Erwin Chemerinsky (*pro hac vice*)
echemerinsky@law.berkeley.edu
Claudia Polsky (CA Bar No. 185505)
cpolsky@law.berkeley.edu
U.C. BERKELEY SCHOOL OF LAW
Law Building
Berkeley, CA 94720-7200
Telephone: 510.642.6483

Elizabeth J. Cabraser (CA Bar No. 83151)
ecabraser@lchb.com
Richard M. Heimann (CA Bar No. 63607)
rheimann@lchb.com
LIEFF CABRASER HEIMANN &
BERNSTEIN, LLP
275 Battery Street, 29th Floor
San Francisco, CA 94111
Telephone: 415.956.1000

Anthony P. Schoenberg (CA Bar No. 203714)
tschoenberg@fbm.com
Linda S. Gilleran (CA Bar No. 307107)
lgilleran@fbm.com
FARELLA BRAUN + MARTEL LLP
One Bush Street, Suite 900
San Francisco, CA 94104
Telephone: 415. 954.4400

Attorneys for Plaintiffs and the Certified Classes

UNITED STATES DISTRICT COURT

NORTHERN DISTRICT OF CALIFORNIA

NEETA THAKUR, et al.,

Plaintiffs,

v.

DONALD J. TRUMP, et al.,

Defendants.

Case No. 3:25-cv-4737

DECLARATION OF ALEXANDER VAN DER BLIEK

DECLARATION OF ALEXANDER VAN DER BLIEK

I, Alexander van der Bliek, declare as follows:

1. I have personal knowledge of the facts contained in this declaration and, if called as a witness, could and would testify competently to those facts.

2. I am a Professor of Biological Chemistry at the University of California Los Angeles (UCLA), where I have been employed for thirty-two years.

3. I earned my PhD at the Netherlands Cancer Institute in the Netherlands in 1988. From 1988-92, I conducted postdoctoral research at CalTech. In 1993, I was hired at UCLA as an Assistant Professor of Biological Chemistry; in 2000, I was promoted to Associate Professor; and in 2006, I was promoted to full Professor.

4. My research honors include fellowships with EMBO (an international membership organization promoting excellence in the life sciences) and HFSPO (the Human Frontiers of Science Organization), and a five-year role as Research Scholar at the American Cancer Society. I also served as member of a grant review panel for the American Heart Association, Western States Division, for six years. During the past two decades I have additionally served as a regular member and a temporary member of multiple NIH study sections. Study sections are groups of 20-25 scientists who review grant applications for the NIH from others in the same field and determine the scores that will help decide who gets funded. This is a crucial aspect of the NIH granting system, as it ensures that limited resources are allocated to the best, most promising, and most feasible projects.

5. My laboratory studies the role of mitochondria—often referred to as the powerhouses of the cell—in neurodegenerative diseases. These diseases include Alzheimer's and peripheral neuropathies (diseases that damage nerves outside the brain and spinal cord, causing chronic pain, tingling, and other symptoms). My lab uncovered the molecular basis of mitochondrial fission, which is essential for cell survival but also plays a critical role in cell death. These findings helped establish mitochondrial dynamics as a central area of cell biology. Today, this vibrant field is recognized not only for its relevance to neurodegenerative diseases but also for its broad impact on conditions with high energy demands, including cancer and diabetes.

6. I have authored more than 75 peer-reviewed publications in scientific journals. A complete list is in my National Library of Medicine bibliography: https://www.ncbi.nlm.nih.gov/myncbi/alexander.van%20der%20bliek.1/bibliography/public/. These publications have been cited more than 22,000 times: https://scholar.google.com/citations?user=AaU_YIcAAAAJ&hl=en.

7. Over the course of many years at UCLA, I have had multiple cycles of four- or five-year NIH grants, as well as grants from non-governmental agencies, which have dependably funded our research on mitochondrial dynamics. The August 2, 2025 notice I received from UCLA informing me of NIH's suspension of my current, already-awarded multi-year RO1 grant is the first grant disruption I have experienced in any context.

8. A true and correct copy of my biographical sketch is attached as Exhibit A.

Application for Grant Funding from NIH

9. On April 29, 2020, I submitted, in conjunction with the UCLA Office of Contract & Grant Administration, an Application for Federal Assistance to the NIH's National Institute of Neurological Disorders and Stroke (NINDS) for a project titled "Control of Calcium Flux and Mitochondrial Fission by the Charcot Marie Tooth Disease Protein Mfn2" (the "R01 Application"). RO1 grants are large, multi-year, highly competitive funding awards that enable research teams to sustain an extended research program and make corresponding significant contributions to scientific knowledge.

10. The Project Summary in the grant application describes our research into the underlying causes of Charcot-Marie-Tooth (CMT) disease. Charcot-Marie-Tooth disease (CMT) is an inherited condition that damages the nerves controlling movement and sensation. In our study, we looked at how mutations in a protein called Mfn2 affect the way mitochondria, the cell's "power plants," divide. Because this process is closely tied to nerve cell health, our work may help explain what causes CMT and point toward new ways to treat it.

11. The R01 Application requested $2,243,240 for a five-year period (1/1/2021 - 12/31/2025). I was identified as the Project Director and Principal Investigator on the Application. The proposal would fund salaries for myself, one lab technician, and two

postdoctoral researchers, as well as supplies and research costs, travel, and publishing.

12. The RO1 Application is attached as Exhibit B.

Award of Grant Funding for R01 Grant

13. My Application was funded in the amount proposed across all years. The NIH Notice of Award is attached as Exhibit C.

14. I received my most recent Notice of Award Action from UCLA grant administrators on January 10, 2025, informing me that I was to receive the last installment of the grant ($342,488) to sustain the project's final year (calendar year 2025). This Notice is attached as Exhibit D.

Suspension of Grant Funding

15. On August 1, 2025, I received from UCLA's research administrators a "Stop Work Notice" for my active NIH grant. The communication explained that NIH-NINDS had issued a "suspension notice" to UCLA that encompassed my project. I was instructed to immediately stop incurring expenses against the grant award. I was also told to submit to the sponsoring agency a report of expenditures through July 31, 2025, as if this were the closeout of a discontinued grant.

16. I was not offered any reason for the suspension of my grant; any means of appealing this suspension; or informed of any other action I could take to reinstate the grant.

17. A true and correct copy of the Stop Work Notice is attached as Exhibit E.

Harm Suffered from Termination of Grant Funding

18. I and my project team have suffered immediate harms as a result of NIH's actions in suspending this grant. These harms are continuing. Specifically:

19. My lab has one technician and two postdoctoral researchers. My only source of funding is the NIH grant that was suspended. As a result, we are no longer able to purchase supplies for our experiments. I will soon have to let my staff go for lack of University support.

20. The disruptions caused by layoffs are twofold. First, research in my lab will grind to a halt. Any temporary lull, even for a few months, has lasting consequences in terms of my research productivity, our laboratory's output, and the publications I produce with postdocs as co-authors.

Related: It took a year and a half to identify the current postdocs who now work with me as collaborators, to wait for them to complete their PhD training, and to bring them into the lab. If I had to repeat this process now, I would be forced to close the lab due to the considerable delay. That would be a real shame, as a lot of exciting new data would be lost.

21. Second, the postdocs will be unable to complete their projects and publish associated papers. These postdocs are highly specialized, having trained for years in this area, and should still have their careers ahead of them. A gap in publications resulting from layoffs will make them far less desirable in the job market and potentially make them unemployable. Thus, the time I have dedicated to their professional mentorship will have mostly been wasted, and the field as a whole will suffer from the loss of these promising researchers. This will have an enduring adverse impact on research in my subfield of biological chemistry.

22. The U.S. public, which ultimately funds NIH grants, will also lose much of the value of their investment if my NIH grant is indefinitely suspended. Thus far, the work supported by this grant has yielded new insights into the root causes of hereditary neuropathies, opening promising directions for future therapies for these often debilitating disorders. Unexpectedly, the research has also revealed toxic cellular mechanisms linked to brain diseases such as Alzheimer's and frontotemporal dementia. With the opportunity to complete the planned studies, our team aims to share these findings through publications and pursue follow-up experiments aimed at developing treatments. In turn, these publications will help advance public understanding of neurodegenerative and metabolic diseases, including Parkinson's, Alzheimer's, diabetes, and cancer.

Executed this 19th day of August 2025, in Los Angeles, California.

Alexander van der Bliek

EXHIBIT A

BIOGRAPHICAL SKETCH

Provide the following information for the Senior/key personnel and other significant contributors.
Follow this format for each person. **DO NOT EXCEED FIVE PAGES.**

NAME
Alexander M. van der Bliek

eRA COMMONS USER NAME (credential, e.g., agency login): VANDB2

POSITION TITLE: Professor

EDUCATION/TRAINING *(Begin with baccalaureate or other initial professional education, such as nursing, include postdoctoral training and residency training if applicable. Add/delete rows as necessary.)*

INSTITUTION AND LOCATION	DEGREE *(if applicable)*	Completion Date MM/YYYY	FIELD OF STUDY
University of Amsterdam, The Netherlands	BSc	09/1979	Biology
University of Amsterdam, The Netherlands	MSc	04/1984	Biochemistry
Netherlands Cancer Institute, The Netherlands	Ph.D.	08/1988	Cancer Biology

A. Personal Statement

My lab is interested in the functions of dynamin and dynamin-related proteins, as well as their impact on various cellular processes. As a postdoctoral fellow, I discovered the role of dynamin in endocytosis, first in *Drosophila* and then in mammalian cells (collaboration with Dr. S. Schmid). My own lab subsequently discovered the role of the dynamin-related protein Drp1 in mitochondrial fission, using *C. elegans* and mammalian cells. Since then, we have successfully utilized both biological systems, leveraging their respective strengths, to study mitochondrial fission and fusion in depth. These complementary systems revealed properties that are conserved in metazoans. A case in point is our discovery of Mff, which is absent from yeast, but turns out to be the main recruitment factor for Drp1 in higher eukaryotes. These combined approaches also allowed us to discover the functions of Fis1 during mitophagy (collaboration with Dr. R. Youle). As a follow-up, we are investigating possible functions of the classic dynamin Dnm2 during mitophagy (Martorell Riera et al. in revision, in bioRxiv). In a separate line of research, we identified the proteases responsible for Opa1 cleavage, which is relevant to the current proposal. Our research also led to the discovery that Mfn2 controls calcium release from mitochondria through NCLX, even though Mfn2 is normally considered to be a fusion protein (Kolitsida et al. manuscript submitted, in bioRxiv). Our current NIH grant focuses on possible connections of Mfn2 and NCLX with neurodegenerative diseases that include peripheral neuropathies and tauopathies. Our work on tauopathies started with a supplement that we received for our CMT NIH grant. I am very excited to work with Dr. Avi Samelson on this new project, which focuses on the mechanisms involved in cell death promoted by late-onset Alzheimer's disease mutations.

Current Research Support

1R01 NS 120690 — 12/01/2020 – 11/31/2025
National Institutes of Health — Role: PI (C. Koehler MPI)
Title: Control of calcium flux and mitochondrial fission by the Charcot Marie Tooth disease protein Mfn2
Goals are to explore the mechanisms by which Mfn2 causes cell death in a peripheral neuropathy.

3R01 NS 120690 03S1 — 07/01/2023 – 06/31/2024
National Institutes of Health — Role: PI (C. Koehler MPI)
Title: Mitochondrial calcium overload and necrosis in tauopathies caused by inhibition of Mfn2 and NCLX
Goals are to investigate a novel mechanism that induces necrosis in tauopathies.

Completed Research Support

No number — 01/01/2019-12/31/2019

Enspire-Bio Role: Scientific Collaborator
This funding was from a startup company at UCLA, Enspore-Bio, with a focus on drug discovery.

U01 GM 109764 08/15/2014 – 08/14/2018
National Institutes of Health Role: PI
Title: Contributions of Mitochondrial Fission Proteins to Mitophagy
Goals were to explore the mechanisms of ROS-induced mitochondrial fission in neuronal cells.

B. Positions and Honors

1988-1992	Postdoctoral research with Prof. Dr. E.M. Meyerowitz, CalTech
1993-2000	Assistant Professor in the Department of Biological Chemistry, UCLA
2000-2006	Associate Professor in the Department of Biological Chemistry, UCLA
2006-present	Professor in the Department of Biological Chemistry, UCLA

1988-1990	EMBO, Long term fellowship
1990-1992	HFSPO, Long term fellowship
2000-2005	American Cancer Society, Research Scholar

2002-2006	Member of review panel for the American Heart Association, Western States Division
2004	Temporary Member of NIH study section CDF4
2009, 2010	Temporary Member of NIH study section MBPP
2011-2015	Member of NIH study section MBPP
2016	Temporary Member of NIH study section ZRG1 CB-L (50) R
2017	Temporary Member of NIH study section ZRG1 MOSS K02

C. Contributions to Science

1. Role of Dynamin in Endocytosis

As a postdoctoral fellow, I cloned the Drosophila *shibire* gene and showed that it encodes Drosophila Dynamin. This led to the realization that Dynamin is involved in endocytosis. In collaboration with Sandra Schmid, I subsequently demonstrated that Dynamin is also instrumental for endocytosis in mammalian cells. In collaboration with Cori Bargmann, my lab then showed that mutations in *C. elegans* Dynamin cause paralysis like in flies. Lastly, we identified a series of intra-molecular binding interactions that helped explain how Dynamin self assembles into higher order structures. These studies opened up new avenues for studying the mechanisms of membrane scission.

van der Bliek, A.M. & E.M. Meyerowitz (1991) Dynamin-like protein encoded by the Drosophila shibire gene associated with vesicular traffic. Nature 351, 411-414. PMID: 1674590

van der Bliek, A.M., T. Redelmeier, H. Damke, E. Tisdale, E. Meyerowitz, & S.L. Schmid (1993) Mutations in human dynamin block an intermediate stage in coated vesicle formation. J. Cell Biol. 122, 553-563. PMID: 8101525

Clark, S., D.L. Shurland, E.M. Meyerowitz, C.I. Bargmann and A.M. van der Bliek (1997) A dynamin GTPase mutation causes a rapid reversible, temperature-inducible locomotion defect in *C. elegans*. *Proc. Natl. Acad. Sci. USA* 94, 10438-10443.

Smirnova, E., Shurland, D.L., Newman-Smith, E.D., Pishvaee, B. and van der Bliek, A.M. (1999) A model for dynamin self-assembly based on binding interactions between three different domains. *J. Biol. Chem.* 274, 14942-14947

2. Role of Dynamin-Related Protein in Mitochondrial Fission

Early on, my lab set out to determine the function of the Dynamin-related protein Drp1. We first thought Drp1 might contribute to vesicular traffic, like Dynamin, but after testing different pathways we found that mitochondrial morphology is affected. We then showed that Drp1 is required for mitochondrial fission in *C. elegans* and in mammalian cells, like it is in yeast. This helped open up the area of mitochondrial membrane

dynamics, which we now know is important for neurodegenerative diseases, cancer and metabolic diseases, such as diabetes.

Smirnova, E., D.L. Shurland, S.N. Ryazantsev and A.M. van der Bliek (1998) A human dynamin-related protein controlling mitochondrial morphology. *J. Cell Biol.* 143, 351-358

Labrousse, A.M., M. D. Zappaterra, D. A. Rube, and A. M. van der Bliek (1999) C. elegans dynamin-related protein DRP-1 controls severing of the mitochondrial outer membrane. Mol. Cell 4, 815-826. PMID: 10619028

Smirnova E., Griparic L., Shurland D.L., van der Bliek A.M. (2001) The Dynamin-Related Protein Drp1 is Required for Mitochondrial Division in Mammalian Cells. Mol. Biol. Cell 12, 2245-2256. PMID: 1151461

3. Functions and Proteolytic Processing of the Mitochondrial Inner Membrane Fusion Dynamin Opa1

Mutations in the mitochondrial inner membrane fusion Dynamin, called Opa1, are a prevalent cause of hereditary optic atrophy through progressive loss of retinal ganglion cells. Our studies with mammalian cells and with C. elegans showed that mild loss of Opa1 changes cristae structures and makes cells hyper-sensitive to ROS. These findings uncovered a novel function for Opa1 and may provide new inroads for treating optic atrophy. While studying Opa1, we and others also observed complex patterns of splicing and proteolytic cleavage in mammalian cells. We found that some Opa1 splice variants are constitutively cleaved by the inner membrane protease YME1L, while other splice variants are rapidly cleaved by the OMA1 protease when mitochondria lose membrane potential or ATP. Tight control of Opa1 provides a novel switch for mitochondrial quality control. We are currently testing whether this switch acts upstream of the mitophagy machinery.

Griparic, L., van der Wel, N., Orozco, I., Peters, P. & van der Bliek, A. M. (2004) The human Mgm1/Opa1 protein is tightly bound to the outer face of the inner membrane where it affects inner membrane morphology. *J. Biol. Chem.* 279, 18792-18798.

Griparic L, Kanazawa T, van der Bliek AM. (2007) Regulation of the mitochondrial dynamin-like protein Opa1 by proteolytic cleavage. J Cell Biol. 178, 757-64.

Kanazawa T, Zappaterra MD, Hasegawa A, Wright AP, Newman-Smith ED, Buttle KF, McDonald K, Mannella CA, van der Bliek AM. (2008) The C. elegans Opa1 homologue EAT-3 is essential for resistance to free radicals. PLoS Genet. 4(2):e1000022.

Head, B., Griparic, L., Amiri, M., Gandre, S. & van der Bliek, A.M. (2009) Inducible proteolytic inactivation of OPA1 mediated by the OMA1 protease in mammalian cells. J. Cell Biol. 187, 959-966. PMID: 20038677

4. Functions of Accessory Proteins in Mitochondrial Fission and Fusion

Only a fraction of Drp1 normally binds to mitochondria, while the rest is cytosolic. Binding to mitochondria is mediated by adaptor proteins. In yeast, the adaptor is a tail-anchored protein, called Fis1. Multicellular animals also have Fis1 homologues, but their functions are different. Our lab discovered a novel role for Fis1 in mitophagy (collaboration with Richard Youle) and we discovered a different protein that serves as the main Drp1 adaptor in multicellular animals (Mff in *C. elegans*, *Drosophila* and mammals). Fis1 acts in sequence with the fission machinery to control the formation and recruitment of autophagic isolation membrane through binding interactions with the Rab7-GAP TBC1D15. These studies have broad implications for the regulation of mitochondrial fission and downstream events, such as mitophagy. Lastly, in collaboration with Phillip Chavrier, we also showed that mitochondrial inner membrane fusion requires an NDP-kinase to sustain high local concentrations of GTP. We are currently testing whether this is also true for mitochondrial fission.

Gandre-Babbe, S. and van der Bliek, A. M. (2008) The Novel Tail-anchored Membrane Protein Mff Controls Mitochondrial and Peroxisomal Fission in Mammalian Cells. Mol. Biol. Cell 19, 2402-2412. PMID: 18353969

Shen, Q., Yamano, K., Head, B. P., Kawajiri, S., Cheung, J. T., Wang, C., Cho, J. H., Hattori, N., Youle, R. J. & van der Bliek, A. M. (2014) Mutations in Fis1 disrupt orderly disposal of defective mitochondria. Mol Biol Cell, 25, 145-59. PMID: 24196833

Yamano, K., Fogel, A.I, Chunxin Wang, C., van der Bliek, A.M. & Youle, R.J. (2014) Mitochondrial Rab GAPs govern autophagosome biogenesis during mitophagy. eLife 3, e01612. PMCID 3930140.

Boissan, M., Montagnac, G., Shen, Q., Griparic, L., Guitton, J., Romao, M., Sauvonnet, N., Lagache, T., Lascu, I., Raposo, G., Schlattner, U., Lacombe, M.-L., Polo, S., van der Bliek, A.M., Roux, A., & Chavrier, P. (2014) Nucleoside Diphosphate Kinases fuel Dynamin Superfamily Proteins with GTP for membrane remodeling. Science 344, 1510-1515. PMID: 24970086

5. Discovery of a Novel Protein Complex at Mitochondrial Contact Sites

While screening for novel mitochondrial fission mutants, we found some new proteins that affect mitochondrial morphology but do not directly control fission. These proteins, called MOMA-1, CHCH-3 and IMMT-1 (a *C. elegans* Mitofilin homologue), showed striking genetic interactions, suggesting that they act together to control a vital mitochondrial function. Soon after our paper came out, a series of yeast studies were published in which it was shown that these proteins form a large complex at the necks of mitochondrial cristae junctions. To avoid confusion, the complex was named MICOS (Mitochondrial Contact Site) and individual proteins were renamed (in our case: Mic27, Mic 19, and Mic60). Our lab no longer studies MICOS function per se, but we are using the complex as a stepping-stone in our continuing search for novel fission proteins.

Head, B.P., Zulaika, M., Ryazantsev, S. & van der Bliek, A.M. (2011) A novel mitochondrial outer membrane protein, MOMA-1, that affects cristae morphology in Caenorhabditis elegans. Mol Biol Cell 22, 831-841. PMID: 21248201

Weber, T. A., Koob, S., Heide, H., Wittig, I., Head, B., van der Bliek, A., Brandt, U., Mittelbronn, M., Reichert, A. S. (2013) APOOL is a cardiolipin-binding constituent of the Mitofilin/MINOS protein complex determining cristae morphology in mammalian mitochondria. PloS one, 2013. **8**(5): p. e63683. PMID: 23704930

Pfanner, N., van der Laan, M., Amati, P., Capaldi, R.A., Caudy, A.A., Chacinska, A., Darshi, M., Deckers, M., Hoppins, S., Icho, T., Jakobs, S., Ji, J., KozjakPavlovic, V., Meisinger, C., Odgren, P.R. Park, S.K., Rehling, P, Reichert, A.S, Sheikh, M.S., Taylor, S.S. Tsuchida, N., van der Bliek, A.M., van der Klei, I.J. Weissman, J.S., Westermann, B., Zha, J., Neupert, W. & Nunnari. J. (2014) Uniform nomenclature for the mitochondrial contact site and cristae organizing system. J. Cell Biol. 204, 1083-1086. doi: 10.1083/jcb.201401006

Complete List of Published Work in MyBibliography:

EXHIBIT B

Electronic Cover Sheet		
PI: **VAN DER BLIEK, ALEXANDER M**	Title: Control of calcium flux and mitochondrial fission by the Charcot Marie Tooth disease protein Mfn2.	
Received: 04/30/2020	Opportunity: PAS-18-483 Clinical Trial:Not Allowed	Council: 10/2020
Competition ID: FORMS-E	FOA Title: Promoting Research in Basic Neuroscience (R01)	
1R01NS120690-01	Dual:	Accession Number: 4431949
IPF: 577505	Organization: UNIVERSITY OF CALIFORNIA LOS ANGELES	
Former Number:	Department: Biological Chemistry	
IRG/SRG: ZRG1 MDCN-B (03)M	AIDS: N	Expedited: N
Subtotal Direct Costs (excludes consortium F&A) Year 1: 250,000 Year 2: 250,000 Year 3: 250,000 Year 4: 250,000 Year 5: 250,000	Animals: Y Humans: N Clinical Trial: N Current HS Code: 10 HESC: N HFT: N	New Investigator: N Early Stage Investigator: N
Senior/Key Personnel:	*Organization:*	*Role Category:*
Alexander van der Bliek PhD	The Regents of the University of California, Los Angeles	PD/PI
Carla Koehler PhD	The Regents of the University of California, Los Angeles	MPI

OMB Number: 4040-0010
Expiration Date: 12/31/2022

APPLICATION FOR FEDERAL ASSISTANCE
SF 424 (R&R)

3. DATE RECEIVED BY STATE	**State Application Identifier**

1. TYPE OF SUBMISSION*

❍ Pre-application ● Application ❍ Changed/Corrected Application

4.a. Federal Identifier

b. Agency Routing Number

c. Previous Grants.gov Tracking Number

2. DATE SUBMITTED 2020-04-30

Application Identifier

5. APPLICANT INFORMATION **Organizational DUNS*:** 092530369

Legal Name*: The Regents of the University of California, Los Angeles
Department:
Division:
Street1*: Office of Contract and Grant Administration
Street2: 10889 Wilshire Boulevard, Suite 700
City*: Los Angeles
County: Los Angeles County
State*: CA: California
Province:
Country*: USA: UNITED STATES
ZIP / Postal Code*: 90095-1406

Person to be contacted on matters involving this application
Prefix: First Name*: Eleanor Middle Name: Last Name*: Forbes Suffix:
Position/Title: Senior Grant Analyst
Street1*: 10889 Wilshire Boulevard, Suite 700
Street2:
City*: Los Angeles
County: Los Angeles County
State*: CA: California
Province:
Country*: USA: UNITED STATES
ZIP / Postal Code*: 90095-1406
Phone Number*: 310-794-6945 Fax Number: Email: eleanor.forbes@research.ucla.edu

6. EMPLOYER IDENTIFICATION NUMBER *(EIN) or (TIN)** 1-956006143-A1

7. TYPE OF APPLICANT* H: Public/State Controlled Institution of Higher Education

Other (Specify):
Small Business Organization Type ❍ Women Owned ❍ Socially and Economically Disadvantaged

8. TYPE OF APPLICATION*

● New ❍ Resubmission
❍ Renewal ❍ Continuation ❍ Revision

If Revision, mark appropriate box(es).

❍ A. Increase Award ❍ B. Decrease Award ❍ C. Increase Duration
❍ D. Decrease Duration ❍ E. Other *(specify)* :

Is this application being submitted to other agencies?* ❍Yes ●No What other Agencies?

9. NAME OF FEDERAL AGENCY*
National Institutes of Health

10. CATALOG OF FEDERAL DOMESTIC ASSISTANCE NUMBER
TITLE:

11. DESCRIPTIVE TITLE OF APPLICANT'S PROJECT*
Control of calcium flux and mitochondrial fission by the Charcot Marie Tooth disease protein Mfn2.

12. PROPOSED PROJECT
Start Date* Ending Date*
12/01/2020 11/30/2025

13. CONGRESSIONAL DISTRICTS OF APPLICANT
CA-033

SF 424 (R&R) APPLICATION FOR FEDERAL ASSISTANCE

14. PROJECT DIRECTOR/PRINCIPAL INVESTIGATOR CONTACT INFORMATION

Prefix: Dr. First Name*: Alexander Middle Name: Last Name*: van der Bliek Suffix: PhD
Position/Title: Professor
Organization Name*: The Regents of the University of California, Los Angeles
Department: Biological Chemistry
Division:
Street1*: 615 Charles E Young
Street2: CHS 27-200N
City*: Los Angeles
County: Los Angeles County
State*: CA: California
Province:
Country*: USA: UNITED STATES
ZIP / Postal Code*: 90095-7070
Phone Number*: (310) 825-9779 Fax Number: (310) 825-6267 Email*: avan@mednet.ucla.edu

15. ESTIMATED PROJECT FUNDING

a. Total Federal Funds Requested*	$1,902,710.00
b. Total Non-Federal Funds*	$0.00
c. Total Federal & Non-Federal Funds*	$1,902,710.00
d. Estimated Program Income*	$0.00

16.IS APPLICATION SUBJECT TO REVIEW BY STATE EXECUTIVE ORDER 12372 PROCESS?*

a. YES ○ THIS PREAPPLICATION/APPLICATION WAS MADE AVAILABLE TO THE STATE EXECUTIVE ORDER 12372 PROCESS FOR REVIEW ON:
DATE:

b. NO ● PROGRAM IS NOT COVERED BY E.O. 12372; OR
○ PROGRAM HAS NOT BEEN SELECTED BY STATE FOR REVIEW

17. By signing this application, I certify (1) to the statements contained in the list of certifications* and (2) that the statements herein are true, complete and accurate to the best of my knowledge. I also provide the required assurances * and agree to comply with any resulting terms if I accept an award. I am aware that any false, fictitious, or fraudulent statements or claims may subject me to criminal, civil, or administrative penalties. (U.S. Code, Title 18, Section 1001)

● I agree*

** The list of certifications and assurances, or an Internet site where you may obtain this list, is contained in the announcement or agency specific instructions.*

18. SFLLL or OTHER EXPLANATORY DOCUMENTATION File Name:

19. AUTHORIZED REPRESENTATIVE

Prefix: Ms. First Name*: Jessica Middle Name: Last Name*: Kim Suffix:
Position/Title*: Contract and Grant Analyst
Organization Name*: The Regents of the University of California, Los Angeles
Department: Office of Contract & Grant Adm
Division:
Street1*: 10889 Wilshire Boulevard, Suite 700
Street2:
City*: Los Angeles
County: Los Angeles County
State*: CA: California
Province:
Country*: USA: UNITED STATES
ZIP / Postal Code*: 90095-1406
Phone Number*: 310-983-3673 Fax Number: Email*: jessica.kim@research.ucla.edu

Signature of Authorized Representative*
Jessica Kim

Date Signed*
04/30/2020

20. PRE-APPLICATION File Name:

21. COVER LETTER ATTACHMENT File Name:cover_letter1060326283.pdf

424 R&R and PHS-398 Specific
Table Of Contents

OMB Number: 4040-0010
Expiration Date: 10/31/2019

Project/Performance Site Location(s)

Project/Performance Site Primary Location

❍ I am submitting an application as an individual, and not on behalf of a company, state, local or tribal government, academia, or other type of organization.

