

METHODS IN MOLECULAR BIOLOGY

Series Editor

John M. Walker

School of Life and Medical Sciences

University of Hertfordshire

Hatfield, Hertfordshire, AL10 9AB, UK

For further volumes:

<http://www.springer.com/series/7651>

The Ubiquitin Proteasome System

Methods and Protocols

Edited by

Thibault Mayor

*Department of Biochemistry and Molecular Biology, Michael Smith Laboratories,
University of British Columbia, Vancouver, BC, Canada*

Gary Kleiger

Department of Chemistry and Biochemistry, University of Nevada Las Vegas, Las Vegas, NV, USA

Editors

Thibault Mayor
Department of Biochemistry
and Molecular Biology
Michael Smith Laboratories
University of British Columbia
Vancouver, BC, Canada

Gary Kleiger
Department of Chemistry
and Biochemistry
University of Nevada Las Vegas
Las Vegas, NV, USA

ISSN 1064-3745 ISSN 1940-6029 (electronic)
Methods in Molecular Biology
ISBN 978-1-4939-8705-4 ISBN 978-1-4939-8706-1 (eBook)
<https://doi.org/10.1007/978-1-4939-8706-1>

Library of Congress Control Number: 2018952458

© Springer Science+Business Media, LLC, part of Springer Nature 2018

This work is subject to copyright. All rights are reserved by the Publisher, whether the whole or part of the material is concerned, specifically the rights of translation, reprinting, reuse of illustrations, recitation, broadcasting, reproduction on microfilms or in any other physical way, and transmission or information storage and retrieval, electronic adaptation, computer software, or by similar or dissimilar methodology now known or hereafter developed.

The use of general descriptive names, registered names, trademarks, service marks, etc. in this publication does not imply, even in the absence of a specific statement, that such names are exempt from the relevant protective laws and regulations and therefore free for general use.

The publisher, the authors, and the editors are safe to assume that the advice and information in this book are believed to be true and accurate at the date of publication. Neither the publisher nor the authors or the editors give a warranty, express or implied, with respect to the material contained herein or for any errors or omissions that may have been made. The publisher remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

This Humana Press imprint is published by the registered company Springer Science+Business Media, LLC part of Springer Nature.

The registered company address is: 233 Spring Street, New York, NY 10013, U.S.A.

Preface

In 1975, a paper was published in the *Proceedings of the National Academy of Sciences* describing a newly discovered polypeptide capable of inducing the differentiation of T cells [1]. The authors named this protein ubiquitous immunopoietic polypeptide (UBIP) “because it is widespread and perhaps even universally represented in living cells. . . .” It soon became clear that this polypeptide, later renamed ubiquitin, is a key signaling molecule in eukaryotic cell biology. Indeed, approximately 5% of human genes are dedicated towards the expression of enzymes and factors that control the ubiquitin proteasome system (UPS), a massive network of integrated pathways responsible for regulating protein degradation, protein localization, and enzyme activity. The pioneering research of Aaron Ciechanover, Avram Hershko, Irwin Rose, and Alex Varshavsky has led to a field that continues to make new and important discoveries regarding how cells function. Greater than 54,000 articles have been indexed in the US National Library of Medicine PubMed database that contain the word “ubiquitin” in either the title or abstract (Fig. 1). The UPS has gifted both biotech and pharmaceutical companies with fresh, new targets for the treatment of various human diseases. Some five drugs have been approved in the United States by the Federal Drug Administration that target enzymes in the UPS, with the promise of many more in the not too distant future. The ubiquitin field continues to grow and change at a dizzying pace with significant advances for both newer methodologies—such as mass spectrometry-based proteomics and cryo-electron microscopy—and the more traditional biochemical, cell biological, and structural analyses and assays. This volume is designed for both veterans in the field wanting to expand their bag of tricks and for novices just getting started.

