

# The Ubiquitin System in Health and Disease

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Editors: S. Jentsch | B. Haendler

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S. Jentsch, B. Haendler  
Editors

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## ***Preface***

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The ubiquitin system has two major functions in eukaryotic cells: it regulates protein degradation, which is essential for normal cellular function and for the removal of potentially harmful, damaged, or misfolded proteins, and it controls protein activity by regulating protein–protein interactions and subcellular localization. The ubiquitin system is thus involved in processes as diverse as cell cycle progression, signal transduction, gene transcription, and DNA repair. Not surprisingly, defects in the ubiquitin system have been linked with numerous diseases such as cancer, inflammation, central nervous system disorders, and metabolic dysfunction.

Ubiquitin is a highly conserved 76-amino acid protein which is transferred to its target protein in an ATP-dependent manner. This post-translational modification takes place in a hierarchical, three-step fashion involving an E1 ubiquitin-activating enzyme, an E2 ubiquitin-conjugating enzyme, and an E3 ubiquitin ligase. Substrate specificity is predominantly controlled by members of a large family of E3 enzymes, which form complexes with the proteins that will be modified. This ultimately leads to the covalent attachment of the C-terminus of ubiquitin to usually an  $\epsilon$ -amino group of a lysine residue in the targeted protein. Additional ubiquitin transfer to lysine-48 of ubiquitin itself will form a polyubiquitin chain, which usually targets the conjugate for degradation by the proteasome. By contrast, mono- or polyubiquityla-

tion involving lysine-63 is normally involved in the control of protein activity. Ubiquitylation can be reverted by deubiquitylating enzymes, of which approximately 95 exist in mammals.

Following the approval of the first proteasome inhibitor for the treatment of multiple myeloma, efforts in both academia and industry have focused on the identification of novel drug targets within the ubiquitin pathway. Since numerous enzymes and co-factors are implicated in the addition or removal of ubiquitin, there is hope that appropriate targets can be found in the near future, opening the way for the identification of selectively blocking compounds.

The remarkable pace of developments in the area of ubiquitin research prompted us to organize a workshop to discuss the relevance of the ubiquitin pathway in health and disease. We believe we were successful in bringing together an outstanding group of international experts in this field. We are grateful to all of them for their excellent presentations and lively discussions. Their contributions to this book are also greatly appreciated. We sincerely hope that the proceedings of the workshop will lead to a better appreciation of the prominent and resourceful role of the ubiquitin system in many physiological processes and in numerous human diseases.

Finally, we wish to express our gratitude to the Ernst Schering Foundation for their excellent organization of the workshop, which undoubtedly helped to make it a great success. Special thanks also go to the Berlin-Brandenburg Academy of Sciences and Humanities and to Prof. G. Stock for hosting the meeting on their premises.

*Bernard Haendler*  
*Stefan Jentsch*

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## ***List of Editors and Contributors***

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### **Editors**

*Jentsch, S.*

Department of Molecular Cell Biology,  
Max-Planck-Institute of Biochemistry,  
Am Klopferspitz 18, 82152 Martinsried/Munich, Germany  
(e-mail: jentsch@biochem.mpg.de)

*Haendler, B.*

Therapeutic Research Group Oncology, Global Drug Discovery,  
Bayer Schering Pharma AG, 13342 Berlin, Germany  
(e-mail: bernhard.haendler@bayerhealthcare.com)

### **Contributors**

*Baumeister, W.*

Department of Molecular Structural Biology,  
Max-Planck-Institute of Biochemistry,  
Am Klopferspitz 18, 82152 Martinsried, Germany  
(e-mail: baumeist@biochem.mpg.de)

*Beutler, J.A.*

Molecular Targets Development Program,  
National Cancer Institute at Frederick, Frederick, MD 21702-1201, USA

*Brooks, C.*

Institute for Cancer Genetics and Department of Pathology  
and Cell Biology, College of Physicians and Surgeons,  
Columbia University, 1130 St. Nicholas Ave,  
New York, NY 10032, USA

*Collins, G.*

Cold Spring Harbor Laboratory, 1 Bungtown Road,  
Cold Spring Harbor, NY 11724, USA

*Daniel-Issakani, S.*

Rigel Pharmaceuticals, Inc. 1180 Veterans Boulevard,  
South San Francisco, CA 94080, USA

*Daulny, A.*

Cold Spring Harbor Laboratory, 1 Bungtown Road,  
Cold Spring Harbor, NY 11724, USA

*Deng, M.*

Department of Molecular Biophysics and Biochemistry,  
266 Whitney Avenue, P.O. Box 208114,  
New Haven, CT 06520-8114, USA

*Eilers, M.*

Institute of Molecular Biology and Tumor Research (IMT),  
Emil-Mannkopff-Str. 2, 35033 Marburg, Germany  
(e-mail: eilers@imt.uni-marburg.de)

*Funakoshi, M.*

Department of Molecular Biophysics and Biochemistry,  
266 Whitney Avenue, P.O. Box 208114,  
New Haven, CT 06520-8114, USA

*Geng, F.*

Cold Spring Harbor Laboratory, 1 Bungtown Road,  
Cold Spring Harbor, NY 11724, USA

*Gu, W.*

Institute for Cancer Genetics and Department of Pathology  
and Cell Biology, College of Physicians and Surgeons,  
Columbia University, 1130 St. Nicholas Ave,  
New York, NY 10032, USA  
(e-mail: wg8@columbia.edu)

*Guzzardo, P.*

Cold Spring Harbor Laboratory, 1 Bungtown Road,  
Cold Spring Harbor, NY 11724, USA

*Hochstrasser, M.*

Department of Molecular Biophysics and Biochemistry,  
266 Whitney Avenue, P.O. Box 208114,  
New Haven, CT 06520-8114, USA  
(e-mail: mark.hochstrasser@yale.edu)

*Hoppe, T.*

Cologne Excellence Cluster on Cellular Stress Responses  
in Aging-Associated Diseases (CECAD) at the Institute for Genetics,  
University of Cologne, Zùlpicher Str. 47, 50674 Cologne, Germany  
(e-mail: thorsten.hoppe@uni-koeln.de)

*Huang, J.*

Rigel Pharmaceuticals, Inc. 1180 Veterans Boulevard,  
South San Francisco, CA 94080, USA  
(e-mail: jhuang@rigel.com)

*Hunter, T.*

Molecular and Cell Biology Laboratory, The Salk Institute,  
10010 North Torrey Pines Road, La Jolla, California 92037-1099, USA  
(e-mail: hunter@salk.edu)

*O'Keefe, B.R.*

Molecular Targets Development Program,  
National Cancer Institute at Frederick, Frederick, MD 21702-1201, USA

*Kitagaki, J.*

Cancer and Developmental Biology Laboratory,  
Center for Cancer Research, National Cancer Institute,  
Frederick, MD 21702, USA

*Kon, N.*

Institute for Cancer Genetics and Department of Pathology  
and Cell Biology, College of Physicians and Surgeons,  
Columbia University, 1130 St. Nicholas Ave,  
New York, NY 10032, USA

*Kreft, S.G.*

Department of Molecular Biophysics and Biochemistry,  
266 Whitney Avenue, P.O. Box 208114,  
New Haven, CT 06520-8114, USA

*Kunjappu, M.*

Department of Molecular Biophysics and Biochemistry,  
266 Whitney Avenue, P.O. Box 208114,  
New Haven, CT 06520-8114, USA

*Kusmierczyk, A.R.*

Department of Molecular Biophysics and Biochemistry,  
266 Whitney Avenue, P.O. Box 208114,  
New Haven, CT 06520-8114, USA

*Leung, A.*

Cold Spring Harbor Laboratory, 1 Bungtown Road,  
Cold Spring Harbor, NY 11724, USA

*Li, M.*

Institute for Cancer Genetics and Department of Pathology  
and Cell Biology, College of Physicians and Surgeons,  
Columbia University, 1130 St. Nicholas Ave,  
New York, NY 10032, USA

*Li, X.*

Department of Molecular Biophysics and Biochemistry,  
266 Whitney Avenue, P.O. Box 208114,  
New Haven, CT 06520-8114, USA

*Liu, Y.-C.*

Division of Cell Biology, La Jolla Institute of Allergy and Immunology,  
9420 Athena Circle, La Jolla, CA 92037, USA  
(e-mail: yuncail@liai.org)

*Müller, J.*

Institute of Molecular Biology and Tumor Research (IMT),  
Emil-Mannkopff-Str. 2, 35033 Marburg, Germany

*Payan, D.G.*

Rigel Pharmaceuticals, Inc. 1180 Veterans Boulevard,  
South San Francisco, CA 94080, USA  
(e-mail: dgpayan@rigel.com)

*Pohl, C.*

Department of Molecular Cell Biology,  
Max-Planck-Institute of Biochemistry,  
Am Klopferspitz 18, 82152 Martinsried, Germany  
(e-mail: cpohl@biochem.mpg.de)

*Qu, K.*

Rigel Pharmaceuticals, Inc. 1180 Veterans Boulevard,  
South San Francisco, CA 94080, USA

*Ravid, T.*

Department of Molecular Biophysics and Biochemistry,  
266 Whitney Avenue, P.O. Box 208114,  
New Haven, CT 06520-8114, USA

*Rockel, B.*

Department of Molecular Structural Biology,  
Max-Planck-Institute of Biochemistry,  
Am Klopferspitz 18, 82152 Martinsried, Germany  
(e-mail: rockel@biochem.mpg.de)

*Sasiela, C.A.*

Molecular Targets Development Program,  
National Cancer Institute at Frederick, Frederick, MD 21702-1201, USA

*Shan, J.*

Institute for Cancer Genetics and Department of Pathology  
and Cell Biology, College of Physicians and Surgeons,  
Columbia University, 1130 St. Nicholas Ave,  
New York, NY 10032, USA

*Sun, H.*

Molecular and Cell Biology Laboratory, The Salk Institute,  
10010 North Torrey Pines Road, La Jolla, California 92037-1099, USA

*Tansey, W.P.*

Cold Spring Harbor Laboratory, 1 Bungtown Road,  
Cold Spring Harbor, NY 11724, USA  
(e-mail: tansey@cshl.edu)

*Tsevtkov, L.*

Rigel Pharmaceuticals, Inc. 1180 Veterans Boulevard,  
South San Francisco, CA 94080, USA

*Weissman, A.M.*

Laboratory of Protein Dynamics and Signaling,  
National Cancer Institute at Frederick, P.O. Box. Bldg. 560, Rm 22-103,  
Frederick, MD 21702-1201, USA  
(e-mail: amw@nih.gov)

*Yang, Y.*

Cancer and Developmental Biology Laboratory,  
Center for Cancer Research, National Cancer Institute,  
Frederick, MD 21702, USA

*Xie, Y.*

Department of Molecular Biophysics and Biochemistry,  
266 Whitney Avenue, P.O. Box 208114,  
New Haven, CT 06520-8114, USA

## ***Crosstalk Between the SUMO and Ubiquitin Pathways***

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T. Hunter<sup>(✉)</sup>, H. Sun

Molecular and Cell Biology Laboratory, The Salk Institute,  
10010 North Torrey Pines Road, 920137-1099 La Jolla, USA  
email: [hunter@salk.edu](mailto:hunter@salk.edu)

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**Abstract.** Several ways in which the SUMO and ubiquitin pathways can intersect and communicate have recently been discovered. This review discusses the principles of crosstalk between SUMOylation and ubiquitination, focusing on the RNF4 family of RING finger E3 ubiquitin ligases, which specifically recognize SUMOylated proteins via their SUMO moiety for ubiquitination.

### **1 The SUMO Pathway**

Post-translational modification adds layers of complexity to the control of protein function (Hunter 2007). Covalent modification of proteins by ubiquitin (Ub) and ubiquitin-like proteins, such as the small ubiquitin-like modifier (SUMO), is an increasingly important post-translational modification. Ub and SUMO are covalently linked through their C-terminal COOH group to an  $\epsilon$ -amine group of a lysine (Lys) in the

modified protein. Higher eukaryotes express four SUMO family members, SUMO1–4, encoded by different genes, with SUMO1 and SUMO2–3/4 forming two distinct groups. Newly synthesized SUMO protein is matured through proteolytic cleavage of a C-terminal peptide to expose a di-Gly motif essential for conjugation. SUMOylation occurs via a biochemical pathway analogous to ubiquitination: mature SUMO is charged via a high-energy thioester bond by the SUMO-activating enzyme (E1), a heterodimeric protein complex, and transferred to the SUMO-conjugating enzyme (E2), which catalyzes the formation of an isopeptide bond between SUMO and the target protein. SUMO ligases (E3s) provide a platform facilitating the conjugation of SUMO from E2 to the target (Gill 2004; Johnson 2004), but in contrast to the greater than 500 E3 Ub ligases in mammals fewer than ten E3 SUMO ligases are known.

In contrast to ubiquitination, where selection of the target Lys in a protein appears to be independent of primary sequence, a significant number (but not all) SUMOylation sites have the consensus sequence  $\Phi$ KxE/D, where  $\Phi$  is a hydrophobic residue preceding the SUMOylation acceptor Lys. This reflects a certain degree of specificity in substrate recognition by the SUMO-conjugating enzyme, Ubc9 (Bernier-Villamor et al. 2002). As with ubiquitination, both mono- and poly-SUMOylation can occur, although mono-SUMOylation is generally the rule. In addition, the branch point in poly-SUMO chains is mainly through a Lys in the N-terminal extension, in contrast to polyubiquitination, where any one of seven Lys can be used for branching and different branch points instruct different fates for the polyubiquitinated protein. While mono-SUMOylation is linked to functional modification of the target protein, the physiological significance of poly-SUMOylation is only just emerging (Hay 2005; Tatham et al. 2008). In budding yeast, SUMO chains are formed but are not essential for cell growth (Bylebyl et al. 2003); the same appears to be true in fission yeast (Prudden et al. 2007). In mammals, SUMO chains can be formed with SUMO2 and SUMO3, but not SUMO1. SUMO2 (and the closely related SUMO3 and 4) has a distinct consensus SUMOylation site ( $_{10}$ VKTE $_{13}$ ) at its N-terminus; SUMO1 lacks this motif, but in principle it may be added to a preformed SUMO2 chain (Tatham et al. 2001). SUMO itself also has the potential of being a ubiquitination

target (Tatham et al. 2008); all SUMOs contain multiple Lys and may prime the formation of ubiquitin chains.

SUMO is essential for normal growth, division, and the maintenance of genome stability in eukaryotic cells. Many SUMOylated proteins are found either in the nucleus (e.g., in nuclear bodies) or at the nuclear periphery, implying an important role for SUMOylation in biological processes in the nucleus (Johnson 2004). Mutations in SUMO conjugation pathway enzymes result in sensitivity to genotoxic challenges. In fission yeast, mutations in *rad31*<sup>+</sup>, encoding a SUMO E1 subunit, and *hus5*<sup>+</sup>, encoding the SUMO E2 (Ubc9 ortholog), render cells sensitive to DNA damage (al-Khodairy et al. 1995; Shayeghi et al. 1997). Deletion of *pmt3*<sup>+</sup>, the only SUMO gene in *Schizosaccharomyces pombe*, results in slow growth, sensitivity to disruption of mitosis, and increased telomere length (Tanaka et al. 1999). Pli1, the *S. pombe* ortholog of PIAS family SUMO E3s, is essential for the stability of centromeres and telomeres (Xhemalce et al. 2004). Nse2/Mms21, a component of the Smc5/6 complex, is also a functional SUMO E3 (Andrews et al. 2005; McDonald et al. 2003; Zhao and Blobel 2005).

Like many other forms of post-translational modification, such as phosphorylation, SUMOylation can tag the modified protein for novel protein–protein interactions. Therefore, SUMO-interacting domains would be expected to play a crucial role in regulating the function of SUMOylated proteins, and the identification of proteins with SUMO-interacting motifs is important for an understanding of the SUMOylation system (Hannich et al. 2005; Hecker et al. 2006). In contrast to the multiple characterized Ub-binding domains, so far only one SUMO-interacting motif (SIM, or SUMO-binding motif, SBM) is known (Minty et al. 2000; Reverter and Lima 2005; Song et al. 2004). The core of the SIM is composed of three hydrophobic aliphatic (I, L, or V) residues, arranged as V/I-V/I-X-V/I/L or V/I-X-V/I-V/I. Structural analysis shows that when a SIM and SUMO interact, these residues form a  $\beta$ -strand and are incorporated into a  $\beta$ -sheet together with SUMO's second  $\beta$ -strand; depending on whether the core sequence is  $\Phi\Phi X\Phi$  or  $\Phi X\Phi\Phi$ , the SIM  $\beta$ -strand binds to SUMO in one or the other orientation, forming either parallel or antiparallel interactions (Song et al. 2005). In this sense, the SIM motif is like SH3 domains, which can bind their peptide ligands in either orientation depending on the

sequence. The SIM-binding surface on SUMO lies between its second  $\beta$ -strand and its  $\alpha$ -helix. A number of hydrophobic residues in SUMO1, including F36 and V38, form a conserved hydrophobic patch to accommodate the hydrophobic side chains of the SIM (Hecker et al. 2006; Reverter and Lima 2005; Song et al. 2004, 2005). Additional residues surrounding the core SIM residues contribute to its association with SUMO, especially through electrostatic interactions involving acidic SIM residues. Residues lying outside the core may also contribute to specificity in recognizing different SUMO isoforms (Hecker et al. 2006). It seems likely that additional specificity in the interaction of SIM-containing proteins with SUMOylated targets is contributed by sequences in the target protein surrounding the SUMOylation site, and potentially interactions elsewhere with regions outside the core SIM.

## **2 The RING Finger Protein 4 Family: SUMO-Dependent E3 Ubiquitin Ligases**

RNF4 (RING finger protein 4, also known as Snurf, small nuclear ring finger protein) was originally identified as an androgen receptor interacting protein and subsequently shown to interact with several transcription factors and regulate their activity (Kaiser et al. 2003; Lyngso et al. 2000; Moilanen et al. 1998; Wu et al. 2004). In mammals, RNF4 is expressed in proliferating tissues, such as testes and tumors, indicating a role in cell cycle and growth control regulation (Cavallo et al. 2005; Galili et al. 2000). Early studies demonstrated that RNF4 was associated with free SUMO-1 and with SUMOylated-PML protein (Hakli et al. 2005). However, the nature of this association was unknown. Recently, we and others found RNF4 to be a member of a conserved protein family with homologs in both mammals and simple eukaryotes that can bind directly to SUMO (Kosoy et al. 2007; Prudden et al. 2007; Sun et al. 2007; reviewed by Perry et al. 2008).

The RNF4 family now includes *Saccharomyces cerevisiae* Hex3 (also known as Slx5), *S. pombe* Rfp1 and Rfp2, *Dictyostelium* MIP1, *Drosophila* CG10981, and mammalian RNF4. So far no ortholog has been identified in *Caenorhabditis elegans*. All RNF4 homologs have an N-terminal SUMO-binding region and a C-terminal RING finger do-

main. Interestingly, the SUMO-binding region contains multiple motifs (SIMs); each resembles a canonical SUMO-interacting motif: Rfp1 and Rfp2, which are closely related, and Slx5 each has two SIMs in tandem, whereas mammalian RNF4 has four distinct SIMs. In the yeast RNF4 homologs, the two SIMs correspond to SIM2 and SIM3 in RNF4. The functional significance of the tandem SIMs in these proteins awaits fuller characterization (see below), but each of the SIMs in fission yeast Rfp1/Rfp2 can interact with SUMO independently, and the same is true for all four SIMs in RNF4 (Tatham et al. 2008). Possibly, the tandem SIMs specifically recognize poly-SUMO chains, in a manner analogous to the tandem Ub-binding motifs in Ataxin3 that specifically recognize poly-Ub. Alternatively, they could recognize two different SUMO residues in a multiply SUMOylated protein.

The RING domains in the RNF4 family proteins can be classified into two groups. Like many RING fingers, the RNF4 RING and MIP1 RING are themselves active Ub E3 ligases. In contrast, the RING domains of the yeast RNF4 homologs – Rfp1, Rfp2, and Slx5 – all lack E3 activity, and instead recruit Slx8, an active RING finger Ub ligase, through a heterodimeric RING–RING interaction, to form a functional E3 complex. As a result, all members of the RNF4 family can act as Ub ligases with the potential to specifically target SUMO-conjugated proteins. Several studies have recently shown that RNF4 family proteins can ubiquitinate target proteins in a SUMO- and SIM-domain-dependent manner *in vitro*, including GST-SUMO fusion proteins, Rad60, Rad52 and PML (Ii et al. 2007a,b; Prudden et al. 2007; Sun et al. 2007; Tatham et al. 2008; Uzunova et al. 2007; Xie et al. 2007). Another characteristic of RNF4 family proteins is a C-terminal motif consisting of three hydrophobic amino acids (L/I-Y/F-L/I/V/Y/F—the central Y/F seems to be critical), located at the end of the RING domain. This motif is present in Rfp1 and Rfp2, Slx5, MIP1, and RNF4. Deletion of these three amino acids abolishes activity *in vivo* (Sun et al. 2007), suggesting that it serves a critical function. One possibility is that this tail is critical for proper RING–RING interactions, as is the case for Mdm2 and MdmX, another heterodimeric RING finger E3 ligase (Poyurovsky et al. 2007; Uldrijan et al. 2007), where the hydrophobic tails of both Mdm2 and MdmX form an essential part of the RING–RING dimer interface (Linke et al. 2008).

In lower eukaryotes, RNF4 family proteins are involved in transcriptional regulation and gene silencing (Darst et al. 2007; Wang et al. 2006) and are crucial for maintaining eukaryotic genome integrity and surviving genotoxic stress, suggesting they act in DNA damage repair pathways (Burgess et al. 2007; Kosoy et al. 2007; Mullen et al. 2001; Prudden et al. 2007; Sun et al. 2007; Torres-Rosell et al. 2007; Xie et al. 2007; Zhang et al. 2006). *S. pombe* Rfp1 and Rfp2 are essential for cell proliferation, and their mutation results in a plethora of phenotypes due to loss of genome integrity (Prudden et al. 2007; Sun et al. 2007). In *S. cerevisiae*, Slx5 and Slx8 are essential for DNA damage repair during cell cycle progression; they interact genetically with Sgs1, a RecQ family DNA helicase (Mullen et al. 2001; Zhang et al. 2006). *slx5* $\Delta$  and *slx8* $\Delta$  mutations are also both synthetically lethal with *sgs1* $\Delta$  (Mullen et al. 2001; Zhang et al. 2006) and interact genetically with the telomerase gene (*tlc1*), such that *slx5* $\Delta$  and *slx8* $\Delta$  enhance the senescence phenotypes of the *tlc1* mutant (Azam et al. 2006). Interestingly, Slx5 associates with components of the Smc5/Smc6 complex in both *S. cerevisiae* and *S. pombe* (Hazbun et al. 2003; Prudden et al. 2007). In addition, Rad60, which associates with Smc5/Smc6 and has two functional C-terminal SUMO-like domains (Boddy et al. 2003; Raffa et al. 2006), was identified in yeast two-hybrid screens for Rfp1/Rfp2 interacting proteins (Sun et al. 2007). Rad60 can be ubiquitinated by Rfp/Slx8 complexes in vitro, suggesting that it may be a physiological target (Prudden et al. 2007). Another DNA repair protein, Rad52, can also be ubiquitinated in vitro by Slx5/Slx8 complexes (Xie et al. 2007). In summary, in lower eukaryotes, RNF4 family proteins are needed for transcriptional regulation and gene silencing (Darst et al. 2007; Wang et al. 2006) and for genotoxic stress survival, suggesting they have a prominent role in DNA damage repair pathways (Kosoy et al. 2007; Mullen et al. 2001; Prudden et al. 2007; Sun et al. 2007; Torres-Rosell et al. 2007; Xie et al. 2007; Zhang et al. 2006). Therefore, SUMOylated subpopulations of DNA repair proteins are likely targets for RNF4/Rfp/Slx5-mediated ubiquitination in vivo. Degradation of SUMOylated forms of these proteins may be necessary to balance their activity and to prevent excessive processing of certain DNA structures generated by the DNA repair machinery, and possibly for restart of replication once repair has been completed.

RNF4 family proteins are linked to the SUMOylation pathway both biochemically and genetically (Burgess et al. 2007; Hannich et al. 2005; Hazbun et al. 2003; Prudden et al. 2007; Sun et al. 2007; Uzunova et al. 2007; Wang et al. 2006). Slx5 interacts with SUMO in yeast two-hybrid screens (Hannich et al. 2005; Uetz et al. 2000). Loss of function mutations in *slx5* and *slx8* suppress a temperature-sensitive *mot1* mutant (*mot1-301*), which encodes an inhibitor of TBP in *S. cerevisiae* (Wang et al. 2006). Significantly, this unbiased genetic approach identified almost exclusively components of the SUMOylation pathway, including both subunits of E1, E2, and two SUMO proteases, together with Slx5 and Slx8. In *Dictyostelium*, DdMIP1, the RNF4 homolog, was found to interact with MEK1 through its SIM in yeast two-hybrid assays. Interestingly, DdMIP1 can promote the ubiquitination of activated MEK1 kinase in a RING finger-dependent manner, and the loss of DdMIP1 results in accumulation of SUMOylated MEK1 during cAMP-induced *Dictyostelium* chemotaxis (Sobko et al. 2002). Therefore, a conserved function of the RNF4 family proteins could be to destabilize SUMOylated transcription factors and activated protein kinases via RING finger-mediated ubiquitination.

In mammalian systems, RNF4 was originally identified through its ability to interact with the androgen receptor and enhance the transcriptional activation by steroid hormone receptors (Moilanen et al. 1998). However, early work demonstrated that mammalian RNF4 was associated with SUMO and SUMOylated proteins, including PML, the major scaffold of nuclear structures known as nuclear bodies. Two recent studies have identified SUMOylated PML as a target for RNF4 (Lallemand-Breitenbach et al. 2008; Tatham et al. 2008). siRNA-mediated depletion of RNF4 in HeLa cells results in accumulation of poly-SUMOylated proteins, containing both SUMO1 and SUMO2. Overexpression of RNF4 results in increased SUMOylation of PML and degradation. Since RNF4 ubiquitinates poly-SUMO1 or poly-SUMO2 chains in vitro more efficiently than mono-SUMO, it has been proposed that RNF4 poly-ubiquitinates PML molecules containing branched SUMO chains. RNF4 stimulates the formation of Ub adducts to multiple Lys in SUMO both in vitro and in vivo, and catalyzes formation of Ub chains branched at several Lys, including K11, K48, and K63; of these, only K48-branched chains are known to be recognized by the proteasome to mediate degra-

dition. Arsenic trioxide is used therapeutically for the treatment of acute promyelocytic leukemia (APL). One form of APL is driven by a chimera between PML and RAR $\alpha$  resulting from a t15;17 chromosome fusion, and arsenic trioxide treatment causes rapid degradation of PML-RAR $\alpha$  in APL cells, as well as PML in normal cells. Recent results show that this depends on SUMOylation of K160 in PML, with a preference for SUMO2 (Lallemand-Breitenbach et al. 2008; Tatham et al. 2008). Degradation of PML in presence of arsenic trioxide requires RNF4, based on the diminished effect of arsenic trioxide in cells depleted for RNF4 or expressing dominant-negative ligase-deficient or SIM-deficient RNF4 mutants. It is currently unclear, however, exactly how arsenic trioxide treatment triggers RNF4-mediated degradation of SUMOylated PML. Overexpressed RNF4 localizes to nuclear bodies, which contain several other SUMOylated proteins, including Daxx, and it is possible that RNF4 also mediates ubiquitination of these proteins. In summary, SUMOylated PML is the first bona fide in vivo target for RNF4, which is ubiquitinated and degraded in a SUMOylation-dependent fashion. Whether other RNF4 targets require polySUMOylation is unclear, and it remains possible that monoSUMOylated proteins can be targeted, perhaps if they contain multiple SUMO residues. Certainly, in fission yeast no phenotype is observed in cells in which SUMO cannot form branches (Prudden et al. 2007), and, in contrast to cells lacking RNF4, SUMOylated proteins do not accumulate, which implies that polySUMOylation is not essential for RNF4 function in mediating metabolism of SUMOylated proteins, at least in this organism.

Consistent with Rfp/Slx8 being responsible for degradation of SUMOylated proteins, genetic depletion of Rfp1/Rfp2 or Slx8 in fission yeast causes accumulation of SUMOylated proteins, that are lost when the Pli1 SUMO E3 ligase is mutated (Kosoy et al. 2007; Prudden et al. 2007; Sun et al. 2007). Nevertheless, there are other possible explanations; for instance, Rfp/Slx8 might regulate activity of the SUMO-specific proteases Ulp1 and Ulp2. Clearly what is needed is the identification of the SUMOylated proteins to which Rfp/Slx8 binds in vivo and a demonstration that the level of one or more of these proteins is elevated in cells lacking Rfp/Slx8 function (e.g., *rfp1*  $\Delta$  *rfp2*  $\Delta$  cells). This demonstration may be difficult, because it is likely that only a very small fraction of the population of any target protein is SUMOylated,

meaning that only a slight change in protein level may be observed. Our original identification of Rfp1 (Sun et al. 2007) came through a yeast two-hybrid interaction with Ark1, the fission yeast ortholog of the Aurora mitotic kinases, and we have preliminary evidence that the level of Ark1 is modestly elevated in *rfp1Δrfp2Δ* fission yeast, suggesting that a population of Ark1 may be a Rfp/Slx8 target. Nevertheless, since monoubiquitination and polyubiquitin chains branched at K63 dictate other fates for the modified proteins, one should not rule out the possibility that RNF4-mediated ubiquitination might have consequences for its target proteins other than proteasomal degradation.

In summary, the RNF4 family shows a remarkably high degree of functional conservation, with human RNF4, being able to complement the combined loss of Rfp1, Rfp2, and Slx8 in fission yeast, in a manner that requires both the SIM region and the RING finger. This implies that SUMOylated proteins are the critical targets, but one should leave open the possibility that RNF4 may also have non-SUMOylated targets for ubiquitination. Finally, in addition to RNF4, it has recently been reported that SUMOylation can also target the HIF-1 $\alpha$  transcription factor for polyubiquitination and degradation by the VHL-containing cullin-Rbx1 E3 ligase (Cheng et al. 2007). The SUMOylated HIF-1 $\alpha$  is directly recognized by the VHL substrate specificity subunit, but whether the SUMO moiety is itself bound by VHL is not known.

### **3 Principles of Crosstalk Between SUMOylation and Ubiquitination**

There are several general principles through which the SUMO and Ub pathways can intersect and communicate.

1. Ub and SUMO E3 ligases can modify the same target Lys in a substrate protein, such that one modification will preclude the other and vice versa. In this manner, SUMOylation might prevent ubiquitination-dependent degradation of a protein or binding by a Ub-binding domain (UBD) protein. This type of Lys target competition appears to be the case for PCNA, I $\kappa$ B $\alpha$ , NEMO, ER $\alpha$ , and p53. In the case of PCNA K164 can either be monoubiquitinated or SUMOylated, and this regulates the type of DNA repair occur-

ring at a lesion (Andersen et al. 2008). Monoubiquitinated PCNA is recognized through UBZ/UBM domain-mediated binding of translesion synthetases, which are needed for DNA replication across residues with large adducts; SUMOylated PCNA is recognized by the Srs2 helicase, whose binding displaces Rad51 and prevents inappropriate homologous recombination. Monoubiquitinated PCNA can also be polyubiquitinated to form K63-branched chains. Whether the stoichiometry of SUMOylation and ubiquitination at a single Lys is ever high enough for competition to be a physiological regulatory mechanism is unclear, but, as is found with PCNA, ubiquitination and SUMOylation of the same Lys can have different consequences, and in this sense they are competitive.

