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*Front Cover: Image by Patrick Lombardi, Johns Hopkins Medical Institute.*
### THE UBIQUITIN FAMILY
Tuesday, April 21 – Saturday, April 25, 2015

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* *Airlie Lawn*, weather permitting

Mealtimes at Blackford Hall are as follows:<br>Breakfast 7:30 am-9:00 am<br>Lunch 11:30 am-1:30 pm<br>Dinner 5:30 pm-7:00 pm<br>Bar is open from 5:00 pm until late
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SESSION 1 CONTROL OF CELLULAR FUNCTIONS BY UBIQUITIN AND UBLs

Chairpersons: D. Barford, MRC Laboratory of Molecular Biology, Cambridge, United Kingdom
D. Komander, MRC Laboratory of Molecular Biology, Cambridge, United Kingdom

KEYNOTE SPEAKER

Brenda Schulman
St. Jude Children’s Research Hospital

Implications for mechanisms of protein ubiquitination from a high resolution structure of the APC/C
Leifu Chang, Ziguo Zhang, Jing Yang, Stephen H. McLaughlin, David Barford.
Presenter affiliation: MRC Laboratory of Molecular Biology, Cambridge, United Kingdom.

Degradation of Ndd1 by APC/C<sub>Cdh1</sub> generates a feed forward loop that times mitotic protein accumulation
Michael Brandeis, Drora Zenvirth, Mor Nitzan, Julia Sajman.
Presenter affiliation: The Hebrew University of Jerusalem, Jerusalem, Israel.

Functional analysis of transcriptional regulation by sumoylation in yeast
John Babu, Veroni S. Sri Theivakadacham, Justin M. Burgener, Akhi Akhter, Emanuel Rosonina.
Presenter affiliation: York University, Toronto, Canada.

Ubiquitin receptor protein UBASH3B mediates a switch-like mechanism of Aurora B localization to microtubules
Ksenia Krupina, Charlotte Kleiss, Kay Hofmann, Olivier Poch, Laurent Brino, Izabela Sumara.
Presenter affiliation: IGBMC, Illkirch, France.
Coping with protein quality control failure  
Anne Bertolotti.  
Presenter affiliation: MRC LMB, Cambridge, United Kingdom.

Inhibition of LUBAC-mediated linear ubiquitination by a virulence factor of Aspergillus, gliotoxin  
Hiroki Sakamoto, Takayoshi Okabe, Tetsuo Nagano, Kazuhiro Iwai.  
Presenter affiliation: Graduate School of Medicine, Kyoto University, Kyoto, Japan.

USP30 and Parkin homeostatically regulate atypical ubiquitin chains on mitochondria  
Christian N. Cunningham, Joshua M. Baughman, Lilian Phu, Joy S. Tea, Christine Yu, Mary Coons, Donald S. Kirkpatrick, Baris Bingol, Jacob E. Corn.  
Presenter affiliation: Genentech, Inc., South San Francisco, California.

Rsp5 and Ubp2 jointly target cytosolic misfolded proteins for proteasome degradation upon heat denaturation  
Nancy N. Fang, Thibault Mayor.  
Presenter affiliation: University of British Columbia, Vancouver, Canada.

Misfolded mitochondrial protein degradation by the ubiquitin-proteasome system  
Meredith B. Metzger, Mitchell Dunklebarger, Allan M. Weissman.  
Presenter affiliation: National Cancer Institute, Frederick, Maryland.

CRL2 ubiquitin ligase eliminates truncated selenoproteins from ambiguous UGA/Sec translation  
Hsiu-Chuan Lin, Hsueh-Chi Sherry Yen.  
Presenter affiliation: Academia Sinica, Taipei, Taiwan; National Taiwan University, Taipei, Taiwan.
The E3 ligase Ubr3 regulates Usher syndrome proteins in
*Drosophila* and zebrafish auditory organs
Tongchao Li, Bernardo Blanco-Sánchez, Nikolaos Giagtzoglou, Shinya
Yamamoto, Manish Jaiswal, Sonal Nagarkar Jaiswal, Daniel F. Eberl,
Dorothea Godt, Monte Westerfield, Andrew K. Groves, Hugo J. Bellen.
Presenter affiliation: Baylor College of Medicine, Houston, Texas;
Texas Children's Hospital, Houston, Texas.

Regulation of ubiquitin metabolism by a direct phosphorylation
mechanism
Sora Lee, Kristin E. Jernigan, Aaron C. Ehlinger, Jessica M. Tumolo,
Walter J. Chazin, Scott D. Emr, Jason A. MacGurn.
Presenter affiliation: Vanderbilt University, Nashville, Tennessee.

The Ubiquitin Code v2.0—Phospho-ubiquitin
David Komander.
Presenter affiliation: MRC Laboratory of Molecular Biology,
Cambridge, United Kingdom.

WEDNESDAY, April 22—2:00 PM

SESSION 3 POSTER SESSION I

An inhibitor of ubiquitin conjugation system
Heeseon An, Alexander V. Statsyuk.
Presenter affiliation: Northwestern University, Evanston, Illinois.

UCHL3 hydrolysis of ubiquitin chains is regulated by allostery as
determined by NMR
Larry J. Anderson, Robert G. Guenette, Kishan M. Patel, Marco
Tonelli, Eric R. Strieter.
Presenter affiliation: University of Wisconsin - Madison, Madison,
Wisconsin.

Development of a drug discovery platform targeting SUMO
specific proteases
Nadia Arnaudo, Yaara Ofir-Rosenfeld, Louise Jones, Jeanine
Harrigan, Lisa Smith, Helen Robinson, Mark Kemp, Xavier Jacq.
Presenter affiliation: MISSION Therapeutics, Cambridge, United
Kingdom.
Proteasome inhibitors increase the growth inhibition of drugs targeting DNA topoisomerase II (TOP2)
Ka Cheong Lee, Rebecca L. Bramley, Ian G. Cowell, Caroline A. Austin.
Presenter affiliation: Newcastle University, Newcastle upon Tyne, United Kingdom.

Alllosteric cross talk between the deubiquitinase Ubp6 and the 26S proteasome
Charlene Bashore, Corey Dambacher, Mary Matsukiela, Gabriel Lander, Andreas Martin.
Presenter affiliation: UC Berkeley, Berkeley, California.

Conjugation of the ubiquitin activating enzyme UBE1 with the ubiquitin-like modifier FAT10 targets it for proteasomal degradation
Johanna Bialas, Marcus Groettrup, Annette Aichem.
Presenter affiliation: University of Konstanz, Konstanz, Germany; Biotechnology Institute Thurgau, Kreuzlingen, Switzerland.

Molluscum contagiosum virus inhibits NF-κB activation by inhibiting ubiquitination of NEMO
Sunetra Biswas, Joanna L. Shisler.
Presenter affiliation: University of Illinois, Urbana-Champaign, Urbana, Illinois.

Ubiquitination of HOIP carboxyl-terminus negatively regulates linear ubiquitination-mediated immune signaling
James W. Bowman, Mary A. Rodgers, Mude Shi, Rina Amatya, Bruce Hostager, Kazuhiro Iwai, Jae U. Jung.
Presenter affiliation: University of Southern California, Los Angeles, California.

The BMI1/RING1A E3 ubiquitin ligase inhibitor PRT4165 increases the growth inhibition of drugs targeting DNA topoisomerase II (TOP2)
Rebecca L. Bramley, Ka Cheong Lee, Ian G. Cowell, Caroline A. Austin.
Presenter affiliation: Newcastle University, Newcastle-upon-Tyne, United Kingdom.
Cezanne (OTUD7B) regulates HIF-1α homeostasis in a proteasome-independent manner  
Anja Bremm, Sonia Moniz, Julia Mader, Sonia Rocha, David Komander.  
Presenter affiliation: Goethe University Frankfurt, Frankfurt am Main, Germany.  

Cdc48 complexes required for multiple steps of SREBP cleavage in *S. pombe*  
Risa Burr, Sumana Raychaudhuri, Diedre Ribbens, Jiwon Hwang, Peter Espenshade.  
Presenter affiliation: Johns Hopkins University School of Medicine, Baltimore, Maryland.  

Studying dimer-dependent Fbw7-substrate interactions: using an ALPHAscreen to quantitate degron cooperativity in full–length substrates  
Shlomo Handelli, Markus Welcker, Bruce E. Clurman.  

Distinct activation of an E2 ubiquitin conjugating enzyme by its cognate E3 ligases  
Itamar Cohen, Yuval Reiss, Reuven Wiener, Tommer Ravid.  
Presenter affiliation: The Hebrew University of Jerusalem, Jerusalem, Israel.  

Muscle denervation, as occur with aging, enhances proteolysis and causes wasting by a mechanism involving the ubiquitin ligase Trim32 and the calcium-dependent protease, Calpain 1  
Inga Rudesky, Eitan Shimko, Shenhav Cohen.  
Presenter affiliation: Technion Institute of Technology, Haifa, Israel.  

HOIP deficiency causes embryonic lethality by aberrant TNFR1-mediated endothelial cell death  
Presenter affiliation: UCL Cancer Institute, University College London, London, United Kingdom.
Building predictive networks of TCGA datasets to identify functional consequences of Fbw7 mutations in human tumors
Ryan J. Davis, Mehmet Gonen, Adam A. Margolin, Bruce E. Clurman.

The p97 small molecule inhibitor CB-5083 causes disruption of the ubiquitin-proteasome pathway, activates the unfolded protein response and has strong anti-cancer effects
Stevan N. Djakovic, Julie Rice, Brajesh Kumar, Szerenke Kiss von Soly, Ronan Le Moigne, Steve Wong, MK Menon, Eduardo Valle, Ferdie Soriano, Jinhai Wang, Bing Yao, Han-Jie Zhou, David Wustrow, Mark Rolfe, Daniel Anderson.
Presenter affiliation: Cleave Biosciences, Burlingame, California.

LUBAC and linear ubiquitin coordinate DUBs recruitment to signaling complexes
Peter Draber, Matthias Reichert, Sebastian Kupka, Helena Draberova, Diego de Miguel, Lisanne Spilgies, Silvia Surinova, Lucia Taraborrelli, Eva Rieser, Henning Walczak.
Presenter affiliation: Centre for Cell Death, Cancer and Inflammation, London, United Kingdom.

Herpesviral tegument proteins interfering with host cell proteins indicate multiple functions in critical pathways of antiviral defense and stress response that are dependent on ubiquitin and related modifiers
Presenter affiliation: Universitaetsklinikum FAU Erlangen, Erlangen, Germany.

Role of E3 ligase TRIM71 in innate immune signaling
Danae Fonseca, Adolfo Garcia-Sastre.
Presenter affiliation: Icahn School of Medicine at Mount Sinai, New York, New York.

ALS-causing mutations in UBQLN2 deregulate its ubiquitin-binding cycle
Bennett W. Fox, Sang Hwa Kim, Mark Scalf, Krystal Obermeier, Randal S. Tibbetts.
Presenter affiliation: University of Wisconsin - Madison, Madison, Wisconsin.
Cancer-associated fibroblasts attenuate the p53 response by increasing ubiquitination of p53 in cancer cells
Emarndeena H. Cheteh, Martin Augsten, Helene Rundqvist, Arne Östman, Klas Wiman.
Presenter affiliation: Karolinska Institutet, Stockholm, Sweden.

Maintaining distance—The E3 ubiquitin ligase Ariadne-1 controls nuclear positioning during muscle development in *Drosophila melanogaster*
Nele A. Haelterman, Kai Li Tan, Sonal Nagarkar, Pei-Tseng Lee, Hugo J. Bellen.
Presenter affiliation: Baylor College of Medicine, Houston, Texas.

Towards understanding mitotic functions of the RanBP2 SUMO E3 ligase complex
Yuqing Hao, Annette Flotho, Hideki Yokoyama, Oliver Gruss, Frauke Melchior.
Presenter affiliation: Heidelberg University, Heidelberg, Germany.

Structural and biochemical characterization of muskelin discoidin domain
Seung Kon Hong, Kook-Han Kim, Eunice E. Kim.
Presenter affiliation: Korea Institute of Science and Technology, Seoul, South Korea.

Ubiquitylation of autophagy receptor optineurin by HACE1 activates selective autophagy for tumor suppression
Ronggui "Cory" Hu.
Presenter affiliation: Shanghai Institutes for Biological Sciences, Shanghai, China.

USP15 regulates SMURF2 kinetics through C-lobe mediated deubiquitination
Prasanna Vasudevan Iyengar, Patrick Jaynes, Dilraj Lama, Chandra Verma, Lim Yoon Pin, Pieter Johan Adam Eichhorn.
Presenter affiliation: National University of Singapore, Singapore.

Discovery of highly selective DUB inhibitors with in vivo pre-clinical anti-tumour activity
Xavier Jacq, Lisa M. Smith, Jeannine Harrigan, Charlotte Knights, Helen Robinson, Yaara Ofir-Rosenfeld, Aaron N. Cranston, Alison Jones, Mark I. Kemp, Stephen P. Jackson, Niall M. Martin.
Presenter affiliation: MISSION Therapeutics Ltd, Cambridge, United Kingdom.
p97 is critical for cytokine-induced IKK activation
Ju-Mei Li, Veronique Schaeffer, Ivan Dikic, Jianping Jin.
Presenter affiliation: The University of Texas Health Science Center at Houston, Houston, Texas.

The role of ubiquitin in the protein quality control of Huntington's disease
Katrin Juenemann, Anne Wiemhoefer, Anne H. Jansen, Bogdan I. Florea, Sabine Schipper-Krom, Eric A. Reits.
Presenter affiliation: Academic Medical Center, University Amsterdam, Amsterdam, Netherlands.

Ubiquitin phosphorylation in mitophagy
Lesley A. Kane, Michael Lazarou, Danielle A. Sliter, Adam I. Fogel, Yan Li, Koji Yamano, Richard J. Youle.
Presenter affiliation: National Institutes of Health, Bethesda, Maryland.

Interaction of proteasome and heat shock proteins in tumor cell response
Betul Karademir, Perinur Bozaykut, Erdi Sozen, Nesrin Kartal Ozer.
Presenter affiliation: Marmara University, Istanbul, Turkey.

Ubiquitin chain elongation requires E3-dependent tracking of the emerging conjugate
Aileen Kelly, Katherine E. Wickliffe, Ling Song, Indro Fedrigo, Michael Rape.
Presenter affiliation: University of California at Berkeley, Berkeley, California.

Identification of novel B cell signal transduction by Pellino 1 E3 ligase gain-of-function
Suhyeon Kim, Hye-Young Park, Kyungrim Hwang, Chang-Woo Lee.
Presenter affiliation: Samsung Biomedical Research Institute, Sungkyunkwan University School of Medicine, Suwon, South Korea.

Ubiquilin-2 is a stress activated proteasomal shuttle
Roland Hjerpe, John Bett, Thimo Kurz.
Presenter affiliation: University of Dundee, Dundee, United Kingdom.

Alpha-ring independent assembly of the 20S proteasome
Dilrajkaur Panfair, Aishwarya Ramamurthy, Andrew R. Kusmierczyk.
Presenter affiliation: Indiana University-Purdue University Indianapolis, Indianapolis, Indiana.
A novel Cullin ubiquitin ligase complex regulates cellular proliferation by modulating biosynthesis of guanine nucleotides
Yejin Lee, Ji-Joon Song, J. Eugene Lee.
Presenter affiliation: Korea Research Institute of Standards and Science, Daejeon, South Korea.

Functions of N-terminal methionine formylation of cytoplasmic proteins in mitochondrial fatty acid synthesis in *Saccharomyces cerevisiae*
Kang-Eun Lee, Jeong-Mok Kim, Cheol-Sang Hwang.
Presenter affiliation: Pohang University of Science and Technology, Pohang, Gyeongbuk, South Korea.

Ubiquitination of Costal 2 by the Ubr3 E3 ligase promotes Hedgehog signaling in *Drosophila*
Tongchao Li, Junkai Fan, Nikolaos Giagtzoglou, Jianhang Jia, Andrew K. Groves, Hugo J. Bellen.
Presenter affiliation: Baylor College of Medicine, Houston, Texas; Texas Children's Hospital, Houston, Texas.

Regulation of the deubiquitinase USP1 by CDKs in DNA damage response
Qin Liang, Zhihao Zhuang.
Presenter affiliation: University of Delaware, Newark, Delaware.

Cytosolic PINK1 is stabilized by NF-κB pathway and promotes non-selective mitophagy
Presenter affiliation: National Neuroscience Institute, Singapore; National University of Singapore, Singapore.

Ubiquitin ligase profiling platform—A novel approach in targeting E3 ligases
Presenter affiliation: AstraZeneca, Macclesfield, United Kingdom.

UBE2O mediates c-Maf ubiquitination and induces myeloma cell apoptosis
Jie Li, Jiefei Tong, Biyin Cao, Zubin Zhang, Michael Moran, Xinliang Mao.
Presenter affiliation: Soochow University, Suzhou, China.
Mechanisms and flexibility of substrate recognition by the proteasome
Kirby Martinez-Fonts, Suzanne Elsasser, Daniel Finley, Andreas Matouschek.
Presenter affiliation: Northwestern University, Evanston, Illinois; The University of Texas at Austin, Austin, Texas.

Development of novel chain selective poly-ubiquitin binding technologies to study the biological roles of specific ubiquitin linkages
Mark Mason, Julia Bellam, James E. Strickler.

The E3 ubiquitin ligase TRIM61 is a regulator of RLRs-mediated type I interferon production
Lisa Miorin, Ricardo Rajsbaum, Adolfo Garcia-Sastre.
Presenter affiliation: Icahn School of Medicine at Mount Sinai, New York, New York.

WEDNESDAY, April 22—4:30 PM
Wine and Cheese Party

WEDNESDAY, April 22—7:30 PM

SESSION 4  CONTROL OF E3 LIGASE ACTIVITY

Chairpersons: C. Lima, Sloan-Kettering Institute, New York, New York
N. Thomä, Friedrich Miescher Institute for Biomedical Research, Basel, Switzerland

E3 ligase complexes reveal activation mechanisms and specificity in the SUMO pathway
Christopher D. Lima.

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SCF Fbx17 mediated ubiquitination of Sufu regulates Hedgehog signaling
Vincenzo D'Angiolella, Madalina Raducu, Rune Toftgard, Csaba Finta, Marie-Laetitia Thézenas, Benedikt M. Kessler.
Presenter affiliation: CRUK/MRC Institute for Radiation Oncology, University of Oxford, Oxford, United Kingdom.

Allosteric activation of UHRF1 ubiquitin ligase activity
Joseph S. Harrison, Scott B. Rothbart, Krzysztof Krajewski, Brian D. Strahl, Brian Kuhlman.
Presenter affiliation: University of North Carolina, Chapel Hill, North Carolina.

Global remodeling of the erythroid proteome by ubiquitination
Anthony T. Nguyen, Miguel A. Prado, Mingwei Min, Yuan Shi, Mona Kawan, Verena Dederer, Paul Schmidt, Dean Campagna, Steven P. Gygi, Mark D. Fleming, Daniel Finley.
Presenter affiliation: Harvard Medical School, Boston, Massachusetts.

The TRIM25 E3 ligase is catalytically active as a tetramer, which matches the signaling active stoichiometry of its substrate, RIG-I
Jacint G. Sanchez, Katarzyna Okreglicka, Michael Chi, Marcin D. Roganowicz, Steven L. Alam, Terri D. Lyddon, Marc C. Johnson, Owen Pomilio.
Presenter affiliation: University of Virginia, Charlottesville, Virginia.

Crystal structure of the Polycomb PRC1 E2-E3 ubiquitylation module bound to its nucleosome substrate
Presenter affiliation: Penn State University, University Park, Pennsylvania.

Plasticity in cullin-RING E3 ligase binding to the COP9 signalosome
Presenter affiliation: Friedrich Miescher Institute for Biomedical Research, Basel, Switzerland.
SESSION 5 REGULATION BY UBIQUITIN AND SUMO

Chairpersons: M. Rape, HHMI/University of California, Berkeley
M. Gyrd-Hansen, University of Oxford, United Kingdom

Ubiquitin-dependent regulation of cell fate decisions
Achim Werner, Shintaro Iwasaki, Colleen McGourty, Nick Ingolia,
Michael Rape.
Presenter affiliation: Howard Hughes Medical Institute, University of
California, Berkeley, California.

CDC-48/p97 dependent DNA replication is governed by conserved
UBX domain proteins
André Franz, Paul A. Pirson, Swagata Halder, Domenic Pilger,
Kristjan Ramadan, Thorsten Hoppe.
Presenter affiliation: University of Cologne, Cologne, Germany.

Sumoylation of Rap1 mediates the recruitment of TFIIID to
promote transcription of ribosomal protein genes
Pierre Chymkowitch, Aurélie Nguéa P, Håvard Aanes, Christian J.
Koehler, Bernd Thiede, Susanne Lorenz, Leonardo A. Meza-Zepeda,
Arne Klungland, Jorrit M. Enserink.
Presenter affiliation: Institute of Microbiology, Oslo University Hospital,
and the University of Oslo, Oslo, Norway.

Internally tagged ubiquitin as a powerful tool to identify novel
linear polyubiquitin-modified proteins
Katarzyna Kliza, Christoph Taeumer, Jaime Lopez-Mosqueda, Mirita
Franz-Wachtel, Boris Macek, Koraljka Husnjak.
Presenter affiliation: Goethe University School of Medicine, Frankfurt
am Main, Germany.

SUMO-2 orchestrates chromatin modifiers in response to DNA
damage
Ivo A. Hendriks, Louise W. Treffers, Matty Verlaan-de Vries, Jesper V.
Olsen, Alfred C. Vertegaal.
Presenter affiliation: Leiden University Medical Center, Leiden,
Netherlands.
Tandem self-association domains of substrate adaptor SPOP mediate formation of liquid nuclear bodies
Presenter affiliation: St. Jude Children's Research Hospital, Memphis, Tennessee.
SUMOylation of the transcription factor Zbtb20 controls neurite growth and branching
Silvia Ripamonti, Kamal Chowdhury, Jeong Seop Rhee, Nils Brose, Marilyn Tirard.
Presenter affiliation: Max Planck Institute of Experimental Medicine, Goettingen, Germany.
New genetic tools to study SUMO/Ub—Identification of Pol III as a functional target of SUMO, implications in neuronal degenerative disease, and development of SUMO inhibitors
Zheng Wang, Catherine Wu, Aaron Aslanian, Vicki Zhou, Annie Mak, Jun Liu, Mike French, Li Ma, Tony Hunter.
Presenter affiliation: The Salk Institute for Biological Studies, La Jolla, California.
Mads Gyrd-Hansen.
Presenter affiliation: Oxford University, United Kingdom.

THURSDAY, April 23—2:00 PM

SESSION 6 POSTER SESSION II

Targeting PIAS1 as a therapeutic for Huntington's disease
Joseph Ochaba, Jacqueline G. O'Rourke, Alex Mas Monteys, John H. Lee, Joan S. Steffan, Beverly L. Davidson, Leslie M. Thompson.
Presenter affiliation: University of California, Irvine, Irvine, California.
Regulation of infection related development in Magnaporthe oryzae by ubiquitination and kinase signaling pathways
Yeonhee Oh, William Franck, Jennifer Parker, David Muddiman, Ralph A. Dean.
Presenter affiliation: North Carolina State University, Raleigh, North Carolina.
Mitochondrially-associated Nrf2 limits the proteotoxicity induced by proteasome inhibition
Gary B. O'Mealey, William L. Berry, Scott M. Plafker.
Presenter affiliation: University of Oklahoma Health Sciences Center, Oklahoma City, Oklahoma; Oklahoma Medical Research Foundation, Oklahoma City, Oklahoma.

Quantifying ubiquitin signaling mechanisms through the PINK1-PARKIN pathway
Presenter affiliation: Harvard Medical School, Boston, Massachusetts.

Proteasome turnover is affected in the absence of E3 ligase, Not4
Olesya O. Panasenko, Susan Liao, Jeffry Corden, Martine A. Collart.
Presenter affiliation: Faculty of Medicine, University of Geneva, Geneva, Switzerland.

Turnover of the yeast inner nuclear membrane protein Asi1 is rapid
Marina Pantazopoulou, Per O. Ljungdahl.
Presenter affiliation: The Wenner-Gren Institute, Stockholm, Sweden.

Characterization of the FUBI-modified proteome in human cells
Presenter affiliation: Novo Nordisk Foundation Center for Protein Research, University of Copenhagen, Copenhagen, Denmark.

Mutually exclusive roles of SHARPIN in integrin inactivation and NF-κB signaling
Nicola de Franceschi, Emilia Peuhu, Maddy Parsons, Christopher S. Potter, Sami Rissanen, Vattulainen Ilpo, Marko Salmi, John P. Sundberg, Johanna Ivaska, Jeroen Pouwels.
Presenter affiliation: University of Turku, Turku, Finland.

Systematic p97-UBXD adaptor network proteomics identifies a role for UBXD3 in regulating ciliogenesis via association with IFT-B
Presenter affiliation: Harvard Medical School, Boston, Massachusetts.
Targeting the SUMO E1-E2 enzyme interaction in *Plasmodium falciparum*
Katherine H. Reiter, Jurgen Bosch, Michael J. Matunis.
Presenter affiliation: Johns Hopkins School of Public Health, Baltimore, Maryland.

Investigation of huntingtin ubiquitination by mass spectrometry
Karen A. Sap, Katrin Juenemann, Anne Wiemhoefer, Bobby Florea, Jeroen Demmers, Eric A. Reits.
Presenter affiliation: Academic Medical Center, Amsterdam, Netherlands.

Maturation of the proteasome core particle induces an affinity switch that controls Pba1-Pba2 and regulatory particle association
Prashant S. Wani, Michael A. Rowland, Alex Ondracek, Eric J. Deeds, Jeroen Roelofs.
Presenter affiliation: Kansas State University, Manhattan, Kansas.

Ltn1/Rkr1-mediated degradation of non-stop endoplasmic reticulum proteins
Presenter affiliation: Ball State University, Muncie, Indiana.

A novel binding domain in KIAA0323 shows a clear preference for NEDD8
Presenter affiliation: Tor Vergata University, Rome, Italy.

Investigating biomarkers of neuropathy initiated by proteasome inhibitor Bortezomib
Gulce Sari, Sravani Musuruni, Grzegorz Wicher, Jia Mi, Jonas Bergquist, Betul Karademir.
Presenter affiliation: Marmara University, Istanbul, Turkey.

Systematic identification of ubiquitin ligase substrates
Darren Saunders, Mary Iconomou, Jessie McKenna, Robert Shearer.
Presenter affiliation: Garvan Institute of Medical Research, Sydney, Australia.
Investigation of the interaction of FAT10 and VCP
Ricarda Schwab, Annette Aichem, Marcus Groettrup.
Presenter affiliation: University of Konstanz, Konstanz, Germany; Biotechnology Institute Thurgau, Kreuzlingen, Switzerland.

The structure and function of parkin E3 ubiquitin ligase
Marjan Seirafi, Guennadi Kozlov, Veronique Sauve, Asparouh Lilov, Kalle Gehring.
Presenter affiliation: McGill University, Montreal, Canada.

Structural basis for catalysis and substrate-induced conformational change of a USP47
Sang Chul Shin, Kook Han Kim, Byung Hak Ha, Eunice E. Kim.
Presenter affiliation: Korea Institute of Science and Technology, Seoul, South Korea.

Dppa3, a maternally derived epigenetic reprogramming factor, is ubiquitinated and partially cleaved in early mouse embryos
Seung-Wook Shin, Jurrien Dean.
Presenter affiliation: NIDDK, National Institutes of Health, Bethesda, Maryland.

Inhibitors of USP30 as a potential treatment of Parkinson’s disease and other mitochondrial dysfunctions
Lisa M. Smith, Martin L. Stockley, Quentin Gueranger, Louise Jones, Aaron N. Cranston, Jeanine A. Harrigan, Mark I. Kemp, Xavier Jacq.
Presenter affiliation: MISSION Therapeutics Ltd, Cambridge, United Kingdom.

Cell cycle regulation by Usp15
Tanuza Das, Joon Kyu Park, Jinyoung Park, Eunji Kim, Eunice EunKyeong Kim, Eun Joo Song.
Presenter affiliation: Korea Institute of Science & Technology, Seoul, South Korea.

Ubiquitin editing of a spliceosome auxiliary factor by TRAF6, a new mechanism linking chronic immune signaling with hematopoietic malignancies
Daniel Starczynowski.
Presenter affiliation: Cincinnati Children's Hospital, Cincinnati, Ohio; University of Cincinnati, Cincinnati, Ohio.
Development of fluorescence based autophagy sensors
Alexandra Stolz, Mateusz Putyrski, Ivana Kutle, Andreas Ernst, Ivan Dikic.
Presenter affiliation: Goethe Universität Frankfurt, Frankfurt, Germany.

Structural insight to specificity for SUMO modification of PCNA by the E3 ligase Siz1
Frederick C. Streich Jr, Christopher D. Lima.
Presenter affiliation: Sloan-Kettering Institute, New York, New York.

DUBs activate isopeptide bonds for cleavage by restricting the conformation of the ubiquitin C-terminus
Larry J. Anderson, Eric R. Strieter.
Presenter affiliation: University of Wisconsin-Madison, Madison, Wisconsin.

The deubiquitinase USP37 promotes efficient S-phase progression and cellular response to replication stress
Presenter affiliation: Cleveland Clinic, Cleveland, Ohio.

UBQLN is essential for quality control of mislocalized proteins
Rigel Suzuki, Hiroyuki Kawahara.
Presenter affiliation: Tokyo Metropolitan University, Hachioji, Japan.

Ubiquitin signalling in the last resort pathway for RNA polymerase II degradation
Kotryna Temcinaite, Michelle Harreman, Jesper Q. Svejstrup.
Presenter affiliation: London Research Institute, London, United Kingdom.

Divergent ubiquitin binding and exosite targeting in the USP family DUBs
Adam H. Tencer, Qin Liang, Yu Peng, Zhihao Zhuang.
Presenter affiliation: University of Delaware, Newark, Delaware.

Misfolding, not ubiquitination, underlies protein targeting to inclusion bodies
Kirill Bersuker, Airlia Thompson, Michael Brandeis, Ron R. Kopito.
Presenter affiliation: Stanford University, Stanford, California.
Conformational switching triggered by a single alpha helix drives completion of proteasome regulatory particle assembly
Robert J. Tomko Jr., David W. Taylor, Zhuo Angel Chen, Juri Rappsilber, Mark Hochstrasser.
Presenter affiliation: The Florida State University College of Medicine, Tallahassee, Florida.

Molecular basis for persistence of botulinum neurotoxin
Yien Che Tsai, Archana Kotiya, Mei Yang, George A. Oyler, Allan M. Weissman.
Presenter affiliation: National Cancer Institute, Frederick, Maryland.

Loss of Ube2W results in increased postnatal lethality accompanied by defects in skin, immune and male reproductive systems
Bo Wang, Amanda K. Huber, Sean A. Merillat, David N. Irani, Kenneth M. Scaglione, Henry L. Paulson.
Presenter affiliation: University of Michigan, Ann Arbor, Michigan.

Nucleus-localized insulin-like growth factor-1 receptor mediates DNA damage tolerance by regulating PCNA phosphorylation and ubiquitination in normal but not in cancer cells
Ahmed Waraky, Yingo Lin, Eiman Aleem, Olle Larsson.
Presenter affiliation: Karolinska Institutet, Stockholm, Sweden.

The ubiquitin E3 ligase TRIM23 regulates adipocyte differentiation via stabilization of the adipogenic activator PPARγ
Masashi Watanabe, Shigetsugu Hatakeyama.
Presenter affiliation: Hokkaido University Graduate School of Medicine, Sapporo, Hokkaido, Japan.

Implications of PINK1-mediated ubiquitin Ser65 phosphorylation
Presenter affiliation: MRC Laboratory of Molecular Biology, Cambridge, United Kingdom.

Unraveling the ubiquitination capacity of the Doa10 ligase in concert with its E2 enzymes Ubc6 and Ubc7
Annika Weber, Ernst Jarosch, Thomas Sommer.
Presenter affiliation: Max Delbrück Center, Berlin, Germany.
Assembly of the elongin A ubiquitin ligase is regulated by genotoxic and other stresses
Presenter affiliation: Stowers Institute for Medical Research, Kansas City, Missouri. 113

A non-destructive interaction of a ubiquitin ligase with its substrate
Markus Welcker, Bruce E. Clurman.
Presenter affiliation: Fred Hutchinson Cancer Research Center, Seattle, Washington. 114

Quality control of a cytoplasmic protein complex—Molecular motors and components of the ubiquitin-proteasome system regulate FAS stoichiometry in yeast
Ingo Amm, Mario Scazzari, Dieter H. Wolf.
Presenter affiliation: University of Stuttgart, Stuttgart, Germany. 115

Structural rearrangements in the proteasome lid activate Rpn11 for isopeptide bond cleavage
Evan J. Worden, Corey M. Dambacher, Gabriel C. Lander, Andreas Martin.
Presenter affiliation: University of California, Berkeley, Berkeley, California. 116

Ubiquitin-like domains can target proteins for proteasome degradation
Houqing Yu, Grace Kago.
Presenter affiliation: The University of Texas at Austin, Austin, Texas; Northwestern University, Evanston, Illinois. 117

Induction of USP25 by viral infection positively regulates innate antiviral responses by mediating the stabilization of TRAF3 and TRAF6
Jie Jin, Dandan Lin, Man Zhang, Yujie Ren, Bo Zhong.
Presenter affiliation: State Key Laboratory of Virology, College of Life Sciences, Wuhan, China. 118
SESSION 7 MECHANISM OF ACTION OF E3 LIGASES

Chairpersons: R. Klevit, University of Washington, Seattle
K. Rittinger, MRC-National Institute for Medical Research, London, United Kingdom

Mechanistic strategies of RING-Between-RING (RBR) E3 ligases
Rachel E. Klevit, Katja K. Dove, Peter S. Brzovic.