Organization Name:	The Regents of the University of California, Los Angeles
Duns Number:	092530369
Street1*:	615 Charles E Young
Street2:	CHS 27-200N
City*:	Los Angeles
County:	Los Angeles County
State*:	CA: California
Province:	
Country*:	USA: UNITED STATES
Zip / Postal Code*:	90095-7070
Project/Performance Site Congressional District*:	CA-033

Additional Location(s) File Name:

OMB Number: 4040-0010
Expiration Date: 12/31/2022

RESEARCH & RELATED Other Project Information

1. Are Human Subjects Involved?* ❍ **Yes** ● **No**

1.a. If YES to Human Subjects

Is the Project Exempt from Federal regulations? ❍ Yes ❍ No

If YES, check appropriate exemption number: _ 1 _ 2 _ 3 _ 4 _ 5 _ 6 _ 7 _ 8

If NO, is the IRB review Pending? ❍ **Yes** ❍ **No**

IRB Approval Date:

Human Subject Assurance Number

2. Are Vertebrate Animals Used?* ● **Yes** ❍ **No**

2.a. If YES to Vertebrate Animals

Is the IACUC review Pending? ● Yes ❍ No

IACUC Approval Date:

Animal Welfare Assurance Number A3196-01

3. Is proprietary/privileged information included in the application?* ❍ **Yes** ● **No**

4.a. Does this project have an actual or potential impact - positive or negative - on the environment?* ❍ **Yes** ● **No**

4.b. If yes, please explain:

4.c. If this project has an actual or potential impact on the environment, has an exemption been authorized or an environmental assessment (EA) or environmental impact statement (EIS) been performed? ❍ Yes ❍ No

4.d. If yes, please explain:

5. Is the research performance site designated, or eligible to be designated, as a historic place?* ❍ **Yes** ● **No**

5.a. If yes, please explain:

6. Does this project involve activities outside the United States or partnership with international collaborators?* ❍ **Yes** ● **No**

6.a. If yes, identify countries:

6.b. Optional Explanation:

	Filename
7. Project Summary/Abstract*	Project_summary1060080743.pdf
8. Project Narrative*	Project_Narrative1060080739.pdf
9. Bibliography & References Cited	references1060326279.pdf
10.Facilities & Other Resources	Facilities1060080740.pdf
11.Equipment	Equipment1060080741.pdf

Funding Opportunity Number: PAS-18-483. Received Date: 2020-04-30T11:45:17.000-04:00

Project summary
Charcot-Marie-Tooth disease (CMT) is a hereditary peripheral neuropathy resulting from demyelination and axon degeneration. Many cases of axon degeneration are caused by mutations in Mitofusin-2 (Mfn2), which is one of two dynamin-like proteins on the surface of mitochondria. Mitofusins primarily mediate mitochondrial outer membrane fusion, but Mfn2 can also promote association between ER and mitochondria in mitochondria associated membranes (the MAM). It is observed in spots that colocalize with the mitochondrial fission dynamin Drp1 and it was proposed to affect axonal transport of mitochondria, raising the possibility that CMT is caused by defects in one of these other functions of Mfn2. Preliminary data show that Mfn2 promotes constriction of mitochondria in $Drp1^{-/-}$ cells and fission in Drp1-Mfn1 DKO cells when treated with the fungal toxin PXA that causes the release of calcium from the mitochondrial matrix. Calcium release and the ability to constrict mitochondria was linked to Mfn2-mediated regulation of NCLX (a Ca/Na exchanger in the mitochondrial inner membrane). It is hypothesized that PXA activates NCLX and that Mfn2 is required for this activity. It is also hypothesized that NCLX-mediated calcium release causes mitochondrial constriction and that the effects of Mfn2 on mitochondrial fission are linked to axonal transport, a process that could be disrupted in CMT patients. These hypotheses will be tested by investigating three aims. **Aim 1. Investigate connections between PXA, NCLX, and Mfn2.** These studies will include comprehensive tests whether PXA triggers calcium release from mitochondria by activating NCLX and investigates of the control of NCLX by Mfn2. **Aim 2. Investigate connections between Mfn2 and mitochondrial fission.** Effects of Mfn2 and NCLX on mitochondrial fission will be tested with knockout cell lines and transfections of fission and fusion protein constructs followed by analyses with a range of imaging techniques. **Aim 3. Investigate the physiological consequences of Mfn2 and NCLX contributions to fission.** Effects on mitochondrial transport proteins will be examined with kymographs of axonal processes in cultured neurons and zebrafish. Alternative functions, such effects on metabolism and a role in mitophagy, will also be considered. Together, these experiments will help establish NCLX as the target of PXA, assess the newly proposed role of Mfn2 in mitochondrial fission, and test possible downstream effects on transport or mitophagy. These experiments may therefore reveal a novel function for Mfn2 and shed new light on the underlying causes of CMT. Possible downstream effects of fission on axonal transport will change the understanding of the underlying causes of CMT and may suggest novel treatment strategies.

Project Narrative

Charcot-Marie-Tooth disease (CMT) is a hereditary neuropathy caused by gene mutations that lead to demyelination and axon degeneration of peripheral neurons. This proposal follows through on data suggesting that mutations in one of the CMT genes called Mfn2 affects mitochondrial fission and axonal transport in unexpected ways. A successful outcome of this project can provide new insight into the underlying causes of the disease and it may point the way towards developing novel treatment strategies.

FACILITIES AND OTHER RESOURCES

Van der Bliek and Koehler Laboratories

Laboratory: Drs. Van der Bliek and Koehler share a 2000 sq. ft. laboratory in the Center for Health Sciences Building at UCLA including bench space for 12 individuals and a 450 sq. ft. equipment room for microscopes. This lab space is part of the "Mitochondria and Metabolism Theme" that consists of shared office space and overall lab space for 10 faculty. The laboratory is fully equipped with fume hoods, chemical benches, and utilities. A sterile culture room with four culture hoods is available adjoining the laboratory.

Computers: Drs. Van der Bliek and Koehler's office and computer rooms (attached to the office) are supplied with PC and Mac computers and printers. The students have portable notebook computers to organize data and run instrumentation in the laboratory. The Department has color scanners and printers.

Office: Drs. Van der Bliek and Koehler each have office space of 130 sq. ft. room next to the laboratory. Desk space for students and postdoctoral fellows is a large office suite near the laboratory.

Core facilities:

(1) A mitochondrial core is housed in the Metabolism theme space. It has several Seahorse XF24 Extracellular Flux Analyzers and a battery of other instruments for measuring mitochondrial functions.
(2) The theme also houses a microscopy core with an Olympus spinning disk confocal and Zeiss LSM 800 with Airyscan equiped for multiphoton capabilities.
(3) The Biochemistry Instrumentation Facility is located in an adjacent building and has instruments for surface plasmon resonance, circular dichroism, thermal denaturation, gel documentation, and analytical ultracentrifugation.
(4) The Molecular Shared Screening Resource core facility is available on campus and operates on a fee-for-service basis; this core is run by Dr. Damoiseaux and has state-of-the-art robotics for high throughput screening.
(5) The UCLA Genotyping and Sequencing Core facility and the Broad Stem Cell Research Core (BSCRC) facility are state-of-the-art laboratory facilities equipped with the latest genomic technologies, including instruments for short-read and long-read next generation sequencing; the facilities operate on a fee-for-service basis.
(6) The Translational Pathology Core Laboratory (TPCL) is a CAP/CLIA certified research facility in the UCLA Department of Pathology and Laboratory Medicine and a UCLA Jonsson Comprehensive Cancer Center Shared Facility. The TPCL provides a reliable and continuous supply of high quality human materials, including fresh-frozen and paraffin-embedded archived human tissues. These tissues are available through an approved protocol. Clinical samples are collected under IRB rules. Diagnostic modalities for characterizing these samples are available and in routine use.
(7) The Department of Medicine has a Statistics Core (DOMStat), which is available to us as members of the JCCC. The primary objective of DOMStat is to provide state-of-the-art study design and statistical analysis collaboration to investigators in the Department of Medicine. DOMStat offers faculty and staff statistical collaboration for grant proposals, data analysis, manuscript preparation, and other research activities requiring statistical input.
(8) The CNSI has an advanced microscopy core, including several confocal microscopes, super-resolution microscopes (PALM, STORM, and STED) and equipment for standard and cryo-EM.

Seminar series: UCLA has a number of departmental and interdepartmental seminar series in the life sciences, including series from the Jonsson Comprehensive Cancer Center, Institute for Molecular Medicine, Broad Stem Cell Research Center, Department of Chemistry and Biochemistry, Molecular Biology Institute, and Embryology Club. There are also numerous special topic symposia, including symposia on mitochondrial biology and metabolism. Dr. Koehler organizes a Mitochondrial Group Meeting where students and post-doctoral fellows present their research; this is scheduled weekly and is attended by nine research groups from UCLA. We also have numerous networking activities and single-day mitochondrial symposia with groups at Cedars Sinai, USC, Cal Tech, and Cal State-LA. Drs. Van der Bliek and Koehler are also integral members of the Mitochondria and Metabolism theme at UCLA; this environment facilitates excellent collaborations in mitochondrial biology and expertise and numerous invited speakers present seminars.

Equipment

The van der Bliek and Koehler laboratories have all of the necessary equipment for the proposed experiments.

The van der Bliek lab has a spinning disc confocal (Mariannis from 3I, consisting of an inverted Zeiss mircoscope with Yokagawa CSU-22 spinning disc and six different lasers controlled by an AOTF), along with a Zeiss Axiovert 200M microscope for epifluorescence, DIC attachments, and Hamamatsu ORCA-ER and C9100-13 EMCCD cameras. The van der Bliek lab is also fully equiped for standard molecular biology, gel electrophoresis, and western blotting. The van der Bliek lab has ample refrigerator and freezer space, and a –80°C freezer. It has its own tissue culture room with hood and CO_2 incubators. The departmental microscopy facility has a Zeiss LSM 880 confocal microscope with Airyscan for super resolutiuon microscopy with a chamber for temperature and CO_2 control.

The Koehler lab is equipped with two high speed J2-HC Beckman centrifuge and rotors, chromatography chamber, refrigerators, freezers, airfuges, fluorescent microscopes, and microcentrifuges. The laboratory is well stocked with power supplies, gel electrophoresis units for protein and nucleic acid separations, a slab gel dryer, shaking and stationary water baths, and incubators. The equipment room houses two –80°C freezers, tetrad dissection microscope, incubators for growing yeast, thermal cycler, and a milliQ water purification system. The required equipment for working with zebrafish are available in the Koehler laboratory. An oxygen electrode is available for measuring oxygen consumption in mitochondria.

The two labs are housed in the metabolism theme space, which also has a metabolism and an imaging core facility, The metabolism core is equipped with six Seahorse XF96 Extracellular Flux Analyzers (Agilent Technologies) and an Operetta High-Content Imaging System (PerkinElmer), which will be used for respirometry. The imaging core has a Zeiss Airyscan microscope, equiped for multiphoton confocal microscopy and fluorescence lifetime imaging. The Department of Biological Chemistry has a Zeiss Airyscan microscope with a fast mode for tracking dynamic processes in live cells.

OMB Number: 4040-0010
Expiration Date: 12/31/2022

RESEARCH & RELATED Senior/Key Person Profile (Expanded)

PROFILE - Project Director/Principal Investigator

Prefix: Dr. First Name*: Alexander Middle Name Last Name*: van der Bliek Suffix: PhD

Position/Title*:	Professor
Organization Name*:	The Regents of the University of California, Los Angeles
Department:	Biological Chemistry
Division:	
Street1*:	615 Charles E Young
Street2:	CHS 27-200N
City*:	Los Angeles
County:	Los Angeles County
State*:	CA: California
Province:	
Country*:	USA: UNITED STATES
Zip / Postal Code*:	90095-7070

Phone Number*: (310) 825-9779 Fax Number: (310) 825-6267

E-Mail*: avan@mednet.ucla.edu

Credential, e.g., agency login: VANDB2

Project Role*: PD/PI Other Project Role Category:

Degree Type: PhD Degree Year: 1988

Attach Biographical Sketch*: File Name: VA_Biosketch1060080735.pdf

Attach Current & Pending Support: File Name:

Tracking Number: GRANT13088859
Funding Opportunity Number: PAS-18-483 . Received Date: 2020-04-30T11:45:17.000-04:00

PROFILE - Senior/Key Person

Prefix: Dr. First Name*: Carla Middle Name M Last Name*: Koehler Suffix: PhD

Position/Title*: Professor
Organization Name*: The Regents of the University of California, Los Angeles
Department: Chemistry & Biochemistry
Division: Biochemistry
Street1*: 4041A Young Hall
Street2: 607 Charles E. Young Dr. East
City*: Los Angeles
County: Los Angeles County
State*: CA: California
Province:
Country*: USA: UNITED STATES
Zip / Postal Code*: 90095-1569

Phone Number*: 310-794-4834 Fax Number: 310-206-4038

E-Mail*: koehlerc@chem.ucla.edu

Credential, e.g., agency login: koehler2

Project Role*: PD/PI Other Project Role Category:

Degree Type: PhD Degree Year: 1995

Attach Biographical Sketch*: File Name: Koehler_Biosketch1060080736.pdf

Attach Current & Pending Support: File Name:

BIOGRAPHICAL SKETCH

Provide the following information for the Senior/key personnel and other significant contributors.
Follow this format for each person. **DO NOT EXCEED FIVE PAGES.**

NAME
Alexander M. van der Bliek

eRA COMMONS USER NAME (credential, e.g., agency login): VANDB2

POSITION TITLE: Professor

EDUCATION/TRAINING *(Begin with baccalaureate or other initial professional education, such as nursing, include postdoctoral training and residency training if applicable. Add/delete rows as necessary.)*

INSTITUTION AND LOCATION	DEGREE *(if applicable)*	Completion Date MM/YYYY	FIELD OF STUDY
University of Amsterdam, The Netherlands	BSc	09/1979	Biology
University of Amsterdam, The Netherlands	MSc	04/1984	Biochemistry
Netherlands Cancer Institute, The Netherlands	Ph.D.	08/1988	Cancer Biology

A. Personal Statement

My lab has a longstanding interest in the functions of dynamin and dynamin-related proteins and how they affect different cellular processes. As a postdoctoral fellow, I discovered the role of dynamin in endocytosis, first in *Drosophila* and then in mammalian cells (collaboration with Dr. S. Schmid). My own lab subsequently discovered the role of the dynamin-related protein Drp1 in mitochondrial fission in animals, using *C. elegans* and mammalian cells. Since then, we have successfully used both biological systems, drawing upon their respective strengths, to study mitochondrial fission and fusion in depth. The use of these complementary systems revealed properties that are conserved in metazoans. A case in point is our discovery of Mff, which is absent from yeast but turns out to be the main recruitment factor for Drp1 in higher eukaryotes. These combined approaches also allowed us to discover the functions of Fis1 during mitophagy (collaboration with Dr. R. Youle). In a separate line of research, we are investigating possible functions of the classic dynamin Dnm2 during autophagy (manuscript submitted, see also Martorell Riera et al. on bioRxiv). Finally, we discovered a possible role of Mfn2 in mitochondrial fission and in controlling calcium release through NCLX from mitochondria, serving as the basis for this proposal. These new roles in fission and calcium release contrast with the traditional role of Mfn2 as a fusion protein. It suggests how Mfn2 and NCLX can modulate metabolism and promote apoptosis, which is important for understanding the effects of these proteins on the progression of Charcot Marie Tooth disease. I am very excited by the prospect of contributing to a better understanding of this disease and possibly providing a new framework for developing drug treatments.

B. Positions and Honors

1988-1992	Postdoctoral research with Prof. Dr. E.M. Meyerowitz, CalTech
1993-2000	Assistant Professor in the Department of Biological Chemistry, UCLA
2000-2006	Associate Professor in the Department of Biological Chemistry, UCLA
2006-present	Professor in the Department of Biological Chemistry, UCLA
1988-1990	EMBO, Long-term fellowship
1990-1992	HFSPO, Long-term fellowship
2000-2005	American Cancer Society, Research Scholar
2002-2006	Member of review panel for the American Heart Association, Western States Division
2004	Temporary Member of NIH study section CDF4
2009, 2010	Temporary Member of NIH study section MBPP
2011-2015	Member of NIH study section MBPP
2016	Temporary Member of NIH study section ZRG1 CB-L (50) R
2017	Temporary Member of NIH study section ZRG1 MOSS K02

C. Contributions to Science

1. Role of Dynamin in Endocytosis

As a postdoctoral fellow, I cloned the Drosophila *shibire* gene and showed that it encodes Drosophila Dynamin. This led to the realization that Dynamin is involved in endocytosis. In collaboration with Sandra Schmid, I then showed that Dynamin also affects endocytosis in mammalian cells. In collaboration with Cori Bargmann, my lab then showed that mutations in *C. elegans* Dynamin cause paralysis in flies. Lastly, we identified a series of intra-molecular binding interactions that helped explain how Dynamin self assembles into higher order structures. These studies opened up new avenues for studying the mechanisms of membrane scission.

- van der Bliek, A.M. & E.M. Meyerowitz (1991) Dynamin-like protein encoded by the Drosophila shibire gene associated with vesicular traffic. Nature 351, 411-414. PMID: 1674590
- van der Bliek, A.M., T. Redelmeier, H. Damke, E. Tisdale, E. Meyerowitz, & S.L. Schmid (1993) Mutations in human dynamin block an intermediate stage in coated vesicle formation. J. Cell Biol. 122, 553-563. PMID: 8101525
- Clark, S., D.L. Shurland, E.M. Meyerowitz, C.I. Bargmann and A.M. van der Bliek (1997) A dynamin GTPase mutation causes a rapid reversible, temperature-inducible locomotion defect in *C. elegans*. *Proc. Natl. Acad. Sci. USA* 94, 10438-10443.
- Smirnova, E., Shurland, D.L., Newman-Smith, E.D., Pishvaee, B. and van der Bliek, A.M. (1999) A model for dynamin self-assembly based on binding interactions between three different domains. *J. Biol. Chem.* 274, 14942-14947

2. Role of Dynamin-Related Protein in Mitochondrial Fission

Early on, my lab set out to determine the function of the Dynamin-related protein Drp1. We first thought Drp1 might contribute to vesicular traffic, like Dynamin, but after testing different pathways we found that mitochondrial morphology is affected. We then showed that Drp1 is required for mitochondrial fission in *C. elegans* and in mammalian cells, like it is in yeast. This helped open up the area of mitochondrial membrane dynamics, which we now know is important for neurodegenerative diseases, cancer, and metabolic diseases, such as diabetes.

- Smirnova, E., D.L. Shurland, S.N. Ryazantsev and A.M. van der Bliek (1998) A human dynamin-related protein controlling mitochondrial morphology. *J. Cell Biol.* 143, 351-358
- Labrousse, A.M., M. D. Zappaterra, D. A. Rube, and A. M. van der Bliek (1999) C. elegans dynamin-related protein DRP-1 controls severing of the mitochondrial outer membrane. Mol. Cell 4, 815-826. PMID: 10619028
- Smirnova E., Griparic L., Shurland D.L., van der Bliek A.M. (2001) The Dynamin-Related Protein Drp1 is Required for Mitochondrial Division in Mammalian Cells. Mol. Biol. Cell 12, 2245-2256. PMID: 1151461

3. Functions and Proteolytic Processing of the Mitochondrial Inner Membrane Fusion Dynamin Opa1

Mutations in the mitochondrial inner membrane fusion Dynamin, called Opa1, are a prevalent cause of hereditary optic atrophy through progressive loss of retinal ganglion cells. Our studies with mammalian cells and with C. elegans showed that mild loss of Opa1 changes cristae structures and makes cells hyper-sensitive to reactive oxygen species. These findings uncovered a novel function for Opa1 and may provide new inroads for treating optic atrophy. While studying Opa1, we and others also observed complex patterns of splicing and proteolytic cleavage in mammalian cells. We found that some Opa1 splice variants are constitutively cleaved by the inner membrane protease YME1L, while other splice variants are rapidly cleaved by the OMA1 protease when mitochondria lose membrane potential or ATP. Tight control of Opa1 provides a novel switch for mitochondrial quality control. We are currently testing whether this switch acts upstream of the mitophagy machinery.

- Griparic, L., van der Wel, N., Orozco, I., Peters, P. & van der Bliek, A. M. (2004) The human Mgm1/Opa1 protein is tightly bound to the outer face of the inner membrane where it affects inner membrane morphology. *J. Biol. Chem.* 279, 18792-18798.

- Griparic L, Kanazawa T, van der Bliek AM. (2007) Regulation of the mitochondrial dynamin-like protein Opa1 by proteolytic cleavage. J Cell Biol. 178, 757-64.
- Kanazawa T, Zappaterra MD, Hasegawa A, Wright AP, Newman-Smith ED, Buttle KF, McDonald K, Mannella CA, van der Bliek AM. (2008) The C. elegans Opa1 homologue EAT-3 is essential for resistance to free radicals. PLoS Genet. 4(2):e1000022.
- Head, B., Griparic, L., Amiri, M., Gandre, S. & van der Bliek, A.M. (2009) Inducible proteolytic inactivation of OPA1 mediated by the OMA1 protease in mammalian cells. J. Cell Biol. 187, 959-966. PMID: 20038677

4. Functions of Accessory Proteins in Mitochondrial Fission and Fusion

Only a fraction of Drp1 normally binds to mitochondria, while the rest is cytosolic. Binding to mitochondria is mediated by adaptor proteins. In yeast, the adaptor is a tail-anchored protein called Fis1. Multicellular animals also have Fis1 homologs, but their functions are different. Our lab discovered a novel role for Fis1 in mitophagy (collaboration with Richard Youle) and we discovered a different protein that serves as the main Drp1 adaptor in multicellular animals (Mff in *C. elegans*, *Drosophila*, and mammals). Fis1 acts in sequence with the fission machinery to control the formation and recruitment of autophagic isolation membrane through binding interactions with the Rab7-GAP TBC1D15. These studies have broad implications for the regulation of mitochondrial fission and downstream events, such as mitophagy. Lastly, in collaboration with Phillip Chavrier, we also showed that mitochondrial inner membrane fusion requires an NDP-kinase to sustain high local concentrations of GTP. We are currently testing whether this is also true for mitochondrial fission.

- Gandre-Babbe, S. and van der Bliek, A. M. (2008) The Novel Tail-anchored Membrane Protein Mff Controls Mitochondrial and Peroxisomal Fission in Mammalian Cells. Mol. Biol. Cell 19, 2402-2412. PMID: 18353969
- Shen, Q., Yamano, K., Head, B. P., Kawajiri, S., Cheung, J. T., Wang, C., Cho, J. H., Hattori, N., Youle, R. J. & van der Bliek, A. M. (2014) Mutations in Fis1 disrupt orderly disposal of defective mitochondria. Mol Biol Cell, 25, 145-59. PMID: 24196833
- Yamano, K., Fogel, A.I, Chunxin Wang, C., van der Bliek, A.M. & Youle, R.J. (2014) Mitochondrial Rab GAPs govern autophagosome biogenesis during mitophagy. eLife 3, e01612. PMCID 3930140.
- Boissan, M., Montagnac, G., Shen, Q., Griparic, L., Guitton, J., Romao, M., Sauvonnet, N., Lagache, T., Lascu, I., Raposo, G., Schlattner, U., Lacombe, M.-L., Polo, S., van der Bliek, A.M., Roux, A., & Chavrier, P. (2014) Nucleoside Diphosphate Kinases fuel Dynamin Superfamily Proteins with GTP for membrane remodeling. Science 344, 1510-1515. PMID: 24970086

5. Discovery of a Novel Protein Complex at Mitochondrial Contact Sites

While screening for novel mitochondrial fission mutants, we found some new proteins that affect mitochondrial morphology but do not directly control fission. These proteins, called MOMA-1, CHCH-3, and IMMT-1 (a *C. elegans* Mitofilin homolog), showed striking genetic interactions, suggesting that they act together to control a vital mitochondrial function. Soon after our paper came out, a series of yeast studies were published that showed that these proteins form a large complex at the necks of mitochondrial cristae junctions. To avoid confusion, the complex was named MICOS (Mitochondrial Contact Site) and individual proteins were renamed (in our case: Mic27, Mic 19, and Mic60). Our lab no longer studies MICOS function per se, but we are using the complex as a stepping-stone in our continuing search for novel fission proteins.

- Head, B.P., Zulaika, M., Ryazantsev, S. & van der Bliek, A.M. (2011) A novel mitochondrial outer membrane protein, MOMA-1, that affects cristae morphology in Caenorhabditis elegans. Mol Biol Cell 22, 831-841. PMID: 21248201
- Weber, T. A., Koob, S., Heide, H., Wittig, I., Head, B., van der Bliek, A., Brandt, U., Mittelbronn, M., Reichert, A. S. (2013) APOOL is a cardiolipin-binding constituent of the Mitofilin/MINOS protein complex determining cristae morphology in mammalian mitochondria. PloS one, 2013. **8**(5): p. e63683. PMID: 23704930
- Pfanner, N., van der Laan, M., Amati, P., Capaldi, R.A., Caudy, A.A., Chacinska, A., Darshi, M., Deckers, M., Hoppins, S., Icho, T., Jakobs, S., Ji, J., KozjakPavlovic, V., Meisinger, C., Odgren, P.R. Park, S.K., Rehling, P, Reichert, A.S, Sheikh, M.S., Taylor, S.S. Tsuchida, N., van der Bliek, A.M., van der Klei, I.J.

Weissman, J.S., Westermann, B., Zha, J., Neupert, W. & Nunnari. J. (2014) Uniform nomenclature for the mitochondrial contact site and cristae organizing system. J. Cell Biol. 204, 1083-1086. doi: 10.1083/jcb.201401006

Complete List of Published Work in MyBibliography:
http://www.ncbi.nlm.nih.gov/sites/myncbi/alexander.van der bliek.1/bibliography/40594909/public/?sort=date&direction=ascending

D. Research Support

Ongoing Research Support
None

Completed Research Support

U01 GM 109764 — 08/15/2014 – 08/14/2018
National Institutes of Health — Role: PI
Title: Contributions of Mitochondrial Fission Proteins to Mitophagy
Goals are to explore the mechanisms of ROS-induced mitochondrial fission in neuronal cells.

BIOGRAPHICAL SKETCH

Provide the following information for the Senior/key personnel and other significant contributors.
Follow this format for each person. **DO NOT EXCEED FIVE PAGES.**

NAME: Koehler, Carla M.

eRA COMMONS USER NAME (credential, e.g., agency login): KOEHLER2

POSITION TITLE: Professor

EDUCATION/TRAINING *(Begin with baccalaureate or other initial professional education, such as nursing, include postdoctoral training and residency training if applicable. Add/delete rows as necessary.)*

INSTITUTION AND LOCATION	DEGREE *(if applicable)*	Completion Date MM/YYYY	FIELD OF STUDY
Univ. Wisconsin River Falls, River Falls, WI			Biochemistry
Iowa State University, Ames, IA	B.S	12/1986	Biochemistry
Iowa State University, Ames, IA	M.S.	12/1989	Biochemistry
Iowa State University, Ames, IA	Ph.D.	05/1995	Biochemistry

A. Personal Statement

I have been a Professor in the Biochemistry Division of the Department of Chemistry and Biochemistry for 20 years at UCLA. My research focuses on understanding the molecular mechanisms of protein and RNA translocation into mitochondria and how mitochondrial dysfunction contributes to disease. My laboratory has pioneered studies characterizing an oxidation-reduction pathway for the import of proteins and a pathway for the assembly of inner membrane proteins. Most recently, we have been developing a chemical biology approach to develop new modulators, termed MitoBloCKs, of mitochondrial biogenesis. To this end, we have conducted numerous small molecule screens and identified inhibitors for most components of the mitochondrial protein translocons. These small molecule inhibitors also modulate protein import in mammalian mitochondria because protein import pathways are highly conserved and the overall structure of proteins components are conserved, even though primary sequences are not. These small molecules are being used for mechanistic studies to understand details in mitochondrial import and for studies to understand the physiologic consequences from a defect in protein import. Specifically, attenuating protein translocation plays an important role in stabilizing Pink1 on the outer membrane, recruiting Parkin, and stimulating mitophagy to remove dysfunctional mitochondria. In the disease primary hyperoxaluria 1, small molecules redirect AGT1 from mitochondria back to peroxisomes and rescue the molecular defect. We are also adapting zebrafish as a new model for mitochondrial diseases, which is an outstanding vertebrate model for testing small molecule modulators. My laboratory relies on methodology in basic biochemistry and cell biology and works in a variety of model organisms to study mitochondrial biogenesis. We are expanding studies to increase the types of small molecule modulators available for mitochondria because of the broad implications of mitochondrial dysfunction in degenerative diseases of muscle and the neural system. In addition, we are expanding our repertoire of experimental systems to investigate the mechanism of RNA import into mitochondria and to understand the role of mitochondria in human embryonic stem cell pluripotency and maintenance**.** Finally, we are developing zebrafish as a model for various mitochondrial diseases.

I have received numerous awards for my research including an Established Investigator Award from the American Heart Association, a Damon Runyon Walter Winchell Scholar Award, a Beckman Young Investigator Award and a Fellow of the American Association for the Advancement of Science. I currently serve on the scientific advisory board of the United Mitochondrial Disease Foundation and the Oxalosis and Hyperoxaluria Foundation. I take an active role in grant review and regularly serve as a panel member for the AHA, DOD, and am currently Chair of the NOMD study section at NIH.

I also actively collaborate with Alex van der Bliek on biochemical and cell biology studies with mitochondrial dynamics and quality control. We share joint laboratory space and have a weekly joint lab meeting. I am also a member of the Mitochondrial and Metabolism theme at UCLA and organize a weekly mitochondrial meeting for the labs for the critical discussion of research projects that are presented by lab personnel.

B. Positions and Honors

PROFESSIONAL POSITIONS:

1/87 - 12/89 M.S. Student, Department of Biochemistry & Biophysics, Iowa State Univ.
advisor: Dr. Donald Beitz

1/90 - 4/95 Ph.D. Student, Department of Biochemistry & Biophysics, Iowa State Univ.
advisor: Dr. Alan Myers

6/95 – 7/99 Post-doctoral fellow, Biochemie, Biozentrum, Basel, Switzerland
advisor: Dr. Gottfried Schatz

8/99 – 7/04 Assistant Professor, Department of Chemistry and Biochemistry, UCLA

8/04 – 6/09 Associate Professor, Department of Chemistry and Biochemistry, UCLA

1/07 – 9/07 Visiting Scientist, Max Planck Institute, Tuebingen, Germany, Nuesslein-Volhard Group

6/12 – 4/13 Visiting Scientist, NINDS, NIH, Richard Youle Group

8/99 – present Member, UCLA Molecular Biology Institute
Member, UCLA Brain Research Institute
Member, Jonsson Comprehensive Cancer Center

7/09 – present Professor, Department of Chemistry and Biochemistry, UCLA

OTHER PROFESSIONAL ACTIVITIES (SELECTED):

Member NIH study section, BRT-A 2006-2012; NOMD, 2014-2020 (Chair 2018-2020); Associate Editor, Current Genetics, 2005-present; Co-Chair, Gordon Research Conference in Protein Translocation, 2007; Co-Chair FASEB Conference on Mitochondrial Biology, 2009; Member AHA study section, 2009-2018; United Mitochondrial Disease Foundation (UMDF) Scientific Advisory Board, 2013-2019; Reviewer, Beckman Young Investigator Program, 2014; Co-Chair, Gordon Research Conference on Mitochondrial Biology, 2019; Co-Chair, UMDF Annual Meeting on Mitochondrial Biology, 2019; Co-Chair, ASBMB Annual Meeting, 2020.

