC Genome Browser. Representative human heart RNA- and Ribo-seq data9 showing translation occurring upstream of the "canonical" ATA. Red box outlines a highly GC-rich region (GC Percent UCSC track) that likely impedes sequencing data acquisition and processing in this region.

B) Screenshots from the UCSC Genome Browser of the human FNDC5 exon 1 region (the most recent GENCODE/Ensembl v44 annotations are shown). EPDnew promoter track is shown with human heart RNA-seq (Burge RNA-seq track, hg19) noting the clear presence of RNA-seq reads mapping immediately downstream of the promoter. Initiating and elongating ribosome profiling from GWIPS-Viz Ribo-seq browser (iPSC:Chen20 tracks) indicates ribosomes initiate translation at an in-frame ATG (cATG) that is upstream of the annotated non-canonical ATA start codon.

C) Screenshots from the UCSC Genome Browser of the mouse FNDC5 exon 1 region (the most recent GENCODE/Ensembl M33 annotations are shown). EPDnew track is shown with representative mouse heart RNA-seq (CSHL Long RNA-seq track, mm9), indicating transcription begins near the corresponding region in humans. Ribosome profiling data from GWIPS-Viz Ribo-seq browser shows the clear presence of translating ribosomes beginning at an in-frame ATG (cATG) that is upstream of the canonical start codon.

D) Screenshots from GWIPS-Viz Ribo-seq browser of the rat FNDC5 exon 1 region. RNA-seq and ribosome profiling tracks (Heart: Shafer 2015 tracks) are shown noting the clear presence or RNA and ribosomes upstream of the annotated transcription and translation initiation sites. Notably, ribosome profiles begin near the cATG, upstream of the annotated start codon.

E) Top, schematic of native Fndc5 cDNA (with annotated protein-coding domains) and various GFP reporter constructs fused to 5' Fndc5 segments. "ATA(ATG)" refers to the previously alleged start codons (ATA for human and ATG for mouse), which are placed into Kozak sequence contexts in "Forced" constructs. ΔcATG-GFP has the upstream cATG deleted. Bottom, GFP reporter constructs were transfected into HT1080 cells and subsequent western blotting of cell lysates indicates that human (left GFP blot) and mouse (right GFP blot) Fndc5 expression is primarily driven from the upstream cATG. Abbreviations and marks: N-term, cATG-derived extended N-terminus; SP, signal peptide; white dotted line, signal peptide cleavage site; TMD, transmembrane domain; C-term, C-terminus.

F) Top, schematic of full-length native Fndc5 cDNA and mutant constructs used to assess Fndc5 protein expression from the cATG and ATA codons. Bottom, transfected HT1080 cell lysates were treated with or without PNGase F (a deglycosylase which removes N-linked oligosaccharides) and blotted using antibodies that recognize the core irisin ectodomain (anti-Fndc5) or the extended N-terminus (Fndc5-NT). A transfection control (GFP, derived from an ires-GFP cassette) and a loading control (Beta-Catenin) are also shown.

G) Top, schematic of GFP reporters used to assess Fndc5 signal peptide (SP) cleavage. Bottom, western blot of transfected HT1080 cells using GFP or custom FNDC5-NT antibodies showing human or mouse cATG- and Forced ATA/ATG-derived Fndc5 N-termini are cleaved to release proteins running at the same size. cSP, cleaved signal peptide reporters. Schematic of the cATG reporter construct is shown above in Figure S1E.

H) Western blot of total heart lysates from wildtype (WT) and global FNDC5 knockout (KO) mice showing non-specific or questionable bands at the expected size. Red arrow indicates a shadow band which could be Fndc5.

I) Western blot of immunoprecipitated Fndc5 from wildtype (WT) or global FNDC5 knockout (KO) mouse hearts showing convincing 34 kDa Fndc5 bands when blotted using an anti-Fndc5

antibody, which are not present in KO samples and not immunoreactive with the custom Fndc5-NT antibody.
J) Western blot of anti-Fndc5 immunoprecipitates from WT mouse or human heart lysates showing the likely presence of 34 kDa Fndc5 band in humans that co-migrates identically to mouse.

Tables

Table 1: Primers used for RT-PCR amplification of the full-length human and mouse FNDC5 5'-UTR and coding region.

Primer Name	**Primer Sequence (5' -> 3')**
hsFNDC5 (F)	AAAAACTCGAGGAGTCGCTGTCCGCGGAGCCGACATG
hsFNDC5-full (R)	AAAAAGCTAGCTTATCATATCTTGCTGCGGAGAAGCCC
mmFNDC5 (F)	AAAAACTCGAGAGTCGCTGTCCGCGGAGCCGATATGCAGG
mmFNDC5-full (R)	AAAAAGCTAGCTTATCATATCTTGCTGCGGAGGAGACCC

Supplemental Experimental Procedures

Bioinformatics and data mining:

Screenshots of the human (hg38 and hg19) or mouse (mm9) *FNDC5* locus were taken from the UCSC genome browser, using the following tracks: EPDnew Promoters, Burge short-read RNA-seq, CSHL long-read RNA-seq. Human heart RNA- and ribosome-sequencing data[9] were viewed using the Integrative Genomics Viewer (version 2.13.2). Human (iPSC:Chen20), mouse (Brain:Cho 2015), and rat (Heart:Shafer 2015) ribosome profiling data were viewed using GWIPS-VIZ. The GC-rich region was identified using the "GC Percent" track (hg38), which shows GC percentage in 5-base windows. Screenshots of the tracks were aligned to the corresponding exons and introns were minimized for clarity.