Deciphering the activation of the E3 ubiquitin ligase parkin—The Pink1 effect
Véronique Sauvé, Asparouh Lilov, Jean-François Trempe, Marjan Seirafi, Kalle Gehring.
Presenter affiliation: McGill University, Montréal, Canada.

Structural and biochemical characterization of RNF4 mediated K63 linked ubiquitin chain synthesis
Emma Branigan, Anna Plechanovová, Ellis Jaffray, James H. Naismith, Ronald T. Hay.
Presenter affiliation: University of Dundee, Dundee, United Kingdom.

Novel insights into SUMO chain formation
Presenter affiliation: Max Planck Institute of Immunobiology and Epigenetics, Freiburg, Germany.

In situ generated activity-based probes for Ub/Ubl E1-E2-E3 enzymes—Structure, activity and applications
Presenter affiliation: Netherlands Cancer Institute, Amsterdam, Netherlands.
RING dimerization links higher-order assembly and E3 ubiquitin ligase activity of the TRIM5α retroviral restriction factor
Presenter affiliation: University of Texas Health Science Center, San Antonio, Texas.

Structural and mechanistic insight into the function of multi-domain E3 ligases
Katrin Rittinger.
Presenter affiliation: MRC-National Institute for Medical Research, London, United Kingdom; Francis Crick Institute, London, United Kingdom.

FRIDAY, April 24—9:00 AM

SESSION 8 REMOVAL OF UBIQUITIN AND UBLs

Chairpersons: T. Sixma, Netherlands Cancer Institute, Amsterdam
M. Nakanishi, Nagoya City University, Japan

Mechanisms of DEUBAD-domain-dependent regulation of UCHL5 and BAP1 DUB activity
Presenter affiliation: Netherlands Cancer Institute, Amsterdam, the Netherlands.

Ubiquitylation/deubiquitylation circuit of histone H3 couples maintenance DNA methylation and DNA replication
Makoto Nakanishi, Luna Yamaguchi, Atsuya Nishiyama.
Presenter affiliation: Nagoya City University, Graduate School of Medical Sciences, Nagoya, Japan.

Structural basis for deubiquitination of histone H2B by the SAGA DUB module
Michael Morgan, Mahmood Haj-Yahya, Ashraf Brik, Cynthia Wolberger.
Presenter affiliation: Johns Hopkins School of Medicine, Baltimore, Maryland.
AUTOPHAGY

Chairpersons:  
R. Youle, National Institutes of Health, Bethesda, Maryland  
F. Melchior, Zentrum für Molekulare Biologie Heidelberg, Germany

KEYNOTE SPEAKER

Ivan Dikic  
Goethe University Medical School, Germany
Ubiquitin signals on cargo promote mitophagy via autophagy receptors
Richard Youle.
Presenter affiliation: National Institutes of Health, Bethesda, Maryland.

Autophagic turnover of the Arabidopsis 26S proteasome involves the dual ubiquitin/ATG8 receptor RPN10
Presenter affiliation: University of Wisconsin-Madison, Madison, Wisconsin.

CUL3-KBTBD6/KBTBD7 ubiquitin E3 ligase cooperates with ubiquitin-like GABARAP proteins to spatially restrict TIAM1-RAC1 signalling
Heide Genau, Jessica Huber, Francesco Baschieri, Masato Akutsu, Volker Dotsch, Hesso Farhan, Vladimir Rogov, Christian Behrends.
Presenter affiliation: Institute of Biochemistry II, Frankfurt, Germany.

Degradation of AMPK by a cancer-specific ubiquitin ligase
Carlos T. Pineda, Saumya Ramanathan, Klementina Fon Tacer, Jenny L. Weon, Malia B. Potts, Yi-Hung Ou, Michael White, Ryan Potts.
Presenter affiliation: UT Southwestern, Dallas, Texas.

Proteomic exploration of ubiquitin and autophagy systems
Malavika Raman, Alban Ordureau, Jin-mi Heo, David Duda, Brenda Schulman, Wade Harper.
Presenter affiliation: Harvard Medical School, Boston, Massachusetts.

FRIDAY, April 24

BANQUET
Cocktails 6:00 PM  Dinner 6:45 PM
SESSION 10  PROTEASOME

Chairpersons: A. Martin, University of California, Berkeley
K.H. Darwin, New York University School of Medicine, New York

Mechanism of degradation-coupled substrate deubiquitination at the 26S proteasome
Andreas Martin, Evan J. Worden, Corey Dambacher, Charlene Bashore, Gabriel C. Lander.
Presenter affiliation: UC Berkeley, Berkeley, California.  138

Key players in proteasome dynamics
Cordula Enenkel.
Presenter affiliation: University of Toronto, Toronto, Canada.  139

N-terminal acetylation and the N-end rule pathway for the control of mammalian G protein signaling
Jeong-Mok Kim, Sang-Eun Park, Ok-Hee Seok, Cheol-Sang Hwang.
Presenter affiliation: Pohang University of Science and Technology, Pohang, South Korea.  140

Deciphering a second code in proteasome targeting
Houqing Yu, Sue Fishbain, Tomo Inobe, Eitan Israeli, Sreenivas Chavali, Madan Babu, Grace Kago, Andreas Matouschek.
Presenter affiliation: The University of Texas at Austin, Austin, Texas; Northwestern University, Evanston, Illinois.  141

Ubiquitin—Gateway to the dynamic proteome
Presenter affiliation: Genentech, South San Francisco, California.  142

Kinetic analysis of substrate degradation by the proteasome
Ying Lu, Byung-hoon Lee, Randall W. King, Daniel Finley, Marc W. Kirschner.
Presenter affiliation: Harvard Medical School, Boston, Massachusetts.  143
Strategies for proteasome inhibition—What do you do, β2?
Presenter affiliation: Takeda Oncology, Cambridge, Massachusetts.

ATP-independent degradation by the Mycobacterium tuberculosis proteasome
K. Heran Darwin, Jordan Jastrab, Lin Bai, Tong Wang, Kuan Hu, Huilin Li.
Presenter affiliation: New York University School of Medicine, New York, New York.
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The anaphase-promoting complex (APC/C) is a large multimeric RING E3 ubiquitin ligase that controls chromosome segregation and mitotic exit. Its regulation by coactivator subunits, phosphorylation, the mitotic checkpoint complex, and interphase inhibitor Emi1 ensures the correct order and timing of distinct cell cycle transitions. Two E2s, UbcH10 and Ubc2S are responsible for assembly of Lys 11-linked polyubiquitin chains on APC/C substrates that include cyclins, mitotic kinases and spindle assembly factors and motors.

We have used single particle cryo-electron microscopy to determine an atomic-resolution structure of the APC/C in complex with its coactivator, Emi1 and a UbcH10–ubiquitin conjugate. I will discuss the implications for understanding mechanisms of protein ubiquitination and regulation by Emi1 and protein phosphorylation.
DEGRADATION OF NDD1 BY APC/CCDH1 GENERATES A FEED FORWARD LOOP THAT TIMES MITOTIC PROTEIN ACCUMULATION

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Ndd1 activates the Mcm1-Fkh2 transcription factor to transcribe mitotic regulators. The Anaphase Promoting Complex/Cyclosome activated by Cdh1 (APC/C^{Cdh1}) mediates degradation of multiple proteins throughout G1. We show that the APC/C^{Cdh1} mediates the degradation of Ndd1, and that APC/C^{Cdh1} activity suppresses accumulation of Ndd1 targets. We confirm putative Ndd1 targets and identify novel ones, many of them APC/C^{Cdh1} substrates. The APC/C^{Cdh1} thus regulates these proteins in a dual manner – both pre-transcriptionally and post-translationally, forming a multi-layered feed-forward loop (FFL). We predict by mathematical modeling and verify experimentally that this FFL introduces a lag between APC/C^{Cdh1} inactivation at the end of G1 and accumulation of genes transcribed by Ndd1 in G2. This regulation generates two classes of APC/C^{Cdh1} substrates early ones that accumulate in S and late ones that accumulate in G2. Our results show how the dual state APC/C^{Cdh1} activity is converted into multiple outputs by interactions between its substrates.
FUNCTIONAL ANALYSIS OF TRANSCRIPTIONAL REGULATION BY SUMOYLATION IN YEAST

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Transcription factors represent one of the largest groups of proteins regulated by post-translational SUMO modifications. Consistent with this, we previously showed by chromatin immunoprecipitation (ChIP) that SUMO is associated with promoters of active genes in yeast. Furthermore, induction of the GAL1, STL1 and ARG1 genes correlates with a striking accumulation of SUMO, specifically on their promoters, and a concomitant recruitment of the sole SUMO E2 conjugating enzyme, Ubc9. This implies that promoter-associated sumoylation plays important roles in regulating gene expression.

To explore functions for sumoylation in regulating transcription and gene expression, we applied the recently developed “anchor away” technique, in which we generated a yeast strain that conditionally and rapidly removes Ubc9 from the nucleus. Under standard conditions, the strain grows normally and chromatin fractionation showed that the vast majority of sumoylated proteins in the cell are associated with chromatin. Nuclear depletion of Ubc9, however, resulted in a dramatic reduction in chromatin-associated sumoylated proteins within 30 min, demonstrating that the anchor away methodology is highly effective, and that most SUMO modifications are probably short-lived. Cells were unable to grow after prolonged nuclear depletion of Ubc9, indicating that nuclear sumoylation plays critical roles. However, a panel of inducible genes (including amino acid starvation-activated ARG1 and CPA2, and osmostress-activated STL1 and GPD1) were fully inducible after 30 min of nuclear Ubc9 depletion. This implies that sumoylation, including sumoylation associated with promoters of induced genes, functions in essential aspects of gene expression or chromatin regulation other than gene activation.

Towards identifying promoter-associated SUMO targets, we examined a panel of gene-specific and general transcription factors (GTFs). In normally growing yeast, we determined that, amongst the transcription factors tested, the osmostress-responsive gene-specific transcription factor Sko1, and the large subunit of TFIIF, Tfg1, are sumoylated to appreciable levels. We found that Sko1 sumoylation, which we mapped to Lys 567, is unaffected upon exposure to osmotic stress, during which Sko1 was previously shown to switch from acting as a transcriptional repressor to an activator. This supports the above conclusion that, paradoxically, although sumoylation is associated with active promoters and promoter-bound factors, it functions outside of the gene activation process.
UBIQUITIN RECEPTOR PROTEIN UBASH3B MEDIATES A SWITCH-LIKE MECHANISM OF AURORA B LOCALIZATION TO MICROTUBULES.

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Mitosis ensures equal segregation of the genome and is controlled by variety of ubiquitylation signals on substrate proteins. However, it remains unexplored how the versatile ubiquitin code can be read during mitotic progression. Here we identify the ubiquitin receptor protein UBASH3B that critically regulates mitosis. UBASH3B interacts with ubiquitylated Aurora B, one of the main kinases regulating chromosome segregation, and controls its subcellular localization but not protein levels. Importantly, UBASH3B is a rate-limiting factor, which is sufficient for targeting Aurora B to microtubules prior to anaphase. Indeed, super-resolution imaging reveals spindle-associated Aurora B in metaphase cells. Moreover, targeting Aurora B to microtubules by UBASH3B determines timing and fidelity of chromosome segregation and thereby euploidy of human cells. Our findings uncover an important mechanism how ubiquitin attachment to a substrate protein is decoded during mitosis, which can be utilized by propagating cancer cells.
While it is well established that accumulation of misfolded proteins is a defining feature of numerous diseases, why this happens remains unclear. Cells normally strive to ensure that proteins get correctly folded and have powerful and sophisticated mechanisms to maintain homeostasis under adverse conditions. However, with age, the cellular defence systems against misfolded proteins seem to fail, leading to the accumulation of misfolded proteins with devastating consequences for cells and organism. In principle, improving the cells’ ability to deal with misfolded proteins should represent a generic approach to reduce the pathology in diverse protein misfolding diseases. I will report on our progress in identifying strategies to improve the cells’ ability to deal with misfolded proteins.
INHIBITION OF LUBAC-MEDIATED LINEAR UBIQUITINATION BY A VIRULENCE FACTOR OF ASPERGILLUS, GLIOTOXIN

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Linear polyubiquitin chains, which are generated by conjugation of the carboxyl group of a ubiquitin monomer to the α-amino group of the first Met of another ubiquitin, play crucial roles in activation of NF-κB, a crucial transcription factor for immune responses, as well as protection from cell death. Aberrant NF-κB activation is reported in in inflammatory diseases and cancer. LUBAC is associated with some forms of B-cell lymphoma and acquisition of resistance to cisplatin, a widely used anti-cancer drug. Since linear chains are exclusively generated by the LUBAC ubiquitin ligase complex, which is composed of HOIP, HOIL-1L, and SHARPIN, inhibition of LUBAC’s ligase activity appears a suitable target for the treatment of those diseases.

We have established a high throughput screening system to identify inhibitors of linear ubiquitination using E1, UbcH5c, and bacterial purified petit-LUBAC or petit-SHARPIN, which contains part of HOIP and HOIL-1L or SHARPIN, respectively. Among 141,137 small chemicals, we identified gliotoxin, which is a major virulence factor of opportunistic pathogen, Aspergillus fumigatus, as an inhibitor of LUBAC-mediated linear ubiquitination. Gliotoxin binds to RING-IBR-RING-LDD region of HOIP, which confers catalytic activity of LUBAC. Gliotoxin inhibits not only activation of IKK, which is triggered by linear ubiquitination, but also expression of NF-κB target genes induced by TNF-α or CD40.

Gliotoxin is suggested to contribute to fungal pathogenicity by targeting primarily the activity of cells involved in innate immunity including neutrophils or other phagocytes. Considering the pivotal roles of NF-κB in the activation of those cells, it is plausible that the linear polyubiquitination activity of LUBAC is a target for enabling opportunistic infection. Moreover, it is the first selective small-molecular inhibitor of LUBAC and could therefore be a valuable tool for studying the functions of linear polyubiquitin chains generated by LUBAC.
USP30 AND PARKIN HOMEOSTATICALLY REGULATE ATYPICAL UBIQUITIN CHAINS ON MITOCHONDRIA

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Multiple lines of evidence indicate that mitochondrial dysfunction is central to Parkinson's Disease. Here we investigate the mechanism by which Parkin, an E3 ubiquitin ligase, and USP30, a mitochondria-localized deubiquitinase, regulate mitophagy. We find that mitochondrial damage stimulates Parkin to assemble Lys6, Lys11, and Lys63 chains on mitochondria, and that USP30 is an ubiquitin-specific deubiquitinase with a strong preference for cleaving Lys6 and Lys11-linked multimers. Using mass spectrometry, we show that recombinant USP30 preferentially removes these linkage types from intact ubiquitinated mitochondria and counteracts Parkin-mediated ubiquitin chain formation in cells. These results, combined with a series of chimera and localization studies, afford insights into the mechanism by which a balance of ubiquitination and deubiquitination regulates mitochondrial homeostasis and suggest a general mechanism for organelle autophagy.
RSP5 AND UBP2 JOINTLY TARGET CYTOSOLIC MISFOLDED PROTEINS FOR PROTEASOME DEGRADATION UPON HEAT DENATURATION.

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Protein quality control pathways monitor the proteome to avoid the accumulation of misfolded proteins. We found that heat-shock, that causes misfolding, leads to the ubiquitination of mainly cytosolic proteins. We showed that both Hul5 and Rsp5 are the two major ubiquitin ligases in this heat-shock response, and that both ligases mainly target different pools of misfolded proteins\(^1\,^2\). Rsp5 directly ubiquitinates heat-induced misfolded proteins \textit{in vitro} and that Nedd4, its closest human homologue, mediates the same heat-shock response in mammalian cells. We showed that a large portion of heat-shock induced Rsp5 substrates are cytosolic and contain short PY Rsp5-interacting motifs. Those are more often located in structured regions, thereby, possibly only interacting with Rsp5 when misfolded. In addition, we found that the Ydj1 Hsp40 protein specifically interacts with Rsp5 upon heat-shock to help mediate substrate ubiquitination and proteasome degradation. We propose a bipartite model in which Rsp5, on one hand, directly targets cytosolic misfolded proteins that expose heat-induced PY-containing degrons, as well as associates to a co-chaperone that functions as a substrate adaptor protein\(^2\). Intriguingly, heat-induced Rsp5 substrates are conjugated to K48 linked ubiquitin chains, while Rsp5 mostly synthesized K63-linked chains\(^2\). After screening for other factors that impact the heat-shock ubiquitination response, we found that the Ubp2 deubiquitinating enzyme, which can antagonize Rsp5 ligase activity, was also required for the proteasome degradation of misfolded proteins targeted by Rsp5. Whereas absence of Ubp2 led to greater ubiquitination levels upon heat-shock, heat-induced Rsp5 substrates were further conjugated to K63-linked ubiquitin chains in this case. Consistent with a role in the heat-shock response, absence of Ubp2 reduced cell fitness upon misfolding stress. Interestingly, Ubp2 was not required for the lysosomal degradation of misfolded plasma membrane proteins, indicating that its role is specific for the cytosolic protein quality control function of Rsp5. Our results indicate that Ubp2 is required for the proteasome degradation of cytosolic misfolded proteins by promoting the conjugation of K48-ubiquitin chains on Rsp5 substrates. Ubp2 may thereby play a major role in the triage of cytosolic and membrane misfolded proteins that are degraded by the proteasome and lysosome, respectively.

MISFOLDED MITOCHONDRIAL PROTEIN DEGRADATION BY THE UBIQUITIN-PROTEASOME SYSTEM

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The ubiquitin-proteasome system (UPS) has been implicated in the degradation of mitochondrial outer membrane (OM) proteins during mitophagy. It also plays a role in the regulated degradation of some OM proteins, such as yeast Fzo1p and mammalian mitofusin 2, during mitochondrial fusion and in response to stress, respectively. To more generally interrogate the role of the UPS in mitochondrial protein turnover, we have used temperature-sensitive (ts) alleles in S. cerevisiae to analyze the degradation of misfolded mitochondrial proteins. Rapidly degraded ts proteins from each mitochondrial sub-compartment (OM, intermembrane space (IMS), inner membrane (IM), and matrix) have been identified, many of which are unstable exclusively when shifted to the non-permissive temperature. We find, not unexpectedly, that all IM- and matrix-localized unstable ts proteins are subject to degradation by mitochondrial proteases and not the proteasome. Interestingly, we have determined that some unstable ts proteins residing in the OM and IMS require the proteasome for their degradation, consistent with involvement of the UPS. We have examined the turnover of unstable ts proteins in a collection of yeast strains mutant for known and putative UPS machinery. These screens have identified several distinct UPS mutants, including E3 ubiquitin ligases and machinery implicated in proteasome targeting, that differentially affect the stability of the various mitochondrial ts proteins. Precise roles for these newly identified UPS components in the degradation of yeast mitochondrial proteins is currently being examined in detail. Identification of novel mitochondrial UPS targets and the characterization of the machinery responsible for their degradation using this tractable system will enable us to interrogate the degradation of the orthologous mammalian proteins. Together, these studies will add to our understanding of how protein homeostasis is maintained in mitochondria.
Selenocysteine (Sec) is translated from the codon UGA, which typically functions as a termination signal. Reassigning UGA to Sec depends on unique cis- and trans-acting factors and is tuned by selenium availability. Codon duality extends the genetic code, but how an organism guards the fidelity of proteome against competition between two UGA-decoding mechanisms is unknown. We found a CRL2 ubiquitin ligase-mediated protein quality control system that specifically eliminates truncated proteins due to failures of the UGA to Sec reassignment. By exposing the peptide immediately upstream of Sec, the CRL2 recognition degron promotes protein degradation. Sec incorporation masks the degron, preventing read-through proteins from detection by CRL2. Our study reveals a coupling between translation ambiguity and proteolysis-assisted protein quality control, as well as a cellular strategy to cope with fluctuations in organismal selenium intake.
To identify novel genes that affect hearing, we screened a collection of mutations in essential genes on the X chromosome for morphological defects in the fly auditory organ, Johnston’s Organ (JO). We isolated two mutations in the gene ubr3, which encodes a conserved, RING type E3 ubiquitin ligase. Loss of ubr3 causes apical detachment of the auditory sensory cells and functional defects in hearing.

Apical detachment of auditory sensory cells is a specific defect that was previously only associated with mutations in the fly homolog of Myosin VIIA (myo7a) gene. myo7a encodes an unconventional myosin. Mutations in this gene cause Usher syndrome in humans, a disease that causes blindness and deafness and accounts for 50% of blind-deaf patients. We found that ubr3 and myo7a genetically interact. In our previous studies, we found that Cullin1 (Cul1), a component of SCF E3 ligase complex, is up-regulated in ubr3 mutant cells. Interestingly, over-expression of Cul1 in wild type cells causes a similar apical detachment in JO, suggesting that Ubr3 may regulate apical attachment of JO through Cul1. Moreover, loss of Cul1, or SkpA, or Roc1a, all components of the SCF E3 ligase complex, results in apical detachment. This indicates that proper Cul1 levels are important for the development of the apical structure of the JO and that Cul1 functions as an E3 ligase in this context. In addition to Myo7a, Ubr3 and Cul1 also show genetic and physical interactions with two other Usher syndrome proteins, Cad99C (PCDH15) and Sans, whose mammalian homologs have been shown to form a protein complex with Myo7a.

To examine whether the regulation of USH proteins by ubr3 is evolutionarily conserved in vertebrates, we characterized the function of Ubr3 in sensory hair cells of zebrafish. Loss of ubr3 causes behavioral and morphological defects typically observed in zebrafish Usher mutants. Interestingly, the localization and levels of Myo7a and two other Usher proteins, Harmonin and PCDH15, are altered in ubr3 morphants. These data suggest that Ubr3 plays a similar function in regulating USH proteins in Drosophila and zebrafish.
REGULATION OF UBIQUITIN METABOLISM BY A DIRECT PHOSPHORYLATION MECHANISM

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There is mounting evidence that many human diseases – particularly diseases related to protein misfolding and aggregation such as neurodegenerative disorders - are associated with diminished function of the ubiquitin-proteasome system and altered ubiquitin homeostasis. Given the emerging consensus that dysregulation of ubiquitin homeostasis is a key feature of neurodegeneration, some have proposed restoring ubiquitin levels as a potential therapeutic strategy. However, very little is known about regulation of ubiquitin metabolism in physiological conditions, during cellular aging, in response to cellular stress, or in states of disease. Thus, there is a critical need to dissect the basic mechanisms responsible for regulating ubiquitin metabolism and to identify new pathways which may be targeted to facilitate precise manipulation of ubiquitin homeostasis in the context of disease cells.

We have identified a yeast phosphatase mutant that exhibits defects in management of ubiquitin pools in response to cellular stress. Through quantitative analysis of the yeast phosphoproteome, we determined that phosphatase deletion mutants exhibit increased levels of ubiquitin phosphorylation at the Ser57 position. Based on these observations, we hypothesize that cellular signaling pathways regulate ubiquitin homeostasis by direct phosphorylation of ubiquitin itself, and that phosphorylation of ubiquitin targets its degradation. Consistent with this hypothesis, we have found that phosphomimetic mutants of ubiquitin are far less stable than wildtype ubiquitin, while phosphorylation-resistant forms of ubiquitin were stabilized relative to wildtype. Unexpectedly, we also found that Ser57 phosphorylation triggers accelerated endocytic trafficking and degradation of endocytic cargo. Using genetics, live cell imaging, and reconstitution biochemistry, we have shown that Ser57 phosphomimetic ubiquitin bypasses recognition by deubiquitylases along the endocytic pathway, leading to degradation of both ubiquitin and cargo. Based on our findings, we propose that direct phosphorylation of ubiquitin at Ser57 prevents the recycling of ubiquitin conjugates from substrates during degradation, and that the cellular concentration of ubiquitin is tightly regulated by a ubiquitin phosphoregulatory cycle. These findings also suggest that specific signaling mechanisms can regulate ubiquitin metabolism and function, providing an interesting example of how global protein stability can be fine-tuned as an adaptation response to cellular stress.
The ubiquitin system is becoming ever more complex, and a new frontier in ubiquitin research is the posttranslational modification of ubiquitin itself, e.g. by phosphorylation. In 2014, PINK1 was shown to be the first protein kinase to phosphorylate ubiquitin at Ser65, and such generated phospho-ubiquitin acts as an activator for the RBR E3 ligase Parkin. Whilst these ‘gain-of-function’ roles of ubiquitin were exciting, we wondered how ubiquitin phosphorylation would affect ubiquitin structure and the ubiquitin system as a whole. Surprisingly, we found that phosphorylation leads to significant structural alterations within ubiquitin, enabling recognition by new binding partners, but more importantly also abrogating known interactions in enzymatic systems. In my talk, I will discuss our latest insights into ubiquitin and Ubl phosphorylation, and how phospho-ubiquitin regulates enzyme function in the ubiquitin system.
AN INHIBITOR OF UBIQUITIN CONJUGATION SYSTEM

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We discovered an inhibitor of the ubiquitin conjugation, and investigated its pharmacological properties. To discover the inhibitor, we synthesized 8 rationally designed molecules and tested their ability to inhibit E1 enzyme families in intact A549 cells using a combination of activity-based profiling and proteomic methods. Among those, ABP A3 has emerged as an inhibitor of the two enzymes that are required to maintain the activity of the entire ubiquitin system, the ubiquitin and Nedd8 E1 enzymes, but not SUMO1-3, Ufm1, ISG15 and LC3 pathways. Paradoxically, ABP A3 induced strong activation of protein sumoylation and ufmylation, which are known markers of cellular stress.

Following the discovery of ABP A3, we used it to study the hypothesis that inhibition of ubiquitin conjugation will induce proteotoxic stress due to the accumulation of mis-folded proteins similar to proteasome inhibitors. Indeed, ABP A3 treatment stimulated phosphorylation of PERK and eIF2α, and induced apoptosis executor CHOP. ABP A3 also induced apoptosis at 3.2 μM in A549 cells. The cell viability assay using MM.1S and A549 cells showed 80 nM and 2.5 μM of IC50, respectively. These results suggest that MM cells are more susceptible to the inhibition of UPS system due to their elevated dependence on protein quality control.

Finally, we hypothesized that ABP A3 would not cause the formation of cytoprotective aggresomes, which are induced by proteasome inhibitors and believed to limit their clinical efficacy. Since aggresome pathway is mediated by HDAC6 that recognizes Ub-tag on the mis-folded proteins, the accumulated proteins during ABP A3 treatment may not be recognized by HDAC6 due to the lack of Ub-tag. Indeed, immuno-staining with LC3, HDAC6 and ubiquitin showed no aggresome formation in ABP A3 treated cells while MG132 (proteasome inhibitor) treated cells did show aggresome formation. Instead, ABP A3 treated cells showed small autophagic puncta scattered over the cytoplasm. Increased level of p62 and LC3 during ABP A3 and chloroquin (lysosome inhibitor) co-treatment suggested that autophagic flux was still activated during ABP A3 treatment. Finally, apoptotic death of A549 cells increased when ABP A3 was co-treated with chloroquin, suggesting that the ABP A3-induced autophagy may contribute to the cell-death.

We envision that ABP A3 will serve as a useful tool compound for exploiting the therapeutic potential of the ubiquitin system. As cullins are the major substrates of Nedd8 system known to date, dual inhibition of Ub and Nedd8 E1s can efficiently inhibit the protein ubiquitylation. The discovery of non-cullin Nedd8 substrates that do not involve ubiquitin E1 pathway will enable us to evaluate possible off-target effects of ABP A3.
UCHL3 HYDROLYSIS OF UBQUITIN CHAINS IS REGULATED BY ALLOSTERY AS DETERMINED BY NMR

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The deubiquitinase activity of ubiquitin C-terminal hydrolase L3 (UCHL3) against ubiquitin chains have been shown to play a significant role in adipogenesis and insulin signaling. Here, we report multiple polyubiquitin substrates of UCHL3 that are hydrolyzed with biologically relevant efficiency (kcat/Km>10⁴ M⁻¹sec⁻¹). To elucidate the mechanism of this substrate recognition and hydrolysis we studied interaction of UCHL3 with various substrates via heteronuclear single quantum coherence NMR (HSQC) and solvent exposed amide transverse relaxation-optimized spectroscopy (SEA-TROSY). These experiments revealed that UCHL3 engages up to three ubiquitin subunits at a time when hydrolyzing polyubiquitin. Furthermore, this unique interaction occurs at distinct sites on UCHL3 termed “exosites”. Upon binding to each exosite, UCHL3 is allosterically activated. Mutational analysis has also revealed that these exosites are necessary for polyubiquitin hydrolysis, but not for cleaving C-terminal adducts. Alterations in the exosites ablate insulin signaling. Our findings establish a novel mechanism of polyubiquitin hydrolysis by UCHL3 involving exosites, and suggest that the ubiquitin C-terminal hydrolase family as a whole may be regulated by a similar model in other signaling pathways.
DEVELOPMENT OF A DRUG DISCOVERY PLATFORM TARGETING SUMO SPECIFIC PROTEASES

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SUMO is a ubiquitin-like protein which is covalently linked to a large number of proteins to control their function and localisation, in a process termed SUMOylation. SUMOylation is a dynamic event that in humans is reversed by Sentrin/SUMO-specific proteases (SENPs). There are nine SENPs in humans, which can be divided into three distinct classes based on their structure. SENPs display differential specificity towards the three SUMO isoforms (SUMO1, SUMO2, SUMO3) and are involved in two distinct activities: the maturation of SUMO precursors (endopeptidase activity) and/or SUMO deconjugation from substrates (isopeptidase activity). Notably, SENPs have been implicated in the pathogenesis of several human diseases, including cancer and neurodegenerative disorders making them attractive targets for drug discovery.

MISSION Therapeutics is a leading expert in DUB drug discovery, with a number of projects in lead optimisation. As part of ongoing efforts to expand its platform, MISSION has recently been awarded a Biomedical Catalyst Feasibility Grant from the UK Technology Strategy Board, aiming at validating SENPs as interesting targets in oncology and developing SENPs inhibitors. Targeting SENPs for drug discovery poses distinct challenges which will be presented in this poster, including the characterization of SENPs enzymatic activity and substrate specificity, and the development of high throughput primary and secondary biochemical assays, as well as cellular assays. These assays will be used for screening a small, focused compound library to identify drug-like starting points for SENPs inhibition.
PROTEASOME INHIBITORS INCREASE THE GROWTH INHIBITION OF DRUGS TARGETING DNA TOPOISOMERASE II (TOP2).

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DNA topoisomerase II is a target for anti-cancer drugs such as etoposide and mitoxantrone. These drugs stabilise the covalent complex of TOP2 on DNA. Repair of these complexes involves proteasomal degradation of the adducted TOP2. To determine if proteasomal inhibition increases the growth inhibitory effect of drugs targeting TOP2 we incubated K562 cells with combinations of a proteasomal inhibitor and a TOP2 drug. Two proteasomal inhibitors were tested MG132 and PS341. K562 cells were incubated for 120 hours with either TOP2 drug alone or TOP2 drug in combination with MG132 or PS341. Growth inhibition was determined using XTT assays and the concentration needed to inhibit growth by 50% (IC50) was calculated. The ratio of the IC50 with and without proteasome inhibitor gave a potentiation factor (PF50). MG132 and PS341 potentiated growth inhibition by most of the drugs targeting TOP2. The highest potentiation was seen with mitoxantrone.
ALLOSTERIC CROSS TALK BETWEEN THE DEUBIQUITINASE UBP6 AND THE 26S PROTEASOME

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The eukaryotic 26S proteasome mediates cell survival through targeted degradation of a wide range of cellular proteins. The ATP-dependent protease plays key roles in maintaining protein quality control and in driving cell signaling by degrading polyubiquitinated proteins. Proteasomal deubiquitinases dismantle ubiquitin chains on a substrate as it is unfolded and translocated into the proteolytic chamber. Efficient substrate degradation requires tight coordination between multiple enzymatic activities and subcomplexes, ultimately manifesting in multiple conformational states.

