HHS Public Access

Author manuscript

Cell Metab. Author manuscript; available in PMC 2024 November 12.

Published in final edited form as:

Cell Metab. 2024 May 07; 36(5): 879–881. doi:10.1016/j.cmet.2024.02.008.

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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The existence of irisin, a putative myokine encoded by *FNDC5*, has been debated since Boström et al. reported that exercise induces Fndc5 protein cleavage to produce secreted irisin.[1] Irisin has become the focus of intense research and controversy,[2–6] with many studies examining its biosynthesis, physiological functions, and therapeutic potential.[7] Much of the controversy centers around the fact that the annotated ATG start codon (in most mammals, including mice) is mutated to ATA in humans and thus cannot translate full-length protein. Raschke et al. used plasmid-based reporters to demonstrate that ATA-mediated Fndc5 translation is virtually undetectable.[2] Nevertheless, Jedrychowski et al. subsequently reported detection of irisin peptides in human plasma and concluded that translation must initiate from the ATA start codon.[5]

We seek to resolve some controversy surrounding the mode of Fndc5 (irisin precursor protein) expression in humans and rodents. While evaluating human *FNDC5* gene architecture and expression in genome browsers (Figure S1A), we noted the presence of RNA sequencing (RNA-seq) and ribosome profiling (Ribo-seq) reads mapping upstream of the primary annotated *FNDC5* transcript 5′ ends. These reads align to a validated transcription start site (TSS) annotated by the Eukaryotic Promoter Database (EPDnew track). Notably since then, the newest human and mouse GENCODE annotations now include transcripts that begin at this TSS; however, these 5′-extended transcripts remain unannotated in most species. Despite observing some upstream RNA reads in humans, there

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AUTHOR CONTRIBUTIONS

R.L.B. conceived and designed the project, supervised research, and analyzed and interpreted the data. N.H.W. designed and executed experiments, analyzed data, and participated in data interpretation. C.R.L. executed experiments. R.L.B. and N.H.W. wrote the manuscript.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.cmet.2024.02.008.

DECLARATION OF INTERESTS

The authors declare no competing interests.

remains a general paucity in RNA-seq read coverage in this region, with a sharp drop-off 5′ of the ATA start codon (Figures S1A and S1B). Notably, this region is extremely GC-rich (>85%), which likely hinders RNA and genome sequencing and/or mapping; e.g., many species lack genome sequence coverage in this region. Similar to humans, we identified mouse and rat heart RNA-seq datasets that also show clear expression of RNA upstream of previous *FNDC5* transcript annotations, with initiation aligning to the same EPDnew promoter (Figures S1C and S1D).

Downstream of this overlooked TSS, we identified a highly conserved start codon (cATG) in-frame with *FNDC5* coding sequence (Figures S1A–S1D), which is now annotated in the newest human and mouse GENCODE releases. This caught our attention given the controversies regarding irisin's existence and how human Fndc5 could be translated with the perceived lack of a suitable ATG start codon. Examination of human Ribo-seq[8] data shows reads (i.e., translation) beginning near this cATG, complemented by a clear signal for initiating ribosomes (GWIPS-viz Ribo-seq browser), which is not the case for the putative ATA start codon (Figures S1A and S1B). Although current human Ribo-seq coverage in this region is rather low, rodent Ribo-seq data show convincing upstream read coverage with prominent initiation at the cATG (Figures S1C and S1D) versus negligible downstream initiation at the previously alleged "canonical" ATG.

To empirically test if *FNDC5* cATG supports translation, we generated reporter constructs fusing GFP in-frame downstream of the first *FNDC5* exon (containing the full extended 5′ UTR, cATG, downstream ATA [human] or ATG [mouse] start codons, and initial coding sequence up to but before the signal peptide cleavage site; cATG-GFP; Figure S1E). Constructs with deletion of the cATG (ΔcATG-GFP) were also made, along with constructs designed to force translation initiation from the downstream ATA (human) or ATG (mouse) start codons by adding Kozak sequences at these positions (e.g., forced ATA-GFP). Constructs were transfected into cells, and western blotting of cell lysates clearly shows banding patterns supporting that translation initiates almost exclusively at the cATG in both humans and mice (Figure S1E). Specifically, cATG constructs only produced larger cATG-derived proteins (N-terminally extended proteins that are lost with ΔcATG) and no detectable expression from downstream ATA/ATG starts sites (no bands co-migrating with those derived from "forced" constructs). Also, with ΔcATG-GFP constructs, we found no evidence for translation initiation from the human ATA and, surprisingly, only minimal expression from the downstream "canonical" mouse ATG versus cATG and "forced" constructs.