RT-PCR:

Total RNA was isolated from ~50mg of snap-frozen human or mouse heart samples using Qiagen miRNeasy Mini Kit and subject to on-column DNase treatment, following the manufacturers protocol. 500ng of purified RNA was subject to first-strand cDNA synthesis using SuperScript IV Reverse Transcriptase and random hexamer primers (ThermoFisher Scientific), following the manufacturer's recommendations. The full-length *FNDC5* 5'-UTR and coding sequence was amplified from human or mouse heart cDNA libraries (to amplify products with extremely high GC-content) using Phusion High-Fidelity DNA polymerase (New England Biolabs). Briefly, a final concentration of 1M Betaine and 5% DMSO was included in the reaction mixture prior to performing a modified Touchdown PCR protocol, beginning at an annealing temperature of 70°C, decreasing by 2°C every 5 cycles until 62°C, and 10 additional cycles at the final annealing temperature. PCR products were analyzed by agarose gel electrophoresis using standard techniques.

Plasmid construction:

Full-length *FNDC5* cDNA expression plasmids were constructed by RT-PCR amplification using primers (**Table 1**) with added restriction enzyme sites and cloned into bicistronic CMV-based expression vectors containing an IRES-GFP cassette (University of Iowa Viral Vector Core). Mutant constructs were generated by PCR amplification using primers containing the desired mutation. The full-length human FNDC5-ΔATA construct was generated by replacing the region between two natively encoded XmaI restriction enzyme sites with the mutant sequence by ligation with Gibson Assembly Master Mix (New England Biolabs). GFP reporters were generated

by fusing FNDC5 fragments, in-frame, to an eGFP cassette in custom CMV-based expression plasmids. All plasmid constructions were verified by Sanger sequencing.

Cell culture and transfection:

Human HT1080 (ATCC, CCL-121) cells were maintained in DMEM with 100 U/ml penicillin, 0.1 mg/ml streptomycin, and 10% FBS at 37°C and 5% CO_2. For transfection, 150,000 cells per well were seeded in 24-well plates and the next day, cells were transfected with 250ng plasmid DNA using lipofectamine 2000 (ThermoFisher Scientific). After 48 hours, cells were harvested in 200ul RIPA buffer containing 1% Triton X-100 for western blot analysis.

Protein deglycosylation:

PNGase F (New England Biolabs) was used to deglycosylate whole cell protein extracts following the manufacturer's recommendations. Briefly, 20ul of protein extract was denatured by adding 1x Glycoprotein Denaturing Buffer (included with the PNGase F enzyme kit) and heating at 95°C for 10mins. After denaturing, 1x GlycoBuffer 2, 1% NP-40, and 1ul PNGase F were added and incubated at 37°C for 1 hour. When glycosylated and de-glycosylated samples were compared, samples were prepared identically except PNGase F was omitted where indicated.

Immunoprecipitation (IP):

Snap-frozen human and mouse hearts were crushed and thawed in lysis buffer (150mM NaCl, 10mM NaF, 5mM EDTA, 5mM EGTA, 50mM Tris pH 7.5, 1% Triton X-100) with fresh protease inhibitor (cOmplete Mini, EDTA-free protease inhibitor cocktail, Roche), followed by shaking in a TissueLyser (Qiagen). Lysates were clarified and total protein was quantified by BCA protein assay (ThermoFisher). For immunoprecipitation, 3ug primary antibody (anti-Fndc5 [AdipoGen, IN102)]) were coupled to 50ul anti-Rabbit IgG coated Dynabeads (ThermoFisher, 11204D), following the manufacturers protocol. Coated beads mixed with with 1mg protein diluted to 1ml in IP buffer (50mM Tris pH 7.5, 150mM NaCl, 10mM NaF, 5mM EDTA, 5mM EGTA, 0.5% Triton X-100) and incubated rotating for 2 hours at 4°C. Samples were washed 3 times in IP buffer containing 0.05% Triton X-100 and eluted in 50ul 1x LDS sample buffer containing reducing agent with heating at 50°C for 10 minutes.

Western blotting:

Protein extracts were prepared by addition of 1x LDS sample buffer containing reducing agent (NuPAGE reagents, ThermoFisher Scientific) and denatured at 95°C for 10 minutes. Proteins were resolved by standard SDS-PAGE on 4-12% Bis-Tris gels (NuPAGE system, ThermoFisher Scientific) before transfer to 0.2 um nitrocellulose membrane (Amersham) using NuPAGE transfer buffer containing 10% ethanol and 0.01% SDS. The membrane was blocked with 5% milk in 1x TBST (0.1% Tween-20) prior to incubation with primary antibodies: anti-Fndc5 (AdipoGen, IN102), anti-Fndc5-NT (custom, BioMatik), anti-GFP (ThermoFisher, A-6455), and anti-Beta-Catenin (Sigma, PLA0230). Membranes were probed with HRP-conjugated goat-anti-rabbit secondary antibody (Jackson ImmunoResearch, 111-035-144) and chemiluminescence detection was performed using West Femto Maximum Sensitivity Substrate (ThermoFisher Scientific). For immunoprecipitation experiments, proteins were detected using HRP-conjugated

Fc-fragment specific goat-anti-rabbit secondary antibody (Jackson ImmunoResearch, 111-036-046) to avoid cross-reactivity with the antibody used for IP, which is eluted.

RESOURCE AVAILABILITY

Plasmids and antibodies generated in this study are available with a completed materials transfer agreement. Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Ryan Boudreau (ryan-boudreau@uiowa.edu).