Here we present how one of the intrinsic deubiquitinases, Ubp6, interacts with and affects the activity of the proteasome. Although previous work has shown that Ubp6 catalytically and allosterically impacts proteasomal substrate degradation, little is known about its location within the proteasome holoenzyme and the mechanisms by which it impacts substrate processing. We have localized Ubp6 and use biochemical approaches to investigate its crosstalk with proteasomal subcomplexes. Our studies give important new insight to how this deubiquitinase participates both catalytically and allosterically in substrate degradation, and further our understanding of the intricate regulatory mechanisms involved in proteome remodeling by the 26S proteasome.
CONJUGATION OF THE UBIQUITIN ACTIVATING ENZYME UBE1 WITH THE UBIQUITIN-LIKE MODIFIER FAT10 TARGETS IT FOR PROTEASOMAL DEGRADATION

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The ubiquitin-like modifier HLA-F adjacent transcript 10 (FAT10) directly targets its substrates for proteasomal degradation by becoming covalently attached via its C-terminal diglycine motif to internal lysine residues of its substrate proteins. The conjugation machinery consists of the bispecific E1 activating enzyme Ubiquitin-like modifier activating enzyme 6 (UBA6), the likewise bispecific E2 conjugating enzyme UBA6-specific E2 enzyme 1 (USE1), and so far unknown E3 ligases. By mass spectrometry analysis the ubiquitin E1 activating enzyme ubiquitin-activating enzyme 1 (UBE1) was identified as putative substrate of FAT10. Here, we confirm that UBE1 and FAT10 form a stable non-reducible conjugate under overexpression as well as under endogenous conditions after induction of endogenous FAT10 expression with pro-inflammatory cytokines. FAT10ylation of UBE1 depends on the diglycine motif of FAT10 and, by specifically downregulating FAT10, UBA6 or USE1 with specific siRNAs, we show that UBE1 modification depends on the FAT10 conjugation pathway. Furthermore, we confirm that UBE1 does not act as a second E1 activating enzyme for FAT10 but that FAT10ylation of UBE1 leads to its proteasomal degradation, implying a putative regulatory role of FAT10 in the ubiquitin conjugation pathway.
MOLLUSCUM CONTAGIOSUM VIRUS INHIBITS NF-KB ACTIVATION BY INHIBITING UBIQUITINATION OF NEMO

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Ubiquitination regulates many biological functions, including protein degradation and protein activation. Viruses use myriad strategies to manipulate the cellular ubiquitin system to benefit virus infection. Molluscum contagiosum virus (MCV) is a dermatotropic poxvirus. It causes persistent skin infections in healthy children, young adults and is an opportunistic infection in immunocompromised individuals. It encodes multiple immune evasion proteins for persistence. One such protein is MC159, a member of the FLIP family of proteins. We have found that MC159 inhibits the NF-κB activation pathway, a pathway that involves many proteins controlled by ubiquitination. One such protein is NEMO, a regulatory subunit of the cytoplasmic IkB Kinase (IKK) complex. Our previous studies show that MC159 interacts with NEMO for its NF-κB inhibitory function. NEMO itself undergoes ubiquitination, phosphorylation, and sumoylation to regulate its function. The goal of my projects is to identify how MC159-NEMO interactions dictate inhibition of NF-κB on a molecular level. My working model is that MC159 blocks NEMO ubiquitination as a means to inhibit NF-κB activation. In support of this hypothesis, I observed that transient MC159 expression greatly inhibits the ubiquitination of NEMO under a variety of stimulating conditions. Our working model is that MC159 masks the NEMO region that is necessary for ubiquitination as its means to inhibit this event. We are now probing the phenotype of mutant MC159 and NEMO proteins for their ability to bind to NEMO and to inhibit NEMO ubiquitination to test this model. MC159 is homologous to other viral and cellular FLIP proteins. As such, the elucidation of the MC159 mode of action will provide new understanding of how viruses can alter the cellular environment to their advantage and how signaling events resulting in ubiquitination occur during normal cellular events.
UBIQUITINATION OF HOIP CARBOXYL-TERMINUS NEGATIVELY REGULATES LINEAR UBIQUITINATION-MEDIATED IMMUNE SIGNALING

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As a unique lysine independent ubiquitin chain, linear ubiquitination is built by the linear ubiquitin assembly complex (LUBAC), containing HOIP, HOIL-1L, and Sharpin, that facilitates NF-κB activation and inflammation upon receptor stimulation by ligating linear ubiquitin chains to critical signaling molecules. While linear ubiquitination is essential for intracellular receptor signaling, this response must be metered and ultimately stopped. Here, we demonstrate that LUBAC activity is terminated through ubiquitination of the catalytic subunit HOIP at the carboxyl-terminal lysine-1056. Ubiquitination of Lys1056 alters HOIP conformation, resulting in the suppression of its linear ubiquitination activity. Consequently, a HOIP Lys1056→Arg mutation not only leads to persistent LUBAC activity but also prolongs canonical NF-κB activation induced by TLR4 stimulation, whereas it shows no effect on NF-κB activation induced by CD40 stimulation. Our study describes a novel post-translational regulation of LUBAC-mediated linear ubiquitin chain formation that is critical for directing specific receptor-mediated NF-κB activation.
THE BMI1/RING1A E3 UBIQUITIN LIGASE INHIBITOR PRT4165 INCREASES THE GROWTH INHIBITION OF DRUGS TARGETING DNA TOPOISOMERASE II (TOP2)

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DNA topoisomerase II (TOP2) is an important anticancer drug target for etoposide, mitoxantrone, mAMSA and anthracyclines such as doxorubicin. TOP2 is degraded by the proteasome in response to TOP2-targeted drugs, and consistently, proteasome inhibitors increase the efficacy of TOP2 drugs. However, the proteasomal degradation of TOP2 is also ubiquitin-dependent, and BMI1/RING1A was recently identified as an E3 ubiquitin ligase complex involved in targeting TOP2A to the proteasome. This suggested that BMI1/RING1A inhibition may also increase the efficacy of TOP2 drugs but with greater specificity. Indeed, we show that TOP2 drugs including etoposide, mitoxantrone and mAMSA are potentiated by a small molecule inhibitor of BMI1/RING1A (PRT4165). Furthermore, TOP2 drug-induced growth inhibition is mostly associated with the TOP2A isoform, suggesting that selectively increasing the levels of TOP2A may improve treatment with TOP2 drugs, while also reducing TOP2B-associated genotoxicity. Nalm-6^{TOP2A+/-} and Nalm-6^{TOP2B-/-} knockout cell lines were used to assess the importance of each TOP2 isoform in the potentiation of TOP2 poisons with PRT4165. The extent of potentiation was lower in Nalm-6^{TOP2A+/-} cells but unaffected in Nalm-6^{TOP2B-/-} cells, suggesting that TOP2A is an important mediator of potentiation by PRT4165. This is consistent with the notion that BMI1/RING1A inhibition improves the cytotoxicity of TOP2 drugs by preventing TOP2A degradation. This also demonstrates that TOP2A and TOP2B are targeted by different E3 ubiquitin ligases, suggesting that targeting the proteasomal degradation TOP2 at the level of the E3 ubiquitin ligases may increase the TOP2A-mediated cytotoxicity of TOP2 drugs, while also decreasing TOP2B-mediated genotoxicity.

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CEZANNE (OTUD7B) REGULATES HIF-1α HOMEOSTASIS IN A PROTEASOME-INDEPENDENT MANNER

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The transcription factor HIF-1α is essential for cells to rapidly adapt to low oxygen levels (hypoxia). HIF-1α protein is mainly regulated by the ubiquitin-proteasome system, and fast turnover of HIF-1α in normoxia is mediated by a well characterized oxygen-dependent enzymatic cascade involving prolyl hydroxylases (PHDs) and a specialized RING E3 ubiquitin ligase complex (CRLVHL). Deregulated HIF-1α levels caused by inactivating VHL mutations predispose humans to a variety of cancers, in which the regulation machinery for HIF-1α degradation has been studied intensively. However, recent reports also showed that HIF-1α is targeted for lysosomal degradation via chaperone-mediated autophagy (CMA).

We demonstrate that the Lys11-linkage specific ovarian tumor (OTU) deubiquitinase Cezanne regulates HIF-1α homeostasis. Loss of Cezanne decreases HIF-1α target gene expression due to a reduction in HIF-1α protein levels, and sensitizes cells for hypoxia-induced apoptosis. Surprisingly, although the Cezanne-regulated degradation of HIF-1α depends on the tumor suppressor pVHL, hydroxylase and proteasome activity are dispensable. Our data suggest that Cezanne is essential for HIF-1α protein stability and that loss of Cezanne stimulates HIF-1α degradation via proteasome-independent routes, potentially via CMA.
CDC48 COMPLEXES REQUIRED FOR MULTIPLE STEPS OF SREBP CLEAVAGE IN S. POMBE

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Schizosaccharomyces pombe regulates sterol and oxygen homeostasis through the movement of the sterol regulatory element binding protein (SREBP) Sre1 to the Golgi under conditions of low oxygen or sterols. There it is cleaved and the cytosolic N-terminal transcription factor domain is released. Fission yeast SREBPs are cleaved in the Golgi through the combined action of the Dsc E3 ligase complex and the rhomboid protease Rbd2. The essential AAA ATPase Cdc48 is also required for SREBP cleavage. Cdc48 is a molecular machine that separates polyubiquitylated substrates from complexes with proteins, nucleic acids, or membranes. This activity is essential for a number of cellular processes and Cdc48 is consequently one of the most abundant cytosolic proteins, comprising about one percent of total protein. Because Cdc48 is involved in multiple pathways, it is targeted to each specific substrate by precise combinations of cofactors.

In the activation of SREBPs, Cdc48 interacts with both Dsc5 and Rbd2, which contain known Cdc48 binding domains (UBX and SHP, respectively). It is unknown what function(s) Cdc48 performs at each of these locations. I have determined that Cdc48 likely plays two different roles during SREBP activation. In the first, Cdc48 promotes ER-to-Golgi transport of the Dsc complex, possibly through extraction of a repressor. I am performing an overexpression screen to identify this repressor. The second role of Cdc48 occurs at the protease Rbd2, although it is not known what function it performs there. To better understand the roles Cdc48 is playing at these two steps, I wanted to understand what cofactors are involved in these processes, because they direct Cdc48’s separase activity to specific functions. First, I performed a pull down and mass spectrometry to identify all the Cdc48 cofactors in S. pombe. From that list, I have identified ufd1 as required for ER-to-Golgi transport of the Dsc complex. Ufd1 does not form a complex with Cdc48 and Rbd2, and is therefore not a cofactor for that step. This suggests that two distinct Cdc48 complexes are involved in SREBP processing. Future work aims to identify additional cofactors bound to Cdc48 and to use these new cofactors to dissect the role of Cdc48 in each process.
STUDYING DIMER-DEPENDENT FBW7-SUBSTRATE INTERACTIONS: USING AN ALPHASCREEN TO QUANTITATE DEGRON COOPERATIVITY IN FULL-LENGTH SUBSTRATES

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The Fbw7 tumor suppressor is an F-box protein that binds to nearly two-dozen known substrates and targets them for ubiquitylation and degradation by SCF^{Fbw7}. Many Fbw7 substrates are critical oncoproteins (e.g. Myc, cyclin E, Notch) and Fbw7 is one of the most commonly mutated genes in human cancers. Fbw7 binds to substrates after they become phosphorylated in phosphodegrons, termed CPDs. Structural studies have revealed the interactions between CPD-derived peptides and the Fbw7 substrate-binding interface, which is formed by its beta-propeller. Multiple CPD residues contact Fbw7 and contribute to binding affinity, which varies greatly among substrates. High affinity (optimal) CPDs contain two phosphorylations (in the 0 and +4 positions) whereas suboptimal substrates may have negatively charged residues instead of the +4 phosphorylation or other unfavorable CPD residues.

We recently found that Fbw7 dimerization results in unexpected complexity in its substrate interactions. Fbw7 dimers may simultaneously bind to two substrate CPDs, and the affinity of these Fbw7-substrate interaction thus reflects the concerted binding of both CPDs to the Fbw7 dimer (avidity). In this way, two low-affinity CPDs may drive substrate ubiquitylation, thereby greatly increasing the control and complexity of substrate ubiquitylation.

Because affinity is the primary determinant of substrate ubiquitylation, it is critical to understand how the avidity of two independent CPDs impacts overall affinity and ubiquitylation. However, because previous approaches to study Fbw7-substrate interactions have utilized CPD-derived peptides and Fbw7 monomers, they cannot be used to address the issues of CPD cooperativity and avidity. We developed an ALPHAScreen-based approach that employs full-length substrates and Fbw7 dimers. Using cyclin E as a model, we show that this assay quantitatively reports the avidity of the two cyclin E CPDs, and represents a facile approach to identifying substrate CPDs and studying their cooperativity. Moreover, because phosphorylated substrates are isolated from cell lysates, our approach is readily adapted to other applications, such as identifying CPD kinases and high throughput screening.
DISTINCT ACTIVATION OF AN E2 UBIQUITIN CONJUGATING ENZYME BY ITS COGNATE E3 LIGASES

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A significant portion of ubiquitin (Ub)-dependent cellular protein quality control takes place at the endoplasmic reticulum (ER), in a process termed ER-associated degradation (ERAD). Yeast ERAD employs two integral ER membrane E3 Ub ligases: Hrd1 (also termed Der3) and Doa10 that recognize a distinct set of substrates. Yet, both E3s bind to and activate a common E2 ubiquitin conjugating enzyme, Ubc7.

Here we describe a yet unexplored feature of the ERAD system that entails differential activation of Ubc7 by its cognate E3s. We found that residues within helix $\alpha_2$ of Ubc7 that interact with donor Ub were essential for polyUb conjugation. Mutagenesis of these residues inhibited the in vitro activity of Ubc7 by preventing the conjugation of donor Ub to the acceptor Ub. Unexpectedly, the defect in Ub chain formation by helix $\alpha_2$ mutant was rescued by the Hrd1 RING domain, but not by the Doa10 RING domain. In a complementary set of experiments, Hrd1 RING also restored polyUb chain formation of Ub mutants that predictably interact with helix $\alpha_2$. In agreement with the in vitro data, expression of mutants of helix $\alpha_2$ in yeast selectively impaired the degradation of Doa10 substrates, but had no apparent effect on the degradation of Hrd1 substrates.

Based on these in vitro and in vivo findings and on previously published structural data, we propose a model for Ubc7 activation by its cognate E3s. According to the model, the RING domain activates Ub transfer by stabilizing a transition state that is determined by non-covalent interactions between the helix $\alpha_2$ of Ubc7 and Ub. This transition state may be further stabilized by some E3 ligases, such as Hrd1, through additional non-covalent interactions outside the RING domain that also compensate for suboptimal helix $\alpha_2$:Ub interaction.

The distinct requirements for E2 activation by Hrd1 and Doa10 imply that it is possible to develop selective ERAD inhibitors. Furthermore, the hierarchical structure of the Ub-conjugation system dictates that similar E3s employ common E2s and thus distinct activation of a single E2 by different E3s, as shown in this study, may be a prevalent.

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Probably the most frequent, but least understood, type of systemic muscle loss is the sarcopenia seen in the elderly. Age-related defects in neuromuscular junctions and death of motor neurons lead to loss of muscle innervation (i.e. denervation), increased protein degradation by the proteasome and ultimately to atrophy. In this study we show that the destruction of the muscle’s contractile unit, the myofibrils, in denervated muscle is preceded and accelerated by the Trim32-dependent loss of the desmin cytoskeleton. This RING E3, is expressed in many cell types and plays a role in neurogenesis, and mutations affecting this enzyme are associated with Limb Gridle Muscular Dystrophy 2H. Using biochemical approaches and a sophisticated in vivo electroporation technique we demonstrate that Trim32 promotes the loss of desmin and actin thin filaments, since its downregulation in denervated atrophying mouse muscles resulted in their accumulation. The muscles expressing shRNA to Trim32 also exhibited a major preservation of mass. Interestingly, upon denervation, the depolymerization of desmin filaments requires the calcium-dependent protease, Calpain-1, since downregulation of this enzyme in denervated muscles prevented the loss of desmin and of the attached myofibrils, and markedly attenuated fiber atrophy. Furthermore, desmin depolymerization was already evident 7 days after denervation, before there was any effect on myofibril content. Thus, upon denervation, Trim32 and Calpain 1 promote myofibril breakdown by initially catalyzing the depolymerization of the desmin cytoskeleton.
HOIP DEFICIENCY CAUSES EMBRYONIC LETHALITY BY ABERRANT TNFR1-MEDIATED ENDOTHELIAL CELL DEATH

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The linear ubiquitin chain assembly complex (LUBAC) is currently the only known E3 ligase capable of generating linearly linked ubiquitin chains. LUBAC is composed of three proteins: HOIL-1, HOIP and SHARPIN with HOIP being the central and catalytically active component of this complex. LUBAC activity is crucial for stability of the TNFR1 signalling complex, the normal gene-inducing capacity of TNF and resistance to cell death induction by this cytokine. We recently showed that both constitutive and endothelial-cell-specific deficiency in HOIP leads to excessive cell death in the yolk sac vasculature resulting in defective vascularization and embryonic lethality at mid-gestation (E10.5). This phenotype resembles that of the deletion of components from the cell death pathway such as capase-8, FADD and FLIP, indicating that LUBAC enables normal development by interfering with aberrant cell death during embryogenesis. Genetic deletion of TNFR1 in HOIP-deficient embryos prevented endothelial cell death and restored normal vascularization rescuing the embryonic lethality at E10.5. Consistently, cells derived from HOIP-deficient embryos aberrantly form a pro-cell death complex in response to TNF and LTα, and are sensitised to cell death induction by these cytokines. Together, this implies that TNFR1-mediated endothelial cell death is the primary cause of embryonic lethality in the absence of linear ubiquitination. We currently aim to understand the mechanisms underlying the lethality of HOIP deficiency in more detail, especially in regards to the involvement of different forms of programmed cell death. Our most recent findings will be presented.
BUILDING PREDICTIVE NETWORKS OF TCGA DATASETS TO IDENTIFY FUNCTIONAL CONSEQUENCES OF FBW7 MUTATIONS IN HUMAN TUMORS

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The Fbw7 tumor suppressor is the substrate receptor of an SCF E3 ubiquitin ligase that ubiquitylates several key oncoproteins including Myc, Notch, Jun, and cyclin E. Fbw7 is correspondingly one of the most frequently mutated genes in human cancers. While the biological functions of individual Fbw7 substrates are well defined, the net effect of loss-of-function Fbw7 mutations—which coordinately stabilize multiple proteins—is less well understood. Therefore, we utilized a novel informatics approach to understand the global impact of Fbw7 mutations in tumors. Briefly, we used The Cancer Genome Atlas (TCGA) to conjointly model multiple tumor types that contain Fbw7 mutations, in order to derive gene expression signatures that predict Fbw7 mutational status. Gene-set enrichment analyses of these predictive signatures revealed highly significant enrichment of specific biological pathways. Some of these modules were common to all organ sites, whereas others exhibited site-specificity. A set of metabolic genes with roles in both glycolysis and mitochondrial function was highly enriched in all tumor types. Follow up studies in isogenic cell lines with engineered Fbw7 mutations as well as colorectal cancer cell lines with naturally occurring Fbw7 mutations have validated both Fbw7’s causal role in the predictive expression signature (increased mitochondrial function) as well as altered central carbon metabolism in Fbw7 mutant cells.

In summary, our informatics approach utilizing primary cancer data from TCGA yielded unique insights into the dominant effects of Fbw7 mutations in cancers, and may be useful in a broader context to leverage high-throughput cancer datasets to understand global consequences of oncogenic mutations in human tumors.
THE P97 SMALL MOLECULE INHIBITOR CB-5083 CAUSES DISRUPTION OF THE UBIQUITIN-PROTEASOME PATHWAY, ACTIVATES THE UNFOLDED PROTEIN RESPONSE AND HAS STRONG ANTI-CANCER EFFECTS

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The ubiquitin-proteasome system (UPS) sustains cancer cell viability by alleviating proteotoxic stress caused by an imbalance of protein synthesis and degradation. While proteasome inhibitors, such as bortezomib, are approved for the treatment of hematological malignancies, relapse following treatment is common. Additionally, these agents have poor efficacy in solid tumors, prompting the need for discovery of novel drug targets within the UPS which might provide a more profound anti-tumor effect. The homo-hexameric AAA-ATPase VCP/p97 functions by converting chemical energy into mechanical force to extract proteins from cell membranes. p97 is closely involved in several facets of protein homeostasis, including ubiquitin-dependent protein degradation, endoplasmic reticulum-associated degradation (ERAD), and autophagy. Therefore p97 inhibition could provide a novel and more potent approach than proteasome inhibitors to exploit cancer cell addiction to protein homeostatic mechanisms. Through a targeted medicinal chemistry effort we have discovered CB-5083, a novel small molecule ATP-competitive inhibitor of p97 ATPase activity with nanomolar enzymatic and cellular potency. Treatment of normal and cancer cells with CB-5083 causes a dramatic increase in poly-ubiquitinated proteins and an accumulation of substrates of the UPS and ERAD. The blockade of ERAD causes induction of a sustained and irresolvable unfolded protein response (UPR), leading to caspase cleavage and apoptosis in a manner that is distinct from proteasome inhibitors. Indeed, UPR and ERAD genes were the most significantly upregulated gene ontologies uncovered from transcriptome profiling. Additionally, knockdown of DR5 protects against CB-5083 mediated cell death, suggesting that death receptor signaling plays a key role in the apoptotic response caused by inhibition of p97 function. In animal models, our p97 inhibitor is orally bio-available and causes rapid and sustained accumulation of poly-ubiquitin and markers of the UPR and apoptosis. Furthermore, strong anti-cancer effects were observed in solid tumor and hematological in vivo models. CB-5083 is currently being tested in phase I clinical trials for relapsed/refractory multiple myeloma and solid tumor patients. Taken together, these results indicate that CB-5083 is a powerful tool for investigating biological functions of p97 with great promise as a novel therapeutic agent for the treatment of cancer.
The linear ubiquitin chain assembly complex (LUBAC) promotes formation of linear ubiquitin chains that play important roles in activation of the NF-kB signaling pathway and in regulation of cell death. The importance of linear (M1) ubiquitination has been well established in TNF, IL-1 and NOD2 signalling. Two deubiquitinases (DUBs) were reported to cleave linear ubiquitin chains - OTULIN and CYLD. Recent work shows that M1-ubiquitination of components within the TNF-R1 signalling complex (TNF-RSC) and the NOD2 signalling complex is highly dynamic and well controlled by DUBs like OTULIN. Surprisingly, LUBAC constitutively interacts with OTULIN and CYLD. The importance of this interaction for the TNF-RSC has been unclear. In this study, we expand the current knowledge of the interplay between LUBAC and TNF-signalling pathway-relevant DUBs at the level of the TNF-RSC and the NOD2 pathway. We show that LUBAC and its activity regulate recruitment of different DUBs to these signalling complexes and present specific functions of different DUBs with respect to M1-ubiquitin-chain regulation.
Several nuclear domain 10 (ND10) components are restriction factors that inhibit herpesviral replication. Different herpesviruses antagonize this restriction by a variety of strategies, including degradation or relocalization of ND10 proteins. We previously identified two rhadinoviral tegument proteins, Kaposi’s Sarcoma-associated human Herpesvirus 8 (KSHV) ORF75 (Full, 2014) and Herpesvirus saimiri ORF3 (Full, 2012), as effector molecules that counteract ND10. The rhesus monkey rhadinovirus (RRV) shares many key biological features with KSHV but according to our data it interferes with ND10 in a manner distinct from both KSHV and HVS. HVS ORF3 selectively effects proteasomal degradation of a single ND10 component, SP100, and this activity critically depends on a single SUMOylation site in ORF3. After KSHV infection, ATRX is degraded and Daxx is dispersed from ND10 early during infection by ORF75, an essential viral gene that is also a homolog to cellular FGARAT (PFAS, EC 6.3.5.3). Upon overexpression in primary cells KSHV ORF75 also induces rearrangement of PML and of Sp100, indicating that KSHV may be able to target multiple ND10 components, presumably by different mechanisms. RRV ORF75 resembles HVS, but it targets a larger set of cellular restriction factors for proteasomal degradation. To identify ORF75-interacting proteins, we employed conventional coimmunoprecipitation as well as proximity-dependent biotinylation using ORF75 fusion proteins to APEX2 peroxidase or a hyperactive BirA* biotin conjugating enzyme (BioID), followed by mass spectrometry analysis. With a focus on proteins independently identified by two methods, several candidate interactions were confirmed. Crispr/Cas9 knockout cells of the corresponding genes were generated for functional characterization of these novel interaction partners in the context of the viral lifecycle. Our preliminary findings support the notion that the viral FGARAT homologs target both well established as well as potentially novel antiviral defense pathways and may be key factors in overcoming host defenses during de-novo infection and lytic replication of the gamma2-herpesviruses.
ROLE OF E3 LIGASE TRIM71 IN INNATE IMMUNE SIGNALING.

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Viral infection triggers a fast and potent cellular response mediated primarily by the production of IFNβ that induces an anti-viral state through a signal cascade. IFNβ induction and subsequent signaling is tightly controlled to allow for a rapid response to infection while preventing unnecessary activation.

There is growing evidence implicating the members of Tripartite-motif (TRIM) protein family of E3 ligases as critical players in this regulation. However, the exact role, mechanism of action, and the physiological relevance of their activity in vivo still remain poorly investigated. Previous work in our lab revealed that an unprecedented large number of TRIMs play critical roles as enhancers in the regulation of innate immune signaling pathways.

Our recent studies focusing on the VII sub-group or NHL sub-family of TRIM proteins showed that 2 members (TRIM32 and TRIM71) have a positive regulatory role on the RIG-I like receptors (RLR) signaling, while the other two members (TRIM2 and TRIM3) neither have positive nor inhibitory activities. Furthermore, detailed work on TRIM71 revealed that its overexpression strongly increased the 2CARD-RIG-I-dependent activation of the IFNβ and ISRE promoters as well as the IFNβ-mediated activation of the ISRE promoter.

Since the E3 ubiquitin ligase activity of many TRIMs has been linked to their antiviral functions, our future studies are aimed to identify TRIM71 interacting factors as potential substrates and delineate the molecular mechanism by which TRIM71-mediated ubiquitin or ubiquitin-like modifications could regulate the response to viral infection.
ALS-CAUSING MUTATIONS IN UBQLN2 DEREGULATE ITS UBIQUITIN-BINDING CYCLE

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Missense mutations in the UBQLN2 gene cause dominantly inherited amyotrophic lateral sclerosis (ALS, also known as Lou Gehrig’s disease) and a mixed ALS/dementia phenotype. UBQLN2 belongs to a family of structurally related ubiquilin (UBQLN) proteins, characterized by an N-terminal ubiquitin (Ub)-like domain and C-terminal Ub-associated domain; UBQLNs have been implicated in proteostasis as shuttling factors for Ub-proteasome- and autophagy-mediated protein degradation. Several distinct disease-causing mutations in UBQLN2 are clustered in a proline-rich-repeat (PRR) domain not present in UBQLN1. In fact, UBQLN1 and UBQLN2 share 95% similarity and otherwise harbor the same type, number, and arrangement of modular domains – suggesting that the PRR may be of particular importance to UBQLN2 function. We discovered that UBQLN2 binds significantly more Ub than UBQLN1 as assessed by co-immunoprecipitation (IP) experiments employing both epitope-tagged and endogenous proteins. UBQLN2ALS mutants exhibit an additional increase in polyUb pulldown by co-IP as compared to UBQLN2, but not by in vitro experiments with recombinant protein, suggesting that UBQLN2ALS mutants might be ubiquitylated in cellulo and/or interacting with a unique cellular cofactor. Further experiments in stable, inducible cell culture models suggest that UBQLN2ALS mutants are unstable proteins, supporting the notion that co-IP experiments detect both UBQLN ubiquitylation and noncovalent association with Ub-modified proteins. Collectively, these findings provide evidence of a functional distinction in Ub-binding affinity between UBQLN1 and UBQLN2, and insight into the pathologic effect of ALS mutations that destabilize the UBQLN2 protein.
CANCER-ASSOCIATED FIBROBLASTS ATTENUATE THE P53 RESPONSE BY INCREASING UBIQUITINATION OF P53 IN CANCER CELLS

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Chemotherapy triggers a DNA damage response that involves activation and accumulation of the tumor suppressor p53. The p53 response serves to halt or eliminate damaged cells and mutation of p53 allows evasion of apoptosis and tumor progression. In addition, growth of tumor cells is influenced by the tumor microenvironment. The microenvironment can mediate drug resistance of tumor cells through soluble factors. Therefore, we have investigated how fibroblasts influence the p53 response to doxorubicin in cancer cells. We have performed co-cultures of prostate cancer cells (LNCaP) with primary fibroblasts derived from prostate tumor areas (CAFs) or cultures of LNCaP in conditioned medium from CAFs. We have found that cancer-associated fibroblasts (CAFs) or CAF-conditioned medium decrease p53 accumulation, p53 phosphorylation and p53 target gene expressions, and increase ubiquitination of p53 in LNCaP cells in response to doxorubicin treatment. As a consequence, this leads to enhanced tumor cell viability. Our observations suggest that CAF-mediated reduction of the p53 response can promote tumor cell survival and enhance drug resistance.
Proper muscle physiology is central to allow an organism to move. Within a multinucleated muscle fiber, nuclei need to be evenly spaced throughout the cytoplasm. Failure to correctly position nuclei leads to muscle dysfunction and disease such as e.g. Emery Dreifuss Muscular Dystrophy. However, the mechanisms that control nuclear muscle spacing remain largely unknown.

Our lab performed a forward genetic mutagenesis screen on the Drosophila X-chromosome and identified novel genes required for proper development and function. From this screen, we identified four mutations in ariadne-1 (ari-1), which encodes a conserved Ring-In Between-Ring E3 ubiquitin ligase.

We found that loss of ari-1 leads to nuclear clustering within larval body wall muscles, a phenotype that is not observed in wild type animals. This nuclear mispositioning appears to be due to a defect in the subcellular localization of Msp-300, which links the outer nuclear membrane to the cytoskeleton to mediate proper nuclear positioning. ari-1 mutants mimic msp-300 mutants as mutant muscles display clustered nuclei, a fragmented microtubular network and a compromised integrity of the nuclear lamina. In addition, removing a single copy of msp-300 in ari-1 mutants enhanced nuclear clustering, strongly suggesting a molecular interaction between Ari-1 and Msp-300.

Together, our data suggest that Ari-1 regulates the function of Msp-300, possibly by mono-ubiquitinating the protein and modulating its ability to connect nuclei to the muscle cytoskeleton. We are currently testing whether Msp-300 is ubiquitinated in wild-type animals and if so will analyze whether its ubiquitination-level is altered in ari-1 mutants. If our hypothesis holds up, Ari-1 would be the first protein to fine-tune Msp-300’s function in controlling nuclear positioning and sustaining muscle function.
Sumoylation is a reversible post-translational modification involved in various cellular processes. One of the few known SUMO E3 ligases is the 358 KD nucleoporin Nup358/RanBP2. Recent work from our lab shows that the RanBP2/RanGAP1*SUMO1/Ubc9 complex, rather than free RanBP2, is the physiologically relevant SUMO E3 ligase in cells. The E3 ligase region overlaps with the binding site for RanGAP1*SUMO1 placing two key enzymatic activities, sumoylation and RanGTPase, in very close proximity. In mitosis, the RanBP2 complex partially localizes to the spindle and kinetochores, where it seems to be involved in microtubule nucleation, attachment, control of RanGTP levels and sumoylation. Only a few interacting proteins and substrates of the RanBP2 complex have been identified so far. Therefore, how the RanBP2 complex contributes to mitotic regulation remains poorly understood.

To better understand the role of the mitotic RanBP2 complex, we identified interacting proteins of RanBP2 by large scale IP from mitotic or interphase Xenopus egg extract followed by mass spectrometry analysis. Out of ~100 proteins identified as potential binding partners of the RanBP2 complex, 14 candidates were enriched specifically in the mitotic IP. Of these, a group of candidates is involved in spindle organization. Here, we focus on one of these candidates, the chromatin remodeling helicase ISWI. Our biochemical studies show that it directly binds to and can be sumoylated by the RanBP2 complex. ISWI is known as a spindle assembly factor that is activated by RanGTP and it is essential for microtubule stability during anaphase. Ongoing functional studies of sumoylated ISWI and the association between ISWI and the RanBP2 complex will contribute to our understanding how the RanBP2 SUMO E3 ligase complex contributes to mitotic progression.
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Muskelin is an intracellular kelch-repeat protein expressed abundantly in diverse tissues including brain, eyes, heart, skeletal muscles and kidney, and is involved in cell adhesion and the regulation of cytoskeleton dynamics as well as being a component of a putative E3 ligase complex. Muskelin is comprised of four domains: discoidin, LisH, CTLH and kelch-repeat domains. Here, the first crystal structure of mouse muskelin discoidin domain (MK-DD) is reported at 1.55 Å resolution, which reveals a distorted eight-stranded β-barrel with two short α-helices at one end of the barrel. Interestingly, the N- and C-termini are not linked by the disulfide bonds found in other eukaryotic discoidin structures. A highly conserved MIND motif appears to be the determinant for MK-DD specific interaction together with the spike loops. Analysis of interdomain interaction shows that MK-DD binds the kelch-repeat domain directly and that this interaction depends on the presence of the LisH domain. This work was supported financially by the Global Research Laboratory Program of the Ministry of Science, ICT and Future Planning of Korea and an institutional grant from the Korea Institute of Science and Technology.
In selective autophagy, receptors are central for cargo selection and delivery. However, it remains yet unclear whether and how multiple autophagy receptors might form complex and function concertedly to control autophagy. Optineurin (OPTN), implicated genetically in glaucoma and amyotrophic lateral sclerosis, was a recently identified autophagy receptor. Here we report that tumor-suppressing ubiquitin ligase, HACE1, ubiquitylates OPTN and promotes its interaction with p62/SQSTM1, thus accelerating cellular autophagic flux. Therefore, ubiquitin signaling could directly regulate selective autophagy through promoting physical interaction between autophagy receptors, suggesting a previously unappreciated mode of regulation for autophagy. Interestingly, the K48-linked polyUbiquitin chains that the E3 conjugates onto OPTN might predominantly target OPTN for autophagic degradation. By demonstrating that the E3-OPTN axis synergistically suppresses growth and tumorigenicity of lung cancer cells, which are otherwise deficient in autophagy, our findings may open a new avenue for developing autophagy-targeted therapeutic intervention into cancer.