2. (Poly)SUMOylated proteins can be targeted for ubiquitination by RNF4 family E3 ligases through direct recognition of the SUMO moiety (Tatham et al. 2008). This can result in proteasomal degradation of SUMO, or possibly other fates.
3. SUMOylation may also target a protein for polyubiquitination through another type of E3 ubiquitin ligase, as has been shown for HIF-1 $\alpha$ , which is polyubiquitinated by the VHL-CRL cullin-RING E3 ligase when it is SUMOylated, through a direct interaction between SUMO-HIF-1 $\alpha$  and the VHL protein (Cheng et al. 2007).
4. SUMO itself can be polyubiquitinated, with the attached Ub chains being branched through K11, K48, and K63 (Tatham et al. 2008). This may be a mechanism for degrading SUMO attached to SUMOylated protein chains or may serve some other purpose.
5. Conversely, enzymes in the ubiquitination pathway can be regulated by SUMOylation. For instance, the ubiquitin-conjugating enzyme E2-25K is SUMOylated at K14, and this interferes with interaction of the upstream E1, and charging of E2-25K with Ub (Pichler et al. 2005). In addition, the USP25 Ub-specific protease contains a SIM, and the binding of SUMO-Ubc9 to the SIM results in SUMOylation close to its two Ub-interaction motifs (UIMs), which are required for efficient hydrolysis of ubiquitin chains, and this SUMO residue thereby impairs binding to and hydrolysis of Ub chains (Meulmeester et al. 2008).

## 4 Conclusions and Challenges

Given that there are more than ten other ubiquitin-like proteins (UBLs) in addition to SUMO, it is entirely possible that analogous crosstalk exists between the Ub pathway and the other UBLs. A major challenge remains the identification of the physiological targets for the highly conserved RNF4 family of proteins, which apparently play roles in many fundamental cellular processes. In addition, since RNF4-ligated Ub chains on SUMO itself and on target SUMOylated proteins are potentially branched at K11, K48, and K63, this raises issues as to which of the RNF4 targets are proteasomally degraded via K48 chains, and which have other fates through K11 and K63 chains. Another unanswered question is how RNF4 recognizes its SUMOylated substrates. Does this require polySUMOylation, and perhaps simultaneous binding of multiple SUMO residues in the chain by the RNF4 multi-SIM, or rather are there monoSUMOylated protein targets, which might be recognized through additional contacts between the substrate and the RNF4 protein. Crosstalk between the ubiquitin and SUMO pathways has emerged only in the past few years, and additional connections between these two pathways are certain to be revealed over the next few years.

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## ***A Tale of Two Giant Proteases***

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B. Rockel, W. Baumeister<sup>(✉)</sup>

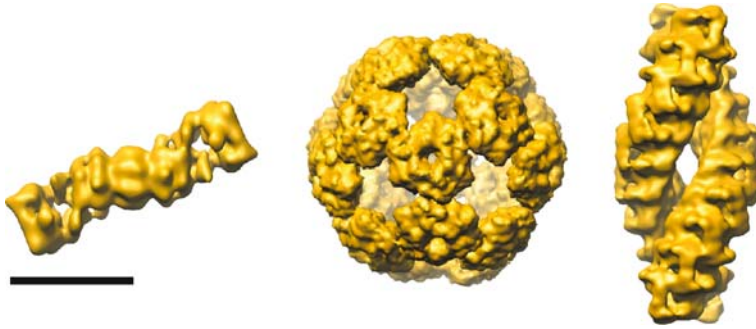
Department of Molecular Structural Biology, Max-Planck Institute of Biochemistry, Am  
Klopferspitz 18, 82152 Martinsried, Germany  
email: [baumeist@biochem.mpg.de](mailto:baumeist@biochem.mpg.de)

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**Abstract.** The 26S proteasome and tripeptidyl peptidase II (TPPII) are two exceptionally large eukaryotic protein complexes involved in intracellular proteolysis, where they exert their function sequentially: the proteasome, a multisubunit complex of 2.5 MDa, acts at the downstream end of the ubiquitin pathway and degrades ubiquitinated proteins into small oligopeptides. Such oligopeptides are substrates for TPPII, a 6-MDa homooligomer, which releases tripeptides from their free N-terminus. Both 26S and TPPII are very fragile complexes refractory to crystallization and in their fully assembled native form have been visualized only by electron microscopy. Here, we will discuss the structural features of the two complexes and their functional implications.

## 1 Intracellular Proteolysis

Maintenance of cellular homeostasis relies upon the spatial and temporal control of protein degradation: regulatory proteins such as transcription factors or components of signal transduction chains need to be degraded at specific moments of their life spans. Misfolded or damaged and, as a consequence, dysfunctional proteins are prone to aggregation and must be removed from the cytoplasm; the immune system relies on the availability of immuno-competent peptides such as obtained by degradation of foreign proteins. Major sites of proteolysis are the cytoplasm and the lysosome, a membrane-bound compartment housing several small proteases. Cytosolic protein degradation in eukaryotes is mainly effected via the ubiquitin pathway: substrates destined for degradation are modified with ubiquitin chains by a cascade of (1) ubiquitin activating, (2) ubiquitin conjugating, and (3) ubiquitin ligating, and these steps are mediated by the enzymes E1, E2, and E3, respectively. Proteins tagged with multiubiquitin chains are then selected by the 26S proteasome and subsequently degraded in an ATP-dependent process (see Ciechanover 2005 for a review). The products of the proteasome's action are peptides of a length of 8–12 amino acids (Kisselev et al. 1999), which subsequently can both be trimmed and presented to the immune system or be degraded into amino acids. The ATP-independent proteases involved in these processes are often referred to as “downstream proteases” and some of them occur as homooligomeric complexes with a size exceeding that of the 26S proteasome. Examples of such “giant proteases beyond the proteasome” (Yao and Cohen 1999) are the tricorn protease (TRI) of the Archaeon *Thermoplasma acidophilum* (Tamura et al. 1996; Walz et al. 1997) and its functional equivalent in eukaryotes, Tripeptidyl peptidase II (TPPII), (Geier et al. 1999; Rockel et al. 2002). The 20S proteasome and TRI have been crystallized (Löwe et al. 1995; Groll et al. 1997; Bosch et al. 2001; Brandstetter et al. 2001), but the holocomplexes of 26S, TRI, and TPPII in their fully assembled and fully functional oligomeric form have thus far only been studied via electron microscopy (Walz et al. 1997, 1998; Rockel et al. 2002; Nickell et al. 2007a) (Fig. 1).



**Fig. 1.** Structures of three giant proteases obtained by electron cryo-microscopy. *Left* 26S proteasome (Nickell et al. 2007a); *center* Tricorn protease (Walz et al. 1999); *right* Tripeptidyl peptidase II (Rockel et al. 2005). *Scale bar*, 25 nm

## 2 The 26S Proteasome

The 26S proteasome links the ubiquitin-pathway with protein degradation and hence is involved in many cellular processes (for reviews see Baumeister et al. 1998; Voges et al. 1999; Pickart and Cohen 2004). In contrast to the cellular function of the 26S proteasome, the better part of its functional mechanism is still only dimly understood, partly due to the lack of a solid structural framework. Aside from its complexity (it consists of more than 30 different subunits) and its fragility, also the plasticity of the 26S proteasome presumably contributes to these difficulties: whereas previously the proteasome population in a cell has been viewed as uniform, it now becomes apparent that a whole array of functionally and structurally distinct complexes might exist and, moreover, that the subunit composition is subject to regulation.

The 26S proteasomes contained in a normal cell can be separated into two subcomplexes; the 20S proteasome—the 700-kDa proteolytic core—and the 19S particle, a 900-kDa regulatory complex required for the recognition of ubiquitinated proteins and their preparation for degradation. To mediate its diverse cellular functions, the 20S proteasome also associates with other specific adaptor complexes, like the PA28/11S complex or the B110/PA200 complex, which function as ac-

tivators of the proteolytic core (for reviews see Glickman and Raveh 2005; Hanna and Finley 2007).

## 2.1 The 20S Proteasome

The 20S proteasome is a barrel-shaped complex consisting of four seven-membered rings. These rings are composed of two distinct but related proteins termed  $\alpha$ - and  $\beta$ -subunits with molecular masses of approximately 25 kDa, which are arranged in  $\alpha$ -rings and  $\beta$ -rings, respectively. The two  $\beta$ -rings enclose the central proteolytic chamber of this barrel-shaped complex, and one  $\alpha$ - and one  $\beta$ -ring jointly form the outer (ante-) chambers. The three chambers of the 20S proteasome are interconnected by a narrow channel. The quaternary structure of 20S proteasomes is the same in all kingdoms, but their level of complexity varies: the simplest 20S proteasomes are found in prokaryotes, which contain only one or two types of  $\alpha$ - and  $\beta$ -subunits, respectively. In eukaryotes, the two subunits have developed into seven different subunits of each type, resulting in seven paralogous  $\alpha$ -subunits and seven paralogous  $\beta$ -subunits (reviewed in Voges et al. 1999; Zwickl et al. 2001).

20S proteasomes from eukaryotes, Archaea, and bacteria have been crystallized; the proteasomal  $\alpha$ - and  $\beta$ -subunits have the fold of Ntn-hydrolases: a pair of five-stranded  $\beta$ -sheets is flanked on both sides by  $\alpha$ -helices (Löwe et al. 1995; Groll et al. 1997; Unno et al. 2002; Groll et al. 2003; Kwon et al. 2004) (Fig. 2). The  $\beta$ -subunits are catalytically active threonine hydrolases in which the N-terminal threonine of the  $\beta$ -subunit of the *Thermoplasma* proteasome acts as both the catalytic nucleophile and the primary proton acceptor. The peptidolytic activity of the *Thermoplasma* proteasome is chymotrypsin-like (Löwe et al. 1995; Seemüller et al. 1995a, 1995b). In eukaryotes, four  $\beta$ -subunits lack the N-terminal threonine residue and, consequently, only three out of the seven  $\beta$ -subunits are proteolytically active. The eukaryotic proteasome possesses three different peptidolytic activities: tryptic, chymotryptic, and postacidic (Kisselev et al. 2006). In higher eukaryotes with an adaptive immune system,  $\gamma$ -interferon excites the expression of three additional active  $\beta$ -subunits. These  $\beta$ -subunits replace the related, constitutively expressed active subunits and the re-

sulting immunoproteasomes are characterized by a modulation of proteolytic specificity (Niedermann 2002).

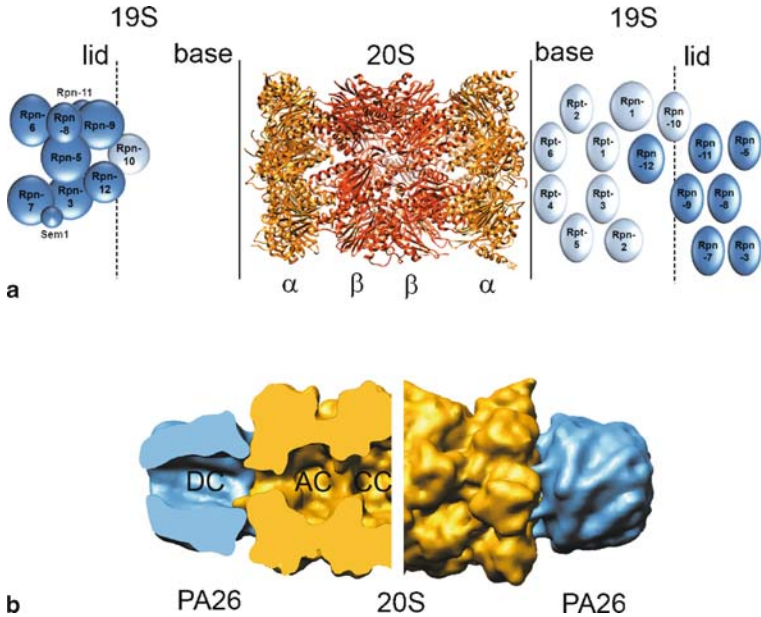
Prior to assembly, the proteolytically active  $\beta$ -subunits contain propeptides. Formation of the active sites requires post-translational removal of these propeptides, which occurs autocatalytically and only after the 20S complex is fully assembled. This delay ensures that the active sites are sequestered within the central chamber, which is only accessible via the antechambers. These again are accessible only through narrow orifices at both ends of the 20S complex. The exact function of the antechambers is currently unknown. Since it has been demonstrated that they can store substrate, their function might be to retain proteins in a partially folded state and—once the previous substrate has been degraded and enough space has become available—to translocate them into the catalytic chamber (Pickart and Cohen 2004; Sharon et al. 2006b).

## 2.2 The Regulatory Complexes

The ports leading into the 20S proteasome are constricted by an annulus built from turn-forming segments of the seven  $\alpha$ -subunits. In the basal state they are shut, but even in their open conformation they are too narrow for folded polypeptides to enter, a feature ensuring that randomly encountered native proteins are denied admission. Thus 20S proteasomes in isolation generally show negligible protease activity. Only by association with adaptor complexes are they transformed into efficient and, dependent of the nature of the particular adaptor complexes, ubiquitin-dependent proteases. The adaptor complexes interact with the terminal  $\alpha$ -rings of the proteasome by mechanisms that open the gate for substrate uptake (Pickart and VanDemark 2000).

### 2.2.1 The 19S Regulatory Complex

In vivo, most eukaryotic 20S proteasomes are flanked on one or both sides by the 19S regulatory complex, which associates with the 20S proteasome in an ATP-dependent manner, and the resulting 2.5-MDa complex is the canonical 26S proteasome (Babbitt et al. 2005). The 19S complex, which comprises approximately 20 different subunits with



**Fig. 2a,b.** The 20S proteasome and its adaptor complexes. **a** 20S and 19S; *left* structural organization of the *Saccharomyces cerevisiae* 19S lid, obtained by mass spectrometry and chemical cross-linking (modified from Sharon et al. 2006a); *center* crystal structure of mammalian 20S (PDB-entry 1IRU); *right* interaction map for the 19S lid of *C. elegans*, obtained by two-hybrid screening (Davy et al. 2001). Lid subunits are *dark blue*, base subunits are *light blue*. **b** Crystal structure of the PA26–20S–PA26 complex (PDB-entry 1fnt), low-pass filtered to 1 nm. *Left* cut open view, *right* surface view. DC dome-shaped cavity, AC antechamber, CC central chamber

a combined mass of 900 kDa, is probably the most important and at the same time the most complicated of the adaptor complexes. It bridges the sites of recognition and degradation, since it contains the recognition sites for the ubiquitinated target proteins as well as chaperone complexes that unfold the substrates and translocate the now unfolded polypeptide chain through the entry ports of the 20S proteasome. The 19S complexes from various organisms have been studied, and differ-

ent nomenclatures exist for the names of their subunits in humans, fruit flies, and yeast (Ferrell et al. 2000). Using biochemical methods, the regulatory particle can be divided into two subcomplexes, the base and the lid (Glickman et al. 1998), which are located proximally and distally in relation to the 20S core, respectively. Among the subunits of the base are the six paralogous AAA-ATPases Rpt1-Rpt6. Like other members of the AAA-family, they exhibit chaperone activity (Braun et al. 1999; Liu et al. 2005) and are thought to form a ring and to be involved in substrate unfolding and translocation. Since only the 26S holoenzyme but not the 20S-base complex is capable of degrading ubiquitinated proteins, recognition and binding, as well as deubiquitylation of ubiquitin-tagged substrates appears to be mediated by the subunits of the lid complex (Glickman et al. 1998). Until now, no high-resolution structure of the 19S regulatory complex has been available and only very few atomic structures of individual subunits exist (Wang et al. 2005; Nakamura et al. 2007; Sanches et al. 2007; Schreiner et al. 2008). Still, by yeast two-hybrid studies (Davy et al. 2001), mass spectrometry (Sharon et al. 2006a) and GST-pulldowns (Chen et al. 2008), a wealth of information on interaction of proteasome subunits has been obtained and topology maps have been constructed (Fig. 2a).

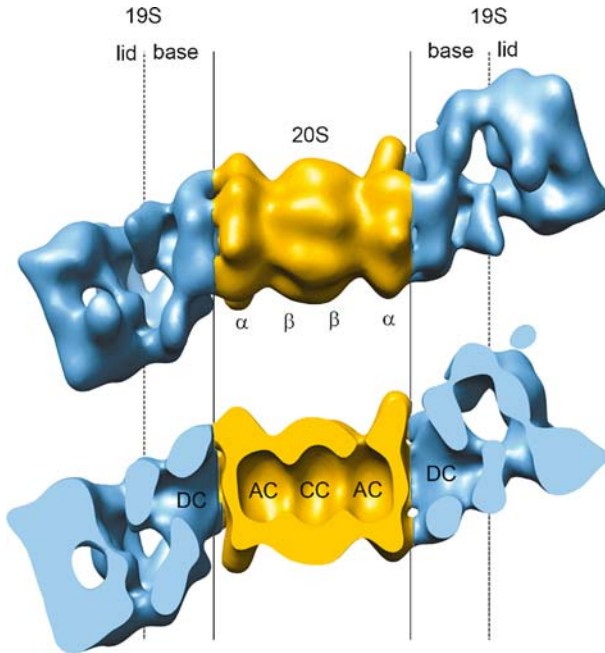
### 2.2.2 Alternative Cap Complexes

Aside from the 19S regulatory particle, alternate structures can also cap the 20S proteasome; all of them are ATP-independent and less complex in composition. Organisms with an adaptive immune system contain the PA28 activator, a 200-kDa cap protein that is induced by interferon and consists of two related subunits of a mass of approximately 28 kDa (Rechsteiner et al. 2000). These  $\alpha$ - and  $\beta$ -subunits assemble into a heteroheptamer and form a dome-shaped structure built of a bundle of alpha-helices. Like the 19S complex, this structure can bind to both ends of the proteasome and also hybrid PA28–20S–19S complexes exist (Cascio et al. 2002). PA26, a PA28-related protein in *Trypanosoma brucei* that also stimulates peptidolytic activity of the 20S proteasome, has been crystallized in complex with 20S, and the interactions that lead to gate opening in the 20S proteasome have been visualized: the N-terminal tails of the alpha subunits are straightened and thus are

moved away from the pore, thereby opening the entrance port (Whitby et al. 2000) (Fig. 2b). PA200/Blm10 is another non-ATPase proteasome cap. It consists of a single polypeptide of 250 kDa, which likewise induces gate opening in the 20S proteasome, as was shown by electron cryomicroscopy (Ortega et al. 2005; Iwanczyk et al. 2006).

### 2.3 The 26S Proteasome from *Drosophila Melanogaster*

In general, structural studies of 26S proteasomes are hampered by their low intrinsic stability, leading to dissociation into various subcomplexes. The 26S complexes from various sources have been isolated and examined by electron microscopy and it turned out that embryos of *Drosophila melanogaster* provide relatively stable 26S particles, comprising a well-defined complement of subunits (Yoshimura et al. 1993; Walz et al. 1998; Hölzl et al. 2000). Still, electron micrographs of *Drosophila*-26S display a degree of structural heterogeneity that complicates image analysis and three-dimensional reconstruction. Two-dimensional averages of negatively stained 26S proteasomes feature the characteristic dragon head, where the 19S complexes in the double-capped particles face in opposite directions, apparently reflecting the C2-symmetry of the eukaryotic 20S proteasome. While negatively stained 26S complexes adsorbed on carbon film are still acceptably intact, cryo-preparation can trigger their disassembly, and in the self-supporting layer of vitrified ice the number of dissociated particles is often relatively high. Despite all these adversities, a structure of the 26S proteasome from *Drosophila melanogaster* has been obtained by cryoelectron microscopy. Double-capped 26S proteasomes were “purified” in silico. Their 3D reconstruction depicts the linear assembly 19S-20S-19S, the regulatory complexes facing opposite directions (Nickell et al. 2007a) (Fig. 3). Most likely, the part of the 19S complex attached to the  $\alpha$ -rings of the 20S proteasome represents the base complex and contains the six paralogous AAA-ATPases. The base complex and the proteasomal  $\alpha$ -rings enclose a dome-shaped compartment next to the antechamber, as is also seen in the ClpAP complex (Ishikawa et al. 2004), and according to current understanding, attachment of the base complex should open the gate to the interior of the 20S proteasome (see Sect. 2.2). How this is carried out mechanically is still an open question. A recent study with PAN



**Fig. 3.** Structure of the 26S proteasome from *Drosophila melanogaster* 26S obtained by single particle electron cryomicroscopy (Nickell et al. 2007a). *Top* surface representation; *bottom* cut-open view. Note the potential substrate entry/exit sites at the 19S–20S interface. *DC* dome-shaped cavity, *AC* antechamber, *CC* central chamber

(proteasome-activating nuclease), an archaeobacterial adaptor complex with homology to the AAA-ATPases of the 19S base, suggests that the C-terminal domains of the ATPases insert into binding-pockets at the 20S  $\alpha$ -rings and thereby trigger gate-opening (Smith et al. 2007).

An interesting feature visible in the present map but also in an earlier reconstruction obtained by electron cryotomography is a sideward channel at the 20S-base interface. This channel connects the cavity underneath the base with the outside and possibly provides an entry or exit site for substrates (Nickell et al. 2007a,b). While features visible in such medium-resolution structures of the 26S proteasome can hint

at the function of certain subcomplexes or building blocks, a detailed understanding of its mechanism will require the atomic structure of the holocomplex. Given the complexity and fragility of 26S proteasomes, it is unlikely that they can be crystallized *in toto*. Furthermore, their dynamics represent an additional problem: besides the set of canonical subunits, there are several variable subunits that modulate proteasome function (Glickman and Raveh 2005). Thus, obtaining a detailed structural model of the 26S proteasome will require a multidisciplinary approach integrating electron microscopic reconstructions of the holocomplex, atomic structures, and interaction maps of its building blocks with all available information from other sources (Robinson et al. 2007).

### 3 Tripeptidyl Peptidase II

In eukaryotes, the proteasome is essential. Its inhibition leads to cell death, although some cells can adapt to proteasome inhibitors. In those cells, the induction of an alternative protease with the capability of substituting for some metabolic functions of the proteasome has been reported (Glas et al. 1998; Geier et al. 1999; Wang et al. 2000; Princiotta et al. 2001). This protease has been identified as Tripeptidyl peptidase II (TPPII) and its basic cellular activity is the removal of tripeptides from the free N-terminus of oligopeptides such as produced by the 26S proteasome (Balow et al. 1983; Tomkinson 1999). In addition to this exopeptidase activity, a much lower endopeptidase activity of the trypsin type was also detected (Geier et al. 1999). TPPII has broad substrate specificity. It has a preference for cleaving after hydrophobic residues but cannot cleave before or after proline residues. As an exopeptidase, it preferentially cleaves after lysine residues but as an endopeptidase, can in fact cleave after proline residues (Geier et al. 1999; Seifert et al. 2003). The endopeptidase activity can create a specific epitope (Nef73-82) of the human immunodeficiency virus (HIV) independently of the proteasome (Seifert et al. 2003).

Like the proteasome, TPPII is involved in the generation of antigenic peptides for presentation by the MHC class I complex (Levy et al. 2002). Here, it appears to trim the N-terminus of some peptides generated by the proteasome and apparently it is the only downstream pro-

tease that can degrade peptides of more than 15 amino acids in length (Reits et al. 2004; York et al. 2006). However, there is some controversy regarding the degree to which TPPII is involved in this process (see van Endert 2008 for a recent review) and its role in MHC class I processing might be that of another cytosolic peptidase mainly destroying epitopes (Firat et al. 2007; Marcilla et al. 2007).

Generally, TPPII is upregulated in diseases that are based on increased or uncontrolled proteolysis such as in septic muscles (Hasselgren et al. 2002; Chand et al. 2005; Stavropoulou et al. 2005) or in malignant cells (Stavropoulou et al. 2006), and inhibition of TPP leads to radiation sensitivity in cancer cells (Hong et al. 2007). Being implicated in tumor cell survival and proliferation, TPPII is discussed as a target for tumor therapy. However, TPPII is also necessary for normal cell survival, since although TPPII knock-out mice are viable, lack of TPPII results in the activation of cell death programs (Huai et al. 2008).

A more specialized task is carried out by a membrane-bound TPPII variant, which inactivates the cerebral neurotransmitter cholecystokinin-(26–33) octapeptide (CCK-8), an endogenous satiety agent (Rose et al. 1996). Its involvement in obesity makes TPPII an interesting target for drug design. A specific inhibitor, butabindide, has been designed and shown to influence the feeding behavior of mice (Rose et al. 1996). Based on the similarity of TPPII to subtilisin, a homology model of its active site has been published and is used for the design of additional inhibitors (De Winter et al. 2005).

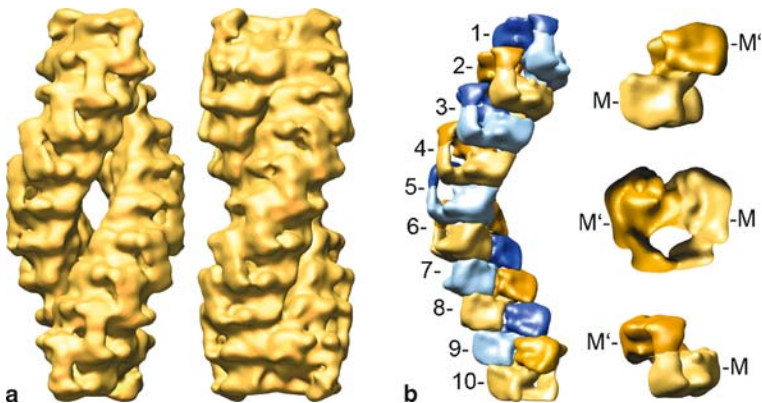
### 3.1 TPPII Structure

TPPII is found in most eukaryotic organisms. The molecular weight of the TPPII monomer ranges from 138 kDa for the mammalian variant to 150 kDa for the plant, worm, and insect homologs. The N-terminal half of the sequence contains the subtilisin core; the catalytic triad in human TPPII has been mapped to Asp-44, His-264, Ser-449, by site-directed mutagenesis (Hilbi et al. 2002). An insert of approximately 200 amino acids interrupts the first two residues of the catalytic triad and has been suggested to be necessary for complex formation (Tomkinson et al. 2002). In contrast to the 26S proteasome, which is a multisubunit complex, TPPII is a large homo-oligomer of 5–6 MDa. TPPII par-

ticles isolated from human red blood cells as well as from *Drosophila melanogaster* embryos have been visualized in the electron microscope, and the only 3D structure available to date is the density map of *Drosophila* TPPII (Macpherson et al. 1987; Geier et al. 1999; Rockel et al. 2002). TPPII complexes are spindle-shaped  $28 \times 60$ -nm particles consisting of two segmented and twisted strands. Each of the two strands is composed of a linear assembly of ten interdigitated segments. These segments are dimers, wherein the globular domains of the comma-shaped monomers are connected by a “handle” formed by the tails (Fig. 4). Whereas intact TPPII complexes isolated from mammals and *Drosophila* are of defined length (Geier et al. 1999; Rockel et al. 2002), TPPII particles heterologously expressed in *Escherichia coli* often possess extensions beyond their spindle poles or occur as single strands of variable lengths. This is presumably a consequence of the comparatively high TPPII concentration in cells overexpressing the protein. Treatment of such extended spindles and single strands with destabilizing agents leads to trimming of extensions and causes disassembly of single strands, and it demonstrates that the spindles observed in native preparations are the thermodynamically favored conformation. This stabilization of the spindles probably results from a double-clamp structure at their poles, where the terminal dimer of one strand locks the two terminal dimers of its neighboring strand (Rockel et al. 2005).

### 3.2 Size–Activity Relationship

TPPII exhibits its highest activity only when assembled into strands; its dissociation (e.g., upon dialysis) results in loss of activity. When human TPPII dissociates into dimers, the specific activity decreases to approximately one-tenth and this activity loss can be reversed by reassociation (Tomkinson 2000). The relationship between size and activity has been studied in more detail with *Drosophila* TPPII (Seyit et al. 2006). Here, assembly studies in conjunction with cross-linking revealed (1) that strand-elongation proceeds by addition of dimers, (2) that the specific activity of TPPII increases with strand length, and (3) that the length distribution of the TPPII strands at equilibrium is dependent on the protein concentration and that high protein concentrations lead to polymorphism. Under conditions favoring dissociation, tetramers are the most

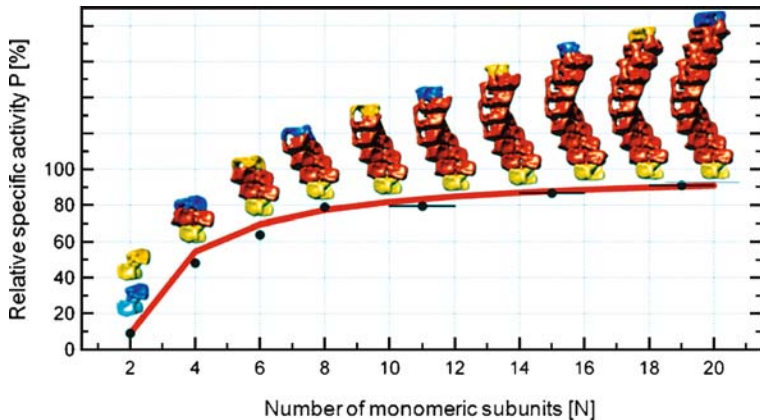


**Fig. 4a,b.** Architecture of TPPII from *Drosophila melanogaster*: **a** Two perpendicular views of the TPPII complex; **b** computationally extracted TPPII dimers; *left* arrangement of the ten dimers of a strand (constituting monomers) are color-coded; *right* TPPII dimer in different orientation (monomers are denoted *M* and *M'*)

stable disassembly product of *Drosophila* TPPII. Whereas dimers of *Drosophila* TPPII have a specific activity of approximately 8% of that of spindles, the activity of a tetramer is as high as 50%. The activation is thought to be triggered by the formation of new monomer–monomer interfaces upon addition of a dimer, which would induce a conformational change at or near the active site. According to the activation model proposed, the increase in specific activity upon strand elongation at equilibrium is described by the equation  $P = ((N - 2) * 100 + 16)/N$ , with  $P$  the specific activity and  $N$  the number of monomers assembled in a strand (Fig. 5) (Seyit et al. 2006).