HONORS AND AWARDS:

1995 MDA Postdoctoral Fellowship (Declined); NSF International Fellowship

1996-1998 Damon Runyon-Walter Winchell Foundation Post-doctoral Fellow

2000-2003 Scholar Award Damon Runyon-Walter Winchell Society

2000-2003 New Investigator Award, Toxicological Sciences from Burroughs Wellcome Foundation

2002-2005 Arnold and Mabel Beckman Young Investigator Award

2003 ISU Outstanding Young Alumna Award

2006-2010 American Heart Association Established Investigator Award

2010 Newby McCoy Research Award

2016 Public Lecture at Balai Sidang University Indonesia

2017 Invited Speaker 2017 Dr. Paul Janssen Award Symposium for Doug Wallace “Mitochondria and Medicine”

2019 Fellow of the American Association for the Advancement of Science

C. Contributions to Science

1. **The TIM22 protein import pathway in mitochondria**—My early publications addressed the characterization of a new import pathway that I identified, the TIM22 import pathway for inner membrane proteins such as the carriers (ATP/ADP carrier, phosphate carrier and import translocation subunits Tim22 and Tim23). Previously, these carriers were thought to rely on the TIM23 translocation system. Our work showed that the TIM22 pathway was quite complex with intermembrane space chaperones (The small Tim proteins, Tim8, Tim9, Tim10, Tim12, and Tim13) and the inner membrane insertion complex consisting of Tim18, Tim22, and Tim54. The small Tim proteins maintain the import competence of the hydrophobic carrier protein and the insertion complex mediates insertion into the inner membrane. I also showed that deafness-dystonia syndrome, caused by a mutation in the import component Tim8, was the first disease related to a defect in mitochondrial protein translocation. My group characterized fibroblasts from patient cell lines and our work indicated that a defect in the import of a subset of carrier proteins was a potential cause for the disease. In these studies, we developed a collection of temperature-sensitive yeast mutants, characterized cultured cell models, and expanded our array of approaches to measure bioenergetics and other mitochondrial activities.
   a. **Koehler, C. M.**, E. Jarosch, K. Tokatlidis, K. Schmid, R. J. Schweyen, and G. Schatz. 1998. Import of mitochondrial carriers mediated by essential proteins of the intermembrane space. Science 279:369-373.

b. Curran, S. P., D. Leuenberger, E. Schmidt, and **C. M. Koehler.** 2002. The role of the Tim8p-Tim13p complex in a conserved import pathway for mitochondrial polytopic inner membrane proteins. J. Cell Biol. 158:1017-1027.
c. Roesch, K., P. H. Hynds, R. Varga, L. Tranebjaerg, and **C. M. Koehler**. 2004. The calcium-binding aspartate/glutamate carriers, citrin and aralar1, are new substrates for the DDP1/TIMM8a-TIMM13 complex. Hum. Mol. Genet. 13:2101-2111.
d. Hwang, D. K., S. M. Claypool, H. D. Tienson, D. Leuenberger, and **C. M. Koehler**. 2007. Tim54p mediates assembly of the i-AAA protease Yme1p. J. Cell Biol.178:1161-75. [PMID: 17893242]

2. **Redox-regulated protein import pathway**—We have identified a new protein import pathway in the intermembrane space in which disulfide bonds are inserted into specific substrates with a twin CX3C or CX9C motif. As my group was characterizing the TIM22 pathway, we showed that the small Tim proteins contained disulfide bonds; this contrasted other studies that suggested the small Tim proteins were Zn^{2+}-binding proteins and opposed the dogma that the mitochondrial intermembrane space was a reducing environment, a redox machinery was lacking, and proteins could not have disulfide bonds. As we showed that substrates in the intermembrane space indeed contained disulfide bonds, other groups identified a machinery consisting of the oxidoreductase Mia40 and the sulfhydryl oxidase Erv1. We have identified novel terminal electron acceptors cytochrome *c* and the fumarate reductase Osm1 that accept electrons from Erv1. We also have taken a chemical biology approach and have specific small molecule modulators that abrogate Erv1 function. With these, we are expanding our mechanistic studies about how electrons are shuttled through the pathway and conducting studies that show the mammalian Erv1 homolog ALR is required for survival of pluripotent stem cells. This small molecule MitoBlock-6 also has translational potential because it selectively kills pluripotent cells that fail-to-differentiate and treatment of a pluripotent cell population that is being induced to differentiate with MitoBloCK-6 may reduce the potential for teratomas. This work confirms our ability to work with stem cells.
a. Bourens, M., D. V. Dabir, H. L. Tienson, I. Sorokina, **C. M. Koehler,** and A. Barrientos. 2012. J. Biol. Chem. Role of twin Cys-Xaa9-Cys cysteines in mitochondrial import of the cytochrome c oxidase biogenesis factor Cmc1. J. Biol. Chem. 287:31258-31269. [PMCID: PMC3438957]
b. Neal, S. E., D. V. Dabir, H. L. Tienson, D. Horn, K. Glaser, R. Loo, A. Barrientos, and **C. M. Koehler**. 2015. Mia40 serves as an electron sink in the Mia40-Erv1 import pathway. J. Biol. Chem. 290:20804-20814. [PMCID: PMC4543643]
c. Thangamani S., M. Maland, H. Mohammad, P. E. Pascuzzi, L. Avramova, **C. M. Koehler**, T.R. Hazbun, and M. N. Seleem. 2017. Repurposing approach identifies auranofin with broad spectrum antifungal activity that tTargets Mia40-Erv1 pathway. Cell Infect. Microbiol. 7:4. [PMCID: PMC5241286]
d. Neal, S. E., D. V. Dabir, J. Wijaya, C. Boon, and **C. M. Koehler.** 2017. Osm1 facilitates the transfer of electrons from Erv1 to fumarate in the redox-regulated import pathway in the mitochondrial intermembrane space. Mol. Biol. Cell. 28:2773-1785. [PMCID: PMC5638582]

3. **Small molecule modulators for protein translocation—**Because genetic studies in yeast limited the number of mutants that could be developed and tools are needed for studying protein translocation in mammalian cells, we have conducted numerous small molecule screens to develop modulators for the TIM22 and TIM23 import pathways, the MIA pathway (see previous section), and the processing proteases, MPP and PreP. These probes are used for mechanistic studies in protein import and also to attenuate protein import in cultured cell and zebrafish models. The probes work across species because the overall structures of the translocation components are conserved. We have used one probe, MitoBloCK-12, to correct mistargeting of alanine:glyoxylate aminotransferase from mitochondria to peroxisomes, resulting in a potential therapeutic strategy to treat patients with primary hyperoxaluria 1. In addition, these probes are useful for characterizing complex translocation pathways of proteins such as Pink1. We collaborate with numerous groups to exploit these probes for understanding how attenuating mitochondrial protein translocation can be useful as a therapeutic strategy. This is a very active research area and numerous publications are forthcoming.
a. Dabir, D. V., S. A. Hasson, K. Setoguchi, M. E. Johnson, P. Wongkongkathep, C. J. Douglas, J. Zimmerman, R. Damoiseaux, M. A.Teitell, and **C. M. Koehler**. 2013. A Small Molecule Inhibitor of Redox-Regulated Protein Translocation into Mitochondria. Dev. Cell. 25:81-92. [PMCID: PMC372622]
b. Miyata, N., J. Steffen, M. E. Johnson, S. Fargue, C. J. Danpure, and **C. M. Koehler**. 2014. Pharmacologic rescue of an enzyme trafficking defect in primary hyperoxaluria 1. Proc. Natl. Acad. Sci.

USA 111:14406-14411. [PMCID: PMC4210028]

c. Miyata, N., Z. Tang, M. A. Conti, M. E. Johnson, C. J. Douglas, S. A. Hasson, R. Damoiseaux, C. A. Chang, and **C. M. Koehler.** 2017. Adaptation of a genetic screen reveals an inhibitor for mitochondrial protein import component Tim44. J. Biol. Chem. In Press. [PMCID: PMC5392686]

d. Filipuzzi, I., J. Steffen, M. Germain, L. Goepfert, M. A. Conti, C. Potting, R. Cerino, M. Pfeifer, P. Krastel, D. Hoepfner, J. Bastien, **C. M. Koehler***, and S. B. Helliwell.* 2017. Stendomycin selectively inhibits TIM23-dependent mitochondrial protein import. Nature Chem. Biol. In Press. 13:1239-1244 [PMCID: In Process]

*co-corresponding authors

4. **RNA import into mammalian mitochondria—**In collaboration with Dr. Michael Teitell at UCLA, we have identified a new RNA import pathway into mitochondria. The protein PNPase is a gatekeeper that localizes to the intermembrane space and binds to a specific loop in substrates including the RNA components of RNase P and MRP1 and the 5S ribosomal RNA. We have identified a targeting sequence that can be used to target any RNA to mitochondria and have shown that this approach can be used to target corrective tRNAs to mitochondria in patient cells lines with mutations in the mitochondrial-coded tRNA (in MELAS and MERRF diseases). Moreover, mutations in PNPase result in inherited deafness, indicating that PNPase is essential for mitochondrial function. In these studies, our models include a conditional knock-out mouse model and knock-out MEF cell lines, and we conduct biochemical analysis of RNA import in vitro and in vivo.

a. Wang, G., H.-W. Chen, Y. Oktay, E. L. Allen, G. M. Smith, K. C. Fan, J. S. Hong, S. W. French, J. M. McCaffery, H. C. Morse III, **C. M. Koehler**, and M. A. Teitell. 2010. PNPASE regulates protein import into mitochondria. Cell 142:456-467. [PMCID: PMC2921675]

b. Wang, G., E. Shimada, **C. M. Koehler**, and M. A. Teitell. 2011. PNPASE and RNA trafficking into mitochondria. Biochim. Biophys. Acta 1819:998-1007. [PMCID: PMC3267854]

c. Wang, G., E. Shimada, J. Zhang, J. S. Hong, G. M. Smith, M. A. Teitell, and **C. M. Koehler**. 2012. Correcting human mitochondrial mutations with targeted RNA import. Proc. Natl. Acad. Sci. USA 109:4840-4845. [PMCID: PMC3323963]

d. von Ameln, S., G. Wang, R. Boulouiz, M. A. Rutherford, G. M. Smith, Y. Li, H. M. Pogoda, G. Nuernberg, B. Stiller, A. D. Volk, G. Borck, J. S. Hong, R. J. Goodyear, O. Abidi, P. Nuernberg, K. Hoffman, G. P. Richardson, M. Hammerschmidt, T. Moser, B. Wollnick, **C. M. Koehler**, M. A. Teitell, A. Barakat, C. Kubish. 2012. A mutation in PNPT1, encoding a mitochondrial RNA import protein PNPase, causes hereditary hearing loss. Am. J. Hum. Genet. 91:919-927. [PMCID: PMC3487123]

5. **Developing new models to study mitochondrial function—**As a result of the collaboration with the Teitell laboratory, we have been characterizing the role of mitochondrial metabolism in human stem cell biology. We manipulate stem cells, study metabolomics, and investigate additional pathways including bioenergetics, dynamics, and ROS generation. Mitochondrial metabolism in stem cells is similar but not identical to that of cancer cells. We find that UCP2 regulates energy metabolism and differentiation potential in human pluripotent cells. Since a sabbatical in the Nuesslein-Volhard lab, my group has been using zebrafish for different studies. Initially, we used them to test specificity of the small molecule modulators in lines in which mitochondria are marked in tissues such as heart, cortical and primary motor neurons, and muscle. As the CRISPR technology has developed, we are generating knock-out lines for a variety of pathways to understand the role of mitochondria in development. To this end, we have been focusing on the role of mitochondria in cortical and primary motor neurons. We also have developed methods to culture the primary cells for ~8 days, facilitating biochemical and cell biology studies. We are generating numerous mutants and are currently characterizing them.

a. Levesque, M. P., J. Krauss, **C. Koehler**, C. Boden, and M. P. Harris. 2013. New Tools for the Identification of Developmentally Regulated Enhancer Regions in Embryonic and Adult Zebrafish. Zebrafish. 10:21-29. [PMCID: PMC3670562]

b. Setoguchi, K., T. TeSlaa, **C. M. Koehler**, and M. A. Teitell. 2016. P53 regulates rapid apoptosis in human pluripotent stem cells. J. Mol. Biol. 428:1465-1475. [PMCID: PMC4733597]

c. Steffen, J., A. A. Vashisht, J. Wan, J. C. Jen, S. M. Claypool, J. A. Wohlschlegel, and C.M. Koehler. 2017. Rapid degradation of mutant SLC25A46 by the ubiquitin-proteasome system results in MFN1/2 mediated hyperfusion of mitochondria. Mol Biol Cell. 28:600-612. [PMCID: PMC5328619]

d. D. Sangwan, S., A. Zhao, K.L. Adams, C. K. Jayson, M. R. Sawaya, E. L. Guenther, A. C. Pan, J. Ngo, D. M. Moore, A. B. Soriaga, T. D. Do, L. Goldschmidt, R. Nelson, M. T. Bowers, C. M. Koehler, D. E.

Shaw, B.G. Novitch, and D. S. Eisenberg. 2017. Atomic structure of a toxic, oligomeric segment of SOD1 linked to amyotrophic lateral sclerosis (ALS). Proc. Natl. Acad. Sci. USA 114: 8770-8775. [PMCID: PMC5565441]

Complete List of Published Work in MyBibliography:
http://www.ncbi.nlm.nih.gov/sites/myncbi/carla.koehler.1/bibliograpahy/40388761/public/?sort=date&direction=descending

D. Additional Information: Research Support and/or Scholastic Performance

Ongoing Research Support

R01GM61721 Koehler (PI) 08/01/15 – 08/01/20 (no-cost extension)
NIH
Biogenesis of the mitochondrial inner membrane
The major goal of this project is to characterize protein import into the mitochondrial inner membrane in S. cerevisiae

FA9550-15-1-0406 (Teitell PI, Koehler, Co-PI) 09/01/15 – 09/01/20
USAF, AFRL
mitoBLAST: A New Opportunity to Reverse Engineer Mitochondria
The project develops technology to deliver isolated mitochondria to cultured cell models.

Completed Research Support

1R01DK101780-01 Koehler (PI) 09/01/15 – 03/30/20 (no-cost extension)
NIH/NIDDK
Small Molecule Probes to Correct AGT Mistargeting in Primary Hyperoxaluria 1
The major goal of this project is to study trafficking of the protein AGT and determine how defective trafficking to mitochondrial leads to the disease. Small molecule probes will be tested for their ability to rescue the trafficking defect.

R01GM073981 Koehler, Teitell (Multi-PI) 07/01/14 – 07/01/19
NIH
RNA import into mitochondria
The major goal of this project is to characterize the import of RNA into mitochondria

W81XWH-17-1-0333
USAMRAA PRMRP Koehler (PI) 07/01/17 – 12/31/18
Developing zebrafish models for interrogating mitochondrial disease, performance, and stress
The major goal of this project is to develop zebrafish models for studying how mitochondrial dysfunction impacts muscle and neural systems.

RT307678 Koehler (PI), Teitell (Co-PI) 06/01/15 – 09/01/18
California Institute of Regenerative Medicine
A small molecule tool for reducing the malignant potential in reprogramming human iPSCs and ESCs.
The major goals of this proposal are to investigate the ability of a mitochondrial small molecule modulator to selective kill pluripotent stem cells but not differentiated lineages and to use the probe as a tool to optimize differentiation protocols for human pluripotent stem cells.

PHS 398 Cover Page Supplement

OMB Number: 0925-0001
Expiration Date: 03/31/2020

1. Vertebrate Animals Section

Are vertebrate animals euthanized? ● Yes ○ No

If "Yes" to euthanasia

Is the method consistent with American Veterinary Medical Association (AVMA) guidelines?

● Yes ○ No

If "No" to AVMA guidelines, describe method and provide scientific justification

2. *Program Income Section

*Is program income anticipated during the periods for which the grant support is requested?

○ Yes ● No

If you checked "yes" above (indicating that program income is anticipated), then use the format below to reflect the amount and source(s). Otherwise, leave this section blank.

*Budget Period	*Anticipated Amount ($)	*Source(s)

Tracking Number: GRANT13088859
Funding Opportunity Number: PAS-18-483 . Received Date: 2020-04-30T11:45:17.000-04:00

PHS 398 Cover Page Supplement

3. Human Embryonic Stem Cells Section

*Does the proposed project involve human embryonic stem cells? ○ Yes ● No

If the proposed project involves human embryonic stem cells, list below the registration number of the specific cell line(s) from the following list: http://grants.nih.gov/stem_cells/registry/current.htm. Or, if a specific stem cell line cannot be referenced at this time, check the box indicating that one from the registry will be used:

☐ Specific stem cell line cannot be referenced at this time. One from the registry will be used.

Cell Line(s) (Example: 0004):

4. Inventions and Patents Section (Renewal applications)

*Inventions and Patents: ○ Yes ○ No

If the answer is "Yes" then please answer the following:

*Previously Reported: ○ Yes ○ No

5. Change of Investigator/Change of Institution Section

☐ Change of Project Director/Principal Investigator

Name of former Project Director/Principal Investigator

Prefix:

*First Name:

Middle Name:

*Last Name:

Suffix:

☐ Change of Grantee Institution

*Name of former institution:

PHS 398 Modular Budget

OMB Number: 0925-0001
Expiration Date: 02/28/2023

Budget Period: 1

Start Date: 12/01/2020 End Date: 11/30/2021

A. Direct Costs

	Funds Requested ($)
Direct Cost less Consortium Indirect (F&A)*	250,000.00
Consortium Indirect (F&A)	0.00
Total Direct Costs*	250,000.00

B. Indirect (F&A) Costs

	Indirect (F&A) Type	Indirect (F&A) Rate (%)	Indirect (F&A) Base ($)	Funds Requested ($)
1.	Research On Campus	56.00	233,111.00	130,542.00
2.				
3.				
4.				

Cognizant Agency (Agency Name, POC Name and Phone Number): DHHS, Janet Turner, 415-437-7820

Indirect (F&A) Rate Agreement Date: 10/12/2018

Total Indirect (F&A) Costs: 130,542.00

C. Total Direct and Indirect (F&A) Costs (A + B)

Funds Requested ($): 380,542.00

PHS 398 Modular Budget

Budget Period: 2

Start Date: 12/01/2021 End Date: 11/30/2022

A. Direct Costs

	Funds Requested ($)
Direct Cost less Consortium Indirect (F&A)*	250,000.00
Consortium Indirect (F&A)	0.00
Total Direct Costs*	250,000.00

B. Indirect (F&A) Costs

	Indirect (F&A) Type	Indirect (F&A) Rate (%)	Indirect (F&A) Base ($)	Funds Requested ($)
1.	Research On Campus	56.00	233,111.00	130,542.00
2.				
3.				
4.				

Cognizant Agency (Agency Name, POC Name and Phone Number): DHHS, Janet Turner, 415-437-7820

Indirect (F&A) Rate Agreement Date: 10/12/2018

Total Indirect (F&A) Costs: 130,542.00

C. Total Direct and Indirect (F&A) Costs (A + B)

Funds Requested ($): 380,542.00

PHS 398 Modular Budget

Budget Period: 3			
Start Date: 12/01/2022 End Date: 11/30/2023			
A. Direct Costs			Funds Requested ($)
		Direct Cost less Consortium Indirect (F&A)*	250,000.00
		Consortium Indirect (F&A)	0.00
		Total Direct Costs*	250,000.00

B. Indirect (F&A) Costs

	Indirect (F&A) Type	Indirect (F&A) Rate (%)	Indirect (F&A) Base ($)	Funds Requested ($)
1.	Research On Campus	56.00	233,111.00	130,542.00
2.				
3.				
4.				

Cognizant Agency (Agency Name, POC Name and Phone Number): DHHS, Janet Turner, 415-437-7820

Indirect (F&A) Rate Agreement Date: 10/12/2018

Total Indirect (F&A) Costs: 130,542.00

C. Total Direct and Indirect (F&A) Costs (A + B)	Funds Requested ($)	380,542.00

PHS 398 Modular Budget

Budget Period: 4

Start Date: 12/01/2023 End Date: 11/30/2024

A. Direct Costs

	Funds Requested ($)
Direct Cost less Consortium Indirect (F&A)*	250,000.00
Consortium Indirect (F&A)	0.00
Total Direct Costs*	250,000.00

B. Indirect (F&A) Costs

	Indirect (F&A) Type	Indirect (F&A) Rate (%)	Indirect (F&A) Base ($)	Funds Requested ($)
1.	Research On Campus	56.00	233,111.00	130,542.00
2.				
3.				
4.				

Cognizant Agency (Agency Name, POC Name and Phone Number): DHHS, Janet Turner, 415-437-7820

Indirect (F&A) Rate Agreement Date: 10/12/2018

Total Indirect (F&A) Costs: 130,542.00

C. Total Direct and Indirect (F&A) Costs (A + B)

Funds Requested ($): 380,542.00

PHS 398 Modular Budget

Budget Period: 5

Start Date: 12/01/2024 End Date: 11/30/2025

A. Direct Costs

	Funds Requested ($)
Direct Cost less Consortium Indirect (F&A)*	250,000.00
Consortium Indirect (F&A)	0.00
Total Direct Costs*	250,000.00

B. Indirect (F&A) Costs

	Indirect (F&A) Type	Indirect (F&A) Rate (%)	Indirect (F&A) Base ($)	Funds Requested ($)
1.	Research On Campus	56.00	233,111.00	130,542.00
2.				
3.				
4.				

Cognizant Agency (Agency Name, POC Name and Phone Number): DHHS, Janet Turner, 415-437-7820

Indirect (F&A) Rate Agreement Date: 10/12/2018

Total Indirect (F&A) Costs: 130,542.00

C. Total Direct and Indirect (F&A) Costs (A + B)

Funds Requested ($): 380,542.00

PHS 398 Modular Budget

Cumulative Budget Information

1. Total Costs, Entire Project Period

Section A, Total Direct Cost less Consortium Indirect (F&A) for Entire Project Period ($)	1,250,000.00
Section A, Total Consortium Indirect (F&A) for Entire Project Period ($)	0.00
Section A, Total Direct Costs for Entire Project Period ($)	1,250,000.00
Section B, Total Indirect (F&A) Costs for Entire Project Period ($)	652,710.00
Section C, Total Direct and Indirect (F&A) Costs (A+B) for Entire Project Period ($)	1,902,710.00

2. Budget Justifications

Personnel Justification	Personnel_Justification1060262418.pdf
Consortium Justification	
Additional Narrative Justification	Additional_Narrative1060262419.pdf

Personnel Justification

Key Personnel:

Drs. van der Bliek and Koehler will work together to administer and oversee the research project, ensuring that all activities are carried out efficiently in accordance with the research plan.

Dr. Alexander M. van der Bliek, Ph.D., Principal Investigator (3.0 calendar months). Dr. van der Bliek is a Professor in the Department of Biological Chemistry with over 25 years of experience with mammalian cells. Dr. van der Bliek will devote much more time to this project than requested in salary support, because it is a main area of focus for the lab. Dr. van der Bliek will meet frequently with Dr. Koehler. Together Drs. Koehler and van der Bliek will provide oversight for experimental design, interpretation of results and preparation of publications. Dr. van der Bliek's department has indicated that they are no longer able to support unfunded programs. Funding for this grant will therefore make it possible to continue the research as proposed.

Dr. Carla M. Koehler, Ph.D., Principal Investigator (1.0 calendar months) is a Professor in the Department of Chemistry and Biochemistry with 25 years of experience in the experimental systems (biochemical analyses, mammalian cells and Zebrafish). She will meet frequently with Dr. van der Bliek and the other personnel to plan experiments and help in their interpretation. She will oversee the preparation and submission of manuscripts for publication. The PI summer salary was calculated with anticipated merit per UCLA academic salary scale, with a limit based on the NIH salary cap.

Non-Key Personnel:

To be named, Post Graduate Researcher (12.0 calendar months). Funding is requested to hire a new postdoctoral fellow for this project. This person should have experience with cell biology and molecular techniques. This postdoctoral fellow will be responsible for continuing the work with mammalian cells in the van der Bliek lab. We request full salary support for this person.

Sean Atamdede, Graduate Student Researcher (4.5 Academic and 3 summer Months), is currently a first year graduate student co-menotred by Drs. Koehler and van der Bliek. Sean worked a s a technician in the lab before and has expertise in developing and characterizing zebrafish models as well as expertise in biochemical approaches to characterize mitochondria.

Salary scales and benefits. Salaries and wages were calculated on the basis of the University of California Academic Salary Schedule for Fiscal Year 2019/2020 and the University of California Staff Personnel Title Pay for Fiscal Year 2019/2020.

Fringe benefits are charged to all senior and other personnel at a composite rate set by the Regents of University of California. PIs Drs. Van der Bliek and Koehler are charged at a rate of 32.2%. The fringe benefit of the graduate student researcher is charged at 4.9% and the Postdoctoral fellow is charged 28.4%. The specific benefits for each individual are determined by strict guidelines set by the University of California and subject to rigorous review. In accordance with their respective eligibility, the benefit rates provide all the individuals within the University the standard fringe benefit package. https://www.finance.ucla.edu/composite-benefit-rate-assessment

Additional Narrative Justification

INDIRECT COSTS

The indirect costs were calculated at 56% of the modified total direct costs (MDTC). These rates were approved by the UC Regents and the Department of Health and Human Services (DHHS) on October 12, 2018. The GSR Tuition/Fee Remissions are excluded from the MTDC Base in the calculation of indirect costs.

PHS 398 Research Plan

OMB Number: 0925-0001
Expiration Date: 02/28/2023

Introduction	
1. Introduction to Application (for Resubmission and Revision applications)	
Research Plan Section	
2. Specific Aims	specific_aims1060326280.pdf
3. Research Strategy*	research_design1060326281.pdf
4. Progress Report Publication List	
Other Research Plan Section	
5. Vertebrate Animals	Vertebrate_Animals1060262392.pdf
6. Select Agent Research	
7. Multiple PD/PI Leadership Plan	multi_pi_plan1060080742.pdf
8. Consortium/Contractual Arrangements	
9. Letters of Support	Letters_of_support1060326268.pdf
10. Resource Sharing Plan(s)	Resource_sharing1060080748.pdf
11. Authentication of Key Biological and/or Chemical Resources	Authentication_key_Biological1060080750.pdf
Appendix	
12. Appendix	

SPECIFIC AIMS

Charcot-Marie-Tooth disease (CMT) is a hereditary peripheral neuropathy resulting from demyelination or axon degeneration[1]. Many cases of CMT with axon degeneration are caused by mutations in Mitofusin-2 (Mfn2)[2], which is one of two dynamin-like proteins on the surface of mitochondria[3]. Mitofusins are known for their roles in mitochondrial outer membrane fusion[4], but Mfn2 also promotes the association between ER and mitochondria in mitochondria-associated membranes (MAM)[5]. Mfn2 is observed in spots that colocalize with the mitochondrial fission dynamin Drp1[6] and was proposed to affect axonal transport of mitochondria[7,8], raising the possibility that CMT results from defects in these other functions of Mfn2. Preliminary data obtained with the fungal toxin PXA shows that Mfn2 controls constriction of mitochondria through calcium release mediated by the mitochondrial inner membrane sodium-calcium exchange protein NCLX. We hypothesize that Mfn2 and NCLX are connected with mitochondrial fission and axonal transport. We propose the following aims to firmly establish NCLX as the target of PXA, as well as the novel role of Mfn2 in mitochondrial fission and its functional consequences for mitochondrial transport:

Aim 1. Investigate the connections between PXA, NCLX, and Mfn2.

We hypothesize that PXA triggers calcium release from mitochondria by activating NCLX and that Mfn2 controls NCLX-mediated calcium release from mitochondria.

a. Is NCLX the target of PXA? We will test whether PXA targets NCLX using a combination of cell biological tests for calcium release and biochemical tests for binding interactions between PXA and NCLX.

b. How does Mfn2 control NCLX? We will investigate the mechanisms by which Mfn2 controls NCLX using genetic analyses, binding assays, biochemical techniques and localization studies with microscopy.

These experiments will determine whether PXA directly targets NCLX and verify that Mfn2 controls NCLX.

Aim 2. Investigate connections between Mfn2 and mitochondrial fission.

We hypothesize that Mfn2 collects at sites of mitochondrial fission, triggering calcium release through NCLX, which may result in localized constriction of mitochondria and fission through Drp1.

a. Does Mfn2 actively contribute to fission? We will use a series of gene deletions and transfections with wildtype and mutant constructs to determine whether Mfn2 plays an active role during fission or acts as an ancillary factor. These functional studies will be complemented by localization studies with microscopy.

b. Upstream regulatory mechanisms. To learn more about the regulatory mechanisms, we will use gene knockouts and transfections to investigate control of Mfn2-dependent functions during mitochondrial fission by signal transduction pathways, Opa1, and the mitochondrial outer membrane protein SLC25A46.

Experiments in this aim will help determine whether Mfn2 plays a novel role in mitochondrial fission.

Aim 3. Investigate the physiological consequences of Mfn2 and NCLX interactions.

We hypothesize that Mfn2 and NCLX affect specific downstream processes in neurons, such as axonal transport, or other processes, such as respiration and apoptosis.

a. Effects of Mfn2 and NLCLX on mitochondrial transport in cultured neuronal cells. The control of transport by Mfn2 and NCLX will be examined in differentiated SH-SY5Y cells using kymographs. Testing CMT mutations in Mfn2 will help determine the relevance of the effects on transport for human disease.

b. Metabolism, apoptosis, and other functions potentially affected by Mfn2 and NCLX. PXA inhibits oxidative phosphorylation and induces apoptosis through excessive calcium release. This calcium release is mediated by NCLX under control of Mfn2, suggesting that CMT mutations in Mfn2 could affect respiration and apoptosis. We will investigate these downstream effects of Mfn2 and NCLX in neuronal cells.

c. Relevance for mitochondrial transport in zebrafish neurons. We will use zebrafish as a vertebrate model to test physiological effects of Mfn2 and NCLX on mitochondrial fission and axonal transport.

These experiments are designed to identify neuronal functions that are affected by Mfn2 and NCLX. Importantly, they will help determine whether axonal transport of mitochondria is affected by interactions between Mfn2 and NCLX rather than by other functions of Mfn2 (mitochondrial fusion and tethering to ER).

Together, the experiments in this proposal will test novel roles of Mfn2 as a fission protein and regulator of calcium release through NCLX. These new functions can be relevant for CMT disease because of their potential effects on axonal transport of mitochondria. Downstream effects on axonal transport will provide new insight into underlying causes of CMT and suggest novel treatment strategies.