The methods have been organized in five parts. In the first part, five methods are described that can be applied to the determination of the mechanisms of action for ubiquitin-conjugating enzymes (E2s), ubiquitin ligases (E3s), and de-ubiquitylating

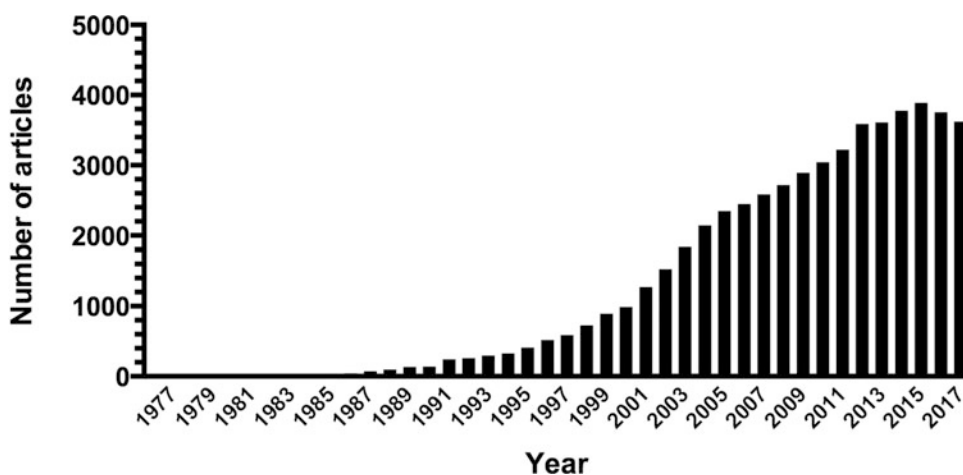


Fig. 1 Publishing in the ubiquitin field: still going strong!

(DUB) enzymes. There are approximately 35 E2s, and perhaps as many as 600 E3s and 90 DUBs in the human proteome, with the vast majority awaiting in-depth characterization. These chapters will be helpful for any researcher aiming to reconstitute *in vitro* ubiquitylation as a means of uncovering E2 and/or E3 function, or to infer the affinity of a DUB for its substrate.

In the second part of this volume, six methods are described for new technological advances that will enable the researcher to (1) assemble and purify poly-ubiquitin chains that are uniform with respect to linkage type in quantities sufficient for biochemical and structural analyses; (2) discover the binding partners of essentially any protein in the UPS; (3) screen for ubiquitin variants with unique properties that can modulate enzymes in the UPS within the context of living cells; (4) discover the unique amino acid sequence signatures that target proteins to be recognized and degraded by the UPS; (5) use flow cytometry towards identifying the UPS enzymes responsible for protein turnover in a high-throughput manner; and (6) identify and characterize ubiquitylation pathways using the easy to work with model organism *E. coli* that, lacking the UPS, optimizes the researcher's chances for success.

In the third part, cutting-edge methods are described for three areas in structural biology: X-ray crystallography, small-angle X-ray scattering (SAXS), and cryo-electron microscopy. Methods to trap dynamic multi-subunit complexes for structural characterization using protein cross-linking are described (while this method is specific to the ubiquitin-like protein SUMO, the similarities between the ubiquitin and SUMO cascades are so great that these methods will undoubtedly prove useful for members of the ubiquitin field). It is often desirable to characterize the structures of UPS enzymes in solution, and SAXS is a powerful and relatively simple method described here to achieve this goal. While cryo-electron microscopy has enabled the determination of the structures of large, multi-subunit complexes to near or at atomic resolution, one challenge has been the preparation of the grids where macromolecules are placed prior to freezing. A detailed protocol for this procedure has been included.

In the fourth part, seven chapters are dedicated to methodologies to study the 26S proteasome, the multi-subunit complex that recognizes and degrades ubiquitylated proteins. These chapters cover (1) the expression and purification of the 19S proteasome regulatory complex in *E. coli*, as well as the ability to introduce chemical modifications to surface residues on any given subunit; (2) the use of native gel electrophoresis to separate and study the proteasome during various stages of assembly; (3) the use of isotopic pulse-chase to directly measure the rates of protein degradation within living cells; (4) three unique and complementary methods to purify proteasomes from cells either grown in isolation or from intact tissues; (5) several quantitative *in vitro* methods for assaying the sequential activities of the 26S proteasome; (6) methods to assess the functional impact of proteasome phosphorylation; and (7) novel proteasome substrates that enable the measurement of protein degradation in a high-throughput manner.

The fifth part is dedicated to cutting-edge proteomic techniques that are first summarized in a review and then covered in three chapters describing methods to (1) identify ubiquitination sites on endogenous proteins from any eukaryotic organism or tissue; (2) enrich for poly-ubiquitin chains with specific linkage types for mass spectrometry analysis; and (3) employ cross-linking to determine domain topology within a multi-subunit complex such as the proteasome.