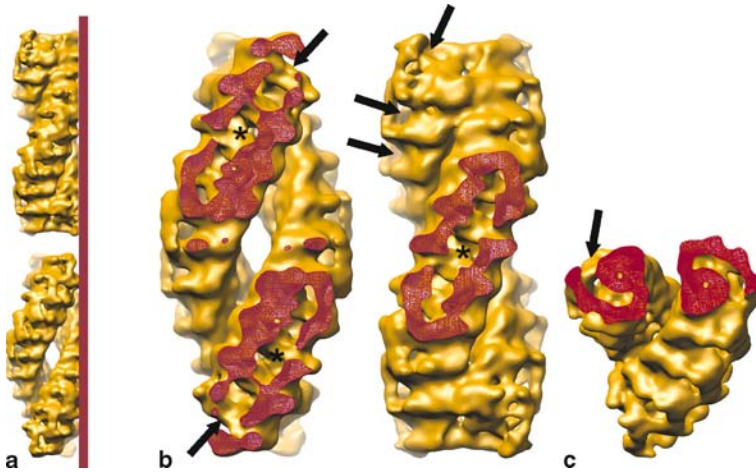
### 3.3 Structure–Function Relationship

While the linear arrangement of the subcomplexes in the 26S proteasome mirrors the sequence of events during its functional cycle (binding, unfolding, translocation, degradation) the functional reason for linearly stacking the subunits of TPPII into strands remains obscure. The 20S proteasome is a self-compartmentalizing protease. Whether this



**Fig. 5.** Size-activity relationship of TPPII. Dependence of the specific activity on the number of subunits assembled. *Black lines* experimental data; *red line*  $P = ((N - 2) * 100 + 16)/N$ ,  $P$  specific activity,  $N$  number of monomers assembled in a strand. Surface representations show TPPII strands of different lengths, activated dimers are colored in *red*. (Adapted from Seyit et al. 2006)

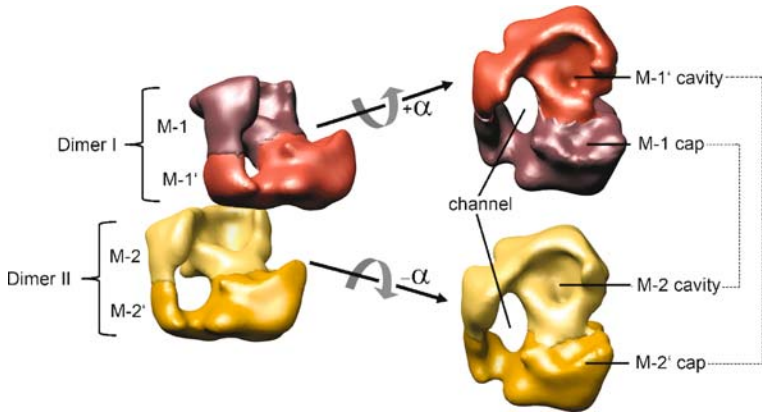
also applies to TPPII remains to be demonstrated and can only be definitely proven when a crystal structure becomes available or when the locations of the active sites within the complex have been determined otherwise. Indeed, the stacking of dimers leads to the formation of a cavity system traversing the strands (Fig. 6). This system includes chambers that result from the stacking of the dimers, where each dimer provides a cavity as well as a cap that seals off the cavity of the subjacent dimer (Fig. 7). Provided the active sites are indeed located within this cavity system, it is not obvious how the relatively small substrates of TPPII should be channeled through the strands. A longitudinal substrate flow appears inefficient with respect to access to the active sites and release of products: while active sites close to the ends of the strands would be easily accessible, at the center of the strands they might be undersaturated because of the diffusion limit. In contrast, a lateral substrate flow through the arcade would lead to equal saturation of all active sites and at the same time protect the substrates from their complete hydrolysis



**Fig. 6a-c.** Channel system of TPPII. **a** Section planes; **b** two perpendicular cut-open views of TPPII; **c** cross-section through TPPII. *Arrows* mark the entry ports to the channel system (labeled with an *asterisk*)

in the cytosol. Such a feature might be important for the role ascribed to human TPPII in peptide trimming (Burri et al. 2002).

Recently, a role in fat metabolism was ascribed to TPPII, and its involvement in adipogenesis appears to be independent of its peptidolytic activity. Both peptidolytically inactive mutants HsTPPIIAsp44Ala and Hs $\Delta$ NTTPPII, where the N-terminal aspartic-acid-containing protease domain was deleted, stimulated adipogenesis in mammalian cell culture to the same extent as wild-type TPPII. As opposed to these mutants, mutants lacking either the N-terminal or the C-terminal domain were not functional (McKay et al. 2007). Since mutations in the TPPII sequence often lead to altered assembly behavior (Tomkinson et al. 2002; Rockel et al. 2005; Seyit et al. 2006), it is tempting to speculate that the loss in function of the latter two TPPII mutations was caused by the loss of their native quaternary structure. If for certain cellular functions of TPPII only its intact structure but not its peptidolytic activity were required, this would allude to a functional relevance of the



**Fig. 7.** Stacked dimers enclose a double chamber. *Left* two dimers (I and II) with color-coded monomers (*M-1*, *M-1'* and *M-2*, *M-2'*). *Right* composition of the double chamber: dimer I was rotated counterclockwise in order to visualize cavity *M-1'* and cap *M-1*, which in their original position are oriented toward the back of the image plane. In the original orientation of the two dimers, cavity *M-1'* is sealed off by the cap *M-2'* and cavity *M-2* by cap *M-1*. Figures were created with the Chimera software package (Pettersen et al. 2004)

spindle architecture of TPPII and imply that the spindle might serve as interaction scaffold.

## 4 Conclusions

The 26S proteasome and TPPII are two major players in eukaryotic cytosolic proteolysis and exert their proteolytic activity mostly consecutively: the 19S caps of the 26S proteasome bind and unfold proteins destined for degradation and feed them into the proteolytic chamber. The products of the 20S proteasome are short peptides of a length suitable for degradation into tripeptides by TPPII. Macromolecular complexes with functions corresponding to 26S and TPPII also occur in other kingdoms of life. In bacteria, the proteases ClpP or ClpQ associate linearly with the AAA-ATPases ClpX, ClpY, or ClpA; adaptor complexes for 20S proteasomes in Archaea are PAN or VAT, which presumably are

associated with the 20S core only transiently. Tricorn is a downstream peptidase with a molecular mass comparable to that of TPPII. Its 121-kDa subunits assemble into a hexamer, which *in vivo* forms an icosahedral capsid of 14.5 MDa. This capsid is thought to be necessary for the docking of the tricorn interacting factors F1, F2, and F3 (Tamura et al. 1998). Most of these large protease complexes are labile, which complicates their isolation in the large amounts and high purity and homogeneity necessary for crystallization. Nevertheless, in some cases it was possible to construct (partial) hybrid models by docking high-resolution crystal structures into the densities obtained by electron microscopy.

Typically, molecular complexes are purified either from their native source or an expression system and subsequently studied *in vitro*. Their functional interplay with other molecules is investigated by interaction studies and the regions of structural interactions are narrowed down by chemical cross-linking. The size of such gargantuan molecules such as 26S proteasomes or TPPII permits a type of functional investigation that is not applicable to smaller molecules: with sizes larger than 30 nm, giant proteases should be easily visible in cellular tomograms and could thus be depicted directly within their functional cellular environment. Admittedly, in the average eukaryotic cell such protease complexes are much less abundant than, for example, ribosomal complexes, but their localization and visualization can be facilitated by the current developments of structure recognition in tomograms of cells and cell sections in combination with correlative microscopy.

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## ***Molecular Genetics of the Ubiquitin-Proteasome System: Lessons from Yeast***

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M. Hochstrasser<sup>(✉)</sup>, M. Deng, A.R. Kusmierczyk, X. Li, S.G. Kreft,  
T. Ravid, M. Funakoshi, M. Kunjappu, Y. Xie

Department of Molecular Biophysics and Biochemistry, Yale University,  
266 Whitney Avenue, P.O. Box 208114, 06520-8114 New Haven, USA  
email: [mark.hochstrasser@yale.edu](mailto:mark.hochstrasser@yale.edu)

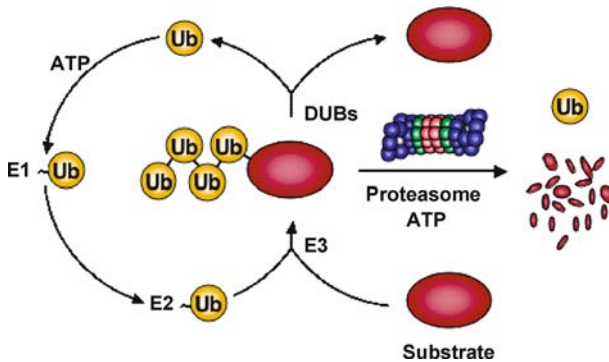
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**Abstract.** Our studies with the yeast *Saccharomyces cerevisiae* have uncovered a number of general principles governing substrate selectivity and proteolysis by the ubiquitin-proteasome system. The initial work focused on the degradation of a transcription factor, the MAT $\alpha$ 2 repressor, but the pathways uncovered have a much broader range of targets. At least two distinct ubiquitination mechanisms contribute to  $\alpha$ 2 turnover. One of them depends on a large integral membrane ubiquitin ligase (E3) and a pair of ubiquitin-conjugating enzymes (E2s). The transmembrane E3 and E2 proteins must travel from their site of synthesis

in the ER to the inner nuclear membrane in order to reach nuclear substrates such as  $\alpha 2$ . The 26S proteasome is responsible for  $\alpha 2$  degradation, and several important features of proteasome assembly and active site formation were uncovered. Most recently, we have delineated major steps in 20S proteasome assembly and have also identified several novel 20S proteasome assembly factors. Surprisingly, alterations in 20S proteasome assembly lead to defects in the assembly of the proteasome regulatory particle (RP). The RP associates with the 20S proteasome to form the 26S proteasome. Our data suggest that the 20S proteasome can function as an assembly factor for the RP, which would make it the first such factor for RP assembly identified to date.

## 1 Introduction

Intracellular proteolysis contributes to many cellular regulatory mechanisms, including cell cycle control, DNA repair, various stress responses, cell differentiation, circadian rhythms, and signal transduction (Gottesman and Maurizi 1992; Hochstrasser 1996). For the majority of short-lived eukaryotic regulatory proteins, conjugation to the polypeptide ubiquitin is a prerequisite for their degradation (Fig. 1) (Pickart 2001; Weissman 2001). In most cases, ubiquitin is joined reversibly to other proteins via an isopeptide linkage between the C-terminus of ubiquitin and  $\epsilon$ -amino groups of lysine residues in the acceptor proteins. In order to be attached to proteins, the C-terminus of ubiquitin must initially be activated in an ATP-dependent reaction catalyzed by the enzyme E1, to which it subsequently becomes linked by a high-energy thioester bond. Ubiquitin then forms a thioester with a second protein, an E2 enzyme. The E2, with the aid of an additional factor, called an E3, or ubiquitin-protein ligase, catalyzes isopeptide bond formation between ubiquitin and the substrate. There are multiple E2 enzymes and often hundreds of different E3 proteins encoded in each eukaryotic genome, and these many variants and their combinations underlie the remarkable range and specificity of protein ubiquitination (Hochstrasser 1996; Smalle and Vierstra 2004). For proteolytic substrates, assembly of a polyubiquitin chain(s) on the protein is usually necessary for rapid degradation by the 26S proteasome, an approximately 2,500-kDa pro-



**Fig. 1.** The ubiquitin–proteasome system. Most if not all ubiquitin–protein ligation events *in vivo* require an E3 factor. Some of these E3s (the HECT-domain class) act as direct donors in ubiquitin transfer to substrate, forming an E3-ubiquitin thioester intermediate (not depicted in figure). Other E3s, such as those with RING domains, act as adaptors between substrate and the E2-ubiquitin thioester conjugate and catalyze transfer of ubiquitin to the substrate. Both ubiquitin conjugation and degradation of ubiquitin–protein conjugates by the 26S proteasome require ATP hydrolysis. *DUBs* deubiquitinating enzymes

tease complex that consists of a catalytic core called the 20S proteasome and a multisubunit regulatory particle, the RP, which confers ATP and ubiquitin dependence on substrate proteolysis by the 20S proteasome.

We began our studies of intracellular proteolysis using the very rapidly degraded MAT $\alpha$ 2 repressor of *Saccharomyces cerevisiae* as a model for a naturally short-lived substrate (Hochstrasser and Varshavsky 1990; Chen et al. 1993; Johnson et al. 1998). *S. cerevisiae* has three cell types: two haploid forms, **a** and  $\alpha$ , and an **a**/ $\alpha$  diploid, produced by mating of haploid cells of opposite cell type (Herskowicz et al. 1992). Cell identity is determined by the information encoded at the mating type, or *MAT*, locus. In homothallic strains, mating type will switch when **a** or  $\alpha$  sequences from one of two unexpressed loci are copied into the *MAT* locus. The change in cellular phenotype is apparent within a single cell cycle, suggesting that the transcriptional regulators encoded by the *MAT* loci may be short-lived. In fact, we found that the

$\alpha 2$  homeodomain protein, which is encoded by the *MAT $\alpha$*  locus, has an in vivo half-life of roughly 4 min in  $\alpha$  cells and that this rapid turnover is essential for efficient switching of the differentiated phenotype (Laney and Hochstrasser 2003, 2004). In other words, this epigenetic switch in cell type is mediated by proteolysis of the master regulators controlling the differentiated state.

The ubiquitin-proteasome system is responsible for  $\alpha 2$  degradation by mechanisms involving at least two degradation signals and at least four E2/Ubc enzymes. From genetic studies, it was established that the E2s Ubc4 and Ubc5 define one proteolytic pathway, while Ubc6 and Ubc7 define a second pathway (Chen et al. 1993). The Ubc6 and Ubc7 E2s function with the Doa10 E3, an integral membrane protein, which localizes to the ER membrane with its cognate E2s (Swanson et al. 2001). Ubc6 has a C-terminal membrane anchor, while Ubc7, a soluble protein, is anchored to the membrane by a transmembrane receptor in the ER called Cue1 (Sommer and Jentsch 1993; Biederer et al. 1997). Doa10/Ubc6/Ubc7/Cue1 appear to form a higher order ubiquitination complex (Neuber et al. 2005; Ravid et al. 2006; Carvalho et al. 2006), and this complex specifically targets the *Deg1* degradation signal (degron) of  $\alpha 2$ , which resides within the first ~60 residues of the repressor (Johnson et al. 1998).

In this contribution, we review recent work from our laboratory on the yeast ubiquitin-proteasome system. Although we initially concentrated much of our analysis on a single naturally short-lived regulatory protein, we have gathered many general insights into how proteins are targeted for ubiquitin modification, how such ubiquitinated proteins are destroyed by the 26S proteasome complex, and how the proteasome itself is assembled. We will provide several illustrative examples of what can be learned about this intricate metabolic regulatory system using a combination of yeast molecular genetics and biochemistry.

## 2 Identification of the Doa10 Ubiquitin Ligase

Although the  $\alpha 2$  repressor was the first naturally short-lived protein shown to be degraded by a ubiquitin-dependent mechanism in vivo, we had failed in our earlier studies to identify any E3 ubiquitin lig-

ase that worked in the known  $\alpha 2$  ubiquitination pathways. As noted in Sect. 1, one of the ubiquitination pathways that targets the  $\alpha 2$  repressor for degradation uses the ER-localized Ubc6 and Ubc7 E2s (Chen et al. 1993; Johnson et al. 1998). However, the presumptive E3(s) that functions with Ubc6 and Ubc7 was not identified in any of our early genetic screens (Hochstrasser and Varshavsky 1990; Chen et al. 1993; Chen and Hochstrasser 1995; Papa and Hochstrasser 1993; Swanson and Hochstrasser 2000). Biochemical approaches were also unsuccessful. Although additional *degradation of alpha2 (doa)* mutants had been isolated for which the corresponding *DOA* genes had not been cloned, these mutants generally did not have the properties expected of the E3 in this pathway. Moreover, the genetic screens had not reached saturation, so additional factors had almost certainly been missed.

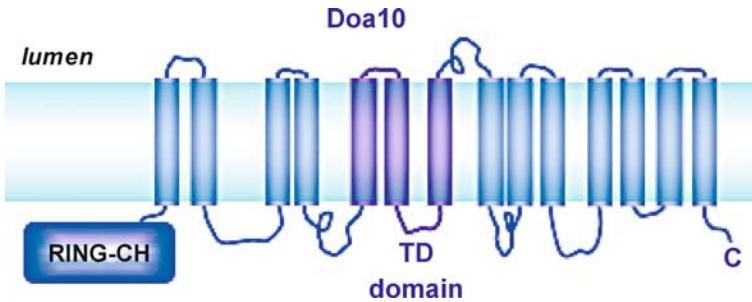
Neither Ubc6 nor Ubc7 is required for normal growth rates, so we reasoned that this was likely to be true for other  $\alpha 2$  ubiquitination factors that acted specifically in this pathway. We therefore designed a genetic selection that demanded rapid growth of mutants in order to bias the search away from essential genes in the ubiquitin-proteasome system, particularly proteasome subunit genes. When *Deg1* is fused to OMP decarboxylase, the protein encoded by *URA3*, the resulting *Deg1-Ura3* fusion is degraded so rapidly that cells with this fusion as the only source of Ura3 activity grow extremely poorly on uracil drop-out media (SD-ura) (Chen et al. 1993). Mutations in *UBC6* or *UBC7* strongly enhance growth of *Deg1-Ura3*-expressing cells on SD-ura (Chen et al. 1993; Swanson et al. 2001).

From a selection of mutagenized cells, 960 colonies that became visible within 1–2 days at 30°C were selected (Swanson et al. 2001). Of the 960 mutants, 602 failed to complement a *ubc6* $\Delta$  *ubc7* $\Delta$  strain, indicating that they had mutations in *UBC6*, *UBC7*, or both. Inter alia crosses among the remaining mutants (and subsequent segregation tests) indicated that 356 of them defined a novel complementation group, which we call *doa10*. Unfortunately, our attempts to clone *DOA10* took nearly 1 year and despite saturated screenings of six different genomic libraries, were ultimately unsuccessful. We therefore used classical genetic mapping to localize the *doa10* mutation. The mutation was fine mapped to a small region on chromosome IX and eventually shown to be in a gene of unknown function called *SSM4* (Mandart et al. 1994).

Extensive molecular genetic analysis of a *doa10/ssm4* deletion mutant has been done to characterize the *in vivo* substrate specificity of the Doa10 enzyme (Swanson et al. 2001; Ravid et al. 2006). The mutant is unimpaired for the degradation of a number of other ubiquitin system substrates including, interestingly, the ER quality control substrate CPY\* (Hiller et al. 1996), whose degradation also depends on Ubc6 and Ubc7. Unlike *ubc7* mutants, *doa10* cells are not hypersensitive to cadmium, nor have we found any other strong phenotypic defects associated with loss of Doa10. Doa10 therefore has an even more restricted specificity than the E2 enzymes implicated in  $\alpha 2$  degradation (Swanson et al. 2001). Single *doa10* $\Delta$  mutants, like *ubc6* $\Delta$ , *ubc7* $\Delta$ , or *ubc4* $\Delta$  mutants, showed only a two- to threefold decrease in  $\alpha 2$  degradation rate. No further stabilization of  $\alpha 2$  occurred in *ubc6* $\Delta$  *doa10* $\Delta$  double mutants. In striking contrast, *ubc4* $\Delta$  *doa10* $\Delta$  double mutants were severely impaired for  $\alpha 2$  turnover, with a half-life approaching 1 h. This is comparable to what had been seen with *ubc4* $\Delta$  *ubc6* $\Delta$  mutants (Chen et al. 1993). Thus, Doa10 has the expected specificity of an E3 Ub ligase for Ubc6/Ubc7-dependent substrates and fits, by epistasis analysis, into the *Deg1*-mediated  $\alpha 2$  degradation pathway.

### 3 Sequence Features of Doa10

Doa10 has an unusual RING domain, which we called RING-CH to highlight the defining Cys and His zinc-coordinating residues at positions 4 and 5, respectively, in the putative RING (Fig. 2) (Swanson et al. 2001). Proteins bearing RING-CH domains are found in most, if not all, eukaryotic organisms as well as a number of viruses, including two small proteins, K3 and K5, in Kaposi's sarcoma-associated herpes virus (Swanson et al. 2001). Originally misclassified as a PHD domain (Boname and Stevenson 2001; Coscoy et al. 2001), the RING-CH in the K3 viral protein was shown by NMR structure determination to fold into a RING structure rather than a PHD fold (Dodd et al. 2004). The well-conserved RING-CH domain defines a subfamily of RING-type E3s that encompasses a number of viral proteins and at least 11 different human proteins (Swanson et al. 2001; Kreft et al. 2006). Nine of the human proteins were noted previously and called membrane-anchored



**Fig. 2.** Model of the topology of Doa10, an integral ER/NE membrane protein. Doa10 has 14 transmembrane segments (TMs) and exposes approximately 63% of its mass on the cytosolic side of the membrane (assuming there are no re-entrant loops)

RING-CH or MARCH proteins (Bartee et al. 2004). We subsequently noted two additional human RING-CH proteins, one that is also predicted to be a transmembrane protein (Genbank XP\_496738) and one that is not (Genbank NP\_689811) (Kreft et al. 2006). Doa10 orthologs are found in almost all fully sequenced eukaryotic genomes. These orthologs include the human protein TEB4 (also called MARCH6).

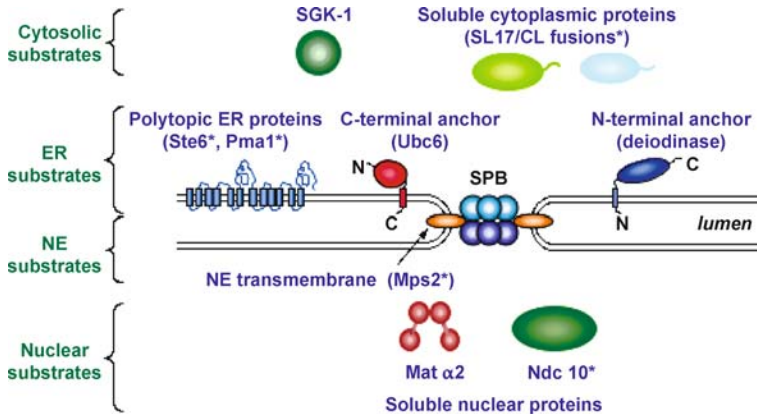
Doa10 was predicted to be a multi-spanning (polytopic) membrane protein based on transmembrane helix (TM) prediction algorithms, and cell fractionation studies confirmed that it behaves as an integral membrane protein (Swanson et al. 2001). We constructed fully functional chromosomal derivatives of *DOA10* that are tagged with sequences encoding either *Aequorea victoria* green fluorescent protein (GFP) or a myc9-epitope tag. By fluorescence microscopy, both Doa10 derivatives localized to the nuclear envelope (NE) and peripheral ER elements. Thus, Doa10 concentrates in the same subcellular structures as Ubc6 and Ubc7. We determined its detailed topology by fusing a dual-topology reporter after various Doa10 segments, from which we were able to infer that Doa10 contains 14 transmembrane helices (TMs) (Fig. 2) (Kreft et al. 2006). Consistent with this analysis, protease digestion of yeast microsomes demonstrated that both the N-terminal RING-CH domain and the C-terminus face the cytosol. Interestingly, the experimentally derived topology was not predicted correctly by any of the

commonly used TM prediction algorithms. We used bioinformatic analysis and *in silico* mutagenesis to guide the topological studies through problematic regions.

Orthologs of Doa10 are defined not only by their N-terminal RING-CH domains and the presence of at least ten TMs but by a conserved approximately 130-residue element dubbed the TD (TEB4-Doa10) domain (Swanson et al. 2001). The conserved TD domain in Doa10 includes three atypical but highly conserved TMs. Among the conserved residues within the TMs of the TD domain are several glycine, proline, and charged residues, which are relatively uncommon in TMs. Such conservation of sequence implies a shared core function of the TD domain within the ER/NE membrane. These TMs might function in co-factor binding or substrate recognition, or they might be part of a retrotranslocation channel (Swanson et al. 2001; Kreft et al. 2006). Doa10 targets not only soluble substrates such as MAT $\alpha$ 2 but also certain integral membrane proteins (see Sect. 4), which are likely to require an exit channel in order to be extracted from the membrane.

#### **4 Diversity of Substrates Targeted by the Doa10 Pathway**

Known substrates of the yeast Doa10 pathway now number over a dozen (Fig. 3) (Ravid et al. 2006). The substrates include membrane proteins, soluble proteins of the cytoplasm and nucleus, naturally short-lived regulators, and aberrant proteins subject to quality control. This represents an unprecedented cross-compartmental diversity of substrates. Given this range of substrates, an obvious question is whether the degrons in these proteins share any common features. For *Deg1* degron recognition, our data suggested that the exposed hydrophobic face of an amphipathic helix was the key recognition determinant (Johnson et al. 1998). Conceivably, Doa10 can directly bind such hydrophobic surfaces in various substrates. In this model, Doa10 recognition of degrons in naturally short-lived regulators and quality control substrates is based on similar principles. The importance of a helical structure in the central degron determinant of *Deg1* was inferred from two observations. First, *Deg1* is largely helical based on CD measurements, and second, the only nontol-



**Fig. 3.** Substrates targeted by the Doa10 pathway in *Saccharomyces cerevisiae*. A surprising variety of proteins can be ubiquitinated by Doa10 in vivo. They reside in multiple cellular compartments and can be either naturally short-lived or aberrant proteins (quality control substrates; indicated by *asterisks*) that are rapidly degraded because of misfolding or failure to assemble properly. *SPB* spindle pole body

erated residues found on the hydrophilic face in the *Deg1* determinant are proline and glycine, both helix-breaking residues (Johnson et al. 1998). Amphipathic helices might be involved in many cases; however, a set of artificial degrons (the SL17/CL series) all share regions of strong hydrophobicity, but only a few are obviously amphipathic (Gilon et al. 2000). We have hypothesized that an exposed hydrophobic helix surface will be the key feature recognized by the Doa10 pathway (Johnson et al. 1998; Ravid et al. 2006).

## 5 Doa10 Traverses the Nuclear Pore Complex Membrane to Access Nuclear Substrates

A notable feature of some of the substrates depicted in Fig. 3 is that they concentrate in the nucleus. How such substrates gain access to the transmembrane Doa10 ligase had been unclear. Either the nuclear substrates are exported out of the nucleus to get to the ER-localized Doa10,

or the E3 enzyme needs to be transported to the inner nuclear membrane (INM) to reach its nuclear substrates. Our recent studies provided compelling evidence for the latter mechanism (Deng and Hochstrasser 2006).

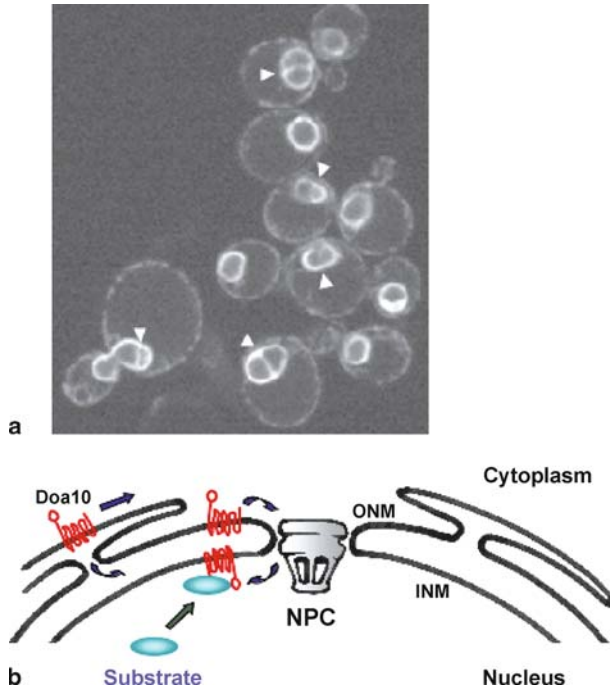
To determine whether the large, polytopic Doa10 protein could reach the INM, we first attempted immunogold labeling and electron microscopy, but this proved unsatisfactory. Therefore, we developed several new assays as alternatives to ultrastructural localization. The first was a targeted silencing assay, which had previously been designed to study the role of nuclear localization in gene silencing (Andrulis et al. 1998). We adapted it to determine if Doa10 expressed at roughly endogenous levels can localize to the INM (Deng and Hochstrasser 2006). This assay uses cells bearing a defective gene silencer element upstream of a convenient reporter gene; silencing can be restored by anchoring the locus to the nuclear periphery where silencing factors are concentrated. By fusing the DNA-binding domain of the Gal4 transcription factor to Doa10, we could measure potential INM tethering of a crippled silencer bearing a triplicated Gal4-binding site. Silencing was indeed observed by a reporter-dependent growth assay (data not shown; see Deng and Hochstrasser 2006). Chromatin immunoprecipitation (ChIP) demonstrated that the Doa10-Gal4 fusion bound to the Gal4-binding sites in the chromosome. These data strongly suggested that Doa10 could reach the inner membrane of the NE.

We developed a second, quantifiable cell-based assay for transmembrane protein localization to the INM. This assay exploited the observation that increased expression of the Nup53 nucleoporin causes the specific proliferation of the INM (Marelli et al. 2001). Based on ultrastructural analysis, the resulting intranuclear membrane lamellae pack against the NE and often also cut across the nuclear interior. If Doa10 or other membrane proteins could enter the INM, fluorescently tagged versions of these proteins in cells overproducing Nup53 should reveal similar structures. Indeed, by examining the abundant ER protein Sec61, which forms part of the translocon for protein import into the ER, we observed that Nup53 overexpression led to the appearance of a distinctive fluorescence signal that transected the nucleus in roughly 25% of the cells (Fig. 4a); the nuclei resembled the Greek letter theta ( $\theta$ ), so we refer to them as theta nuclei (Deng and Hochstrasser 2006). A sim-

ilar frequency of theta nuclei was observed when Doa10 was tagged with GFP. Not all ER membrane proteins can readily enter the INM. Most tellingly, the other major transmembrane E3 of the yeast ER, Hrd1/Der3, showed little if any localization to the INM, indicating that INM protein localization is selective (Deng and Hochstrasser 2006). The three major proteins that function with Doa10 in Doa10-dependent ubiquitination (Ubc6, Ubc7, and Cue1) could also reach the INM, supporting the possibility that a complex of Doa10 with these proteins exists in the INM. Our results demonstrate that the Doa10 and Hrd1 E3 complexes concentrate in different subdomains of the continuous ER-NE membrane system.

Prior to our study, little was known about what trans-acting factors were required for trafficking polytopic membrane proteins to the inner NE. INM proteins had been postulated to diffuse from the ER through the lipid bilayer to the INM via lateral channels in the NPC (Worman and Courvalin 2000). Preliminary evidence for NPC involvement came from microinjection of animal cells with antibodies or lectins that bind NPC subunits; this had modest effects on INM localization of a model membrane protein (Ohba et al. 2004). To test the involvement of the NPC in the INM targeting of Doa10, we examined Doa10-GFP by the theta nuclei assay in a panel of nucleoporin mutants (Deng and Hochstrasser 2006). Out of the ten mutants tested, deletion of two specific nucleoporins, Pom152 and Nup188, partially but significantly reduced theta nuclei formation. Pom152 is one of only three integral membrane NPC proteins, and it therefore could be in close proximity to, or a component of, the NPC lateral channels. Pom152 directly binds Nup188 as well. The most straightforward interpretation of our data is that Nup188 and Pom152 form part of an NPC structure necessary for efficient membrane protein movement through the NPC pore membrane.

Interestingly, an independent study (King et al. 2006) published at about the same time as our study on Doa10 localization (Deng and Hochstrasser 2006) also demonstrated NPC involvement in the movement of membrane proteins to the INM. However, the pathway uncovered in this case depended on karyopherins and nuclear localization sequences (NLSs) in the two closely related substrates, which are highly concentrated in the INM, and required distinct components of the NPC



**Fig. 4a,b.** Doa10 is trafficked to the inner nuclear membrane (INM). **a** Over-expression of the Nup53 nucleoporin induces INM proliferation that can be visualized by fluorescence microscopy. In this confocal image, the ER translocon subunit Sec61 is tagged with GFP. INM lamellae that cross the nucleus (*arrowheads*) create stained structures resembling the Greek letter “θ”. **b** Doa10 must traverse the lateral channels in the nuclear pore complex (*NPC*) in order to get from its site of synthesis in the ER/outer nuclear membrane (*ONM*) to the INM, its site of action against nuclear substrates

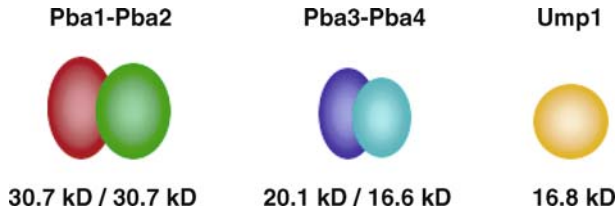
(King et al. 2006). Doa10 has no obvious NLSs. Therefore, there are likely to be at least two distinct routes for integral membrane protein trafficking to the INM. For Doa10, which is broadly employed in the ER-NE membrane system, strong localization to the INM would not be expected.