A. SIGNIFICANCE

Charcot Marie Tooth (CMT) disease is a heterogeneous group of peripheral neuropathies caused by demyelination and axonal degeneration. The disease is associated with loss of sensory and motor neurons, muscle weakness, and foot deformities[1]. The overall prevalence is 1 in 3,500 adults[9]. Mutations in over 80 genes are associated with CMT[10]. This proposal focuses on Mitofusin 2 (Mfn2), which is often mutated in axonal CMT[2]. Mfn2 is one of two mitofusins (Mfn1 and Mfn2), which are large GTPases that mediate mitochondrial outer membrane fusion[3]. Defects in mitochondrial fusion are a possible explanation of the neurodegenerative effects of CMT disease, as over time fusion-defective mitochondria lose bioenergetic functions[4]. There is, however, also evidence suggesting that defects in other functions of Mfn2 cause CMT disease[7,8,11-13]. These alternative functions connect Mfn2 with axonal transport of mitochondria, either indirectly through the role of Mfn2 as an ER tether[13], or directly through interactions with Miro, which is a protein on the surface of mitochondria that participates in axonal transport[7].

Table 1. Key discoveries forming the basis for this proposal:

1. The fungal toxin PXA activates NLCX, triggering calcium release from mitochondria.
2. NCLX activation constricts mitochondria, even causing fission in Mfn1-Drp1 DKO cells.
3. Mfn2 is required for NCLX activation.

Preliminary data, summarized in Table 1, now provide evidence for additional connections between Mfn2 and mitochondrial fission, which can also provide a link to axonal transport. We show that a fungal toxin, called PXA, selectively activates the mitochondrial Na/Ca exchanger NCLX, causing rapid calcium release and mitochondrial fragmentation in wildtype cells and mitochondrial constrictions in Drp1$^{-/-}$ cells. Drp1-independent constrictions were previously also observed in neuronal mitochondria as a result of localized calcium oscillations, suggesting that this is a prelude to mitochondrial fission and subsequent axonal transport[14]. The PXA-induced constrictions are suppressed in Mfn2-Drp1 DKO cells, showing that NCLX is controlled by Mfn2. These constrictions may contribute to fission, because mitochondria rapidly fragment, even in Mfn1-Drp1 DKO cells but not in Mfn2-Drp1 DKO cells. We conclude that Mfn2 affects calcium release and mitochondrial fission, which are both important for axonal transport and therefore potential causes of CMT disease.

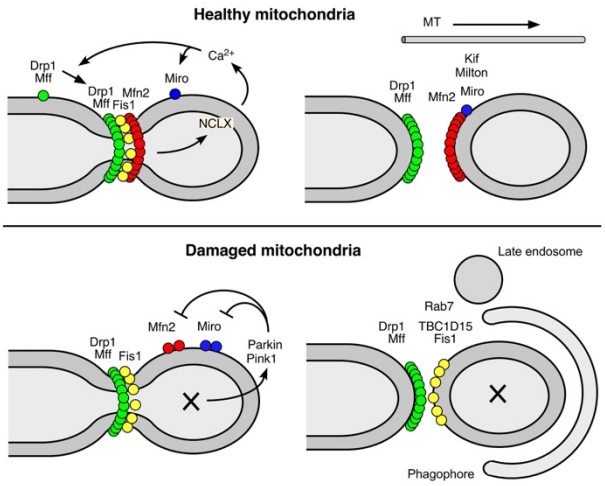


Fig. 1. Fission may help determine mitochondrial fate. (A) Fission of healthy mitochondria connected with axonal transport. Calcium release through NCLX activates the fission machinery, while interactions with Miro connect this process with microtubule dependent transport. (B) Asymmetric fission that leads to degradation of faulty mitochondria through mitophagy, showing steps that are closely associated with fission during mitophagy. Mfn2 and Miro are selectively extracted from the phagophore and degraded by the proteasome at an early stage of mitophagy.

We hypothesize that there are two modes of mitochondrial fission (Fig. 1): (A) Fission of healthy mitochondria produces daughters that can be connected to axonal transport proteins, such as Miro. We propose to test whether this connection is disrupted by CMT disease mutations. (B) Asymmetric fission that leads to degradation of faulty mitochondria through mitophagy[15]. Experiments in this proposal focus on fission of healthy mitochondria, aiming to strengthen the observation that Mfn2 constricts mitochondria and test for connections with axonal transport. This new hypothesis could aid the development of drug screens for treating CMT.

Mitochondrial dynamics: Fission and fusion are integral parts of the mitochondrial life cycle. Fusion is needed to sustain mitochondrial health by mixing and matching functioning parts of mitochondria, while fission is needed to distribute mitochondria in cells during growth and development (Fig. 1A). Fission also helps cleanse mitochondria when asymmetry is introduced by sorting damaged components into one of the daughter mitochondria (Fig. 1B). This daughter mitochondrion can then be targeted for degradation by mitophagy[15,16]. Fission is mediated by Drp1, while fusion is mediated by the Mitofusins Mfn1 and Mfn2 on the outer membrane and by Opa1 on the inner membrane (Fig. 2)[17,18]. These four proteins are members of the dynamin family, a group of structurally related large GTP-binding proteins that form multimeric assemblies[19,20]. Dynamins undergo conformational changes that are driven by GTP hydrolysis. Fission dynamins, like Drp1, form spirals that wrap around membrane tubules and further constrict to sever the membrane after subunits from adjacent rungs of the spiral contact each other[21]. Although several SNARE-like models have been proposed[22-24], it is still unclear how Mitofusins and Opa1 mediate fusion.

Dynamin family members have an N-terminal GTPase domain, a middle domain, a variable domain, and a GTPase effector domain (GED). The middle domain and the GED fold back on each other to form a

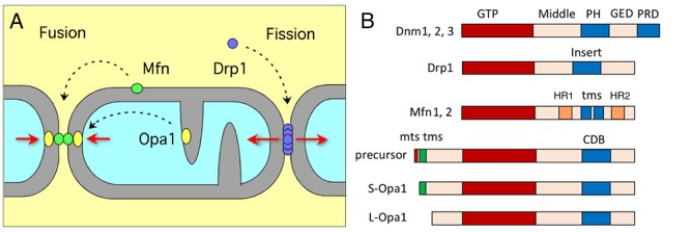


Fig. 2. Dynamin family members that mediate mitochondrial fission and fusion. (A) Fusion is mediated by the Mitofusins (Mfn1 and 2) on the outer membrane and Opa1 on the inner membrane, while fission is mediated by Drp1. (B) Protein domains in the different dynamin family members. Dynamin (Dnm1, 2 and 3) is shown for comparison. HR1 and HR2 are coiled-coil domains in the Mitofusins. S- and L-Opa1 are short and long isoforms of Opa1. CDB is a cardiolipin binding domain in Opa1.

stalk[25-27], which makes multiple contacts with adjacent subunits in the dynamin spiral[27]. Variable domains bring the protein to the target membrane, through interactions with receptors on the surface of mitochondria (Drp1), transmembrane anchors (Mitofusins), or cardiolipin binding (Opa1). Opa1 resides in the mitochondrial intermembrane space. It has an N-terminal mitochondrial targeting sequence that is cleaved during import, giving rise to a long form (L-Opa1) that retains an N-terminal transmembrane segment. Certain isoforms are then cleaved by Yme1L, giving rise to the short form (S-Opa1)[28,29]. When mitochondria lose their membrane potential, the remaining L-Opa1 variants are cleaved by Oma1 to make more S-Opa1[30,31]. Drp1 and mitofusins are regulated by phosphorylation and other protein modifications[32,33]. Altogether, a complex picture of the interplay between fission and fusion proteins and their upstream regulatory pathways has emerged.

Mfn1 and Mfn2 have similar sequences (63% identity in humans) and both can mediate outer membrane fusion. Although neurodegenerative defects in a mouse Mfn2 mutant were rescued by an Mfn1 transgene[34], the two proteins are only partially redundant[35]. Mfn1 mediates specific forms of stress-induced fusion and it is required for fusion induced by overexpression of Opa1, while Mfn2 is connected to diseases such as CMT and diabetes[36]. Immunofluorescence and binding studies suggest that Mfn2 is close to or in the mitochondrial fission complex at ER-mitochondrial junctions in mitochondria-associated membranes (MAM)[5,6,37], associates with Miro (a regulatory GTPase in the MAM)[7], and promotes contacts between the ER and mitochondria[5]. Together, these data suggest that Mfn2 is tied to functions at the MAM, for example coordinating fusion and fission when they occur in rapid succession, while Mfn1 is responsible for stress-induced fusion[38] and fusion when mitochondria bump into each other randomly. There are also important differences between Mfn1 and 2 functions in the developing cerebellum[39]. Dendritic outgrowth, spine formation, and survival of Purkinje cells as well as dopaminergic neurons require Mfn2, but not Mfn1[39,40]. Mfn2 is therefore critical for mitochondrial dynamics in neurons.

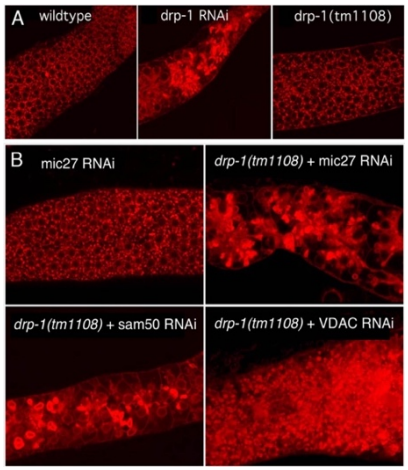


Fig. 3. Drp1-independent fission of mitochondria in C. elegans gonads. (A) Gonads from WT, *drp-1* RNAi and *drp-1(tm1108)* worms stained with Mitotracker. Gonad nuclei (holes in the honeycomb structure) are surrounded by mitochondria (red), which are disrupted by *drp-1* RNAi but not by *drp-1* deletion. (B) Gonads from a WT worm with mic-27 RNAi, *drp1-(tm1108)* with mic-27 RNAi, *drp-1(tm1108)* with Sam50 RNAi and *drp-1(tm1108)* with Porin RNAi.

Reports on the roles of different Opa1 isoforms vary. It was argued that inner membrane fusion requires heteromultimers of S- and L-Opa1 homologs[41,42]. There is, however, more L-Opa1 in cristae and more S-Opa1 on the rim, suggesting functional differences[43]. L-Opa1 could, for example, be needed to maintain cristae morphology, as also suggested by the opening up of cristae with loss of Opa1[44]. L-Opa1 also works together with Mfn1 during stress-induced mitochondrial hyperfusion[38], while S-Opa1 promotes a single round of inner membrane fusion after each cleavage event[45]. These data show that S-Opa1 and L-Opa1 affect different kinds of fusion. It was even suggested that S-Opa1 can promote mitochondrial fission when overexpressed[46]. Our project does not address the different roles of mitofusins and Opa1 during fusion. Instead, it focuses on their potential roles in mitochondrial fission and effects in CMT.

Interactions between Mfn2 and NCLX: This proposal focuses on two novel functions of Mfn2: a possible role in mitochondrial fission and control of calcium release through interactions with the mitochondrial inner membrane sodium-calcium exchange protein NCLX. These interactions were discovered with suppression of NCLX-mediated calcium release from mitochondria in $Mfn2^{-/-}$ cells. NCLX was previously identified by our collaborators in Israel (Sekler lab)[47]. They showed that NCLX is regulated by PKA phosphorylation and by membrane potential[48,49]. Our collaborators in Germany (Stork lab) found that the fungal toxin PXA releases calcium from mitochondria, but mechanisms for this release were unknown[50]. We now show that PXA induces calcium release through NCLX and that NCLX is controlled by Mfn2 on the mitochondrial outer membrane, as previously suggested by others[51]. Connection between NCLX and Mfn2 are a major focus of this proposal.

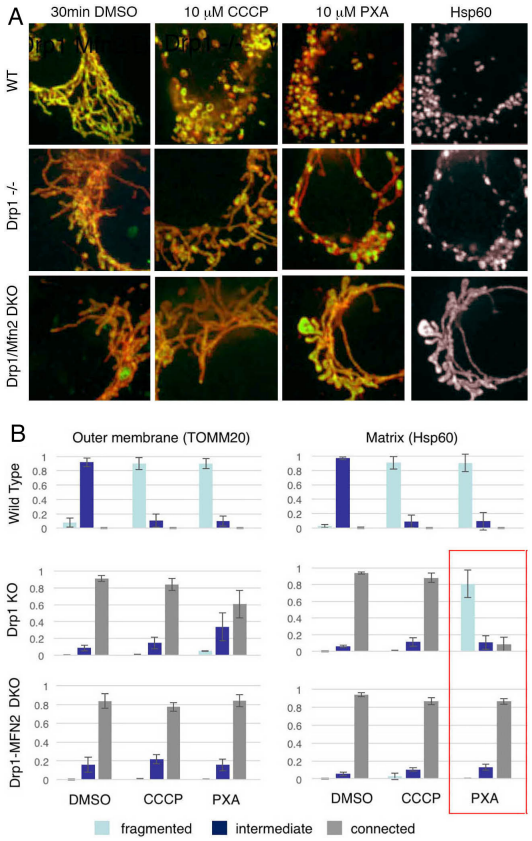


Fig. 4. Effects of CCCP and PXA on mitochondria in WT, Drp1 -/- and Drp1-Mfn2 DKO cells. (A) Confocal images after 30 min with DMSO, 10 μM CCCP or 10 μM PXA. IF for HSP60 (matrix, green) and TOMM20 (outer membrane, red). Separate images of HSP60 clearly show the differences between Drp1 -/- and Drp1-Mfn2 DKO cells. (B) Cells were classified according to mitochondrial morphologies (200 cells/condition, n=3; SD). Red box shows the shift from constricted to non-constricted caused by mutating Mfn2.

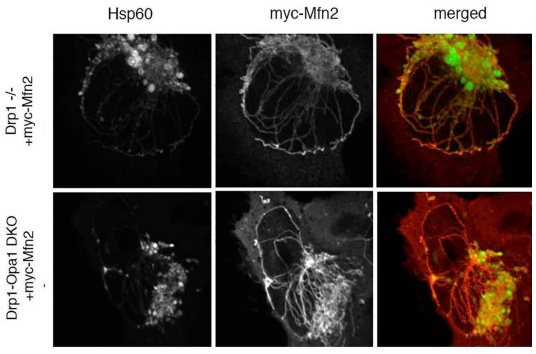


Fig. 5. Overexpressed myc-Mfn2 constricts mitochondria and forms a closed net. Drp1 -/- and Drp1-Opa1 DKO cells were transiently transfected with myc-Mfn2, fixed and stained for myc-Mfn2 (red) and matrix (Hsp60, green

Preliminary data: Some years ago, we noticed that *C. elegans* with deletions in the *drp-1* gene looked surprisingly healthy. Their brood-size was also sizeable (average, 119 progeny/worm vs. 302 for wildtype animals). Oogenesis is one of the main places for expanding mitochondrial mass in worms because all eggs need to be populated with mitochondria. In addition, mitochondria in the gonads of the *drp1-(tm1108)* deletion strain looked wildtype while those in *drp-1* RNAi worms were tangled in tight clusters, consistent with a fission defect (Fig. 3A). Mitochondria in somatic cells of the deletion strain have a fission defect. These results suggest that gonads in the deletion strain are capable of Drp1-independent mitochondrial fission. To identify proteins that affect this process, we tested numerous candidates for redundancy using RNAi. Synthetic lethality and disrupted mitochondria were observed in double mutants of Drp1 with Micos components, such as Mic27 and Sam50, which form cristae junctions, but not with VDAC, which serves as control (Fig. 3B). In addition, we observed synthetic effects with *fzo-1* RNAi (*C. elegans* mitofusin): 119±16 progeny/worm for *drp-1(tm1108)* alone; 180±36 for *fzo-1* RNAi alone; and 17±8 for both). Unfortunately, the surviving synthetic progeny were too sick for imaging, but these data do show that Drp1 redundancy requires FZO-1 on the surface of mitochondria.

Effects of PXA on Drp1 and Mfn2 mutant cells: To further investigate Drp1-independent fission, we made CRISPR/Cas9-mediated deletions in *Drp1*, *Mfn1*, *Mfn2*, and *Opa1* genes in HeLa cells and MEFs (complete lack of expression was verified with westerns and sequencing). Drp1$^{-/-}$ cells had closed mitochondrial nets, while Mfn1, Mfn2, and Opa1 deletions cause fragmentation. Loss of mitochondrial membrane potential upon treatment with CCCP did not induce fission in Drp1$^{-/-}$ cells, unlike wildtype cells, where mitochondria become fragmented (Fig. 4A). There was, however, a notable change in morphology when tested with a potent new fission-inducing chemical, called PXA. In collaboration with Bjorn Stork (Dusseldorf), we showed that PXA induces fission in wildtype cells and induces inner membrane but not outer membrane fission in Drp1$^{-/-}$ cells (Fig. 4A)[50]. Additional studies showed that PXA-induced constriction no longer occurs in Drp1-Mfn2 double knock out (DKO) cells, showing that Mfn2 is required for PXA-induced constriction of the mitochondrial outer membrane (Figs. 4A, B).

We tested the constriction properties of Mfn2 by overexpressing this protein in HeLa cells. Immunofluorescence shows thin mitochondrial tubules coated with Mfn2 (Fig. 5). These tubules are highly connected, which could be due to inhibition of mitochondrial fission or excessive fusion. The extensive constriction of mitochondrial tubules caused by Mfn2 overexpression shows that Mfn2 can constrict mitochondria, possibly by activating NCLX or by another mechanism.

Opposite effects of Mfn1 and 2: To test whether Mfn1 and Mfn2 have similar suppressive effects on PXA-induced constrictions in a Drp1$^{-/-}$ background, we generated Drp1-Mfn1 DKO cells. To our surprise, knocking out Mfn1 had the opposite effect. Instead of preventing constrictions, it aggravated the effects of PXA treatment to the point that mitochondria were severed through Drp1-independent fission (Fig. 6). These data show that Mfn1 and Mfn2 have very different effects on PXA-induced constrictions in Drp1$^{-/-}$ cells. Mfn1 and Mfn2 can work together in a heterotypic complex during fusion[52], but these new results also suggest that Mfn1 suppresses the fission properties of Mfn2 when present in a heterotypic complex.

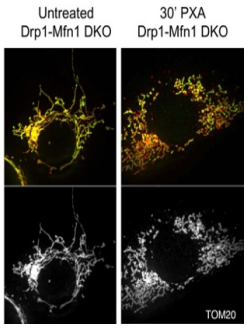


Fig. 6. Drp1-independent mitochondrial fission induced by PXA in Drp1-Mfn1 DKO cells. Confocal images show effects of 30 min treatment with DMSO or 10 μM PXA. Cells were stained with antibodies for HSP60 in the matrix (green) and TOMM20 (red) on mitochondrial outer membrane. Separate images of TOMM20 more clearly show the fragmentation induced by PXA in Drp1-Mfn1 DKO

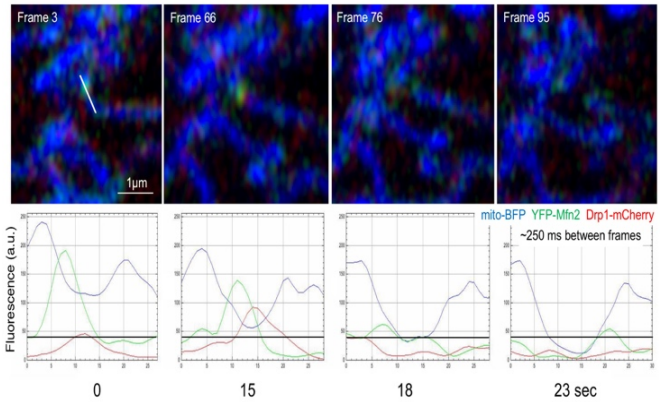


Fig. 7. Assembly of Mfn2 at constricted sites just before Drp1 assembles during normal fission. MEF cells were transfected with YFP-Mfn2, Drp1-mCherry and mitoBFP. Overexpression artifacts were avoided with low concentrations of Mfn2 DNA (0.2 g/ml). Images were acquired with a Zeiss Airyscan microscope equipped with a fast-mode. The line in the first image shows where tracings were made.

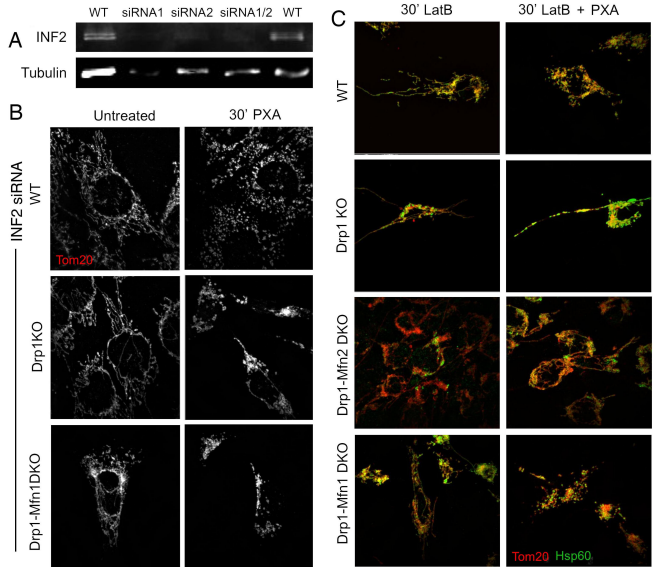


Fig. 8. No effects of INF2 siRNA and Latrunculin B on PXA-induced constrictions. MEFs were transfected with two INF2 siRNA oligonucleotides. Effects on INF2 levels are shown with western blot (A) and the lack of an effect on mitochondrial constriction or fission induced with PXA is shown with IF images of Tom20 (B). Similarly, incubations with Latrunculin B did not prevent the constriction or fission with PXA (C)

Live cell imaging of Mfn2: To determine whether Mfn2 spots, previously observed at sites of mitochondrial fission during apoptosis[6], are also present during homeostatic fission, we conducted live cell imaging with fluorescent proteins in untreated cells (Fig. 7). We observed accumulation of Mfn2 in spots on mitochondria before Drp1 spots formed. The mitochondria then constrict and are severed when an adjacent Drp1 spot is formed. We conclude that Mfn2 first assembles into a complex at the constriction site, followed by assembly of Drp1 and completion of the fission process.

No effects of actin: It was previously shown that INF2 and actin assembly on ER contributes to the initial constriction of mitochondria[53], while Mfn2 helps tether ER to mitochondria[5]. Fission often occurs at these sites[54]. To test whether the effects of Mfn2 on fission that we observed with PXA are also due to these connections with actin and INF2, we transfected cells with INF2 siRNA or treated them with the actin assembly inhibitor Latrunculin B with or without PXA (Fig. 8). The results suggest that neither INF2 siRNA nor Latrunculin B prevent PXA-induced constriction and fragmentation. Although these data still need to be quantified, it does seem likely that PXA acts through a different mechanism.

Effects of PXA on NCLX: The effects of PXA could be due to calcium release through NCLX, as suggested by combining the previously observed effects of PXA on mitochondrial calcium[50] with the potential role of Mfn2 in controlling NCLX function[51] and our data on reversal of PXA-induced constrictions in Drp1-Mfn2 DKO cells. With help from the Sekler lab in Israel, we tested more directly whether PXA affects NCLX-mediated calcium release by transducing Drp1$^{-/-}$ MEF cells with NCLX shRNA or treating them with the NCLX inhibitor CGP37157 followed by incubation with PXA. Levels of soluble mitochondrial calcium were determined by microscopy with Rhod-2 AM fluorescence. The results show that each of these

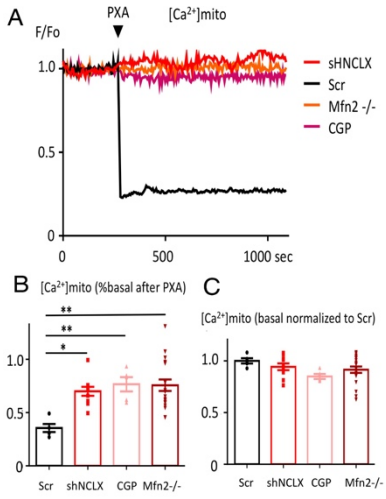


Fig. 9. Suppression of PXA-induced calcium release by NCLX siRNA or CGP37157 in Drp1$^{-/-}$ cells and in Drp1-Mfn2 DKO cells. (A) Time course of mitochondrial calcium concentrations determined with Rhod-2 AM dye, which binds to soluble unbuffered mitochondrial calcium. Cells were transduced with scrambled shRNA (SCR) or shNCLX, pretreated with CGP37157, or Mfn2 Drp1 double KO MEFs (labeled Mfn2$^{-/-}$). (B) Quantification of mitochondrial calcium after PXA treatment. (C) Basal calcium levels (no PXA treatment) were similar in the different cells.

treatments suppress the effects of PXA, thus strongly suggesting that PXA triggers calcium release by activating NCLX and that this activation requires Mfn2 (Fig. 9). Experiments in Aim 1 of this proposal are designed to test whether NCLX is directly targeted by PXA.

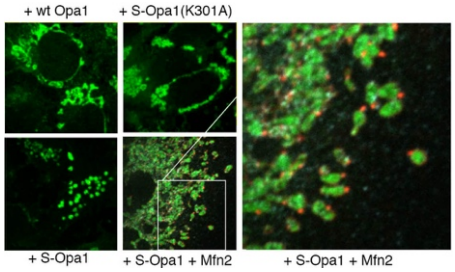


Fig. 10. Transfection of Drp1-Opa1 DKO cells with Opa1 variants. Overexpressed wt Opa1 or S-Opa1(K301A) induces constriction but matrix fragments remain connected by outer membrane (top left). Overexpression of S-Opa1 induces fragmentation (bottom left). Cotransfection of mycMfn2 and S-Opa1 shows Mfn2 redistribution into spots (red). S-Opa1 construct has a MICU targeting sequence.

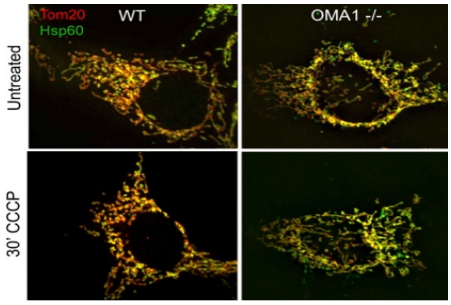


Fig. 11. Lack of CCCP-induced fission in Oma1$^{-/-}$ cells.

Effects of Opa1 on fission: Because S-Opa1 was implicated in fission and fusion processes, we tested whether overexpression of the different Opa1 cleavage products altered the patterns of overexpressed Mfn2. We did this with Drp1-Opa1 DKO cells so there would be no interference from endogenous Opa1. Overexpression of WT Opa1 in Drp1-Opa1 DKO cells gave rise to swollen mitochondria, but those mitochondria remained connected by thin tubules (Fig. 10). Similar effects were observed with S-Opa1(K301A), which has a point mutation in the GTP binding site. In contrast, overexpression of S-Opa1 gave rise to fragmented mitochondria. Co-transfected Mfn2 was in spots instead of coating thin tubes, suggesting that S-Opa1 rearranges Mfn2 into foci while inducing fragmentation. In conclusion, Mfn2 induces mitochondrial constriction in Drp1$^{-/-}$ cells, while S-Opa1 induces Drp1-independent fission and Mfn2 redistribution. These data reveal a novel form of fission.

It was previously shown that CCCP-induced fission requires Opa1 cleavage by Oma1[55]. We confirmed this with Oma1$^{-/-}$ MEFs generated with CRISPR/Cas9 (Fig. 11). CCCP-induced cleavage of Opa1 triggers Drp1-dependent fission, but does not induce constrictions or fission in Drp1$^{-/-}$ cells (Fig. 4), while overexpression of S-Opa1 along with but Mfn2 does cause fission and constriction (Fig. 10). We conclude that high levels of S-Opa1 can induce Drp1-independent fission, while the lower levels generated by Oma1 induce Drp1-dependent fission.

B. INNOVATION

Discovery of a role for Mfn2 and NCLX in mitochondrial fission. This unexpected function was made apparent with Drp1-independent fission, a phenomenon that has been observed by others but with no known mechanism (Table 2). Importantly, this novel function may also contribute to conventional Drp1-dependent fission, since we observe clusters of Mfn2 at sites of fission in wildtype cells. We propose that Mfn2 assemblies promote fission by localized activation of NCLX, which in turn constricts mitochondria by releasing calcium. This newly discovered function will be compared and contrasted with alternative explanations, such as effects of Mfn2 on ER tethering.

Table 2. Earlier observations of Drp1-independent mitochondrial constrictions and fission.

- Defects in the mitochondrial calcium-proton exchanger Letm1 promote Drp1-independent fission[56].
- Holes in the plasma membrane formed by Listeriolysin trigger Drp1-independent fission[57].
- Calcium oscillations induce localized constrictions in mitochondria without Drp1[14].
- Ionomycin induces Drp1-independent constrictions in mitochondria[58].
- Bit by bit mitophagy can remove small portions of mitochondria without Drp1[59].
- Mitochondria derived vesicles (MDVs) are formed without require Drp1[60].
- We observe Drp1-independent fission of mitochondria in *C. elegans* gonads (fig. 3).

Additional innovations include:

1. Identification of NCLX as the main target of the fungal toxin PXA. Our data strongly support the idea that PXA triggers calcium release from the mitochondrial matrix by activating NCLX (Fig. 9). This discovery opens up new avenues for studying calcium regulation in mitochondria.
2. Expression levels of Mfn2 control mitochondrial diameters. Overexpression of Mfn2 in Drp1$^{-/-}$ cells decreases mitochondrial diameters, while loss of Mfn2 in Mfn2-Drp1 DKO cells appears to cause an increase in diameter (Fig. 4, 5). These results are consistent with downstream effects of NCLX on matrix calcium, which controls swelling and crimping of mitochondria, as previously observed by others[14].
3. Mfn1 and Mfn2 act antagonistically in Drp1-independent mitochondrial fission. This antagonism suggest that Mfn1 sequesters a portion of Mfn2 in a heteromeric complex that does not contribute to fission. Effects of PXA on fusion have not been formally ruled out, but they seem unlikely because of the speed of PXA-induced fission in Drp1-Mfn1 DKO cells (within minutes) (Fig. 4, 6).
4. Ectopically expressed S-Opa1 induces the formation of Mfn2 clusters at fission sites. This result provides an explanation for CCCP-induced mitochondrial fission because that correlates with Oma1-mediated cleavage of Opa1 and it is blocked in Oma1$^{-/-}$ cells[55]. If this is the case, then inducible proteolytic cleavage of

endogenous L-Opa1 by Oma1 to form S-Opa1 could be used to translate metabolic parameters, such as ATP levels[61] and membrane potential, into triggers for mitochondrial fission.

5. Mfn2 clusters coincide with sites of mitochondrial fission prior to the formation of Drp1 clusters. This clustering is consistent with an active role in fission.