We next generated a similar panel of plasmids to assess protein output from constructs expressing native full-length *FNDC5* (Figure S1F), as well as a downstream GFP reporter to monitor transfection efficiency (driven by an internal ribosomal entry site). Notably, we performed RT-PCR to clone these *FNDC5* cDNAs from human and mouse heart tissue RNA samples, confirming that the 5′-extended transcripts are naturally expressed. Constructs were transfected into cells, and western blotting using a commercially available anti-Fndc5 antibody corroborates that Fndc5 translation initiates primarily at cATG in both humans and mice (Figure S1F). No human Fndc5 was detected when the cATG is deleted (ΔcATG), and

deletion of the ATA (ΔATA) did not influence Fndc5 expression, furthering support against ATA-driven translation.

Unexpectedly, we found that "forced" ATA(ATG)-FNDC5 and native *FNDC5* (cATG-*FNDC5*) constructs produced similarly sized proteins (20 or 34 kDa with or without deglycosylation, respectively; Figure S1F), suggesting that extended Fndc5 protein may be cleaved at or near the signal peptide. To address this, we generated an antibody against extended Fndc5 N terminus (anti-Fndc5-NT; immunogen sequence upstream of ATA [conserved in mice]) and validated its use for western blotting, demonstrating that it clearly detects prominent bands from human and mouse cATG-GFP constructs lacking the signal peptide cleavage site (shown in Figure S1G). As suspected, western blotting indicated that proteins derived from human and mouse cATG-*FNDC5* constructs are not immunoreactive with anti-Fndc5-NT (Figure S1F), suggesting that the N terminus is likely efficiently co-translationally cleaved and degraded similar to other signal peptides.[9–11] To test this, we generated extended human and mouse GFP reporter constructs containing the known signal peptide and cleavage site (cSP-cATG-GFP; Figure S1G). Western blotting of transfected cell lysates indicates that inclusion of the signal peptide causes cATG-derived reporters to be cleaved to generate proteins that migrate identically to "forced" ATA-(human)- or ATG-(mouse)-derived reporters (cSP-forced-ATA-GFP), with no immunoreactivity to anti-Fndc5-NT (Figure S1G).

Our data support that Fndc5 is translated from the upstream cATG and cleaved to produce a protein that migrates at 34 kDa (glycosylated state). We assessed if we could detect 34 kDa Fndc5 in mouse and human protein lysates. Western blot of mouse heart lysates from global *FNDC5* knockout (KO) and wild-type (WT) mice using anti-Fndc5 antibody showed non-specific bands in WT and KO samples at the expected size (Figure S1H), consistent with reports citing challenges with detecting endogenous Fndc5/irisin.[4,6,7,12] We next performed immunoprecipitation (IP) and western blotting using anti-Fndc5 antibody, which showed clear 34 kDa Fndc5 bands in WT samples that were absent in KO samples and not immunoreactive with anti-Fndc5-NT (Figure S1I). Finally, we performed anti-Fndc5 IP and western blotting on WT mouse and human cardiac lysates, which revealed the likely presence of 34 kDa Fndc5 in humans (Figure S1J).

Together, our findings uncover the most probable mode of Fndc5/irisin precursor protein synthesis in humans (and other species) and should help to resolve a decade of controversy.[2–6] Future research will be needed to better characterize the complex biogenesis and processing of endogenous human Fndc5/irisin precursor protein, while addressing (1) the biological rationale for an extended N terminus (perhaps generating a cleaved functional peptide, the fate of which remains unknown; we have yet to detect cleaved N-terminal fragments) and (2) the functional significance and timing of potential sequential cleavage events (perhaps to ensure proper folding and/or post-translational modification).[9–11] These and other studies aimed at firmly establishing that N-terminally extended Fndc5 is subsequently cleaved to release the irisin myokine will contribute to a broader field working to further establish its likely translational relevance to human health and disease.

Limitations of the study: Our data strongly support our main conclusions; however, we primarily employ plasmid-based expression vectors, and obtaining unequivocal evidence of endogenous upstream Fndc5 translation in human (and mouse) cells may require follow-up work with CRISPR editing to generate KO samples to ensure antibody specificity, as well as cATG mutations to verify loss of Fndc5/irisin precursor protein.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGMENTS

We thank Bruce Spiegelman (Harvard Medical Center) for generously providing global *FNDC5* knockout and wild-type littermate mouse heart tissues. We thank Dr. Kenneth Margulies (UPenn Human Heart Tissue Bank) for providing human heart tissue. This work was supported by the University of Iowa Carver College of Medicine (Distinguished Scholars Program to R.L.B.), NIH NHLBI (HL148796, HL144717, and HL150557 to R.L.B.), NIH NIGMS (predoctoral fellowship T32 GM067795 to N.H.W.), American Heart Association (20IPA35360150 to R.L.B. and 23PRE1011277 to N.H.W.), and University of Iowa Office of Undergraduate Research (fellowship to C.R.L.).

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