To determine whether INM localization of Doa10 was functionally relevant, we tethered Doa10 to the cell periphery by fusing it to the actin-binding domain of coronin (Crn1) (Deng and Hochstrasser 2006). Crn1 binds actin filaments and localizes to cortical actin patches (Goode et al. 1999). The Doa10-Crn1 domain fusion localized to foci at the cell cortex, resembling what is seen with native Crn1. Most importantly, the fusion failed to localize to the NE and did not form theta nuclei when Nup53 was overexpressed. Degradation of nuclear Doa10 substrates, but not cytosolic ones, was found to be impaired. Moreover, when an actin filament assembly inhibitor was added to cells to disassemble their cortical actin patches, the Doa10-Crn1 fusion partially relocalized from cortical sites to the NE, and, in parallel with this partial restoration of NE localization, we observed a partial recovery of nuclear protein degradation (Deng and Hochstrasser 2006). Therefore, Doa10 localization to the inner NE is necessary for efficient targeting of its nuclear substrates (Fig. 4b).

## **6 Stepwise Subunit Addition and Rate-Limiting Steps for 20S Proteasome Assembly In Vivo**

In addition to studying early steps in substrate targeting to the ubiquitin-proteasome system, we have been engaged for some time in analyzing the proteasome itself (Chen and Hochstrasser 1995; Chen and Hochstrasser 1996; Arendt and Hochstrasser 1997, 1999; Velichutina et al. 2004). The 20S proteasome (also called the core particle or CP) is a barrel of four co-axially stacked rings of seven subunits each. Two structurally related classes of subunits make up the rings. The outermost rings are composed of  $\alpha$ -type subunits and the inner rings of  $\beta$ -type subunits, each the product of a different gene in eukaryotes. Three distinct protease centers exist in the proteasome interior and are formed by specific  $\beta$  subunits.

Our recent work has concentrated on proteasome assembly, particularly that of the 20S proteasome or CP. Data presented in several recent studies from our lab argue that yeast 20S proteasome assembly proceeds *in vivo* by a series of discrete intermediates and in association with at least three conserved assembly chaperones dedicated to 20S proteasome

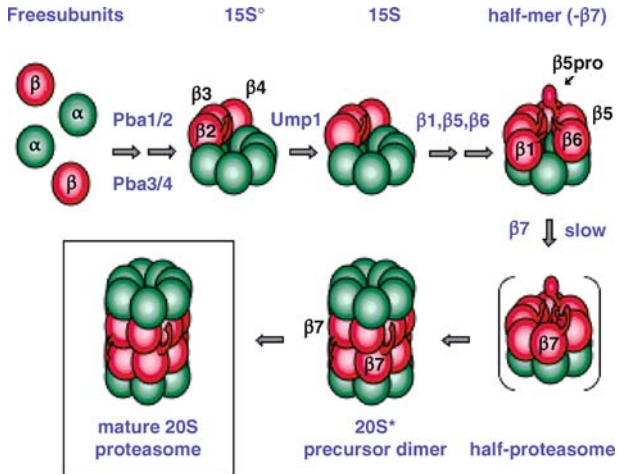


**Fig. 5.** Eukaryotes use at least three distinct assembly factors dedicated to 20S proteasome assembly. The yeast Pba1-Pba2 heterodimer, Pba3-Pba4 heterodimer, and Ump1 protein are shown in cartoon form in rough proportion to their relative sizes. Pba3 and Pba4 also appear to be able to form tetramers under some conditions. The oligomeric state of Ump1 is not known

assembly (Li et al. 2007; Kusmierczyk et al. 2008). These assembly factors—Ump1 (Ramos et al. 1998), Pba1-Pba2, and Pba3-Pba4—are depicted schematically in Fig. 5. Our understanding of the exact mechanisms by which these proteins facilitate proteasome biogenesis is still very limited.

In Fig. 6, we present a model for the stepwise assembly of eukaryotic 20S proteasomes based on analyses of various proteasome intermediates that were purified from yeast and whose compositions were then determined by tandem mass spectrometry (Li et al. 2007). In the model, the  $\beta 5$  propeptide helps to bring together and align half-proteasome precursor complexes and to stabilize the resulting precursor dimer during the  $\beta$ -subunit precursor cleavages and conformational rearrangements that lead to mature 20S proteasomes. This essential function of the  $\beta 5$  propeptide, which was previously unknown, is linked to the  $\beta 7$  C-terminal tail and can be largely bypassed when  $\beta 7$  is present in high amounts. Ump1 is proposed to inhibit stable dimerization until the rate-limiting insertion of the  $\beta 7$  subunit into the half-mer. The  $\beta 7$  tail helps overcome the Ump1 checkpoint and stabilize the precursor dimer during maturation (20S\* to mature 20S in Fig. 6). This tail is normally not essential if functional  $\beta 5$  propeptide is present. By our model, the absence of Ump1 allows aberrant off-pathway half-mer association, thereby impeding subsequent assembly and maturation steps.

This simple model can explain an otherwise surprising array of genetic interactions seen between various  $\beta$ -subunit mutants as well as the



**Fig. 6.** Model for 20S proteasome assembly. Proteasomes assemble stepwise from 14 individual subunits. An early assembly intermediate is thought to be an  $\alpha$ -ring heteroheptamer, although such a structure has not yet been isolated from yeast. Both Pba1-Pba2 and Pba3-Pba4 function early in assembly, facilitating  $\alpha$ -ring formation or preventing formation of off-pathway  $\alpha$ -ring dimers. The  $\alpha$ -ring serves as a template for  $\beta$ -subunit addition, with  $\beta 2$ ,  $\beta 3$ , and  $\beta 4$  entering early and  $\beta 7$  entering last. Addition of  $\beta 7$  is tightly linked with half-proteasome dimerization, followed by  $\beta$ -subunit propeptide processing and degradation of Ump1 and Pba1-Pba2. Ump1 facilitates proper half-mer dimerization and 20S proteasome maturation, but it may also have earlier roles

paradoxical effects of Ump1 mutations on assembly (Li et al. 2007). Ump1 can be viewed as an assembly checkpoint protein that helps ensure the proper order of proteasome assembly events. This ultimately enhances productive proteasome assembly by reducing flux through slow or dead-end assembly pathways. A particularly simple way by which Ump1 might work would be to limit directly  $\beta 7$  insertion into the half-mer until all the other  $\beta$  subunits have incorporated.

Why might  $\beta 2$ ,  $\beta 3$ , and  $\beta 4$  associate with the  $\alpha$  ring prior to the other  $\beta$  subunits, leading to accumulation of the so-called 15S intermediate even in wild-type cells (Fig. 6)? Consideration of the surface area buried between subunits suggests a potential solution (Li et al. 2007).

Of all the  $\beta$  subunits,  $\beta 2$  buries the largest surface area against the  $\alpha$  ring (2676  $\text{\AA}^2$  vs the next largest, which is  $\beta 7$  at 1931  $\text{\AA}^2$ ). It also has by far the largest cis- $\beta$  contact, at 3130  $\text{\AA}^2$  compared to 1812  $\text{\AA}^2$  for the next nearest; this is primarily due to the long C-terminal arm that wraps around  $\beta 3$ . Either  $\beta 2$  or a  $\beta 2$ - $\beta 3$  heterodimer, which would bring the amount of surface area buried against the  $\alpha$  ring to 4600  $\text{\AA}^2$ , could serve to nucleate  $\beta$ -ring assembly on the  $\alpha$ -ring template. The  $\alpha$  subunits have roughly double the amount of buried surface area between subunits relative to  $\beta$ -cis- $\beta$  contacts, which should allow  $\alpha$ -ring assembly without any  $\beta$  subunits. Notably,  $\beta 2$ ,  $\beta 3$ , and  $\beta 4$  have three of the four largest surface areas buried against the  $\alpha$  ring, whereas the two subunits flanking this trio,  $\beta 1$  and  $\beta 5$ , have the two lowest, and neither makes extensive cis-contacts with  $\beta 2$  and  $\beta 4$ , respectively. Therefore, stable addition of these two subunits might normally be limiting, causing 15S precursors to accumulate.

## 7 Identification of the Pba1-Pba2 Proteasome Assembly Chaperone

We consistently observe two additional polypeptides, Pba1 and Pba2 (proteasome biogenesis-associated factors 1 and 2), in proteasomal precursors (Li et al. 2007). Notably, a previous study with mammalian cells identified a heterodimeric complex, PAC1-PAC2, which facilitates early stages of proteasome assembly, possibly by limiting off-pathway reactions such as  $\alpha$ -ring dimerization (Hirano et al. 2005). Yeast Pba1 and Pba2 show significant, albeit limited, sequence similarity to PAC1 and PAC2, respectively (Li et al. 2007). Purified Pba1 and Pba2 form a stable heterodimer as well. The inference that Pba1-Pba2 participates in yeast 20S proteasome assembly derived from the finding that the complex was found within multiple precursor particles but never in mature 20S proteasomes. Both proteins are induced during the ER unfolded-protein response, as are other ubiquitin-system components, and *pba2* (*add66*) mutants have a mild defect in the degradation of some ER substrates (Palmer et al. 2003). Finally, Pba1-Pba2 associates with very early proteasome precursors (Li et al. 2007), similar to

what has been reported for the human heterodimer (Hirano et al. 2005). These data make it likely that the mechanism of action of Pba1-Pba2 is also conserved.

Proteasome assembly does not absolutely require Pba1-Pba2 since the corresponding deletion mutants are viable, but genetic interactions with proteasome mutants are consistent with a role in proteasome biogenesis (Li et al. 2007). It is particularly intriguing that loss of Pba1 or Pba2 partially suppresses defects associated with *ump1*  $\Delta$ . Deletion of *Ump1* causes an accumulation of aberrant late assembly products. By slowing proteasome biogenesis at an earlier step, *pba1*  $\Delta$  or *pba2*  $\Delta$  might limit the build-up of such dead-end complexes.

## 8 The Pba3-Pba4 Assembly Chaperone Controls 20S Proteasome Composition

Generally, eukaryotic proteasome assembly has been assumed to generate a universal 20S proteasome core, with a dyad-symmetric  $\alpha_{1-7}\beta_{1-7}\beta_{1-7}\alpha_{1-7}$  architecture. However, some species have several closely related isoforms of specific 20S subunits, potentially enabling subtle modulation of proteasome composition and activity (Yuan et al. 1996; Fu et al. 1998). Organisms with an adaptive immune system also express three alternative  $\beta$ -subunits in response to  $\gamma$ -interferon stimulation; these substitute for their constitutive counterparts, thereby enhancing processing of certain antigens for MHC class I presentation (Rock et al. 2002). Another alternative  $\beta 5$  subunit is expressed specifically in the thymus of mammals, and it is also believed to modulate cellular immunity (Murata et al. 2007).

Positional substitution of a much more divergent subunit occurs in *S. cerevisiae* cells that lack  $\alpha 3$  (Pre9), the only 20S proteasome subunit not required for viability (Emori et al. 1991; Velichutina et al. 2004). In  $\alpha 3$   $\Delta$  cells, a second copy of  $\alpha 4$  (Pre6), with a sequence only roughly 33% identical to  $\alpha 3$ , takes the position normally occupied by  $\alpha 3$  (Velichutina et al. 2004). In wild-type cells, the  $\alpha 3$  subunit is incorporated to the virtual exclusion of  $\alpha 4$  at this position, at least under standard growth conditions. No mechanism for the alternative assembly

of  $\alpha$ -subunit rings had been identified previously, but our recent discovery of the Pba3-Pba4 assembly chaperone revealed that it acts to ensure the exclusive incorporation of  $\alpha 3$  between the  $\alpha 2$  and  $\alpha 4$  subunits in the  $\alpha$  ring (Kusmierczyk et al. 2008). Mutation of Pba3-Pba4 leads to cells accumulating distinct subpopulations of 20S proteasomes. Unexpectedly, assembly of the regulatory particle (RP) is also perturbed in 20S proteasome assembly mutants, suggesting that the 20S proteasome functions as an assembly factor during RP biogenesis.

We first identified *PBA3* (*YLR021W*) and *PBA4* (*YPL144W*) by a targeted bioinformatic analysis of published genomic-scale functional studies (Kusmierczyk et al. 2008). We then determined that yeast mutants lacking the corresponding proteins had traits commonly associated with proteasomal defects, such as hypersensitivity to amino acid analogs and temperature-sensitive growth. A *pba3* $\Delta$  *pba4* $\Delta$  double mutant showed growth deficiencies comparable to the single mutants. Interestingly, no enhancement of  $\alpha 3$  $\Delta$  growth defects was observed when combined with either *pba3* $\Delta$  or *pba4* $\Delta$ , but when the latter deletions were combined with a partial loss-of-function point mutation in  $\alpha 5$ , *doa5-1* (Chen and Hochstrasser 1995), the double mutants grew much more poorly than the single mutants. The lack of synthetic growth defects when *pba3* $\Delta$  or *pba4* $\Delta$  was combined with  $\alpha 3$  $\Delta$  suggested that mutations in Pba3/Pba4 and  $\alpha 3$  might affect a common aspect of proteasome assembly or function.

An earlier proteomic analysis of yeast protein complexes suggested that Pba3 and Pba4 could associate in vivo (Krogan et al. 2006). We expressed the two proteins in *Escherichia coli* and found that they interacted directly in a stoichiometric complex. Moreover, when we co-expressed hexahistidine-tagged Pba3 (Pba3-his) and Pba4 with each of the  $\alpha$  subunits in bacterial cells, Pba3-Pba4 bound strongly to  $\alpha 5$  and, to a much more limited extent,  $\alpha 1$ , but none of the other subunits. The interaction with  $\alpha 5$  could also occur when  $\alpha 5$  was in complexes with other  $\alpha$  subunits.

To determine if Pba3-Pba4 function was related to proteasome biogenesis, we examined proteasomal particles from *pba3* $\Delta$  and *pba4* $\Delta$  yeast lysates resolved by nondenaturing gel electrophoresis, followed by in-gel peptidase assays using a fluorogenic peptide substrate or by immunoblotting. These experiments suggested a primary defect in 20S

proteasome assembly rather than RP assembly. However, when we analyzed native gel-separated proteins by immunoblotting with antibodies to RP base (Rpt5) or lid (Rpn5), changes were also observed in these RP subcomplexes. There had been no published data showing that the 20S proteasome was necessary for assembly of the RP *in vivo*, and the intact RP can readily dissociate from and reassociate with the 20S proteasome *in vitro*. Nevertheless, in all the 20S assembly mutants we examined, aberrant sets of RP subparticles accumulated. A greatly increased amount of free lid (i.e., not associated with the base of the RP) was seen, and a complex set of RP base subparticles was observed with antibodies to the Rpt5 base subunit. Intriguingly, the pattern of Rpt5-containing particles from the *pba3* $\Delta$  and *pba4* $\Delta$  lysates was strikingly similar to the pattern observed in  $\alpha3$  $\Delta$ .

Collectively, these results suggested first, that Pba3-Pba4 is a 20S proteasome assembly factor whose action may be linked to the  $\alpha3$  subunit, and second, that formation of 20S proteasomes influences assembly of the RP *in vivo*, potentially acting as a template that facilitates assembly of the latter complex.

The similarities in the patterns of proteasomal subcomplexes that accumulated in *pba3* $\Delta$ /*pba4* $\Delta$  and  $\alpha3$  $\Delta$  mutants and the absence of synthetic growth defects when  $\alpha3$  $\Delta$  was combined with *pba3* $\Delta$ /*pba4* $\Delta$  led us to hypothesize that the Pba3-Pba4 complex might normally facilitate incorporation of  $\alpha3$  into the  $\alpha$  ring. As mentioned above, we had found that in  $\alpha3$  $\Delta$  cells, a second copy of  $\alpha4$  occupies the position normally taken by  $\alpha3$  (Velichutina et al. 2004). Using both genetic suppression analysis and protein-protein crosslinking through structure-based disulfide engineering, we found that  $\alpha4$  can also occupy the  $\alpha3$  positions of the 20S proteasome in cells lacking Pba3-Pba4 (Kusmierczyk et al. 2008). In *pba4* $\Delta$  cells, an estimated 20%–50% of 20S proteasome  $\alpha$  rings were in the  $\alpha4$ - $\alpha4$  configuration. Therefore, Pba3 and Pba4 function to ensure that  $\alpha3$  is incorporated into 20S proteasomes. In this regard, the Pba3-Pba4 complex is a true proteasomal chaperone since it controls the proper assembly of wild-type 20S proteasomes.

The ability of yeast Pba3-Pba4 to modulate assembly of specific isoforms of the proteasome raises the question of whether the formation of proteasomes with alternative  $\alpha$  rings is relevant in other eukaryotes. Although this mechanism remains to be tested in other organisms, there

is reason to suspect that it is conserved. First, orthologs of both Pba3 and Pba4 can be found throughout the Eukarya (Le Tallec et al. 2007; Kusmierczyk et al. 2008; Yashiroda et al. 2008; Hoyt et al. 2008). Second, it appears that the ability of  $\alpha 4$  to take two positions in the  $\alpha$  ring is likely to be widespread as well. This inference is based primarily on the earlier observation that  $\alpha 4$  from the plant *Arabidopsis* can also insert at both the  $\alpha 4$  and  $\alpha 3$  positions when expressed in yeast (Velichutina et al. 2004). The conserved ability of  $\alpha 4$  to substitute for  $\alpha 3$  suggests that the singular dispensability of  $\alpha 3$  in yeast might also be true for other organisms. A recently reported *Aspergillus nidulans* mutant encodes an  $\alpha 3$  subunit that lacks the C-terminal third of the protein, and is likely to be a null allele, but the mutation is not lethal (Lee and Shaw 2007). All other 20S proteasome subunits appear to insert into a single unique proteasomal location.

Is there a selective advantage to being able to form these alternative  $\alpha 4$ - $\alpha 4$  proteasomes and/or to lower levels of proteasomes? Under most conditions, yeast mutants lacking Pba3 or Pba4 grow more poorly than wild-type cells (Kusmierczyk et al. 2008). Interestingly, however, formation of the alternative  $\alpha 4$ - $\alpha 4$  proteasome correlated with enhanced growth of cells subjected to specific environmental stresses. In particular, upon exposure to high levels of the heavy metal cadmium, *pba3* $\Delta$ /*pba4* $\Delta$  and  *$\alpha 3$*  $\Delta$  mutants grew better than otherwise identical wild-type cells. Cadmium induces oxidative stress in yeast (Brennan and Schiestl 1996). Cells might be able to downregulate Pba3-Pba4 activity under certain conditions so that alternative or lower amounts of proteasomes are synthesized. Degradation of toxic oxidatively damaged proteins in yeast is enhanced when 26S proteasome assembly from 20S and RP components is impaired (Inai and Nishikimi 2002). The  $\alpha 4$ - $\alpha 4$  proteasome, which should have a constitutively open  $\alpha$ -ring gate (Groll et al. 2000), may also enhance such degradation.

Our in vitro data demonstrated direct binding between Pba3-Pba4 and the proteasome  $\alpha 5$  subunit and show that Pba3-Pba4 can associate with specific  $\alpha$ -subunit subcomplexes. The major in vivo consequence of Pba3/4 activity is the stimulation of  $\alpha 3$  insertion into the  $\alpha$ -ring, even though the chaperone does not appear to bind strongly to  $\alpha 3$  itself. We propose that Pba3-Pba4 functions as a scaffolding complex that enhances  $\alpha 5$  binding to its neighbors, including  $\alpha 4$ . Indeed, the re-

cent solution of a Pba3-Pba4- $\alpha 5$  cocrystal structure and its modeling onto the known 20S proteasome structure suggests such a scaffolding role is feasible (Yashiroda et al. 2008). If  $\alpha 3$  and  $\alpha 4$  generally insert last into the nascent  $\alpha$  ring, Pba3-Pba4 might help prevent  $\alpha 4$  insertion at the  $\alpha 3$  position and this in turn could favor the final insertion of  $\alpha 3$  at its normal position. Loss of Pba3-Pba4 will stimulate formation of proteasomes with  $\alpha 4$ - $\alpha 4$   $\alpha$ -rings, but  $\alpha 4$  would still have a disadvantage relative to  $\alpha 3$  for insertion next to  $\alpha 2$ , so overall proteasome assembly will be less efficient. The net result will be a heterogeneous population of proteasomes containing  $\alpha 4$ - $\alpha 4$  and  $\alpha 3$ - $\alpha 4$  rings along with a reduction in total 20S proteasome levels. Experimental tests of these ideas are underway.

Although often viewed as a fairly rigid and invariant complex, the eukaryotic 20S proteasome may have evolved a substantial degree of structural and regulatory flexibility. Besides alternative subunit isoforms, such as those that incorporate when the immune system is stimulated, many posttranslational modifications have also been documented (Froment et al. 2005). In addition, increasing evidence suggests that 20S proteasomes do not function exclusively as part of 26S proteasomes (Liu et al. 2003). The ability of a dedicated chaperone to modulate proteasome composition represents a unique mechanism for providing structural flexibility to the ubiquitin-proteasome system.

## 9 Perspectives

Despite its relative morphological simplicity, the single-celled model eukaryote *Saccharomyces cerevisiae* uses many of the same basic biochemical regulatory mechanisms as more complex eukaryotes, including humans. The ubiquitin-proteasome system is a prime example of this. All of the ubiquitin system enzymes that we have been studying in yeast have clear orthologs in humans and most other eukaryotes. Understanding of their biochemical mechanisms and physiological functions can be gained much more readily with the well-developed yeast model, but insights gained from studies of the yeast ubiquitin-proteasome system will also be relevant for humans and other metazoans. Future work using an interplay of yeast molecular genetics, biochemical analysis,

cell biological approaches, and structural studies promises a much more sophisticated comprehension of how substrates are recognized by the ubiquitin-conjugation machinery, how they acquire their polyubiquitin chain modifications and are directed to the proteasome, and how the proteasome itself assembles and operates in the crowded interiors of the cell.

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## ***Less Is More: How Protein Degradation Regulates Muscle Development***

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T. Hoppe<sup>(✉)</sup>

Cologne Excellence Cluster on Cellular Stress Responses in Aging-Associated Diseases (CECAD) at the Institute for Genetics, University of Cologne, Zùlpicher Str. 47, 50674 Cologne, Germany  
email: [thorsten.hoppe@zmnh.uni-hamburg.de](mailto:thorsten.hoppe@zmnh.uni-hamburg.de)

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**Abstract.** The organization of sarcomeric structures during muscle development involves regulated multistep assembly pathways. The myosin assembly factor UNC-45 functions both as a molecular chaperone and as an Hsp90 co-chaperone for myosin throughout muscle thick-filament formation. Consequently, mutations in *unc-45* result in paralyzed worms with severe myofibril disorganization in striated body wall muscles. Our data suggest that functional muscle formation in *Caenorhabditis elegans* is linked to ubiquitin-dependent UNC-45 turnover, regulated by the E3 enzymes UFD-2 and CHN-1 in cooperation with the ubiquitin-selective chaperone CDC-48 (also known as p97 in human). Missense mutations in the gene encoding p97 are known to cause a dominant, late-onset hereditary inclusion body myopathy. Remarkably, we identified a conserved role of CDC-48/p97 in the process of myofiber differentiation and maintenance, which appears to have important implications for understanding defects in muscle formation and maintenance during pathological conditions.

The assembly of myosin into thick filaments during muscle development is still a largely unexplored phenomenon (Barral and Epstein 1999). Recent data suggest that the organization of myosin into sarcomeric structures is the result of a regulated multistep assembly pathway that requires additional factors. Candidates for this process are

members of a protein family containing a UCS (*UNC-45/CRO1/She4p*) domain, which have been indicated to be necessary for proper myosin function (Hutagalung et al. 2002). One founding member of this family is *UNC-45*, for which homologs have been identified in a variety of organisms, from yeast to humans (Hutagalung et al. 2002). It was demonstrated that the UCS domain of *UNC-45* interacts with muscle myosin and exerts chaperone activity onto the myosin head, whereas its N-terminal TPR domain (tetratricopeptide repeat) binds the general molecular chaperone Hsp90 (Barral et al. 2002). Thus, *UNC-45* functions both as a molecular chaperone and as an Hsp90 co-chaperone for myosin during muscle thick-filament assembly. Consequently, mutations in *C. elegans unc-45* (Epstein and Thomson 1974) result in paralyzed animals with severe myofibril disorganization in striated body wall muscles (Barral et al. 1998).

Our recent work revealed that protein levels of the myosin chaperone *UNC-45* are subject to stringent regulation, which appears to be dependent on *UFD-2* and *CHN-1* ubiquitylation activity (Hoppe et al. 2004; Janiesch et al. 2007). *UFD-2* is an ortholog of yeast *UFD2* known to bind oligoubiquitylated substrates to catalyze the addition of further ubiquitin moieties in the presence of E1, E2, and E3 enzymes. Thus, *UFD2* defines a novel enzymatic activity that mediates multiubiquitin chain assembly, needed for subsequent proteasomal degradation and thus was termed E4 enzyme (Hoppe 2005; Koegl et al. 1999). The human *CHN-1* ortholog *CHIP* was identified both as a co-chaperone of Hsc70 and Hsp90 and to be an E3 enzyme (Ballinger et al. 1999; Connell et al. 2001). Thus, *CHIP* probably acts as a protein quality-control ubiquitin ligase that selectively leads abnormal proteins recognized by molecular chaperones to degradation by the 26S proteasome (Cyr et al. 2002; Murata et al. 2003).

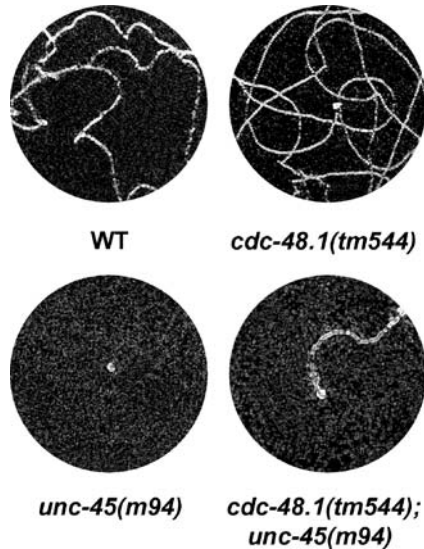
We were able to show that either *UFD-2* or *CHN-1* alone, in collaboration with E1 and E2, conjugates *UNC-45* with one to three ubiquitin moieties. Therefore, both *CHN-1* and *UFD-2* work independently as E3 enzymes in this pathway. However, in combination, *CHN-1* and *UFD-2* increase the ubiquitylation of *UNC-45* (Hoppe et al. 2004). Movement defects of *unc-45* thermosensitive (*ts*) mutants are suppressed in animals lacking *CHN-1* or *UFD-2* most likely due to stabilization of the corresponding *UNC-45* (*ts*) proteins. Interestingly, analysis of body-

wall muscle cells by polarized light microscopy showed that the muscle structure of *chn-1* and *ufd-2* knockout worms is comparable to that of wild-type; however, overexpression of transgenic *unc-45* leads to strong sarcomeric assembly defects (Janiesch et al. 2007). Therefore, the amount of UNC-45 protein present in the muscle cells is critical for proper thick filament function.

Another factor that we identified to be involved in targeting the myosin assembly chaperone UNC-45 for degradation is the ubiquitin-selective chaperone CDC-48 (Fig. 1). Its homologs Cdc48p in yeast and p97 in mammals belong to the family of AAA-type ATPases and form homohexameric rings with chaperone-like activity (Rouiller et al. 2000). CDC-48/p97 is intimately linked to the ubiquitin pathway because its central role is to bind and segregate ubiquitylated proteins to extract these from their binding partners for substrate recruitment and ubiquitin chain assembly (Rape et al. 2001; Ye 2006). In *C. elegans*, we found that CDC-48 forms a complex together with UFD-2 and CHN-1 to regulate UNC-45 protein levels. This trimeric complex links turnover of UNC-45 to functional muscle formation. Our recent work showed an upregulation of *ufd-2*, *chn-1*, and *cdc-48* transcripts during larval stages in which body-wall muscle development mainly occurs (Janiesch et al. 2007). This observation suggests that the formation of the CDC-48/UFD-2/CHN-1 complex could be developmentally regulated by muscle-specific co-expression.

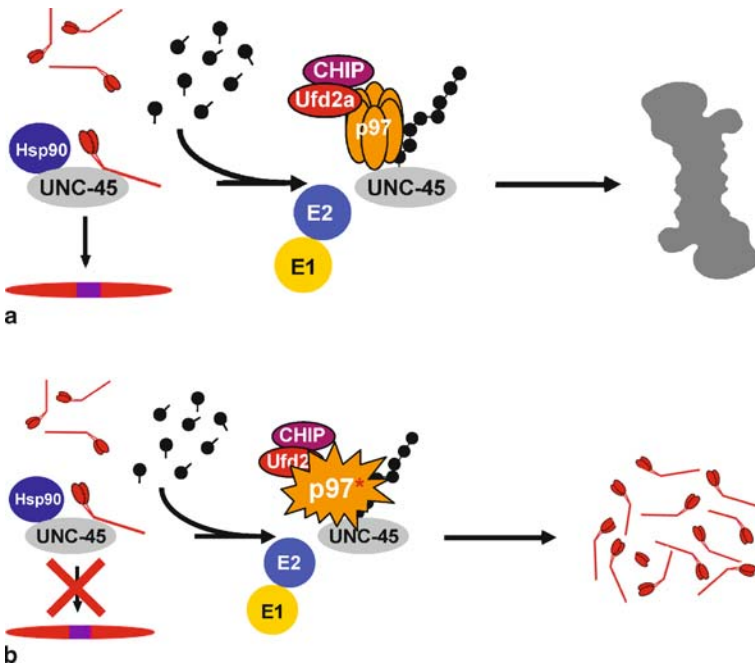
Intriguingly, a similar pathway required for muscle development might exist in humans as well, since mutations in p97 are known to cause a dominantly inherited form of inclusion body myopathy (IBM) (Watts et al. 2004). Direct binding and co-localization between p97 and the mammalian UFD-2 and CHN-1 homologs, Ufd2a and CHIP, indicate regulation of myosin assembly by an evolutionarily conserved p97/Ufd2a/CHIP complex (Fig. 2a) (Janiesch et al. 2007). Consistent with the hypothesis that such a complex could be required for vertebrate muscle formation, Ufd2a and CHIP have been implicated in cardiac and skeletal myogenesis or cardiotoxic resistance, respectively (Ballinger et al. 1999; Kaneko et al. 2003; Mahoney et al. 2002).

IBM associated with Paget disease of bone and frontotemporal dementia (IBMPFD) is an inherited disorder that produces adult-onset muscle wasting and weakness and is characterized by muscle pathology



**Fig. 1.** CDC-48 regulates the myosin chaperone UNC-45, suppressing the movement defect of temperature-sensitive *unc-45(m94)* worms. The bacterial lawns on the plates show traces of temperature shifted worms, *cdc-48.1(tm544)*, *unc-45(m94)*, double mutants, and wild-type (*WT*), after crawling for 1 h at 22 °C. Ten young adults were assayed for each strain and all displayed similar motility

including cytoplasmic and nuclear aggregates in skeletal and cardiac muscle (Watts et al. 2004). We demonstrated that in contrast to wild-type, mutations in p97 known to cause myopathy are not able to replace CDC-48 throughout the UNC-45-dependent myosin assembly pathway in worms. Moreover, the degradation of human UNC-45 is abrogated by the same IBMPFD-associated p97 mutations, resulting in severely disorganized myofibrils and sarcomeric defects (Janiesch et al. 2007). Therefore, p97 seems to regulate UNC-45 levels during the process of myofiber differentiation and muscle maintenance, which is abolished during pathological conditions, resulting in the accumulation of aggregated proteins.