Together, these data suggest that NCLX promotes mitochondrial fission by inducing constrictions through calcium release. Clustering of Mfn2 at sites of fission may help localize calcium release, but an active role of Mfn2 in fission through dynamin-like spirals is also possible. The effects of S-Opa1 on Mfn2 and fission provide potential connections between Oma1-mediated stress sensing and fission.

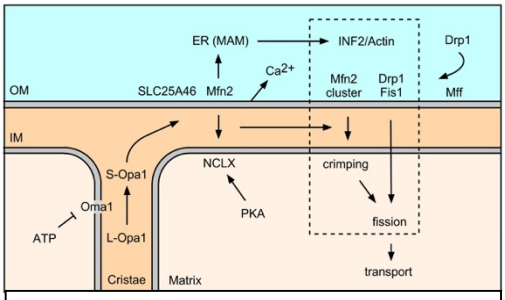


Fig. 12. Proposed connections between Mfn2, NCLX and mitochondrial fission.

Broad implications:

- Our data identify a novel pathway connecting mitochondrial fission with bioenergetics. It has been known for some time that rates of mitochondrial fission and fusion are influenced by the metabolic status of cells, for example through phosphorylation of Drp1 and Mff[62,63]. There is also evidence for the converse, namely control of mitochondrial bioenergetics by fission and fusion, but the mechanisms for that are unknown[63-65]. Connections between fission, Mfn2, and NCLX provide an avenue for this type of control (Fig. 12). Mfn2 regulation of NCLX-mediated calcium release can control the TCA cycle through matrix calcium concentrations. NCLX function is also governed by mitochondrial membrane potential and PKA[48,49], providing a feedback loop for this process.
- Role of Mfn2 in fission provides a new explanation for effects of CMT mutations. Effects of Mfn2 on fission can be important for understanding the disease mechanisms of CMT, because CMT mutations in Mfn2 affect mitochondrial transport[7,12] and transport proteins are connected with mitochondrial fission proteins[66,67].

Motivation for this collaboration and division of labor between the labs: This proposal draws on complementary strengths of the two labs: Organelle dynamics in the van der Bliek lab and biochemical analysis as well as work with zebrafish in the Koehler lab. This range of approaches will help tackle the complexity of Mfn2 functions. The two labs recently moved into renovated shared space, providing an excellent collaborative environment. The Multi-PI format strengthens this collaboration, accelerating the research and increasing the likelihood of success. Aims 1 and 2 have cell biological experiments, which will be conducted in the van der Bliek lab, and biochemical experiments, which will be conducted in the Koehler lab. Aim 3 has Zebrafish experiments, which will be conducted in the Koehler lab.

C. APPROACH

Aim 1. Investigate connections between PXA, NCLX, and Mfn2.

Hypothesis: We propose that PXA triggers calcium release from mitochondria by activating NCLX and that Mfn2 controls NCLX-mediated calcium release from mitochondria.

a. Is NCLX the target of PXA?

PXA-induced calcium release depends on NCLX: It was previously shown that PXA does not target the mitochondrial permeability transition pore mPTP or the mitochondrial calcium uniporter MICU[50]. NCLX has remained a viable candidate as target for PXA and this suspicion is further strengthened by our discovery of the suppressive effects of Mfn2 deletions on PXA-induced calcium release, consistent with a report of Mfn2-mediated control of NCLX[51]. Our preliminary data also show that NCLX siRNA and CGP37157 both suppress PXA-induced calcium release from mitochondria in WT and Drp1$^{-/-}$ cells. Effects of CGP37157 on cell surface calcium exchange proteins have been reported[68], and off target effects of siRNA remain possible. We will therefore confirm these observations with NCLX$^{-/-}$ cells and NCLX-Drp1 DKO cells, which will be generated from MEFs with CRISPR/Cas9 as a third line of evidence.

Effects of PXA on NCLX mediated calcium release will initially be assessed with Fura-2 for cytosolic calcium and Rhod-2 AM for matrix calcium, as in Fig. 9. Titrations with increasing concentrations of PXA will be used to determine the half-maximal dose. More rigorous analyses will include measurements of cytosolic, mitochondrial, and ER calcium levels, using organelle targeted Ca^{2+}-sensitive fluorescent proteins[50]. For this, we will generate stable transfectants with the ratiometric calcium imaging protein mito-GEM-GECO (Addgene)[69,70] or with single fluorescence using GCaMPf6 (Addgene) targeted to the matrix for increased sensitivity[71,72]. The kinetics of calcium release will then be studied in cells with NCLX siRNA or overexpression with NCLX constructs (Origene) with or without PKA-site mutations that constitutively activate or inactivate the protein (from the Sekler lab)[48,49]. We will use DMSO, ionomycin, and thapsigargin as negative and positive

controls and we will use treatments with CCCP and oligomycin or DNP and oligomycin to fully deplete mitochondrial calcium stores, thus establishing a plateau for maximal calcium release.

Possible indirect effects: It is conceivable that PXA reduces mitochondrial membrane potential independent of NCLX. This reduction in membrane potential acidifies the mitochondrial matrix, which in turn solubilizes $CaPO_4$ precipitates that could then be released by NCLX (calcium stores in the matrix mostly consist of these precipitates)[73]. This explanation for the effects of PXA seems unlikely because loss of membrane potential with CCCP does not have the same effects on calcium release and it has opposite effects on respiration. It, nevertheless, remains possible that the actual target of PXA is not NCLX. To test this, we will monitor effects of PXA on membrane potential in NCLX-deficient cells using the fluorescent dye TMRE. To this end, we will incubate NCLX-Drp1 DKO cells or $Drp1^{-/-}$ cells transfected with NCLX siRNA with PXA, TMRE, and Mitotracker as a reference and determine effects with quantitative fluorescence microscopy, as routinely used in the Shirihai lab on our floor. These experiments will determine whether there is an indirect effect of PXA on membrane potential or if effects of PXA fully depend on NCLX.

Potential for further enhancement of calcium release: Runaway activation of NCLX could also increase the pool of releasable calcium by lowering the pH of the mitochondrial matrix through ion exchange. NCLX increases the sodium concentration in the matrix through an exchange of 2 or 3 Na^+ per Ca^{2+}, while excess Na^+ will be released into the cytosol by the Na+/H+ exchanger NHE, thus lowering the pH and solubilizing calcium. To determine the contribution of this exchange to the total amount of calcium released by PXA, we will inhibit NHE, for example with BIX NHE1 inhibitor (Tocris), or reduce NHE expression with siRNA[74], followed by treatment with PXA and mitochondrial calcium measurements as described above.

Biochemical tests for PXA interactions with NCLX: The proteolytic protection assay DARTS[75,76] will initially be used to test whether NCLX is a target of PXA. As shown for other drug target interactions, we expect that NCLX will be stabilized in the presence of PXA and show increased protease resistance. A variety of proteases (pronase, trypsin, etc.) will first be tested with detergent-solubilized mitochondria (typically a 10–30 min time course of degradation at 4°C) and sensitivity will be verified with western blots. When a protease is identified that shows a reproducible degradation pattern, stability will be tested in the presence and absence of PXA to determine whether NCLX binds the drug directly.

Expected results and alternative approaches: Together, these experiments will help determine whether NCLX is a direct target of PXA and will undoubtedly be valuable for further studies of mitochondrial calcium-dependent processes. Classic biochemical approaches will be pursued as an alternative at a later stage. These approaches include affinity chromatography[77,78], after structure-activity relationship (SAR) studies to identify nonessential sites for attaching an affinity tags, followed by IP, gel electrophoresis, and western blots. Several related compounds have been synthesized[79]. Additional compounds will be synthesized by the UCLA Bioscience Synthetic Chemistry Core (Dr. Mike Jung). Alternatively, we will test effects of PXA on purified NCLX after reconstitution of fractions from beef heart mitochondria in proteoliposomes as described[80,81]. These proteoliposomes will be incubated with calcium and sodium dyes (Fura-2 and SBFI) to measure flux with PXA or CGP37157. Together, these experiments will help determine whether NCLX is a direct target of PXA.

b. How does Mfn2 control NCLX?

Genetic interactions: We previously showed numerous constrictions in mitochondria of cells that overexpress the GTPase defective mutant Mfn2(K109T) and were co-transfected with Drp1, Mff, or Fis1 siRNA to prevent fission[82]. These constrictions may have resulted from activation of NCLX, but if that is the case, then GTP hydrolysis by Mfn2 might not be necessary for this to occur. We will test this by replacing endogenous Mfn2 with Mfn2(K109T) in our existing Mfn2-Drp1 DKO cells and treat these cells with PXA or CGP37157 to determine whether constriction depends on calcium efflux.

Physical interactions: We will conduct coIP experiments with NCLX and Mfn2 antibodies and epitope-tagged constructs to test for physical interactions and possible changes in these interactions during fission of healthy mitochondria. If necessary, we will use cross-linkers and fission-inducing conditions, such as CCCP, to improve the signal and look for dynamic changes in associations between NCLX and Mfn2. These experiments will provide critical tests for the constriction properties of Mfn2 and its potential association with NCLX during fission. Commercial NCLX antibodies are unfortunately unsuitable for IP, but we have a Myc-tagged construct from the Sekler lab, as well as epitope-tagged of Mfn2 constructs. We will test whether interactions are promoted by fission inducing treatments (CCCP), with NCLX activation using PXA titrations or with NCLX phosphorylation (forskolin) or NCLX constructs with mutations in PKA phosphorylation sites[49].

Localization: Regardless of whether Mfn2 and NCLX directly bind to each other, it seems likely that these two proteins are in close proximity because of the regulatory interactions suggested by functional data.

We will first test for colocalization with fluorescence microscopy after staining for endogenous proteins with commercial antibodies and for epitope-tagged versions with or without inducing fission with CCCP. We will then test for displacement, following up on the preliminary data showing that S-Opa1 overexpression converts overexpressed Mfn2 from an even distribution along thin connected mitochondria in Drp1$^{-/-}$ cells into spots at the tips of fragmented mitochondria. We will determine whether the distribution of NCLX is also affected by S-Opa1. Similar overexpression experiments will also be used to determine whether Mfn2 and NCLX levels influence localization of either protein.

Expected results and alternative approaches: These experiments will further test the connections between NCLX and Mfn2, thus providing novel mechanistic insight into the regulation of NCLX. The coIP experiments will be complemented with blue native gel electrophoresis (BNGE), which we have used in the past to detect multimeric complexes on mitochondria[82]. Advanced studies of colocalization using proximity ligation assay (PLA) or FRET are unfortunately not feasible, because the epitopes for Mfn2 and NCLX are too far apart (opposite sides of inner and outer membranes). Instead, we will use immuno-EM through an ongoing collaboration with Dr. McCaffery (Johns Hopkins). Additional structure-function studies with Mfn1 and Mfn2 chimeras will provide support for the specificity of the interactions with NCLX. Alignment of Mfn1 and Mfn2 shows highly conserved segments, such as the GTPase domain, while other segments are more variable. We will determine the sequence requirements for constriction and fission by systematically swapping portions of Mfn1 and Mfn2. These chimeras could potentially also separate the fission and fusion functions of Mfn2, which would enable further in-depth analysis of these processes.

Aim 2. Investigate connections between Mfn2 and mitochondrial fission.

Hypothesis: We propose that Mfn2 collects at sites of mitochondrial fission, where it triggers calcium release through NCLX resulting in localized constriction of mitochondria. These constrictions may assist Drp1 assembly and mitochondrial fission. Alternative mechanisms: Mfn2 tethering of mitochondria to ER could passively contribute to fission by supporting the formation of INF2-induced actin filaments. If Mfn2 forms large spirals like other dynamins, then Mfn2 tethers could also guide ER around the mitochondrial circumference. An active role in fission is possible if Mfn2 spirals constrict through GTP hydrolysis. These mechanisms may complement the proposed role of Mfn2 in promoting localized constrictions through NCLX-calcium release.

a. Does Mfn2 actively contribute to fission?

Functional studies: To corroborate the observed effects of Mfn2 on PXA-induced constrictions in mitochondria of MEF cells, we will generate Drp1, Drp1-Mfn2, and Drp1-Mfn1 knockouts in human cell lines as well (HeLa and U2OS, because these are large flat cells, and SH-SY5Y cells as an example of neurons). CRISPR/Cas9 knockouts will be verified by sequencing and western blots. Effects on constriction will be quantified by classification of mitochondrial morphologies as in Fig. 4 and by measuring the widths of individual mitochondria using ImageJ. Adequate numbers for statistical analysis will be obtained with independent experiments. Mitochondrial inner membrane fission will be verified by discontinuities in diffusion detected with photo-activated PAGFP targeted the matrix. PAGFP will also be used to monitor fusion[83]. Drp1-Mfn1 DKO cells will be used to determine whether Mfn1 masks possible fission effects of Mfn2. Drp1-Mfn1-Mfn2 TKO MEFs[84] will serve as a control. Lastly, we will determine whether fusion defects in Mfn2$^{-/-}$ MEFs are suppressed by Mfn1 overexpression, as shown for neurons[34], while effects on fission might not be suppressed by Mfn1. These experiments will help determine whether Mfn2 affects mitochondrial constriction in cells other than MEFs.

Localization studies: Our preliminary data show assembly of Mfn2 in spots during the initial constriction phase, after which Drp1 also forms a spot and fission occurs. These phenomena will be studied more extensively to determine whether the same sequence of events always occurs during fission or is associated with specific kinds of fission. In addition, connections with other fission-associated proteins will be investigated, including Mff, Fis1, and ER proteins such as INF2, as well as nucleoids, which were proposed to segregate during mitochondrial fission[85]. These experiments will be done with live imaging of fluorescent proteins using a Zeiss Airyscan microscope in fast-mode. Care will be taken to express low levels of fluorescent proteins, since overexpression of Mfn2 causes artificial constriction. To this end, we will transfect low amounts of DNA or use a weak promoter (MMLV instead of CMV) and a bright GFP variant (mClover3) (Addgene)[86] or constructs with tandem GFP to increase the signal[87]. As an alternative approach, we will introduce a FLAG- or Myc-tag into the endogenous locus with CRISPR. We anticipate observing increased colocalization of Drp1 and Mfn2 during fission of healthy mitochondria. As a follow-up, we will test mutations that slow constriction of Drp1 spirals modeled after the ts1 mutation in Drosophila dynamin[88] or Q40E in human Dnm2[89]. Lastly, we will use super-resolution microscopy (STED, PALM, and SIM are all available on campus) to determine whether Mfn2 can be observed in rings or spirals, like dynamin, or is concentrated in spots on one side of the mitochondria.

Potential roles of INF2 and actin: INF2 and actin filaments constrict mitochondria and promote Drp1 recruitment prior to mitochondrial fission[53,90]. Preliminary experiments with INF2 siRNA and Latrunculin B did not inhibit PXA-induced constrictions (Fig. 8). More conclusive tests will be possible with INF2$^{-/-}$ cells, which we will generate with CRISPR/Cas9 as described for INF2 in U2OS cells[58]. These experiments will help determine with greater certainty whether PXA-induced constrictions depend on INF2 and actin.

Expected results and alternative approaches: These experiments will help determine whether Mfn2 actively contributes to mitochondrial fission or acts as a passive tether to the ER. In both cases, we will have uncovered a novel function for a mitochondrial fusion protein, but an active role in fission would imply a radical departure from prevailing views of Mfn2 functions and asks for further exploration of the underlying mechanisms. As an alternative, additional support for a role of Mfn2 in fission will be sought by testing potential synthetic effects of mild Drp1 and Mfn2 deficiencies (heterozygous mutations generated with CRISPR/Cas9[91] or partial loss of function (short transfections or low concentrations of siRNA monitored with western blots). Synthetic effects in *C. elegans* (see preliminary results) suggest that this is a viable approach. As an extension, we will also test effects of a series of FLAG-tagged Mfn2 constructs with CMT mutations (R400Q, R94Q, T105M, K109A, M376A mutations) obtained from the Dorn lab (WUSTL). If time permits, microscopy will be complemented with biochemical fractionations as described[92], to determine whether Mfn2 and NCLX are present in MAM fractions when fission is induced. Results will be compared with earlier experiments where it was shown that NCLX is separate from contact sites with sarcoplasmic reticulum in mitochondrial fractions from muscle cells[93]. Our experiments will further test whether NCLX association with the MAM is inducible.

b. Upstream regulatory mechanisms.

Protein modifications: It seems likely that interactions between Mfn2 and NCLX are controlled by physiological stimuli that induce fission, specifically when coupled with fluctuations in calcium concentrations. Changes in local calcium concentrations are critical regulators of numerous biological processes[94]. Several pathways trigger NCLX-mediated calcium release from mitochondria, including pathways activated by extracellular norepinephrine and ATP. These stimuli trigger the production of cytoplasmic IP3 and cAMP, which then activate calcium release from the ER and mitochondria, respectively[95]. PKA phosphorylates Mfn2 at Ser442[96] and NCLX at Ser258, triggering calcium release[49]. However, the effects of these modifications on interactions between Mfn2 and NCLX are unknown. Direct effects of cAMP on calcium release from mitochondria were observed in brown adipose tissue[97], where Mfn2 also plays a critical regulatory role[98]. These pathways activate NCLX by PKA phosphorylation[99], which additionally requires Mfn2[98] and mitochondrial membrane potential for calcium release[48]. We obtained phospho-mutant NCLX constructs and will generate mutant Mfn2 constructs. These mutants will be used to determine whether they affect constriction in Drp1$^{-/-}$ cells. As controls, we will test effects of the PKA inhibitors KT5720 and H89 on cells transfected with mutant or wildtype Mfn2 and NCLX constructs. Additional regulation of Mfn2 by other mechanisms, such as disulfide bond formation and glutathionylation at Cys684[100,101], will be investigated if time permits.

S-Opa1 and SLC25A46: These two proteins are potential regulators of Mfn2 and NCLX. Control of Mfn2 by S-Opa1 is suggested by preliminary data with overexpressed S-Opa1 changing Mfn2 from an even distribution along the surface of mitochondria into spots that may coincide with fission events (Fig. 10). This form of control is consistent with the effects of CCCP-induced cleavage of Opa1, because that also triggers fission (Fig. 11). Additional control is potentially mediated by SLC25A46, which is a mitochondrial outer membrane protein that was shown to coIP the Mitofusins and Opa1[102]. Mutations in SLC25A46 cause lethal congenital pontocerebellar hypoplasia[103], but they can also cause CMT or optic atrophy[104,105], similar to the effects of mutations in Mfn2 and Opa1. The yeast homolog of SLC25A46 is Ugo1, which can coIP yeast Opa1 and Mitofusin homologs[106], but Ugo1 affects fusion[107-109], while SLC25A46 may affect fission[103,104,110].

We will determine whether S-Opa1 and SLC25A46 interact with each other and with Mfn2 during mitochondrial fission using coIP with tagged SLC25A46, Mfn2, and S-Opa1, and DSP cross-linking[102]. We will compare and contrast conditions with or without fission with CCCP or with other triggers (low ATP caused by oligomycin and antimycin A), metabolic triggers (shift between glycolysis and respiration), or external triggers such as adding ATP (Aim 3a). L-Opa1 will be used as a negative control for S-Opa1. We will use an Opa1 construct lacking Oma1 and Yme1L cleavage sites (Opa1 Isoform-1 delta-S1)[29]. These experiments will be complemented with BNGE analysis of protein complexes that are formed by endogenous Mfn2, SLC25A46, and Opa1 proteins. Additionally, we will investigate effects on mitochondrial fission using fluorescence microscopy and monitor the distributions of GFP-Mfn2 in transfected cells. Together, these experiments will help determine whether S-Opa1 and SLC25A46 interactions help regulate Mfn2 during mitochondrial fission.

Expected results and alternative approaches: These experiments will help identify triggers for Mfn2- and NCLX-dependent functions as part of mitochondria fission. Alternative explanations for the observed effects of Mfn2 and NCLX will also be considered. The speed with which constrictions are induced by PXA in Drp1$^{-/-}$ cells and fragmentation is induced in Drp1-Mfn1 DKO cells suggest that fission is the predominant factor, but additional effects on fusion remain possible. Mitochondrial fission and fusion rates will be measured in live cells using photo-activated GFP[111]. Fluorescent proteins like Dendra and Kaede shift colors, which make fused compartments even more apparent because they have mixtures of colors. These are effective markers for mitochondrial fission and fusion events[112]. These proteins will be targeted to the mitochondrial matrix with a mitochondrial leader sequence. We will use our spinning disc confocal microscope, equipped with a UV laser and a point scanner for photo-activation of fluorescent proteins, to track mitochondrial fission and fusion in live cells. We will use this setup to measure fission and fusion rates.

We will also consider an alternative topology proposed for Mfn2 with only one membrane-spanning segment and the C-terminus in the intermembrane space [101]. This new topology disagrees with structures of mitofusins and yeast Fzo1[23,113] and a study showing that the C-terminus tethers mitochondria[22]. It is also unlike structures of other dynamins[23]. Although this topology is unlikely, it will be considered when interpreting our results. Lastly, we will assess a proposed role of the archetypal dynamin Dnm2 in mitochondrial fission[114]. Several labs, including our own, were unable to reproduce effects on fission[115-117], but this novel function could still be interesting because mutations in Dnm2 and Mfn2 both cause axonal CMT[118]. If time permits, we will test contributions of Dnm2 to fission with Dnm2-Drp1 DKO cells using our existing gRNAs for CISPR/Cas9[117].

Aim 3. Investigate the physiological consequences of Mfn2 and NCLX collaborative functions.

Hypothesis: We hypothesize that Mfn2 interactions with NCLX are important for mitochondrial transport. Effects of genetic interactions on transport will be studied with double mutants or partial knockdown with siRNA for one gene in a mutant background for the other gene. Enhancement of phenotypes is indicative of genetic interactions[119]. Interestingly, CMT mutations in Mfn2 and in GDAP1 (another mitochondrial outer membrane protein) are much more severe in patients with mutations in the two genes than in patients with mutations in either one of those genes alone, suggesting that these proteins act sequentially or in parallel to affect the same process[120-123]. We expect similar synergies if Mfn2 and NCLX collaboratively affect mitochondrial transport.

a. Effects of Mfn2 and NLCLX on mitochondrial fission and transport in processes of cultured neurons.

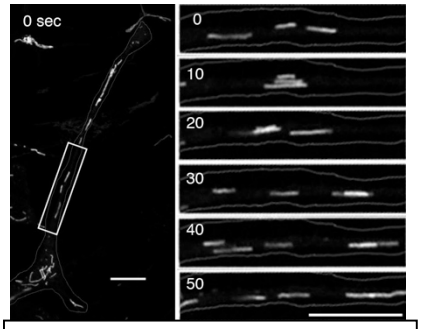


Fig. 13. Mitochondrial motility in processes of differentiated SH-SY5Y cells. Cells were grown in differentiation media and labeled with mCherry-GFP-Fis1. Bar, 10 µM.

Effects on transport: We will test the effects of Mfn2 and NLCLX on transport in neuronal processes of SH-SY5Y cells that are differentiated in serum-free neurobasal medium with B27 supplement, GlutaMAX, and 10 µM retinoic acid (Fig. 13). If necessary, neurite outgrowth will be further enhanced by transfecting PEA3 transcription factor (Addgene)[124]. We will first confirm that Mfn2 but not Mfn1 or Opa1 is required for transport, as previously described for Mfn2 deficiencies in neurons[7,12]. Knockdown with siRNA will be monitored with western blots, mitochondria will be labeled with mitoDsRed, and movement along neuronal processes will be tracked with time-lapse images using NIH ImageJ software to generate kymographs. We will then test interactions between Mfn2 and NCLX by transfecting siRNA for one gene into homozygous or heterozygous mutant cells for the other gene. Heterozygotes will be generated with adapted CRISPR/Cas9 protocols[91]. Expression after siRNA will be monitored with Western blots. At a later stage, we will generate double mutants (homo and heterozygotes) for Mfn2 and NCLX. Synthetic effects of heterozygous mutations in Mfn2 or NCLX with a mutation in the other gene will support the hypothesis that interactions between these genes lead to transport defects in CMT disease. We will then generate mutations in Mfn2 modeled after existing CMT mutations (for example, R400Q or R94Q), with protocols for homology directed recombination[125-127]. These experiments will be further expanded with drug studies, using NCLX inhibition with CGP37157 and activation with a low dose of PXA in an Mfn2 mutant background to test for potential enhancement or suppression of transport defects.

Effects on mitochondrial morphology and calcium efflux: Although this aim is focused on downstream effects such as axonal transport, we will also monitor potential effects on mitochondrial fission, fusion, and calcium efflux in SH-SY5Y cells because those are critical intermediary steps in preparation for transport. Mitochondrial fission and fusion will be monitored with time lapse images while making the kymographs described above. Numbers of fission and fusion events will be tallied in 10 or more processes and at least three independent experiments for statistical analysis. Parallel experiments to verify complete fission and

fusion will use Tomm20-GFP to detect outer membrane fission or photo-activated fluorescent proteins (PA-GFP or Kaede) to detect mixing of matrix content after fusion. Effects of Mfn2 and NCLX mutations on mitochondrial calcium release will also be monitored in neuronal processes using fluorescent dyes and proteins, as in Aim 1a. If there are too few fission events within timeframes that are achievable with live cell imaging, we will induce more fission and calcium release using physiological triggers such as extracellular ATP or norepinephrine, as in Aim 2c. These experiments will help correlate localized changes in mitochondrial physiology with transport, as described above.

Interactions with other proteins: To gain further insight into the mechanisms that couple Mfn2 with axonal transport, we will investigate interactions with other proteins such as Miro, which couples mitochondria to dynein and kinesins. Humans have two Miro homologs (Rhot1 and Rhot2), which are anchored in the mitochondrial outer membrane proteins, each with two cytosolic Rho-like GTPases and an EF hand domain that binds calcium. Mfn2 and Miro coIP when overexpressed in HEK293 cells[7]. We will determine whether Mfn2 and Miro also coIP with SH-SY5Y cells (with or without DSP cross-linker). Colocalization will be tested with IF and proximity ligation assay (Duolink by Sigma) using antibodies for endogenous proteins. We have ample experience with these techniques and commercial antibodies for Mfn2 and mammalian Miro.

Expected results and alternative approaches: These experiments will help determine whether Mfn2 connects fission of healthy mitochondria to axonal transport. Follow-up experiments will test for functional requirements using Miro constructs with mutations in the GTPases and calcium binding sites (Addgene) and potential interactions with fission proteins, such as Drp1, Fis1 and Mff. If time permits, we will investigate possible effects of Mfn2 and NCLX on interactions with calpastatin, which is a cytosolic inhibitor of calpain. Calpastatin was recently shown to be transported in axons through binding interactions with Mfn2, thereby providing yet another potential effect of CMT mutations in Mfn2 on neuronal survival[128].

b. Metabolism, apoptosis, and other functions potentially affected by Mfn2 and NCLX.

Metabolism: Results obtained with NCLX inhibition[129,130] and the putative NCLX activator PXA[50] suggest that this protein regulates oxidative phosphorylation through changes in matrix calcium or other parameters such as pH. Mitochondrial fission and fusion proteins also affect respiration, but how this might work was unclear[131]. Our new data suggest that mitochondrial dynamics controls respiration through the regulation of NCLX by Mfn2. To test this, we will conduct respirometry using differentiated SH-SY5Y cells (mutant and siRNA, subaim 1a). Oxygen consumption rates (OCR) will be measured with a Seahorse XF96 Extracellular Flux Analyzer, using cells grown in microplates with assay media added on the day of the experiment as described by the manufacturer (Agilent Technologies). Tracings will be made with additions of oligomycin, FCCP, and rotenone/antimycin A. Extracellular acidification rates (ECAR) will be determined under glycolytic conditions with sequential addition of glucose, oligomycin, and 2-deoxyglucose. To normalize activities per well, we will measure cell numbers at the end of assays by fixing the cells with 4% paraformaldehyde, staining with Hoechst, and counting nuclei with an Operetta High-Content Imaging System (PerkinElmer).

Apoptosis: Different effects of Mfn2 on apoptosis have been reported[132-134] and there are connections with apoptotic proteins during mitochondrial fusion[52,135]. Activation of NCLX also triggers apoptosis, as shown with PXA-induced apoptosis[50,136]. It is therefore possible that NCLX and Mfn2 affect apoptosis in ways that could be relevant for CMT. Indeed, Mfn2 was previously shown to be instrumental for glutamate-induced excitotoxicity in neurons[137], while the NCLX inhibitor CGP37157 protects neurons from excitotoxicity[138]. We will test this by inducing apoptosis in differentiated SH-SY5Y cells (mutant and siRNA, see subaim 1a), using glutamate[137,138], staurosporine and actinomycin D[29,82,139]. Effects on apoptosis will be determined with fluorescence assays (cytochrome c release from mitochondria, formation of pycnotic nuclei) and biochemical markers (cleavage of PARP and caspase 9). Release of cytochrome c from mitochondria will be detected by immunofluorescence. Adding the pan-caspase inhibitor z-VAD-fmk helps obtain reliable counts of cells with cytochrome c release by inhibiting further progression of apoptosis, which prevents detachment from the substrate[82]. Apoptosis will also be quantified with the numbers of pycnotic nuclei (Hoechst staining), PARP cleavage, and caspase 9 cleavage[29,139]. These experiments will allow us to determine to what extent the functional interactions between Mfn2- and NCLX-interacting proteins affect apoptosis.

Expected results and alternative approaches: There is ample evidence for effects of Mfn2 and NCLX on metabolism and apoptosis. Experiments in this subaim will expand on this by determining potential effects in neurons. Possible effects on mitophagy are less likely because mitophagy does not normally involve Mfn2 (Fig. 1), but if necessary this will be tested with our MEFs and SH-SY5Y cells. Mitophagy will be induced with CCCP or oxidative damage with rotenone or H_2O_2. Induction, progression, and rates of autophagy will be monitored with western blot analysis of LC3-lipidation, optineurin, and p62 turnover, turnover of target proteins, and RFP-

GFP-LC3 fusions to monitor progression of autophagy and bafilomycin to inhibit lysosome fusion in order to distinguish LC3 accumulation due to increased flux from inhibition at a late stage of autophagy[140-142].

c. Relevance for transport in zebrafish neurons.

Overview: Zebrafish will serve as a vertebrate model for physiological effects, focusing on the question whether Mfn2 control NCLX affects axonal transport. These experiments will expand on studies showing that axonal transport in zebrafish is affected by Mfn2 morpholinos, mRNA injections and mutations in the Mfn2 gene[11,12,143]. There are no studies yet of NCLX effects on axonal transport, but our collaborator at UCLA, Dr. Jau-Nian Chen (see letter), has unpublished data showing that NCLX morpholinos cause embryonic cardiac arrhythmia. Her lab also generated fish with an NCLX gene deletion. These fish survive but manifest various cardiac defects. Different combinations of morpholinos and fish mutants will allow us to study potential genetic interactions between Mfn2 and NCLX and their effects on axonal transport.

