Creating Optimal Conditions for OPA1 isoforms by Western Blot in Muscle Tissue

Dominique C. Stephens[1,2*] Amber Crabtree[1*], Heather K. Beasley[1]*, Edgar Garza-Lopez[3],, Margaret Mungai[3], Kit Neikirk[1], Larry Vang[1], Zer Vue[1], Neng Vue[1], Andrea G. Marshall[1], Kyrin Turner[2], Jianqiang Shao[6], Sandra Murray[5], Jennifer A. Gaddy[7,8] Celestine Wanjalla[9], Jamaine Davis[4], Steven Damo[2#] Antentor O. Hinton Jr[1#]

1 Department of Molecular Physiology and Biophysics, Vanderbilt University, Nashville, TN, 37232, USA

2 Department of Life and Physical Sciences, Fisk University, Nashville, TN, 37232, USA

3 Department of Internal Medicine, University of Iowa, Iowa City, IA, 52242, USA

4 Department of Biochemistry and Cancer Biology. Meharry Medical College, Nashville, TN, 37208, USA

5 Department of Cell Biology, College of Medicine, University of Pittsburgh, Pittsburgh, PA, 15260, USA

6 Central Microscopy Research Facility, University of Iowa, Iowa City, IA, 52242, USA

7 Division of Infectious Diseases, Vanderbilt University School of Medicine, Nashville, TN, 37232, USA

8 Tennessee Valley Healthcare Systems, U.S. Department of Veterans Affairs, Nashville, TN, 37212, USA

9 Vanderbilt University Medical Center: Department of Medicine, Division of Infectious Disease, Nashville, TN, USA

*Co-first Authors

[#]Corresponding Authors:

Jamaine Davis, PhD
Meharry Medical College
jdavis@mmc.edu

Steven Damo, PhD
Fisk University
sdamo@fisk.edu

Antentor O. Hinton, Jr, PhD
Vanderbilt School of Medicine Basic Sciences
antentor.o.hinton.jr@vanderbilt.edu

Summary

OPA1 is a dynamin-related GTPase that modulates various mitochondrial functions and is involved in mitochondrial morphology. There are eight different isoforms of OPA1 in humans that are expressed as short or long-form isoforms. These isoforms contribute to OPA1's ability to control mitochondrial functions. However, isolating OPA1 isoforms through western blot has been a difficult task. To address this issue, we outline an optimized western blot protocol to isolate different isoforms of OPA1 on the basis of different antibodies. This protocol can be used to study changes in mitochondrial structure and function.