We are, first and foremost, most grateful to all of our colleagues that contributed to this volume. While it is undoubtedly true that many researchers consider their research areas as

special, we are constantly taken aback by the generosity and good will of the investigators that make the ubiquitin field their home. We are grateful to Springer for the opportunity to feature our work through this book series and to John Walker for his dedicated help in assembling this book. Lastly, we would like to dedicate this volume to our postdoctoral advisor, Ray Deshaies, as well as his long-time research associate, Rati Verma. This volume would not have been possible without their expert mentorship and friendship.

Vancouver, BC, Canada
Las Vegas, NV, USA

Thibault Mayor
Gary Kleiger

Reference

1. Goldstein G, Scheid M, Hammerling U, Schlesinger DH, Niall HD, Boyse EA (1975) Isolation of a polypeptide that has lymphocyte-differentiating properties and is probably represented universally in living cells. Proc Natl Acad Sci U S A 72(1):11–15

Contents

<i>Preface</i>	<i>v</i>
<i>Contributors</i>	<i>ix</i>

PART I METHODS TO UNCOVER THE MECHANISMS OF ACTION FOR ENZYMES THAT ASSEMBLE OR DISASSEMBLE POLY-UBIQUITIN CHAINS

1	Characterization of RING-Between-RING E3 Ubiquitin Transfer Mechanisms	3
	<i>Katherine H. Reiter and Rachel E. Klevit</i>	
2	Single-Turnover RING/U-Box E3-Mediated Lysine Discharge Assays	19
	<i>Lori Buetow, Mads Gabrielsen, and Danny T. Huang</i>	
3	Methods for NAD-Dependent Ubiquitination Catalyzed by <i>Legionella pneumophila</i> Effector Proteins	33
	<i>Jiazhang Qiu and Zhao-Qing Luo</i>	
4	Using In Vitro Ubiquitylation Assays to Estimate the Affinities of Ubiquitin-Conjugating Enzymes for Their Ubiquitin Ligase Partners	39
	<i>Spencer Hill, Connor Hill, and Gary Kleiger</i>	
5	Competition Assay for Measuring Deubiquitinating Enzyme Substrate Affinity	59
	<i>Michael T. Morgan and Cynthia Wolberger</i>	

PART II TOOLS TO STUDY UBIQUITYLATION

6	Enzymatic Assembly of Ubiquitin Chains	73
	<i>Martin A. Michel, David Komander, and Paul R. Elliott</i>	
7	Ubiquitin-Activated Interaction Traps (UBAITs): Tools for Capturing Protein-Protein Interactions	85
	<i>Hazel F. O'Connor, Caleb D. Swaim, Larissa A. Canadeo, and Jon M. Huibregtse</i>	
8	Generating Intracellular Modulators of E3 Ligases and Deubiquitinases from Phage-Displayed Ubiquitin Variant Libraries	101
	<i>Wei Zhang and Sachdev S. Sidhu</i>	
9	Integrated Proteogenomic Approach for Identifying Degradation Motifs in Eukaryotic Cells	121
	<i>Yifat Geffen, Alon Appleboim, Richard G. Gardner, and Tommer Ravid</i>	
10	A Method to Monitor Protein Turnover by Flow Cytometry and to Screen for Factors that Control Degradation by Fluorescence-Activated Cell Sorting	137
	<i>Sophie A. Comyn and Thibault Mayor</i>	