**Fig. 2a,b.** Model for UNC-45-dependent myosin assembly. The myosin-directed chaperone UNC-45 binds myosin and Hsp90 simultaneously in muscle thick-filament assembly. **a** The conserved p97/Ufd2a/CHIP complex directly multiubiquitylates UNC-45, leading to subsequent degradation by the 26S proteasome. Development specific assembly of the multiubiquitylation complex seems to connect UNC-45 turnover to functional muscle formation. **b** IBMPFD-causing mutations in human p97 disrupt the ubiquitylation process, resulting in increased levels of UNC-45. The stabilization of UNC-45 probably disturbs the integration of myosin into sarcomeric structures or supports their disassembly. High amounts of unassembled myosin might then induce protein aggregation in muscle cells

The pathogenic mechanisms that cause muscle weakness in IBM, and IBMPFD in particular, might be related to the aggregation of stabilized or misassembled proteins. How these protein aggregates and finally inclusion bodies are formed in the presence of p97 mutations is

not clear. Interestingly, another dominantly-inherited form of IBM is caused by mutations in the head region of fast myosin IIa (MYH2), which render MYH2 to aggregate (Martinsson et al. 2000; Tajsharghi et al. 2005). Consistent with such a myosin-based inclusion body formation, stabilization of UNC-45 may disturb the integration of myosin into sarcomeric structures or support their disassembly. The resulting accumulation of unassembled myosin in the cytosol might then induce protein aggregation in both skeletal and cardiac muscle (Fig. 2b). Our future studies will address the molecular mechanism underlying the process of inclusion-body formation and hopefully help us understand the connection between protein degradation and muscle development.

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## ***Transcriptional Control and the Ubiquitin–Proteasome System***

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A. Leung, F. Geng, A. Daulny, G. Collins,  
P. Guzzardo, W.P. Tansey<sup>(✉)</sup>

Cold Spring Harbor Laboratory, 1 Bungtown Road, 11724 Cold Spring Harbor, USA  
email: [tansey@cshl.edu](mailto:tansey@cshl.edu)

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**Abstract.** Regulation of transcription is a critically important process that controls development, differentiation, and the maintenance of cellular homeostasis. Cells have evolved numerous mechanisms to keep gene transcription tightly in check, some of which involve the ubiquitin–proteasome system. In this chapter, we review evidence supporting the concept that ubiquitin and the proteasome not only control transcription, but provide the biochemical means to drive key steps in the transcription process forward.

## 1 Introduction

Correct regulation of gene activity is essential for organismal development and the maintenance of cellular homeostasis. Accordingly, one of the most highly regulated processes within the cell is gene expression. For a gene to be expressed, numerous molecular processes have to occur in an appropriate sequence. The first step in gene expression is gene transcription. For transcription of an mRNA-type gene to occur, a set of sequence-specific transcription factors must descend on regulatory DNA elements (promoters, enhancers, etc.) surrounding the gene to be transcribed. These transcription factors work by recruiting proteins that both alter the local chromatin structure and recruit core components of the transcriptional machinery to promoter DNA. The net effect of these interactions between transcriptional regulators and the core transcriptional machinery is to recruit RNA polymerase II (pol II) to the gene and to initiate transcription. Given that these are very early events in gene expression, it is not surprising that they are subject to some of the most stringent levels of regulation.

After transcription is initiated, however, many other processes must be both facilitated and regulated for a functional transcript to be made. For example, polymerase must escape the vicinity of promoter DNA and convert to a form that is competent for synthesis of full-length transcripts. Along the way, polymerase must coordinate transcription with pre-messenger RNA processing, negotiate chromatin structure and DNA sequence that may retard its progress, sense for DNA damage, and terminate transcription when appropriate. Commensurate with these processes, mRNA must be coordinately exported from the nucleus for translation. Given the extraordinarily complex set of events required to produce an active mRNA in the cytoplasm, it is clear that deregulation of any of these steps could profoundly modify the gene expression profile of a cell. Conversely, it is also clear that, for normal cell growth and function to occur, each stage in gene transcription must be tightly regulated.

Cells have evolved numerous ways to control transcription. Many of the cellular strategies regulating gene activity involve control of transcription factor localization or modification state, or by having relevant factors function only in the presence of a particular ligand. In this chap-

ter, however, we argue that one important way in which gene activity is regulated is by ubiquitin (Ub) and the proteasome.

Although it is difficult to imagine two processes that are more dissimilar than gene regulation and Ub-mediated proteolysis, a growing body of evidence suggests that multiple steps in the expression of genetic information are controlled by Ub-dependent transactions. In some cases, the main function of the Ub–proteasome system (UPS) is exerted via proteolysis; in others, the main function appears to be exerted via nonproteolytic activities, both of Ub and the proteasome. Much of the work that is covered in this review focuses on how the UPS influences gene transcription, an area of particular interest to the Tansey laboratory, although it should be stressed that numerous groups have made significant contributions to understanding connections between the transcription and Ub–proteasome systems. Here, we will attempt to cover the most significant developments in recent years, speculate about the fundamental mechanisms through which the UPS can impact transcription, and discuss the implications of the transcription/UPS connection for human diseases, particularly cancer.

## **2 Historical Ties Between the Transcription and Ubiquitin–Proteasome Systems**

Although the extent to which the UPS influences transcription has only recently been appreciated (Collins and Tansey 2006; Lipford and Deshaies 2003; Muratani and Tansey 2003), it should be noted that clear links to transcription emerged at the birth of the Ub–proteasome field. In their characterization of the nuclear protein A24, Busch and colleagues (Goldknopf and Busch 1977; Goldknopf et al. 1975) described an isopeptide linkage between lysine 119 (K119) of histone H2A and the protein that is now known as ubiquitin (Ub). Later, studies in *Drosophila* (Levinger and Varshavsky 1982), *Tetrahymena* (Davie et al. 1991; Davie and Murphy 1990; Nickel et al. 1989; Vavra et al. 1982), and mammalian cells (Huang et al. 1986) demonstrated that this ubiquitylated form of H2A was specifically associated with actively transcribed genes, making histone H2A ubiquitylation one of the first recognized markers of transcriptionally active chromatin. Subsequently, ubiq-

uitylated forms of each of the histones have been described (Muratani and Tansey 2003) and clear roles for this histone modification established in both gene activation (e.g., Kao et al. 2004) and silencing (e.g., de Napoles et al. 2004; Fang et al. 2004).

Intriguingly, early links between the transcription and Ub-proteasome systems were not confined only to histone ubiquitylation, but also extended to the proteasome itself. In 1992, Johnston and colleagues (Swaffield et al. 1992) reported the results of a genetic screen for mutations that would allow a form of Gal4—missing a piece of its transcriptional activation domain (TAD)—to activate transcription. This screen identified allele-specific recessive mutations in two genes: *Sug1* and later *Sug2* (Russell et al. 1996). For a while in the 1990s, *Sug1* was recognized as a transcriptional mediator (Kim et al. 1994; Lee et al. 1995; Melcher and Johnston 1995; Swaffield et al. 1995; vom Baur et al. 1996; Xu et al. 1995). This recognition came to an end, however, when genetic (Ghislain et al. 1993), evolutionary (Akiyama et al. 1995), and biochemical (Rubin et al. 1996) evidence identified *Sug1* as a component of the proteasome. The realization that *Sug1* was a part of the proteasome led to the idea that its effects on transcription were indirect, and resulted not from a direct role in transcription, but rather changes in the levels of some important transcription-control proteins. As discussed later, the subsequent realization that Ub plays a significant role in transcription refueled interest in the proteasome as a transcriptional regulator, and it is now clear that proteasome components interact with chromatin and can function at multiple steps in transcription.

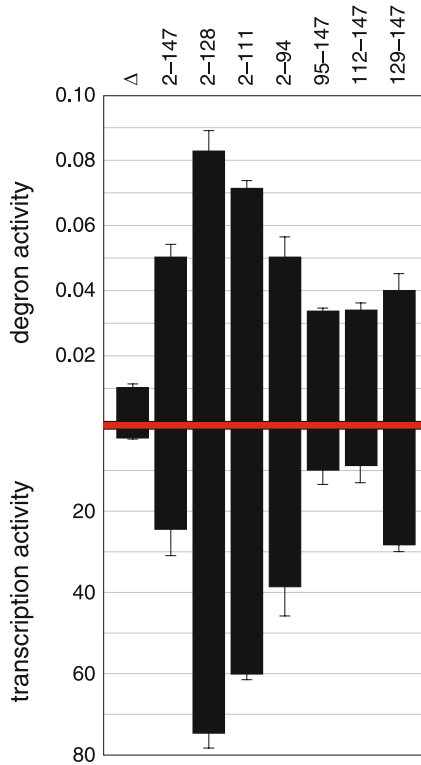
### **3 Relationship Between Transcription Factor Activity and Destruction**

When considering the role that proteolysis generally plays in regulating protein function, it is reasonable to imagine that proteolysis occurs at a point when the function of that protein is no longer needed. In this model, proteins will be most abundant when they are most active. During our analysis of Ub-mediated proteolysis of the oncoprotein transcription factor Myc (Salghetti et al. 1999), however, we made an observation that suggested that such an arrangement may not hold true

for all proteins. Specifically, when we mapped the Myc degron—the region that signals Myc ubiquitylation—we found that it coincided with the region in Myc that allows it to activate transcription (transcriptional activation domain or TAD). Indeed, the TAD and degron of Myc did not simply coincide, but they were functionally connected. Mutational analysis revealed that the degron function of this region of Myc correlated very tightly with activation function (Fig. 1; data originally published in Salghetti et al. 1999): without exception, segments of Myc that are better able to activate transcription are better able to signal Ub-mediated proteolysis, and vice-versa. The close correlation between activity and destruction in this domain led us to conclude that the Myc TAD and degron were essentially the same element. Our subsequent studies with other TADs showed (Salghetti et al. 2000; W. Tansey, unpublished observations) that degron function was a general feature of activation domains that are rich in acidic-type residues (as for the Myc TAD), and that transcriptional activation potential correlates closely with degron function. Importantly, the rate of destruction of Myc, and other transcription factors, is inversely correlated with their activity, and these proteins tend to be least abundant precisely when they are most active.

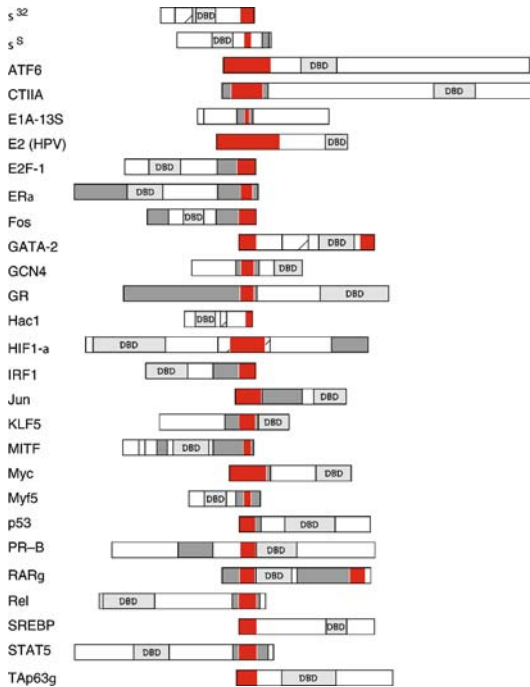
Inspection of the literature in 2000 revealed that Myc was not the only transcription factor where TADs and degrons had been mapped to the same region of the protein. At the time, there were six examples of proteins that fell into this class: E2F-1, Fos, GCN4, Jun, Myc, and p53 (see Muratani and Tansey 2003 for references), suggesting that the overlap of TADs and degrons in natural proteins may not be confined to Myc. As of February 2008, there are at least 27 examples in the literature of transcription factors with overlapping TADs and degrons (Fig. 2), revealing that the functional connection between activation and destruction is widespread. Interestingly, although eubacteria do not have Ub-mediated proteolysis, they do possess ATP-dependent protein turnover, and for at least two bacterial sigma factors (Fig. 3), overlapping of TADs and destruction elements occurs. This finding suggests that these two types of element became coincident early in evolution.

What is the basis for the extensive overlap of TADs and degrons? Two possible models could explain this relationship. In the first model, activation and degron function are connected, but there is no direct relationship between proteolysis and gene activation. In other words, TADs



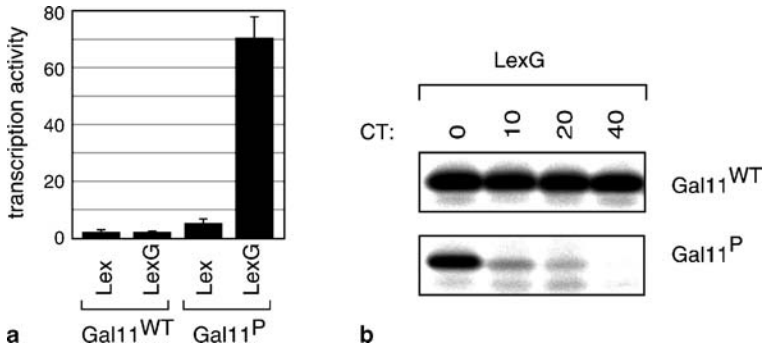
**Fig. 1.** Myc TAD and degron function are tightly correlated. The indicated segments from the Myc activation domain (residues 1–147) were fused to the Gal4 DNA-binding domain (DBD;  $\Delta$ ). We then measured the metabolic stability of each fusion protein in human HeLa cells using pulse-chase analysis. Degrone activity is represented as the inverse of the half-life of each protein (*top section*). The Gal4DBD, for example, has a half-life of 100 min, whereas the Gal4DBD(2–147) fusion has a half-life of 20 min. We also measured the ability of each protein to activate a reporter gene in HeLa cells (transcription activity; *bottom*). Note the close correlation between the two activities. (This figure was constructed from data originally published by Salghetti et al. 1999)

signal both proteolysis and gene activation, and they rely on similar residues for both activities, but proteolysis is disconnected from the



**Fig. 2.** TADs and degrons overlap in many transcription factors. The figure shows the domain structure of 27 transcription factors—2 bacterial ( $\sigma^{32}$  and  $\sigma^S$ ) and 25 eukaryotic—that have overlapping TADs and degrons. TADs are *gray*, degrons are *striped*, and *red* indicates an overlap between the two. (This figure was compiled from references listed in Muratani and Tansey 2003 and additional references: Chen et al. 2005; Minegishi et al. 2005; Pierson-Mullany and Lange 2004; Rasti et al. 2006; Salghetti et al. 2000; Schnappauf et al. 2003; Sundqvist and Ericsson 2003; Ying et al. 2005)

process of transcriptional regulation. In the second model, the activator is destroyed as part of the events that lead to gene induction. The latter model, which we favor, posits that activating transcription per se is a signal for activator destruction, and that the correlation between TAD and degron function reflects the fact that activators are destroyed



**Fig. 3a,b.** Activating transcription is sufficient to signal protein destruction. In this experiment, the bacterial DNA-binding protein LexA was fused to a 17 amino acid segment from the Gal4 dimerization domain (LexG; Barberis et al. 1995). In cells expressing wild-type Gal11, the LexG protein cannot activate transcription (**a**) and is metabolically stable (**b**; *top panel*). In cells expressing a mutant form of Gal11 that allows LexG to activate transcription (**a**), however, the LexG protein is unstable (**b**; *lower panel*). This result demonstrates that, in the absence of a bona-fide TAD, activation of transcription per se is a signal for activator turnover

at a rate that depends on the potency with which they stimulate transcription.

There is certainly support for the latter model, particularly from studies of Ericsson and colleagues, who have shown that destruction of the SREBP transcription factor requires not only its potent TAD but also the ability of SREBP to bind promoter DNAs (Sundqvist and Ericsson 2003). In addition, Deshaies and colleagues (Chi et al. 2001) have demonstrated that destruction of the yeast transcription factor GCN4 is dependent on its phosphorylation by Srb10, a kinase that is intimately tied to the basal transcriptional machinery. But there is always the possibility in these types of studies that transcription factors could be interacting separately with the transcription and Ub–proteasome systems. To ask, therefore, whether activating transcription is a sufficient signal for protein destruction, we examined a system in which an inert DNA-binding domain (DBD) can be converted into a transcriptional activator (Fig. 3), as described by Ptashne and colleagues (Barberis et al.

1995; Himmelfarb et al. 1990). In this system, the bacterial LexA DNA-binding protein is fused to a 17 amino-acid sequence from the Gal4 protein (G). This Gal4 sequence is not a TAD—it is derived from within the Gal4 dimerization domain—and the fusion protein (LexG) will not activate a reporter containing LexA-binding sites in yeast that express the wild-type version of Gal11, a component of the pol II mediator complex (Kim et al. 1994). If, however, Gal11 carries a single point mutation (Gal11<sup>P</sup>) that creates a fortuitous, high-affinity interaction with the Gal4 dimerization domain, the LexG protein can recruit Gal11<sup>P</sup> to promoter DNAs, which results in the recruitment of the pol II holoenzyme and activation of transcription (Fig. 3a). Thus, by making a single point mutation, the LexG protein can be converted from a DNA binding protein incapable of activating transcription to a potent transcriptional activator.

We compared the metabolic stability of LexG in cells that expressed either the Gal11<sup>WT</sup> or Gal11<sup>P</sup> proteins (Fig. 3b). In WT cells, where LexG was unable to activate transcription, it was metabolically stable, with a predicted half-life of well over 40 min. In the presence of the Gal11<sup>P</sup> protein, however, LexG was very unstable, with a half-life of less than 5 min. Thus a single amino acid difference between the two cell types not only converts LexG into an efficient activator of transcription, but also makes it a highly unstable protein. This result shows that the ability to activate transcription is sufficient to signal proteolysis. We conclude from these studies, and others, that TADs and degrons overlap in many transcription factors because the act of stimulating transcription is obligatorily coupled activator proteolysis. This conclusion leads to the somewhat paradoxical realization that potent transcription factors may be present at their lowest amounts when they are their most active.

#### **4 How Ub-Dependent Proteolysis Contributes to Transcription Factor Activity**

From a teleological perspective, the metabolic instability of potent transcriptional activators makes sense. Regulation of gene transcription is critical for the maintenance of cellular homeostasis, and it is important that the regulators of transcription are themselves highly regulated. Destroying proteins in a way that depends on their activity offers the most

potential for controlling their function. There is, however, another significant implication of the tight connection between TADs and degrons. If the UPS system has the ability to sense the activity of transcriptional regulators, then it is very likely that, at some point, direct connections between the transcription and Ub–proteasome systems must occur. This implication prompted us to ask whether the UPS is simply an enforcer of transcription factor regulation, or whether it is mechanistically involved in the process of gene activation.

Our strategy was to probe the role that Ub ligases play in the activity of their cognate transcription factors. Traditional views would argue that loss of a Ub ligase would stabilize its transcription factor target, resulting in an increase in transcription. While studying the synthetic activator LexA–VP16 in yeast (Salghetti et al. 2001), we demonstrated that its ubiquitylation and destruction was dependent on the F-box protein Met30. Deletion of the *Met30* gene stabilized LexA–VP16 and promoted its destruction. Importantly, deletion of *Met30* also blocked the ability of LexA–VP16 to activate transcription (Salghetti et al. 2001), while leaving the activity of other activators (whose destruction was not dependent on Met30) intact. We found that we could rescue LexA–VP16 activity in  $\Delta met30$  yeast by simply fusing Ub in-frame at the amino-terminus of LexA–VP16, arguing that the sole essential function of Met30 in LexA–VP16 activity is to ubiquitylate the protein. The requirement for activator ubiquitylation in VP16 TAD function is consistent with the close relationship between TAD and degron function and led us to propose a licensing model for transcriptional regulation. In this model, ubiquitylation of the activator directly couples its activity to its destruction, essentially granting a license to the activator for a limited period of activity before it is destroyed. A similar requirement for Ub ligases in the activity of other transcription activators (Kim et al. 2003; Lipford et al. 2005; Muratani et al. 2005; von der Lehr et al. 2003)—and co-activators (Barboric et al. 2005; Kurosu and Peterlin 2004; Wu et al. 2007)—has been described.

We originally reported that fusion of a single Ub moiety was sufficient to rescue LexA–VP16 function in *met30*-null cells. This finding has led to the notion that monoubiquitylation stimulates the activity of transcription factors without signaling their proteolysis. In the case of Gal4, for example, it has been argued that monoubiquitylation of the

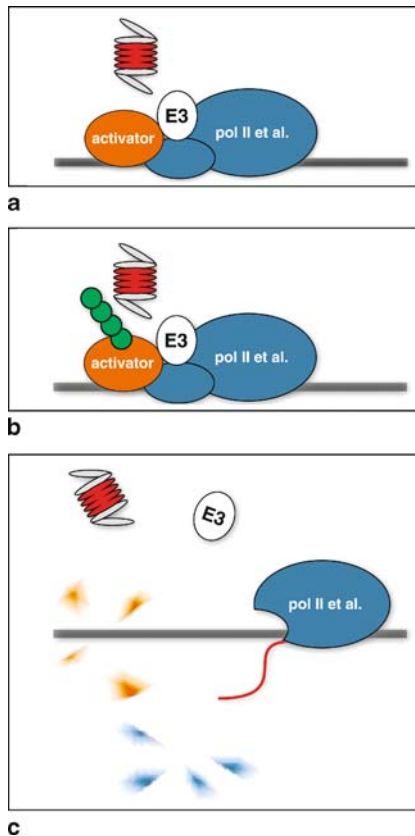
protein allows it to resist stripping from promoter DNAs by the 19S proteasome (Ferdous et al. 2007), thereby promoting its function. It has also been argued that inhibition of the proteolytic activity of the proteasome by MG132 in yeast does not effect Gal4 activity (Nalley et al. 2006), bolstering the notion that there is a disconnect between activation by ubiquitylation vs destruction by ubiquitylation.

We do not find the above argument compelling for a number of reasons. First, we have found that attachment of a single Ub moiety to LexA–VP16, as reported in 2001 (Salghetti et al. 2001) triggers a substantial amount of LexA–VP16 polyubiquitylation, some of which occurs within the fused Ub moiety (W. Tansey, unpublished observations). Thus the concept that Ub–LexA–VP16 is exclusively mono-ubiquitylated is invalid, and it could be possible that the effects of a single Ub fusion could be mediated via a polyubiquitin chain. Second, Gal4 has not been demonstrated to be monoubiquitylated in yeast cells. Rather, the Ferdous et al. study showed that Gal4 was monoubiquitylated *in vitro* in extracts from human HeLa cells (Ferdous et al. 2007). Third, there are few, if any, examples, of activators accumulating in their monoubiquitylated forms. Fourth, there is a tight relationship between activator activity and destruction, with the destruction elements of most transcription factors overlapping with the TADs (see Sect. 2). The extent of this relationship implies that a destruction-associated type of ubiquitylation (i.e., polyubiquitylation) is connected to activity more closely than a regulatory monoubiquitylation event. Fifth, we have shown that activating transcription per se is a signal for activator turnover (Fig. 3): if monoubiquitylation is connected to activity, then we would not expect the LexG protein to be so unstable in Gal11<sup>P</sup> cells. Finally, the role of the proteolytic activity of the proteasome in GAL gene activation is controversial. Although Nalley et al. (2006) reported that MG132 has little if any effect on Gal4 activity, we have not been able to reproduce this result (W. Tansey, unpublished data). Moreover, the Deshaies laboratory (Lipford et al. 2005) has previously reported that MG132 inhibits activation by both the Gal4 and Gcn4 activators. It is for these reasons that we favor a model in which proteolysis is mechanistically coupled to activator function.

Our view of the relationship between transcription factor activity and destruction is depicted in Fig. 4. In this model, transcription factors re-

cruit core components of the transcriptional machinery, as well as a Ub ligase(s) and the 26S proteasome, to promoter DNAs. Coincident with initiation, the activator is ubiquitylated and destroyed, promoting the disassembly of the preinitiation complex and transition to complexes that are competent for productive elongation of transcription. Destruction of the activator prevents subsequent rounds of activator-dependent initiation and keeps promoter function responsive to regulatory cues from outside the nucleus. By analogy to the cell cycle, proteolysis not only provides the energy for alterations in protein subunit composition, but also drives the process in a single, highly regulated, direction. This model is based on ones originally proposed by Deshaies and colleagues (Lipford and Deshaies 2003) and makes a number of predictions that we are currently investigating.

As a final point, it must be emphasized that the licensing model cannot apply to all examples of unstable transcription factors. There are clear examples in the literature of transcription factors that are inhibited by their cognate Ub ligases: HIF1 (Maxwell et al. 1999) and p53 (Fuchs et al. 1998; Haupt et al. 1997) are inhibited by the VHL and Mdm2 Ub ligases, respectively, even though they have overlapping TADs and degrons. How can ubiquitylation activate a transcription factor under one set of circumstances and antagonize its function under another? Our study of Gal4 (Muratani et al. 2005) provided insight into how this may occur. We showed that Gal4 is under the control of two distinct F-box proteins, Grr1 and Mdm30, which act on Gal4 under different conditions. When Gal4 is inactive (growth on raffinose-containing media), its destruction is mediated by Grr1. Deletion of Grr1 causes Gal4 to accumulate and promotes ectopic GAL gene activation. This scenario is consistent with classic views of the role proteolysis plays in protein function. When Gal4 is activated, however (in the presence of galactose), the Grr1 pathway is shut down, and Gal4 destruction is now mediated by Mdm30. Deletion of Mdm30 causes Gal4 to accumulate and promotes ectopic GAL gene activation, consistent with the licensing model. The simplest way to reconcile these observations is to imagine that the role ubiquitylation plays depends on where and when it occurs. If an activator is ubiquitylated under nonactivation conditions (e.g., unbound to promoter DNA or actively repressed, etc.) its levels will be reduced and its function blocked. If, however, an activator is in the process of stimu-



**Fig. 4a–c.** A model for how activator ubiquitylation and proteolysis impacts transcription. **a** In the process of stimulating gene expression, the activator recruits core components of the transcriptional machinery (*pol II et al.*), a Ub ligase (*E3*), and the proteasome to chromatin to form a preinitiation complex. **b** As a result of its actions, the activator is ubiquitylated. **c** The activator is then destroyed by the proteasome, possibly together with some other component of the transcriptional machinery. Destruction of the activator in this way not only terminates the signal to activate, but also promotes disassembly of the preinitiation complex, allowing *pol II* to transcribe the gene to produce a functional messenger RNA

lating transcription, a scenario similar to one outlined in Fig. 4 will play out, and the destruction of the protein will ultimately be required for its function. Thus, activators can interact with the UPS in two distinct modes, with two distinct outcomes.

## 5 Role for the Proteasome in Transcription

As described above, connections were made between the proteasome and transcription early in the characterization of the proteasome itself. Although the tide of popular opinion turned toward the notion that Sug1 and Sug2 mutants affected transcription in an indirect way, it now seems clear that there is a direct role for the proteasome in multiple aspects of gene transcription. We recently reviewed the transcriptionally relevant activities of proteasome components (Collins and Tansey 2006), so we will not go into depth on this subject here. But it is worth emphasizing that both proteolytic and nonproteolytic components of the proteasome associate with genes in a manner that correlates with gene activity (Auld et al. 2006), and that both of its key functions—as an ATP-dependent protein chaperone and a protease—are implicated in gene regulation.

The licensing model, and its variants, predict that proteolysis by the proteasome is inexorably coupled to gene activation. In this model, proteasomal proteolysis is required to disassemble preinitiation complexes by destruction of the activator and, conceivably, other proteins. Such models are supported by reports showing that chemical inhibition of the proteasome attenuates transcriptional activation by the progesterone receptor, Gcn4, Gal4, and others (Collins and Tansey 2006; A. Leung et al., unpublished data). They are also supported by the finding that multiple 20S subunits of the proteasome are recruited to chromatin (Gillette et al. 2004; Morris et al. 2003; A. Leung et al., unpublished data) in a manner that depends on both transcriptional activators and histone H2B ubiquitylation (Ezhkova and Tansey 2004; A. Leung et al., unpublished data). We argue, therefore, that the proteolytic activities of the 20S proteasome are likely to contribute to gene activation.

There is also a significant body of work demonstrating that the ATPases of the 19S base complex are also important for transcription. The Sug mutants are both AAA-type ATPases that possess classic protein

chaperone activity (Braun et al. 1999), giving rise to the notion that this chaperone function serves to remodel protein complexes that participate in multiple steps in transcription, including transcriptional elongation (Ferdous et al. 2001) and histone modification (Ezhkova and Tansey 2004). Although a compelling model for how Sug mutants suppress partial loss of the Gal4 TAD has not been proposed, it is reasonable to conclude that these mutants reflect the direct involvement of 19S ATPase function in one or more key aspects of transcription. Indeed, in collaboration with the Workman laboratory (Lee et al. 2005), we demonstrated that the ATPase activity of the 19S base complex can stabilize the interaction of the Gal4 TAD with one of its key transcriptional targets—the SAGA complex—in vitro, revealing that, in a defined biochemical system, the 19S proteins can indeed function as a transcriptionally-relevant ATPase.

There is some indication that the 19S ATPases function in a complex, referred to as APIS (Sun et al. 2002), that is distinct from the 26S proteasome. The relevance of APIS is supported by chromatin immunoprecipitation (ChIP) experiments showing differential temporal and spatial patterns of interaction of 19S and 20S subunits with the *GAL* genes (Gillette et al. 2004). APIS also receives support from our finding that the 19S base complex is sufficient for stabilization of the Gal4–SAGA interaction (Lee et al. 2005). It is critical to emphasize, however, that APIS has not been demonstrated to exist as a distinct complex in cells, and that ChIP experiments can be influenced profoundly by epitope accessibility; a protein could be bound to chromatin in such a way that the relevant epitope was masked at specific stages in transcription, making it invisible in the ChIP analysis. It is also critical to emphasize that there is no need to develop models that posit distinct 19S and 20S complexes. The ATPase activities of the 19S base complex are clearly functional within the context of the intact proteasome, and they are likely to unfold proteins on the outer surface of the ATPase ring (Navon and Goldberg 2001). In this way, even if the 19S ATPases act at the critical, rate-limiting, step in transcription, they could do so as part of the full 26S complex.

Finally, one particularly intriguing aspect of the 26S proteasome is that it carries with it a number of distinct biochemical activities. In addition to performing protein unfolding and proteolysis, the proteasome

is also a deubiquitylating enzyme, a Ub-binding protein, a DNA helicase (Fraser et al. 1997), and a Ub ligase (Crosas et al. 2006). Although transcriptional roles for these other activities have not been established, it is easy to imagine how these diverse functions could modulate multiple steps in transcription. We predict that studies to date have only scratched the surface of ways in which this unique multipurpose protein machine contributes to gene regulation.

## 6 Implications for Cancer

Perhaps one of the strongest testimonials of the critical role of gene regulation in eukaryotes is the relationship between deregulation of transcription and cancer. Many oncogenes and tumor suppressors encode proteins that regulate transcription, such as p53, Myc, Jun, Fos, and Rb, and aberrant patterns of gene expression are a hallmark of cancer. Given that transcription factors are linked to cancer, and that they are regulated by the UPS, we expect that the intersection of transcription and the Ub–proteasome systems will also be important for oncogenesis.

At present, there are only a handful of studies that have implicated the transcription/UPS connection as important in human cancer. The Eilers group has reported that the HectH9 Ub ligase builds nonproteolytic K63-linked poly-Ub chains on the oncoprotein transcription factor Myc (Adhikary et al. 2005). In addition, we and others (Kim et al. 2003; von der Lehr et al. 2003) have provided evidence that the regulation of Myc ubiquitylation by the F-box protein Skp2 is important for its transcriptional activity. Consistent with the licensing model, Skp2-mediated ubiquitylation of Myc stimulates Myc function while also targeting Myc for enhanced destruction. The role of Skp2 in Myc activation is intriguing because, like Myc, Skp2 is an oncoprotein, suggesting that upregulation of Skp2 in certain cancers (Gstaiger et al. 2001) drives oncogenesis by increasing the activity of another oncoprotein, Myc. Consistent with this idea, we found that Myc is essential for the ability of Skp2 to drive ectopic cell proliferation *in vitro* (Kim et al. 2003). Together, these observations reveal that ubiquitylation of at least Myc is important for its oncogenic activities. Given that Myc is responsible for about one-third of all cancer deaths in the United States, the

UPS/transcription connection via Myc alone could have a profound impact on human cancer.