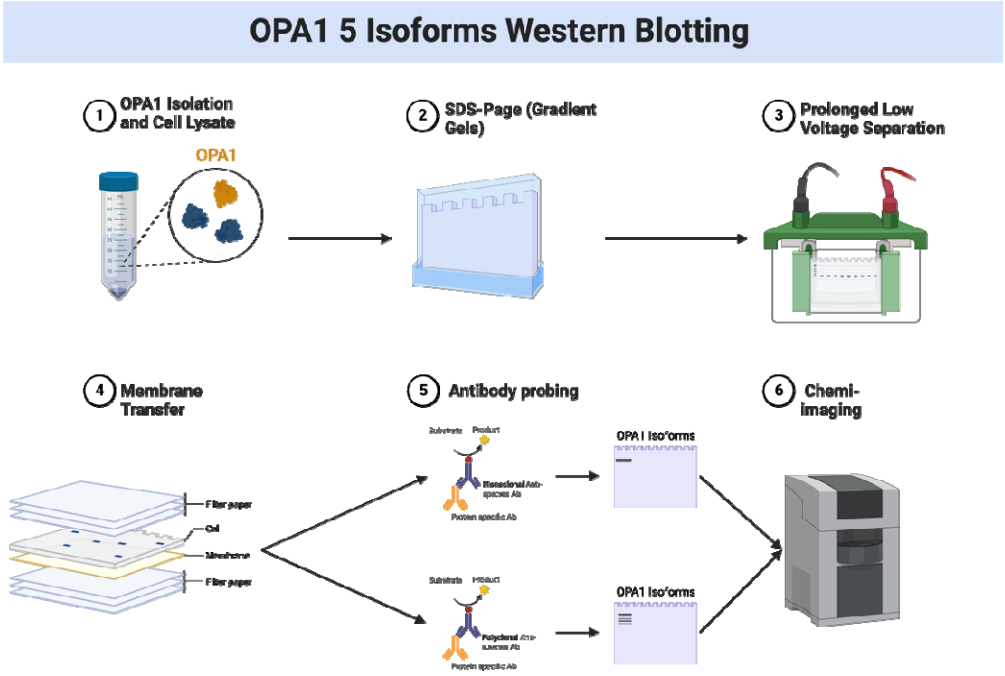
Tweetable Abstract

Western blot protocol optimization to visualize OPA1 isoforms.

Highlights:

- Protocol for isolating OPA1 isoforms in skeletal muscle tissue
- Steps for running isolated skeletal muscle cells from muscle tissue on a gel
- How to collect samples in preparation for western blotting
- Detection of OPA1 isoforms

Graphical Abstract



Key Resources Table		
Reagent or Resource	Source	Identifier
Antibodies		
Anti-OPA1 antibody (for 5 isoforms)	abcam	ab42364
Anti-OPA1 antibody (for 2 isoforms)	BD Biosciences	#612606
Rabbit Anti-Mouse IgG H&L	abcam	ab6728
Experimental Models: Cell Lines		
C2C12	ATCC	CRL-1772
Chemicals		
NaCl	Thermo Scientific	7647-14-5
Tris Base	Millipore Sigma	77-86-1
SDS	Millipore Sigma	151-21-3
Triton X-100	Thermo Scientific	9002-93-1
Sodium deoxycholate monohydrate	Thermo Scientific	145224-92-6
EDTA	Millipore Sigma	60-00-4
Protease inhibitor tablet	Roche	COEDTAF-RO
Glycerol	Millipore Sigma	56-81-5
β-mercaptoethanol	Millipore Sigma	60-24-2
Bromophenol blue	Thermo Scientific	115-39-9
DTT	Thermo Scientific	R0861
Glycine	Millipore Sigma	56-40-6
Methanol	Millipore Sigma	67-56-1
Tween-20	Millipore Sigma	9005-64-5

Dulbecco's Modified Eagle Medium (DMEM)	Thermo Scientific	11965084
Fetal bovine serum (FBS)	Thermo Scientific	16000044
PBS	Thermo Scientific	10010023
Penicillin/streptomycin (Pen/Strep) 100×	Thermo Scientific	10378016
w/v nonfat dry milk	Biotium	22012
BSA	Millipore Sigma	9048-46-8
TBS	Bio-Rad	1706435
2X Laemmli sample buffer	Bio-Rad	1610737
Quantification and Software		
GraphPad	GraphPad Software, San Diego, California USA	www.graphpad.com
Image J	Schindelin et al.5	https://imagej.net/
Critical Commercial Assays		
SuperSignal™ West Pico PLUS Chemiluminescent Substrate	Thermo Scientific™	34579
Other		
100 mm plate	Corning	353003
Microcentrifuge tube	Thermo Scientific	AM12450
22 G needle	Amazon	LY-999
Tris-glycine Mini Gels	Novex	XP00100BOX
Vertical Electrophoresis System	Bio-Rad	1658004
Nitrocellulose membrane	Bio-Rad	1620115
Microcentrifuge	Thermo Scientific	75002492

Method Summary

Samples for western blot analysis are isolated from lysed cells, loaded onto a gel, and ran using optimized conditions to better isolate OPA1 isoforms. Samples are transferred to a membrane to for incubation and protein detection using OPA1 antibodies.

Keywords: Muscle Tissue, Mitochondria, Optic atrophy-1 (OPA1), Western Blot, isoforms, isolation

Background

Optic atrophy-1 (OPA1) is a GTPase located in the inner membrane of mitochondria and is a central player in mitochondrial fusion [1]. OPA1 is a widely expressed protein localized to the inner mitochondrial membrane, which regulates mitochondrial fusion and cristae morphology and protects against apoptosis [2]. OPA1 is also associated with changes in mitochondrial structure, respiratory chain and membrane potential maintenance, cristae organization, apoptosis regulation, mitochondrial DNA maintenance, and neurodegenerative diseases [1,3]. The connection between OPA1 and so many different cellular functions have the potential to lead to future

therapies. However, additional exploration is needed to understand OPA1-associated mechanisms and pathologies.