11	<i>E. coli</i> -Based Selection and Expression Systems for Discovery, Characterization, and Purification of Ubiquitylated Proteins	155
	<i>Olga Levin-Kravets, Tal Keren-Kaplan, Ilan Attali, Itai Sharon, Neta Tanner, Dar Shapira, Ritu Rath, Avinash Persaud, Noa Shohat, Anna Shusterman, and Gali Prag</i>	
PART III STRUCTURAL APPROACHES AS APPLIED TO ENZYMES THAT PARTICIPATE IN THE UBIQUITIN PROTEASOME SYSTEM		
12	Strategies to Trap Enzyme-Substrate Complexes that Mimic Michaelis Intermediates During E3-Mediated Ubiquitin-Like Protein Ligation.	169
	<i>Frederick C. Streich Jr. and Christopher D. Lima</i>	
13	Small-Angle X-Ray Scattering for the Study of Proteins in the Ubiquitin Pathway	197
	<i>Jean-François Trempe and Kalle Gebring</i>	
14	Methods for Preparing Cryo-EM Grids of Large Macromolecular Complexes	209
	<i>Leifu Chang and David Barford</i>	
PART IV METHODS TO STUDY 26S PROTEASOME FUNCTION		
15	Recombinant Expression, Unnatural Amino Acid Incorporation, and Site-Specific Labeling of 26S Proteasomal Subcomplexes	219
	<i>Jared A.M. Bard and Andreas Martin</i>	
16	Native Gel Approaches in Studying Proteasome Assembly and Chaperones	237
	<i>Jeroen Roelofs, Anjana Suppahia, Kenrick A. Waite, and Soyeon Park</i>	
17	Measuring the Overall Rate of Protein Breakdown in Cells and the Contributions of the Ubiquitin-Proteasome and Autophagy-Lysosomal Pathways	261
	<i>Zhe Sha, Jinghui Zhao, and Alfred L. Goldberg</i>	
18	Methods to Rapidly Prepare Mammalian 26S Proteasomes for Biochemical Analysis	277
	<i>Chueh-Ling Kuo, Galen Andrew Collins, and Alfred L. Goldberg</i>	
19	Measurement of the Multiple Activities of 26S Proteasomes.	289
	<i>Hyoung Tae Kim, Galen Andrew Collins, and Alfred L. Goldberg</i>	
20	Exploring the Regulation of Proteasome Function by Subunit Phosphorylation	309
	<i>Jordan J.S. VerPlank and Alfred L. Goldberg</i>	
21	Scalable In Vitro Proteasome Activity Assay	321
	<i>Amit Kumar Singh Gautam, Kirby Martinez-Fonts, and Andreas Matouschek</i>	

PART V PROTEOMIC METHODS TO STUDY THE UBIQUITIN
 PROTEASOME SYSTEM

22 Exploring the Rampant Expansion of Ubiquitin Proteomics 345
Amalia Rose and Thibault Mayor

23 Ubiquitin diGLY Proteomics as an Approach to Identify
 and Quantify the Ubiquitin-Modified Proteome..... 363
Amit Fulzele and Eric J. Bennett

24 Interpreting the Language of Polyubiquitin with Linkage-Specific
 Antibodies and Mass Spectrometry..... 385
*Marissa L. Matsumoto, Erick R. Castellanos, Yi Jimmy Zeng,
 and Donald S. Kirkpatrick*

25 Dissecting Dynamic and Heterogeneous Proteasome Complexes
 Using In Vivo Cross-Linking-Assisted Affinity Purification
 and Mass Spectrometry 401
Xiaorong Wang and Lan Huang

Index 411

Contributors

- ALON APPLEBOIM • *Department of Biological Chemistry, The Alexander Silberman Institute of Life Sciences, The Hebrew University of Jerusalem, Jerusalem, Israel; School of Computer Science and Engineering, The Hebrew University of Jerusalem, Jerusalem, Israel*
- ILAN ATTALI • *Department of Biochemistry and Molecular Biology, Institute of Structural Biology, George S. Wise Faculty of Life Sciences, Tel Aviv University, Tel Aviv, Israel*
- JARED A. M. BARD • *Department of Molecular and Cell Biology and California Institute for Quantitative Biosciences, University of California at Berkeley, Berkeley, CA, USA*
- DAVID BARFORD • *MRC Laboratory of Molecular Biology, Cambridge, UK*
- ERIC J. BENNETT • *Section of Cell and Developmental Biology, Division of Biological Sciences, University of California, San Diego, La Jolla, CA, USA*
- LORI BUETOW • *Cancer Research UK Beatson Institute, Glasgow, UK; Institute of Cancer Sciences, University of Glasgow, Glasgow, UK*
- LARISSA A. CANADEO • *Department of Molecular Biosciences, University of Texas at Austin, Austin, TX, USA*
- ERICK R. CASTELLANOS • *Department of Structural Biology, Genentech Inc., South San Francisco, CA, USA*
- LEIFU CHANG • *MRC Laboratory of Molecular Biology, Cambridge, UK*
- GALEN ANDREW COLLINS • *Department of Cell Biology, Harvard Medical School, Boston, MA, USA*
- SOPHIE A. COMYN • *Department of Biochemistry and Molecular Biology, Michael Smith Laboratories, University of British Columbia, Vancouver, BC, Canada*
- PAUL R. ELLIOTT • *Division of Protein and Nucleic Acid Chemistry, MRC Laboratory of Molecular Biology, Cambridge, UK*
- AMIT FULZELE • *Section of Cell and Developmental Biology, Division of Biological Sciences, University of California, San Diego, La Jolla, CA, USA*
- MADS GABRIELSEN • *Cancer Research UK Beatson Institute, Glasgow, UK; Institute of Cancer Sciences, University of Glasgow, Glasgow, UK*
- RICHARD G. GARDNER • *Department of Pharmacology, University of Washington, Seattle, WA, USA*
- YIFAT GEFFEN • *Department of Biological Chemistry, The Alexander Silberman Institute of Life Sciences, The Hebrew University of Jerusalem, Jerusalem, Israel; School of Computer Science and Engineering, The Hebrew University of Jerusalem, Jerusalem, Israel*
- KALLE GEHRING • *Department of Biochemistry, McGill University, Montréal, QC, Canada*
- ALFRED L. GOLDBERG • *Department of Cell Biology, Harvard Medical School, Boston, MA, USA*
- CONNOR HILL • *Department of Chemistry and Biochemistry, University of Nevada, Las Vegas, NV, USA*
- SPENCER HILL • *Department of Chemistry and Biochemistry, University of Nevada, Las Vegas, NV, USA*
- DANNY T. HUANG • *Cancer Research UK Beatson Institute, Glasgow, UK; Institute of Cancer Sciences, University of Glasgow, Glasgow, UK*
- LAN HUANG • *Department of Physiology and Biophysics, University of California, Irvine, CA, USA*