Key words: HACE1, OPTN, autophagy receptor, p62, selective autophagy, tumor suppression
USP15 REGULATES SMURF2 KINETICS THROUGH C-LOBE MEDIATED DEUBIQUITINATION

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Transforming growth factor-β (TGFβ) is a multifunctional cytokine that acts as a tumour suppressor in normal cells and as an oncogene in advanced cancer cells. Ubiquitin modification of the TGFβ pathway components has emerged as a key mechanism in regulating its signaling such as SMURF2 (an E3 ubiquitin ligase) which negatively regulates the signaling output. Also, ubiquitination is a reversible process and we show that Ubiquitin Specific Peptidase 15 (USP15) directly targets SMURF2 resulting in decreased TGFβ receptor ubiquitination. We show both in vitro and in vivo evidence that suggest that SMURF2 is de-ubiquitinated by USP15. Using CRISPR technology, we further validated the role of USP15 through SMURF2 deubiquitination. Furthermore, we employed mass spectrometric strategy and identified lysine residues on SMURF2 that are de-ubiquitinated by USP15. By using site-directed mutagenesis screening of potential de-ubiquitinating sites, we identified a key lysine residue at the extreme C-terminus of SMURF2, which turned out to be not only a de-ubiquitinating site of USP15 but also a critical ubiquitination site. We further found that mutating this site affected the function of SMURF2 by increasing the migratory potential in advanced breast cancer cells. With the help of 3D-molecular modeling and trans-thiolation assay, we were able to predict the importance of this lysine site with respect to the biochemical function of SMURF2. In the TGFβ pathway, USP15 opposes R-SMAD (transcription factor) mono-ubiquitination and SMURF2 mediated poly-ubiquitination, permitting SMAD promoter recognition. Also, USP15 binds to the SMAD7-SMURF2-TGFβ receptor complex, regulating TGFβ receptor ubiquitination and stability. In our present study, we show that USP15 targets SMURF2 and may explain how USP15 targets multiple nodes in the TGFβ pathway.
DISCOVERY OF HIGHLY SELECTIVE DUB INHIBITORS WITH IN VIVO PRE-ClinICAL ANTI-TUMOUR ACTIVITY

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Ubiquitin proteasome pathways are emerging as a growing source for novel anti-cancer therapeutics. In this respect, by depleting a number of deubiquitylating enzymes (DUBs) through a combination of synthetic lethality screens and isogenic cell line models, MISSION has identified several novel DUBs essential for a range of different tumour types, including platinum-resistant ovarian cancers, DNA damage response (DDR) pathway deficient tumours (e.g. ATM, ATR or BRCA2 defective) and haematological tumours such as multiple myeloma. Illustrating the diversity of DUBs as oncology targets, we identified USP11 and UCHL1 using our target validation platform. USP11 was identified as essential for proliferation of platinum-resistant cells but not platinum-sensitive tumour cells in an isogenic model derived from patient tumours before and after development of resistance. In addition, USP11 depletion displayed synthetic lethality in isogenic models of BRCA2 or ATR loss, supporting USP11’s previously identified association with homologous recombination proteins as well as the importance such processes in platinum resistance mechanisms. UCHL1 on the other hand is a prototypical oncogene target that selectively drives proliferation in many tumour types, such as multiple myeloma and lung cancer. UCHL1 depletion leads to selective killing of a number of tumours whose proliferation is driven by UCHL1. Validation of targets like USP11 or UCHL1 for the selective treatment of cancer bearing genetic deficiencies or resistant to standard-of-care supports the rationale of developing DUB inhibitors for cancers with unmet medical need. A broad drug discovery platform combining unique biochemical, cellular, biophysical and structural assays was designed by MISSION to identify and optimise potent and selective DUB inhibitors. Early selective DUB hits, developed for USP11 and UCHL1, are successfully recapitulating target validation biology, including synthetic lethality in matched isogenic backgrounds. Lead optimisation of chemical series has recently been translated into compounds with adequate properties for in vivo proof-of-concept studies. The challenges and advances in demonstrating in vivo DUB target engagement will be discussed. MISSION’s integrated drug discovery methodologies exemplify the tractability of DUBs to pharmacologic intervention and the potential scope for DUB inhibitors in a number of cancer types, including those characterised by DDR deficiencies.

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NF-κB is a family of transcription factors that regulate gene expression in response to stimuli, such as stress, cytokines and pathogen infections. By binding to its inhibitor, IκBα, NF-κB is sequestered in the cytoplasm and remains inactive. Upon extracellular stimulation, the IKK protein kinase is activated to phosphorylate IκBα. This phosphorylation event is required for ubiquitination and proteolysis of IκBα. Consequently, NF-κB is free to translocate into the nucleus where it induces the expression of a spectrum of genes which are critical for inflammatory response. Previously, we found that the p97-UFD1L-NPL4 protein complex plays an essential role in cytokine-induced IκBα turnover by regulating the post-ubiquitinational events of IκBα proteolysis. Such a post-ubiquitinational regulation of IκBα is crucial for cytokines to activate NF-κB. Here we report that p97 and its ATPase activity are also critical for fully activation of the IKK protein kinase under cytokine stimuli. However, neither UFD1L nor NPL4 seems to be important to such an event. Our data indicate that p97 regulates ubiquitination of RIP1 and NEMO by maintaining the proper formation of the TNF-R1 signaling complex (TNF-RSC). In p97-inactivated cells, the recruitment of both the LUBAC ubiquitin ligase and TRAF2 to the TNF-RSC upon cytokine treatment is largely defective. Conversely, p97 also could not associate with TNFR1 under cytokine stimuli, if LUBAC is ablated. We are currently dissecting the mechanism by which p97 affects the TNF-RSC formation.
THE ROLE OF UBIQUITIN IN THE PROTEIN QUALITY CONTROL OF HUNTINGTON’S DISEASE.

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Huntington’s disease (HD) is one of nine diseases caused by an expanded polyglutamine repeat within the affected protein and is hallmarked by intracellular inclusion bodies composed of aggregated N-terminal huntingtin (Htt) fragments and other sequestered proteins. HD is an autosomal-dominant inherited, progressive and fatal neurodegenerative disorder caused by expansion of a CAG repeat in the htt gene, coding for polyglutamine. The expansion of the polyglutamine stretch in the Htt protein to a length greater than 36 consecutive glutamines is causing HD. Apparently, the common cellular degradation machineries are unable to recognize and degrade these mutant Htt protein fragments efficiently, causing protein aggregation and neuronal dysfunction.

Here we study the intracellular ubiquitylation pattern of the N-terminal fragment Htt-exon1 depending on the length of the polyglutamine stretch and the Htt-exon1 cellular localization. Detection of soluble and insoluble, formic acid-treated, ubiquitylated species of the mutant Htt-exon1 protein reveals mono- and poly-ubiquitylation dependent on the lysine residues in the N17 domain. Our data show that poly-ubiquitylation of mutant Htt-exon1 is dependent on its cellular localization. Expression of the mutant Htt-exon1 protein with and without a nuclear localization signal shows different ubiquitylation pattern of cytoplasmic versus nuclear mutant Htt-exon1 in aggregates. Intriguingly, intracellular mutant Htt aggregates are positive for catalytically active DUBs, suggesting a role in HD protein quality control.

Furthermore, we study the interactome of wildtype and mutant Htt in neuronal cells. After intracellular protein crosslinking and immunoprecipitation of Htt we identified several components of ubiquitin-independent pathways by mass spectrometry analysis, which might be implicated in the protein quality control of Htt.
PINK1 and Parkin are two proteins mutated in early onset Parkinson’s disease and implicated in mitochondrial quality control. Parkin, an E3 ligase, is normally cytosolic, but is recruited to damaged mitochondria by the kinase activity of PINK1. PINK1 was known to phosphorylate the ubiquitin-like domain of Parkin, but mutation of this site, or any other conserved serine or threonine in Parkin, does not block its PINK1-mediated translocation. This indicated that other PINK1 substrates must be required for Parkin activation. Using CRISPR-mediated genomic editing we generated PINK1 knockout cells and then used an unbiased proteomics approach to study mitochondrial outer membrane proteins in these and wild-type cells following mitochondrial damage (Kane, et al. J. Cell Biol. 205, 143-153 (2014)). We identified ubiquitin as a novel PINK1 substrate, uniquely phosphorylated at Ser65. This mirrored the phosphorylation of the ubiquitin-like domain of Parkin by PINK1 at the conserved position, Ser65. Recombinant PINK1 phosphorylates ubiquitin in vitro, proving that this is a direct phosphorylation event. Phosphorylated ubiquitin binds to and activates the E3 ligase function of Parkin, playing a role in its recruitment to damaged mitochondria. This was the first identification of a ubiquitin kinase and the first function assigned to phosphorylated ubiquitin. Follow-up analyses are underway to identify other phospho-ubiquitin binding proteins in mitophagy and beyond. Preliminary data indicates that though there is a large common set of proteins that bind all forms of ubiquitin, there are select proteins that preferentially bind different phospho-ubiquitin species and likely regulate ubiquitin signaling. These discoveries suggest that ubiquitin post-translational modification is extensive and likely mediates many cellular pathways.
INTERACTION OF PROTEASOME AND HEAT SHOCK PROTEINS IN TUMOR CELL RESPONSE

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Proteasomal degradation of oxidized proteins is a crucial mechanism to prevent the accumulation of cellular damage. The removal of the damage is generally a required process for healthy organisms to keep the integrity while in cancer cells the situation may be different. In normal conditions, cancer cells have higher proteasome activity compared to normal cells. During cancer treatment, cellular damage by chemotherapy is an expected process to be able to kill the tumor cells. And the accumulation of this damage accompanied by the decrease in protein repair and removal systems may increase the efficacy of the cancer therapy.

Heat shock proteins (Hsp) as molecular chaperones are involved in the folding, activation and assembly of a variety of proteins. Among these Hsp40, Hsp70 and Hsp90 are believed to act as a chaperone system to regulate the proteasomal degradation.

In this study, we tested the role of heat stress response on the proteasomal degradation of oxidized proteins. We used two different cell lines to observe the difference in normal and tumor cells. First the effect of heat stress (42 °C, 1h) were tested in terms of protein oxidation tested by protein carbonyl formation and proteasomal degradation. The results were extremely different in normal cells and tumor cells. In the same direction, the expressions of Hsp40, Hsp70 and Hsp90 were affected in a different manner in two cell lines, will be discussed in detail. On the other hand, proteasomal response and autophagy have been tested in colon carcinoma cells which shows the possible heat shock protein involvement for the chemotherapy resistance.

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Modification of proteins with ubiquitin chains is an essential signaling event catalyzed by E3 ubiquitin ligases, most of which contain a RING domain to recruit a ubiquitin-charged E2 enzyme and a distinct domain to recruit substrates. How RING-E3s build polymeric ubiquitin chains on substrates while binding the substrate and the E2 at defined interfaces is not well understood. The RING-E3 APC/C catalyzes chain elongation by increasing the affinity of its E2 for the acceptor ubiquitin at the end of the growing chain. This chain tracking ability requires the APC/C coactivator Cdc20 and conserved residues in the E2 and the acceptor ubiquitin. This activity of the APC/C is necessary for degradation of APC/C substrates in cells and for accurate cell cycle progression. These results suggest that RING-E3s can tether the distal end of the growing ubiquitin chain in close proximity to the active site of the E2 and thus promote chain formation while maintaining interactions with both substrate and E2.

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IDENTIFICATION OF NOVEL B CELL SIGNAL TRANSDUCTION BY PELLINO 1 E3 LIGASE GAIN-OF-FUNCTION

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Pellino (Peli) 1 is highly conserved in the course of evolution and contains C3HC4 RING-like motives in its C-terminal domains and catalyzes ubiquitin chains of several key molecules linked to lysine 48 (K48) or 63 (K63) in cell signaling pathway. Peli 1 has been recently found to regulate Toll-like receptor (TLR) and T-cell receptor (TCR) signaling to NF-κB, and thus contributes to the maintenance of self-tolerance and the production of pro-inflammatory cytokines. In addition, activation of B cell and T cell receptor-mediated signaling induces Peli 1 expression and activation. However, their physiological roles in other cell types and cell signaling pathways remain unclear. Recently, we have generated genetically engineered mouse (GEM) models to examine the molecular lesion caused by Peli 1 gain-of-function and loss-of-function. This study also includes the data from studies investigating the mutation and expression of Peli 1 in the different types of cancers.
UBIQUILIN-2 IS A STRESS ACTIVATED PROTEASOMAL SHUTTLE

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Recognition of polyubiquitylated proteins at the proteasome is either mediated by any one of three stoichiometric subunits of the 26S proteasome (Rpn10, Rpn13 and Dss1) or by extrinsic UBL-UBA domain proteins, which can shuttle on and off the proteasome to deliver their ubiquitylated cargo.

The ubiquilins (UBQLNs) are a family of UBL-UBA domain proteins that comprises 4 members in mammals (UBQLN1-4). In addition to the UBL and UBA domains, the UBQLNs contain a series of tandem STI1 motifs, which bind to members of the HSP70 chaperone family, suggesting an important role of UBQLNs in cellular quality control pathways. UBQLNs have indeed been shown to play a role in ERAD and were also reported to regulate aggresome formation.

UBQLN2 has been the focus of recent studies since it was found to harbor pathogenic mutations leading to familial Amyotrophic Lateral Sclerosis (ALS) and more recently a severe early onset neurodegeneration syndrome. UBQLN2 is distinguished from the other UBQLNs by the presence of a collagen-like repeated PXX motif prior to the C-terminal UBA domain (P = proline and X = any amino acid). While the vast majority of the familial-ALS mutations occur in the PXX motif, the function of this domain remains unknown. UBQLN2 was found to co-localize to inclusion bodies in the motor neurons both of ALS cases directly caused by PXX-mutated UBQLN2, but also in other cases of ALS where UBQLN2 was normal, strongly supporting a role of UBQLN2 in cellular quality control degradation pathways. However, precisely how UBQLN2 functions to regulate cellular quality control and how mutations in the PXX domain lead to disease remain unknown.

We report a novel function of UBQLN2 as a stress activated proteasome shuttle. UBQLN2 forms stress-inducible degradation complexes with the proteasome and chaperones and is required for the clearance of ubiquitylated, aggregated cellular proteins. Depletion of UBQLN2 is toxic to cells undergoing protein folding stress but not to resting cells, suggesting an essential function of UBQLN2 in the clearance of aggregation-prone proteins. We also provide evidence that mutant UBQLN2 is defective in its ability to degrade disease-linked aggregated proteins in vivo, which we attribute to defects in the formation of the degradation complex. Our data provides evidence that the activity of proteasomal shuttles is inducible and also gives an explanation for how mutations in UBQLN2 cause neurodegeneration.

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Archaeal proteasomes are extremely useful models in structural and functional studies of the proteasome; many of the lessons learned have been directly applicable to eukaryotic proteasomes which share the same architecture. Proteasome assembly has received considerable attention over the past decade and here too archaea and eukaryotes exhibit many similarities. In general, α subunits are thought to form rings first, which serve as a platform for the entry of β subunits leading to the formation of a half-proteasome; this half-proteasome quickly dimerizes and undergoes maturation into the functional 20S proteasome. It is noteworthy that 20S proteasomes are also found in a subset of bacteria, presumably the result of a horizontal gene transfer from archaea. However, it is peculiar that their assembly mechanism is unlike that of archaea (or eukaryotes) because it does not involve α ring formation. We investigated the process that leads to the formation of α rings using a targeted mutagenesis approach with recombinantly produced proteasomes from the archaeon Methanococcus maripaludis as a model. Among a number of findings, we demonstrate that mutants defective in the ability to form α rings result in efficient formation of fully functional proteasomes. We suggest that the currently held “α-ring first” view of proteasome assembly should be revised to include a parallel assembly pathway highly reminiscent of bacterial proteasome assembly. Our results reconcile the seemingly separate bacterial assembly mechanism with the rest of the proteasome realm. Moreover, they demonstrate that the absolutely conserved architecture of proteasomes across all domains of life is underpinned by common mechanisms of assembly, further underscoring their common evolutionary origins.
A NOVEL CULLIN UBIQUITIN LIGASE COMPLEX REGULATES CELLULAR PROLIFERATION BY MODULATING BIOSYNTHESIS OF GUANINE NUCLEOTIDES

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Inosine monophosphate dehydrogenase (IMPDH) plays a crucial role in cellular proliferation by catalyzing the rate-limiting step in de novo biosynthesis of guanine nucleotides. IMPDH is over-expressed in various cancer cells, making the enzyme an attractive target for therapeutic intervention. Although the cellular function of IMPDH has been studied extensively, how the activity of IMPDH is controlled remains largely ambiguous. Here, we find an ankyrin-repeat domain protein (ANK) that binds IMPDH and promotes its degradation via ubiquitin-proteasome system. Using quantitative proteomic methodologies in conjunction with immuno-blotting and in vitro binding assays, we detected interactions among IMPDH, rbx2, elongin B, elongin C, Cul5, and ANK. Proteasome inhibitor MG132 or neddylation inhibitor MLN4924 increased the steady-state level of IMPDH in HEK293 cells. In addition, the crystals of the quaternary complex composed of elongin B, elongin C, N-terminus of Cul5, and ANK were prepared. Taken together, we propose a novel model for the regulation of cellular proliferation—ANK functions as a substrate receptor for a cullin-based ubiquitin ligase complex composed of Cul5, elongin B and C, and rbx2, which down-regulates guanine nucleotide biosynthesis and cellular proliferation through the proteasomal degradation of a key metabolic enzyme IMPDH.
FUNCTIONS OF N-TERMINAL METHIONINE FORMYLATION OF CYTOPLASMIC PROTEINS IN MITOCHONDRIAL FATTY ACID SYNTHESIS IN SACCHAROMYCES CEREVISIAE

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Bacteria and eukaryotic organelles (mitochondria and chloroplasts) contain Nα-terminally formylated methionine on nascent proteins. The formyl methionine stems from the initiator formyl methionyl-tRNA at the moment of protein synthesis. Methionyl-tRNA transformylase (MTF) transfers a formyl moiety from N10-formyltetrahydrofolate to initiator methionine-tRNA. According to Ramesh et al’s paper (Mol Cell Biol, 2002), the heterologous overexpression of Escherichia coli MTF increased the levels of initiator formyl methionyl-tRNA, and thus resulting in the retarded growth of Saccharomyces cerevisiae cells. To decipher the E. coli MTF-mediated impaired S. cerevisiae growth in more details, we performed synthetic genetic array (SGA) analysis through which E. coli MTF was overexpressed in ~4,800 S. cerevisiae mutants with the deletions of non-essential genes, and then identified 43 mutants showing synthetic dosage lethality. Out of the 43 genes, we paid attention to MCT1 (malonyl-CoA:ACP transferase), CEM1 (mitochondrial beta-keto-acyl synthase) and ETR1 (2-enoyl thioester reductase), because of their functions in mitochondrial fatty acid synthesis (FAS II). In addition, we directly verified the synthetic dosage lethality of other mitochondrial FAS II-related genes such as OAR1 (mitochondrial 3-oxoacyl-[acyl-carrier-protein] reductase), HTD2 (mitochondrial 3-hydroxyacyl-thioester dehydratase), LIP2 (lipoyl ligase), LIP3 (lipoyl-protein ligase), LIP5 (lipoyl synthase), CBP6 (cytochrome B pre-mRNA-processing protein 6) and COX5a (cytochrome c oxidase polypeptide 5A). Taken together, these results suggested that N-terminal methionine formylation of cellular proteins might functionally influence mitochondrial fatty acid, lipoic acid and cytochrome synthesis.
UBQUITINATION OF COSTAL 2 BY THE UBR3 E3 LIGASE PROMOTES HEDGEHOG SIGNALING IN DROSOPHILA

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Hedgehog (Hh) signaling is an important pathway regulating multiple developmental processes including cell proliferation, cell differentiation and wound healing. Loss of Hh signaling leads to developmental disorders including holoprosencephaly, craniofacial defects, polydactyly and skeletal malformations. Aberrant activation of Hh signaling can also cause polydactyly and multiple cancers including basal cell carcinoma (BCC), medulloblastoma, and rhabdomyosarcoma. Here we report the isolation of the Drosophila homolog of UBR3, a member of the UBR superfamily of E3 ubiquitin ligases, from a forward genetic screen designed to identify genes that affect eye development. Generation of ubr3 mutant mosaic clones in the eye show that loss of ubr3 causes a loss of Hh signaling in the developing eye imaginal discs. Interestingly, loss of ubr3 results in the upregulation of Costal2 (Cos2), a kinesin-related motor protein that negatively regulates Hh signaling. Previous studies have shown that loss of Cost2 and over-expression of Cos2 cause activation and inactivation of Hh signaling respectively, indicating the importance of controlling Cos2 levels. We found that Ubr3 binds to the amino terminal motor domain of Cos2 with its substrate binding domain and that it can polyubiquitinate Cos2 through lysine 48 of ubiquitin, promoting its degradation. Interestingly, Hh signaling activation strongly enhances poly-ubiquitination of Cos2 by inducing the transcription of Ubr3 and promoting the binding of Ubr3 to Cos2, thus forming a positive feedback regulatory loop. In summary, we identified a novel E3 ligase that acts as a positive regulator of Hh signaling and defined a regulatory mechanism that controls the levels of Cos2 protein.
Ubiquitin specific protease 1 (USP1) plays critical roles in DNA damage responses, including DNA translesion synthesis (TLS), Fanconi anemia (FA) and homologous recombination (HR). In addition, USP1 also functions in preserving the stem cell-like characteristics in osteosarcoma cells by stabilizing inhibitor of DNA binding (IDs) proteins. Tight regulation of USP1’s activity is critical for maintaining genome stability and regulating stem cell commitment and differentiation. Herein, we showed that in human cells DNA damage inflicted by cisplatin induced the activation of USP1 through increasing the phosphorylation level of USP1 at Ser313. And the phosphorylation of Ser313 activated USP1 by enhancing its interaction with UAF1 in cells. We found that both CDK1 and CDK2 are responsible for the phosphorylation of USP1 at Ser313, but at different stages of the cell cycle. Our study revealed a DNA damage response pathway that leads to the activation of an essential deubiquitinase complex, USP1/UAF1, to cope with the lesions induced by DNA crosslinkers. Our finding supports an important role of deubiquitination by USP1/UAF1 in the proper DNA damage response in humans.
CYTOSOLIC PINK1 IS STABILIZED BY NF-κB PATHWAY AND PROMOTES NON-SELECTIVE MITOPHAGY.

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The potential cellular function of the 53kDa cytosolic form of PINK1 (PINK1-53) is often overlooked due to its rapid degradation by the proteasome upon its production. Although a number of recent studies have suggested various roles for PINK1-53, how this labile PINK1 species attain an adequate expression level to fulfil these roles remains unclear. Here, we demonstrated that PINK1-53 is stabilized in the presence of enhanced K63-linked ubiquitination and identified TRAF-6-related NFκB activation as a novel pathway involved in this. We further showed that a mimetic of PINK1-53 promotes mitophagy but curiously in apparently healthy mitochondria. We speculate that this “non-selective” form of mitophagy may potentially help to counteract the build-up of reactive oxygen species in cells undergoing oxidative stress and as such represent a cytoprotective response.
Ubiquitylation is an enzymatic cascade with E3 ubiquitin ligases divided into two major classes RING and HECT domain, together playing a central signalling role in health and disease. Despite a clear opportunity for the development of a new class of therapeutic agents, the pharmacological industry has been restricted in targeting E3 ligases, which is mainly due to the lack of technologies and the significant complexity inherent to targeting enzymes lacking ‘kinase-like’ catalytic domains.

We developed a novel generic approach enabling us to target catalytic activity of RING domain E3 ubiquitin ligases within the natural cellular context. This enabled us to design a scalable and reliable cellular assay for a high throughput screen against RNF8 ubiquitin ligase that plays an important role in recruiting DNA repair factors to damaged DNA. Our orthogonal cascade included cellular assays that utilize identical technology but different E3 ubiquitin ligases, this way identifying compounds that do not target technology and common upstream signalling pathways but instead demonstrate selectivity against RNF8 cellular assay. Furthermore, a subset of these compounds were demonstrated to block signalling downstream of RNF8 by blocking the recruitment of 53BP1 repair factor, to the sites of DNA damage, in response to ionizing radiation. However, upstream RNF8 signalling was not affected, as evidenced by the presence of phospho-H2AX nuclear foci, demonstrating a functional relevance of the identified compounds. Therefore, the developed novel technology could be applied to a large class of E3 ubiquitin ligases, accelerating tool compound and drug discovery in this area.
UBE2O MEDIATES C-MAF UBIQUITINATION AND INDUCES MYELOMA CELL APOPTOSIS

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c-Maf is a transcription factor frequently overexpressed in multiple myeloma, a fatal hematological malignancy of plasma cells. By binding to the specific recognition element, c-Maf modulates the expression of a panel of critical genes such as cyclin D2 and integrin beta 7 which are involved in the pathophysiology of myeloma. We and other groups found that c-Maf stability could be processed by the ubiquitin-proteasome pathway. Protein ubiquitination is usually achieved under a guidance of E1 activating, E2 conjugating and E3 ligase enzymes, but it is not clear which enzymes mediate c-Maf ubiquitination. To find out such enzymes, we performed an affinity purification-coupled tandem mass spectrometry (AP-MS) which has successfully assisted identification of specific ubiquitination enzymes. In our study, hemagglutinin (HA)-tagged c-Maf was transiently expressed in HEK293T cells. After immunoprecipitation (IP) with an anti-HA antibody, the co-immunoprecipitated protein complex was resolved by denaturing gel electrophoresis, subjected to in-gel trypsin digestion and then LC-MS/MS (LTQ-Orbitrap, Thermo Fisher) analysis. Using protein identification parameters exceeding 99% probability, and a minimum of 2 unique peptides for protein identification, 102 proteins were identified as specifically associated with c-Maf. A further unbiased analysis for KEGG-defined pathways revealed a set of 7 proteins annotated for “ubiquitin-mediated proteolysis”, one of which was UBE2O, a ubiquitin conjugating/ligase enzyme. To find out whether UBE2O mediates c-Maf ubiquitination, we co-transfected c-Maf and UBE2O plasmids into HEK293T cells followed by immunoprecipitation/Western blotting analyses. In this cell-based study, UBE2O was also found to interact with c-Maf and increases the ubiquitination level of c-Maf. Further studies revealed that UBE2O mediates c-Maf degradation which could be abolished by MG132, a proteasome inhibitor. Interestingly, UBE2O mediates the degradation of both c-Maf and MafB but not MafA, suggesting UBE2O probably specifically targets c-Maf and MafB proteins. Furthermore, UBE2O lentivirus led to c-Maf decease in myeloma cells and induced myeloma cell apoptosis. Therefore, through an AP/MS strategy and subsequent studies, we identified UBE2O as a c-Maf ubiquitination enzyme, which could be used as a modulator for myeloma therapy.
Proteins are targeted to different cellular processes by polyubiquitin chains. Polyubiquitin chains can be of different lengths and are formed by the attachment of the C-terminus of one ubiquitin to any of the seven lysines or to the N-terminus of substrate-bound ubiquitin. The linkage of ubiquitin chains in part directs substrate fate. Modification with lysine 48-linked ubiquitin chains targets substrates to the proteasome for degradation, whereas lysine 63-linked ubiquitin chains are primarily thought to direct membrane sorting \textit{in vivo}. Here we investigate how the proteasome interprets ubiquitin modifications. The proteasome contains at least two intrinsic receptors that recognize ubiquitin chains: Rpn10, located near the substrate translocation channel, and Rpn13, located more distally. We are exploring modes of proteasomal recognition of various ubiquitin chains by using kinetic degradation assays to evaluate the proteasomal affinity for free ubiquitin chains and ubiquitinated substrates. In the assays we combine an array of defined ubiquitin chains with reassembled proteasome complexes bearing all combinations of wild type and mutant variants in Rpn10 and Rpn13.
DEVELOPMENT OF NOVEL CHAIN SELECTIVE POLY-UBIQUITIN BINDING TECHNOLOGIES TO STUDY THE BIOLOGICAL ROLES OF SPECIFIC UBIQUITIN LINKAGES

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Purpose of study: This work presented here focuses on developing easy to use tools to distinguish between specific ubiquitin chain linkages, which are involved in a multitude of cellular functions.

Ubiquitylation is a common post translational protein modification that occurs through an isopeptide linkage between the C-terminus of ubiquitin (Ub) and the \( \varepsilon \)-amino group of a lysine residue on the target substrate. Ub itself has seven Lys residues (K6, K11, K27, K29, K33, K48, and K63), each of which can participate in further ubiquitylation, generating poly-Ub chains. The ability of Ub to form polymers through various lysines as well as its NH2-terminus appears to be central to the versatility of this system in regulating a variety of cellular processes. Emerging evidence suggests that different polyUb linkages regulate a variety of cellular processes. K48 polyUb chains regulate protein levels by signaling a target protein for degradation by the proteosome. K63 chains are involved with processes such as endocytic trafficking, inflammation and DNA repair while K11-linked polyUb chances are implicated in mitotic regulation and endoplasmic reticulum associated degradation (ERAD). Therefore, it is increasingly clear that different polyUb linkages convey different information to the cell. A major hurdle determining the role of specific Ub linkages in cancer related pathways is the fact that there is no straight forward method to determining the exact nature of ubiquitylation of a target protein. Development of polyUb selective tools will revolutionize the field of ubiquitin. We and our collaborators are constructing a series of tools designed to identify specific Ub chains by Far Western blotting and pull-down experiments. These tools will have a major impact on our understanding of the functioning of ubiquitin proteasome and signaling pathways related to a wide variety of human disease.

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THE E3 UBIQUITIN LIGASE TRIM61 IS A REGULATOR OF RLRS-MEDIATED TYPE I INTERFERON PRODUCTION

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The prompt and tightly controlled induction of type I interferon (IFN) is a central event of the immune defense against viral infection. Members of the tripartite motif-containing (TRIM) protein family recently emerged as key components of the host cell innate immune response. However, the mechanism of action of most of these factors and the physiological relevance of their activity in vivo are yet to be established.

By systematic analysis of all known human TRIMs for their ability to activate IFN-β, NF-κB-, and ISG54-related luciferase reporters we identified TRIM61 as a new regulator of pattern-recognition receptors signaling. Overexpression of TRIM61 significantly enhances the activation of all the reporters tested upon stimulation with the 2CARD domains of RIG-I, but does not affect ISG54 and NF-κB promoter induction triggered by IFN-β and TNFα respectively. Mass spectrometry analysis and co-immunoprecipitation studies suggest that TRIM61 acts at the level of the mitochondria to promote downstream signaling and subsequent induction of type I IFN.

Further work in the lab is currently being performed in order to investigate the role of TRIM61 in vivo, as well as to better characterize the molecular mechanism by which TRIM61-synthetized polyubiquitin chains regulate mitochondria-mediated antiviral immunity.
Ubiquitin (Ub) and ubiquitin-like (Ubl) pathways rely on distinct enzymes for Ub/Ubl conjugation to substrates. Among these factors are E3 ligases that can activate the E2-Ub/Ubl for discharge and promote conjugation to specific lysine residues on the target substrate. Ongoing work will be presented that addresses activation mechanisms and specificity determinants for diverse members of the SUMO E3 ligase family.

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SCF FBXL17 MEDIATED UBIQUITINATION OF SUFU REGULATES HEDGEHOG SIGNALING

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F-box proteins recruit Skp1 (S phase kinase-associated protein 1), Cul1 (Cullin 1) and Rbx1 (RING-box protein 1) in order to assemble a functional SCF (Skp1-Cul1-F-box protein) E3 ubiquitin ligase complex. SCF ubiquitin ligases can control protein ubiquitination in time and space and are involved in the modulation of signal transduction pathways required for cell survival. We show that Fbxl17 (F-box and leucine-rich repeat protein 17) forms a functional SCF complex and targets the tumor suppressor Sufu (Suppressor of fused) for proteolysis upon Hedgehog (Hh) pathway activation. Sufu is an essential gene and controls the activity and levels of the transcription factors Gli1, Gli2, and Gli3, for the regulation of Hedgehog (Hh) signalling pathway transcriptional output. Maintaining balanced levels of Sufu is essential to coordinate cell proliferation and differentiation through Hedgehog signalling and Sufu mutations and low expression are described in multiple cancer types, including Medulloblastoma, prostate cancer and pancreatic cancer.