Morpholinos and fish strains: Genetic interactions will be tested by crossing Mfn2 and NCLX mutant fish lines to generate transheterozygotes and by injecting single mutant lines with morpholinos or mRNAs for the opposing gene. The Koehler lab has extensive experience with zebrafish[103,144-146] and materials for these experiments, including stable lines with mitochondrial matrix GFP in neurons (Fig. 14). Neuronal promoters (enolase or gata2)[147] will be used for additional constructs, such as a mitochondrial outer membrane marker. Mfn2 mutant fish were available[11] and our collaborator at UCLA, Dr. Chen, will provide NLCX morpholinos and knockout fish (see letter). Additional experiments will use mRNA injections for transient expression of Mfn2 with CMT mutations (L76P and R94Q) that are dominant and thus affect mitochondrial transport[12]. If time permits, we will test genetic interactions with Opa1 and SLC25A46. Mutations in zebrafish SLC25A46 or Opa1 genes have not yet been described, but morpholinos for these genes are effective: SLC25A46 morpholinos lead to elongated mitochondria, as expected for a fission defect[104], while Opa1 morpholinos lead to fragmented mitochondria[148]. We are currently generating SLC25A46 knockouts with CRISPR/Cas9.

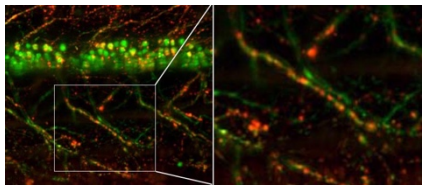


Fig. 14. Light sheet image of Zebrafish motor-neurons labeled with cytosolic GFP and mitochondrial DsRed. Mitochondria are detected in individual axons.

Axonal transport and calcium: We will study axonal transport in single and double mutants for Mfn2 or NCLX, or alternatively with morpholinos for one gene injected in mutants for the other gene. Kymographs of mitochondria in motor neurons of the spinal cord will be made as described[12,149]. Briefly, larvae at 48 h post fertilization will be anesthetized with 0.02% Tricaine and transferred to droplets of agarose on coverslips in MakTek dishes. Images will be collected with a spinning disk confocal microscope at 100 msec intervals and analyzed with ImageJ software to generate kymographs. If necessary, this approach will be adapted for the light-sheet microscope. Calcium imaging in live animals is routinely done in the lab of our collaborator at UCLA Dr. Chen, using matrix GCaMP and vital dyes, such as Fura-2[150]. If necessary, we will use other genetically encoded calcium indicators, such as matrix pericam as used by the Chen lab in zebrafish[150-152]. Lastly, we will challenge mitochondria by inducing fragmentation with CCCP as also used in zebrafish[153] and we will challenge them with chemicals that affect calcium release in neurons. It is not yet clear whether PXA or CGP37157 are effective in fish embryos, but other calcium reagents, such as thapsigargin, have worked before[154].

Expected results and alternative approaches: Zebrafish will help investigate genetic interactions between Mfn2 and NCLX in the neurons of an intact organism. Importantly, this will help decide which of the different processes involving Mfn2 are responsible for CMT. Some evidence suggests that mutations in Mfn2 cause CMT disease by disrupting mitochondrial fusion[34], but there is a larger body of literature suggesting that disruptions in axonal transport are responsible[8,11,12,155,156]. These disruptions are likely to occur when mitochondria connect with microtubule motor proteins during mitochondrial fission. Mfn2 can affect fission by controlling NCLX as proposed here or alternatively by tethering to ER, which then promotes mitochondrial constrictions through INF2. Although mutations in INF2 also cause CMT, they do this by demyelination instead of axon degeneration[53 157 158]. We will, nevertheless, consider Mfn2 tethering to the ER and the downstream consequences of this connection, as an alternative explanation for disruptions in axonal transport. We can test this with morpholinos for INF2 and drugs affecting calcium release from ER. Zebrafish will thus provide critical functional tests for the effects of CMT mutations. Eventually, the need may arise for testing newly discovered functions of Mfn2 and NCLX in mice. We will develop tools for this as the project progresses.

Prospects: This proposal addresses fundamental questions about the mechanistic relationships between calcium release, fission and transport. These new insights will help understand the underlying causes of CMT disease. The heterozygous cell lines and methods of analysis that will be developed here can be used in the long term to screen for therapeutic drugs.

PHS Human Subjects and Clinical Trials Information

OMB Number: 0925-0001 and 0925-0002
Expiration Date: 03/31/2020

Are Human Subjects Involved	❍ Yes ● No
Is the Project Exempt from Federal regulations?	❍ Yes ❍ No
Exemption Number	❑ 1 ❑ 2 ❑ 3 ❑ 4 ❑ 5 ❑ 6 ❑ 7 ❑ 8
Does the proposed research involve human specimens and/or data	❍ Yes ● No
Other Requested information	

Vertebrate Animals

Our Institution has the full accreditation from the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) International. The vertebrate animal protocol for use in this investigation is pending until this application is approved by our Institutional Animal Care and Use Committee (IACUC). However, the general protocols with zebrafish have been approved by our IACUC.

1. **Description of Procedures—** The provision for the care, use, and treatment of animals found in the NIH "Guide for the Care and Use of Laboratory Animals" will be followed. The proposed research will use strains of zebrafish derived from the A/B line. Age will range from embryos through 1.2 years of life. Both male and females will be used. Each female can produce approximately 50–100 eggs per spawn. Approximately 50 mating pairs of A/B fish will be available as a stock. All studies utilizing embryos will be terminated during the embryonic (0–3 days post-fertilization) and larval life-stages (3–5 days post-fertilization). We may also use animals through the early life-stage (up to 5 days). Crispr/Cas9 technology will be used to knock-out genes and transgenes will be inserted using the TOL2 system. Standard published protocols will be used.

2. **Justification of the use of animals—**Because we are interested in studying mitochondrial dynamics in vertebrates, zebrafish is the lowest vertebrate model that is amenable to imaging and studying organ development, such as the muscle, heart, and neurons. The signal transduction mechanisms, anatomy, and physiology of zebrafish are homologous to those of humans. Zebrafish possess all the classical sense modalities, including vision, olfaction, taste, touch, balance, and hearing. Zebrafish studies provide a quick and inexpensive way to test hypotheses and generate strategies for complementary integrative research with rodent models and with humans. Because fertilization is external, organ development can be watched easily. In contrast, it is more difficult in mice, because organ development occurs *in utero*. In addition, cardiac function is not needed until day 5 in zebrafish, because oxygen is supplied to tissues by exchange with the surrounding media. Therefore, the heart can develop completely until it is needed after day 5 and the embryo typically survives. It is easier to generate many genetic models with fish embryos than mice because embryos can be injected at the one-cell stage and the germline can be transformed. Finally, housing and care of zebrafish is less expensive than housing a mouse colony.

3. **Veterinary care—**Veterinary care is provided as needed and fish are housed in a central core facility. The fish are inspected by veterinarians on a monthly basis. We inspect the tanks twice daily for sick and dead fish. If we see signs of sickness, the fish are promptly euthanized with a high dose of pharmaceutical grade anesthesia (tricaine). The PI, Dr. Carla Koehler, has spent a year sabbatical in the laboratory of Dr. Nuesslein-Volhard, who established zebrafish as a research model, and has extensive experience with the handling and care of zebrafish. Any fish that are introduced into the colony are brought in as embryos that have been bleached; a standard protocol that is used by all fish labs to prevent the spread of contamination and disease. In addition, there is quarantine system in which adult fish that are provided from outside labs can be raised. Should there be signs of infection/disease, the staff will contact veterinary services immediately.

4. **Provisions to minimize discomfort, distress, pain, and injury—**It is not anticipated that the zebrafish will experience discomfort, distress, pain, or injury from these studies. Zebrafish will be monitored on a daily basis to prevent any potential adverse outcomes. Fish that are in pain are euthanized with a lethal dose of pharmacologic-grade anesthesia, tricaine.

5. **Euthanasia—**Fish are euthanized with a lethal dose of pharmacologic-grade anesthesia, tricaine. This method is consistent with the recommendation of the Panel on Euthanasia of the American Veterinary Medical Association and has been approved by the UCLA Animal Research Committee.

Project Leadership Plan

Drs. van der Bliek and Koehler have been collaborating formally and informally since 2000, when Carla joined UCLA. Previously we co-authored a “Highlight” article (van der Bliek, A. M., and C. M. Koehler 2003. A mitochondrial rhomboid protease. Dev. Cell. 4:769-770). As our research directions are merged, we are now collaborating closely to investigate mitochondrial quality control pathways. We are currently co-mentoring a graduate student and our students and post-docs are working closely together. Two years ago, we moved to shared lab space as part of the larger “Mitochondrial and Metabolism Theme.” This has been very beneficial, fostering collaborations between our labs and with other labs with similar interests on our floor, including interactions with other mitochondrial colleagues, Orian Shirihai, Marc Liesa, and Ajit Divakaruni.

As our research areas have merged, we are now collaborating closely and have a joint manuscript that is being submitted shortly and a second one that is being completed. This grant application has grown out of this collaboration. Importantly, our labs have complementing areas of expertise. Dr. Koehler is an expert in mitochondrial protein and RNA import and biochemical methodology with model systems of yeast, zebrafish, and cultured cells. Dr.van der Bliek is an is an expert in mitochondrial dynamics and quality control pathways and cell biology/microscopy approaches with model systems of cultured cells and worms. Drs. Koehler and van der Bliek share reagents and generate cell lines for both labs.

In this project, van der Bliek has the expertise for the studies in cell models, including microscopy techniques; and Koehler has the expertise in biochemical methods including co-immunoprecipitation assays. Koehler and van der Bliek are using new technologies and tools to develop new approaches in studying mitochondrial stress pathways. Therefore, our active, long-term collaboration represents a perfect example of groups with different strengths joining resources to work together to advance a new area, using different experimental systems and a broad range of approaches. It also creates an exciting, interdisciplinary environment in which to train students and post-docs. Our close collaboration has stimulated a weekly Mitochondrial Group Meeting with presentations by our trainees; this meeting is attended by nine PIs and their labs.

In this project, both van der Bliek and Koehler will provide oversight of the entire program and development and implementation of all policies, procedures, and processes. In these roles, Drs. van der Bliek and Koehler are responsible for the implementation of the Scientific Agenda, the Leadership Plan, and the specific aims and ensure that systems are in place to guarantee institutional compliance with US laws, DHHS, and NIH policies including biosafety, human and animal research, data, and facilities. Specifically, van der Bliek and Koehler will oversee all aims. Koehler will be responsible for all animal research approvals. van der Bliek will serve as the contact PI and will assume fiscal and administrative management duties, including maintaining communication among Koehler and lab personnel through weekly meetings and daily interactions. van der Bliek will be responsible for communication with NIH and submission of annual reports. The budget of this grant will be shared based on contributions of van der Bliek and Koehler; as this project grows out of van der Bliek’s research, he will use ~65% of the budget and Koehler will use ~35% of the budget. Publication authorship will be based on the relative scientific contributions of the PIs and laboratory personnel. Finally, as Koehler and van der Bliek are excited to share a joint lab, our daily interactions have definitely helped stimulate the research development.

In the rare event of a conflict over administrative or scientific issues of the project, Professors Orian Shirihai (Professor in the Department of Medicine, UCLA), Sabeeha Merchant (Professor in the Department of Biochemistry, Biophysics and Structural Biology, UC Berkeley) and Jau Nian Chen (Professor in the Department of Molelular Cellular and Developmental Biology, UCLA) will meet together with the PIs to resolve the conflict. Dr. Shirihai is an expert in metabolism and mitochondrial dynamics. Dr. Merchant is an expert in biochemical analysis of subcecullar organelles. Dr. Chen is an expert in cellular analyses of calcium handling and muscle function, primarily working with zebrafish. Dr. Shirihai who has known both PIs professionally for some time will be the primary arbitrator.

References:

1. Pareyson, D. & C. Marchesi (2009), *Diagnosis, natural history, and management of Charcot-Marie-Tooth disease.* Lancet Neurol 8, 654-67.
2. Stuppia, G., F. Rizzo, G. Riboldi, R. Del Bo, M. Nizzardo, C. Simone, G.P. Comi, N. Bresolin & S. Corti (2015), *MFN2-related neuropathies: Clinical features, molecular pathogenesis and therapeutic perspectives.* J Neurol Sci 356, 7-18.
3. Chandhok, G., M. Lazarou & B. Neumann (2017), *Structure, function, and regulation of mitofusin-2 in health and disease.* Biol Rev Camb Philos Soc.
4. Chen, H., A. Chomyn & D.C. Chan (2005), *Disruption of fusion results in mitochondrial heterogeneity and dysfunction.* J Biol Chem 280, 26185-92.
5. de Brito, O.M. & L. Scorrano (2008), *Mitofusin 2 tethers endoplasmic reticulum to mitochondria.* Nature 456, 605-10.
6. Karbowski, M., Y.J. Lee, B. Gaume, S.Y. Jeong, S. Frank, A. Nechushtan, A. Santel, M. Fuller, C.L. Smith & R.J. Youle (2002), *Spatial and temporal association of Bax with mitochondrial fission sites, Drp1, and Mfn2 during apoptosis.* J Cell Biol 159, 931-8. PMC2173996
7. Misko, A., S. Jiang, I. Wegorzewska, J. Milbrandt & R.H. Baloh (2010), *Mitofusin 2 is necessary for transport of axonal mitochondria and interacts with the Miro/Milton complex.* The Journal of neuroscience : the official journal of the Society for Neuroscience 30, 4232-40. 2852190
8. Misko, A.L., Y. Sasaki, E. Tuck, J. Milbrandt & R.H. Baloh (2012), *Mitofusin2 mutations disrupt axonal mitochondrial positioning and promote axon degeneration.* The Journal of neuroscience : the official journal of the Society for Neuroscience 32, 4145-55. 3319368
9. Pareyson, D., P. Saveri & C. Pisciotta (2017), *New developments in Charcot-Marie-Tooth neuropathy and related diseases.* Curr Opin Neurol 30, 471-480.
10. Timmerman, V., A.V. Strickland & S. Zuchner (2014), *Genetics of Charcot-Marie-Tooth (CMT) Disease within the Frame of the Human Genome Project Success.* Genes (Basel) 5, 13-32. PMC3978509
11. Chapman, A.L., E.J. Bennett, T.M. Ramesh, K.J. De Vos & A.J. Grierson (2013), *Axonal Transport Defects in a Mitofusin 2 Loss of Function Model of Charcot-Marie-Tooth Disease in Zebrafish.* PLoS One 8, e67276. PMC3694133
12. Bergamin, G., D. Cieri, G. Vazza, F. Argenton & M.L. Mostacciuolo (2016), *Zebrafish Tg(hb9:MTS-Kaede): a new in vivo tool for studying the axonal movement of mitochondria.* Biochim Biophys Acta 1860, 1247-55.
13. Bernard-Marissal, N., G. van Hameren, M. Juneja, C. Pellegrino, L. Louhivuori, L. Bartesaghi, C. Rochat, O. El Mansour, J.J. Medard, M. Croisier, C. Maclachlan, O. Poirot, P. Uhlen, V. Timmerman, N. Tricaud, B.L. Schneider & R. Chrast (2019), *Altered interplay between endoplasmic reticulum and mitochondria in Charcot-Marie-Tooth type 2A neuropathy.* Proc Natl Acad Sci U S A 116, 2328-2337. PMC6369737
14. Cho, B., H.M. Cho, Y. Jo, H.D. Kim, M. Song, C. Moon, H. Kim, K. Kim, H. Sesaki, I.J. Rhyu, H. Kim & W. Sun (2017), *Constriction of the mitochondrial inner compartment is a priming event for mitochondrial division.* Nat Commun 8, 15754. PMC5472732
15. Twig, G., A. Elorza, A.J. Molina, H. Mohamed, J.D. Wikstrom, G. Walzer, L. Stiles, S.E. Haigh, S. Katz, G. Las, J. Alroy, M. Wu, B.F. Py, J. Yuan, J.T. Deeney, B.E. Corkey & O.S. Shirihai (2008), *Fission and selective fusion govern mitochondrial segregation and elimination by autophagy.* EMBO J 27, 433-46. PMC2234339
16. Youle, R.J. & A.M. van der Bliek (2012), *Mitochondrial fission, fusion, and stress.* Science 337, 1062-5.
17. van der Bliek, A.M., Q. Shen & S. Kawajiri (2013), *Mechanisms of mitochondrial fission and fusion.* Cold Spring Harb Perspect Biol 5. PMC3660830
18. Chan, D.C. (2012), *Fusion and fission: interlinked processes critical for mitochondrial health.* Annu Rev Genet 46, 265-87.
19. Schmid, S.L. & V.A. Frolov (2011), *Dynamin: functional design of a membrane fission catalyst.* Annu Rev Cell Dev Biol 27, 79-105.

20. Ferguson, S.M. & P. De Camilli (2012), *Dynamin, a membrane-remodelling GTPase.* Nat Rev Mol Cell Biol 13, 75-88. PMC3519936
21. Antonny, B., C. Burd, P. De Camilli, E. Chen, O. Daumke, K. Faelber, M. Ford, V.A. Frolov, A. Frost, J.E. Hinshaw, T. Kirchhausen, M.M. Kozlov, M. Lenz, H.H. Low, H. McMahon, C. Merrifield, T.D. Pollard, P.J. Robinson, A. Roux & S. Schmid (2016), *Membrane fission by dynamin: what we know and what we need to know.* EMBO J 35, 2270-2284. PMC5090216
22. Koshiba, T., S.A. Detmer, J.T. Kaiser, H. Chen, J.M. McCaffery & D.C. Chan (2004), *Structural basis of mitochondrial tethering by mitofusin complexes.* Science 305, 858-62.
23. Cao, Y.L., S. Meng, Y. Chen, J.X. Feng, D.D. Gu, B. Yu, Y.J. Li, J.Y. Yang, S. Liao, D.C. Chan & S. Gao (2017), *MFN1 structures reveal nucleotide-triggered dimerization critical for mitochondrial fusion.* Nature 542, 372-376. PMC5319402
24. Brandt, T., L. Cavellini, W. Kuhlbrandt & M.M. Cohen (2016), *A mitofusin-dependent docking ring complex triggers mitochondrial fusion in vitro.* Elife 5. PMC4929004
25. Ford, M.G., S. Jenni & J. Nunnari (2011), *The crystal structure of dynamin.* Nature 477, 561-6. PMC4075756
26. Chappie, J.S., S. Acharya, M. Leonard, S.L. Schmid & F. Dyda (2010), *G domain dimerization controls dynamin's assembly-stimulated GTPase activity.* Nature 465, 435-40. 2879890
27. Gao, S., A. von der Malsburg, S. Paeschke, J. Behlke, O. Haller, G. Kochs & O. Daumke (2010), *Structural basis of oligomerization in the stalk region of dynamin-like MxA.* Nature 465, 502-6.
28. Song, Z., H. Chen, M. Fiket, C. Alexander & D.C. Chan (2007), *OPA1 processing controls mitochondrial fusion and is regulated by mRNA splicing, membrane potential, and Yme1L.* J Cell Biol 178, 749-55.
29. Griparic, L., T. Kanazawa & A.M. van der Bliek (2007), *Regulation of the mitochondrial dynamin-like protein Opa1 by proteolytic cleavage.* J Cell Biol 178, 757-64. PMC2064541
30. Ehses, S., I. Raschke, G. Mancuso, A. Bernacchia, S. Geimer, D. Tondera, J.C. Martinou, B. Westermann, E.I. Rugarli & T. Langer (2009), *Regulation of OPA1 processing and mitochondrial fusion by m-AAA protease isoenzymes and OMA1.* J Cell Biol 187, 1023-36. PMC2806285
31. Head, B., L. Griparic, M. Amiri, S. Gandre-Babbe & A.M. van der Bliek (2009), *Inducible proteolytic inactivation of OPA1 mediated by the OMA1 protease in mammalian cells.* J Cell Biol 187, 959-66. PMC2806274
32. Chang, C.R. & C. Blackstone (2010), *Dynamic regulation of mitochondrial fission through modification of the dynamin-related protein Drp1.* Ann N Y Acad Sci 1201, 34-9. PMC5585781
33. Chen, H. & D.C. Chan (2010), *Physiological functions of mitochondrial fusion.* Ann N Y Acad Sci 1201, 21-5.
34. Zhou, Y., S. Carmona, A. Muhammad, S. Bell, J. Landeros, M. Vazquez, R. Ho, A. Franco, B. Lu, G.W. Dorn, 2nd, S. Wang, C.M. Lutz & R.H. Baloh (2019), *Restoring mitofusin balance prevents axonal degeneration in a Charcot-Marie-Tooth type 2A model.* J Clin Invest 130, online, no page numbers yet.
35. Chen, H., S.A. Detmer, A.J. Ewald, E.E. Griffin, S.E. Fraser & D.C. Chan (2003), *Mitofusins Mfn1 and Mfn2 coordinately regulate mitochondrial fusion and are essential for embryonic development.* J Cell Biol 160, 189-200. PMC2172648
36. Nasrallah, C.M. & T.L. Horvath (2014), *Mitochondrial dynamics in the central regulation of metabolism.* Nat Rev Endocrinol 10, 650-8.
37. Huang, P., C.A. Galloway & Y. Yoon (2011), *Control of mitochondrial morphology through differential interactions of mitochondrial fusion and fission proteins.* PLoS One 6, e20655. PMC3103587
38. Tondera, D., S. Grandemange, A. Jourdain, M. Karbowski, Y. Mattenberger, S. Herzig, S. Da Cruz, P. Clerc, I. Raschke, C. Merkwirth, S. Ehses, F. Krause, D.C. Chan, C. Alexander, C. Bauer, R. Youle, T. Langer & J.C. Martinou (2009), *SLP-2 is required for stress-induced mitochondrial hyperfusion.* EMBO J 28, 1589-600. PMC2693158
39. Chen, H., J.M. McCaffery & D.C. Chan (2007), *Mitochondrial fusion protects against neurodegeneration in the cerebellum.* Cell 130, 548-62.

40. Pham, A.H., S. Meng, Q.N. Chu & D.C. Chan (2012), *Loss of Mfn2 results in progressive, retrograde degeneration of dopaminergic neurons in the nigrostriatal circuit.* Hum Mol Genet 21, 4817-26. PMC3607482
41. Herlan, M., C. Bornhovd, K. Hell, W. Neupert & A.S. Reichert (2004), *Alternative topogenesis of Mgm1 and mitochondrial morphology depend on ATP and a functional import motor.* J Cell Biol 165, 167-73.
42. Sesaki, H., S.M. Southard, A.E. Hobbs & R.E. Jensen (2003), *Cells lacking Pcp1p/Ugo2p, a rhomboid-like protease required for Mgm1p processing, lose mtDNA and mitochondrial structure in a Dnm1p-dependent manner, but remain competent for mitochondrial fusion.* Biochem Biophys Res Commun 308, 276-83.
43. Zick, M., S. Duvezin-Caubet, A. Schafer, F. Vogel, W. Neupert & A.S. Reichert (2009), *Distinct roles of the two isoforms of the dynamin-like GTPase Mgm1 in mitochondrial fusion.* FEBS Lett 583, 2237-43.
44. Frezza, C., S. Cipolat, O. Martins de Brito, M. Micaroni, G.V. Beznoussenko, T. Rudka, D. Bartoli, R.S. Polishuck, N.N. Danial, B. De Strooper & L. Scorrano (2006), *OPA1 Controls Apoptotic Cristae Remodeling Independently from Mitochondrial Fusion.* Cell 126, 177-89.
45. Mishra, P., V. Carelli, G. Manfredi & D.C. Chan (2014), *Proteolytic cleavage of Opa1 stimulates mitochondrial inner membrane fusion and couples fusion to oxidative phosphorylation.* Cell Metab 19, 630-41. PMC4018240
46. Anand, R., T. Wai, M.J. Baker, N. Kladt, A.C. Schauss, E. Rugarli & T. Langer (2014), *The i-AAA protease YME1L and OMA1 cleave OPA1 to balance mitochondrial fusion and fission.* J Cell Biol 204, 919-29. PMC3998800
47. Palty, R., W.F. Silverman, M. Hershfinkel, T. Caporale, S.L. Sensi, J. Parnis, C. Nolte, D. Fishman, V. Shoshan-Barmatz, S. Herrmann, D. Khananshvili & I. Sekler (2010), *NCLX is an essential component of mitochondrial Na+/Ca2+ exchange.* Proc Natl Acad Sci U S A 107, 436-41. PMC2806722
48. Kostic, M., T. Katoshevski & I. Sekler (2018), *Allosteric Regulation of NCLX by Mitochondrial Membrane Potential Links the Metabolic State and Ca(2+) Signaling in Mitochondria.* Cell Rep 25, 3465-3475 e4.
49. Kostic, M., M.H. Ludtmann, H. Bading, M. Hershfinkel, E. Steer, C.T. Chu, A.Y. Abramov & I. Sekler (2015), *PKA Phosphorylation of NCLX Reverses Mitochondrial Calcium Overload and Depolarization, Promoting Survival of PINK1-Deficient Dopaminergic Neurons.* Cell Rep 13, 376-86. PMC4709126
50. Bohler, P., F. Stuhldreier, R. Anand, A.K. Kondadi, D. Schlutermann, N. Berleth, J. Deitersen, N. Wallot-Hieke, W. Wu, M. Frank, H. Niemann, E. Wesbuer, A. Barbian, T. Luyten, J.B. Parys, S. Weidtkamp-Peters, A. Borchardt, A.S. Reichert, A. Pena-Blanco, A.J. Garcia-Saez, S. Itskanov, A.M. van der Bliek, P. Proksch, S. Wesselborg & B. Stork (2018), *The mycotoxin phomoxanthone A disturbs the form and function of the inner mitochondrial membrane.* Cell Death Dis 9, 286. PMC5833434
51. Samanta, K., G.R. Mirams & A.B. Parekh (2018), *Sequential forward and reverse transport of the Na(+) Ca(2+) exchanger generates Ca(2+) oscillations within mitochondria.* Nat Commun 9, 156. PMC5765001
52. Hoppins, S., F. Edlich, M.M. Cleland, S. Banerjee, J.M. McCaffery, R.J. Youle & J. Nunnari (2011), *The soluble form of Bax regulates mitochondrial fusion via MFN2 homotypic complexes.* Mol Cell 41, 150-60. PMC3072068
53. Korobova, F., V. Ramabhadran & H.N. Higgs (2013), *An actin-dependent step in mitochondrial fission mediated by the ER-associated formin INF2.* Science 339, 464-7. PMC3843506
54. Friedman, J.R., L.L. Lackner, M. West, J.R. DiBenedetto, J. Nunnari & G.K. Voeltz (2011), *ER tubules mark sites of mitochondrial division.* Science 334, 358-62. 3366560
55. Quiros, P.M., A.J. Ramsay, D. Sala, E. Fernandez-Vizarra, F. Rodriguez, J.R. Peinado, M.S. Fernandez-Garcia, J.A. Vega, J.A. Enriquez, A. Zorzano & C. Lopez-Otin (2012), *Loss of mitochondrial protease OMA1 alters processing of the GTPase OPA1 and causes obesity and defective thermogenesis in mice.* The EMBO journal 31, 2117-33. 3343468
56. Dimmer, K.S., F. Navoni, A. Casarin, E. Trevisson, S. Endele, A. Winterpacht, L. Salviati & L. Scorrano (2008), *LETM1, deleted in Wolf-Hirschhorn syndrome is required for normal mitochondrial morphology and cellular viability.* Hum Mol Genet 17, 201-14.
57. Stavru, F., A.E. Palmer, C. Wang, R.J. Youle & P. Cossart (2013), *Atypical mitochondrial fission upon bacterial infection.* Proc Natl Acad Sci U S A 110, 16003-8. PMC3791707

58. Chakrabarti, R., W.K. Ji, R.V. Stan, J. de Juan Sanz, T.A. Ryan & H.N. Higgs (2018), *INF2-mediated actin polymerization at the ER stimulates mitochondrial calcium uptake, inner membrane constriction, and division.* J Cell Biol 217, 251-268. PMC5748994
59. Yamashita, S.I., X. Jin, K. Furukawa, M. Hamasaki, A. Nezu, H. Otera, T. Saigusa, T. Yoshimori, Y. Sakai, K. Mihara & T. Kanki (2016), *Mitochondrial division occurs concurrently with autophagosome formation but independently of Drp1 during mitophagy.* J Cell Biol 215, 649-665. PMC5147001
60. Neuspiel, M., A.C. Schauss, E. Braschi, R. Zunino, P. Rippstein, R.A. Rachubinski, M.A. Andrade-Navarro & H.M. McBride (2008), *Cargo-selected transport from the mitochondria to peroxisomes is mediated by vesicular carriers.* Curr Biol 18, 102-8.
61. Baricault, L., B. Segui, L. Guegand, A. Olichon, A. Valette, F. Larminat & G. Lenaers (2007), *OPA1 cleavage depends on decreased mitochondrial ATP level and bivalent metals.* Exp Cell Res 313, 3800-8.
62. van der Bliek, A.M., M.M. Sedensky & P.G. Morgan (2017), *Cell Biology of the Mitochondrion.* Genetics 207, 843-871. PMC5676242
63. Wai, T. & T. Langer (2016), *Mitochondrial Dynamics and Metabolic Regulation.* Trends Endocrinol Metab 27, 105-117.
64. Chen, H. & D.C. Chan (2017), *Mitochondrial Dynamics in Regulating the Unique Phenotypes of Cancer and Stem Cells.* Cell Metab 26, 39-48. PMC5539982
65. Roy, M., P.H. Reddy, M. Iijima & H. Sesaki (2015), *Mitochondrial division and fusion in metabolism.* Curr Opin Cell Biol 33, 111-8. PMC4380865
66. Fu, H., H. Zhou, X. Yu, J. Xu, J. Zhou, X. Meng, J. Zhao, Y. Zhou, A.D. Chisholm & S. Xu (2020), *Wounding triggers MIRO-1 dependent mitochondrial fragmentation that accelerates epidermal wound closure through oxidative signaling.* Nat Commun 11, 1050. PMC7044169
67. Ding, L., Y. Lei, Y. Han, Y. Li, X. Ji & L. Liu (2016), *Vimar Is a Novel Regulator of Mitochondrial Fission through Miro.* PLoS Genet 12, e1006359. PMC5065127
68. Thu le, T., J.R. Ahn & S.H. Woo (2006), *Inhibition of L-type Ca2+ channel by mitochondrial Na+-Ca2+ exchange inhibitor CGP-37157 in rat atrial myocytes.* Eur J Pharmacol 552, 15-9.
69. Ye, L., M.A. Haroon, A. Salinas & M. Paukert (2017), *Comparison of GCaMP3 and GCaMP6f for studying astrocyte Ca2+ dynamics in the awake mouse brain.* PLoS One 12, e0181113. PMC5524333
70. Kettunen, P. (2020), *Calcium Imaging in the Zebrafish.* Adv Exp Med Biol 1131, 901-942.
71. Zhang, N. & S. Ding (2018), *Imaging of Mitochondrial and Cytosolic Ca(2+) Signals in Cultured Astrocytes.* Curr Protoc Neurosci 82, 2 29 1-2 29 11. PMC5779630
72. Chen, T.W., T.J. Wardill, Y. Sun, S.R. Pulver, S.L. Renninger, A. Baohan, E.R. Schreiter, R.A. Kerr, M.B. Orger, V. Jayaraman, L.L. Looger, K. Svoboda & D.S. Kim (2013), *Ultrasensitive fluorescent proteins for imaging neuronal activity.* Nature 499, 295-300. PMC3777791
73. Wolf, S.G., Y. Mutsafi, T. Dadosh, T. Ilani, Z. Lansky, B. Horowitz, S. Rubin, M. Elbaum & D. Fass (2017), *3D visualization of mitochondrial solid-phase calcium stores in whole cells.* Elife 6. PMC5703638
74. Sanhueza, C., J. Araos, L. Naranjo, F. Toledo, A.R. Beltran, M.A. Ramirez, J. Gutierrez, F. Pardo, A. Leiva & L. Sobrevia (2017), *Sodium/proton exchanger isoform 1 regulates intracellular pH and cell proliferation in human ovarian cancer.* Biochim Biophys Acta Mol Basis Dis 1863, 81-91.
75. Lomenick, B., R. Hao, N. Jonai, R.M. Chin, M. Aghajan, S. Warburton, J. Wang, R.P. Wu, F. Gomez, J.A. Loo, J.A. Wohlschlegel, T.M. Vondriska, J. Pelletier, H.R. Herschman, J. Clardy, C.F. Clarke & J. Huang (2009), *Target identification using drug affinity responsive target stability (DARTS).* Proceedings of the National Academy of Sciences of the United States of America 106, 21984-9. 2789755
76. Pai, M.Y., B. Lomenick, H. Hwang, R. Schiestl, W. McBride, J.A. Loo & J. Huang (2015), *Drug affinity responsive target stability (DARTS) for small-molecule target identification.* Methods Mol Biol 1263, 287-98. PMC4442491
77. Sleno, L. & A. Emili (2008), *Proteomic methods for drug target discovery.* Curr Opin Chem Biol 12, 46-54.
78. Sato, S., A. Murata, T. Shirakawa & M. Uesugi (2010), *Biochemical target isolation for novices: affinity-based strategies.* Chem Biol 17, 616-23.