There are eight OPA1 isoforms expressed in humans [4]. These isoforms can present themselves as long-form or short-form isoforms and are associated with different mitochondrial functions. While long-form and short-form isoforms are able to restore cristae structure, mitochondrial DNA (mtDNA) abundance, and energetic efficiency [4], long and short isoforms have differential roles. More specifically, in a membrane potential-dependent manner, long isoforms are cleaved to short isoforms by OMA1, to cause increased fission [5]. Notably, certain experimental conditions, typically associated with cellular stress such as cold exposure, have been illustrated to cause increased cleavage of OPA1 to short isoforms [6]. However, short isoforms still play important roles in energetic efficiency, requiring a balance of short and long isoforms [4]. While traditional monoclonal antibodies allow for seeing these two principal long and short isoforms, they do not allow for additional isoforms to be observed beyond these two [7]. Recently, researchers found that OPA1-specific isoforms may have functional roles, such as 1 and 7 which have therapeutic potential for diseases caused by mitochondrial dysfunction [8]. Therefore, it is highly important to be able to efficiently isolate and study OPA1 isoforms. Here, we show optimal conditions for isolating OPA1 isoforms by western blot.

Materials and Methods

Buffers

To lyse the cell, we used a RIPA buffer:

Reagent	Stock Concentration	Final Concentration	Amount
NaCl	5M	150 mM	320ul
Tris pH 7.2	1.0M	10 mM	100ul
SDS	10%	0.1%	100ul
Triton X-100	100%	1.0%	100ul
deoxycholate	10%	1%	80ul
EDTA	0.5M	5 mM	40ul
Di H2O	N/A	N/A	9.26mL
Protease inhibitor tablet	N/A	N/A	1 Tablet
Total	N/A	N/A	10mL

Milder lysis buffer (a non-denaturing buffer):

Reagent	Stock Concentration	Final Concentration	Amount
NaCl	5M	150 mM	320ul
Tris pH 7.5	1.0M	20 mM	200ul
NP40	10%	1%	80ul
EDTA	0.5M	5 mM	40ul
Di H2O	N/A	N/A	9.36mL
Protease inhibitor tablet	N/A	N/A	1 Tablet
Total	N/A	N/A	10mL

Once cell lysate samples are obtained, we mixed our samples with 6X sample buffer before loading:

Reagent	Stock Concentration	Final Concentration	Amount
Tris pH 6.8	1.5 M	0.375 M	2.5mL
Glycerol	100%	48%	4.8mL
SDS	N/A	6%	0.6g
β-mercaptoethanol	100%	6%	0.6mL
Bromophenol blue	N/A	0.06%	0.006g
DTT	N/A	0.6 M	0.930g
DI H2O	N/A	N/A	2.1mL
Total	N/A	N/A	10mL

After the samples were loaded onto an SDS-PAGE gel, we used 1X Tris-glycine buffer as our running buffer:

Reagent	Stock Concentration	Final Concentration	Amount
Tris base	N/A	25 mM	12.1g
Glycine	N/A	250 mM	75.06g

SDS	N/A	0.1%	4g
DI H2O	N/A	N/A	4L

To transfer to a membrane, we used a 1X Tris-glycine transfer buffer:

Reagent	Stock Concentration	Final Concentration	Amount
Tris base	N/A	25 mM	12.1g
Glycine	N/A	192 mM	57.6g
Methanol	100%	20%	800mL
SDS	N/A	0.02%	0.8g
DI H2O	N/A	N/A	4L

The following buffers needed for antibody incubations: Blocking buffer

Reagent	Stock Concentration	Final Concentration	Amount
TBS	10X	1X	10mL
Tween-20	100%	0.1%	0.1mL
w/v nonfat dry milk	N/A	5%	5g
DI H2O	N/A	N/A	89.9mL
Total	N/A	N/A	100mL

Antibody dilution buffer:

Reagent	Stock Concentration	Final Concentration	Amount
TBS	10X	1X	10mL
Tween-20	100%	0.1%	0.1mL
BSA	N/A	5%	5g
DI H2O	N/A	N/A	89.9mL
Total	N/A	N/A	100mL