We identify the mechanism of localisation of Fbxl17-Sufu complex and observe that the ubiquitination of Sufu mediated by Fbxl17 induces Gli1 release for full activation of the Hh signalling pathway. In addition, depletion of Fbxl17 arrests cell proliferation by preventing Sufu degradation. Our findings reveal Fbxl17 as a novel regulator of Hh signaling pathway and provide initial evidence of Fbxl17 as a target in the therapy of cancers that rely on Hh pathway activation.
ALLOSTERIC ACTIVATION OF UHRF1 UBIQUITIN LIGASE ACTIVITY

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UHRF1 is a multi-domain RING E3 ligase that facilitates the faithful epigenetic inheritance of DNA methylation patterns by recruiting DNA methyltransferase 1 (DMNT1) to sites of newly synthesized DNA during replication. We show that UHRF1 associates with chromatin through the coordinated action of three reader modules: 1) a SET- and RING-associated domain (SRA) that binds DNA with strong selectivity for hemi-methylated CpG dinucleotides, 2) a plant homeodomain (PHD) that interacts with the N-terminus of histone H3, and 3) a tandem Tudor domain (TTD) that selectively recognizes di- and tri-methylated lysine 9 on H3. We further show that the trivalent interaction of UHRF1 with this defined chromatin signature allosterically activates the ubiquitin ligase activity of UHRF1, promoting ubiquitylation on the H3 N-terminus – a post-translational modification previously identified to recruit DNMT1 to chromatin. We will present our recent findings elucidating the structural features that contribute to this novel mechanism of E3 ligase regulation, and we will discuss how allostery contributes to UHRF1 cellular function as a key regulator of DNA methylation inheritance.
GLOBAL REMODELING OF THE ERYTHROID PROTEOME BY UBIQUITINATION

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Global remodeling of the proteome consists of the programmed elimination of most generic constituents of the cell in parallel with abundant synthesis of a small number of new, cell-type-specific proteins such as globin. The scale and speed of these changes are remarkable in the case of terminally differentiated cells such as erythrocytes, spermatozoa, and others. Reticulocytes are a canonical example of a proteome in rapid transition during terminal erythroid differentiation. However, the mechanisms that drive rapid turnover of normally stable proteins remain largely unknown. Ube2O/E2-230K is a ubiquitin-conjugating enzyme that is highly and selectively upregulated in the erythroid lineage. A null mutation in the murine Ube2O gene, known as hem9, produces a specific phenotype of hypochromic, microcytic anemia, with no known sequelae outside of the erythroid system. All major ubiquitin-protein conjugate bands of reticulocytes that are of low molecular weight are greatly reduced in levels in hem9 mutants, an unusual phenotype suggesting that Ube2O is a dominant ubiquitinating factor in the reticulocyte. When reticulocyte lysates from null mutants were treated with recombinant Ube2O, ribosomal proteins proved to be a major class of targets. These data support the hypothesis that Ube2O functions as an E2-E3 hybrid, first suggested to account for the exceptional size of Ube2O in comparison to other E2 enzymes. In agreement with the in vitro data, hem9 reticulocytes have elevated ribosome levels and aberrant polysome profiles with an exaggerated 80S ribosome peak by polysome gradient analysis. Moreover, during the ex vivo maturation of reticulocytes into erythrocytes, there is a major defect in the elimination of ribosomes, thus accounting for the phenotype of elevated ribosome abundance. In contrast, mitochondrial elimination is not noticeably impaired in the mutants, indicating the specificity of the effect on ribosomes. Ube2O overexpression in non-erythroid 293T cells appears to drive the degradation of ribosomes, suggesting that Ube2O is sufficient to induce ribosomal degradation. In summary, we propose that Ube2O serves to selectively ubiquitinate ribosomes and thus plays a central role in the elimination of ribosomes during terminal erythroid differentiation. These insights into the mechanism of erythroid cellular remodeling may have therapeutic implications for hematologic diseases, particularly β-thalassemia and Diamond Blackfan Anemia.
THE TRIM25 E3 LIGASE IS CATALYTICALLY ACTIVE AS A TETRAMER, WHICH MATCHES THE SIGNALING ACTIVE STOICHIOMETRY OF ITS SUBSTRATE, RIG-I

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In the RIG-I signaling pathway of the innate immune system, the signal recognition complex, composed of the RIG-I protein bound to viral RNA, seeds the formation of filamentous signal amplification assemblages, containing many hundreds of MAVS protein molecules. K63-linked polyubiquitin chains regulate the seeding mechanism, by wrapping around four RIG-I 2CARD molecules and stabilizing a “lock washer” tetramer, which then acts as template for long helical filaments of the MAVS CARD. These polyubiquitin chains are synthesized by the E3 ligase, TRIM25, which is itself a self-assembling protein. We hypothesized that high-order assembly of the ligase regulates catalysis, perhaps in a manner that is linked to assembly of the 2CARD tetramer. We have previously shown that the TRIM25 coiled-coil domain is a stable, antiparallel dimer that positions two catalytic RING domains on opposite ends of a 170 Å long rod. We now show that the RING domain is a separate dimerization motif, and that RING dimerization is required for catalysis. The combined coiled-coil and RING mediated interactions generate the catalytically active oligomeric state of the enzyme, which is a tetramer. Given that the catalytically active form of TRIM25 matches the stoichiometry of the ubiquitin-stabilized state of its substrate, RIG-I 2CARD, ongoing experiments are exploring the possibility that TRIM25 is an effector that nucleates, prior to stabilizing with polyubiquitin, the 2CARD lock washer tetramer that seeds MAVS assembly. These studies are likely to provide a molecular explanation for why TRIM25 is an essential component of the RIG-I pathway.

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The Polycomb group proteins control cell fate by regulating expression of cell lineage developmental genes. They form multicomponent complexes that alter chromatin structure by installing and removing post-translational modifications on the histone components of chromatin. The Polycomb Repressive Complex 1 (PRC1) uses its Ring1b and Bmi1 E3 ligase subunits to add ubiquitin to histone H2A Lys119. Together with the E2 ubiquitin-conjugating enzyme UbcH5c, Ring1b and Bmi1 form the PRC1 ubiquitylation module that ubiquitylates histone H2A only in the context of the nucleosome.

We have crystallized the PRC1 ubiquitylation module in complex with its nucleosome substrate, and determined the crystal structure of this 300 kDa complex. This is the first structure of a ubiquitin E2-E3-substrate complex, as well as the first structure of a histone-modifying enzyme bound to the nucleosome. The structure provides structural insights into how the three subunits of the PRC1 ubiquitylation module recognize key features of the nucleosome to promote ubiquitylation of nucleosomal histone H2A. Surprisingly, the structure shows that the E2 ubiquitin-conjugating enzyme interacts directly with nucleosomal DNA, suggesting a novel role for an E2 in substrate recognition.
Specificity in the ubiquitin-proteasome system is largely conferred by ubiquitin E3 ligases (E3s). Cullin-RING ligases (CRLs), constituting ~1/3 of all E3s in humans, mediate the ubiquitination of ~20% of the proteins degraded by the proteasome. CRLs are divided into six families based on their cullin constituent (either CUL1, CUL2, CUL3, CUL4A/B or CUL5). Each cullin binds a RING-domain containing protein (RBX1 or RBX2) and a vast repertoire of adaptor/substrate receptor modules, collectively creating more than 200 distinct CRLs. All CRLs are regulated by the COP9 signalosome (CSN), an eight-protein isopeptidase (CSN1-8) that removes the covalently attached activator, NEDD8, from the cullin. Independent of NEDD8 cleavage, CSN forms protective complexes with CRLs that prevent autoubiquitination, limiting their turnover. CSN inhibition is overcome once the CRL binds a substrate. How CSN recognizes structurally diverse CRLs and releases them in response to a multitude of different substrates remains unclear. Here, we present single-particle electron microscopy (EM) structures for CSN in complex with representatives of the CRL families Despite striking differences among CRLs, CSN engages the cullin C-terminal domain (CUL_{CTD}) and RBX1 in a conserved manner. Beyond the cullin, CSN-CRL interactions involving the adaptor/substrate receptor modules differ. However, we find substrate receptors are uniformly spaced ~15-20 Å from CSN. CRL substrates not accommodated in the gap between receptor and CSN dissociate the CSN-CRL complex. The size of the bound CRL substrate, not its identity prevents CSN binding. Our studies provide the molecular basis for CSN binding and inhibition across CRLs, and reveal a shared mechanism through which CRL substrates relieve CSN inhibition.
Metazoan development depends on accurate execution of differentiation programs that allow pluripotent stem cells to adopt specific fates. Differentiation is brought about by global changes to chromatin architecture and transcriptional networks, yet whether other regulatory events support cell fate determination is less well understood. Using human embryonic stem cell and Xenopus models, we identified a vertebrate-specific ubiquitin ligase as an essential regulator of neural crest specification. We find that ubiquitylation alters the translational program of differentiating cells to support the generation of neural crest cells. Thus, ubiquitin-dependent regulation of ribosome function is an important feature of cell fate determination.
Faithful transmission of genomic information is a highly dynamic process that involves ubiquitin dependent regulation. In the licensing step of DNA replication CDT-1 is loaded onto chromatin to subsequently promote the recruitment of additional replication factors including CDC-45 and GINS. The chaperone-like ATPase CDC-48/p97 is central for chromatin-associated degradation of the licensing factor CDT-1, which is linked to disassembly of the CDC-45/GINS complex. *C. elegans* embryos lacking CDC-48 or its cofactors UFD-1/NPL-4 accumulate CDT-1 on mitotic chromatin, indicating a novel function of CDC-48 in CDT-1 turnover. Strikingly, CDC-48(UFD-1/NPL-4) deficient embryos show persistent chromatin association of CDC-45/GINS, which is a consequence of CDT-1 stabilization. This process is conserved in humans; however, the spatiotemporal control of CDC-48/p97 recruitment and substrate selection remained unclear. Here, we report that CDC-48 dependent replication events are coordinated by UBXN-3. In addition to CDC-48, UBXN-3 directly binds CDT-1 and additional ubiquitylated proteins, thereby recruiting CDC-48 for turnover and disassembly of DNA replication factors. Consequently, *C. elegans* embryos with reduced UBXN-3 and CDC-48 level stabilize CDT-1 and GINS at the chromatin. Since depletion of the human UBXN-3 ortholog FAF1 severely affects progression of the replication fork, our work identifies a conserved substrate selection module required for chromatin-associated degradation of DNA replication factors.


SUMOYLATION OF RAP1 MEDIATES THE RECRUITMENT OF TFIID TO PROMOTE TRANSCRIPTION OF RIBOSOMAL PROTEIN GENES

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Transcription factors are abundant Sumo targets, yet the global distribution of Sumo along the chromatin and its physiological relevance in transcription are poorly understood. Using Saccharomyces cerevisiae, we determined the genome-wide localization of Sumo along the chromatin. We discovered that Sumo-enriched genes are almost exclusively involved in translation, such as tRNA genes and ribosomal protein genes (RPGs). Whole-genome expression analysis showed that Sumo positively regulates their transcription. We also discovered that the Sumo consensus motif at RPG promoters is identical to the DNA binding motif of the transcription factor Rap1. We demonstrate that Rap1 is the molecular target of Sumo and that sumoylation of Rap1 is important for cell viability. Furthermore, Rap1 sumoylation promotes recruitment of the basal transcription machinery, and sumoylation of Rap1 cooperates with the target of rapamycin kinase complex 1 (TORC1) pathway to promote RPG transcription. Strikingly, our data reveal that sumoylation of Rap1 functions in a homeostatic feedback loop that sustains RPG transcription during translational stress. Taken together, Sumo regulates the cellular translational capacity by promoting transcription of tRNA genes and RPGs.

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INTERNALLY TAGGED UBIQUITIN AS A POWERFUL TOOL TO IDENTIFY NOVEL LINEAR POLYUBIQUITIN-MODIFIED PROTEINS

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Ubiquitination is an essential post-translational protein modification, which regulates numerous cellular processes. Besides mono- and multiple monoubiquitination, proteins can also be modified by polyubiquitin, formed through either isopeptide linkages between any seven internal lysine (Lys) residues of ubiquitin (Ub), or through peptide linkages between C-terminal glycine (Gly) and N-terminal methionine (Met) of Ub (linear ubiquitination). While Lys48- and Lys63-linked polyubiquitin chains have been implicated in protein degradation and DNA repair, respectively, the function of other ubiquitin chain types and their cognate substrates still remains elusive. Existing methods for the study of Lys-based polyubiquitination (Tandem ubiquitin binding entity/TUBEs; N-terminally tagged Ub mutants; GlyGly antibodies combined with mass spectrometry) are neither optimal nor suitable for the detection of linearly polyubiquitinated proteins. Henceforth, we generated internally tagged Ub variants with all Lys residues unchanged (INT-Ub.wt) or mutant variants with all Lys residues replaced with arginine (Arg; INT-Ub.7KR), the latter being able to be incorporated solely into linear polyubiquitin chains. We characterized the functionality of INT-Ub.wt in vitro and in vivo. Our data show that INT-Ub.wt is functional as it can be incorporated into polyubiquitin chains in vitro and in vivo. Furthermore, immunoprecipitation of INT-Ub allows the detection of endogenously ubiquitinated proteins. Thus we hypothesized that immunoprecipitation with the INT-Ub.7KR variant would allow us to trap linearly polyubiquitinated substrates in vivo. Towards this end, we performed mass spectrometry analysis of SILAC-labelled HEK293T cells that can inducibly express either INT-Ub.wt, INT-Ub.7KR or INT-UbΔGG variants. Several putative linearly modified proteins were further validated (for the ability of binding to LUBAC components, immunoprecipitation with linear Ub-specific antibody upon LUBAC overexpression etc). Among others, we could show that the E3 ligase TRAF6 is modified by linear ubiquitination and we are currently studying the physiological significance of that modification. Overall, our data show that we have established a powerful method allowing for the sensitive detection of novel linearly polyubiquitinated proteins. Our findings will contribute towards our understanding of the broad spectrum of linear polyubiquination, both in healthy cellular physiology and in disease conditions.
SUMO-2 ORCHESTRATES CHROMATIN MODIFIERS IN RESPONSE TO DNA DAMAGE

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Small Ubiquitin-like Modifiers play critical roles in the DNA Damage Response (DDR). To increase our understanding of SUMOylation in the mammalian DDR, we employed a quantitative proteomics approach to identify dynamically regulated SUMO-2 conjugates and modification sites upon treatment with the DNA damaging agent MMS. We have uncovered a dynamic set of 20 upregulated and 33 downregulated SUMO-2 conjugates, and 755 SUMO-2 sites, of which 362 were dynamic in response to MMS. In contrast to yeast, where a response is centered on homologous recombination, we identified dynamically SUMOylated interaction networks of chromatin modifiers, transcription factors, DNA repair factors and nuclear body components. SUMOylated chromatin modifiers include JARID1B/KDM5B, JARID1C/KDM5C, p300, CBP, PARP1, SetDB1 and MBD1. Whereas SUMOylated JARID1B was ubiquitylated by the SUMO-targeted ubiquitin ligase RNF4 and degraded by the proteasome in response to DNA damage, JARID1C was SUMOylated and recruited to the chromatin to demethylate histone H3K4.
TANDEM SELF-ASSOCIATION DOMAINS OF SUBSTRATE ADAPTOR SPOP MEDIATE FORMATION OF LIQUID NUCLEAR BODIES

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Cells segregate components into specialized compartments to enrich function. In contrast to membrane-bound organelles, the functions, properties, and biophysical basis underlying formation of membrane-less cellular bodies are still incompletely understood. Speckle-type POZ protein (SPOP), a tumor suppressor frequently mutated in cancers, is a cullin3-RING ligase (CRL) substrate adaptor that is localized to nuclear puncta. Herein, we demonstrate that these nuclear bodies have liquid-like properties and that their formation depends on the ability of SPOP to form higher-order oligomers through tandem self-association domains. We describe the size distribution of SPOP, which self-associates through an isodesmic mechanism, providing for the first time quantitative analysis of protein assemblies participating in cellular body formation. Higher-order oligomerization of SPOP stimulates CRL-SPOP ubiquitination efficiency, suggesting that multivalent interactions of SPOP create highly concentrated, liquid nuclear bodies that are hotspots of ubiquitination. Mutations within both oligomerization domains have been observed in a variety of cancers (see www.cBioPortal.org), supporting our conclusion that SPOP self-association is important for its biological function. We propose that a new protein domain architecture, i.e. tandem self-association domains, promotes formation of membrane-less organelles with liquid-like character. This work will drive future investigations into the properties of membrane-less organelles and how their disruption contributes to disease.
SUMOYLATION OF THE TRANSCRIPTION FACTOR ZBTB20 CONTROLS NEURITE GROWTH AND BRANCHING

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Multiple recent studies have shown that SUMOylation contributes substantially to a wide range of cellular signaling events in neurons - from nuclear transport to synaptic transmission. Transcription factors and other nuclear components are the most abundant SUMO substrates in cells, including neurons. In this context, SUMOylation regulates various aspects of gene expression, from the DNA-binding of transcriptional complexes to epigenetic mechanisms, and therefore, SUMOylation is poised to play a critical role during development. In a systematic search for endogenous SUMOylation substrates using His6-HA-SUMO1 knock-in mice, we identified Zbtb20 (Zinc finger and BTB domain containing 20) as a novel SUMO1-conjugate in the brain of young mice (Tirard et al. 2012; Proc. Natl. Sci. U.S.A. 109, 21122-21127). Zbtb20 is a transcription factor with a key role in the development of the mouse hippocampus (Rosenthal et al., 2012; Hippocampus 22, 2144-2156). We now show that primary hippocampal neurons that lack Zbtb20 (Zbtb20 KO) exhibit reduced dendrite complexity and axon branching, as compared to wild-type (WT) control neurons. Accordingly, whole-cell patch-clamp recordings showed reduced synaptic transmission in Zbtb20 KO neurons as compared to WT control neurons, indicating that Zbtb20 is required for proper neurite branching and synaptic signaling. Interestingly, the morphological changes in Zbtb20 KO neurons are rescued by re-expression of WT-Zbtb20 while a non SUMOylatable form of Zbtb20 (Zbtb20-2KR) has no rescue activity, indicating that SUMOylation of Zbtb20 is required for its proper function. Furthermore, our data show that while SUMOylation of Zbtb20 does not affect Zbtb20 dimers formation, SUMOylation of Zbtb20 is crucial for its capacity to bind DNA. Accordingly, RT-qPCR analysis indicates that expression levels of several Zbtb20 targeted genes are altered in KO hippocampi as compared to WT, such as Neurexin1, SNAP25, Contactin4, FEZ family zinc finger 2, Sox5 and EphrinA4.

Taken together, our data indicate that SUMOylation of Zbtb20 is crucial for its function as a key transcription regulator of genes that control the development of hippocampal neurons.
NEW GENETIC TOOLS TO STUDY SUMO/UB: IDENTIFICATION OF POL III AS A FUNCTIONAL TARGET OF SUMO, IMPLICATIONS IN NEURONAL DEGENERATIVE DISEASE, AND DEVELOPMENT OF SUMO INHIBITORS

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Post-translational modifications by SUMO and ubiquitin target a large number of proteins in almost all cellular pathways. However, the significance of SUMOylation/ubiquitylation on their target proteins is hard to determine, given the fact that abolishment of these modifications on a particular substrate very often causes no phenotype. Therefore, phenotype-based genetic approaches are needed in order to efficiently identify genes/pathways for which SUMO/Ub is functionally important. To address this issue, we designed a reverse suppressor screen in budding yeast, Saccharomyces cerevisiae, looking for lethal/sick mutations that can be rescued by partial loss of SUMOylation. As anticipated, the screen identified mutations in the SUMO pathway components (SMT3, AOS1, and ULP2) and a known SUMO target, MOT1. Strikingly, the remaining mutated genes identified are all in the RNA polymerase III (Pol III) machinery, including two Pol III subunits (RPC160 and RPC128), one TFIIB subunit (BRF1), and two TFIIC subunits (TFCl and TFC6).

Subsequent studies showed 1) that SUMO represses Pol III-mediated transcription by modification of a Pol III subunit, Rpc53, which may block its interactions with other Pol III factors, such as Tfc4, 2) that Pol III SUMOylation may play a role in stress response, where Pol III transcription is turned down, and 3) that this mechanism is very likely to be conserved in humans, because SUMO is highly enriched at Pol III target genes in mammalian cells and RPC4 (human Rpc53) is extensively SUMOylated in vivo. More importantly, mutations in Pol III were recently found to cause two types of neuronal degenerative diseases in humans. Our preliminary data indicated that the defects caused by some of the disease mutations can be rescued at least partially by inhibiting SUMOylation, suggesting SUMO is a potential therapeutic target for the disease. We therefore developed a high throughput screen based on a yeast growth assay, looking for small-molecule inhibitors against SUMOylation, and have successfully identified several novel SUMO inhibitors, which are potentially useful for both research and medicine purposes. These results also emphasized that yeast genetics is a powerful tool to study SUMO/Ub, particularly for identification of their functional targets and development of small-molecule inhibitors.
TARGETING PIAS1 AS A THERAPEUTIC FOR HUNTINGTON'S DISEASE

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Huntington’s disease (HD) is caused by an expanded CAG repeat in the HD gene leading to expanded polyglutamines in the Huntingtin (HTT) protein. Diverse cellular processes are impacted by expression and accumulation of mutant HTT (mHTT) and post-translational modifications of HTT contribute to normal and aberrant protein levels and function. SUMOylation involves covalent attachment of SUMO (Small Ubiquitin-like Modifier) to specific lysine residues and modulates protein activity and clearance. The SUMO pathway is highly conserved and is implicated in HD and other neurodegenerative diseases. We have previously shown that a single E3 SUMO ligase, PIAS1, modulates both SUMO-1 and 2/3 modification of mHTT and accumulation of insoluble HTT species in cells, raising the possibility that modulation of PIAS1 may provide a selective therapeutic target (O'Rourke et al 2013). In recent studies, we now show that intrastriatal viral delivery of PIAS1 miRNA in a robust fragment mouse model of HD, the R6/2 mouse, significantly reduces HD-like behavioral phenotypes. Further, biochemical analysis reveals a reduction of accumulated SUMO-1/2-modified proteins, Ubiquitin modified proteins, and insoluble HMW HTT species following knockdown. In complementary studies, overexpression of PIAS1 exacerbates mHTT phenotypes. It is well recognized that PIAS proteins modulate various cellular processes including cell proliferation, DNA damage responses, and inflammatory responses and are relevant in transcriptional regulation of pathways including proinflammatory cytokine signaling and the innate immune response. Our findings indicate that PIAS1 displays specificity in proinflammatory cytokine signaling and regulation of microglial activation in R6/2 mice by restoring several pathways that are dysfunctional in HD. PIAS1 interacts with a wide range of proteins and structurally diverse molecules and we show that it may act as an important regulator of signaling cascades that regulate SUMOylation cascades, cytokine signaling, protein clearance networks, and inflammation in HD. The pathways identified here in the context of HD and targeting gene-specific regulators such as PIAS1 may represent novel cytokine and immune-based therapeutic strategies that impact mutant HTT levels and provide insight into mechanisms underlying HD.
REGULATION OF INFECTION RELATED DEVELOPMENT IN MAGNAPORTHE ORYZAE BY UBIQUITINATION AND KINASE SIGNALING PATHWAYS

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Rice blast is the most important disease of rice worldwide, and is caused by the filamentous ascomycete fungus, Magnaporthe oryzae. Protein ubiquitination, which is highly selective, regulates many important biological processes including cellular differentiation and pathogenesis in fungi. Gene expression analysis revealed that a number of genes associated with protein ubiquitination were developmentally regulated during spore germination and appressorium formation. We identified an E3 ubiquitin ligase, MGG_13065, which is induced during infection-related development. MGG_13065 is homologous to fungal F-box proteins including Saccharomyces cerevisiae Grr1, a component of the Skp1-Cullin-F-box protein (SCFGrr1) E3 ligase complex. Targeted gene deletion of MGG_13065 resulted in pleiotropic effects on M. oryzae including abnormal conidia morphology, reduced growth and sporulation, reduced germination and appressorium formation and the inability to cause disease. Our study suggests that MGG_13065 mediated ubiquitination of target proteins plays an important role in nutrient assimilation, morphogenesis and pathogenicity of M. oryzae. Moreover, ubiquitination by F-box E3 ligases is phosphorylation dependent. In analyses of our global proteomics data from cells undergoing appressorium formation, we found extensive evidence of key regulatory proteins being both phosphorylated and ubiquitinated, which are under further investigation.
MITOCHONDRIALLY-ASSOCIATED NRF2 LIMITS THE PROTEOTOXICITY INDUCED BY PROTEASOME INHIBITION

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Proteasome inhibition initiates a cascade of responses aimed at preventing the accumulation of toxic aggregates that propagate proteotoxicity. Here, we demonstrate that the mitochondrial network forms microtubule-dependent, juxtanuclear clusters as an early response to this stress. Clustering facilitates the delivery of a stable, mitochondrial population of the anti-stress transcription factor Nrf2 to the nucleus to induce chaperone expression and suppress the impending proteotoxicity. Nrf2 is anchored at the mitochondria in complex with the CUL3 E3 ligase substrate adaptor KEAP1 and the mitochondrial phosphatase PGAM5. Disrupting this complex reduces clustering and the mitochondrial delivery of Nrf2 to the nucleus, which in turn delays expression of the Nrf2 target gene, Hsp70, a key chaperone in the maintenance of proteostasis. PGAM5-KEAP1-Nrf2 complex disruption abrogates clustering by giving rise to a neomorphic population of KEAP1 on mitochondria that leads to destabilization of Miro2, a mitochondrial GTPase required for mitochondrial translocation along microtubules. Collectively, our data identify a novel function for Nrf2 at the mitochondria and show that the mitochondria function as a beacon of proteotoxic stress. These findings could have pathophysiological implications, as Nrf2 loss is associated with numerous age-dependent diseases.
QUANTIFYING UBIQUITIN SIGNALING MECHANISMS THROUGH THE PINK1-PARKIN PATHWAY

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Our understanding of kinase-driven ubiquitin (UB) ligase signaling pathways is extensive and numerous regulatory paradigms have been illuminated. The ultimate goal in such studies is to elucidate how kinases activate substrate ubiquitylation and to understand the consequences of ubiquitylation of specific substrates in vivo. The vast majority of such studies employ immunoblotting with anti-UB antibodies to detect substrate ubiquitylation, or with chain specific antibodies for a limited subset of the 8 UB linkage types. However, these approaches are inherently non-quantitative and do not provide readout for all UB chain linkage types and often rely on overexpression of UB mutants, which are prone to artifacts.

We have developed a highly quantitative experimental framework for understanding ligase activation parameters and chain-linkage specificity both in vitro and in vivo, as well as understanding the dynamic interplay between ligase and substrate. We employed this framework to investigate the PINK1-PARKIN system, a signal-driven pathway for mitochondrial quality control that is defective in those affected by Parkinson’s disease. Using Absolute Quantification (AQUA)-based proteomics, we define the kinetics of UB chain synthesis by PINK1-activated PARKIN on mitochondria in vivo and the kinetics of chain synthesis by PARKIN in vitro, activated either by PARKIN phosphorylation within PARKIN’s UBL domain or by phospho-UB. Additionally, we determined the kinetics and absolute stoichiometries of PARKIN and UB phosphorylation by PINK1. These studies reveal that PARKIN has the capacity to assembly 4 different UB chain linkages both in vivo and in vitro, which is a very unusual property for a RING-HECT hybrid ligase. Finally, we apply all of these approaches to a collection of patient and structural-based mutants in PARKIN, allowing a quantitative unbiased assessment of how PARKIN mutants affect multiple aspects of its function in mitochondrial quality control.

We propose a feed-forward mechanism that explains how, upon mitochondrial damage, PINK1 phosphorylation of both PARKIN and poly-UB chains synthesized by PARKIN drives upon mitochondrial damage a program of PARKIN recruitment exclusively onto damaged mitochondria, leading to mitochondrial ubiquitylation which is an important first step for subsequent clearance of these “flagged” damaged mitochondria.

Application of this novel framework to other kinase-ligase systems has the potential to transform our mechanistic understanding of these pathways from a quantitative perspective.
PROTEASOME TURN OVER IS AFFECTED IN THE ABSENCE OF E3 LIGASE, NOT4

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Not4 is an RING E3 ligase and a subunit of the Ccr4-Not complex. Not4 is essential for cellular protein solubility and has been proposed to be involved in co-translational quality control. Our previous studies revealed that Not4 is important for the effective proteasome assembly and normal level of proteasomal chaperones. Moreover in the absence of Not4 a salt-resistant interaction between the proteasome RP and CP is observed.

To determine whether Not4 is important during de-novo synthesis of the proteasome, we performed pulse-chase experiments. Here we demonstrate that newly synthesized RP subunits are faster incorporated into the RP subcomplex in wild type cells than in the absence of Not4, and also turn over faster compared to not4Δ. In contrast in not4Δ cells, newly synthesized CP subunits are incorporated into CP faster and turn over slower compared to the wild type.

Subcomplexes smaller than the proteasome and containing mostly lid subunits, accumulate in wild type cells but not in not4Δ. Par-CliP experiments with tagged Not4 revealed an enriched association of Not4 with mRNAs coding proteasomal lid subunits. This data indicates that Not4 might imprint these mRNAs to ensure that their translation occurs under conditions of optimal lid assembly.
TURNOVER OF THE YEAST INNER NUCLEAR MEMBRANE
PROTEIN ASI1 IS RAPID

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The nuclei of eukaryotic cells are bounded by a nuclear envelope, a double
membrane with outer and inner membranes. The outer nuclear membrane
faces the cytoplasm and is continuous with and biochemically similar to the
endoplasmic reticulum. The inner nuclear membrane (INM) contacts the
nucleoplasm and contains a specific set of integral membrane proteins that
participate in a variety of important processes including maintenance of
nuclear architecture, chromatin organization, signaling and gene expression.
Asi1, Asi2, and Asi3 are integral components of the INM of the yeast
Saccharomyces cerevisiae. Asi1 and Asi3 are homologous proteins with
RING-finger domains at their C-termini. Asi1 and Asi3 function together as
an E3-ubiquitin ligase complex that functions together with E2-ubiquitin
conjugating enzymes Ubc6 and Ubc7 as components of a novel quality
control pathway that functions at the INM. This INM associated degradative
(INMAD) pathway plays an essential role in clearing the nuclear
compartment from mislocalized soluble and integral membrane proteins.
Here we report that Asi1 exhibits rapid turnover (t1/2 ≤ 20 min). Notably,
Asi1 turnover is ubiquitin and proteasome dependent, but occurs
independent of the other known INMAD components Asi3 and Ubc6. By
contrast, the turnover of Asi1 exhibits a clear dependence on Ubc7 and
partial dependence on the E3 ubiquitin ligase Doa10. These findings
suggest the involvement of additional E3 ubiquitin ligase(s), which despite
extensive efforts have not been identified. Finally, Asi1 exhibits enhanced
stability in cells lacking STS1, a gene required for proper localization of
proteosomal subunits to the nucleus. Together our data suggest that the
stability of Asi1 is controlled by a novel pathway of the cellular ubiquitin
proteasome system localized and functioning inside the nucleus.
CHARACTERIZATION OF THE FUBI-MODIFIED PROTEOME IN HUMAN CELLS

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FUBI is the ubiquitin-like protein most closely related to ubiquitin at the sequence and structural level. It is ubiquitously expressed in metazoan cells as a precursor, FAU, in which FUBI is fused with the ribosomal protein S30 as its C-terminal extension. In its proteolytically processed mature form, FUBI contains a conserved C-terminal di-glycine motif, suggesting that it can be covalently conjugated to cellular proteins. Despite its similarity to ubiquitin, however, the cellular function of FUBI remains virtually unknown and few, if any, substrates of FUBI-dependent modification have been identified. Using cell lines expressing wild type or mutant forms of FUBI, we confirmed that FUBI indeed forms covalent conjugates with cellular proteins in a manner dependent on the di-glycine motif. Moreover, a K25R mutation that removes the single lysine residue in FUBI reduced cellular FUBI-protein conjugates, suggesting that FUBI is capable of undergoing chain formation similar to ubiquitin and SUMO. To address the cellular function of FUBI, we generated a FUBI-specific antibody and a human cell line, in which the expression of FUBI is selectively ablated while leaving that of S30 intact. Using these tools, we performed mass spectrometry-based surveys of the FUBI-modified proteome, revealing a range of candidate cellular targets of FUBI conjugation as well as potential components of the underlying enzymatic machinery. At the meeting, we will present our latest insights into the emerging mechanistic basis and biological ramifications of FUBI-dependent signaling in human cells.
MUTUALLY EXCLUSIVE ROLES OF SHARPIN IN INTEGRIN INACTIVATION AND NF-KB SIGNALING

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SHARPIN-deficient (Shadincpdm) mice display multi-organ inflammation and chronic proliferative dermatitis. SHARPIN inhibits integrin activity and is a component of the linear ubiquitin chain assembly complex (LUBAC), which enhances signal-induced NF-κB activity. However, it is currently unclear whether these seemingly different roles for SHARPIN are functionally related and how they contribute to the Shadincpdm phenotype. Here we present the first comprehensive analysis of the functional contribution of SHARPIN to integrin and NF-κB regulation. Treatment with an integrin beta 1 (ITGB1) function blocking antibody reduced epidermal hyperproliferation and caused an associated decrease in epidermal thickness, suggesting that ITGB1s contribute to dermatitis in Shadincpdm mice. Furthermore, the integrin and LUBAC binding sites were mapped to partly overlapping regions within the conserved ubiquitin-like domain of SHARPIN. Consistently, we demonstrate that SHARPIN binds integrin and LUBAC in a mutually exclusive manner. Altogether, our data indicate that SHARPIN-dependent regulation of both integrin signaling and LUBAC function contributes to the complex phenotype of Shadincpdm mice and that SHARPIN could potentially mediate the longstanding interdependence between integrin and NF-κB signaling.
SYSTEMATIC P97-UBXD ADAPTOR NETWORK PROTEOMICS IDENTIFIES A ROLE FOR UBXD3 IN REGULATING CILIogenesis VIA ASSOCIATION WITH IFT-B

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The AAA-ATPase p97 uses ATP hydrolysis to “segregate” ubiquitinated proteins from binding partners. p97 acts via UBX-domain containing adaptors that likely provide target specificity, but targets and functions of UBXD proteins remain poorly understood. Through systematic proteomic analysis of UBXD proteins, we reveal a network of over 195 interacting proteins, implicating p97 in diverse cellular pathways. We have explored one such complex between an unstudied adaptor UBXD3 and the intraflagellar transport B (IFT-B) complex, which regulates anterograde transport into cilia. UBXD3 localizes to cilia in a p97-dependent manner and both p97 and UBXD3 are required for ciliogenesis. Pharmacological inhibition of p97 destabilized the IFT-B complex and increased trafficking rates. Depletion of UBXD3 in zebrafish embryos causes defects in left-right asymmetry, which depends on functional cilia. This study provides a resource for exploring the landscape of UBXD proteins in biology and identifies an unexpected requirement for p97-UBXD3 in ciliogenesis.
Resistance to first-line anti-malarial drugs has accelerated the need for new drugs with novel targets for effective malaria treatment. Many of the current anti-malarial drugs work in part by overwhelming the parasite stress response. In eukaryotes, the oxidative stress response machinery includes antioxidants as well as SUMOylation, a post-translational modification that involves the covalent attachment of small ubiquitin-related modifiers (SUMOs) to proteins. We hypothesize that SUMOylation plays an essential role in parasite cell stress survival, and that parasite-specific SUMOylation inhibitors could prove effective in combination with current anti-malarial drugs. We have previously demonstrated that human and *Plasmodium falciparum* (*Pf*) SUMO conjugation systems have biochemically distinct E1-E2 enzyme interactions, suggesting a target for drug development. Here, we use structural studies and mutational analysis to further define and compare the human and *Pf* E1-E2 interaction. We have identified non-conserved and structurally distinct surface residues that will help guide the identification of parasite-specific inhibitors of SUMOylation.
INVESTIGATION OF HUNTINGTIN UBIQUITINATION BY MASS SPECTROMETRY

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Huntington’s disease (HD) is a neurodegenerative disorder hallmarked by the aggregation of polyglutamine (polyQ)-expanded N-terminal Huntingtin protein fragments (mHtt) in both the cytoplasm and nuclei of affected neurons. Aggregation of mHtt causes cellular toxicity hence clearance of mHtt prior to aggregation would be an interesting target to prevent or delay the onset of HD.