79. Frank, M., H. Niemann, P. Bohler, B. Stork, S. Wesselborg, W. Lin & P. Proksch (2015), *Phomoxanthone A--From Mangrove Forests to Anticancer Therapy.* Curr Med Chem 22, 3523-32.
80. Li, W., Z. Shariat-Madar, M. Powers, X. Sun, R.D. Lane & K.D. Garlid (1992), *Reconstitution, identification, purification, and immunological characterization of the 110-kDa Na+/Ca2+ antiporter from beef heart mitochondria.* J Biol Chem 267, 17983-9.
81. Paucek, P. & M. Jaburek (2004), *Kinetics and ion specificity of Na(+)/Ca(2+) exchange mediated by the reconstituted beef heart mitochondrial Na(+)/Ca(2+) antiporter.* Biochim Biophys Acta 1659, 83-91.
82. Gandre-Babbe, S. & A.M. van der Bliek (2008), *The novel tail-anchored membrane protein Mff controls mitochondrial and peroxisomal fission in mammalian cells.* Mol Biol Cell 19, 2402-12. PMC2397315
83. Karbowski, M., M.M. Cleland & B.A. Roelofs (2014), *Photoactivatable green fluorescent protein-based visualization and quantification of mitochondrial fusion and mitochondrial network complexity in living cells.* Methods Enzymol 547, 57-73. PMC4508014
84. Song, M., A. Franco, J.A. Fleischer, L. Zhang & G.W. Dorn, 2nd (2017), *Abrogating Mitochondrial Dynamics in Mouse Hearts Accelerates Mitochondrial Senescence.* Cell Metab 26, 872-883 e5. PMC5718956
85. Lewis, S.C., L.F. Uchiyama & J. Nunnari (2016), *ER-mitochondria contacts couple mtDNA synthesis with mitochondrial division in human cells.* Science 353, aaf5549. PMC5554545
86. Bajar, B.T., E.S. Wang, A.J. Lam, B.B. Kim, C.L. Jacobs, E.S. Howe, M.W. Davidson, M.Z. Lin & J. Chu (2016), *Improving brightness and photostability of green and red fluorescent proteins for live cell imaging and FRET reporting.* Sci Rep 6, 20889. PMC4754705
87. Genove, G., B.S. Glick & A.L. Barth (2005), *Brighter reporter genes from multimerized fluorescent proteins.* Biotechniques 39, 814-818.
88. Damke, H., T. Baba, A.M. van der Bliek & S.L. Schmid (1995), *Clathrin-independent pinocytosis is induced in cells overexpressing a temperature-sensitive mutant of dynamin.* J Cell Biol 131, 69-80.
89. Liu, Y.W., J.P. Mattila & S.L. Schmid (2013), *Dynamin-catalyzed membrane fission requires coordinated GTP hydrolysis.* PLoS One 8, e55691. PMC3561337
90. Lynch-Day, M.A., D. Bhandari, S. Menon, J. Huang, H. Cai, C.R. Bartholomew, J.H. Brumell, S. Ferro-Novick & D.J. Klionsky (2010), *Trs85 directs a Ypt1 GEF, TRAPPIII, to the phagophore to promote autophagy.* Proceedings of the National Academy of Sciences of the United States of America 107, 7811-6. 2867920
91. Paquet, D., D. Kwart, A. Chen, A. Sproul, S. Jacob, S. Teo, K.M. Olsen, A. Gregg, S. Noggle & M. Tessier-Lavigne (2016), *Efficient introduction of specific homozygous and heterozygous mutations using CRISPR/Cas9.* Nature 533, 125-9.
92. Wieckowski, M.R., C. Giorgi, M. Lebiedzinska, J. Duszynski & P. Pinton (2009), *Isolation of mitochondria-associated membranes and mitochondria from animal tissues and cells.* Nat Protoc 4, 1582-90.
93. De La Fuente, S., J.P. Lambert, Z. Nichtova, C. Fernandez Sanz, J.W. Elrod, S.S. Sheu & G. Csordas (2018), *Spatial Separation of Mitochondrial Calcium Uptake and Extrusion for Energy-Efficient Mitochondrial Calcium Signaling in the Heart.* Cell Rep 24, 3099-3107 e4. PMC6226263
94. De Stefani, D., R. Rizzuto & T. Pozzan (2016), *Enjoy the Trip: Calcium in Mitochondria Back and Forth.* Annu Rev Biochem 85, 161-92.
95. White, N. & G. Burnstock (2006), *P2 receptors and cancer.* Trends Pharmacol Sci 27, 211-7.
96. Zhou, W., K.H. Chen, W. Cao, J. Zeng, H. Liao, L. Zhao & X. Guo (2010), *Mutation of the protein kinase A phosphorylation site influences the anti-proliferative activity of mitofusin 2.* Atherosclerosis 211, 216-23.
97. Assali, E.A., A.E. Jones, M. Veliova, M. Taha, N. Miller, M. Shum, M.F. Oliveira, G. Las, M. Liesa, I. Sekler & S. Orian (2020), *NCLX prevents cell death during adrenergic activation of the brown adipose tissue.* bioRxiv.
98. Boutant, M., S.S. Kulkarni, M. Joffraud, J. Ratajczak, M. Valera-Alberni, R. Combe, A. Zorzano & C. Canto (2017), *Mfn2 is critical for brown adipose tissue thermogenic function.* EMBO J 36, 1543-1558. PMC5452040
99. Kostic, M. & I. Sekler (2019), *Functional properties and mode of regulation of the mitochondrial Na(+)/Ca(2+) exchanger, NCLX.* Semin Cell Dev Biol 94, 59-65.

100. Thaher, O., C. Wolf, P.N. Dey, A. Pouya, V. Wullner, S. Tenzer & A. Methner (2017), *The thiol switch C684 in Mitofusin-2 mediates redox-induced alterations of mitochondrial shape and respiration.* Neurochem Int.
101. Mattie, S., J. Riemer, J.G. Wideman & H.M. McBride (2017), *A new mitofusin topology places the redox-regulated C terminus in the mitochondrial intermembrane space.* J Cell Biol. PMC5800796
102. Steffen, J., A.A. Vashisht, J. Wan, J.C. Jen, S.M. Claypool, J.A. Wohlschlegel & C.M. Koehler (2017), *Rapid degradation of mutant SLC25A46 by the ubiquitin-proteasome system results in MFN1/2-mediated hyperfusion of mitochondria.* Mol Biol Cell 28, 600-612. PMC5328619
103. Wan, J., J. Steffen, M. Yourshaw, H. Mamsa, E. Andersen, S. Rudnik-Schoneborn, K. Pope, K.B. Howell, C.A. McLean, A.J. Kornberg, J. Joseph, P.J. Lockhart, K. Zerres, M.M. Ryan, S.F. Nelson, C.M. Koehler & J.C. Jen (2016), *Loss of function of SLC25A46 causes lethal congenital pontocerebellar hypoplasia.* Brain 139, 2877-2890. PMC5840878
104. Abrams, A.J., R.B. Hufnagel, A. Rebelo, C. Zanna, N. Patel, M.A. Gonzalez, I.J. Campeanu, L.B. Griffin, S. Groenewald, A.V. Strickland, F. Tao, F. Speziani, L. Abreu, R. Schule, L. Caporali, C. La Morgia, A. Maresca, R. Liguori, R. Lodi, Z.M. Ahmed, K.L. Sund, X. Wang, L.A. Krueger, Y. Peng, C.E. Prada, C.A. Prows, E.K. Schorry, A. Antonellis, H.H. Zimmerman, O.A. Abdul-Rahman, Y. Yang, S.M. Downes, J. Prince, F. Fontanesi, A. Barrientos, A.H. Nemeth, V. Carelli, T. Huang, S. Zuchner & J.E. Dallman (2015), *Mutations in SLC25A46, encoding a UGO1-like protein, cause an optic atrophy spectrum disorder.* Nat Genet 47, 926-32. PMC4520737
105. Terzenidou, M.E., A. Segklia, T. Kano, F. Papastefanaki, A. Karakostas, M. Charalambous, F. Ioakeimidis, M. Papadaki, I. Kloukina, M. Chrysanthou-Piterou, M. Samiotaki, G. Panayotou, R. Matsas & E. Douni (2017), *Novel insights into SLC25A46-related pathologies in a genetic mouse model.* PLoS Genet 13, e1006656. PMC5380310
106. Sesaki, H. & R.E. Jensen (2004), *Ugo1p links the Fzo1p and Mgm1p GTPases for mitochondrial fusion.* J Biol Chem.
107. Sesaki, H. & R.E. Jensen (2001), *UGO1 encodes an outer membrane protein required for mitochondrial fusion.* J Cell Biol 152, 1123-34.
108. Sesaki, H., S.M. Southard, M.P. Yaffe & R.E. Jensen (2003), *Mgm1p, a Dynamin-related GTPase, Is Essential for Fusion of the Mitochondrial Outer Membrane.* Mol. Biol. Cell 14, 2342-2356.
109. Wong, E.D., J.A. Wagner, S.V. Scott, V. Okreglak, T.J. Holewinske, A. Cassidy-Stone & J. Nunnari (2003), *The intramitochondrial dynamin-related GTPase, Mgm1p, is a component of a protein complex that mediates mitochondrial fusion.* J Cell Biol 160, 303-11.
110. Janer, A., J. Prudent, V. Paupe, S. Fahiminiya, J. Majewski, N. Sgarioto, C. Des Rosiers, A. Forest, Z.Y. Lin, A.C. Gingras, G. Mitchell, H.M. McBride & E.A. Shoubridge (2016), *SLC25A46 is required for mitochondrial lipid homeostasis and cristae maintenance and is responsible for Leigh syndrome.* EMBO Mol Med 8, 1019-38. PMC5009808
111. Twig, G., S.A. Graf, J.D. Wikstrom, H. Mohamed, S.E. Haigh, A. Elorza, M. Deutsch, N. Zurgil, N. Reynolds & O.S. Shirihai (2006), *Tagging and tracking individual networks within a complex mitochondrial web with photoactivatable GFP.* Am J Physiol Cell Physiol 291, C176-84.
112. Arimura, S., J. Yamamoto, G.P. Aida, M. Nakazono & N. Tsutsumi (2004), *Frequent fusion and fission of plant mitochondria with unequal nucleoid distribution.* Proc Natl Acad Sci U S A 101, 7805-8. PMC419687
113. De Vecchis, D., L. Cavellini, M. Baaden, J. Henin, M.M. Cohen & A. Taly (2017), *A membrane-inserted structural model of the yeast mitofusin Fzo1.* Sci Rep 7, 10217. PMC5578988
114. Lee, J.E., L.M. Westrate, H. Wu, C. Page & G.K. Voeltz (2016), *Multiple dynamin family members collaborate to drive mitochondrial division.* Nature 540, 139-143. PMC5656044
115. Kamerkar, S.C., F. Kraus, A.J. Sharpe, T.J. Pucadyil & M.T. Ryan (2018), *Dynamin-related protein 1 has membrane constricting and severing abilities sufficient for mitochondrial and peroxisomal fission.* Nat Commun 9, 5239. PMC6286342
116. Fonseca, T.B., A. Sanchez-Guerrero, I. Milosevic & N. Raimundo (2019), *Mitochondrial fission requires DRP1 but not dynamins.* Nature 570, E34-E42.

117. Martorell-Riera, A., C. Iriondo-Martinez, S. Itskanov, J. Steffen, B. Roach, C.M. Koehler & A.M. van der Bliek (2019), *Dynamin-2 controls phagophore maturation.* bioRxiv doi: https://doi.org/10.1101/241901.
118. Niemann, A., P. Berger & U. Suter (2006), *Pathomechanisms of mutant proteins in Charcot-Marie-Tooth disease.* Neuromolecular medicine 8, 217-42.
119. Boucher, B. & S. Jenna (2013), *Genetic interaction networks: better understand to better predict.* Front Genet 4, 290. PMC3865423
120. Anghelescu, C., B. Francou, R. Cardas, A. Guiochon-Mantel, P. Aubourg, L. Servais & T. Gidaro (2017), *Targeted exomes reveal simultaneous MFN2 and GDAP1 mutations in a severe Charcot-Marie-Tooth disease type 2 phenotype.* Eur J Neurol 24, e15-e16.
121. Kostera-Pruszczyk, A., J. Kosinska, A. Pollak, P. Stawinski, A. Walczak, K. Wasilewska, A. Potulska-Chromik, P. Szczudlik, A. Kaminska & R. Ploski (2014), *Exome sequencing reveals mutations in MFN2 and GDAP1 in severe Charcot-Marie-Tooth disease.* J Peripher Nerv Syst 19, 242-5.
122. Vital, A., P. Latour, G. Sole, X. Ferrer, M. Rouanet, F. Tison, C. Vital & C. Goizet (2012), *A French family with Charcot-Marie-Tooth disease related to simultaneous heterozygous MFN2 and GDAP1 mutations.* Neuromuscular disorders : NMD 22, 735-41.
123. Cassereau, J., C. Casasnovas, N. Gueguen, M.C. Malinge, V. Guillet, P. Reynier, D. Bonneau, P. Amati-Bonneau, I. Banchs, V. Volpini, V. Procaccio & A. Chevrollier (2011), *Simultaneous MFN2 and GDAP1 mutations cause major mitochondrial defects in a patient with CMT.* Neurology 76, 1524-6.
124. Kandemir, B., B. Caglayan, B. Hausott, B. Erdogan, U. Dag, O. Demir, M.S. Sogut, L. Klimaschewski & I.A. Kurnaz (2014), *Pea3 transcription factor promotes neurite outgrowth.* Front Mol Neurosci 7, 59. PMC4072091
125. Song, F. & K. Stieger (2017), *Optimizing the DNA Donor Template for Homology-Directed Repair of Double-Strand Breaks.* Mol Ther Nucleic Acids 7, 53-60. PMC5363683
126. Li, G., X. Zhang, C. Zhong, J. Mo, R. Quan, J. Yang, D. Liu, Z. Li, H. Yang & Z. Wu (2017), *Small molecules enhance CRISPR/Cas9-mediated homology-directed genome editing in primary cells.* Sci Rep 7, 8943. PMC5566437
127. Maruyama, T., S.K. Dougan, M.C. Truttmann, A.M. Bilate, J.R. Ingram & H.L. Ploegh (2015), *Increasing the efficiency of precise genome editing with CRISPR-Cas9 by inhibition of nonhomologous end joining.* Nat Biotechnol 33, 538-42. PMC4618510
128. Wang, L., J. Gao, J. Liu, S.L. Siedlak, S. Torres, H. Fujioka, M.L. Huntley, Y. Jiang, H. Ji, T. Yan, M. Harland, P. Termsarasab, S. Zeng, Z. Jiang, J. Liang, G. Perry, C. Hoppel, C. Zhang, H. Li & X. Wang (2018), *Mitofusin 2 Regulates Axonal Transport of Calpastatin to Prevent Neuromuscular Synaptic Elimination in Skeletal Muscles.* Cell Metab 28, 400-414 e8. PMC6125186
129. Nita, II, M. Hershfinkel, C. Kantor, G.A. Rutter, E.C. Lewis & I. Sekler (2014), *Pancreatic beta-cell Na+ channels control global Ca2+ signaling and oxidative metabolism by inducing Na+ and Ca2+ responses that are propagated into mitochondria.* FASEB J 28, 3301-12. PMC6101212
130. Nita, II, M. Hershfinkel, E.C. Lewis & I. Sekler (2015), *A crosstalk between Na(+) channels, Na(+)/K(+) pump and mitochondrial Na(+) transporters controls glucose-dependent cytosolic and mitochondrial Na(+) signals.* Cell Calcium 57, 69-75.
131. Westermann, B. (2012), *Bioenergetic role of mitochondrial fusion and fission.* Biochim Biophys Acta 1817, 1833-8.
132. Leboucher, G.P., Y.C. Tsai, M. Yang, K.C. Shaw, M. Zhou, T.D. Veenstra, M.H. Glickman & A.M. Weissman (2012), *Stress-induced phosphorylation and proteasomal degradation of mitofusin 2 facilitates mitochondrial fragmentation and apoptosis.* Molecular cell 47, 547-57. 3526191
133. Suen, D.F., K.L. Norris & R.J. Youle (2008), *Mitochondrial dynamics and apoptosis.* Genes Dev 22, 1577-90.
134. Xiong, Y., Q. Zhao, L. Gu, C. Liu & C. Wang (2018), *Shenqi Fuzheng Injection Reverses Cisplatin Resistance through Mitofusin-2-Mediated Cell Cycle Arrest and Apoptosis in A549/DDP Cells.* Evid Based Complement Alternat Med 2018, 8258246. PMC6206574
135. Karbowski, M., K.L. Norris, M.M. Cleland, S.Y. Jeong & R.J. Youle (2006), *Role of Bax and Bak in mitochondrial morphogenesis.* Nature 443, 658-62.

136. Wang, C., L. Engelke, D. Bickel, A. Hamacher, M. Frank, P. Proksch, H. Gohlke & M.U. Kassack (2019), *The tetrahydroxanthone-dimer phomoxanthone A is a strong inducer of apoptosis in cisplatin-resistant solid cancer cells.* Bioorg Med Chem 27, 115044.
137. Martorell-Riera, A., M. Segarra-Mondejar, J.P. Munoz, V. Ginet, J. Olloquequi, J. Perez-Clausell, M. Palacin, M. Reina, J. Puyal, A. Zorzano & F.X. Soriano (2014), *Mfn2 downregulation in excitotoxicity causes mitochondrial dysfunction and delayed neuronal death.* EMBO J 33, 2388-407. PMC4253527
138. Ruiz, A., E. Alberdi & C. Matute (2014), *CGP37157, an inhibitor of the mitochondrial Na+/Ca2+ exchanger, protects neurons from excitotoxicity by blocking voltage-gated Ca2+ channels.* Cell Death Dis 5, e1156. PMC5424111
139. Griparic, L., N.N. van der Wel, I.J. Orozco, P.J. Peters & A.M. van der Bliek (2004), *Loss of the intermembrane space protein Mgm1/OPA1 induces swelling and localized constrictions along the lengths of mitochondria.* J Biol Chem 279, 18792-8.
140. Mizushima, N., T. Yoshimori & B. Levine (2010), *Methods in mammalian autophagy research.* Cell 140, 313-26. PMC2852113
141. Yamano, K., A.I. Fogel, C. Wang, A.M. van der Bliek & R.J. Youle (2014), *Mitochondrial Rab GAPs govern autophagosome biogenesis during mitophagy.* Elife 3, e01612. PMC3930140
142. Shen, Q., K. Yamano, B.P. Head, S. Kawajiri, J.T. Cheung, C. Wang, J.H. Cho, N. Hattori, R.J. Youle & A.M. van der Bliek (2014), *Mutations in Fis1 disrupt orderly disposal of defective mitochondria.* Mol Biol Cell 25, 145-59. PMC3873885
143. Vettori, A., G. Bergamin, E. Moro, G. Vazza, G. Polo, N. Tiso, F. Argenton & M.L. Mostacciuolo (2011), *Developmental defects and neuromuscular alterations due to mitofusin 2 gene (MFN2) silencing in zebrafish: a new model for Charcot-Marie-Tooth type 2A neuropathy.* Neuromuscular disorders : NMD 21, 58-67.
144. Curado, S., E.A. Ober, S. Walsh, P. Cortes-Hernandez, H. Verkade, C.M. Koehler & D.Y. Stainier (2010), *The mitochondrial import gene tomm22 is specifically required for hepatocyte survival and provides a liver regeneration model.* Dis Model Mech 3, 486-95. 2898538
145. Dabir, D.V., S.A. Hasson, K. Setoguchi, M.E. Johnson, P. Wongkongkathep, C.J. Douglas, J. Zimmerman, R. Damoiseaux, M.A. Teitell & C.M. Koehler (2013), *A small molecule inhibitor of redox-regulated protein translocation into mitochondria.* Dev Cell 25, 81-92. PMC3726224
146. Levesque, M.P., J. Krauss, C. Koehler, C. Boden & M.P. Harris (2013), *New tools for the identification of developmentally regulated enhancer regions in embryonic and adult zebrafish.* Zebrafish 10, 21-9. PMC3670562
147. Sager, J.J., Q. Bai & E.A. Burton (2010), *Transgenic zebrafish models of neurodegenerative diseases.* Brain Struct Funct 214, 285-302.
148. Rahn, J.J., K.D. Stackley & S.S. Chan (2013), *Opa1 is required for proper mitochondrial metabolism in early development.* PLoS One 8, e59218. PMC3597633
149. Drerup, C.M. & A.V. Nechiporuk (2016), *In vivo analysis of axonal transport in zebrafish.* Methods Cell Biol 131, 311-29.
150. Shimizu, H., J. Schredelseker, J. Huang, K. Lu, S. Naghdi, F. Lu, S. Franklin, H.D. Fiji, K. Wang, H. Zhu, C. Tian, B. Lin, H. Nakano, A. Ehrlich, J. Nakai, A.Z. Stieg, J.K. Gimzewski, A. Nakano, J.I. Goldhaber, T.M. Vondriska, G. Hajnoczky, O. Kwon & J.N. Chen (2015), *Mitochondrial Ca(2+) uptake by the voltage-dependent anion channel 2 regulates cardiac rhythmicity.* Elife 4. PMC4293673
151. Shimizu, H., A.D. Langenbacher, J. Huang, K. Wang, G. Otto, R. Geisler, Y. Wang & J.N. Chen (2017), *The Calcineurin-FoxO-MuRF1 signaling pathway regulates myofibril integrity in cardiomyocytes.* Elife 6. PMC5576919
152. Xie, Y., M. Ottolia, S.A. John, J.N. Chen & K.D. Philipson (2008), *Conformational changes of a Ca2+-binding domain of the Na+/Ca2+ exchanger monitored by FRET in transgenic zebrafish heart.* Am J Physiol Cell Physiol 295, C388-93. PMC2518419
153. Kim, M.J., K.H. Kang, C.H. Kim & S.Y. Choi (2008), *Real-time imaging of mitochondria in transgenic zebrafish expressing mitochondrially targeted GFP.* Biotechniques 45, 331-4.
154. Kreiling, J.A., Z.L. Balantac, A.R. Crawford, Y. Ren, J. Toure, S. Zchut, L. Kochilas & R. Creton (2008), *Suppression of the endoplasmic reticulum calcium pump during zebrafish gastrulation affects left-right asymmetry of the heart and brain.* Mech Dev 125, 396-410.

155. Baloh, R.H., R.E. Schmidt, A. Pestronk & J. Milbrandt (2007), *Altered axonal mitochondrial transport in the pathogenesis of Charcot-Marie-Tooth disease from mitofusin 2 mutations.* J Neurosci 27, 422-30.
156. Palau, F., A. Estela, D. Pla-Martin & M. Sanchez-Piris (2009), *The role of mitochondrial network dynamics in the pathogenesis of Charcot-Marie-Tooth disease.* Advances in experimental medicine and biology 652, 129-37.
157. Mathis, S., B. Funalot, O. Boyer, C. Lacroix, P. Marcorelles, L. Magy, L. Richard, C. Antignac & J.M. Vallat (2014), *Neuropathologic characterization of INF2-related Charcot-Marie-Tooth disease: evidence for a Schwann cell actinopathy.* J Neuropathol Exp Neurol 73, 223-33.
158. Boyer, O., F. Nevo, E. Plaisier, B. Funalot, O. Gribouval, G. Benoit, E.H. Cong, C. Arrondel, M.J. Tete, R. Montjean, L. Richard, A. Karras, C. Pouteil-Noble, L. Balafrej, A. Bonnardeaux, G. Canaud, C. Charasse, J. Dantal, G. Deschenes, P. Deteix, O. Dubourg, P. Petiot, D. Pouthier, E. Leguern, A. Guiochon-Mantel, I. Broutin, M.C. Gubler, S. Saunier, P. Ronco, J.M. Vallat, M.A. Alonso, C. Antignac & G. Mollet (2011), *INF2 mutations in Charcot-Marie-Tooth disease with glomerulopathy.* The New England journal of medicine 365, 2377-88.

UNIVERSITY OF CALIFORNIA, LOS ANGELES

UCLA

BERKELEY • DAVIS • IRVINE • LOS ANGELES • MERCED • RIVERSIDE • SAN DIEGO • SAN FRANCISCO

SANTA BARBARA • SANTA CRUZ

JAU-NIAN CHEN
PROFESSOR
DEPARTMENT OF MOLECULAR, CELL,
AND DEVELOPMENTAL BIOLOGY
UNIVERSITY OF CALIFORNIA
LOS ANGELES, CALIFORNIA 90095-1606

April 19, 2020

Dear Alex,

This letter is to confirm my enthusiasm for your proposal to investigate connections between Mfn2, MCLX and mitochondrial fission as outlined in your grant application titled “Control of calcium flux and mitochondrial fission by the Charcot Marie Tooth disease protein Mfn2“. I am excited to work with you in a collaborative effort to study the effects of interactions between Mfn2 and NCLX in zebrafish.

As you know, my lab has a longstanding interest in zebrafish heart muscle development with a focus on the role of calcium signaling in this process. We have the necessary probes and experience with calcium imaging in zebrafish to help you with your project. In our 2015 eLife paper (DOI: 10.7554/eLife.04801), we showed the feasibility of tracking calcium transients in zebrafish heart. Similar techniques have been applied by others for calcium transients in neurons. Of particular relevance for your research, we have developed and worked with NCLX morpholinos as well as NCLX knockout fish. I am happy to share our experiences, reagents and fish lines with you in what I expect to be a fruitful collaborative effort to learn more about possible connections between Mfn2 and NCLX. This is an exciting new area, which will undoubtedly have powerful and sustained impact on the field.

I very much look forward to collaborating with you on this project. I wish you the very best with your application. Please let me know if there is anything else I can provide you with as we move forward with this project.

Sincerely,

Jau-Nian Chen, Ph.D.

JOHNS HOPKINS
UNIVERSITY
Department of Biology
144 Mudd Hall/3400 N. Charles St.
Baltimore, MD 21218-2685

Integrated Imaging Center

April 24, 2020

Alex van der Bliek, Ph.D.
Department of Biological Chemistry
UCLA School of Medicine
Los Angeles, CA 90095-1737

Dear Alex,

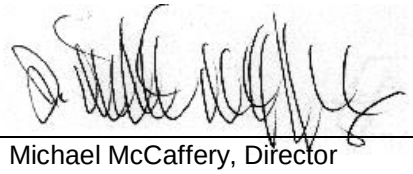
I am pleased to write this letter confirming my willingness to assist with your project exploring the "Control of calcium flux and mitochondrial fission by the Charcot Marie Tooth disease protein Mfn2". Your novel findings regarding the possible roles of this protein in calcium flux and fission are exciting and could potentially lead to some important discoveries.

As you know, my laboratory has extensive experience with conventional and immuno-electron microscopy. We have also had numerous successful collaborations with other cell biology labs in the past, supporting them with essential EM analysis. Our expertise therefore fits the needs of your laboratory very well.

To assist you, my laboratory will gladly provide the EM analysis and intellectual support necessary to fulfill the research objectives of this project. I look forward to working with you on what will certainly be a productive and successful collaboration.

Good luck with your application!
Best wishes,

Michael



J. Michael McCaffery, Director
The Integrated Imaging Center

Research Professor
Department of Biology,
Engineering in Oncology Center, and
The Institute for NanoBioTechnology

Ben-Gurion University of the Negev
P.O. Box 653, 84 105 Beer Sheva, Israel

Prof Israel Sekler, Ph.D.
Department of Physiology and cell Biology
Faculty of Health Sciences

Tel: (8) 647-7328
Fax: (8) 647-7628
Email: sekler@bgu.ac.il

25,4,2020

Dear Alex,

I write this letter to provide strong and enthusiastic support for the project described in your grant application titled "Control of calcium flux and mitochondrial fission by the Charcot Marie Tooth disease protein Mfn2". This project aimed at investigating connections between Mfn2, MCLX and mitochondrial fission dovetails wonderfully with the work in my lab on NCLX functions. I am excited to support this as a mutually beneficial collaborative effort.