It is also tempting to imagine that the clinical success of the proteasome inhibitor Velcade (Kane et al. 2003) is in some way due to effects on transcription. Although this is highly speculative, inhibition of the proteasome does affect the activity of several cancer-relevant transcription factors such as the estrogen (Nawaz et al. 1999) and progesterone receptors (Lonard et al. 2000). Perhaps, therefore, treating patients with Velcade not only retards the progress of cancer by blocking bulk protein turnover, but also by inhibiting the activity of key transcriptional regulators. Determining if such a phenomenon exists, and revealing the factors that are involved, may have potential for the design of specific Ub-ligase inhibitors that can selectively kill cancer cells by targeting specific subsets of transcriptional regulators.

## 7 Summary

It appears clear that transcription, like most critical cellular processes, is regulated at many levels by the Ub–proteasome system. Although we focused here mostly on proteolytic roles for Ub, it should be stressed that there are many excellent examples in which Ub functions nonproteolytically to influence gene activity (e.g., Gwizdek et al. 2006; Sun and Allis 2002). Given the pace with which research in this area is gaining momentum, it is very likely that additional examples, of proteolytic and nonproteolytic activities of the UPS in transcription will surface in the near future.

What are the key questions that need to be addressed at this stage? One important priority is to understand how Ub ligases are recruited to transcriptional activators: what are the mechanisms that allow Ub ligases to sense when a transcription factor is activator? Another priority is to clearly define the role that activator ubiquitylation plays vs proteolysis and to define the steps in transcription that are controlled by this process. As for the proteasome, it is critical that the nature of the proteasome that participates in transcription (i.e., 19S, 20S, or 26S) is defined, as are the relevant targets of the proteasome components. It will also be important to determine whether additional biochemical activities

of the proteasome contribute to gene regulation. Answering these questions will require identification of additional transcription factors that are controlled by the UPS, isolation of mutants that can tease apart proteolytic vs nonproteolytic activities of the various components, and biochemical reconstitution experiments to allow underlying mechanisms to be deciphered. We have our work cut out for us.

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## ***Ubiquitination of Myc: Proteasomal Degradation and Beyond***

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J. Müller, M. Eilers<sup>(✉)</sup>

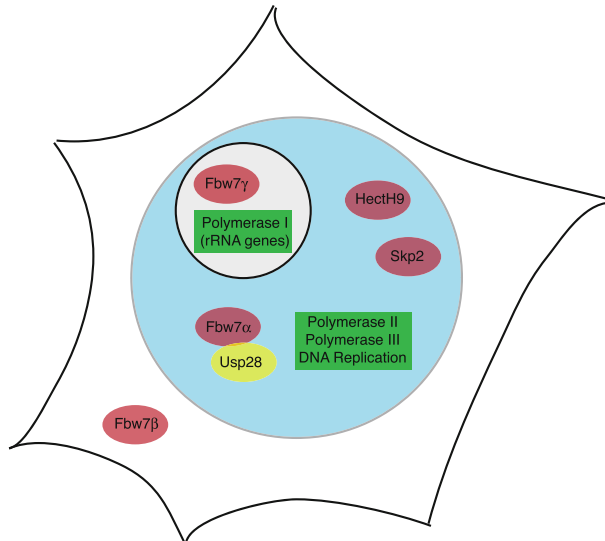
Institute of Molecular Biology and Tumor Research, Philipps-University Marburg,  
Marburg, Germany  
email: [eilers@imt.uni-marburg.de](mailto:eilers@imt.uni-marburg.de)

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**Abstract.** The level of Myc proteins is a critical determinant of cellular proliferation and apoptosis. Ubiquitination of Myc plays a key role in controlling protein levels by stimulating proteasomal degradation of the protein. Some experiments suggest that ubiquitination may also regulate Myc function in addition to turnover. This review attempts to summarize current knowledge about this field.

### **1 Introduction**

Mammalian *MYC* genes comprise a small gene family that has five members, three of which have been implicated in the genesis of multiple human tumors; these are called *c-MYC*, *MYCN*, and *MYCL* and are thought to differ mainly in their expression pattern, although some functional differences have been reported. All three genes encode phospho-



**Fig. 1.** Intracellular localization of Myc function and turnover. The diagram shows the intracellular localization of ubiquitin ligases and deubiquitinating enzymes that have been implicated in the turnover of Myc proteins and the functions of Myc proteins in each compartment

proteins that are predominantly localized in the nucleus (Adhikary and Eilers 2005; Oster et al. 2002). FRAP experiments show, however, that c-Myc, the most intensely studied of the Myc proteins, rapidly shuttles between nucleolus, nucleus, and the cytosol, arguing that the steady-state distribution of Myc proteins is the result of a highly dynamic and probably also a highly regulated process (Arabi et al. 2003) (see Fig. 1).

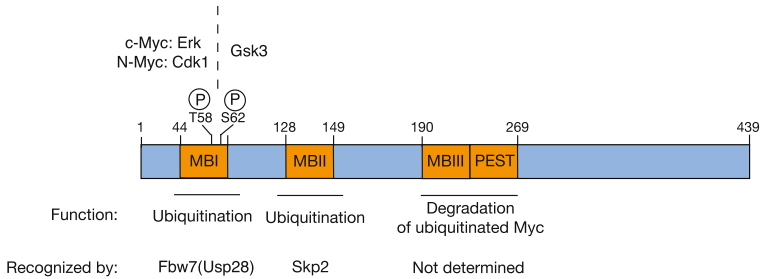
Myc proteins are transcription factors that both activate and repress distinct sets of target genes. In addition, they appear to have direct functions in controlling DNA replication (Dominguez-Sola et al. 2007). As transcription factors, Myc proteins appear to be unusual in two respects: first, they occupy a staggeringly large number of binding sites, recently estimated to be well over 10,000 sites per haploid genome (Chen et al. 2008; Kim et al. 2008). This generates a somewhat paradox situation in the sense that the number of Myc molecules in nontransformed cells

is probably significantly lower than the number of binding sites in the genome; if so, each binding site is likely to be occupied only transiently. Second, Myc proteins can activate transcription mediated by all three eukaryotic RNA polymerases, including RNA polymerase I, which transcribes genes encoding the ribosomal RNA precursor in the nucleolus (Arabi et al. 2005; Grandori et al. 2005). Consistently, Myc proteins accumulate in the nucleolus, most notably in cells treated with inhibitors of the proteasome (Arabi et al. 2003). The mechanisms, by which Myc affects the expression of its target genes, and the identity and relevance of target genes have been reviewed recently and will not be discussed here.

In nontransformed cells, Myc proteins are thought to translate growth factor-dependent signals into changes in gene expression, thereby altering cell behavior (e.g., by stimulating cell proliferation and cell growth). This notion is supported by several key findings: first, expression of the different *MYC* mRNAs is tightly regulated by external growth factors in nontransformed cells and this control is frequently disrupted by oncogenic mutations during transformation (He et al. 1998). Second, the activation of conditional alleles of Myc in tissue culture and in vivo is often sufficient to induce strong changes in cell proliferation, cell growth, and also in apoptosis (Pelengaris et al. 2002). Third, Myc proteins are very short-lived in vivo and growth factor-dependent and cell cycle-dependent pathways regulate their turnover (Lüscher and Eisenman 1988). This latter aspect is the central topic of this review.

## 2 Turnover of Myc

In nontransformed cells, (c-)Myc rapidly turns over with a half-life of approximately 20–30 min; similar values have been reported for N-Myc. Degradation of Myc proteins is mediated by the proteasome, as documented by numerous observations showing that inhibition of the proteasome greatly extends the half-life of Myc (Bonvini et al. 1998; Gross-Mesilaty et al. 1998). The proteasome usually recognizes ubiquitinated proteins; consistent with this observation, Myc proteins are extensively ubiquitinated in vivo. The lysine residues of Myc that are ubiquitinated have not been determined in vivo, although it has been



**Fig. 2.** MycBoxes and their function in Myc turnover. Localization of four sequence elements in Myc proteins that have been implicated in Myc ubiquitination and turnover. The primary sequence of PEST element defined in c-Myc is evolutionarily poorly conserved and therefore not considered a MycBox

described that a mutant allele of Myc lacking six lysine residues is less ubiquitinated by one of the ubiquitin ligases of Myc, HectH9 (Adhikary et al. 2005). The half-life of Myc proteins can be extended in tumor cells, arguing that degradation of Myc is a target for oncogenic mutations (Malempati et al. 2006). Indeed, by now different mutations have been found that impinge on Myc turnover; several of these will be discussed below.

Three domains of the Myc protein are necessary to mediate its turnover: since they were initially discovered as short, evolutionarily highly conserved stretches of amino acids in an otherwise less conserved family of proteins, they were termed MycBoxes (see Fig. 2).

The best understood domain in terms of degradation is MycBoxI, located close to the amino-terminus of Myc: this is the binding site for one of the ubiquitin ligases that mediate Myc turnover, Fbw7 (Welcker et al. 2004b; Yada et al. 2004). Point mutations within MycBoxI therefore stabilize the Myc protein and reduce its ubiquitination (Bahram et al. 2000). Such point mutations in MycBoxI are frequently found in lymphomas; in contrast, no point mutations of Myc have been found in solid tumors.

In addition, both MycBoxII and MycBoxIII affect Myc turnover. Deletion of MycBoxIII stabilizes Myc and leads to the accumulation of ubiquitinated Myc in tissue culture experiments (Herbst et al. 2004,

2005). This would argue that MycBoxIII, in contrast to MycBoxI, promotes turnover at a postubiquitination step; similar observations have also been made for the deletion of a PEST sequence that is adjacent to MycBoxIII (Gregory and Hann 2000). The mechanism(s) that prevents degradation of ubiquitinated Myc in both deletion mutants have not been resolved. Finally, MycBoxII serves as a binding site for Skp2, an F-box ubiquitin ligase that has been implicated in Myc turnover (Kim et al. 2003; von der Lehr et al. 2003).

One of the most interesting and also puzzling aspects of the Myc biology is the tight link between Myc turnover and its biological functions. One example for this is provided by the mutations in MycBoxI, which are found in lymphomas (Hemann et al. 2005). Such mutations not only enhance Myc stability as described above, but also strongly decrease the ability of Myc to induce apoptosis. As a result, lymphomas that are generated using these mutant alleles of Myc lack mutations in either the p53 gene or its upstream regulators that are invariably found in lymphomas arising with wild-type Myc. Two explanations can account for these observations: first, it is possible that the phosphodegron in MycBoxI is also recognized by other proteins that mediate effector functions of Myc. Consistent with this suggestion, phosphorylation at S62 not only regulates Myc stability, but also controls the spectrum of target genes that are regulated by Myc: for example, upregulation of gamma-glutamyl-cysteine synthetase expression by Myc requires Erk-dependent phosphorylation of S62 (Benassi et al. 2006). Alternatively, ubiquitinated Myc may itself have effector functions that differ from nonubiquitinated Myc.

### 3 Ubiquitin Ligases and Their Regulation

There are three ubiquitin ligases for which there is evidence that they can ubiquitinate Myc *in vivo*.

Best understood is the SCF<sup>Fbw7</sup> complex; this complex binds to the phosphodegron that is contained in MycBoxI and, upon binding, targets Myc for degradation. Several pieces of evidence document a role for the Fbw7 subunit of the SCF<sup>Fbw7</sup> complex in Myc turnover: for example, cells deficient for Fbw7 show elevated steady state levels of Myc

and an increased half-life of the protein (Onoyama et al. 2007; Yada et al. 2004). Consistent with this role, human *FBW7* has properties of a tumor suppressor gene; point mutations in the substrate recognition domain and missense mutations are found in colon carcinoma and other tumor entities (Mao et al. 2004). These mutations often affect only one of the two *FBW7* alleles, suggesting that *FBW7* may be a haploinsufficient tumor suppressor gene. Since Fbw7 proteins form homodimers, an alternative model proposes that the protein encoded by the mutated allele might have dominant-negative properties since it sequesters the wild type protein onto nonfunctional heterodimers (Welcker and Clurman 2008). A third possibility is to suggest that some degree of ubiquitination by Fbw7 has a positive function in Myc biology.

Notably, there are three isoforms of Fbw7, which arise by alternative splicing of the same primary RNA transcript (Grim et al. 2008; Welcker et al. 2004a). All three isoforms share a common carboxyl-terminus, which contains the substrate-recognition domain and therefore are identical in their ability to bind phosphorylated substrates. They differ, however, in their amino-terminus and subcellular localization: Fbw7 $\alpha$  is localized in the nucleus, Fbw7 $\beta$  localizes to the cytosol and Fbw7 $\gamma$  is found in the nucleolus; furthermore, the isoforms differ in their ability to interact with the ubiquitin-specific protease, Usp28 (see the next section). The specific localization of Fbw7 isoforms may also be responsible for the observation that Myc proteins accumulate to very high levels in the nucleolus after inhibition of the proteasome. This has been taken as evidence that the rate of turnover of Myc in the nucleolus is very high.

To initiate degradation by Fbw7, a threonine residue (T58) in Myc-BoxI needs to be phosphorylated. In both c-Myc and N-Myc, glycogen synthase kinase 3 (Gsk3) is the kinase responsible for the phosphorylation of this residue (Welcker et al. 2004b; Yada et al. 2004). Since Gsk3 in turn is phosphorylated and inhibited by Akt, the stability of Myc proteins depends on an active PI3kinase/Akt pathway (Sears et al. 1999). Conversely, there is evidence that maintaining Myc stability is a key function of this pathway during oncogenic transformation (Yeh et al. 2004). Gsk3 needs a priming phosphorylation and this is provided by phosphorylation of serine 62. The mitotic cyclin B/Cdk1 complex phosphorylates S62 in N-Myc, and as a result degradation of N-Myc is

initiated during mitosis (Sjostrom et al. 2005). This mitotic degradation of N-Myc is important in terminating the proliferation of neuroblasts during the development of the central and most likely also the peripheral neural system (Otto et al., unpublished observations). In contrast, Map-kinases phosphorylate S62 in c-Myc (Benassi et al. 2006).

One important and still unresolved issue is the exact phosphorylation status of Myc proteins that are degraded by Fbw7; if differentially phosphorylated forms of Myc regulate different groups of target genes, the selective degradation of distinct phosphorylated Myc-species by Fbw7 may be an important tool to regulate Myc function *in vivo*. For c-Myc, several pieces of evidence suggest that isomerization of the Proline 61-peptide bond by the prolyl-isomerase Pin1 and subsequent dephosphorylation of S62 by protein phosphatase 2A (PP2A) is required for degradation by Fbw7, arguing that the substrate for Myc turnover is exclusively phosphorylated at T58 (Arnold and Sears 2006; Yeh et al. 2004). However, Fbw7 also recognizes a peptide corresponding to the doubly phosphorylated site *in vitro* (Yada et al. 2004). *In vivo*, Fbw7 recognizes both the mono- and the biphosphorylated phosphodegron of cyclin E, another target of the ligase, yet the mode of recognition is different (Welcker and Clurman 2007). The biphosphorylated degron has a higher affinity for Fbw7 and can be degraded by monomeric Fbw7, whereas the monophosphorylated degron binds Fbw7 with lower affinity and needs Fbw7 dimers to be degraded; this would argue that dephosphorylation at S62 reduces the affinity of Fbw7 for Myc. Furthermore, Fbw7-dependent degradation of N-Myc does not require Pin1, although the sequence of the phosphodegron is identical (Sjostrom et al. 2005). Clearly, more work appears to be necessary to resolve this issue.

The second ubiquitin ligase that can both ubiquitinate and induce proteasomal degradation of Myc is Skp2. Binding of Skp2 to Myc does not require MycBoxI; instead, the integrity of MycBoxII is required for this interaction, although the binding may be indirect (Kim et al. 2003; von der Lehr et al. 2003). Consistent with the idea that Skp2 and Fbw7 recognize Myc via different domains, both proteins regulate Myc turnover independently and additively, as witnessed by the analysis of cells that are either singly or doubly depleted for Fbw7 and Skp2 (Yada et al. 2004). The analysis of the interaction of Skp2 with Myc provides a second clear example for the close link between ubiquitination and

Myc function: on one hand, Skp2 has clearly been implicated in Myc degradation; on the other hand, Skp2 itself has oncogenic properties. For example, deregulated expression of Skp2 is sufficient to transform primary cells in conjunction with oncogenic alleles of Ras (Gstaiger et al. 2001). A possible explanation for this paradox could be that Skp2 is also essential for both transcriptional activation and repression by Myc, suggesting that ubiquitinated Myc has a specific role in both processes (Kim et al. 2003; von der Lehr et al. 2003).

The third ubiquitin ligase implicated in Myc ubiquitination is the Hect-domain protein HectH9/Huwe1/Arf-Bp1/Mule (Adhikary et al. 2005). HectH9 binds both endogenous c- and N-Myc proteins at an yet unidentified binding site.

HectH9 polyubiquitinates c-Myc via lysine 63 (K63) linkage and appears not to be involved in turnover. Instead, ubiquitination of c-Myc is required to enhance transcriptional activation of several Myc target genes and to recruit the p300 co-activator protein to target promoters (Adhikary et al. 2005). Consistent with these observations, expression of HectH9 is strongly upregulated in multiple human tumors and depletion of HectH9 leads to inhibition of tumor cell proliferation in all phases of the cell cycle (Adhikary et al. 2005). Surprisingly, this situation is different in primary cells that depend on N-Myc for proliferation, such as ES cells. In contrast to its interaction with c-Myc, HectH9 assembles a predominantly K48-linked chain on N-Myc and has a role in catalyzing N-Myc turnover; as a result, inhibition of HectH9 enhances proliferation of these cells (Zhao et al. 2008).

One puzzling aspect of the biology of this ubiquitin ligase is that it has been isolated in a diverse number of biological contexts: initially described as a protein that degrades histones during spermatogenesis (Liu et al. 2005), HectH9 has been identified as (a) a protein that interacts with the Arf tumor suppressor protein and degrades p53 (Chen et al. 2005) (b) as a ubiquitin ligase that degrades the anti-apoptotic Mcl1 protein during DNA damage-induced apoptosis (Zhong et al. 2005), (c) as a substrate for the Atm protein kinase (Mu et al. 2007), and (d) as a ubiquitin ligase that degrades Cdc6 after DNA damage (Hall et al. 2007). Whether these diverse functional descriptions, which also imply that HectH9 can be active in different subcellular compartments, reflect a common biochemical pathway or whether they indicate that the same

protein carries out largely unrelated biological functions is currently an open question.

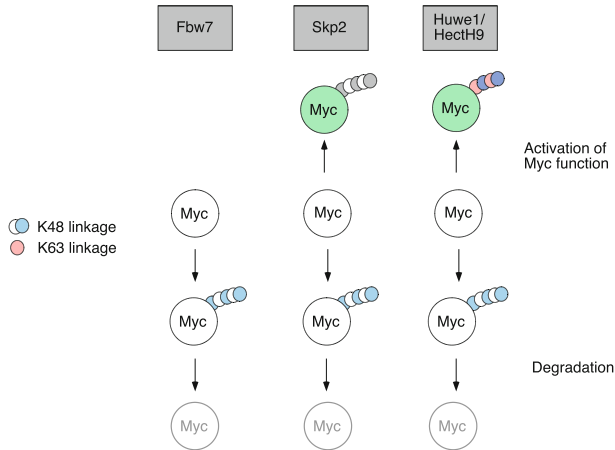
#### **4 Reversibility of Ubiquitination**

Ubiquitination of Myc by Fbw7 can be reverted by the ubiquitin-specific protease, Usp28, which was identified in a siRNA-screen searching for proteins that are required for Myc function (Popov et al. 2007b). Depletion of Usp28 destabilizes Myc by facilitating turnover by Fbw7. Usp28 is localized in the nucleus, but not in the nucleolus, and can form a ternary complex with Myc and Fbw7 $\alpha$ , but not with Fbw7 $\gamma$ , providing a potential explanation why Myc is highly unstable in the nucleolus. The findings suggest that in proliferating cells nuclear Myc may undergo one or more futile cycles of ubiquitination and deubiquitination, before it is degraded by the proteasome. This model is similar to what has been proposed for the regulation of p53 by Mdm2 ubiquitin ligase and the Hausp deubiquitinating enzyme (Li et al. 2004). In both cases, the suggestion would be that this ensures a rapid regulation of protein levels in response to environmental changes. In support of this notion, regulation of Myc protein levels in response to DNA damage involves Usp28 (Popov et al. 2007a). Notably, expression of Usp28 itself is regulated in a proliferation-dependent manner in normal colon and strongly upregulated during carcinogenesis; tissue culture experiments suggest that *USP28* may be a transcriptional target of the Wnt pathway (Popov et al. 2007b). Upregulation of Usp28 may therefore be one mechanism explaining how Myc stability is extended during tumor development.

A second ubiquitin-specific protease that has been linked to Myc function is Usp22. Usp22 is a transcriptional co-activator of Myc, which reverses the monoubiquitination of histone H2B at Myc target genes (Zhang et al. 2008).

#### **5 Ubiquitination of Myc: Beyond Turnover?**

As indicated throughout this review, a number of observations demonstrate that ubiquitination of Myc is tightly coupled to the regulation of



**Fig. 3.** Evidence for functional roles of ubiquitinated Myc. The diagram shows the three main ubiquitin ligases that have been implicated in turnover of Myc proteins together with a summary of what is known about nonproteasomal roles of Myc

its function. There appear to be two major issues that remain to be resolved (see Fig. 3).

First, two ubiquitin ligases, Skp2 and HectH9, are capable of both activating Myc's transcriptional functions and promoting Myc turnover (Adhikary et al. 2005; Kim et al. 2003; von der Lehr et al. 2003). In the case of HectH9, the assembly of K63-linked polyubiquitin chain on Myc does not target Myc to the proteasome; instead, it leads to enhanced activation. In contrast, HectH9 mediates degradation of N-Myc via the assembly of a K48-linked chain (Zhao et al. 2008); the topology of the chain that is assembled by Skp2 has not been determined. Two alternate models can explain these observations. first, assembly of a nondegradable polyubiquitin chain may prevent turnover and thereby the removal of chromatin-bound Myc from specific promoters. Consequently, this may enhance the time-of-residence of Myc at a given site on DNA. Under conditions where the total number of Myc molecules is smaller than the total number of binding sites (and therefore these sites would not be immediately refilled once Myc is removed), alterations in

residence time may have a major impact on gene regulation. Alternatively, ubiquitinated Myc may have direct effector functions that differ from nonubiquitinated, such as recruitment of the p300 histone acetyl transferase, which binds to polyubiquitin chains (Grossman et al. 2003).

Second, mutations in MycBoxI affect Myc function in a manner that does not simply reflect their effect on Myc protein levels (Hemann et al. 2005). One possible explanation would be that proteins other than Fbw7 recognize MycBoxI in a phosphorylation-dependent manner and that these proteins mediate transcriptional effector functions of Myc. This view is supported by two observations: first, the residues in Myc that are mutated in lymphomas include the phosphodegron residues that are recognized by Fbw7. However, other residues that are not thought to be involved in Fbw7 binding are also mutated, albeit at lower frequencies (Bahram et al. 2000). This would argue that the critical interaction that is disrupted by the mutations may not be the Myc/Fbw7 interaction. Second, phosphorylation at S62 in response to oxidative stress has widespread effects on the spectrum of Myc-target genes (Benassi et al. 2006). Although this observation does not rule out a model in which altered turnover has differential effects on Myc target genes, both observations together suggest that critical effector proteins bind to MycBoxI in a phosphorylation-dependent manner. If so, the specific removal of Myc species that are phosphorylated at T58 by Fbw7 might generate different functional states of Myc in vivo, a model first proposed by Amati and colleagues (Amati 2004).

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## ***Regulation of Apoptosis and Cytokinesis by the Anti-apoptotic E2/E3 Ubiquitin-Ligase BRUCE***

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C. Pohl, S. Jentsch<sup>(✉)</sup>

Department of Molecular Cell Biology, Max Planck Institute of Biochemistry,  
Am Klopferspitz 18, 82152 Martinsried, Germany  
email: [Jentsch@biochem.mpg.de](mailto:Jentsch@biochem.mpg.de)

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**Abstract.** Members of the inhibitor of apoptosis protein (IAP) family are key regulators of apoptosis as they bind and inhibit caspases and other pro-apoptotic factors. Recent findings suggest that these proteins play additional roles, e.g., in cell cycle regulation, angiogenesis, and carcinogenesis. Here, we review the function of BRUCE (BIR repeat-containing ubiquitin-conjugating enzyme), an unusual 528-kDa IAP with ubiquitin ligase activity, and describe its role in apoptosis and cytokinesis. Additionally, we discuss how these seemingly unrelated functions might be linked.

## **1 Apoptosis Regulation by Inhibitor of Apoptosis Proteins**

Programmed cell death, or apoptosis, is driven by proteases of the caspase family, which initiate and execute cell death by cleaving crucial cellular proteins (Salvesen and Duckett 2002). Caspases can be inhibited by so-called BIRPs, proteins that harbor baculovirus inhibitor of apoptosis repeats (BIRs). BIRPs also protect cells from apoptosis by ubiquitylation and degradation of pro-apoptotic factors (Verhagen et al. 2001). The BIR domain represents a zinc-binding fold of approximately 70 amino acids harboring a CX<sub>2</sub>CX<sub>6</sub>WX<sub>3</sub>DX<sub>5</sub>HX<sub>6</sub>C consensus sequence (Hinds et al. 1999). Notably, linker sequences at the boundaries of BIR domains have been shown to bind to activated caspases, thereby sterically preventing access to substrates (Riedl et al. 2001; Huang et al. 2001; Chai et al. 2001).

In mammalian cells, after loss of mitochondrial integrity, apoptosis-inducing factors such as Smac and the serine protease HtrA2 are released through permeability transition pores. These factors can bind to BIRPs via a short N-terminal sequence on the same surfaces where caspases bind. This results in a competition of Smac/HtrA2 and caspases for BIRP binding. Therefore, as soon as the cytoplasmic concentration of Smac/HtrA2 rises above a critical level, caspases are relieved from repression by BIRPs and can initiate apoptosis (Ditzel and Meier 2002).

## **2 BRUCE Is a Cell Death Regulator**

BIRPs of the IAP class usually contain several BIR domains and a C-terminal RING finger domain, which endows the protein with E3 ubiquitin ligase activity. BRUCE is an unusual BIRP/IAP because of its enormous size (528 kDa) and the presence of a single N-terminally located BIR domain and a C-terminally located ubiquitin-conjugating enzyme (UBC) domain. BRUCE was discovered in a screen using degenerate primers for ubiquitin-conjugating (UBC) enzymes (the E2 enzymes of the ubiquitin-conjugation pathway) (Hauser 1992). In vitro BRUCE can form a thioester with ubiquitin and can transfer ubiquitin to substrate proteins, demonstrating that it functions as a chimeric

E2/E3 ubiquitin ligase (Hauser et al. 1998; Bartke et al. 2004; Hao et al. 2004). Cell fractionation and membrane flotation experiments revealed that BRUCE is a peripheral, membrane-associated protein (Bardoff 1997). Furthermore, BRUCE was found to localize mainly to the TGN and perinuclear vesicles. It was also reported that cancer cells expressing high levels of BRUCE are resistant to apoptosis-inducing agents, and that conversely downregulation of BRUCE results in sensitivity to these agents (Chen et al. 1999).

The anti-apoptotic function of BRUCE is well studied in *Drosophila*. The BRUCE homolog, dBRUCE, was found to inhibit apoptosis when executed by the Smac/HtrA2-related pro-apoptotic factors Reaper and Grim, but not Hid (Vernooy et al. 2002), suggesting that dBRUCE plays a more specialized role in cell death regulation. Indeed, dBRUCE is crucial for the regulation of the concluding step of spermatogenesis in *Drosophila* when spermatids remove their bulk cytoplasm during spermatid individualization. This process is accompanied by an apoptosis-like caspase activation, which has to be locally and temporally restricted (Arama et al. 2003). Strikingly, in *dbruce*<sup>-/-</sup> flies, which are male sterile, spermatids acquire hypercondensed nuclei and finally degenerate, indicating uncontrolled apoptosis. During spermatid individualization dBRUCE was shown to interact with a testis-specific multisubunit ubiquitin ligase complex (Arama et al. 2007). dBRUCE directly binds to the substrate-recruiting subunit Khl10 of this ligase complex composed of Cullin-3 and Roc1b. Notably, the Cul3/Roc1b/Khl10 complex is required for the transient caspase activation in fly testis, and therefore probably also for dBRUCE degradation.

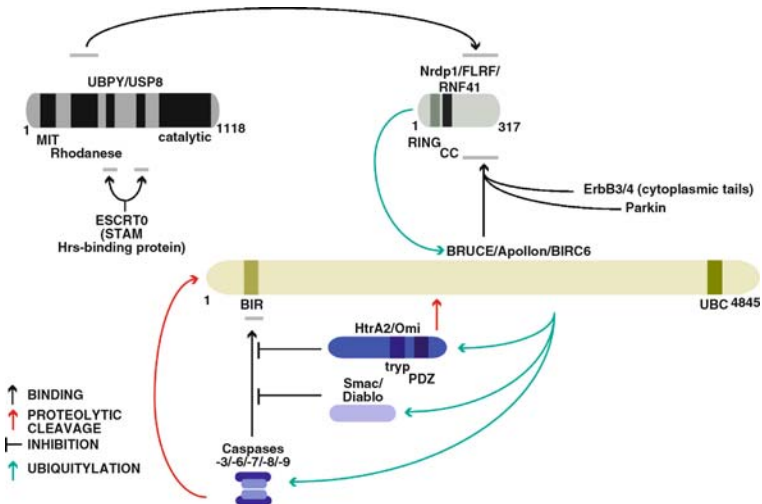
In mammalian cells, BRUCE functions as a typical inhibitor of apoptosis protein (Bartke et al. 2004; Hao et al. 2004). It can bind and inhibit activated initiator caspases-8 and -9 and executioner caspases-3, -6, and -7. Furthermore, both Smac and HtrA2 are able to compete for BRUCE-bound caspases. Interestingly, BRUCE is also a substrate of caspases and the serine protease HtrA2, pointing to a role in regulating apoptosis at early stages when proteolytic activity mediated by these enzymes is still low. Furthermore, BRUCE can ubiquitylate both Smac and HtrA2 and probably also caspase-9, a caspase initiating apoptosis after mitochondrial permeabilization (Bartke et al. 2004; Hao et al. 2004). Although only a monoubiquitylation on the substrate Smac was observed

in vitro, BRUCE might collaborate with other E3 ligases for polyubiquitylation and proteasomal degradation. This is likely also the case for other BRUCE substrates such as HtrA2 or caspases. However, since BRUCE is localized in interphase cells mainly to the TGN in interphase cells and endosomes, its anti-apoptotic activity is most likely restricted to these sites (Hauser et al. 1998). Notably, BRUCE is also downregulated by the ubiquitin-proteasome system. Nrdp1/FLRF, a RING finger-containing ubiquitin ligase initially found to be involved in the degradation of the receptor tyrosine kinases ErbB3/4 (Diamonti et al. 2002; Qiu and Goldberg 2002), can act as a ubiquitin ligase for BRUCE, leading to its proteasome-dependent degradation (Qiu et al. 2004). Nrdp1 also interacts with a deubiquitylating enzyme USP8 (UBPY) (Wu et al. 2004), a cysteine protease with the highest similarity to yeast Doa4. USP8 is implicated in cell cycle regulation, downregulation of the EGF receptor, and the regulation of the ESCRT (endosomal sorting complex required for transport) components Hrs and STAM (Clague and Urbé, 2006). Depletion of USP8 leads to an increase in the size and number of multivesicular bodies (MVBs) and an accumulation of ubiquitin on their surface (Row et al. 2006). Figure 1 illustrates the interaction of BRUCE with some of its binding partners.