The ubiquitin-proteasome system (UPS) plays a key role in the degradation of proteins in the cell. Proteins are targeted for proteasome-dependent degradation by K48-linked polyubiquitin chains. Recent data suggests that mHtt polyubiquitination is less abundant in the nucleus as compared to the cytoplasm, consequently nuclear Htt might not be efficiently recognized and degraded by the UPS. In this project we will use mass spectrometry to identify covalent interactions of ubiquitin and N-terminal Htt protein fragments; both of wildtype Htt and polyQ-expanded mHtt, and compare soluble and aggregated Htt in both the nucleus and cytoplasm. We are interested in both the Htt ubiquitination sites and the ubiquitin-linkage types.

Identification of Htt ubiquitination patterns might reveal circumstances under which Htt is improperly targeted for proteasome-mediated degradation, which in turn may become targets for intervention.
MATURATION OF THE PROTEASOME CORE PARTICLE INDUCES AN AFFINITY SWITCH THAT CONTROLS PBA1-PBA2 AND REGULATORY PARTICLE ASSOCIATION

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The formation of proteasome holoenzymes is a complex process where the assembly of 66 subunits is guided by at least ten proteasome-specific chaperones. A crucial step in this assembly process is the association of the core particle (CP) with the regulatory particle (RP). RP-specific chaperones have been shown to regulate this association, by preventing RP from interacting with the face of the cylindrically shaped CP. Interestingly, the yeast CP chaperone dimer Pba1-Pba2 also binds to the face of the CP. In vivo, Pba1-Pba2 is found associated exclusively with immature CP, while RP binds exclusively with mature CP. It is unclear how these binding events are coordinated. Here, we used biochemical purifications to show that Pba1-Pba2 binds tightly to immature CP and prevents RP from binding to immature CP in vivo. We also observed a strongly reduced affinity of Pba1-Pba2 for mature CP, indicating that the maturation of the CP induces a switch in the affinity of Pba1-Pba2 for CP. Unlike the human orthologs PAC1-PAC2, Pba1 and Pba2 are stable proteins. Thus, Pba1 and Pba2 are not degraded in the process of CP-RP association. Instead, RP can replace Pba1-Pba2 bound to mature CP. In sum, our data suggest that conformational changes upon CP maturation change the affinity of Pba1-Pba2 for CP, thereby allowing the release of Pba1-Pba2 from mature CP and association of RP with CP. Mathematical modeling indicates that this “affinity switch” mechanism has likely evolved to improve assembly efficiency by preventing the accumulation of stable, non-productive intermediates. Our work thus provides mechanistic insights into a crucial step in proteasome biogenesis.
LTN1/RKR1-MEDIATED DEGRADATION OF NON-STOP ENDOPLASMIC RETICULUM PROTEINS

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Non-stop proteins arise from aberrant translation of mRNA molecules beyond the coding sequence into the 3' untranslated region. If a stop codon is not fortuitously encountered, translation continues into the poly(A) tail, resulting in C-terminal appendage of a poly-lysine tract. Such proteins remain associated with the ribosome until cellular quality control machinery triggers ribosome subunit dissociation and degradation of the associated mRNA and non-stop protein molecules. Conditions known to increase the frequency of non-stop protein generation reduce cellular fitness in the absence of functional quality control pathways. Mutation of the mammalian non-stop protein quality control ubiquitin ligase Listerin cause significant neurological pathology. Further, mutant, non-stop alleles of multiple genes (including at least one encoding an ER-targeted protein) are known to cause human disease. In Saccharomyces cerevisiae, the ubiquitin ligase Ltn1/Rkr1 has been implicated in the proteasomal degradation of soluble, cytosolic non-stop proteins. Whether and how non-stop proteins targeted to the endoplasmic reticulum (ER) are detected and degraded is unclear. Therefore, we generated and characterized model ER-targeted soluble (co- and post-translationally translocated) and transmembrane non-stop proteins (and non-stop mimetic proteins containing internal poly-lysine tracts). We found that these proteins are subject to proteasomal degradation. We tested three candidate ubiquitin ligases (Ltn1 and the ER-resident ubiquitin ligases Doa10 and Hrd1) for roles in regulating abundance of these proteins. Our results indicate that, of these three ubiquitin ligases, Ltn1 plays the primary role in the degradation of the model non-stop soluble and transmembrane ER-targeted proteins analyzed in this study. These data expand the known catalog of Ltn1 substrates and highlight a previously unappreciated role for the ubiquitin ligase at the ER membrane.
A NOVEL BINDING DOMAIN IN KIAA0323 SHOWS A CLEAR PREFERENCE FOR NEDD8.

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Introduction
The covalent attachment of ubiquitin (Ub) and ubiquitin-like molecules to target proteins is a widely used strategy to control protein function. NEDD8 is the closest in sequence of Ub (58% identity) and shares with Ub the hydrophobic patch typically involved in the recognition by ubiquitin binding domains (UBDs) (1). To date, domains that selectively bind NEDD8 have not been reported. On the contrary, there is evidence of some promiscuity between the mechanisms responsible for the recognition of ubiquitinated and neddyylated proteins.

Results
By employing the phage-display technique, we identified a list of Ubiquitin/NEDD8 binding domains showing different binding preferences. To determine the basis of this specificity, we performed site-directed mutagenesis at earlier reported molecular determinants of NEDD8 specific recognition by conjugating and deconjugating enzymes (1,2). We found that Ala72 in NEDD8, which corresponds to Arg72 in Ubq, contributes to the weak binding of NEDD8 towards the majority of the UBDs, since the A72R mutation in NEDD8 substantially increases binding efficiency. Conversely, we identified two binding domains mapping at the C-terminal ends of RHBDD1 and KIAA0323 proteins, whose NEDD8 recognition is not affected by the substitution A72R. These domains bind to neddyylated cullins and show a clear preference for monomeric NEDD8. Here, we used domain swapping, binding assays, isothermal titration calorimetry (ITC) and nuclear magnetic resonance spectroscopy (NMR) to describe the biochemical and structural properties of the KIAA0323 NEDD8 binding domain.

Discussion
Our data reveal that the NEDD8 binding ability of KIAA0323 is conferred by the presence of an “atypical” CUE domain lacking the canonical Phe-Pro motif. Moreover, they show for the first time that, despite the high conservation between Ubiquitin and NEDD8 in the binding surfaces recognized by the UBDs, a selective mechanism exists. And the position 72 is a molecular discriminant through which the preference versus NEDD8 can be highlighted.

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INVESTIGATING BIOMARKERS OF NEUROPATHY INITIATED BY PROTEASOME INHIBITOR BORTEZOMIB

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Bortezomib, which is a peptide boronate proteasome inhibitor, belongs to the synthetic analog proteasome inhibitors group. Bortezomib inhibits proteasome activity via binding beta5 subunit which has the chymotrypsin-like activity; and it shows enzyme specificity as well as metabolic stability compared to other proteasome inhibitors. Although bortezomib has shown potent anticancer activity against a variety of cancer cell lines both in vitro and in vivo, the side effects of bortezomib are limiting both dosage and usage of bortezomib. Like other chemotherapeutic agents, bortezomib also can cause neuropathy; specifically called chemotherapy induced peripheral neuropathy (CIPN) or bortezomib induced peripheral neuropathy (BIPN).

The aim of our study was to identify the possible proteins involved in the bortezomib induced neuropathy. In this direction, we treated the E14 mouse embryo derived neural cells with bortezomib and analyzed the neural proteome using nanoLC-MS/MS for the probable prospective targets. Also, we treated neural cells with another proteasome inhibitor carfilzomib which is believed to be less neurotoxic compared to bortezomib. With the help of these analyses, we identified the differences between bortezomib-treated and carfilzomib-treated neural cell proteomes.

Obtained data with the different expressions and existence of some proteins pointed out the mitotoxicity; cytoskeleton formation, myelination and neuronal synaptic transmission hypothesis that may have roles in apoptotic cell death.

Key words: proteasome inhibitors, bortezomib, carfilzomib, neuropathy
Identification of E3 ubiquitin ligase substrates is key to defining their biological function and understanding their roles in disease. However, even with advances in proteomics and in vitro assays, substrate identification remains a significant challenge. We have developed an integrated approach to define the E3 ligase substrates, combining genetic mouse and cellular models with orthogonal proteomics approaches to identify interacting proteins and ubiquitylation targets and high-throughput BiFC to validate substrates in situ. We have now applied this approach to identify substrates of a number of E3 ligases, including UBR5 - which has been implicated in cancer progression and chemoresistance.

UBR5-interacting proteins were isolated using GFP-Trap affinity purification followed by nanoLC-MS/MS identification and label-free quantitation. We identified ~300 UBR5-interacting proteins, many dependent on the UBR and HECT functional domains for binding. Analysis of differentially ubiquitylated proteins in breast cancer cells depleted of UBR5 by shRNA identified ~1100 proteins with altered ubiquitylation when UBR5 is depleted. Intriguingly, a large number of Ubiquitin-Proteasome System components have altered ubiquitylation in UBR5-depleted cells, suggesting co-ordinate regulation of this system.

Integration of the UBR5 interactome and ubiquitome generates a set of 25 high-confidence ligase substrates. We have validated a number of these using BiFC and have mapped the role of novel UBR5 functional domains in mediating these interactions using disease-specific mutants. These orthogonal but complementary approaches are providing interesting new insights into the function UBR5, suggesting a role in mediating crosstalk between DNA damage response and transcriptional regulation.
HLA F associated transcript 10 (FAT10) is encoded in the MHC class I locus and is expressed only in mammals. It is an ubiquitin-like modifier as its two domains have 29% and 36% homology to ubiquitin. FAT10 is highly expressed in organs of the immune system such as the thymus or lymph nodes, but it can also be induced by the proinflammatory cytokines IFN-γ and TNF-α in various cell types.

FAT10 targets proteins for proteasomal degradation as it can be conjugated via its free diglycin motif to lysine residues of other proteins via a dedicated enzyme cascade. In contrast to ubiquitin which is getting recycled, FAT10 is probably degraded together with its substrate. FAT10 plays a role in several cellular processes, e.g. it interacts with the cell cycle control protein MAD2 and with HDAC6, which mediates the transport of polyubiquitylated proteins to aggresomes.

Valosin containing protein (VCP, p97, CDC48) is a ring-shaped hexameric ATPase of the AAA family. It segregates ubiquitinated proteins from complexes and mediates the degradation of some ER associated degradation (ERAD)-substrates via the ubiquitin-proteasome-system (UPS). VCP is involved in many cellular processes such as the fusion of membranes or cell cycle progression.

In a mass spectrometry based screening VCP was found as putative interaction partner of FAT10. We confirm this interaction and show that FAT10 and VCP interact directly. Data on the influence of VCP on FAT10-mediated degradation and on the effect of FAT10 on VCP activity will be presented.
Mutations in the Parkin or PINK1 genes are the leading cause of autosomal recessive form of Parkinson’s disease (PD). The gene products parkin and PINK1 are involved in a common pathway regulating clearance of depolarized mitochondria by mitophagy. Parkin is a 465-residue E3 ubiquitin ligase involved in ubiquitination pathway. Ubiquitination typically marks proteins for degradation via the proteasome. It can also act as a signal for autophagy as well as alter substrate protein activity or location. Parkin ubiquitinates mitochondrial proteins on the damaged organelle leading to their autophagic elimination. Ubiquitination is carried out through the sequential action of three enzymes: E1 ubiquitin-activating enzymes, E2 ubiquitin-conjugating enzymes, and E3 ubiquitin ligases. Parkin consists of a ubiquitin-like domain (Ubl) at its N-terminus and four zinc-coordinating RING-like domains: RING0, RING1, In-Between-RING (IBR), and RING2. It is believed that parkin may function as a RING/HECT hybrid, where ubiquitin is first transferred by the E2 enzyme onto parkin active cysteine and then to the substrate. Compared to other ubiquitin ligases, the parkin protein exhibits low basal activity and requires activation both in vitro and in cells. Recently, we reported the crystal structure of full-length parkin using X-ray crystallography. This structure shows parkin in a compact auto-inhibited conformation and provides insight into how it is activated. In the structure RING0 occludes the ubiquitin acceptor site Cys431 in RING2 whereas a novel repressor element of parkin (REP) binds RING1 and blocks its E2-binding site. The ubiquitin-like domain (Ubl) binds adjacent to the REP through the hydrophobic surface centered around Ile44 and regulates parkin activity. Mutagenesis and NMR titrations verified interactions observed in the crystal. We also proposed the putative E2 binding site on RING1 and confirmed it by mutagenesis and NMR titrations. In mitophagy pathway, PINK1 acts upstream of parkin and is required for both parkin activation and recruitment to depolarized mitochondria. In this pathway, PINK1 phosphorylates parkin Ubl domain and ubiquitin, both regulating Parkin ligase activity. In cells, expression of the non-phosphorylatable S65A ubiquitin delays parkin recruitment to the depolarized mitochondria, and mutation of both parkin and ubiquitin at Ser65 entirely abolishes parkin activation. Current work is directed towards obtaining structure of parkin in the active conformation in complex with E2 and phospho-ubiquitin. It is my hope that understanding how parkin functions will lead to new therapeutic strategies for treating and ultimately preventing PD.
STRUCTURAL BASIS FOR CATALYSIS AND SUBSTRATE-INDUCED CONFORMATIONAL CHANGE OF A USP47

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USP47 is ubiquitin-specific protease that specifically deubiquitinates monoubiquitinated DNA polymerase beta (POLβ), stabilizing POLβ thereby playing a role in base-excision repair (BER). It acts as a regulator of cell growth and genome integrity, and may also indirectly regulates CDC25A expression at a transcriptional level. It consists of the N-terminal ubiquitin-like domain, catalytic domain and five ubiquitin-like domains at the C-terminal. Phylogenetic analysis shows that it is closely related to USP7 which is associated with prostate cancer and other refractory solid malignancies and thereby considered as a potential cancer therapeutic target. We have determined the crystal structure of the catalytic domain of USP47 alone and in complex with ubiquitin. The overall structure is the same as that of USP7 as expected. However, it differs in parts, and the details will be described.

This work was supported by grants from the Korea Institute of Science and Technology Institutional Program, and the GRL program form National Research Foundation of Korea.
DPPA3, A MATERNALLY DERIVED EPIGENETIC REPROGRAMMING FACTOR, IS UBIQUITINATED AND PARTIALLY CLEAVED IN EARLY MOUSE EMBRYOS

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After mammalian fertilization, maternal control of gene expression is coordinately transferred to the newly established embryonic program in totipotent 1-cell zygotes (Zhou and Dean, 2015, Trends Cell Biol, in press). This maternal-to-zygotic transition (MZT) is accompanied by degradation of maternal proteins by the ubiquitin-proteasome system (UPS) and activation of the embryonic genome (Shin et al., Open Biol 2:170, 2013). However, mechanisms underlying this changeover remain largely unknown. Here, we identify maternal proteins that are regulated by ubiquitin and investigate the effect of UPS cleavage on their localization and function following fertilization. To detect candidate maternal proteins, we isolated and parthenogenetically activated 4000 eggs. Half were treated with epoxomicin, a proteasome specific inhibitor, and half were untreated controls. Ubiquitinated proteins were isolated from each sample with agarose-TUBEs (Tandem ubiquitin binding entities) and differentially labeled with stable-isotope prior to microscale tandem mass spectrometry. A total of 627 ubiquitinated proteins were identified including maternal proteins and those associated with the UPS. In initial studies, we focused on Dppa3 (Developmental pluripotency associated 3), a maternal-effect protein also known as PGC7 or Stella. Dppa3 has been implicated in protecting embryonic DNA from TET3-mediated demethylation and maternal genetic ablation of Dppa3 results in cleavage-stage embryonic lethality. The Dppa3 protein is highly expressed during the MZT, but its abundance dramatically decreases after the 4-cell stage of embryogenesis. We document that Dppa3 is partially cleaved in 2- and 4-cell embryos and determine the cleavage sites by Edman degradation after 20S proteasome digestion of purified recombinant Dppa3 protein. Using mutant RNA constructs microinjected into 1-cell zygotes, we demonstrate that the normal export of Dppa3 from pronuclei to cytoplasm is prevented by point mutations that preclude cleavage and result in Dppa3 persistence in the nuclei of 2- and 4-cell embryos. These results suggest that after proteasome cleavage, maternal Dppa3 is exported from pronuclei to the cytoplasm, and this mechanism may be essential for loss of DNA methylation and epigenetic chromatin remodeling during cleavage-stage mouse embryogenesis. Currently, we are focusing on the whether the embryonic arrest at 4-cells can be rescued by normal and mutant Dppa3 isoforms and plan to determine epigenetic effects by comparing global DNA methylation patterns of transgenic embryos expressing control and mutant maternal Dppa3.
INHIBITORS OF USP30 AS A POTENTIAL TREATMENT OF PARKINSON’S DISEASE AND OTHER MITOCHONDRIAL DYSFUNCTIONS

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Parkinson’s disease (PD) affects around 10 million people worldwide (Parkinson’s disease Foundation) and is characterised by the loss of dopaminergic neurons in the substantia nigra. The exact mechanisms underlying PD remain unclear, however, mitochondrial dysfunction is thought to be one key factor. While most cases are linked to defects in alpha-synuclein, 10% of Parkinson’s cases are linked to specific genetic defects, one of which is in the ubiquitin E3 ligase parkin. Parkin and the protein kinase PINK1 cooperate to ubiquitylate mitochondrial membrane proteins on damaged mitochondria resulting in a process called mitophagy. Dysregulation of mitophagy results in increased oxidative stress, which has been described as a characteristic of PD.

USP30 is the only reported mitochondrial associated deubiquitylating enzyme (DUB) and has been shown to have a role in maintenance of mitochondrial morphology (Nakamura and Hirose 2008). Recently it has been demonstrated that USP30 opposes parkin-mediated mitophagy and that reduction of USP30 activity can rescue parkin-mediated defects in mitophagy (Bingol et al., 2014). Thus inhibition of USP30 is a potential therapeutic strategy for treatment of PD. Using our proprietary DUB screening platform UbiSphere™ we have discovered novel, potent inhibitors of USP30. In a high-throughput biochemical assay, we have identified a number of chemical series with low nanomolar potencies. These inhibitors have been shown to inhibit USP30 catalytic activity in cellular activity probe assays that measure target engagement with IC₅₀s in the 100 nM range. Here we will present the development and in vivo characterisation of these inhibitors as potential therapeutics for PD or other mitochondrial dysfunctions.

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CELL CYCLE REGULATION BY USP15

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Ubiquitination is a post-translational modification that involves the covalent attachment of ubiquitin (Ub) to cellular proteins. This process is carried out by three enzymes referred to as E1, E2 and E3. Specific deubiquitinating enzymes (DUBs) can remove ubiquitin from substrates. Ub-mediated modification has been shown to regulate the stability, function or localization of their modified target proteins. In this study, we identified DUBs which cause mitotic defect. We focused on DUB candidates whose depletion lead to significant spindle checkpoint bypass in response to taxol treatment. Subsequently, we selected and analyzed the role of Usp15, depletion of which showed mitotic defect such as chromosome missegregation and multiple poles. We isolated Usp15 complex using mass spectrometry to identify substrates and studied functional mechanism. We found that Usp15 regulate spliceosomal activity by inhibiting the modification of spliceosomal proteins with ubiquitin chains. Loss of Usp15 interferes with the accumulation of correctly spliced mRNAs. Therefore, these findings indicate that Usp15 are involved in cell cycle progression through regulating the modification of spliceosomal proteins. [This work is supported by GRL Program (20110021713) from the National Research Foundation of Korea and an institutional grant from Korean Institute of Science and Technology]
Toll-like receptors (TLR) are known for regulating myeloid homeostasis and response to infection, but chronic stimulation of TLR signaling can also lead to hematopoietic stem and progenitor cell (HSPC) dysfunction. Furthermore, mutations that lead to activation of TLR pathways contribute to premalignant hematologic conditions, such as myelodysplastic syndromes (MDS); however, the underlying cellular and molecular mechanisms are unknown. As a means of chronically activating TLR signaling within HSPC, we generated a mouse model to elevate expression of TRAF6, a downstream TLR-effector with ubiquitin (Ub) ligase activity that is overexpressed in MDS HSC. Elevating TRAF6 expression resulted in myeloid skewing, reduced HSPC function, and bone marrow failure. Intriguingly, TRAF6-expressing HSPC exhibited discrete and durable alterations in splicing patterns. Aberrant splicing of Arhgap1, a GTPase activating protein, resulted in constitutive Cdc42 GTPase activation and accounts for several HSPC defects. To explore the function of TRAF6 in alternative splicing we employed a global Ub-enrichment screen for novel substrates. The approach uncovered hnRNPA1, an RNA-binding protein that regulates exon usage. hnRNPA1 underwent TRAF6-ubiquitination adjacent to its RNA-binding domain, that resulted in enhanced affinity of hnRNPA1 for pre-mRNA substrates and alternative exon utilization. Our findings uncover a novel mechanism by which sustained TLR signaling, via TRAF6-mediated ubiquitination of hnRNPA1, alters RNA splicing and contributes to MDS-associated HSPC defects in part by activating Cdc42. These results indicate a novel function for Ub signaling in coordinating transcriptional initiation and alternative splicing by TLR within the immune system and in premalignant hematologic diseases, such as MDS.
Selective autophagy is a major quality control pathway that sequesters diverse cytosolic components and delivers them to the lysosome for degradation. Several types of selective autophagy have been described, including the breakdown of aggregated proteins (aggrephagy), mitochondria (mitophagy), peroxisomes (pexophagy), ribosomes (ribophagy), endoplasmic reticulum (reticulophagy) and pathogens (xenophagy). The molecular mechanisms behind cargo recognition and transport are complex. They require the inducible assembly of the autophagy proteins on membranes of diverse origins as well as specific autophagy receptors. Both processes are dependent on a ubiquitin-like protein family named LC3/GABARAPs. Members of this family are fused to the lipid phospatidyl ethanolamine and decorate autophagosomal membranes. The genome of humans contains six members of the LC3/GABARAP family, which are essential for autophagosome formation and the recruitment of autophagy receptors during selective autophagy. Despite their central function in autophagy, we have no knowledge of their actual involvement and their redundancy in different autophagy pathways. To overcome this lack of knowledge we are developing fluorescence based sensors specific for one subfamily and one family member, respectively. Developed sensors consist of specific peptides fused to a fluorophore. I will give an update on received specific sensors, their sensitivity and biophysical attributes.
Dynamic modification of cellular proteins by ubiquitin (Ub) and ubiquitin-like proteins such as SUMO regulate many cellular functions including chromosome maintenance and the response to DNA damage. During S phase, yeast Proliferating Cell Nuclear Antigen (PCNA) can be modified with SUMO on two lysine residues to promote interactions with the Srs2 helicase to suppress recombination during replication. During DNA damage, one of the same PCNA lysine residues can be modified with monoubiquitin and polyubiquitin chains to provide signals to recruit DNA repair factors. SUMO modification of Lys127 is stimulated by SUMO E3 ligases however it can be modified by the E2_{ubc9}~SUMO thioester alone because Lys127 resides within a SUMO consensus motif that is recognized by the SUMO E2. Unlike Lys127, Lys164 does not reside within a SUMO consensus site and its modification is strictly dependent on the E3 ligase Siz1. Much progress has been made in our understanding of RING domain activation of E2~Ub. While the SP-RING domain is a structural homologue to the RING domain and constitutes the core of the catalytic module within Siz/PIAS SUMO E3 ligases, the mechanism by which SP-RING containing E3s activate the E2_{ubc9}~SUMO thioester is not known. Furthermore, it remains unclear how substrate recognition domains dictate substrate specificity and guide particular lysine residues to the E2 active site.

Presented is our structural and biochemical progress toward an atomic resolution understanding of Siz1 mediated activation of E2_{ubc9}~SUMO and substrate recognition, details that will inform a general understanding of how E3 ligases can dictate specificity.
DUBS ACTIVATE ISOPEPTIDE BONDS FOR CLEAVAGE BY
RESTRICTING THE CONFORMATION OF THE UBIQUITIN C-
TERMINUS

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Through the dynamic process of conjugation and deconjugation, ubiquitin (Ub) and ubiquitin-like (Ubl) proteins regulate nearly every aspect of cellular function. Structural studies have revealed that upon interacting with an isopeptidase, the Ub/Ubl C-terminal glycine of the scissile bond adopts a cis-like conformation. Whether conformation affects the overall reactivity of the amide bond, however, remains unclear. We sought to address this problem by examining the relationship between $\phi$, $\psi$ torsion angles and the stereoelectronic effects of the scissile bond. Using computational methods we discovered that resonance stabilization is significantly reduced as $\psi$ approaches 0°. Concomitant with this reduction, the carbonyl carbon undergoes pyramidalization. These stereoelectronic effects were then corroborated with 13C NMR and site-specifically 13C-labeled Ub conjugates. Consistent with pyramidalization, the addition of saturating amounts of catalytically inactive deubiquitinases results in a significant chemical shift change for the Ub C-terminal carbonyl carbon. This same shift is not observed for the penultimate Ub C-terminal residue, Gly75, nor does it occur for Ub-Gly76 when conjugated to aminomethylcoumarin (AMC), which is predicted by computational analysis. Our work reveals important insights into the molecular details of Ub/Ubl deconjugating enzymes by demonstrating how conformational changes in the substrate lead to an increase in the reactivity of the scissile bond. We will discuss how these findings have implications for the design of potent active site DUB inhibitors.
the deubiquitinase USP37 promotes efficient S-phase progression and cellular response to replication stress.

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Ubiquitin-mediated proteolysis is a key regulatory process in cell cycle progression. Recently, we identified the deubiquitinase USP37 as a regulator of S-phase entry. Here we report that depletion of USP37 leads to diminished cellular proliferation and loss of viability. USP37-depleted cells exhibit altered replication kinetics and increased levels of DNA damage markers γH2AX and 53BP1. Moreover, USP37-depleted cells display significantly increased sensitivity to replication stress. Underlying the increased sensitivity, we find that USP37 interacts with the checkpoint kinase Chk1 and its binding partner Claspin to promote Chk1 activity. In turn, activation of Chk1 promotes the down-regulation of APC<sup>c<sub>cdh1</sub></sup> activity, which requires checkpoint mediated weakening of the USP37-APC<sup>c<sub>cdh1</sub></sup> interaction. Our data suggest a model whereby temporally regulated interplay of USP37, APC<sup>c<sub>cdh1</sub></sup>, and the replication checkpoint machinery determines the timing of replication checkpoint activity that sharpens the G1/S transition and promotes the efficient completion of replication. These data provide an improved understanding of the replication checkpoint, control of APC<sup>c<sub>cdh1</sub></sup>, and maintenance of genome stability.
UBQLN IS ESSENTIAL FOR QUALITY CONTROL OF MISLOCALIZED PROTEINS.

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Many membrane proteins are translocated into the endoplasmic reticulum (ER). However, the insertion of membrane protein is not perfectly efficient. Mislocalized proteins (MLP) that fail to assemble into the ER are rapidly degraded to avoid its aggregation and the disruption of cell homeostasis. Recently, it has been reported that BAG6 is involved in the metabolism of MLP. Nevertheless, how BAG6 and its associated proteins determine the fate of MLP is not adequately elucidated. In this study, we identified UBQLN as a new BAG6 binding protein.

To examine whether UBQLN is associated with the MLP degradation mechanism, we prepared the signal sequence (SS) deletion mutant of IL-2Rα as a model of nascent defective mislocalized protein. IL-2Rα is a transmembrane protein which possesses SS at N-terminus and transmembrane (TM) domain at C-terminus. We confirmed that IL-2Rα ΔSS is not translocated into the ER membrane because it exists in the cytosol as a non-glycosylated form. We next verified whether this model is rapidly degraded. The result showed that this substrate is degraded within an hour. However, this MLP model is stabilized and ubiquitinated in MG 132 treated cells. These data suggest that the MLP model we prepared is rapidly degraded by the ubiquitin-proteasome system. These results indicated that IL-2Rα ΔSS could be a nice model for MLP. In order to examine whether UBQLN is involved in the degradation of MLP, we compared the rate of turnover of MLP in the presence or absence of UBQLN. We show that the MLP model is stabilized in UBQLN knock down cells. This result suggested that UBQLN is essential for the degradation of MLP. Next, to reveal how UBQLN regulates the turnover of MLP, we examined its interaction with the MLP model. Immunoprecipitation of UBQLN resulted in co-precipitation of IL-2Rα ΔSS, but not IL-2Rα WT and IL-2Rα ΔSSΔTM. This result indicated that UBQLN interacts with the TM domain of IL-2Rα ΔSS that exposed to cytosol. To determine the domain in UBQLN required for MLP model recognition, we used a series of UBQLN deletion mutants. We found the important region of UBQLN to interact with MLP model. These results suggest that UBQLN recognizes TM domain of MLP, and is involved in the turnover of this model defective substrate.
UBIQUITIN SIGNALLING IN THE LAST RESORT PATHWAY FOR RNA POLYMERASE II DEGRADATION

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Ubiquitin signalling plays a crucial role in the last resort pathway for RNA polymerase II (RNAPII) degradation, although the exact mechanism is still being defined. The last resort pathway is activated when RNAPII becomes arrested at sites of DNA damage, and the lesion cannot be repaired by the transcription-coupled nucleotide excision repair pathway. It leads to the removal of stalled, ubiquitylated RNAPII from DNA by the ubiquitin-dependent chaperone Cdc48, and proteasome-mediated degradation of its largest subunit, Rpb1, thus clearing the DNA for other repair factors.

The last resort pathway is comprised of several ubiquitin-dependent steps. Rpb1 undergoes two sequential ubiquitylation events. The second of these is dependent on the activation of the RNAPII degradation factor Def1. Interestingly, Def1 itself is activated by partial proteasomal processing in a ubiquitin-dependent manner (Wilson et al., 2013). Here we describe how Cdc48, together with a UBX domain adaptor protein, is also required for the normal activation of Def1. Our observations provide an outline of a more general mechanism for partial proteasomal processing in yeast. Additionally, they raise an important question about UBX domain protein specificity.