Wash buffer TBS-T:

Reagent	Stock Concentration	Final Concentration	Amount
TBS	10X	1X	10mL
Tween-20	100%	0.1%	0.1mL
DI H2O	N/A	N/A	89.9mL
Total	N/A	N/A	100mL

Western Blot Protocol for Isolating OPA1 Isoforms in Skeletal Muscle Tissue

You can run a two-band blot of OPA1 by running a standard western blot protocol and then blotting for OPA1 (**Figure 1**). However, if you happen to see a change in your OPA1 protein level, we outline a protocol to look at all isoforms of OPA1 (**Figure 2**).

CRITICAL: The antibody utilized determines the number of bands observed. Monoclonal antibody allows for earlier detection of proteins, yet polyclonal antibody is necessary to see more than 2 isoforms of OPA1 [7].

1. Day 1:

CRITICAL: Everything must be chilled in advance

This section describes how to collect and prepare samples.

a. Aspirate media
b. Wash cells with ice-cold PBS to remove residual media
c. Aspirate PBS
d. Add RIPA buffer directly to cells.
e. Scrap cells from plate
f. Transfer scraped cells to a microcentrifuge tube.

Note: We used 1 mL in a 100 mm plate. Scale up or down as necessary.

g. Incubate the microcentrifuge tubes on ice for 10 mins (vortexing every few minutes)

Note: Lysates can also be passed through a 22 G needle to aid in solubilization.

h. Centrifuge the microcentrifuge tubes at 17,000 rpm for 10 mins.

This section describes how to run protein samples on an SDS-PAGE gel

i. Load 20-50 µg of lysate with loading dye (Bio-Rad 2X Laemmli sample buffer and add BME)
j. Run lysate samples on **Novex 4-20% Tris-glycine Mini Gels** (pre-chilled for one hour in cold running buffer before use).

Note: You can make your own gels also, but it is important to use a gradient gel, as they provide better separation than normal gel for the close molecular weights of OPA1 isoforms.

Note: It is important to ensure that all materials and reagents, especially the Novex Mini Gels, are chilled for an hour prior to use.

i. Run gel for **10 mins at 100 V** (small gel boxes) to get the sample through the wells.

Note: For larger gel boxes, 170-200 V can be used, but multiple isoforms may not be seen.

k. Reduce the voltage to **50 V for 5-7 hrs**.

Note: Gels can be run overnight at **35 V**.

2. Day 2:

This section describes how to transfer proteins from a gel to a membrane.

a. Transfer the Tris-glycine gel to a **nitrocellulose membrane** for **12 hours at 35 mV** in a cold room.

Antibody Incubations

b. Wash the membrane in 1X TBS for 5 mins.

c. Cover the membrane with the blocking buffer.

d. Shake membrane in blocking buffer overnight at 4 □ or 1 hr at room temperature.

3. Day 3:

a. Wash the membrane three times with TBST for 5 mins each.

b. Incubate membrane with primary antibody for 1 hr at room temperature,

c. Wash three times with TBST for 5 mins each.

d. Incubate and shake the membrane with the appropriate conjugated secondary antibody for 1 hr at room temperature.

e. Wash the membrane three times with TBST for 5mins each.

Protein Detection

f. Prepare ECL detection reagent by mixing solutions A & B in a 1:1 ratio.

g. Cover membrane with ECL solution

h. Incubate for 5 mins at room temperature with gentle rocking.

i. Remove the membrane from the solution.

j. Wrap the membrane in plastic wrap, (while eliminating air bubbles)

k. Place the membrane in an exposure cassette.

l. Expose to film for different amounts of time.