- JON M. HUIBREGTSE • *Department of Molecular Biosciences, University of Texas at Austin, Austin, TX, USA*
- TAL KEREN-KAPLAN • *Department of Biochemistry and Molecular Biology, Institute of Structural Biology, George S. Wise Faculty of Life Sciences, Tel Aviv University, Tel Aviv, Israel*
- HYOUNG TAE KIM • *Department of Cell Biology, Harvard Medical School, Boston, MA, USA*
- DONALD S. KIRKPATRICK • *Department of Microchemistry, Proteomics and Lipidomics, Genentech, Inc., South San Francisco, CA, USA*
- GARY KLEIGER • *Department of Chemistry and Biochemistry, University of Nevada, Las Vegas, NV, USA*
- RACHEL E. KLEVIT • *Department of Biochemistry, University of Washington, Seattle, WA, USA*
- DAVID KOMANDER • *Division of Protein and Nucleic Acid Chemistry, MRC Laboratory of Molecular Biology, Cambridge, UK*
- CHUEH-LING KUO • *Department of Cell Biology, Harvard Medical School, Boston, MA, USA*
- OLGA LEVIN-KRAVETS • *Department of Biochemistry and Molecular Biology, Institute of Structural Biology, George S. Wise Faculty of Life Sciences, Tel Aviv University, Tel Aviv, Israel*
- CHRISTOPHER D. LIMA • *Structural Biology Program, Sloan Kettering Institute, Memorial Sloan Kettering Cancer Center, New York, NY, USA; Howard Hughes Medical Institute, New York, NY, USA*
- ZHAO-QING LUO • *Department of Biological Sciences, Purdue Institute for Inflammation, Immunology and Infectious Disease, Purdue University, West Lafayette, IN, USA*
- ANDREAS MARTIN • *Department of Molecular and Cell Biology and California Institute for Quantitative Biosciences, University of California at Berkeley, Berkeley, CA, USA; Howard Hughes Medical Institute, University of California at Berkeley, Berkeley, CA, USA*
- KIRBY MARTINEZ-FONTS • *Department of Molecular Biosciences, The University of Texas at Austin, Austin, TX, USA*
- ANDREAS MATOUSCHEK • *Department of Molecular Biosciences, The University of Texas at Austin, Austin, TX, USA*
- MARISSA L. MATSUMOTO • *Department of Structural Biology, Genentech Inc., South San Francisco, CA, USA*
- THIBAUT MAYOR • *Department of Biochemistry and Molecular Biology, Michael Smith Laboratories, University of British Columbia, Vancouver, BC, Canada*
- MARTIN A. MICHEL • *Division of Protein and Nucleic Acid Chemistry, MRC Laboratory of Molecular Biology, Cambridge, UK*
- MICHAEL T. MORGAN • *Department of Biophysics and Biophysical Chemistry, Johns Hopkins University School of Medicine, Baltimore, MD, USA*
- HAZEL F. O'CONNOR • *Department of Molecular Biosciences, University of Texas at Austin, Austin, TX, USA*
- SOYEON PARK • *Department of Molecular, Cellular, and Developmental Biology, University of Colorado Boulder, Boulder, CO, USA*
- AVINASH PERSAUD • *Department of Biochemistry and Molecular Biology, Institute of Structural Biology, George S. Wise Faculty of Life Sciences, Tel Aviv University, Tel Aviv, Israel*
- GALI PRAG • *Department of Biochemistry and Molecular Biology, Institute of Structural Biology, George S. Wise Faculty of Life Sciences, Tel Aviv University, Tel Aviv, Israel*