DIVERGENT UBIQUITIN BINDING AND EXOSITE TARGETING IN THE USP FAMILY DUBS

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Deubiquitinating enzymes (DUBs) are responsible for reversing mono- and poly-ubiquitination of proteins and play essential roles in numerous cellular processes. Close to 100 human DUBs have been identified and are classified into six families, with the ubiquitin-specific protease (USP) family being the largest one (> 50 members). The binding of ubiquitin (Ub) to USP is strikingly different from that observed for the DUBs in the ubiquitin C-terminal hydrolase (UCH) and ovarian tumor domain protease (OTU) families. Unlike UCH and OTU that bind ubiquitin largely through its C-terminal peptide, USPs bind Ub more extensively at both the N-terminal region and the body of ubiquitin in addition to the ubiquitin C-terminal peptide. We generated a panel of mutant ubiquitins and used them to probe the ubiquitin binding to a number of USPs. Our results revealed a remarkable divergence of USP-Ub interactions despite a high level of sequence similarity among the USP catalytic cores. Our double mutant cycle analysis targeting the ubiquitin residues located in the N-terminal region, the body, and the C-terminal tail of ubiquitin also demonstrated different crosstalk among the USP-Ub interactions. This work uncovered intriguing divergences in the ubiquitin-binding mode in the USP family DUBs and raised the possibility of targeting the ubiquitin-binding exosites on USPs for selective inhibition of USPs by small molecule antagonists.
MISFOLDING, NOT UBIQUITINATION, UNDERLIES PROTEIN TARGETING TO INCLUSION BODIES

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Inclusion bodies (IB) containing aggregated forms of disease-associated proteins and polyubiquitin conjugates are universal histopathological features of neurodegenerative disease. A detailed understanding of how this aggregation alters the ubiquitin-modified proteome, however, is lacking. Moreover, the mechanisms that govern recruitment of ubiquitinated proteins to IB are not well understood. Ubiquitin (Ub) has previously been proposed to target proteins to IB, possibly to facilitate degradation via selective autophagy. To construct a detailed map of alterations in the ubiquitin-modified proteome in response to the aggregation of different aggregation-prone proteins, we have used affinity capture of diglycine isopeptides together with mass spectrometry. Additionally, we use conditionally destabilized reporters that undergo misfolding and ubiquitination upon removal of stabilizing ligand to examine the role of Ub conjugation in targeting proteins to IB composed of an N-terminal fragment of mutant huntingtin (htt), the causative protein in Huntington’s disease (HD). We show that reporters are excluded from IB in the presence of stabilizing ligand, but are recruited to IB following ligand washout. However, we find that Ub conjugation is neither necessary nor sufficient to target reporters to IB. Moreover, misfolded proteins and Ub conjugates are stable at IB. These data indicate that compromised folding states, not conjugation to Ub, specifies recruitment to IBs.
CONFORMATIONAL SWITCHING TRIGGERED BY A SINGLE ALPHA HELIX DRIVES COMPLETION OF PROTEASOME REGULATORY PARTICLE ASSEMBLY

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Many intracellular processes are conducted by large multiprotein machines. These machines frequently contain dozens of individual and often highly similar polypeptide subunits. This is certainly true of the proteasome, a 2.5 megadalton ATP-dependent protease complex responsible for most intracellular protein degradation. The proteasome consists of 66+ individual protein subunits, each of which must occupy specific positions in the final structure for its proper function. In principle, numerous subunit assembly pathways could be followed to form the proteasome holoenzyme, but in vivo, proteasome biogenesis appears to follow defined, stepwise assembly paths. The molecular mechanisms enforcing this ordered assembly remain very poorly understood.

We have previously found that biogenesis of the proteasome lid subcomplex follows an ordered, hierarchical assembly pathway. In this path, incorporation of the final lid subunit, Rpn12, licenses the lid to stably bind to the base subcomplex, in turn forming the proteasomal regulatory particle. Structural studies by others indicate that Rpn12 makes minimal contact with the base; thus, how it regulates lid-base joining from such a peripheral position has remained unclear. Using biochemical reconstitutions, quantitative crosslinking-mass spectrometry, and electron microscopy, we reveal that Rpn12 incorporation triggers a large-scale conformational remodeling of the nascent lid that relieves steric clash with the base. Surprisingly, this remodeling is triggered by a single, highly conserved Rpn12 alpha helix. This assembly-coupled conformational switching is reminiscent of viral capsid maturation, and may represent a commonly used mechanism to enforce hierarchical assembly in multisubunit complexes.
Botulinum neurotoxins, comprising seven distinct serotypes, are the most potent natural toxins. The holotoxin consists of two subunits, a heavy chain that binds its cell surface receptor on motor neurons, promoting internalization of the holotoxin, and a catalytic light chain protease that cleaves target presynaptic SNARE proteins thereby inhibiting synaptic vesicle fusion and neurotransmitter release. Serotypes A (BoNT/A) and E (BoNT/E) both target the same presynaptic protein, SNAP25, yet exhibit vastly different durations of muscle paralysis. Previous studies have suggested that for BoNT/A, which can paralyze muscles for months (and is the most potent BoNT serotype), the protease activity remains detectable for weeks following toxin exposure. In contrast, for BoNT/E, which causes fleeting muscle paralysis, the protease activity vanishes shortly after toxin exposure. It has now become evident, through our studies, that the basis for this difference in the longevity of protease activity lies in the fact that the catalytic light chain of BoNT/A is stable whereas the catalytic light chain of BoNT/E is rapidly degraded by the ubiquitin-proteasome system (UPS). The rapid degradation of BoNT/E light chain is dependent on the RING ubiquitin ligase TRAF2. We have also established that both of these serotypes can be targeted for degradation by the UPS using “designer ligases.” We are presently focusing our attention on the more toxic BoNT/A and, through a siRNA screen, have identified a deubiquitinating enzyme (DUB) responsible for its longevity. We have also identified an ubiquitin ligase that, when the function of the DUB is compromised, leads to rapid degradation of BoNT/A. This strongly supports the idea that protection of BoNT/A catalytic light chain from the UPS leads to the long half-life of the toxin. Our findings provide a mechanistic basis for the development of inhibitors for BoNT/A protease activity as well as establishing a rationale for developing inhibitors of DUBs for the treatment of botulism.
LOST OF UB2W RESULTS IN INCREASED POSTNATAL LETHALITY ACCOMPANIED BY DEFECTS IN SKIN, IMMUNE AND MALE REPRODUCTIVE SYSTEMS.

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Ube2W mono-ubiquitinates the amino-terminus of proteins rather than internal lysine residues, showing a preference for substrates with intrinsically disordered amino-termini. The in vivo functions and intracellular behavior of this intriguing E2 remain unknown. To explore its in vivo function, we generated Ube2W knock-out (KO) mice. Ube2W germ-line KO mice are prone to early postnatal lethality: 58% of Ube2W null mice die between postnatal day 0 and day 2 (19 of 33) without obvious developmental abnormalities in major internal organs assessed histopathologically. The basis of early death is uncertain, but several organ systems manifest changes in Ube2W KO mice. Strikingly, 3 of 7 Ube2W KO mice examined histologically at age P0 showed marked dermatological abnormalities: complete loss of the stratum granulosum and defects in the stratum spinosum layers of the skin. Mirroring a higher expression level of Ube2W in testis and thymus, Ube2W KO mice testis showed a disproportional decrease in weight of these two organs (~50%), suggesting a function role for Ube2W in the immune and male reproductive system. Indeed, Ube2W KO mice displayed sustained neutrophilia accompanied by increased G-CSF signaling, and testicular vacuolation associated with decreased fertility. The availability of KO mice further allowed us to establish that, in mice, Ube2W is ubiquitously expressed as a single major isoform localized primarily to the cytoplasm. Our results to date indicate that Ube2W, while not essential for life, is an important protein for early postnatal survival and proper functioning of the skin, immune and male reproductive systems. Further studies are needed to define the link between Ube2W’s unusual enzymatic properties and its in vivo functions.
NUCLEUS-LOCALIZED INSULIN-LIKE GROWTH FACTOR-1 RECEPTOR MEDIATES DNA DAMAGE TOLERANCE BY REGULATING PCNA PHOSPHORYLATION AND UBIQUITINATION IN NORMAL BUT NOT IN CANCER CELLS.

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Since our discovery of nuclear translocation of the insulin-like growth factor-1 receptor (IGF-1R) in year 2010 several other studies have implicated biological functions of it in normal and cancer cells. Here we demonstrate that nuclear IGF-1R (nIGF-1R) associates with and tyrosine phosphorylates the proliferating cell nuclear antigen (PCNA) in embryonic stem cells and normal fibroblasts but not in cancer cells. In turn this targets PCNA for mono- and poly-ubiquitination through E2 and E3 ligases mediating DNA damage tolerance, involving both polymerase and template switching. These responses were not observed in IGF-1R knockout cells but increased in ligand-stimulated and UV-treated IGF-1R-expressing cells. Our results suggest that the interaction between nIGF-1R and PCNA may play a role in maintaining genomic stability in normal and stem cells.
Adipocyte differentiation is a strictly controlled process regulated by a series of transcriptional activators. Adipogenic signals activate early adipogenic activators and facilitate the transient formation of early enhanceosomes at target genes. These enhancer regions are subsequently inherited by the late enhanceosomes. PPARγ is one of late adipogenic activators and is known as a master regulator of adipogenesis. However, the factors that regulate PPARγ expression remain to be elucidated. Here, we show that a novel ubiquitin E3 ligase, tripartite motif protein 23 (TRIM23), stabilizes PPARγ protein and mediates M1 and K27-linked atypical polyubiquitin conjugation. TRIM23 knockdown caused a marked decrease in PPARγ protein abundance during preadipocyte differentiation, resulting in a severe defect in late adipogenic differentiation, whereas it did not affect the formation of early enhanceosomes. Our results suggest that TRIM23 plays a critical role in the switching from early to late adipogenic enhanceosomes by stabilizing PPARγ protein via M1 and K27-linked atypical polyubiquitin conjugation.
The protein kinase PINK1 was recently shown to phosphorylate ubiquitin (Ub) on Ser65, and phosphoUb activates the E3 ligase Parkin allosterically. We show that PINK1 can phosphorylate every Ub in Ub chains. Moreover, Ser65 phosphorylation alters Ub structure, generating two conformations in solution. A crystal structure of the major conformation resembles Ub but has altered surface properties. NMR reveals a second phosphoUb conformation in which β5-strand slippage retracts the C-terminal tail by two residues into the Ub core. We further show that phosphoUb has no effect on E1-mediated E2 charging but can affect discharging of E2 as well as E3 enzymes to form polyUb chains. Moreover 10 out of 12 deubiquitinases (DUBs), including USP8, USP15 and USP30, are impaired in hydrolyzing phosphoUb chains. I will show published as well as unpublished data to discuss the implications of phosphoUb on the ubiquitin system and its role in the regulation of Parkin.
The yeast ubiquitin ligase Doa10 is an integral membrane protein, which targets misfolded polypeptides of the endoplasmic reticulum and the nucleus for proteasomal degradation. In contrast to other ubiquitin ligases, Doa10 acts in concert with two ubiquitin-conjugating enzymes Ubc6 and Ubc7 for substrate processing. In course of my PhD studies, I aim to characterize the molecular interplay and regulation of these proteins. My data suggest a novel two-step ubiquitination mechanism for Doa10 substrate ubiquitination. In an initial step Ubc6 targets Doa10 client proteins by the attachment of single ubiquitin-moieties. Subsequently, Ubc7 in concert with its co-factor Cue1 then modifies these primed substrates by elongating the conjugated mono-ubiquitin with K48-linked poly-ubiquitin chains. The balanced activity of both enzymes is required for Doa10 substrate processivity: the cellular amount of Ubc6 is highly controlled by auto-ubiquitination, resulting in a permanent turnover of Ubc6. Overexpression of Ubc6 impairs the degradation of Doa10 substrates to a similar extend as a Ubc6 knockout. My data will contribute to expand our limited knowledge on substrate processing by ubiquitin ligases and shed light on differences in catalytically and regulatory control mechanisms employed by them.
ASSEMBLY OF THE ELONGIN A UBIQUITIN LIGASE IS
REGULATED BY GENOTOXIC AND OTHER STRESSES

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Elongin A performs dual functions in cells as a component of RNA
polymerase II (Pol II) transcription elongation factor Elongin and as the
substrate recognition subunit of a Cullin-RING (CRL) E3 ubiquitin ligase
that targets Pol II stalled at sites of DNA damage. Here we show that
assembly of the Elongin A ubiquitin ligase is a tightly regulated process.
Whereas Elongin A is predominately present as part of Pol II elongation
factor Elongin in unstressed cells, assembly of Elongin A into the ubiquitin
ligase is strongly induced by genotoxic and transcriptional stresses that lead
to accumulation of stalled Pol II and by other stresses that activate Elongin
A-dependent transcription. Taken together, our findings reveal a new mode
of regulation of the Elongin A ubiquitin ligase and suggest that it may play
a role in Elongin A-dependent transcription.
A NON-DESTRUCTIVE INTERACTION OF A UBIQUITIN LIGASE WITH ITS SUBSTRATE

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The SCFFbw7 ubiquitin ligase controls the abundance of a number of important proto-oncogenes, including cyclin E, Myc and Notch. Fbw7 recruits substrates via its WD40 beta-propeller, which binds to a well-defined linear degron motif in substrates, called CPD (Cdc4 phospho-degron). This interaction usually results in the rapid degradation of the substrate. However, in recent years, we have reported Fbw7-substrate interactions that apparently lead to stable binding rather than substrate turnover, despite of the canonical nature of the interaction (SV40 Large T antigen and Ebp2). We now describe a novel and 'non-productive' interaction between a c-Myc CPD and Fbw7, that is canonical in every respect except that is does not lead to Myc turnover. Thus, in addition to Myc's regulation by Fbw7 via the T58 degron, we have identified a second Myc CPD which mediates potent Fbw7 binding, yet is dispensable and insufficient for Myc degradation by Fbw7. Possible functions of this interactions will be discussed.
QUALITY CONTROL OF A CYTOPLASMIC PROTEIN COMPLEX: MOLECULAR MOTORS AND COMPONENTS OF THE UBQUITIN-PROTEASOME SYSTEM REGULATE FAS STOICHIOMETRY IN YEAST

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The stoichiometry of protein complexes has to be strictly regulated in cells. Any surplus of a subunit has to be avoided as such a subunit might undergo non-productive and harmful interactions with other cellular proteins. Furthermore it might aggregate in a non-physiological fashion harming cellular physiology. The fatty acid synthase (FAS) complex of yeast is composed of 12 subunits of two types: alpha (Fas2) and beta (Fas1). Six of the Fas2 subunits form a wheel-like structure onto which three Fas1 subunits each dock axially from both sides, respectively. We disturbed build up of the mature complex by deleting the FAS1 gene. This resulted in formation of a Fas2 complex, devoid of Fas1, most likely assembled as the wheel-like structure. While Fas2 is stable in its wild type environment, it rapidly disappears when being an orphan protein. This disappearance is triggered by the ubiquitin ligase Ubr1, the ubiquitin conjugating enzymes Ubc2 and Ubc4 and the proteasome. The Hsp70 chaperone Ssa1 keeps orphan Fas2 soluble and is required for ubiquitination to occur. The AAA-ATPase Cdc48 acts after ubiquitination of Fas2 and is most likely responsible for dissociation of the Fas2 complex prior to degradation by the proteasome.

* Ingo Amm and Mario Scazzari contributed equally to the work.
Polyubiquitin chains target proteins to the 26S proteasome, where the ubiquitin modifications are removed by the deubiquitinase Rpn11 during substrate processing. Despite years of study, the mechanistic details of Rpn11’s regulation and function at the proteasome have remained largely mysterious. Using cryo-electron microscopy we determined the structure of the isolated proteasome lid complex. Our structure reveals that the Rpn11-Rpn8 dimer is in an orientation that occludes the proximal substrate binding site of Rpn11 and stabilizes its flexible Ins-1 loop in an inhibitory conformation that is unable to accommodate the C-terminus of a bound ubiquitin. Mutations in lid subunits that release the Rpn11-Rpn8 dimer from this sterically occluded orientation stimulate the DUB activity of the enzyme to levels comparable to the Rpn11-Rpn8 dimer in isolation. These findings explain the mechanism of Rpn11 inhibition in the isolated lid complex and allow us to propose a model for Rpn11 activation upon lid incorporation into the proteasome.
UBIQUITIN-LIKE DOMAINS CAN TARGET PROTEINS FOR PROTEASOME DEGRADATION

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Ubiquitin-like (UbL) domains share the same β-grasp fold and between 10 and 50% sequence identity with ubiquitin. They either form domains in larger proteins or serve as reversibly post-translational modifications and play roles in cellular processes such as autophagy, DNA repair, and mRNA splicing. Despite the structural and sequence similarity between UbL domains and ubiquitin, only a few of them are known to interact with the proteasome and none of the proteins containing UbL domains are thought to be degraded. Here we investigate all 11 UbL domains identified in S. cerevisiae. We find that the UbL domains bind to the proteasome with affinities ranging from 0.5 µM to 65 µM in vitro and that they are all able to target model proteins for proteasomal degradation in vitro and in vivo. We go on to investigate why two natural UbL proteins, Ubp6 and Mdy2, nevertheless resist degradation in yeast. Ubp6 escapes the proteasome because it lacks a disordered region at which the proteasome can initiate degradation. Mdy2 does contain a disordered domain but this region is buried by tightly bound Get4 protein. Removing Get4 or attaching artificial disordered regions to Ubp6 or Mdy2 leads to degradation by the proteasome. Thus, the fate of UbL proteins is determined by their proteasome affinity and the compactness of their structure.
INDUCTION OF USP25 BY VIRAL INFECTION POSITIVELY REGULATES INNATE ANTIVIRAL RESPONSES BY MEDIATING THE STABILIZATION OF TRAF3 AND TRAF6

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Host pathogen-recognition receptors (PRRs) detect nucleic acid from invading viruses and initiate a series of signaling pathways that lead to the production of type I interferons (IFNs) and pro-inflammatory cytokines. Here, we found that a viral infection-induced deubiquitinase (DUB), ubiquitin-specific protease 25 (USP25) was required for host defense against RNA and DNA viruses. The activation of transcription factors IRF3 and NF-κB was impaired and the production of type I IFNs and pro-inflammatory cytokines was inhibited in various types of Usp25-/- cells compared to the wild-type counterparts after stimulation by RNA or DNA viruses. In addition, USP25 deficient mice produced decreased amount of antiviral cytokines after RNA or DNA virus challenge and were more susceptible to the influenza virus H5N1 or herpes simplex virus 1 (HSV-1) infection compared to the wild-type mice. USP25 was associated with tumor necrosis factor receptor-associated factor 3 (TRAF3) and TRAF6 after infection by RNA or DNA viruses and inhibited proteasome – dependent or independent degradation of TRAF3 and TRAF6, respectively. Moreover, reconstitution of TRAF3 and TRAF6 into Usp25-/- MEFs restored virus-triggered production of type I IFNs and pro-inflammatory cytokines. Our findings thus reveal a previously uncovered positive feedback regulation of innate immune responses against RNA and DNA viruses through posttranslational modification of TRAF3 and TRAF6 by USP25.
“RING-Between-RING” (RBR) E3s are a small family of ubiquitin ligases, with approximately a dozen members in the human genome. Although originally thought to be a sub-class of the large RING family of E3s, the RBRs are mechanistically more akin to the HECT family. RBRs use a RING1 domain to bind an E2~Ub and transfer activated Ub to an active-site Cys residue in a RING2 domain, leading to their “RING-HECT hybrid” moniker. RBRs provide a unique platform to compare and contrast structural and mechanistic features with “real” RING and HECT E3s in the ongoing effort to understand the fundamental principles of Ub transfer and signaling.
Parkin is an E3 ubiquitin ligase responsible for some autosomal recessive forms of Parkinson’s disease. Even though parkin is a RING-type E3 ligase, it uses a hybrid RING/HECT mechanism for its activity. The crystal structures of full-length and the RING0-RING1-In-Between-RING-RING2 module of parkin reveal a conformation of parkin in which its E2 binding site is too far from its catalytic cysteine for the transfer of ubiquitin [1-3]. Many intramolecular interactions occur between the different RING domains, notably between RING2 and RING0, as well as with a repressor element which stacks against RING1. This element, along with the RING0 domain, are unique to parkin. Mutations of residues involved in those interactions lead to an increase of parkin activity. This suggests that parkin adopts an auto-inhibited state in basal conditions. Therefore, under stress-response conditions, parkin needs to undergo molecular rearrangements, modulated by post-translational modification and/or interactions with other proteins, to become active. The phosphorylation of serine 65 of in the Ubl of parkin by Pink1, a kinase also found mutated in some Parkinson’s patients, was shown to increase the activity of parkin [4]. More recent publications have demonstrated that ubiquitin is also phosphorylated by Pink1 and, furthermore, that phosphorylated ubiquitin could activate parkin [5-7]. We have used different techniques of structural biology and protein-protein interactions to further characterize the interaction of phosphorylated ubiquitin with parkin and the effects of phosphorylation on parkin structure. Our work, including a high-resolution structure of full-length parkin, provides insight into the mechanism of activation and the mitochondrial translocation of parkin. This contributes to a better understanding of Parkinson’s disease and, therefore, to the design of future therapies.

STRUCTURAL AND BIOCHEMICAL CHARACTERIZATION OF RNF4 MEDIATED K63 LINKED UBIQUITIN CHAIN SYNTHESIS.

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RNF4 is a SUMO-targeted ubiquitin E3 ligase that plays a crucial role in the degradation of PML in response to arsenic treatment. It is also involved in the mammalian DNA damage response, facilitating DNA repair by homologous recombination. SUMO chains bind SUMO interaction motifs of RNF4 inducing dimerization of the RING domain, which is required for its function as an E3 ligase. Thus, RNF4 is recruited to DNA damage sites by recognition of SUMO-modified DNA damage associated proteins such as MDC1. In the presence of a ubiquitin-primed, SUMO modified substrate and RNF4, Ubc13/Ube2V2 generates K63 linked ubiquitin chains, that are involved in signaling DNA damage repair. In this work, we have determined a structure of the RING domain dimer of RNF4 in complex with a Ubc13–Ub conjugate and Ube2V2. The structure has captured K63 in the acceptor ubiquitin poised to attack the active site of Ubc13 that is linked to the donor ubiquitin. The structure reveals a conformation of the Ubc13–Ub conjugate similar to that of the UbcH5a–Ub conjugate in complex with the RING domain, while specificity for K63 is guided by Ube2V2 binding the acceptor ubiquitin. Biochemical analysis suggests that in addition to positioning the acceptor ubiquitin Ube2V2 is required for RNF4 catalyzed substrate ubiquitination by Ubc13. Mutational analysis indicates that arginine 85 in the active site loop of Ubc13 plays a critical role in maintaining the active configuration of the Ubc13–Ub, Ube2V2, RNF4 complex.
NOVEL INSIGHTS INTO SUMO CHAIN FORMATION

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The role of SUMO-chains is best understood as signal for ubiquitin mediated proteasomal degradation. Chain assembly is described by the hierarchical action of E1-activating, E2-conjugating and E3-ligating enzymes but currently no specific mechanism for SUMO-chain elongation is characterized. Such enzymes, discussed as E4-elongases, are specialized E3-ligases which efficiently extend a chain but are inefficient in the initial conjugation of the modifier. We identified ZNF451 as novel class of SUMO enzymes with overlapping (catalysis) but clearly distinct (substrate recognition) E3-ligase and E4-elongase activities. We discovered a tandem SIM (SUMO-interaction-motif) region executing catalysis by involving one SIM for donor SUMO priming, while the second SIM binds SUMO in the backside of the E2-enzyme. Surprisingly, this short region is sufficient to extend a backside anchored linear TetraSUMO fusion mimicking a short SUMO-chain (E4-elongase function). Chain initiation requires in addition a zinc-finger containing region to anchor the acceptor SUMO (E3-ligase function). Finally, we show four human proteins that share the E4 elongase activity.
IN SITU GENERATED ACTIVITY-BASED PROBES FOR UB/UBL E1-E2-E3 ENZYMES: STRUCTURE, ACTIVITY AND APPLICATIONS

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E1, E2 and E3 enzymes play a critical role in a wide range of biological processes by regulating the ligation of ubiquitin (Ub) and ubiquitin-like proteins (Ubl) onto protein substrates. As such they have emerged as potential drug targets.1 This is further underscored by the clinical use of the E3 ligase inhibitors thalidomide, lenalidomide, and pomalidomide for multiple myeloma,2 and the current clinical validation of Ub E1 and Nedd8 E1 inhibitors for the treatment of cancer.1

Whereas deubiquitylating enzymes have been studied for over a decade with a variety of activity-based probes (ABPs),3 activity probes for Ub and Ubl ligating enzymes have only recently emerged, targeting E1 enzymes4a,b or E3 enzymes.4b Here we present for the first time the design and characterization of full-length Ub and Ubl based ABPs that target the entire E1-E2-E3 cascade.5 These ABPs are processed as native substrates and at the same time allow covalent trapping of the active site cysteine residue of all players in the E1-E2-E3 cascade (both HECT and RBR type E3). These novel suicide ligase activity probes will support visualization and characterization of the Ub and Ubl conjugation machineries and will be useful tools in exploring the therapeutic potential of Ub and Ubl conjugating enzymes.

References:
RING DIMERIZATION LINKS HIGHER-ORDER ASSEMBLY AND E3 UBIQUITIN LIGASE ACTIVITY OF THE TRIM5α RETROVIRAL RESTRICTION FACTOR

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Several members of the tripartite motif (TRIM) protein family of RING E3 ubiquitin (Ub) ligases promote innate immunity responses by catalyzing synthesis of K63-linked polyubiquitin chains. In this study we investigate the mechanism, by which the TRIM5α retroviral restriction factor activates Ubc13, the K63-linkage specific E2 Ub-conjugating enzyme. Structural, biochemical and functional characterization of the TRIM5α:Ubc13-Ub interactions reveals that activation of the Ubc13-Ub conjugate requires dimerization of the TRIM5α RING domain. The activation mechanism is analogous to the one previously described for UbcH5, whereby the RING dimer stabilizes the “closed” conformation of the E2-Ub conjugate. Our findings suggest that this is a general mechanism of E2-Ub activation irrespective of the type of ubiquitin linkage generated by a particular RING:E2 pair. Most importantly, the mechanism explains how higher-order oligomerization TRIM5α promoted by the interaction with the retroviral capsid, activates the E3 ligase activity of this protein. The two RING domains within the TRIM dimer are located on the opposite ends of the approximately 170 angstrom-long rod-shaped molecule, thus the RING dimer is most likely formed as the result of ligand-templated higher-order oligomerization of TRIM5α at the surface of the retroviral capsid. The mechanism we describe is likely to be conserved in many other members of the TRIM family and may have evolved to facilitate recognition of repetitive epitope patterns associated with infection.
E3 ubiquitin ligases are key mediators of the ubiquitination process and select the substrate to be modified and in some cases determine the topology of the ubiquitin chain synthesized. They can be divided into 3 families including RING ligases, HECT ligases and RBR (RING-between-RING) ligases that adopt a RING/HECT hybrid mechanism. RBR ligases share a common tripartite catalytic domain structure and different domains recognize the ubiquitin-loaded E2 and subsequently form a ubiquitin-thioester intermediate before the final transfer of ubiquitin onto a substrate protein.

TRIM ligases belong to the RING family of E3s and are characterized by a conserved tripartite domain structure that consists of a canonical RING domain, one or two B-boxes and a coiled-coil region (“RBCC”). They constitute a unique subfamily of RING ligases and structural integrity of the RBCC is believed to be required for their proper regulation and catalytic activity. The coiled coil domain mediates homo- and hetero-oligomerization, which has been suggested to be necessary for catalytic activity and in some cases seems to be enhanced by the adjacent B-box. On the other hand it has been proposed that B-boxes may also have E3 ligase activity or otherwise contribute to the regulation of ligase activity.

I will discuss work from my lab aimed at understanding the interplay between the individual domains in these ligases and their contribution to the regulation of catalytic activity.

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MECHANISMS OF DEUBAD-DOMAIN-DEPENDENT REGULATION OF UCHL5 AND BAP1 DUB ACTIVITY

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Deubiquitinating enzymes (DUBs) control vital processes in eukaryotes by hydrolyzing ubiquitin adducts. Their activities are tightly regulated but the mechanisms remain elusive. In particular the regulation of BAP1 and UCHL5 by related DEUBAD domains is poorly understood.

UCHL5 is an essential gene, that forms part of the proteasome lid and the INO80 remodelling complex. Both BAP1 and INO80 are important for DNA damage responses. BAP1 is a tumor suppressor and germ-line loss of BAP1 predisposes to cancer. The BAP1 enzyme activity is stimulated by ASXL1 to deubiquitinate H2A in nucleosomes. For UCHL5, activation by the proteasome subunit RPN13 (ADRM1) and inactivation by INO80G (NFRKB) have been observed. These regulators share a related DEUBAD domain.

Here we report crystal structures of UCHL5 complexes with DEUBAD domains. These show how the DEUBAD domain in RPN13 activates UCHL5 by positioning its C-terminal ULD domain and cross-over loop to promote substrate binding and catalysis. The related DEUBAD domain in INO80G inhibits, employing molecular mimicry to block ubiquitin docking. In this process large conformational changes create small but highly specific interfaces that modulate UCH-L5 activity by altering the affinity for substrates. Using mutagenesis and biochemistry we show that BAP1 activation follows equivalent mechanisms, relying on ASXL1 DEUBAD domain to bind to ubiquitinated nucleosomal H2A.

Our results establish how related domains can exploit enzyme conformational plasticity to switch DUB activity on and off. These allosteric sites may present novel insights for pharmaceutical intervention in DUB activity.
UBIQUITYLATION/DEUBIQUITYLATION CIRCUIT OF HISTONE H3 COUPLES MAINTENANCE DNA METHYLATION AND DNA REPLICATION

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Transmission of epigenetic cell memory to daughter cells is an essential for maintaining specific function of the individual differentiated cells, and deregulation of this system likely leads to malignant transformation. Transmission of epigenetic information is regulated at least in part by inheritance of DNA methylation patterns during DNA replication. Dnmt1 is a DNA methyltransferase responsible for the maintenance of global DNA methylation patterns in the genome. Importantly, Dnmt1 is present at functional replication foci with a strong preference for hemi-methylated DNA, coupling replication of genetic and epigenetic information. However, the mechanisms underlying this coupling remain to be elusive. By means of in vitro cell free system using Xenopus egg extracts, we successfully reproduced maintenance DNA methylation in vitro. Dnmt1 depletion resulted in a dramatic accumulation of Uhrf1-dependent ubiquitylation of histone H3 at lysine 23. Dnmt1 preferentially associated with ubiquitylated H3 in vitro. In mammalian cells, we also found Uhrf1-dependent ubiquitylation of H3 specifically during S phase. The RING finger mutant of Uhrf1 failed to recruit Dnmt1 to DNA replication sites and maintain DNA methylation in mammalian cultured cells. Importantly, Dnmt1 formed a complex with deubiquitylation enzymes toward ubiquitylated H3, suggesting the existence of ubiquitylation/deubiquitylation circuit that regulates the transient recruitment of Dnmt1 to DNA replication sites. Surprisingly, suppression of deubiquitylation of ubiquitylated H3 resulted in a defect in maintenance DNA methylation, indicating the molecular coupling between deubiquitylation of H3 and conversion of hemimethylated DNA to fully methylated DNA.

Our findings are thus the first evidence of the mechanistic link between DNA methylation and DNA replication through histone H3 ubiquitylation/deubiquitylation circuit.
STRUCTURAL BASIS FOR DEUBIQUITINATION OF HISTONE H2B
BY THE SAGA DUB MODULE

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DNA in eukaryotic cells is packaged into chromatin, whose fundamental unit comprises genomic DNA is wrapped around an octamer containing histones H2A, H2B, H3, and H4 to form the nucleosome core particle (NCP). Post-translational modifications of histone proteins annotate the genome with distinct patterns constituting a “histone code”, which regulates chromatin dynamics as well as the recruitment of transcriptional machinery. H2B monoubiquitinated at K123 (in yeast; K120 in humans) is a mark of actively transcribed chromatin that is required for stimulating histone H3 trimethylation, recruitment of H2A/H2B chaperones, and appropriate activation and elongation. H2B is ubiquitinated at inducible genes by Rad6/Bre1 and deubiquitinated by the Spt-Ada-Gcn5 acetyltransferase (SAGA) complex, a 1.8MDa multi-protein transcriptional co-activator conserved from yeast to humans. We previously determined the structure of the yeast SAGA deubiquitinating module (DUBm), a subcomplex composed of Ubp8, Sgf11, Sgf73, and Sus1. In order to understand the molecular details of how the DUBm recognizes and appropriately targets H2B-Ub, we determined the crystal structure of the DUBm bound to a ubiquitinated nucleosome (NCP-Ub). The structure reveals that the four-protein DUBm engages the nucleosome almost entirely through the H2A/H2B dimer, in addition to contacting the conjugated ubiquitin. The arginine-rich Sgf11 zinc finger, which is located adjacent to the Ubp8 active site makes multiple contacts with a conserved acidic patch on H2A/H2B that has been previously observed to be a recruitment site for other nucleosome-binding proteins. Biochemical assays with nucleosomes containing semi-synthetic H2B-Ub with a native isopeptide linkage confirm the relevance of the structure to deubiquitination of H2B. We find that the DUBm can efficiently remove ubiquitin from H2B in the context of either the intact nucleosomes or from an H2A/H2B dimer, consistent with the fact that DUBm almost exclusively contacts H2A/H2B only. We discuss these findings in light of the putative role of dynamic ubiquitination and deubiquitination during cycles of nucleosome disassembly and reassembly during the transcription cycle.
ATXN7L3 AND ENY2: MASTER CONTROLLERS OF MULTIPLE H2B DUBs IN MAMMALIAN CELLS

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Defects associated with ubiquitinating and deubiquitinating enzymes have been implicated in a number of human pathologies, including neurological diseases and cancer. Ubiquitin Specific Protease 22 (USP22) has been identified as a member of an 11 gene “death-from-cancer” signature that serves as a predictor for treatment resistance, tumor aggressiveness and metastatic probability in cancer patients. This gene encodes a deubiquitinating enzyme, which is part of a large, multi-subunit chromatin modifying complex called SAGA. USP22 activity is believed to be a major regulator of global histone H2B ubiquitin levels, removing the ubiquitin moiety from monoubiquitinated H2B to regulate gene transcription. Interestingly, USP22 is not an active enzyme on its own, it interacts with two adapter proteins, ATXN7L3 and ENY2, to gain full activity. These three proteins form the deubiquitinating core of the SAGA complex, called SAGA deubiquitinating module (DUBm).