As you know, my laboratory has worked extensively with calcium exchange proteins with a strong focus on the calcium exchange that occurs in mitochondria. Ten years ago, we showed that the long sought after sodium-calcium exchange protein in mitochondria is NCLX (PNAS 107:436-41, 2010). Since, then, we have published a series of studies on the cellular processes affected by NCLX and the regulation of NCLX by different cellular parameters (EMBO J 36, 797-815, 2017; Cell Rep 25, 3465-3475, 2018; Cell Rep 13, 376-86, 2015). Your studies of NCLX regulation by Mfn2 and the potential use of PXA as an activator of NCLX provide exciting new directions for this research.

I am happy to share our expertise, constructs, cell lines and assays in order to advance this project as a collaborative effort. I look forward to a productive partnership with you and wish you success with this proposal.

Israel Sekler PhD
Professor of Physiology and Cell Biology

Ben-Gurion University of the Negev

David Geffen School of Medicine

Department of Medicine
Division of Endocrinology, Diabetes & Hypertension
900 Veteran Ave, 24-130 Warren Hall
Los Angeles, CA 9095-7073
(310) 794-7555
Work cell phone 617-230-8570
Fax: (310) 794-7654

Alex Van der Bliek,
Professor of Biological Chemistry
UCLA

April 25, 2020

Dear Alex,

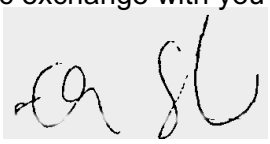
I am pleased to write this letter confirming my willingness to assist you with your project on the "Control of calcium flux and mitochondrial fission by the Charcot Marie Tooth disease protein Mfn2". You are a pioneer in our field and it is an honor for me to provide support for your group and be your collaborator in any way I can help. Your ideas about triggers for apoptosis and their connections with mitochondrial dynamics are exciting and likely will lead to some key discoveries in the field.

As you know, my laboratory recently moved to UCLA where I became director of UCLA Metabolism Theme, a group of 30 laboratories sharing an interest in mitochondria and metabolism. We organize meetings, foster collaborations within the theme and sponsor numerous other activities meant to facilitate scientific interactions. In addition, I am directing a new ***mitochondrial core facility*** here at UCLA. This core facility includes various pieces of equipment for measuring mitochondrial function, such as ***six Seahorse extracellular flux analyzers*** for OCR and a Perkin Elmer Operetta system for high throughput image analysis. The core also has broad expertise for many types of mitochondrial analyses. You will have ready access to this facility as well as to the ***superresolution confocal microscope*** that I have acquired recently and includes 2-Photon as well as fluorescence life time capabilities.

Lastly, my laboratory and I myself will gladly assist you with the reagents, as well as the equipment and the expertise that we have in the lab. I am convinced that results from your project will have lasting impact and look forward to many years of stimulating Best Wishes and scientific exchange with you and members of your lab.

Best Wishes,

Orian Shirihai



Orian Shirihai, M.D., Ph.D.
Professor of Medicine
Dep. of Medicine (Endocrinology) and Dep. of Pharmacology, David Geffen School of Medicine at UCLA
Director of UCLA Metabolism Theme
Director of UCLA Mitochondria & Bioenergetics Core
Head of Mitochondria Research lab

University Hospital Düsseldorf
Prof. Dr. rer. nat. Björn Stork
Institute of Molecular Medicine I

Universitätsstr. 1,
Building 23.12
D-40225 Düsseldorf

Phone: +49 (0)211 81-11954
Fax: +49 (0)211 81-11611954
E-Mail: bjoern.stork@hhu.de

Alexander van der Bliek
Department of Biological Chemistry
David Geffen School of Medicine at UCLA
615 Charles E Young Drive South
Los Angeles, CA 90095-1737
USA

April 24th, 2020

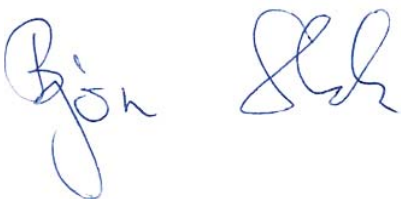
Support Letter

Dear Alex,

I will be delighted to support your project titled "Control of calcium flux and mitochondrial fission by the Charcot Marie Tooth disease protein Mfn2". As you contributed to this study, you know my lab recently published an extensive analysis of the cell biological effects of phomoxanthone PXA. This study showed that PXA triggers calcium release from mitochondria and it triggers apoptosis, independent of MICU or mPTP functions. Your new data suggesting that PXA activates NCLX and that calcium-release through NCLX is controlled by Mfn2 is truly exciting. These discoveries open up new ways to manipulate the effects of calcium in mitochondria and, as also suggested by earlier studies with PXA, these discoveries may lead to new approaches to cancer chemotherapy. We will be more than happy to continue sharing reagents and information so that this project will be successful.

I look forward to continuing this exciting collaboration.

Sincerely,



Björn Stork

Resource Sharing Plan

Sharing results and critical reagents is an essential part of the scientific endeavor. Our results will be made available to the scientific community through publications and presentations at meetings. Newly generated reagents will be made available as soon as they are published in a peer reviewed journal.

No mouse strains, large data sets, or antibodies will be generated.

“Other Research Resources” generated with funds from this grant, such as stable or knockout cells, zebrafish strains, and DNA constructs will be freely distributed to academic investigators for non-commercial research.

Requests to use novel reagents for commercial purposes will be handled by the UCLA Technology Development Group (http://tdg.ucla.edu/transferring-research-materials).

We will adhere to the NIH Grant Policy on Sharing of Unique Research Resources including the Sharing of Biomedical Research Resources Principles and Guidelines for Recipients of NIH Grants and Contracts issued in December, 1999. All “model organisms” generated by this project will be distributed freely or deposited into a repository/stock center making them available to the broader research community, either before or immediately after publication. Our lab has demonstrated its commitment to sharing over the past 24 years. If we assume responsibility for distributing the newly generated model organisms, we fill requests in a timely fashion. In addition, we will provide relevant protocols and published genetic and phenotypic data upon request. Material transfers will be made with no more restrictive terms than in the Simple Letter Agreement (SLA) or the Uniform Biological Materials Transfer Agreement (UBMTA) and without reach through requirements. Should any intellectual property arise which requires a patent, we will ensure that the materials remain widely available to the research community in accordance with the NIH Principles and Guidelines document.

Authentication of Key Biological and/or Chemical Resources

Van der Bliek lab and Koehler labs, UCLA

Scientific methods are used to validate our results. Reproducibility is ensured by including tests for statistical significance. Investigator bias is counteracted by including double blind experiments. Conclusions are validated with multiple independent approaches.

1. Key Biological Resources.

   a. Antibodies: Purchased antibodies come with spec sheets, but they nevertheless often need further validation. Antibodies are validated by western blot analysis (for size and specificity) and immunofluorescence (for localization), using knockdown cells and positive and negative controls where applicable. No new antibodies will be generated by us for this project. Some antibodies were generated by a rabbit farm (Robert Sargent, Ramona, CA) for other projects in the lab. Those antibodies have also been validated by western blot analysis and immunofluorescence, using knockdown cells and positive and negative controls.

   b. Plasmids: DNA constructs for transfections and transgenic worms are validated by sequence analysis and by western blots analysis, immunofluorescence, or functional tests.

   c. Zebrafish Lines: The zebrafish transgene lines will be screened via standard PCR genotyping and visualized using fluorescent microscopy to confirm that expression is as expected in the correct subcellular location and cell type. If necessary, co-localization to ER will be done with an antibody against Sec61. Germline transmission will be verified by inheritance of the transgene to embryos. Morpholino studies will be controlled by (1) testing that the target protein is not detected with antibodies, (2) testing that the mRNA is down-regulated by RT-PCR, (3) verifying specificity by using both ATG and splice morpholinos as well as negative morpholino controls, and (4) testing rescue of the morpholino knockdown by co-injection of the WT mammalian mRNA for the specific gene. Knockout lines generated by CRISPR/Cas9 will be verified by PCR-based genotyping around the target locus and immunoblot analysis to verify the protein is not produced. Germline transmission will be verified by outcrossing the line to WT A/B fish and using PCR-based genotyping to confirm transmission. To confirm specificity of mutant phenotype: (1) at least two independent mutants will be generated, (2) the mutant phenotype will be transiently rescued by co-injection of the WT mRNA when possible, and (3) outcrossing to WT A/B fish to insure that CRISPR/Cas9 only targeted the intended locus.

   d. Mammalian cell lines: The lab has MEFs, HeLa cells, C2C12 cells, HEK 293T cells, and several other cell lines that are recognizable by their appearances. They have been tested for mycoplasma. New CRIPSR/Cas9 deletion mutants are validated by PCR, western blot analysis, and phenotypic characterization.

   e. Oligonucleotides for siRNA and dsRNA for injection-mediated RNAi or bacterial strains for feeding RNAi are validated by qPCR, western blot analysis, and phenotypic characterization.

2. Key Chemical Resources.

   a. All chemicals are purchased from reputable suppliers (Fisher Scientific, Sigma-Aldrich, Tocris, etc.).

   b. Inhibitors of specific enzymes, such as kinases or phosphatases, and pathways, such as oxidative phosphorylation, are validated by the phenotypes that they elicit.

EXHIBIT C

Department of Health and Human Services
National Institutes of Health
NATIONAL INSTITUTE OF NEUROLOGICAL DISORDERS AND STROKE

Notice of Award
FAIN# R01NS120690
Federal Award Date
12/19/2024

Recipient Information

1. Recipient Name
UNIVERSITY OF CALIFORNIA, LOS ANGELES
10889 WILSHIRE BLVD STE 700
LOS ANGELES, CA 90024

2. Congressional District of Recipient
36

3. Payment System Identifier (ID)
1956006143A1

4. Employer Identification Number (EIN)
956006143

5. Data Universal Numbering System (DUNS)
092530369

6. Recipient's Unique Entity Identifier
RN64EPNH8JC6

7. Project Director or Principal Investigator
ALEXANDER M VAN DER BLIEK, PHD
(Contact)
Professor
AVAN@MEDNET.UCLA.EDU
310-825-9779

8. Authorized Official
Eleanor Forbes
eleanor.forbes@research.ucla.edu
310-794-6945

Federal Agency Information

9. Awarding Agency Contact Information
Kerry Gastley

NATIONAL INSTITUTE OF NEUROLOGICAL DISORDERS AND STROKE
kerry.gastley@nih.gov
(240) 276-5472

10. Program Official Contact Information
Glen H. Nuckolls
Program Official
NATIONAL INSTITUTE OF NEUROLOGICAL DISORDERS AND STROKE
nuckollg@mail.nih.gov
301-496-5745

Federal Award Information

11. Award Number
5R01NS120690-05

12. Unique Federal Award Identification Number (FAIN)
R01NS120690

13. Statutory Authority
42 USC 241 42 CFR 52

14. Federal Award Project Title
Control of calcium flux and mitochondrial fission by the Charcot Marie Tooth disease protein Mfn2.

15. Assistance Listing Number
93.853

16. Assistance Listing Program Title
Extramural Research Programs in the Neurosciences and Neurological Disorders

17. Award Action Type
Non-Competing Continuation

18. Is the Award R&D?
Yes

Summary Federal Award Financial Information	
19. Budget Period Start Date 01/01/2025 **– End Date** 12/31/2025	
20. Total Amount of Federal Funds Obligated by this Action	$342,488
20 a. Direct Cost Amount	$225,000
20 b. Indirect Cost Amount	$117,488
21. Authorized Carryover	
22. Offset	
23. Total Amount of Federal Funds Obligated this budget period	$342,488
24. Total Approved Cost Sharing or Matching, where applicable	$0
25. Total Federal and Non-Federal Approved this Budget Period	$342,488
26. Project Period Start Date 01/01/2021 **– End Date** 12/31/2025	
27. Total Amount of the Federal Award including Approved Cost Sharing or Matching this Project Period	$2,243,240

28. Authorized Treatment of Program Income
Additional Costs

29. Grants Management Officer - Signature
Nia Pree

30. Remarks

Acceptance of this award, including the "Terms and Conditions," is acknowledged by the recipient when funds are drawn down or otherwise requested from the grant payment system.

Notice of Award

RESEARCH
Department of Health and Human Services
National Institutes of Health

NATIONAL INSTITUTE OF NEUROLOGICAL DISORDERS AND STROKE

SECTION I – AWARD DATA – 5R01NS120690-05

Principal Investigator(s):
Carla M Koehler, PHD
ALEXANDER M VAN DER BLIEK (contact), PHD

Award e-mailed to: awards@research.ucla.edu

Dear Authorized Official:

The National Institutes of Health hereby awards a grant in the amount of $342,488 (see "Award Calculation" in Section I and "Terms and Conditions" in Section III) to UNIVERSITY OF CALIFORNIA LOS ANGELES in support of the above referenced project. This award is pursuant to the authority of 42 USC 241 42 CFR 52 and is subject to the requirements of this statute and regulation and of other referenced, incorporated or attached terms and conditions.

Acceptance of this award, including the "Terms and Conditions," is acknowledged by the recipient when funds are drawn down or otherwise requested from the grant payment system.

Each publication, press release, or other document about research supported by an NIH award must include an acknowledgment of NIH award support and a disclaimer such as "Research reported in this publication was supported by the National Institute Of Neurological Disorders And Stroke of the National Institutes of Health under Award Number R01NS120690. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health." Prior to issuing a press release concerning the outcome of this research, please notify the NIH awarding IC in advance to allow for coordination.

Award recipients must promote objectivity in research by establishing standards that provide a reasonable expectation that the design, conduct and reporting of research funded under NIH awards will be free from bias resulting from an Investigator's Financial Conflict of Interest (FCOI), in accordance with the 2011 revised regulation at 42 CFR Part 50 Subpart F. The Institution shall submit all FCOI reports to the NIH through the eRA Commons FCOI Module. The regulation does not apply to Phase I Small Business Innovative Research (SBIR) and Small Business Technology Transfer (STTR) awards. Consult the NIH website http://grants.nih.gov/grants/policy/coi/ for a link to the regulation and additional important information.

If you have any questions about this award, please direct questions to the Federal Agency contacts.

Sincerely yours,

Nia Pree
Grants Management Officer
NATIONAL INSTITUTE OF NEUROLOGICAL DISORDERS AND STROKE

Additional information follows

Cumulative Award Calculations for this Budget Period (U.S. Dollars)

Federal Direct Costs	$225,000
Federal F&A Costs	$117,488
Approved Budget	$342,488
Total Amount of Federal Funds Authorized (Federal Share)	$342,488
TOTAL FEDERAL AWARD AMOUNT	$342,488
AMOUNT OF THIS ACTION (FEDERAL SHARE)	$342,488

SUMMARY TOTALS FOR ALL YEARS (for this Document Number)		
YR	**THIS AWARD**	**CUMULATIVE TOTALS**
5	$342,488	$342,488

Fiscal Information:
Payment System Identifier: 1956006143A1
Document Number: RNS120690A
PMS Account Type: P (Subaccount)
Fiscal Year: 2025

IC	CAN	2025
NS	8472428	$342,488

NIH Administrative Data:
PCC: NUCKOGNG / **OC**: 41025 / **Released**: 12/19/2024
Award Processed: 12/20/2024 12:24:06 AM

SECTION II – PAYMENT/HOTLINE INFORMATION – 5R01NS120690-05

For payment and HHS Office of Inspector General Hotline information, see the NIH Home Page at http://grants.nih.gov/grants/policy/awardconditions.htm

SECTION III – STANDARD TERMS AND CONDITIONS – 5R01NS120690-05

This award is based on the application submitted to, and as approved by, NIH on the above-titled project and is subject to the terms and conditions incorporated either directly or by reference in the following:

a. The grant program legislation and program regulation cited in this Notice of Award.
b. Conditions on activities and expenditure of funds in other statutory requirements, such as those included in appropriations acts.
c. 45 CFR Part 75.
d. National Policy Requirements and all other requirements described in the NIH Grants Policy Statement, including addenda in effect as of the beginning date of the budget period.
e. Federal Award Performance Goals: As required by the periodic report in the RPPR or in the final progress report when applicable.
f. This award notice, INCLUDING THE TERMS AND CONDITIONS CITED BELOW.

(See NIH Home Page at http://grants.nih.gov/grants/policy/awardconditions.htm for certain references cited above.)

Research and Development (R&D): All awards issued by the National Institutes of Health (NIH) meet the definition of "Research and Development" at 45 CFR Part§ 75.2**.** As such, auditees should identify NIH awards as part of the R&D cluster on the Schedule of Expenditures of Federal Awards (SEFA). The auditor should test NIH awards for compliance as instructed in Part V, Clusters of Programs. NIH recognizes that some awards may have another classification for purposes of indirect costs. The auditor is not required to report the disconnect (i.e., the award is classified as R&D for Federal Audit Requirement purposes but non-research for indirect cost rate purposes), unless the auditee is charging indirect costs at a rate other than the rate(s) specified in the award document(s).

This institution is a signatory to the Federal Demonstration Partnership (FDP) Phase VII Agreement which requires active institutional participation in new or ongoing FDP demonstrations and pilots.

An unobligated balance may be carried over into the next budget period without Grants Management Officer prior approval.

This grant is subject to Streamlined Noncompeting Award Procedures (SNAP).

This award is subject to the requirements of 2 CFR Part 25 for institutions to obtain a unique entity identifier (UEI) and maintain an active registration in the System for Award Management (SAM). Should a consortium/subaward be issued under this award, a UEI requirement must be included. See http://grants.nih.gov/grants/policy/awardconditions.htm for the full NIH award term implementing this requirement and other additional information.

This award has been assigned the Federal Award Identification Number (FAIN) R01NS120690. Recipients must document the assigned FAIN on each consortium/subaward issued under this award.

Based on the project period start date of this project, this award is likely subject to the Transparency Act subaward and executive compensation reporting requirement of 2 CFR Part 170. There are conditions that may exclude this award; see http://grants.nih.gov/grants/policy/awardconditions.htm for additional award applicability information.

In accordance with P.L. 110-161, compliance with the NIH Public Access Policy is now mandatory. For more information, see NOT-OD-08-033 and the Public Access website: http://publicaccess.nih.gov/.

This award represents the final year of the competitive segment for this grant. See the NIH Grants Policy Statement Section 8.6 Closeout for complete closeout requirements at: http://grants.nih.gov/grants/policy/policy.htm#gps.

A final expenditure Federal Financial Report (FFR) (SF 425) must be submitted through the Payment Management System (PMS) within 120 days of the period of performance end date; see the NIH Grants Policy Statement Section 8.6.1 Financial Reports, http://grants.nih.gov/grants/policy/policy.htm#gps, for additional information on this submission requirement. The final FFR must indicate the exact balance of unobligated funds and may not reflect any unliquidated obligations. There must be no discrepancies between the final FFR expenditure data and the real-time cash drawdown data in PMS. NIH will close the awards using the last recorded cash drawdown level in PMS for awards that do not require a final FFR on expenditures. It is important to note that for financial closeout, if a grantee fails to submit a required final expenditure FFR, NIH will close the grant using the last recorded cash drawdown level.

A Final Invention Statement and Certification form (HHS 568), (not applicable to training, construction, conference or cancer education grants) must be submitted within 120 days of the expiration date. The HHS 568 form may be downloaded at: http://grants.nih.gov/grants/forms.htm. This paragraph does not apply to Training grants, Fellowships, and certain other programs—i.e., activity codes C06, D42, D43, D71, DP7, G07, G08, G11, K12, K16, K30, P09, P40, P41, P51, R13, R25, R28, R30, R90, RL5, RL9, S10, S14, S15, U13, U14, U41, U42, U45, UC6, UC7, UR2, X01, X02.

Unless an application for competitive renewal is submitted, a Final Research Performance Progress Report (Final RPPR) must also be submitted within 120 days of the period of performance end date. If a competitive renewal application is submitted prior to that date, then an Interim RPPR must be submitted by that date as well. Instructions for preparing an Interim or Final RPPR are at: https://grants.nih.gov/grants/rppr/rppr_instruction_guide.pdf. Any other specific requirements set forth in the terms and conditions of the award must also be addressed in the Interim or Final RPPR. *Note that data reported within Section I of the Interim and Final RPPR forms will be made public and should be written for a lay person audience*.

NIH requires electronic submission of the final invention statement through the Closeout feature in the Commons.

NOTE: If this is the final year of a competitive segment due to the transfer of the grant to another institution, then a Final RPPR is not required. However, a final expenditure FFR is required and must be submitted electronically as noted above. If not already submitted, the Final Invention Statement is required and should be sent directly to the assigned Grants Management Specialist.

Recipients must administer the project in compliance with federal civil rights laws that prohibit discrimination on the basis of race, color, national origin, disability, age, and comply with applicable conscience protections. The recipient will comply with applicable laws that prohibit discrimination on the basis of sex, which includes discrimination on the basis of gender identity, sexual orientation, and pregnancy. Compliance with these laws requires taking reasonable steps to provide meaningful access to persons with limited English proficiency and providing programs that are accessible to and usable by persons with disabilities. The HHS Office for Civil Rights provides guidance on complying with civil rights laws enforced by HHS. See https://www.hhs.gov/civil-rights/for-providers/provider-obligations/index.html and https://www.hhs.gov/.

- Recipients of FFA must ensure that their programs are accessible to persons with limited English proficiency. For guidance on meeting the legal obligation to take reasonable steps to ensure meaningful access to programs or activities by limited English proficient individuals, see https://www.hhs.gov/civil-rights/for-individuals/special-topics/limited-english-proficiency/fact-sheet-guidance/index.html and https://www.lep.gov.
- For information on an institution's specific legal obligations for serving qualified individuals with disabilities, including providing program access, reasonable modifications, and to provide effective communication, see http://www.hhs.gov/ocr/civilrights/understanding/disability/index.html.
- HHS funded health and education programs must be administered in an environment free of sexual harassment; see https://www.hhs.gov/civil-rights/for-individuals/sex-discrimination/index.html. For information about NIH's commitment to supporting a safe and respectful work environment, who to contact with questions or concerns, and what NIH's expectations are for institutions and the individuals supported on NIH-funded awards, please see https://grants.nih.gov/grants/policy/harassment.htm.
- For guidance on administering programs in compliance with applicable federal religious nondiscrimination laws and applicable federal conscience protection and associated anti-discrimination laws, see https://www.hhs.gov/conscience/conscience-protections/index.html and https://www.hhs.gov/conscience/religious-freedom/index.html.

In accordance with the regulatory requirements provided at 45 CFR 75.113 and Appendix XII to 45 CFR Part 75, recipients that have currently active Federal grants, cooperative agreements, and procurement contracts with cumulative total value greater than $10,000,000 must report and maintain information in the System for Award Management (SAM) about civil, criminal, and administrative proceedings in connection with the award or performance of a Federal award that reached final disposition within the most recent five-year period. The recipient must also make semiannual disclosures regarding such proceedings. Proceedings information will be made publicly available in the designated integrity and performance system (currently the Federal Awardee Performance and Integrity Information System (FAPIIS)). Full reporting requirements and procedures are found in Appendix XII to 45 CFR Part 75. This term does not apply to NIH fellowships.

Treatment of Program Income:
Additional Costs

SECTION IV – NS SPECIFIC AWARD CONDITIONS – 5R01NS120690-05

Clinical Trial Indicator: No
This award does not support any NIH-defined Clinical Trials. See the NIH Grants Policy Statement Section 1.2 for NIH definition of Clinical Trial.

STANDARD TERMS AND CONDITIONS OF AWARD

This award provides funding in response to PAS -18-483. These funds are to be administered in accordance with the guidelines described in this specific funding opportunity.

The funds in this award shall not be used to pay the salary of an individual at a rate in excess of Executive Level II. Please see the Salary Cap Summary (FY 1990 - Present) for rates and effective dates.

In accordance with the NINDS Funding Strategy, NINDS does not provide funds for inflationary increases. Where applicable, future years have been adjusted.

The recipient is required to follow the model organism sharing plan included in the grant application and may not implement any changes in the plan without NINDS staff approval.

FUNDING

NIH is currently funded through a Continuing Resolution. This award has been issued at 90% of the level indicated in the previous Notice of Award. Upward adjustments to awarded levels will be considered after our FY 2025 appropriations are enacted.

FOREIGN COMPONENTS

This award includes collaboration with Björn Stork in Germany. Contact the Grants Management Specialist listed below if the collaboration changes.

This award includes collaboration with Israel Sekler in Israel. Contact the Grants Management Specialist listed below if the collaboration changes.

SPREADSHEET SUMMARY
AWARD NUMBER: 5R01NS120690-05

INSTITUTION: UNIVERSITY OF CALIFORNIA LOS ANGELES

Budget	Year 5
TOTAL FEDERAL DC	$225,000
TOTAL FEDERAL F&A	$117,488
TOTAL COST	$342,488

Facilities and Administrative Costs	Year 5
F&A Cost Rate 1	56%
F&A Cost Base 1	$209,800
F&A Costs 1	$117,488

EXHIBIT D

Outlook

OCGA Notice of Award Action: Van Der Bliek, Alexander M [20204267]

From UCLA Research Admin <DoNotReply@research.ucla.edu>
Date Fri 1/10/2025 1:02 AM
To Van der Bliek, Alexander <AVan@mednet.ucla.edu>; Biol Chem ORA <BiolChemORA@mednet.ucla.edu>
Cc awardsafe@research.ucla.edu <awardsafe@research.ucla.edu>; EFMTeam3@research.ucla.edu <EFMTeam3@research.ucla.edu>; Alabastro, Kady <KAlabastro@mednet.ucla.edu>; PATSRecords@research.ucla.edu <PATSRecords@research.ucla.edu>

1 attachment (27 KB)
UCLA_RA_CGA_logo.png;

UCLA OCGA Notification of Award Action

Principal Investigator: Van Der Bliek, Alexander M
Department: BIOLOGICAL CHEMISTRY[1445]
Project Title: Control of Calcium Flux and Mitochondrial Fission by the Charcot Marie Tooth Disease Protein Mfn2.

Sponsor: NIH-NINDS National Institute of Neurological Disorders and Stroke
Sponsor Award Number: 5R01NS120690-05
Fund Number:
UCLA PATS Record Number: 20204267
Award Type: Grant
Action: Continuation

Funds Awarded this Action: $342,488
Total Funds Awarded to Date: $2,243,240
Current Budget Period: 1/1/2025 - 12/31/2025
Project Period: 1/1/2021 - 12/31/2025

On behalf of the Office of Contract and Grant Administration (OCGA) within the Office of Research Administration (ORA), the above-referenced document is fully executed.

Please review and adhere to all terms and conditions in your award document, paying particular attention to the technical and financial reporting requirements in the grant, contract and/or sponsor guidelines.

Please also use our additional on-line tools to view valuable information about your award:

- Research Portal (http://portal.research.ucla.edu): You can now view a real-time updated copy of your award snapshot and award terms and conditions using this tool.

- PI Portal (http://piportal.research.ucla.edu): You can view expense information, including a real time fund balance for your award using this tool.

Please note, for awards involving more than one F&A rate, you may receive a separate notification related to the portion of the award associated with each rate.

If you have any questions, please contact:

Perez, Samuel
sperez@research.ucla.edu
--

If you have any non-financial post-award related questions, please contact your OCGA Analyst or Officer directly: http://ora.research.ucla.edu/OCGA/Pages/Contact-Us/contact-us-home.aspx

Thank you,

OCGA Award Intake Team
Office of Contract and Grant Administration
10889 Wilshire Boulevard, Suite 700
Los Angeles, CA 90095
ora.research.ucla.edu/ocga

EXHIBIT E

Outlook

Grant Suspension Notice - Stop Work Order [PATS 20204267]

From UCLA Research Admin <DoNotReply@research.ucla.edu>
Date Fri 8/1/2025 6:24 PM
To Van der Bliek, Alexander <avan@mednet.ucla.edu>
Cc Alabastro, Kady <kalabastro@mednet.ucla.edu>; Shuch, Maria [ORA/OCGA] <maria.shuch@research.ucla.edu>; nancy.torres@research.ucla.edu <nancy.torres@research.ucla.edu>; PATSRecords@research.ucla.edu <PATSRecords@research.ucla.edu>

Stop Work Notice

Award #: R01NS120690
Title: Control of Calcium Flux and Mitochondrial Fission by the Charcot Marie Tooth Disease Protein Mfn2.
PATS #: 20204267
Fund #(s): 31574

Professor Van Der Bliek,

UCLA has received a suspension notice from NIH-NINDS National Institute of Neurological Disorders and Stroke for the above referenced project.

This email is to notify you to ***immediately stop incurring costs/expenditures on the grant(s) referenced above effective July 31, 2025***.

If your grant includes active subawards, OCGA will be writing to the subawardee's administrative contact with formal notice of the subaward suspension and the requirement to stop immediately all expenditures against the subaward. You may also want to separately reach out to your collaborator to provide additional context.

UCLA is required to submit to the sponsor, within 30 days of this suspension, a financial report of expenditures through July 31, 2025. OCGA will request that the subawardee submit to you, within 15 days of the notice, an invoice for expenses incurred to date so that we can include those expenses in our report to the sponsor. Extramural Fund Management (EFM) will seek the support of your fund manager to prepare a complete and accurate financial report of expenses incurred through July 31, 2025.

We are saddened that this has happened and echo the sentiments expressed in the recent communications from Chanceller Frenk and Vice Chancellor for Research Wakimoto. Campus leadership is actively engaged in working to resolve these issues. Updates will be shared as they become available. For questions regarding the suspension, please contact awards@research.ucla.edu or reach out to me directly. For financial or reimbursement-related inquiries, reach out to your EFM contact.

ACTION REQUIRED

Please:

1. Forward any communications you may receive from the federal sponsor related to this suspension to OCGA at awards@research.ucla.edu.
2. Work with your fund manager or financial staff to ensure all expenditures are reported and subaward invoices are approved.

We understand this is a stressful time, and we appreciate your dedication to research excellence at UCLA.

Tracey Fraser
Senior Director
UCLA Office of Contract & Grant Administration
10889 Wilshire Boulevard, Suite 700
Los Angeles, CA 90095-1406
T: (310) 825-0671 | **E:** tracey.fraser@research.ucla.edu
https://ocga.research.ucla.edu/