Expected Outcomes

Notably, while OPA1 deficiency causes a loss of mitochondrial morphology it also promotes insulin sensitivity in a fibroblast growth factor 21-dependent manner [9]. Here we used C2C12 myoblasts and myotubes and, following serum starvation overnight, performed insulin treatment (10 nM/L) in 2-hour increments. To begin with, we performed a standard WB procedure with a monoclonal antibody (Figure 1A), which showed a progressive increase of OPA1 levels across 4 hours due to insulin stimulation in myoblasts (Figure 2B). Given that the AKT pathway aids in regulating Opa1 cleavage [10], it was expected that there would be concomitant changes in AKT and the active phosphorylated form of AKT. Furthermore, it is well established that insulin stimulation increases the expression of AKT [11]. Notably, recent studies in cardiomyocytes have elucidated that insulin stimulation activates the Akt-mTOR-NFκB pathway to increase OPA1 [7]. We saw a similar increase in AKT as OPA1, marked by significant progressive increases after 2 and 4 hours (Figure 1C-D).

From there, we utilized this protocol to result in the visualization of 5 isoform bands of OPA1 (Figure 2). We used 2 different factors to allow for normalization and as an internal control gene, GAPDH and alpha-tubulin. When looking at the 5 OPA1 isoforms across insulin stimulation (Figure 2A), we noticed again an increase in OPA1 levels, but conversely, there was a more significant increase after 2 hours, while 4 hours showed a smaller increase compared to the control (Figure 2B). Paralleling this was a similar change in AKT which was additionally consistent across 6 hours of insulin stimulation (Figure 2C-D). Previously in cardiomyocytes, it was elucidated that both 2 isoform bands of OPA1 and 5 isoforms of OPA1 show a similar change in expression due to insulin stimulation [7]. Here, we show that changes in the total expression of OPA1 can be noted by looking at 5 isoforms of OPA1 as opposed to only long and short isoforms.

Quantification and statistical analysis

Bands from the blot are quantified using ImageJ. Statistical analyses were performed using GraphPad software.

Limitations

The main limitation of western blotting is that researchers are confined to using commercially available primary antibodies for detection. Beyond this, cross-reactivity with other proteins or nonspecific binding can lead to false-positive or false-negative results. Here, specific antibodies are utilized depending on the OPA1 isoform desired, but polyclonal antibodies in certain cell types such as cardiomyocytes may not detect modifications in the levels of the Opa-1 protein at an early stage [7]. Beyond that, it is unclear if the number of OPA1 isoforms is dependent on the cell type and if certain models have more or less isoforms. We have only noted 5 isoforms using this protocol, but it may be possible to optimize it to look at all 8 of the known OPA1 isoforms [4,8]. Specifically, future advances may try to look at relative abundance of specific bands, as the top two bands are hypothesized to be long forms of OPA1 while the bottom 3 bands are thought to be short isoforms [12]. Elucidating expression of these specific isoforms may be important given their differential role [4].

Looking at other proteins such as AKT to confirm associated changes may be important. Finally, it is possible that, depending on the experimental conditions, cleavage of Opa1 to C-terminal fragments is happening causing bands not to occur [13]. Given that this C-cleavage is dependent on Mfn2, and not common cleavage factors like the metalloprotease OMA1, it can be more difficult to look at upstream factors in such scenarios [5].

Troubleshooting

Problem 1

You do not detect any bands on your membrane.

Potential Solution

This can be fixed through several ways to increase the retention time. One may Increase the primary antibody inoculation time to O/N rocking in a cold room. Alternatively, increase the secondary antibody to no longer than 2hrs. Increase the amount of film exposure time. This lack of detection could be a result of the antibody not having enough time to bind to the proteins on the membrane. Also, we recommended being sure that the primary antibody has not expired, as that would decrease its effectiveness.

Problem 2

Your protein did not transfer from the gel to the membrane.

Potential Solution

This can be checked by using a bromo blue stain on the gel. If bands show up then the proteins were not transferred. Additionally, it is important to ensure that the stacking order is correct to avoid potential issues. Finally, it is important to optimize transfer to avoid weak or undetectable signals, without overtransfer, which can lead to nonspecific binding.

Problem 3

You are unable to tell how many isoforms are on the band

Potential Solution

Smaller separation means a smaller distribution of the different isoforms. We have found that 5-7 hour separation at 50V is key to allowing for all isoforms to be distinguishable. However, tweaking of experimental design to increase retention time may be necessary.