In an attempt to characterize the mammalian SAGA DUB module and monitor the impact of ablation of different module components on global H2B ubiquitination, we discovered that only the adapter proteins ATXN7L3 and ENY2 are indispensable for global H2B deubiquitination in mammalian cells. Surprisingly, depletion of the deubiquitinating enzyme in the module, USP22, led to a slight reduction, rather than an increase, in global H2B-Ub levels. We further discovered that ATXN7L3 and ENY2 cooperate with other deubiquitinating enzymes highly similar to USP22 (USP27x and USP51) to regulate global levels of H2B-Ub in mammalian cells. These alternative DUB modules are formed independently of SAGA, and USP27x and USP51 compete with USP22 for adaptor proteins. Nevertheless, USP27x and USP51 both require interaction with ATXN7L3 and ENY2 for full DUB activity, as does USP22.

Ablation of USP27x and USP51 impacts severely cell proliferation, cell adhesion, and tumor progression as tested by mouse xenograft models. Because of the common features that USP27X and USP51 share with the cancer associated USP22, it is likely that these DUBs also have crucial roles during tumorigenesis. Ongoing studies will reveal how the balance between these DUBs affects normal cell growth, and how imbalances might impact cancer formation or progression.
SARS PLPRO, A PROCESSING PROTEASE FROM THE SEVERE ACUTE RESPIRATORY SYNDROME HUMAN CORONAVIRUS (SARS HCoV), IS A UNIQUE K48-SPECIFIC DI-DISTRIBUTIVE DEUBIQUITINATING ENZYME

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Ubiquitin (Ub) and the ubiquitin-like modifier, interferon stimulated gene 15 (ISG15), participate in the host defense of viral infections. Viruses, including the Severe Acute Respiratory Syndrome human coronavirus (SARS hCoV), have co-opted Ub/ISG15-conjugation pathways for their own advantage or have evolved effector proteins to counter pro-inflammatory properties of Ub/ISG15-conjugated host proteins. Here, we compare the Ub and ISG15 cleavage specificity of the papain-like protease (PLpro) from the recently emerged Middle Eastern Respiratory Syndrome (MERS) hCoV to the related protease from SARS, SARS PLpro. We show through biochemical assays that like SARS PLpro, MERS PLpro is both a deubiquitinating and deISGylating enzyme; and show MERS PLpro to be a broad-linkage-specific deubiquitinating enzyme (DUB), which cleaves ubiquitin chains in a distributive manner, one ubiquitin at a time. Unexpectedly, we uncover a preference of SARS PLpro to cleave K48-linked poly-Ub chains by sensing a di-Ub moiety as a minimal recognition element within a longer poly-Ub chain. This unique mechanism for SARS PLpro can result in the accumulation and stabilization of diUb units and of monoUb-conjugated substrates, which could have functional consequences in the role of immune suppression by SARS PLpro. Furthermore, this “di-distributive” mechanism of Ub chain sensing and cleavage is unique to SARS PLpro as no USP-family member human DUB shares such recognition of K48-linked Ub chains. Thus, our study reveals mechanistic differences between related viral proteases in their ability to deconjugate ubiquitinated substrates. We propose that these intrinsic enzymatic differences between SARS and MERS PLpro will help identify pro-inflammatory substrates of these viral DUBs and can guide in the design of therapeutics to combat infection by coronaviruses.
Ubiquitin Specific Protease 7 (USP7) is a deubiquitinase that regulates the stability or localization of proteins involved in apoptosis, epigenetics, cell proliferation and viral infection. USP7 consists of an N-terminal TRAF-like domain, a catalytic domain and five C-terminal ubiquitin-like (Ubl) domains. USP7 was initially discovered by its strong and specific interaction with herpes simplex virus infected cell protein 0 (ICP0) and is alternatively named ‘herpesvirus-associated ubiquitin-specific protease’ (HAUSP). ICP0 is an E3 ligase that promotes the degradation of promyelocytic leukemia proteins (PML), Sp100, DNA-PKcs, RNF8 and RNF168; proteins that are important for cellular antiviral responses. Furthermore, ICP0 functions depend on its interaction with USP7, which stabilizes ICP0 by interfering with its auto-ubiquitylation. USP7 interacts with several proteins including ICP0, GMPS, FOXO4 and claspin through its C-terminal Ubl domains. Previous studies on the mechanism of the USP7-ICP0 interaction showed that ICP0 residues 594-633, and K620, in particular, are important for USP7 binding. However, the structural details of the USP7-ICP0 interaction and USP7 residues important for interaction are still unknown. We mapped the binding site between USP7 and ICP0 using a combination of GST pull-downs and fluorescence polarization studies. We also determined the crystal structure of the USP7 Ubl domains with an ICP0 peptide and identified USP7 residues mediating the interaction with ICP0. Mutagenesis studies of these residues disrupted the interaction with ICP0. Details and analysis of the crystal structure will be presented.
Modification with proteins of the SUMO family serves to regulate the functional state of hundreds of proteins in numerous pathways. Considering that all SUMO targets compete with each other for the rate-limiting modifiers, SUMOylation seems ideally suited to coordinate cellular functions. We thus speculate that remodeling of the SUMO proteome in response to physiological and pathophysiological signals is an important means to switch cellular states. Consistent with this idea, significant remodeling of the SUMO proteome has been observed in response to severe stresses, but also at the physiological transition from interphase to mitosis. To follow up on this idea, we decided to analyze changes of the endogenous HeLa SUMO proteome in response to EGF. Epidermal growth factor (EGF) receptor signaling is one of the best-studied pathways in mammalian cells. It functions in a wide range of cellular processes, including cell proliferation, migration and apoptosis, and it plays a crucial role in various cancer developments. EGF receptor activation leads to global changes in the phospho-proteome and is known to alter ubiquitylation of numerous proteins. Whether SUMOylation contributes to EGF receptor signaling is currently unknown. To address this question, we compared the endogenous SUMO proteome of HeLa cells with or without exposure to 100 ng EFG for 10 min via a SILAC-based mass spectrometric approach (collaboration with Dr. Henning Urlaub, Göttingen). While most SUMO targets remained unchanged, a small number of proteins associated with genome organization and transcription changed in their levels of sumoylation. Amongst those were three related transcriptional regulators that were not known to be sumoylated and had not previously been linked to EGF receptor signaling. We confirmed their sumoylation, identified the SUMO acceptor sites and are currently investigating the role of their EGF-dependent desumoylation. First results from ChipSeq experiments (in collaboration with Dr. Nati Ha and Dr. Michael Brunner, Heidelberg) indicate that these proteins may significantly contribute to global changes in EGF-dependent transcription.
UBIQUITIN SIGNALS ON CARGO PROMOTE MITOPHagy VIA AUTOPHagy RECEPTORS

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Biochemical and genetic studies reveal that the products of two genes that are mutated in autosomal recessive parkinsonism, PINK1 and Parkin, normally work together in the same pathway to govern mitochondrial quality control, bolstering previous evidence that mitochondrial damage is involved in Parkinson's disease. PINK1 accumulates on the outer membrane of damaged mitochondria where it phosphorylated ubiquitin chains occurring on the outer mitochondrial membrane. These Phospho-S65 Ubiquitin chains bind to cytosolic Parkin and activate Parkin’s E3 ubiquitin ligase activity. This builds more chains on the mitochondrial surface for PINK1 to phosphorylate yielding a feedback amplification loop the drives mitophagy to completion. How ubiquitin chains or phospho-ubiquitin chains induce autophagy remains less clear. We have used the CRISPR/Cas9 system to knock out in HeLa cells a series of autophagy receptors including p62, NBR1, NDP52, Tax1BP1 and Optineurin. These studies reveal the hierarchy of autophagy receptors involved in mitophagy and identify a link between them and those known to be involved in xenophagy. How the autophagy receptors recruit autophagy machinery to mitochondria will be discussed.
Autophagic turnover of intracellular constituents is critical for cellular housekeeping, nutrient recycling, and various aspects of growth and development in eukaryotes. Here, we show that autophagy also impacts the other major degradative route involving the ubiquitin-proteasome system by eliminating 26S proteasomes in a process we termed proteophagy. Using Arabidopsis proteasomes tagged with GFP, we observed their deposition into vacuoles via a route requiring components of the autophagy machinery. This transport can be separately induced by nitrogen starvation through the ATG1 kinase sensor, and by chemical or genetic inhibition of the proteasome, implying that multiple mechanisms converge. Proteasome inhibition stimulates comprehensive ubiquitylation of the complex and increased association of the ubiquitin receptor RPN10 to the 26S particle via the added ubiquitin moieties. The ensuing proteophagy then uses this bound RPN10, which can independently bind ATG8 to form a tripartite ATG8-RPN10-ubiquitin bridge, to tether proteasomes to autophagic vesicles. Collectively, we propose that Arabidopsis RPN10 acts as a selective autophagy receptor that targets inactive 26S proteasomes for breakdown by simultaneous interaction with ubiquitylated proteasome subunits/targets and lipiddated ATG8 lining the enveloping autophagic membranes. Preliminary studies indicate that similar proteophagic pathways exist in yeast but appear independent of RPN10.
CUL3-KBTBD6/KBTBD7 UBIQUITIN E3 LIGASE COOPERATES WITH UBIQUITIN-LIKE GABARAP PROTEINS TO SPATIALLY RESTRICT TIAM1-RAC1 SIGNALLING

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Ubiquitin and the ubiquitin-like family of human ATG8 proteins are covalently attached to their respective targets. Whereas bound ubiquitin alters the stability, localization and/or activity of proteins, LC3 and GABARAP proteins are conjugated to the phospholipid phosphatidylethanolamine (PE). Their best understood function is during autophagy where LC3B serves as multipurpose docking sites for cargo receptors and regulatory factors. Here, we report an unexpected link between GABARAP proteins and the ubiquitin proteasome system. Briefly, we found that GABARAPs bind the BTB and Kelch proteins KBTBD6 and KBTBD7 (hereafter referred to as KBTBD6/7) in an ATG8 family interaction motif (AIM)-dependent manner. Both proteins share ~90% sequence identity, heterodimerize and function as substrate adaptors for a Cullin3-RING ubiquitin ligase (CRL3). As substrate of CUL3-KBTBD6/KBTBD7, we identified T-lymphoma and metastasis gene 1 (TIAM1), a peripheral membrane protein that functions as specific guanine exchange factor (GEF) to activate the small GTPase RAC1 at membranes. Ubiquitylation targets TIAM1 to proteasomal degradation and subsequently negatively controls the activity of RAC1 thereby altering actin morphology, focal adhesions, proliferation, cell invasion and morphology. Intriguingly, this signal cascade critically depends on the membrane binding domain of TIAM1, the AIM motifs of KBTBD6/7 and the conjugation of GABARAP proteins to PE. On the contrary, autophagosomal degradation or localization to autophagosomes are not involved in this process. Collectively, we provide strong evidence that GABARAP proteins recruit the ubiquitin E3 ligase complex CUL3-KBTBD6/7 to control the abundance of TIAM1 at specific membrane sites, thereby contributing to the spatial-restricted control of RAC1 activity. Besides their role in autophagy and trafficking, we uncovered a previously unknown function of GABARAP proteins as membrane localized signaling scaffolds.
AMP-activated protein kinase (AMPK) is a master sensor and regulator of cellular energy status. Upon metabolic stress, AMPK suppresses anabolic and promotes catabolic processes to regain energy homeostasis. Cancer cells can occasionally suppress the growth restrictive AMPK pathway by mutation of an upstream regulatory kinase. Here, we describe a widespread mechanism to suppress AMPK through its ubiquitination and degradation by the cancer-specific MAGE-A3/6-TRIM28 ubiquitin ligase. MAGE-A3 and MAGE-A6 are highly similar proteins normally expressed only in the male germline, but frequently (up to 75% of tumors) re-activated in human cancers. MAGE-A3/6 are necessary for cancer cell viability and sufficient to drive tumorigenic properties of non-cancerous cells, including xenograft tumor growth. MAGE-A3/6 are part of the large MAGE protein family that function as regulators of specific E3 RING ubiquitin ligases, TRIM28 in the case of MAGE-A3/6. Screening for targets of the MAGE-A3/6-TRIM28 ligase revealed that it ubiquitinates and degrades AMPKα1. Mechanistically, MAGE-A3/6 function as substrate adapters that directly bind AMPKα1 and recruit it to the TRIM28 E3 ubiquitin ligase for ubiquitination and subsequent proteasomal degradation. Degradation of AMPKα1 by this cancer-specific ubiquitin ligase leads to numerous changes in cellular metabolism, including inhibition of tumor suppressive autophagy and activation of pro-growth mTOR signaling. Furthermore, expression of MAGE-A3/6 hypersensitizes cells to AMPK agonists, such as metformin, suggesting important therapeutic implications to this work. Overall, these findings elucidate a germline mechanism commonly hijacked in cancer to suppress AMPK and reveal novel activities of the MAGE family of E3 ubiquitin ligase regulators as substrate adapters.
The ubiquitin and autophagy systems control proteostasis in health and disease. We are using proteomic and cell biological approaches to understand the architecture and interactions of components within both of these systems. One area of focus is the The AAA-ATPase p97 uses ATP hydrolysis to “segregate” ubiquitinated proteins from binding partners. p97 acts via UBX-domain containing adaptors that likely provide target specificity, but targets and functions of UBXD proteins remain poorly understood. Through systematic proteomic analysis of 16 UBXD proteins, we reveal a network of over 195 interacting proteins, implicating p97 in diverse cellular pathways. These interactions suggest specialized roles for UBSX proteins in diverse p97-dependent processes, involving nuclear cytoplasmic and organellar-specific functions. We have explored one such complex between a unstudied adaptor UBXD3 and the intraflagellar transport B (IFTB) complex, which regulates anterograde transport into cilia, and have demonstrated a role for UBXD3 in ciliagenesis. A second major interest is the application of quantitative proteomics to elucidate molecular mechanisms of ubiquitin signaling. We have used this approach to understand the kinetics, stoichiometry, and chain linkage types built on mitochondria in response to activation of the PINK1-PARKIN pathway. These types of approaches are providing us with an ever-increasing understanding of the mechanisms of complex ubiquitination pathways, in this case in the context of phosphorylation of both PARKIN and ubiquitin by PINK1 to promote mitophagy. Finally, using quantitative proteomics, we are searching for novel cargo and adaptors for autophagy. We recently identified the NCOA4 protein as a cargo adaptor for ferritinophagy, and are pursuing this through multiple approaches. New work on genetic dissection of autophagosomal components will also be discussed.
MECHANISM OF DEGRADATION-COUPlED SUBSTRATE DEUBIQUITINATION AT THE 26S PROTEASOME.

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Protein substrates are targeted for degradation by the eukaryotic 26S proteasome through the attachment of poly-ubiquitin chains that have to be removed by intrinsic proteasomal deubiquitinases (DUBs) to allow efficient substrate translocation into the internal degradation chamber. The highly promiscuous DUB Rpn11 is localized in the proteasome lid subcomplex right above the entrance to the processing pore and removes ubiquitin chains en bloc from translocating substrates. Our recent structural and mutational studies revealed how the DUB activity of Rpn11 is regulated during proteasome assembly, switching from an inhibited state in the isolated lid to an active state when the lid is incorporated into the proteasome holoenzyme. Conformational changes in Rpn11 upon ubiquitin binding to its catalytic groove suggest potential mechanisms for translocation-coupled deubiquitination. Furthermore, we were able to localize the second intrinsic DUB, Ubp6, within the proteasome complex and characterize its crosstalk with the proteasomal translocation machinery. Together, our studies provide important new insights into the coordination and coupling between mechanical substrate translocation and efficient removal of poly-ubiquitin chains at the proteasome.
KEY PLAYERS IN PROTEASOME DYNAMICS

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Proteasomes are the key enzymes in regulating the proteolysis in cell cycle progression and protein quality control\(^1\). Most of our body’s cells do not proliferate and remain in quiescence during aging. Studies in quiescent yeast, a well-established organism of eukaryotic cells, revealed that metabolic enzymes including proteasomes are sequestered into reversible and motile cytosolic granuli, which are not surrounded by membranes. The organizing principle of these granuli remains a mystery. Proteasome granuli are proposed to function as (i) proteolytic centers for the degradation of unwanted proteins or (ii) as storage compartments, thus named **proteasome storage granuli** (PSG)\(^2,3\). Their presence strengthens the resilience of quiescent yeast towards genotoxic stress. In dividing cells, proteasome holo-enzymes are primarily localized in the nucleus, where they are assembled from new precursor complexes. The transition from proliferation to quiescence is accompanied by metabolic changes and stalled de novo synthesis of proteasomal precursors. Old proteasomes migrate to the nuclear envelope and accumulate within the PSG, which exits the nucleus during quiescence. Once the cells receive signals for growth resumption, the PSG rapidly dissolves and proteasomes re-localize to the nucleus. To determine which proteins orchestrate PSG formation and clearance, we performed a comprehensive survey of the yeast null mutant collection\(^4\). Blm10, a proteasome-dedicated nuclear transporter, emerged as the most promising candidate\(^5\). Twenty additional genes were also identified which are responsible for post-translational modifications such as phosphorylation and ubiquitylation, the maintenance of the cellular energy budget and the response to genotoxic stress. These activities reflect environmental challenges and their impact on proteasome dynamics. None of the hit proteins of our array colocalizes with the PSG, thus cannot scaffold the PSG. To identify PSG components, we enriched for in vivo cross-linked PSGs by sucrose cushion ultracentrifugation. Mass spectrometry analysis using affinity purification\(^6\) suggests that proteasome-intrinsic proteins are responsible for PSG organization. Our working hypothesis is that the PSG comprises the proteasome cargo with its nuclear transport receptor. Structural features of proteins allowing the motility and reversibility of PSG-like organelles are currently under investigation.

References and acknowledgements:

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The N-end rule pathway recognizes proteins containing N-terminal degradation signals for the proteolytic system, thus playing a wide range of biological functions. The N-end rule pathway comprises two branches: the Arg/N-end rule pathway and the Ac/N-end rule pathway. The Arg/N-end rule pathway targets proteins with specific unacetylated N-terminal residues. In contrast, the Ac/N-end rule pathway targets proteins with Nα-terminally acetylated residues (Hwang et al, Science, 2010). The Ac/N-end rule pathway has been identified in *S. cerevisiae* (Shemorry A et al, Mol Cell, 2013; Kim HK et al, Cell, 2014), but its presence in mammals has been conjectural so far.

Rgs2, a regulator of G proteins, lowers blood pressure by decreasing signaling through Gαq. Human patients expressing Met-Leu-Rgs2 (ML-Rgs2) or Met-Arg-Rgs2 (MR-Rgs2) are hypertensive compared to people expressing wild-type Met-Gln-Rgs2 (MQ-Rgs2). Here we found that wild-type MQ-Rgs2 and its mutant MR-Rgs2 were destroyed by the Ac/N end rule pathway. The shortest-lived mutant ML-Rgs2 was targeted by both the Ac/N-end rule and Arg/N-end rule pathways. Thus, the Nt-acetylated Ac MX-Rgs2 (X=Q, L, R) proteins are specific substrates of the mammalian Ac/N-end rule pathway. Furthermore, the Ac/N-degron of Ac-MQ-Rgs2 was conditional, and Teb4, an endoplasmic reticulum membrane-embedded ubiquitin ligase, was able to regulate G-protein signaling by targeting Ac-MX-Rgs2 proteins for degradation through their Nα-terminal acetyl group.

*These authors contributed equally to this work.

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The ubiquitin proteasome system (UPS) adjusts cellular protein concentrations by selecting specific proteins for destruction and hydrolyzing them into small peptides. At the center of this system is the proteasome, a protein degradation machine that proteolyses a wide range of proteins with exquisite specificity. Proteins are targeted to the proteasome through a two-part degradation signal or degron. The degron consists of a proteasome-binding tag, typically a polyubiquitin chain, and a proteasome initiation site in the form of an unstructured region within the substrate. Surprisingly, the proteasome has pronounced preferences for the amino acid sequence of the initiation region. Initially, we studied 15 sequences and compared how well they initiated proteasomal degradation. The ability of the proteasome to initiate degradation correlated with the complexity of the sequence both in vitro and in vivo. The correlation is consistent over multiple model substrates and at distinct protein expression levels. Additionally, the sequence preferences are conserved between *S. cerevisiae*, *S. pombe*, and *H. sapiens*. The ability of the proteasome to initiate degradation is correlated with the binding affinity between initiation regions and proteasome. To investigate sequence preferences more systematically, we developed a fluorescence-based screen and have begun to use it to analyze 400,000 initiation sequences representing a large part of the human proteome.
The enzymes of the ubiquitin system serve to maintain the levels of individual proteins and the activities of signal transduction networks in a state of equilibrium. Through mechanisms involving regulated protein degradation and inducible protein-protein interactions, the ubiquitin system functions as an overarching regulator that controls cell division, cell death and nearly every process in between. Recent advances in mass spectrometry (MS) proteomics and immunoaffinity enrichment (IAE) now make it possible to profile thousands of site specific ubiquitination events in a quantitative manner. Moreover, methods for performing multiplexed proteomic analysis provide a means of performing temporal profiling studies to unmask the dynamic nature of ubiquitination in response to both activating and inhibitory events. Based on a series of IAE-MS experiments using label-free and NeuCode SILAC approaches, this work describes how ubiquitin substrate profiling can provide a means to identify the pathways targeted by small molecule modulators that do not directly engage the ubiquitin system. Included among these agents are mitochondrial modulators, apoptotic inducers, targeted kinase inhibitors, and regulators of the innate immune system. This talk will focus on implementation of IAE-MS proteomics methods toward ubiquitin and their use in the context of small molecule drug discovery.
Regulated protein degradation through the ubiquitin-proteasome system (UPS) plays a key role in most cellular functions. The supreme regulatory potency of the degradation machinery relies on its specificity in substrate recognition. While specificity of the UPS most likely originates from the ubiquitin configurations on substrates, we still do not understand the underlying rules by which the proteasome recognizes ubiquitin configurations during the process of substrate engagement and degradation. Complicating this problem is that many targets of the UPS, including most substrates of the Anaphase-Promoting Complex (APC), carry multiple ubiquitylated lysine residues and therefore possess complex ubiquitin configurations with unknown degeneracy. To address how the configuration of conjugated ubiquitins determines the recognition of substrates by the proteasome, we studied the kinetics of degradation of substrates with chemically defined ubiquitin configurations. Contrary to a prevailing view that a tetra-ubiquitin chain is the minimal signal for efficient proteasomal degradation, we find that, for all tested substrates, multiple di-ubiquitin chains having the same overall level of ubiquitylation as tetra-ubiquitin chains are more efficient degradation signals. To understand how kinetic features in the ubiquitylation and degradation pathways may be exploited to establish substrate specificity, we developed single-molecule fluorescent assay with enough precision to distinguish transient intermediates in the ubiquitylation and degradation reactions, and applied it to the study of molecular mechanisms of human 26S proteasome. Most substrates require ubiquitin chains for proteasomal degradation. This function of ubiquitin chains is usually thought as due to a role in promoting interaction with proteasome. We find that, the affinity of substrate binding to the proteasome is mainly determined by the total number, but not the chain structure of ubiquitins on a substrate. The study of the processive deubiquitylation by the proteasome-bound deubiquitylating enzyme Rpn11 shows that the chain structure of ubiquitin on substrates specifically promotes initiation of translocation through the axial channel of the proteasome, in an ATP-dependent process. These results provide a new understanding of the mechanism and selectivity of proteasomal degradation, and would benefit the study of cellular processes regulated by the UPS.
The 20S proteasome core contains two copies of three catalytic subunits, each with differing proteolytic specificity (β1: caspase-like; β2: trypsin-like and β5: chymotrypsin-like). Bortezomib (VELCADE®) and ixazomib (Takeda’s oral proteasome inhibitor, currently in clinical trials) primarily inhibit β5 (~5 nM IC50), with 10-fold lesser potency against β1, while β2 inhibition is much weaker (>1 µM IC50). We therefore investigated whether targeting of β2 could represent an alternate approach to inhibit the proteasome and have an anti-cancer effect.

We present a novel class of non-covalent dipeptide inhibitors possessing nano-molar potency for the β2 site in vitro with high selectivity over the β1 and β5 sites. Although activity in cells was reduced, selectivity was retained. Interestingly, while β2 inhibitors alone were not cytotoxic, a synergistic combination with ixazomib or other β5 inhibitors was observed. β2 inhibition alone did not result in protein stabilization or apoptotic markers, whereas ixazomib treatment stabilized a variety of proteins and resulted in cell death. Co-treatment with a selective β2 inhibitor and a low concentration of ixazomib resulted in stabilization of a similar set of proteins as the high concentration of ixazomib alone. Finally, bortezomib-resistant cell lines containing active site mutations in β5 were re-sensitized by co-treatment with β2 inhibitors. Compromises in β5 may render the mutant proteasomes susceptible to inhibition at β2. Taken together, this suggests that inhibition of β2 can potentiate inhibition of β5 with bortezomib or ixazomib, suggesting a strategy for the application of a combination of proteasome inhibitors in disease settings with limited sensitivity to these agents.
ATP-INDEPENDENT DEGRADATION BY THE *MYCOBACTERIUM TUBERCULOSIS* PROTEASOME

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*Mycobacterium tuberculosis* (*M. tuberculosis*) encodes a proteasome that is highly similar to eukaryotic proteasomes and is required to cause lethal infections in animals. The only pathway known to target proteins for proteasomal degradation in bacteria is pupylation, which is functionally analogous to eukaryotic ubiquitylation. However, evidence suggests that the *M. tuberculosis* proteasome contributes to pupylation-independent pathways as well. To identify new proteasome cofactors that might contribute to such pathways, we isolated proteins that bound to proteasomes overproduced in *M. tuberculosis* and found a previously uncharacterized protein, PafE, which formed rings and capped *M. tuberculosis* proteasome core particles. PafE enhanced peptide and protein degradation by proteasomes in an adenosine triphosphate (ATP)-independent manner. We identified putative PafE-dependent proteasome substrates. Importantly, an *M. tuberculosis* *pafE* mutant had a general growth defect and was attenuated for growth in mice. Collectively, these data demonstrate that ATP-independent proteasome activators are not confined to eukaryotes, and can contribute to the virulence of one the world's most devastating pathogens.
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Career Options for Biomedical Scientists


The majority of PhDs trained in biomedical sciences do not remain in academia. They are now presented with a broad variety of career options, including science journalism, publishing, science policy, patent law, and many more. This book examines the numerous different careers that scientists leaving the bench can pursue, from the perspectives of individuals who have successfully made the transition. In each case, the book sets out what the job involves and describes the qualifications and skill sets required.

2015, 232 pp., illustrated, index
Hardcover $45 ISBN 978-1-936113-72-9
VISITOR INFORMATION

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<tr>
<th>EMERGENCY</th>
<th>CSHL</th>
<th>BANBURY</th>
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<tr>
<td>Fire</td>
<td>(9) 742-3300</td>
<td>(9) 692-4747</td>
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<tr>
<td>Ambulance</td>
<td>(9) 742-3300</td>
<td>(9) 692-4747</td>
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<tr>
<td>Poison</td>
<td>(9) 542-2323</td>
<td>(9) 542-2323</td>
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<td>Police</td>
<td>(9) 911</td>
<td>(9) 549-8800</td>
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<td>Safety-Security</td>
<td>Extension 8870</td>
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**Emergency Room**
Huntington Hospital
270 Park Avenue, Huntington
631-351-2300 (1037)

**Dentists**
Dr. William Berg
631-271-2310
Dr. Robert Zeman
631-271-8090

**Doctor**
MediCenter
234 W. Jericho Tpke., Huntington Station
631-423-5400 (1034)

**Drugs - 24 hours, 7 days**
Rite-Aid
391 W. Main Street, Huntington
631-549-9400 (1039)

**Free Speed Dial**
Dial the four numbers (****) from any tan house phone to place a free call.

**GENERAL INFORMATION**

**Books, Gifts, Snacks, Clothing, Newspapers**
BOOKSTORE 367-8837 (hours posted on door)
Located in Grace Auditorium, lower level.

**Photocopiers, Journals, Periodicals, Books, Newspapers**
Photocopying – Main Library
Hours: 8:00 a.m. – 9:00 p.m. Mon-Fri
10:00 a.m. – 6:00 p.m. Saturday
Helpful tips – Use PIN# 62260 to enter Library after hours.
See Library staff for photocopier code.

**Computers, E-mail, Internet access**
Grace Auditorium
Upper level: E-mail only
Lower level: Word processing and printing.
STMP server address: mail.optonline.net
To access your E-mail, you must know the name of your home server.

**Dining, Bar**
Blackford Hall
Breakfast 7:30–9:00, Lunch 11:30–1:30, Dinner 5:30–7:00
Bar 5:00 p.m. until late
Helpful tip - If there is a line at the upper dining area, try the lower dining room

**Messages, Mail, Faxes**
Message Board, Grace, lower level
Swimming, Tennis, Jogging, Hiking
  June–Sept. Lifeguard on duty at the beach. 12:00 noon–6:00 p.m.
  Two tennis courts open daily.

Russell Fitness Center
  Dolan Hall, east wing, lower level
  PIN#: Press 62260 (then enter #)

Concierge
  On duty daily at Meetings & Courses Office.
  After hours – From tan house phones, dial x8870 for assistance

Pay Phones, House Phones
  Grace, lower level; Cabin Complex; Blackford Hall; Dolan Hall, foyer

CSHL's Green Campus

Cold Spring Harbor Laboratory is pledged to operate in an environmentally responsible fashion wherever possible. In the past, we have removed underground oil tanks, remediated asbestos in historic buildings, and taken substantial measures to ensure the pristine quality of the waters of the harbor. Water used for irrigation comes from natural springs and wells on the property itself. Lawns, trees, and planting beds are managed organically whenever possible. And trees are planted to replace those felled for construction projects.

Two areas in which the Laboratory has focused recent efforts have been those of waste management and energy conservation. The Laboratory currently recycles most waste. Scrap metal, electronics, construction debris, batteries, fluorescent light bulbs, toner cartridges, and waste oil are all recycled. For general waste, the Laboratory uses a “single stream waste management” system, removing recyclable materials and sending the remaining combustible trash to a cogeneration plant where it is burned to provide electricity, an approach considered among the most energy efficient, while providing a high yield of recyclable materials.

Equal attention has been paid to energy conservation. Most lighting fixtures have been replaced with high efficiency fluorescent fixtures, and thousands of incandescent bulbs throughout campus have been replaced with compact fluorescents. The Laboratory has also embarked on a project that will replace all building management systems on campus, reducing heating and cooling costs by as much as twenty-five per cent.

Cold Spring Harbor Laboratory continues to explore new ways in which we can reduce our environmental footprint, including encouraging our visitors and employees to use reusable containers, conserve energy, and suggest areas in which the Laboratory's efforts can be improved. This book, for example, is printed on recycled paper.
1-800 Access Numbers

AT&T  9-1-800-321-0288
MCI  9-1-800-674-7000

Local Interest
Fish Hatchery   631-692-6768
Sagamore Hill   516-922-4447
Whaling Museum  631-367-3418
Heckscher Museum 631-351-3250
CSHL DNA Learning  x 5170
Center

New York City
Helpful tip -
Take Syosset Taxi to Syosset Train Station
($9.00 per person, 15 minute ride), then catch Long Island Railroad to Penn Station (33rd Street & 7th Avenue).
Train ride about one hour.

TRANSPORTATION

Limo, Taxi
Syosset Limousine  516-364-9681 (1031)
Super Shuttle  800-957-4533 (1033)
   To head west of CSHL - Syosset train station
   Syosset Taxi  516-921-2141 (1030)
   To head east of CSHL - Huntington Village
Orange & White Taxi  631-271-3600 (1032)
Executive Limo  631-696-8000 (1047)

Trains
Long Island Rail Road  822-LIRR
Schedules available from the Meetings & Courses Office.
Amtrak  800-872-7245
MetroNorth  800-638-7646
New Jersey Transit  201-762-5100

Ferries
Bridgeport / Port Jefferson  631-473-0286 (1036)
Orient Point/ New London  631-323-2525 (1038)

Car Rentals
Avis  631-271-9300
Enterprise  631-424-8300
Hertz  631-427-6106

Airlines
American  800-433-7300
America West  800-237-9292
British Airways  800-247-9297
Continental  800-525-0280
Delta  800-221-1212
Japan Airlines  800-525-3663
Jet Blue  800-538-2583
KLM  800-374-7747
Lufthansa  800-645-3880
Northwest  800-225-2525
United  800-241-6522
US Airways  800-428-4322