### 3 Regulation of Cytokinesis

Besides apoptosis regulation, some BIRPs such as survivin (Li et al. 1999) and cIAP1 (Samuel et al. 2005) also participate in cell cycle events and cytokinesis. Survivin is a 17-kDa protein, which harbors an N-terminal BIR domain (which resembles the BIR of BRUCE) and a C-terminal coiled-coil domain. It is a core component of the chromosomal passenger complex and is hence essential for cytokinesis. Similar to BRUCE, survivin can firmly associate with Smac but shows only limited potential in inhibiting caspases in vivo (Song et al. 2003; Banks et al. 2000).

cIAP1, a predominantly nuclear protein in interphase, has recently been shown to participate in cell cycle regulation as well (Samuel et al. 2005). After nuclear reaccumulation in telophase, a small pool of cIAP1 associates with the midbody in a complex with survivin. Cells over-



**Fig. 1.** Protein interaction network of BRUCE. Schematic diagram depicting the multifunctional BRUCE (alias Apollon or BIRC6), the RING E3-ligase Nrdp1 (alias FLRF or RNF41), and the de-ubiquitylating enzyme USP8 (alias UBPY). The domain architectures and sizes (in amino acids) of the proteins are shown. Protein-protein interactions are indicated. *tryp* trypsin domain, *CC* coiled-coil, *MIT* domain in microtubule interacting and trafficking proteins, *BIR* baculovirus inhibitor of apoptosis repeat, *UBC* ubiquitin conjugating enzyme domain, *ESCRT* endosomal sorting complex required for transport

expressing cIAP1 exhibit an accumulation in G2/M, grow slower, and exhibit cytokinesis defects.

Interestingly, BRUCE-knockout mice die perinatally not because of apoptosis induction, but rather impaired placental development, which can be attributed to insufficient differentiation (Lotz et al. 2004). Indeed, we recently discovered that BRUCE, besides its role in apoptosis regulation, is a novel player of cytokinesis and central for abscission (Pohl and Jentsch 2008).

Cytokinesis is the final step of cell division in which daughter cells physically separate. The earliest discernible event during this process is the formation of a cortical actomyosin ring. Constriction of this ring leads to furrowing that generates a narrow intercellular bridge. Con-

comitantly with furrow ingression, another cytokinesis-specific structure, the midbody, assembles by bundling of spindle-midzone microtubules. In the midpoint of the intercellular bridge that results from furrowing, midbody microtubules embrace a phase-dense circular structure called the midbody ring (also called the Flemming body). Finally, during abscission, the tubular bridge is cleaved and two daughter cells are formed.

Recent work demonstrated that the midbody serves as a rigid but dynamic platform on which different processes that drive cytokinesis converge, e.g., kinase signaling, degradation of cell cycle regulators, rearrangements of membranes and of the cytoskeleton. It became clear that traffic-regulating GTPases, such as Arf1, Arf6, and Rab11, play an important role in cytokinesis (Albertson et al. 2005). These proteins seem to cooperate with the multisubunit exocyst membrane-targeting complex to deliver endosomal vesicles to the site of abscission. Interestingly, a proportion of vesicles seem to arrive at the midbody ring chiefly from only one of the prospective daughter cells (Gromley et al. 2005), suggesting an intrinsic asymmetric element in cytokinesis. Besides these proteins, only a few additional components required for membrane remodeling at the midbody are currently known. One such factor is centriolin, a protein that binds to the maternal centriole and is needed for the proper localization of the exocyst complex to the midbody ring (Gromley et al. 2005).

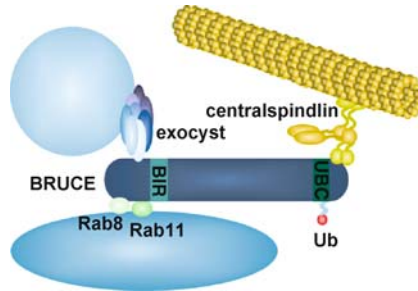
Previous studies also point to a role of ubiquitylation and proteasomal activity in cytokinesis regulation (Pines 2006). Notably, components of the ubiquitin–proteasome system are concentrated at midbodies (Grenfell et al. 1994; Wojcik et al. 1995), and several proteins that are crucial regulators of mitosis (e.g., the chromosomal passenger proteins aurora B and survivin, Polo-like kinase 1 (Plk1), and the actin-associated protein anillin) are degraded during cytokinesis (Pines 2006). Hence, the activity of the proteasome also seems to be important after anaphase onset. Interestingly, combined inhibition of Cdk1 and proteasomes with a subsequent release of inhibition in late cytokinesis can revert cells into a presumably pre-anaphase state (Potapova et al. 2006). Since the ubiquitin-controlled ESCRT pathway is also necessary for abscission (Carlton and Martin-Serrano 2007), nonproteolytic functions of ubiquitin play a role in late cytokinesis as well.

#### **4 Final Stages of Cytokinesis Are Controlled by BRUCE**

During cytokinesis, BRUCE localizes to the midbody ring where it binds mitotic regulators and components of the vesicle-targeting machinery (Pohl and Jentsch 2008). Localization to the midbody ring depends on a targeting domain in BRUCE (MTD) that comprises approximately 150 amino acids. This domain can mediate direct interactions with the midbody ring resident mitotic kinesin-like protein 1 (MKLP1). Analysis of cells that overexpress this targeting domain (which acts as a dominant-negative BRUCE construct specific for this localization), or cells that were treated with BRUCE-specific siRNAs, revealed that BRUCE is involved in the delivery of membranes to the site of abscission. Thus, the phenotypes that occur upon BRUCE depletion are most likely caused by a failure of membrane delivery and defective recruitment of mitotic regulators to the midbody ring. Notably, these phenotypes resemble those caused by centriolin depletion (Gromley et al. 2005; see above).

Several regulators of vesicular trafficking associate with the N-terminal region of BRUCE, including the GTPases Rab8/Rab11 and components of the exocyst (Fig. 2). Notably, BRUCE relocates during cell division, which appears to be driven by vesicle movements along microtubules. The majority of these vesicular structures where BRUCE localizes are large pleiomorphic traffic intermediates (Peränen et al. 1996; Urbé et al. 1993). In interphase cells, BRUCE localizes to the TGN and tubular/recycling endosomes, but during cytokinesis, a fraction travels to the midzone where it arrives specifically at the midbody. Notably, BRUCE associates with the midbody ring concomitant with its appearance in telophase, travels after completed abscission into one daughter cell together with the midbody ring, and remains bound to the discarded midbody ring until it dissolves.

BRUCE depletion also leads to cytokinesis-coupled apoptosis and the formation of elongated syncytia. Remarkably, in HeLa cells, BRUCE depletion leads to apoptosis precisely when the dividing cells attempt abscission. It should be noted, however, that BRUCE depletion in other cell types than HeLa did not induce cytokinesis-associated apoptosis but variable failures in cytokinesis. It is thus likely that cytokinesis-associated apoptosis might be restricted to cell types that require



**Fig. 2.** BRUCE as a protein-binding platform at the midbody ring. BRUCE physically links membrane targeting and midbody ring components. In addition, it might also ubiquitylate proteins of the midbody ring. The complex of MKLP1 and MgcRacGAP is described as centralspindlin. *BIR* baculovirus inhibitor of apoptosis repeat, *UBC* ubiquitin-conjugating enzyme domain, *Ub* ubiquitin

specialized IAPs or a balanced ratio of different IAPs during cytokinesis such as HeLa cells (Crnkovic-Mertens et al. 2006). Indeed, BRUCE is part of a mixed BIRP/IAP complex with survivin, suggesting that they cooperate in apoptosis regulation. BRUCE not only co-localizes with survivin at the midzone and physically interacts with this BIRP, but also monoubiquitylates the protein *in vitro*. This suggests that BRUCE might trigger survivin degradation or that ubiquitylation might control midbody dynamics.

Intriguingly, cytokinesis is accompanied by a dramatic relocation of ubiquitin. Ubiquitin first appears as striking ball-like accumulations flanking the midbody ring symmetrically. Then, after a period of perceptible absence, ubiquitin reappears directly on the midbody ring (Pohl and Jentsch 2008). Fluorescence redistribution after photobleaching (FRAP) experiments showed that ubiquitin on the midbody ring is very static, suggesting that midbody ring ubiquitylation occurs only once per cell cycle, possibly because it plays a structural role. The crucial substrates for ubiquitylation are currently unknown, but BRUCE and MKLP1 are ubiquitylated *in vivo*. Moreover, USP8, which associates with BRUCE and the midbody ring, may function as their deubiquitylating enzyme. Remarkably, partial BRUCE depletion interferes with the ubiquitin dynamics at the midbody (Pohl and Jentsch

2008), suggesting that BRUCE may directly participate in the observed ubiquitylation events.

## 5 Conclusion

The midbody ring represents a unique cellular structure (about 1.5  $\mu\text{m}$  in diameter) which requires several processes for abscission to converge. BRUCE appears to be an integral part of the ring and is required for its normal formation and integrity. Since the midbody ring is densely ubiquitylated and BRUCE possesses E2/E3 ubiquitin ligase activity (Bartke et al. 2004), it seems likely that BRUCE's ubiquitylation activity is directly involved in midbody ring function or integrity. Furthermore, BRUCE also functions as an anti-apoptotic IAP, suggesting that the observed cytokinesis-associated apoptosis of BRUCE-depleted HeLa cells is a direct consequence of its absence. This suggests that BRUCE, through the combination of different activities and binding sites, is ideally suited to coordinate multiple functions during cytokinesis. It remains to be seen how this "molecular Swiss army knife" coordinates cytokinesis events at the molecular level and what the crucial substrates are.

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## ***Dissecting Roles of Ubiquitination in the p53 Pathway***

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J. Shan, C. Brooks, N. Kon, M. Li, W. Gu<sup>(✉)</sup>

Institute for Cancer Genetics and Department of Radiation Oncology, College of Physicians and Surgeons, Columbia University 1130 St. Nicholas Ave, 10032 New York, USA  
email: wg8@columbia.edu

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**Abstract.** Posttranslational modification of proteins by mono- or polyubiquitination represents a central mechanism to modulate a wide range of cellular functions like protein stability, intracellular transport, protein interactions, and transcriptional activity. Analogous to other posttranslational modifications, ubiquitination is a reversible process counteracted by deubiquitinating enzymes (DUBs), which cleave the isopeptide linkage between protein substrate and the ubiquitin residue. The p53 tumor suppressor is a sequence-specific DNA-binding transcriptional factor that plays a central role in regulating growth arrest and apoptosis during the stress response. Notably, recent studies indicate that both the stability and the subcellular localization of p53 are tightly regulated by ubiquitination; p53 is mainly ubiquitinated by Mdm2 but other ubiquitin ligases such as ARF-BP1/HectH9/MULE are also involved in p53 regulation in vivo. Moreover, a deubiquitinase HAUSP was initially identified in p53 deubiquitination but more recent studies showed that both Mdm2 and Mdmx are also bona fide substrates of HAUSP. In this article, we review our latest understanding of ubiquitination in modulating the p53 tumor suppression pathway.

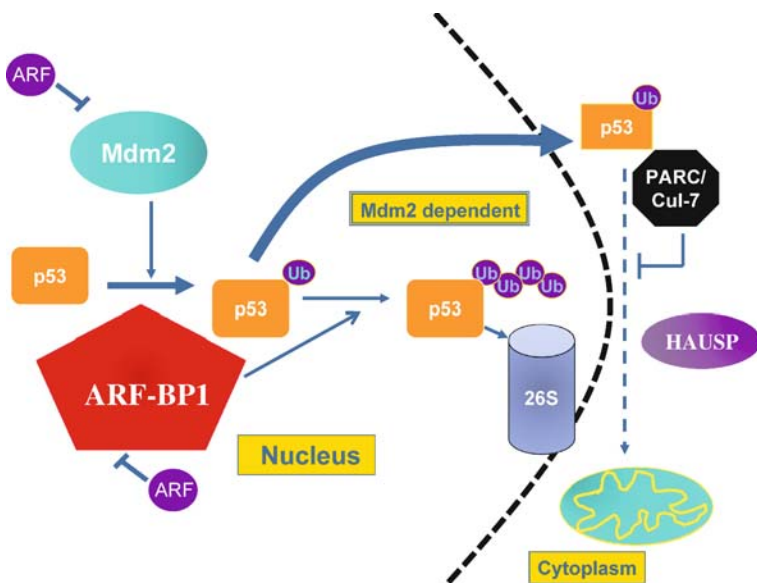
## 1 Introduction

Tumor development is a multistep process that depends upon the successive activation of oncogenes and inactivation of tumor suppressor genes (Vogelstein et al. 2000). Numerous studies demonstrate that inactivation of the p53 pathway is a pivotal event in tumorigenesis of all kinds of human cancers (Prives and Hall 1999; Vousden and Lane 2007). Indeed, the germline p53 mutations of Li-Fraumeni patients confer a high risk of cancer, and more than 50% of the tumors have also been shown to contain somatic p53 mutations. The p53 tumor suppressor exerts antiproliferative effects, including growth arrest, apoptosis, and cell senescence, in response to various types of stress (Brooks and Gu 2003, 2006). p53 is also critical for maintenance of genomic stability; aberrant ploidy, gene amplification, increased recombination, and centrosomal dysregulation have been observed in cells lacking functional p53. Wild-type p53 has been called the guardian of the genome, as p53 responds to DNA damage or checkpoint failure by either arresting the cell in the G1 phase for damage repair or through the initiation of an apoptotic pathway to eliminate the damaged cell entirely (Vousden and Lane 2007). The molecular function of p53 that is required for tumor suppression involves its ability to act as a transcriptional factor in regulating endogenous gene expression (Prives and Hall 1999). A number of genes that are critically involved in either cell growth arrest or apoptosis have been identified as p53 direct targets, including p21<sup>CIP1/WAF1</sup>, Mdm2, GADD45, Cyclin G, 14-3-3 $\sigma$ , Noxa, p53AIP1, PUMA and others (Vogelstein 2000). Accumulating evidence further indicates that in cells that retain wild-type p53, other defects in the p53 pathway also play an important role in tumorigenesis (Chen et al. 2005). For example, mutations of the ARF tumor suppressor are observed in tumor cells that retain wild-type p53 (Sherr 2006; Lowe and Sherr 2003). While the precise mechanisms of p53 activation are not fully understood, they are generally thought to involve posttranslational modifications of the p53 polypeptide. Ubiquitination regulates a diverse spectrum of cellular processes by providing a specific signal for intracellular protein degradation as well as some degradation-independent functions. It is well accepted that the ubiquitin-proteasome pathway plays a major part in the scope of p53 regulation; however, it is becoming more appar-

ent that the role of ubiquitination in the balance of p53 is not as simple as once thought.

## **2 Ubiquitination of p53 Is a Pivotal Event for Its Regulation**

Protein ubiquitination, including both mono- and polyubiquitination, is involved in a broad spectrum of cellular processes. While polyubiquitination can serve to target proteins for degradation by providing a recognition signal for the 26S proteasome, monoubiquitination has been implicated in a number of degradation-independent processes, including endocytosis, virus budding, and transcriptional regulation (Hicke and Dunn 2003). p53 is a short-lived protein whose activity is maintained at low levels in normal cells. Tight regulation of p53 is essential for its effect on tumorigenesis as well as maintaining normal cell growth. The cellular functions of p53 are rapidly activated in response to stress. Ubiquitination of p53 was first discovered in papillomavirus-infected cells, where p53 degradation is mediated by the viral E6 protein and a cellular ubiquitin ligase called E6-AP. In normal cells, Mdm2 acts as a specific E3 ubiquitin ligase for p53, which, if malignantly activated, has the potential to counteract the tumor suppressor functions of p53 (see Fig. 1). The oncoprotein Mdm2 physically interacts with the N-terminus of p53 and counteracts the tumor suppressor activity of p53. The binding strongly induces p53 ubiquitination both *in vitro* and *in vivo* (Michael and Oren 2003). Importantly, by acting as p53-specific E3 ligase, Mdm2 promotes both degradation and nuclear export of monoubiquitinated p53 (Li et al. 2003). Notably, the p53 activity is downregulated in many human tumors by overexpressing the Mdm2 protein. For example, the Mdm2 gene is amplified in 30% of osteosarcomas and in 20% of soft tissue tumors in general. Interestingly, transcription of the Mdm2 gene is activated by p53, setting up an autoregulatory loop in which increased Mdm2 production limits p53 induction in response to a variety of cell stresses (Michael and Oren 2003; Prives and Hall 1999). The critical role of mdm2 in inhibiting p53 is best illustrated by studies carried out in mice where inactivation of p53 was



**Fig. 1.** Critical roles of ubiquitination in the regulation of the p53 pathway. p53 is ubiquitinated by Mdm2 and ARF-BP1/HectH9/MULE. Polyubiquitination of p53 leads to protein degradation by the 26S proteasome; monoubiquitination of p53 by Mdm2 promotes its nuclear export. *Ub* ubiquitination, 26S 26S proteasome

shown to completely rescue the embryonic lethality caused by the loss of Mdm2 function.

Although the importance of Mdm2 in p53 regulation is well established, the precise mechanisms of ubiquitination-mediated effects remain unclear. We found that Mdm2 differentially catalyzes monoubiquitination and polyubiquitination of p53 in a dosage-dependent manner (Li et al. 2003; Brook and Gu 2006). As a consequence, low levels of Mdm2 activity induce monoubiquitination and nuclear export of p53, whereas high levels promote polyubiquitination and nuclear degradation of p53 (Fig. 1). It is likely that these distinct mechanisms are exploited under different physiological settings. For example, Mdm2-mediated polyubiquitination and nuclear degradation may play a critical

role in suppressing p53 function during the later stages of a DNA damage response or when Mdm2 is malignantly overexpressed. On the other hand, Mdm2-mediated monoubiquitination and subsequent cytoplasmic translocation of p53 may represent an important means of p53 regulation in unstressed cells, where Mdm2 is maintained at low levels. These results, together with other developments in the field (Vousden and Lane 2007), suggest the Mdm2-p53 pathway is regulated in a dynamic fashion during the DNA damage response. Nevertheless, our study also raises several critical questions. First, what is the molecular role of p53 ubiquitination in nuclear export? Does monoubiquitination act as a specific signal for nuclear export? Can we identify monoubiquitination-dependent factors that are required for nuclear export of p53?

### **3 ARF-BP1 Is a Potential Therapeutic Target in Tumors Regardless of p53 Status**

ARF was originally identified as an alternative transcript of the *Ink4a/ARF* tumor suppressor locus (Sherr 2006; Lowe and Sherr 2003). Numerous studies indicate that ARF suppresses aberrant cell growth in response to oncogene activation, mainly by inducing the p53 pathway. The ARF induction of p53 appears to be mediated through Mdm2, since overexpressed ARF interacts directly with Mdm2 and inhibits its ability to promote p53 degradation. Interestingly, ARF also has tumor suppressor functions that do not depend on p53 or Mdm2 (Sherr 2006). For example, ARF can induce cell growth arrest in tumor cells that lack a functional *p53* gene. To elucidate novel factors and the mechanisms in ARF-mediated tumor suppression, we isolated naturally formed ARF-containing nuclear complexes from human cells and identified a novel 500-kDa ubiquitin ligase ARF-BP1, as a major ARF binding partner in human cells (Chen et al. 2005; Zhong et al. 2005). ARF-BP1 harbors a signature HECT (homolog to E6-AP C-terminus) motif and its ubiquitin ligase activity is inhibited in the presence of ARF. Notably, inactivation of ARF-BP1, but not Mdm2, suppresses the growth of p53-null cells in a manner reminiscent of ARF induction. Surprisingly, in p53 wild-type cells, ARF-BP1 directly binds and ubiquitinates p53 (Fig. 1). Thus, our study modifies the current view of ARF-mediated p53 activa-

tion and reveals that ARF-BP1 is a critical mediator of both the p53-independent and p53-dependent tumor suppressor functions of ARF. However, it also raises more general questions regarding the role of ARF-BP1 in ARF-mediated tumor suppression function. For example, (1) what are additional targets mediating p53-independent cell growth arrest and (2) how are the ARF-ARF-BP1 and ARF-BP1-p53 interactions regulated? Further analysis of this process should clarify the precise role of ARF-BP1 and yield broader insights into the mechanisms of ARF-mediated tumor suppression. First, we test whether the ARF-BP1-p53 and ARF-ARF-BP1 interactions are regulated upon oncogene activation or other types of stress. Second, to precisely understand how ARF mediates its tumor suppression effects in p53-null cells, we have isolated cellular factors that specifically interact with and mediate p53-independent functions of ARF-BP1. Finally, to define the physiological role of ARF-BP1 in normal development and tumorigenesis, we have established a knock out mouse model of ARF-BP1 to dissect its roles in vivo.

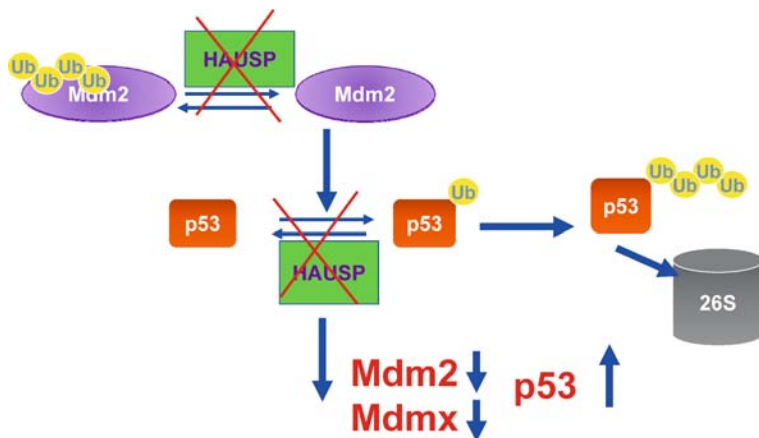
#### **4 Ubiquitination of p53 Is Reversible**

Originally, the ubiquitin-proteasome pathway was thought to have a one-way direction from substrate ubiquitination to degradation by the 26S proteasome. However, the discovery and emergence of deubiquitination enzymes (DUBs) changed the global view of the enzymatic process and quickly showed the incredible dynamics of this pathway. Our early finding that the herpesvirus-associated ubiquitin-specific protease (HAUSP) interacts and stabilizes p53 by deubiquitination (Li et al. 2002; Hu et al. 2002) was one of the first indications that DUBs exhibited a specific role in the p53 pathway (Fig. 2). Surprisingly, the simple linear model was obscured, however, with the subsequent findings that HAUSP deubiquitinates Mdm2 and is essential for controlling the Mdm2 stability in vivo (Li et al. 2004; Cummins and Vogelstein 2004; Meulmeester et al. 2005). In addition to ubiquitinating p53, Mdm2 elicits high levels of self-ubiquitination which makes Mdm2 itself very liable in cells. Our studies demonstrate that HAUSP expression can rescue Mdm2 from self-ubiquitination and is required for maintaining Mdm2-mediated func-

tion. Moreover, SiRNA-mediated inactivation of endogenous HAUSP leads to unmanageable self-ubiquitination and destabilization of Mdm2, which indirectly results in p53 activation. These findings were further supported by the study of somatic HAUSP knockout human cells (HCT116-HAUSP<sup>-/-</sup>) in Bert Vogelstein's lab (Cummins and Vogelstein 2004) and more recently confirmed in mouse HAUSP<sup>-/-</sup> embryos by our lab (N. Kon and W. Gu, unpublished data). In summary, these studies identify HAUSP as a critical regulator involved in p53 activation and implicate a dynamic role of the HAUSP deubiquitinase in regulating the p53/Mdm2 pathway (Hu et al. 2006; Brooks et al. 2007). These studies also suggest that HAUSP is a potential therapeutic target for activating p53 function by downregulating both Mdm2 and MdmX in cancer cells (Fig. 2). However, it also raises more interesting questions regarding the precise function of HAUSP *in vivo*. For example, what are the precise molecular mechanisms by which p53 is stabilized during the DNA damage response? Is deubiquitination the most efficient way to stabilize p53? How are the p53-HAUSP and Mdm2-HAUSP interactions regulated by DNA damage? To answer these questions, we have used biochemical methods to characterize the composition, stoichiometry, and subcellular localization of p53-HAUSP and HAUSP-Mdm2 complexes upon DNA damage, as well as the posttranslational modifications of their polypeptide components. By defining the status of these complexes with respect to these parameters during, for example, different stages of the stress response, we expect to learn when and where these complexes function and how their activities are regulated.

## 5 Identification of Novel Deubiquitinases in Cancer Pathways

A growing number of substrate-specific mammalian deubiquitinases (DUBs) involved in tumorigenesis are continually being revealed (Russell and Wilkinson 2005; Nijman et al. 2005; Amerik and Hochstrasser 2004; D'Andre and Pellman 1998). Considering the enzymatic process of deubiquitination does not require the cascade of enzymes needed for ubiquitination (e.g., E1, E2, and E3), DUBs may be simpler and better targets for therapeutic purpose. The deubiquitination enzyme fam-



**Fig. 2.** A model for a dynamic role of HAUSP in regulating Mdm2 and p53. HAUSP can induce p53 deubiquitination; however, Mdm2 is also highly self-ubiquitinated and very unstable. HAUSP is required for rescuing Mdm2 from self-ubiquitination. Moreover, Mdmx stability is also tightly regulated by HAUSP. Thus, inactivation of HAUSP by RNAi or potential small molecular inhibitors will induce downregulation of the functions of both Mdm2 and Mdmx, which indirectly leads to p53 activation

ily (DUBs) falls within two classes of proteases—the metalloproteases and cysteine proteases—though most DUBs fall within the latter. Further classification subdivides the cysteine proteases into four subclasses: ubiquitin C-terminal hydrolases (UCH), ubiquitin-specific proteases (USP), Machado-Joseph disease proteases (MJD), and Otubain proteases (OUT). There are a total of 89 deubiquitinase genes in the data base (Nijman et al. 2005; Amerik and Hochstrasser 2004). Our work on HAUSP in the p53 pathway clearly validates the role of deubiquitinases in modulating cancer pathways. Recent studies also show important roles of ubiquitination in modulating other cancer pathways such as PTEN, c-Myc, Ras, and EGFR; however, specific deubiquitinases for modulating these proteins remain unknown. We will use two different approaches to identify specific deubiquitinases in these pathways. The first one is the RNAi-base screen assays. Moreover, since most of Dubs

are stable proteins, the levels of Dubs cannot be sufficiently knocked down by RNAi-base screen assays. To compensate this, the second approach is the protein-based screen assay. We have cloned and expressed 80 members out of the 89 deubiquitinase library. We will first use in vitro deubiquitination assays to identify candidates and then use these candidates to confirm the biological consequence with in vivo assays. Indeed, our preliminary studies have identified several interesting candidates as specific deubiquitinase for PTEN, cyclin D1, r-H2AX, BRCA1, and c-Myc. Further characterizations of these findings will elucidate crucial roles of these novel deubiquitinases in tumorigenesis.

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## **Regulation of T Cell Differentiation and Allergic Responses by the E3 Ubiquitin Ligase Itch**

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Y.-C. Liu<sup>(✉)</sup>

Division of Cell Biology, La Jolla Institute for Allergy and Immunology, 9420 Athena  
Circle, 92037 La Jolla, USA  
email: [yuncail@liai.org](mailto:yuncail@liai.org)

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**Abstract.** Itch is an E3 ubiquitin ligase that is originally identified by genetic analysis of a mutant mouse with aberrant immunological phenotypes and constant itching in the skin. Itch<sup>-/-</sup> T cells are biased toward the differentiation of T helper type 2 cells with augmented interleukin-4 cytokine production and serum IgE level. One of the mechanisms for Itch E3 ligase to regulate T cell responses is the induction of T cell anergy in which T cells become unresponsive upon restimulation. However, the detailed mechanisms underlying Itch-mediated protein ubiquitination and allergic responses remain to be investigated. Here we provide evidence that Itch is involved in the regulation of transforming growth factor (TGF)- $\beta$  signaling in naïve T cells and TGF- $\beta$ -induced expression of the tran-

scription factor Foxp3, a master regulator in regulatory T cells. Itch promotes ubiquitin conjugation to TGF- $\beta$  inducible early gene 1 product (TIEG1). Moreover, monoubiquitinated TIEG1 positively modulates the transcription of *Foxp3* gene. The results suggest a novel mechanism by which Itch regulates regulatory T cells and subsequent allergic responses.

## 1 The Ubiquitin Conjugation System

The ATP-dependent protein degradation via conjugation to a protein substrate with a 76 amino acid peptide, ubiquitin, was discovered in early 1980s (Hershko et al. 1980). It has now been well established that this conjugation process, or protein ubiquitination, is carried out by a cascade of enzymatic reactions (Pickart 2001; Weissman 2001). The C-terminal glycine residue of ubiquitin is first activated by the ubiquitin activating enzyme or E1 to form a high-energy thiol-ester bond between the C-terminal glycine residue of ubiquitin and the active cysteine of E1. The activated ubiquitin is then transferred to one of the ubiquitin conjugating enzymes, or E2s, via a similar thiol-ester bond formation. The ubiquitin ligases, or E3s, recruit a protein substrate and bind to the ubiquitin-E2 complex and thereby help transfer ubiquitin from E2 to the lysine residue of a substrate via a covalent isopeptide bond formation. Based on the structure of E2 binding, the E3 ubiquitin ligases can be generally divided into two families, the really interesting new gene (RING)-type E3s, and the homologous to E6-associated protein C-terminus (HECT)-type E3s. In addition to E2 binding, the E3 ligases also contain well-defined protein interaction domains, through which the E3s confer the specificity of the substrate targeting in the ubiquitin system.

A substrate can be tagged with a single ubiquitin molecule to one lysine residue of a substrate, called monoubiquitination, or to multiple lysine residues to form multiple monoubiquitination (Hicke 2001). A substrate can also be tagged with more ubiquitin molecules via successive conjugation of one ubiquitin to another by utilizing lysine residues of the ubiquitin to form polyubiquitination. Depending on the usage of different lysine residues on a ubiquitin, the polyubiquitin chain can be

formed via the lysine 48 (K48) linkage, or K63 linkage. Evidence has been accumulated that monoubiquitinated protein occurs more often on cell surface receptors, which results in lysosome-dependent downmodulation, whereas K48-linked polyubiquitination leads to proteasome-dependent degradation, and K63-linked polyubiquitination is related to protein complex formation (Pickart 2001; Weissman 2001). In addition to ubiquitin, ubiquitin-like molecules such as SUMO (small ubiquitin-like modifier), or ISG15, are also tagged to the substrate in a manner similar to the ubiquitin conjugation pathway (Liu et al. 2005).

## 2 Ubiquitination in Immune Regulation

The process of protein ubiquitination has been implicated in various aspects of immune regulation, including the development, differentiation, activation, and tolerance induction of lymphocytes, viral and bacterial infection, antigen presentation, and immune evasion (Liu 2004). The identification of Cbl family proteins as RING-type E3 ubiquitin ligases clearly indicates that the lymphocyte function is tightly controlled by protein ubiquitination. Particularly, loss of Cbl-b results in excessive IL-2 cytokine production and T cell proliferation even without the necessity of CD28-mediated costimulation (Bachmaier et al. 2000; Chiang et al. 2000). Cbl-b was identified as an E3 ligase for the p85 subunit of PI3-kinase, which affects its association with upstream signaling molecules without directly affecting its degradation (Fang and Liu 2001). One of the recent findings is the involvement of Cbl-b in the induction of T cell anergy, a process of unresponsiveness upon T cell restimulation (Heissmeyer et al. 2004; Jeon et al. 2004). Cbl-b<sup>-/-</sup> T cells are not susceptible to either ionomycin-induced or high-dose soluble antigen-induced tolerance induction. In addition, in a mouse model of collagen-induced arthritis, Cbl-b<sup>-/-</sup> mice display early onset and severe joint inflammation in response to collagen immunization (Jeon et al. 2004). In addition to Cbl-b, other E3 ubiquitin ligases such as the RING finger protein Grail or the HECT-type E3 ligase Itch are upregulated during T cell anergy induction (Heissmeyer et al. 2004). Indeed, Grail E3 ligase is a critical player in T cell tolerance, as revealed by both in vitro and in vivo experimental approaches (Anandasabapathy et al. 2003).