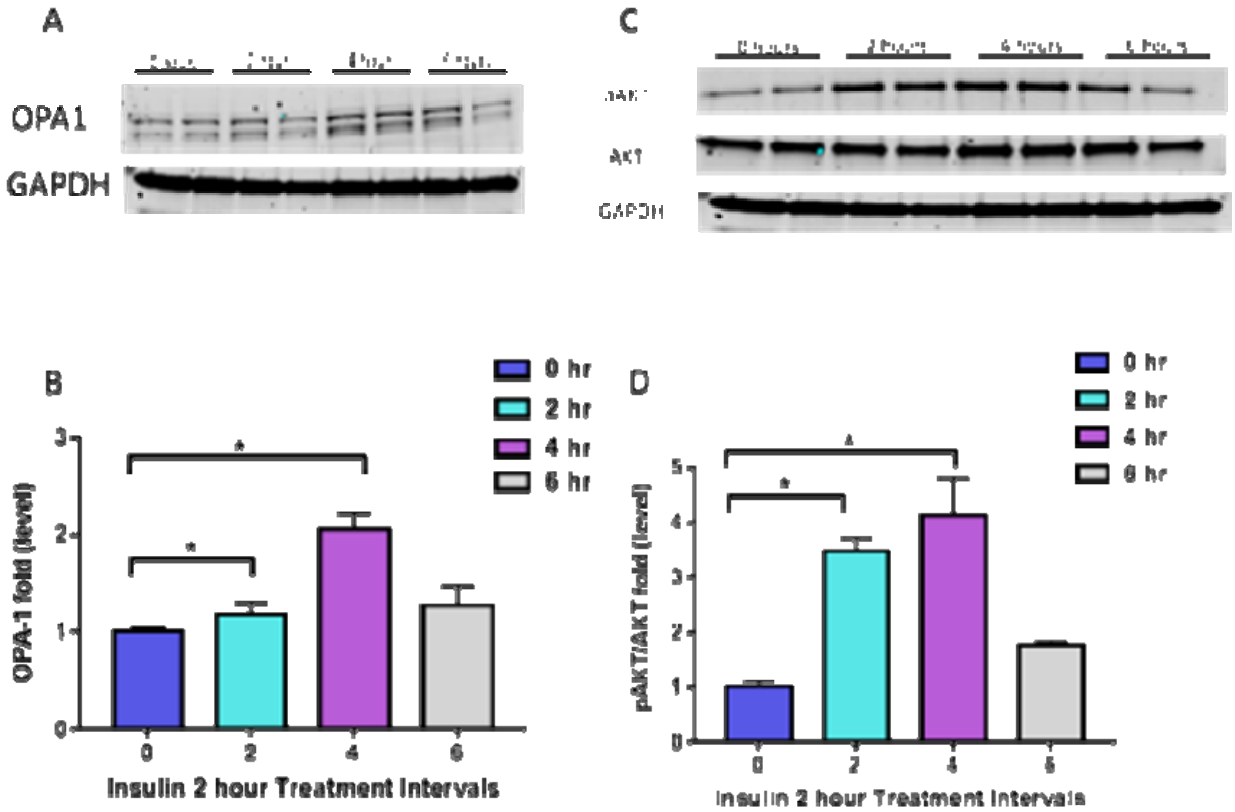


Figure 1. Example of two OPA1 bands. Post-2 hours and 4 hours Insulin Stimulation Increases OPA1 levels in C2C12 myoblasts **(A)** Western blot expression of OPA1 and GAPDH (Glyceraldehyde-3-phosphate dehydrogenase), following treatment with insulin for 0 – 6 hours. (**B**) OPA1 levels normalized to GAPDH expression across insulin stimulation intervals. (**C**) Western blot of OPA1, AKT/Protein Kinase B, and phosphorylated AKT, the active form of AKT, following treatment with insulin for 0 – 6 hours. (**D**) AKT and pAKT levels normalized to GAPDH expression across insulin stimulation intervals. N = 6 per treatment with triplicates, and * indicates p-value < .05