- JIAZHANG QIU • *Key Laboratory of Zoonosis, Ministry of Education, College of Veterinary Medicine, Jilin University, Changchun, China*
- RITU RATHI • *Department of Biochemistry and Molecular Biology, Institute of Structural Biology, George S. Wise Faculty of Life Sciences, Tel Aviv University, Tel Aviv, Israel*
- TOMMER RAVID • *Department of Biological Chemistry, The Alexander Silberman Institute of Life Sciences, The Hebrew University of Jerusalem, Jerusalem, Israel*
- KATHERINE H. REITER • *Department of Biochemistry, University of Washington, Seattle, WA, USA*
- JEROEN ROELOFS • *Division of Biology, Kansas State University, Manhattan, KS, USA*
- AMALIA ROSE • *Department of Biochemistry and Molecular Biology, Michael Smith Laboratories, University of British Columbia, Vancouver, BC, Canada*
- ZHE SHA • *Department of Cell Biology, Harvard Medical School, Boston, MA, USA*
- DAR SHAPIRA • *Department of Biochemistry and Molecular Biology, Institute of Structural Biology, George S. Wise Faculty of Life Sciences, Tel Aviv University, Tel Aviv, Israel*
- ITAI SHARON • *Department of Biochemistry and Molecular Biology, Institute of Structural Biology, George S. Wise Faculty of Life Sciences, Tel Aviv University, Tel Aviv, Israel*
- NOA SHOHAT • *Department of Biochemistry and Molecular Biology, Institute of Structural Biology, George S. Wise Faculty of Life Sciences, Tel Aviv University, Tel Aviv, Israel*
- ANNA SHUSTERMAN • *Department of Biochemistry and Molecular Biology, Institute of Structural Biology, George S. Wise Faculty of Life Sciences, Tel Aviv University, Tel Aviv, Israel*
- SACHDEV S. SIDHU • *Donnelly Centre for Cellular and Biomolecular Research, University of Toronto, Toronto, ON, Canada; Banting and Best Department of Medical Research, University of Toronto, Toronto, ON, Canada; Department of Molecular Genetics, University of Toronto, Toronto, ON, Canada*
- AMIT KUMAR SINGH GAUTAM • *Department of Molecular Biosciences, The University of Texas at Austin, Austin, TX, USA*
- FREDERICK C. STREICH JR. • *Structural Biology Program, Sloan Kettering Institute, Memorial Sloan Kettering Cancer Center, New York, NY, USA*
- ANJANA SUPPAHIA • *Division of Biology, Kansas State University, Manhattan, KS, USA*
- CALEB D. SWAIM • *Department of Molecular Biosciences, University of Texas at Austin, Austin, TX, USA*
- NETA TANNER • *Department of Biochemistry and Molecular Biology, Institute of Structural Biology, George S. Wise Faculty of Life Sciences, Tel Aviv University, Tel Aviv, Israel*
- JEAN-FRANÇOIS TREMPÉ • *Department of Pharmacology and Therapeutics, McGill University, Montréal, QC, Canada*
- JORDAN J. S. VERPLANK • *Harvard Medical School, Boston, MA, USA*
- KENRICK A. WAITE • *Division of Biology, Kansas State University, Manhattan, KS, USA*
- XIAORONG WANG • *Department of Physiology and Biophysics, University of California, Irvine, CA, USA*
- CYNTHIA WOLBERGER • *Department of Biophysics and Biophysical Chemistry, Johns Hopkins University School of Medicine, Baltimore, MD, USA*
- YI JIMMY ZENG • *Department of Microchemistry, Proteomics and Lipidomics, Genentech, Inc., South San Francisco, CA, USA*
- WEI ZHANG • *Donnelly Centre for Cellular and Biomolecular Research, University of Toronto, Toronto, ON, Canada*
- JINGHUI ZHAO • *Department of Cell Biology, Harvard Medical School, Boston, MA, USA; AbbVie, Cambridge, MA, USA*