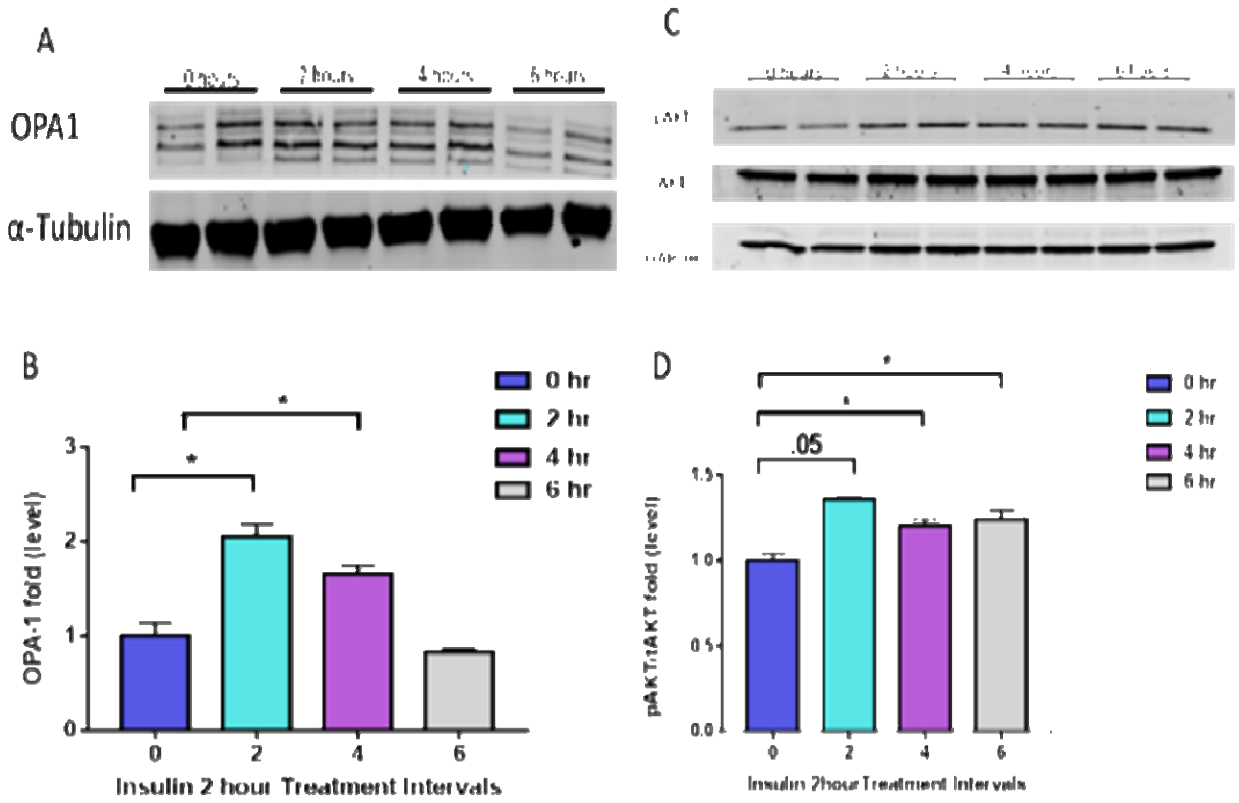


Figure 2. Example of five OPA1 bands. Post-2 hours and 4 hours Insulin Stimulation Increases OPA1 levels in C2C12 Myotubes **(A)** Western blot expression of OPA1 and alpha-tubulin, following treatment with insulin for 0 – 6 hours. (**B**) OPA1 levels normalized to alpha-tubulin expression across insulin stimulation intervals. (**C**) Western blot of OPA1, AKT/Protein Kinase B, and phosphorylated AKT, the active form of AKT, following treatment with insulin for 0 – 6 hours. (**D**) AKT and pAKT levels normalized to GAPDH expression across insulin stimulation intervals. N = 6 per treatment with triplicates, and * indicates p-value < .05

Resource Availability:

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Antentor Hinton (antentor.o.hinton.jr@Vanderbilt.Edu).

Materials availability

All generated materials, if applicable, are created in methods highlighted in the text above.

Data and code availability

Full data utilized and requests for data and code availability should be directed to and will be fulfilled by the lead contact, Antentor Hinton (antentor.o.hinton.jr@Vanderbilt.Edu).

Financial & Competing Interests' Disclosure

All authors have no competing interests.

This project was funded by the UNCF/Bristol-Myers Squibb E.E. Just Faculty Fund, BWF Career Awards at the Scientific Interface Award, BWF Ad-hoc Award, NIH Small Research Pilot Subaward to 5R25HL106365-12 from the National Institutes of Health PRIDE Program, DK020593, Vanderbilt Diabetes and Research Training Center for DRTC Alzheimer's Disease Pilot & Feasibility Program. CZI Science Diversity Leadership grant number 2022- 253529 from the Chan Zuckerberg Initiative DAF, an advised fund of Silicon Valley Community Foundation (to A.H.J.). NSF EES2112556, NSF EES1817282, NSF MCB1955975, and CZI Science Diversity Leadership grant number 2022-253614 from the Chan Zuckerberg Initiative DAF, an advised fund of Silicon Valley Community Foundation (to S.D.) and National Institutes of Health grant HD090061 and the Department of Veterans Affairs Office of Research award I01 BX005352 (to J.G.). Additional support was provided by the Vanderbilt Institute for Clinical and Translational Research program supported by the National Center for Research Resources, Grant UL1 RR024975–01, and the National Center for Advancing Translational Sciences, Grant 2 UL1 TR000445–06 and the Cell Imaging Shared Resource.

References

1. Chen, L., Gong, Q., Stice, J. P. & Knowlton, A. A. Mitochondrial OPA1, apoptosis, and heart failure. *Cardiovascular Research* **84**, 91–99 (2009).
2. Frezza, C. *et al.* OPA1 controls apoptotic cristae remodeling independently from mitochondrial fusion. *Cell* **126**, 177–189 (2006).
3. Belenguer, P. & Pellegrini, L. The dynamin GTPase OPA1: More than mitochondria? *Biochimica et Biophysica Acta (BBA) - Molecular Cell Research* **1833**, 176–183 (2013).
4. Del Dotto, V. *et al.* OPA1 Isoforms in the Hierarchical Organization of Mitochondrial Functions. *Cell Rep* **19**, 2557–2571 (2017).
5. Gilkerson, R., De La Torre, P. & St. Vallier, S. Mitochondrial OMA1 and OPA1 as Gatekeepers of Organellar Structure/Function and Cellular Stress Response. *Frontiers in Cell and Developmental Biology* **9**, (2021).

6. Pereira, R. O. *et al*. OPA1 deletion in brown adipose tissue improves thermoregulation and systemic metabolism via FGF21. *Elife* **10**, e66519 (2021).
7. Parra, V. *et al.* Insulin stimulates mitochondrial fusion and function in cardiomyocytes via the Akt-mTOR-NFκB-Opa-1 signaling pathway. *Diabetes* **63**, 75–88 (2014).
8. Maloney, D. M. *et al.* Optimized OPA1 Isoforms 1 and 7 Provide Therapeutic Benefit in Models of Mitochondrial Dysfunction. *Front Neurosci* **14**, 571479 (2020).
9. Pereira, R. O. *et al*. OPA 1 deficiency promotes secretion of FGF 21 from muscle that prevents obesity and insulin resistance. *The EMBO journal* **36**, 2126–2145 (2017).
10. Cai, W. J. *et al.* AKT-GSK3β Signaling Pathway Regulates Mitochondrial Dysfunction-Associated OPA1 Cleavage Contributing to Osteoblast Apoptosis: Preventative Effects of Hydroxytyrosol. *Oxid Med Cell Longev* **2019**, 4101738 (2019).
11. Kim, Y.-B., Nikoulina, S. E., Ciaraldi, T. P., Henry, R. R. & Kahn, B. B. Normal insulin-dependent activation of Akt/protein kinase B, with diminished activation of phosphoinositide 3-kinase, in muscle in type 2 diabetes. *J Clin Invest* **104**, 733–741 (1999).
12. Song, Z., Chen, H., Fiket, M., Alexander, C. & Chan, D. C. OPA1 processing controls mitochondrial fusion and is regulated by mRNA splicing, membrane potential, and Yme1L. *J Cell Biol* **178**, 749–755 (2007).
13. Sood, A. *et al.* A Mitofusin-2–dependent inactivating cleavage of Opa1 links changes in mitochondria cristae and ER contacts in the postprandial liver. *Proceedings of the National Academy of Sciences* **111**, 16017–16022 (2014).