Protein ubiquitination is a reversible process that is catalyzed by deubiquitinating enzymes. Analysis using a genomic bioinformatics approach suggests that more than 100 deubiquitinating enzymes are present in the human genome (Nijman et al. 2005). Both genetic and biochemical studies have implicated some of the deubiquitinating enzymes such as A20 or CYLD in the regulation of both innate and adaptive immune responses (Boone et al. 2004; Reiley et al. 2006).

### 3 The E3 Ligase Itch

Itch was originally described by Neal G. Copeland and Nancy A. Jenkins's group from studies on mouse coat color alterations (Hustad et al. 1995). Mutations in the *agouti* locus on mouse chromosome 2 that either upregulate or downregulate the expression of *agouti* protein cause the color changes in the hair shaft. One of these mutations,  $\alpha 18H$ , results from the decreasing expression of *agouti*, which leads to darker than normal coats. Interestingly, unlike mutations in other alleles, this mutation also causes a skin (in the back and neck) scratching phenotype and immunological disorders, manifested by hyperplasia of lymphoid organs and inflammation in the lung and digestive tract. Due to the constant itching in the skin, the mutant mice were also called itchy mice. It was hypothesized that in addition to the *agouti* gene, there was another mutation in the  $\alpha 18H$  allele, which is responsible for the itchy and immunological abnormality.

Subsequent genetic studies by the same group confirmed this hypothesis and revealed that the  $\alpha 18H$  mutation results from a chromosomal inversion that deletes 18 and 20 base pairs from the proximal and distal inversion breaks, respectively (Perry et al. 1998). This inversion affects the expression of *agouti* and disrupts the expression of a novel gene, named *Itch*. Sequencing of the *Itch* cDNAs identified an open reading frame of 2,562 base pairs, which encodes 854 amino acids with a molecular mass of approximately 113 kDa. Homology alignments of the predicted amino acid sequences showed that the *Itch* protein contains a carboxyl-terminal E3 ligase domain, preceded by four protein-interacting WW domains, with high homology with E3 ligases such as the yeast *Rsp5* or the mammalian *Nedd4* proteins. This genetic study

suggests for the first time that Itch may act as an E3 ubiquitin ligase in regulating immune responses.

#### 4 Itch Regulates Th2 Development

Given the immunological disorder in itchy mice, we initiated the study of Itch in immune regulation by identifying its potential target proteins. A genetic study on Notch signaling in *Drosophila* revealed a novel gene product, suppressor of Deltex or Su(dx), a negative regulator of Notch signaling (Cornell et al. 1999). Su(dx) belongs to the family of HECT-type E3 ligases similar to Itch. We found that Itch indeed is an E3 ligase for Notch by promoting Notch ubiquitination both in test tube and in transfected cells (Qiu et al. 2000). Itch<sup>-/-</sup> T cells displayed enhanced cell proliferation and chronic activation (Fang et al. 2002). Particularly, the mutant T cells produce more Th2 cytokines like interleukin-4 (IL-4) and IL-5 and sera from Itch<sup>-/-</sup> mice contain higher levels of IgG1 and IgE compared with wild-type mice. At the molecular level, Itch WW domains bind to a PPXY motif in JunB, a member of Jun family proteins and Itch promotes ubiquitin conjugation to JunB. Our results are consistent with previous publications in that JunB has been shown to be an important regulator in the differentiation of Th2 cells both in JunB transgenic mice and *JunB* gene-targeted mice (Hartenstein et al. 2002; Li et al. 1999).

Although those studies suggest that Itch is important in modulating critical signaling pathways by targeting specific substrates for ubiquitination, the mechanisms by which Itch-induced protein ubiquitination is regulated remain largely unclear. A recent study from Michael Karin's group, in collaboration with us, may shed light on this aspect, in which a MEKK1-JNK-mediated signaling pathway controls the turnover of Jun proteins via the serine/threonine phosphorylation of Itch and its subsequent activation (Gao et al. 2004). More recently, we showed that Itch is also regulated by Fyn-mediated tyrosine phosphorylation. Unlike the serine/threonine phosphorylation, tyrosine phosphorylation of Itch does not affect its ligase activity, rather it negatively modulates its association with the substrate JunB (Yang et al. 2006). These studies indicate that Itch is regulated by both tyrosine and serine/threonine kinases, but with

opposing effects, suggesting that Itch-mediated JunB ubiquitination is tightly controlled by upstream kinases via counterbalancing tyrosine vs serine/threonine phosphorylation.

## **5 Self-tolerance in the Immune System**

Mature T cells are capable of mounting robust immune responses against invading pathogens, but at the same time are tolerant of self-tissues. The induction of T cell tolerance involves many mechanisms at different stages of T cell development. At first, self-reactive T cells are eliminated during thymocyte maturation via negative selection, a process called central tolerance. In addition to thymus-derived antigens, many antigens from other tissues or organs are expressed in the thymus antigen-presenting cells, which causes clonal deletion of T cells specific for self-peptide–MHC complexes. Evidence supporting the central tolerance mechanism includes the finding of AIRE, a transcription factor as well as an E3 ubiquitin ligase, which promotes the expression of many peripheral tissue antigens in thymus medullary epithelial cells (Anderson et al. 2002).

However, the central tolerance mechanism is not sufficient, since autoreactive T cells can escape into the secondary lymphoid organs, where the peripheral tolerance mechanisms take effect to keep them under control. Several mechanisms have been proposed to account for peripheral T cell tolerance to self-antigens, which include ignorance, activation-induced cell death, T cell anergy, and suppression by regulatory T cells (Tregs) (Walker and Abbas 2002).

## **6 Itch in Th2 Tolerance Induction**

T cell anergy represents one of the peripheral tolerance mechanisms in which T cells lose the ability to proliferate and produce IL-2 upon restimulation (Schwartz 2003). Early studies have documented that T cell anergy is due to defective TCR signal transduction starting from partial or reduced phosphorylation of upstream Src kinases, decreased Erk phosphorylation, or diminished activation of AP-1 transcription factors (Fields et al. 1996; Gajewski et al. 1994; Li et al. 1996). Recent stud-

ies have shown that E3 ubiquitin ligases such as GRAIL, Cbl-b, and Itch, play a critical role in the process of T cell anergy induction (Anandasabapathy et al. 2003; Heissmeyer et al. 2004; Jeon et al. 2004). Up-regulation of these E3 ligases results in the downmodulation of critical signal molecules such as PLC- $\gamma$ 1, or PKC $\theta$ , that blocks T cell activation even upon effective stimulation.

We went on to investigate the *in vivo* biological function of Itch during soluble antigen-induced tolerance induction. In this tolerance model, mice were injected systematically with high-dose soluble antigen, followed by immunization with the same antigen plus either alum adjuvant to elicit Th2 response, or CFA adjuvant to induce Th1 response (Venuprasad et al. 2006). Itch is primarily involved in the Th2 tolerance induction, since Itch<sup>-/-</sup> T cells continue to produce Th2 type cytokines and Itch<sup>-/-</sup> mice develop severe airway inflammation. In addition, mice deficient in either MEKK1 kinase domain or JNK1 displayed similar resistance to Th2 tolerance induction, supporting a notion that MEKK1-JNK1 signaling converges with Itch-mediated ubiquitination to regulate Th2-mediated allergic responses.

## 7 Regulatory T Cells

Regulatory T cells, or Tregs, are a unique subset of the T cell population, characterized by the cell surface expression of CD4 and the IL-2 cytokine receptor alpha chain CD25, play a pivotal role in maintaining self-tolerance via actively suppressing the effector function of other T cells (Sakaguchi 2004). The transcription factor Foxp3 is a master regulator of Treg development and function, since loss or mutation of Foxp3 is linked to abnormal T cell responses and the development of autoimmune diseases. Tregs are generated in the early stage of thymic development, which become the naturally occurring Foxp3<sup>+</sup>CD4<sup>+</sup>CD25<sup>+</sup> Tregs in the periphery. In addition, peripheral CD4<sup>+</sup> T cells can be converted into Foxp3<sup>+</sup> Tregs by either tolerogenic antigen stimulation *in vivo* or TGF- $\beta$  stimulation *in vitro* (Apostolou and von Boehmer 2004; Chen et al. 2003; Fantini et al. 2004; Kretschmer et al. 2005; Li et al. 2006; Wan and Flavell 2005). However, the molecular mechanisms for such conversion remain largely unclear.

Historically, instead of a systematic antigen injection as described above, oral or nasal antigen administration has been used to induce Th2 tolerance (McMenamin et al. 1994). Unlike the Th2 tolerance induced by high-dose antigen injection, tolerance via the oral or nasal route has been shown to result from the generation of Tregs (Mucida et al. 2005; Ostroukhova et al. 2004). Repeated exposure to inhaled low-dose antigen results in the generation of Foxp3<sup>+</sup>CD4<sup>+</sup> Tregs that also express membrane-bound TGF- $\beta$  (Ostroukhova et al. 2004). These Tregs from the tolerized mice inhibit the proliferation of normal CD4<sup>+</sup> T cells and suppress Th2-mediated allergic responses when adoptively transferred into a naïve host. A more recent study suggests that antigen-specific Tregs could be generated via oral tolerance in the absence of naturally occurring Tregs (Mucida et al. 2005). Like the inhaled antigen-induced Tregs, the oral tolerance-generated Tregs suppress CD4<sup>+</sup> T cell proliferation *in vitro* and inhibit IgE production and lung inflammation *in vivo*. In both studies, TGF- $\beta$  was shown to be involved in the proper function of Treg-mediated suppression of the Th2 response, since administration of anti-TGF- $\beta$  antibody abrogated the tolerance induction and hence the restoration of allergic responses. It remains unknown whether Itch affects Treg-regulated allergic responses.

## 8 TGF- $\beta$ Signaling in Immune Regulation

TGF- $\beta$  signaling is involved in diverse cellular responses such as cell proliferation, differentiation, apoptosis, and migration (Attisano and Wrana 2002). TGF- $\beta$  binding to the type II receptor induces the complex formation with type I receptor, which results in the phosphorylation of the type I receptor serine/threonine kinase. The activation of the receptor complex in turn phosphorylates the intracellular transducers, Smad2/3, which then form complex with Smad4 and are translocated into the nucleus to regulate the transcription of target genes. One of the target gene products is Smad7, an inhibitory Smad, which negatively modulates TGF- $\beta$  signaling by directly competing with Smad2/3 for receptor interaction. In addition to the Smad-dependent signaling pathways, TGF- $\beta$  also activates Smad-independent signaling pathways (Derynck and Zhang 2003).

The intracellular signal transduction induced by TGF- $\beta$  is regulated by the ubiquitin system, as E3 ligases such as Smurfs directly associate with Smad proteins and affects the stability of Smads or their binding partners (Izzi and Attisano 2004). We found that Itch<sup>-/-</sup> fibroblasts are resistant to TGF- $\beta$ -induced proliferative inhibition (Bai et al. 2004). Itch E3 ligase directly promotes the ubiquitination to Smad2/3 and affects their phosphorylation by the receptor. However, whether Itch is involved in the TGF- $\beta$  signaling in T cells remains unclear.

Previous studies have established that TGF- $\beta$  signaling is important in regulating immune responses. Ablation of either TGF- $\beta$  or the TGF- $\beta$  receptor is linked to abnormal T cell responses and onset of autoimmunity (Kulkarni et al. 1993; Li et al. 2006; Marie et al. 2006; Shull et al. 1992). TGF- $\beta$  signaling regulates both Th1 and Th2 cell differentiation (Gorelik et al. 2000, 2002). As described earlier, TGF- $\beta$  also plays an important role in Treg generation and maintenance (Chen et al. 2003; Fantini et al. 2004; Li et al. 2006; Wan and Flavell 2005). In addition, recent studies have demonstrated a critical role of TGF- $\beta$  in the development of Th17, a new subset of T helper cells, which are involved in autoimmune and inflammatory responses (Weaver et al. 2007). However, the detailed intracellular signaling pathways that TGF- $\beta$  initiates in diverse processes of different types of T cells remain to be investigated.

## 9 Itch in the Development of Regulatory T Cells

To understand how Itch is involved in the regulation of airway inflammation, we set up an intranasal tolerance protocol. Consistent with a previous report (McMenamin et al. 1994), wild-type mice that inhaled the aerosolized antigen did not show airway inflammation. However, the same treatment failed to inhibit the lymphocyte infiltration in the lung of Itch<sup>-/-</sup> mice (Venuprasad et al. 2008). It seems that although Itch is not involved in the development of naturally occurring CD25<sup>+</sup>CD4<sup>+</sup> Tregs, it affects the generation of TGF- $\beta$ <sup>+</sup> adaptive Tregs during tolerance induction.

Next we examined the responsiveness of Itch<sup>-/-</sup> T cells to Treg- or TGF- $\beta$ -mediated suppression and found that Itch<sup>-/-</sup> CD4<sup>+</sup>CD25<sup>-</sup> T cells

were resistant to the suppression by both Tregs and TGF- $\beta$ . TGF- $\beta$  treatment resulted in an upregulation of both *Foxp3* gene transcription and protein expression in wild-type T cells, but to a much less degree in *Itch*<sup>-/-</sup> T cells. The in vitro converted Tregs from wild-type mice showed suppressive activity toward CD4<sup>+</sup>CD25<sup>-</sup> T cells, whereas TGF- $\beta$ -treated *Itch*<sup>-/-</sup> T cells were much less inhibitory. The results collectively suggest that loss of *Itch* alters TGF- $\beta$  signaling in T cells and affects TGF- $\beta$ -induced *Foxp3* expression.

## 10 *Itch* Promotes Ubiquitination of TIEG1

To understand the mechanisms underlying the hyporesponsiveness of *Itch*<sup>-/-</sup> T cells to TGF- $\beta$  treatment, we looked for further downstream signaling molecules that may act as the target protein(s) for *Itch*. One of the putative targets is the TGF- $\beta$ -induced early gene 1 (TIEG1) protein, which contains multiple proline-rich sequences and is rapidly induced upon TGF- $\beta$  stimulation and functionally mimics TGF- $\beta$ -mediated transcriptional events in transiently transfection systems (Hefferan et al. 2000). A series of biochemical studies suggested that *Itch* associates with TIEG1 in vitro and in cells via *Itch* WW domains (Venuprasad et al. 2008). Importantly, *Itch* promotes ubiquitin conjugation to TIEG1 in both the mono- and polyubiquitinated forms.

Functionally, coexpression of *Itch* and TIEG1 induces an augmented transactivation of *Foxp3* promoter. In addition, TIEG1 directly binds to the *Foxp3* promoter as released by the DNA binding gel shift assay and chromatin immunoprecipitation assay. To examine a direct role of TIEG1 in *Foxp3* expression, we expressed TIEG1 in mouse CD4<sup>+</sup> T cells and found that TIEG1 expression resulted in *Foxp3* expression in wild-type CD4<sup>+</sup> T cells. However, the induction of *Foxp3* was much less in *Itch*<sup>-/-</sup> T cells. The results pointed out that TIEG1 is a positive regulator of *Foxp3* expression, whose activity is dependent on *Itch*-mediated ubiquitination.

To further understand the involvement of TIEG1 in *Foxp3* expression, we compared the responsiveness of wild-type and TIEG1<sup>-/-</sup> T cells to TGF- $\beta$  treatment. Like *Itch*<sup>-/-</sup> T cells, loss of TIEG1 in T cells resulted in a resistance to TGF- $\beta$ -induced proliferative inhibition. Such

defect in *Foxp3* expression could be reversed by TIEG1 reconstitution of TIEG1<sup>-/-</sup> T cells. In addition, TGF- $\beta$ -treated TIEG1<sup>-/-</sup> CD4<sup>+</sup> T cells displayed much less inhibitory effect to the responder T cells in comparison with TGF- $\beta$ -treated wild-type CD4<sup>+</sup> T cells. The results provided solid genetic evidence that TIEG1 is involved in TGF- $\beta$ -induced *Foxp3* expression and the suppressive function of adaptive Tregs.

## 11 Perspectives

Previous studies have identified several substrates for the Itch E3 ubiquitin ligase, such as JunB, or c-FLIP (Chang et al. 2006; Fang et al. 2002; Gao et al. 2004), which go through proteasome-dependent degradation. The recent identification of TIEG1 as another target for Itch provides a novel mechanism of regulation, in which ubiquitin-conjugation to TIEG1 leads to its transcriptional activation of *Foxp3* promoter. In this case, Itch most likely induces monoubiquitination of TIEG1, which in turn directly binds to the promoter region of *Foxp3* gene. How exactly the monoubiquitination of TIEG1 exerts its biological function, either via formatting complex with other transcription factors, or affecting its binding affinity to a particular DNA binding motif, remains to be investigated. Another issue is to fully comprehend the functional overlap of the Itch E3 ubiquitin ligase in differentially regulates Th2 vs Treg development. It is quite possible that the two mechanisms are not mutually exclusive: the regulation of Th2 differentiation by Itch may have an impact on its effect on *Foxp3* expression in the Tregs, or vice-versa. Interestingly, it was recently shown that *Foxp3* expression is correlated with Th2 cytokine production: *Foxp3* deficiency results in a conversion of conventional CD4<sup>+</sup> T cells into Th2 type effector T cells (Wan and Flavell 2007). Further investigation of the correlation of the two different T cell differentiation pathways may shed light on the mechanistic insight into the regulation of Th2-mediated allergic responses, which will eventually lead to the discovery of new therapeutic approaches for allergic and other immunological abnormalities.

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## ***Approaches to Discovering Drugs that Regulate E3 Ubiquitin Ligases***

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J. Huang<sup>(✉)</sup>, L. Tsvetkov, K. Qu, S. Daniel-Issakani, D.G. Payan

Rigel Pharmaceuticals, Inc., 1180 Veterans Boulevard, 94080 South San Francisco, USA  
email: [jhuang@rigel.com](mailto:jhuang@rigel.com)

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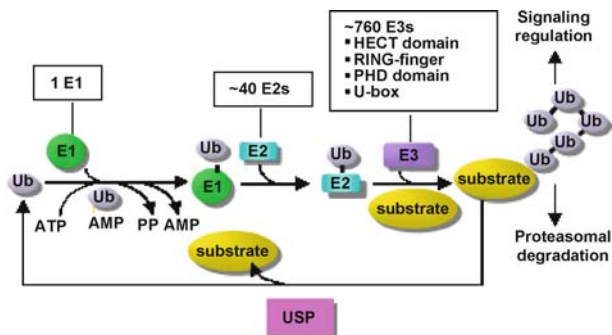
**Abstract.** The ubiquitin-proteasome system (UPS) plays an essential role in a wide variety of cell regulatory signaling pathways. The clinical effectiveness of the proteasome inhibitor Velcade in the treatment of several human cancers underscores the importance of the UPS as a novel target area for pharmaceutical intervention. E3 ubiquitin ligases are key enzyme complexes that regulate and determine the ubiquitination of specific substrates, whose abnormal regulation has been implicated in multiple disease phenotypes. Targeting a selective E3 ligase may allow specific manipulation of distinct pathways and eventually lead to a better therapeutic index with reduced nonspecific side effects. Here, we aim to discuss the challenges of interfering with small molecules in this target class, as well as current strategies and progress in E3 ligase drug discovery.

## 1 Introduction

The UPS has been implicated in the regulation of many human disease activities, including oncogenesis, inflammation, viral infection, CNS disorders, and metabolic dysfunction. Recent evidence that pharmacological interference with the proteasome by Velcade® can be efficacious in the treatment of human cancers has elicited significant interest in the development of small molecules that selectively target the activities of disease-specific components of the UPS.

Ubiquitin is a small protein (76 amino acids) that functions through covalent attachments to many substrate proteins targeted for proteasome degradation and alternation in cellular localization as a means to regulate their activity. The ubiquitin conjugation cascade is initiated by an ATP-dependent activating enzyme (E1) to generate an E1-ubiquitin thioester linkage. Following the activation step, the ubiquitin is transferred to the active site cysteine of the conjugating enzyme (E2). Subsequently, the E3 ligase promotes the transfer of the ubiquitin from an E2 thioester intermediate to an isopeptide linkage with a selected substrate (Fig. 1). While there is a common ubiquitin E1 and an intermediate number of E2s, the ubiquitin E3s represent the largest and most diverse class of enzymes, including RING-finger E3s, HECT-domain E3s, and U-box E3s, and are the primary determinants of substrate specificity. E3 ligases exist and act as single polypeptides (MDM2, IAP, TRAF6, etc.) or as multiple enzyme complexes composed of several subunits (SCF and APC). Some E2s can work with different E3s, and some E3s can form complexes with different adaptor proteins to recognize functionally and structurally distinct substrates. Thus, the tightly regulated pattern of interactions between E2s, E3s, and their targets, provide the necessary specificity for appropriate substrate ubiquitination, degradation, or functional modification.

The clinical success of the proteasome inhibitor Velcade ((R)-3-methyl-1-((S)-3-phenyl-2-(pyrazine-2-carboxamido)propanamido)butylboronic acid) in the treatment of several types of cancers suggests that inhibition of the ubiquitin system at a number of intervention points with varying degrees of substrate specificity, such as E1 inhibitors, may also have potential for regulating certain types of tumors. For example, ubiquitin E1, an ATP-dependent enzyme, might be an attractive target



**Fig. 1.** Ubiquitin conjugating/deconjugating system. In the presence of ATP, ubiquitin is first activated and attached to E1 ubiquitin activating enzyme through a thioester linkage. The activated ubiquitin is then transferred to the active site of the E2 conjugating enzyme. The E3 ubiquitin ligase recruits the substrate protein and promotes the ubiquitin transfer from the E2 to substrate. Ubiquitin-specific protease (USP) reverses this process by hydrolyzing ubiquitin from the substrate

for small molecule therapeutics, given the history of successful targeting the kinase ATP pocket in the current generation of new therapeutics in oncology. In fact, identification of a novel ubiquitin E1 inhibitor has recently been reported, which reinforces the principle for the capacity to differentially kill transformed cells (Yang et al. 2007). However, it remains to be determined whether a sufficient therapeutic window can be obtained by selectively inhibiting ubiquitin E1. In contrast, as the primary determinants of substrate specificity, E3 ligases have gained increasing attention as drug targets. Modulating a single ligase may allow for selective stabilization of a subset of ubiquitinated proteins, resulting in specific manipulation of distinct signaling pathway, and eventually lead to a better therapeutic index with reduced nonspecific side effects.

With the understanding of ubiquitin biology progressing rapidly, diverse ubiquitin-regulated biological pathways have been associated with multiple disease mechanisms. A few examples include: (1) Mdm2: Mdm2 binds and ubiquitinates p53 for proteasome degradation (Haupt et al. 1997). Overexpression of Mdm2 in many human tumors effectively impairs p53 function. Inhibition of p53 ubiquitination by Mdm2

can stabilize p53 in cells and offer a novel strategy for cancer therapy. (2) SCF<sup>Skp2</sup> complex: SCF<sup>Skp2</sup> mediates ubiquitination and degradation of the tumor suppressor p27 (Carrano et al. 1999; Tsvetkov et al. 1999). Decreased p27 levels in cancer cells have been associated with enhanced protein degradation and linked to poor prognosis. Restoration of p27 by inhibiting SCF<sup>Skp2</sup> has anti-tumor potential. (3) Atrogin-1 and MuRF1: both are upregulated in muscle wasting and play a critical role in the loss of muscle proteins (Gomes et al. 2001; Bodine et al. 2001). Targeting Atrogin-1 and MuRF1 could have therapeutic potential in the treatment of cancer cachexia and/or sarcopenia.

However, because E3s are unconventional enzymes, the development of specific inhibitors represents a significant challenge in drug discovery and design. Potential approaches for blocking E3 ligase activities include targeting the RING-finger domain to block E3 interactions with E2s and disrupting substrate–E3 interactions. Crystal structures of several E3 ligases, together with their adaptor proteins or substrates, have been solved and should provide important insights into the potential for targeting these E3s with small molecule inhibitors. Current strategies and assays developed in recent years for drug discovery of E3 ligase inhibitors, together with a few successful examples will be described below.

## **2 Targeting E3 RING Finger Domains: Substrate-Independent Ubiquitination Assays**

Many RING finger E3 ligases catalyze their own autoubiquitination and/or promote formation of free polyubiquitin chains in an *in vitro* assay, especially in the absence of a substrate. This feature can be adapted to establish a rapid, simple, and reliable substrate-independent assay for the screening of small molecules targeting the RING finger domain enzymatic activity, in which the reaction measures a self-polyubiquitylation of the E3 ligase. The assay is particularly useful for high-throughput screening of multicomponent E3s with unusual size and subunit complexity (such as the anaphase promoting complex, APC), which makes it challenging to develop a holoenzyme assay. Traditional methods for measuring the formation of ubiquitin conjugation have relied primar-

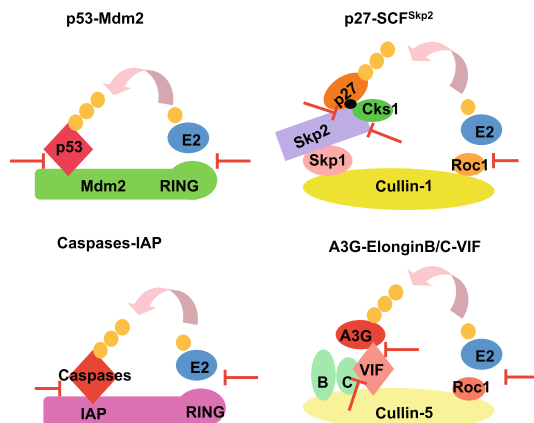
ily upon radiolabeled ubiquitin and anti-ubiquitin immuno-blots for detection. Both methods are labor-intensive and low-throughput, and neither is sensitive enough to determine the potency of inhibitors within a dynamic range. In recent years, several assays for high-throughput drug screening of E3 ligase inhibitors have been reported that are based on ELISA (enzyme-linked immunosorbent assay), FRET (fluorescence resonance energy transfer), or HTRF (homogeneous time-resolved fluorescence) technologies, including those targeting APC2/APC11, Mdm2, and TRAF6 mediated polyubiquitylation (Huang et al. 2005; Davydov et al. 2004; Hong et al. 2003).

As an example of these efforts, one group has reported the screening of a chemical library of 10,000 compounds using an *in vitro* Mdm2 autoubiquitination assay. A family of small molecules (HLI98) was identified to inhibit Mdm2's E3 activity and reduce p53 ubiquitination by Mdm2 (Yang et al. 2005). These compounds showed some specificity for Mdm2 *in vitro*, although effects on unrelated RING and HECT domain E3s were detectable at higher concentrations. These effects could possibly be due to the inhibitions of E2-ubiquitin thioester enzyme activities. In cell-based assays, HLI98 stabilized both Mdm2 and p53, and evidence for the p53 dependence of the growth-inhibitory effect was seen in multiple human tumor cell lines. However, at high concentrations, these compounds also inhibited cell growth regardless of the p53 status, probably reflecting the off-target activities such as the inhibition of other E3s or perhaps a subset of E2s.

Small molecules that inhibit APC2/APC11, the E3 core subunits of the APC have also been identified (Huang et al. 2005). These proof-of-concept studies suggest that it is possible to identify a novel class of small molecules as inhibitors of the E3 ligases. Because of the homologies within the E3 RING finger domains, identification of a ligase-friendly pharmacophore that selectively inhibits one E3 in a structural region that may be a common motif to the family as a whole should enable the rapid design of inhibitors of other therapeutically important members of the E3 target class.

Assays that monitor E3 ligase activity involve multiple component enzymatic cascades of E1 ubiquitin charging, E2 ubiquitin conjugating, and E3 ubiquitin polymerizing of specific substrates. Although the assay can be set up for E3 ligase activity with the amount of E3 protein be-





**Fig. 3.** Strategies to target E3 ligase mediated protein ubiquitination. Inhibition of substrate ubiquitination can be achieved through targeting the E3 RING finger domain enzymatic activity or disrupting the E3-substrate interaction

surement of a specific substrate's ubiquitination event by its E3 ligase will be a better approach to achieving eventual functional selectivity. This is critical since the ubiquitination status of the substrate is the ultimate determinant of its biological activity. Strategies to target E3 ligase-mediated protein ubiquitination are shown in Fig. 3.

As an example, compounds targeting p53 ubiquitination by Mdm2 have been reported (Lai et al. 2002). Three chemically distinct types of inhibitors were identified from the *in vitro* assay monitoring Mdm2-catalyzed ubiquitin transfer from pre-conjugated ubiquitin-Ubc4 to p53. All three types of compounds displayed selective inhibition of Mdm2 E3 ligase activity, with little or no effect on other ubiquitin regulating enzymes: E1, Nedd4, or SCF. Most strikingly, these compounds did not inhibit the autoubiquitination activity of Mdm2. Although no cell-based activity was reported, these compounds established the feasibility of selectively blocking Mdm2-mediated ubiquitination of p53 by small molecule inhibitors.

Substrate-dependent ubiquitination assays are especially valuable for the screening of multicomponent E3 ligases for selective inhibitors. For example, SCF E3 ligases ubiquitinate a variety of cellular pro-

teins with substrate specificity determined by the different F-box proteins (p27 via Skp2, I $\kappa$ B $\alpha$  via  $\beta$ -TrCP,  $\beta$ -Catenin via  $\beta$ -TrCP). A general inhibitor against the core E3 subunit of ROC1/CUL1 may not have the desirable specificity against a different complex. Therefore, biochemical assays to detect specific substrate ubiquitination events, such as SCF<sup>Skp2</sup>-mediated p27 ubiquitination, and SCF <sup>$\beta$ TrCP1</sup>-mediated I $\kappa$ B $\alpha$  ubiquitination have been established for HTS (Tsvetkov et al. 2008; Xu et al. 2005). In the SCF<sup>Skp2</sup>-mediated p27 ubiquitination assay, purified E1, Ubc3, ROC1/CUL1, Skp1/Skp2, Cks1, p27, CDK2/Cyclin E (for p27 phosphorylation), and ubiquitin were used to generate polyubiquitinated p27. This assay can be screened to discover inhibitors of p27 ubiquitination, which would selectively increase p27 levels in cells and inhibit tumor cell growth to provide a new approach to treating selected malignancies where growth is driven by low p27 levels.

It is a significant challenge to develop and conduct an *in vitro* HTS of an assay monitoring substrate ubiquitination by the holoenzyme complex. The assay complexity is primarily due to presence of multiple components: ten proteins in the assay system measuring SCF<sup>Skp2</sup>-mediated p27 ubiquitination. The hits resulting from this type of HTS will need to go through several deconvolution assays (Fig. 2). Selectivity of the inhibitors can be followed up by secondary assays through biochemical counter screens, as well as cell-based assays to compare their effects on protein substrates of various F-box components. However, unlike kinases or proteases, the multicomponent E3 complex does not contain an evident enzymatically active site to which small molecules could bind. With the complexity of the reaction, the biggest challenge is to determine the site(s) where the inhibitor binds. In the absence of functional subunit assays and SBDD, this challenge will be a significant obstacle to lead optimization.

#### **4 Targeting Protein–Protein Interaction Interfaces: E3–Substrate Interactions, Adaptor Protein Interactions**

Interruption of protein–protein interactions has proven to be a challenge for drug discovery, especially for small molecule inhibitors. However,

the fact that many point mutations disrupt protein interactions and that an amino acid side chain may be structurally similar to peptide ligands and equal to or smaller in size than many small molecules, suggest that many protein interfaces do contain a “hot pocket” susceptible to inhibition by small molecular compounds (Cardozo and Abagyan 2005). In fact, several recent success stories indicate that protein–protein interfaces might be more tractable than has been previously thought (Wells and McClendon 2007). These studies discovered small molecules that bind with drug-like potencies to hotspots on the contact surfaces regulating protein interactions. Some of these molecules are now making their way through clinical trials.

In the absence of a central enzymatic site, E3 ligases seem to promote ubiquitination by cooperative protein interactions between E2s and their substrates. Therefore, inhibition of E3 ligase–substrate interaction is likely to be the most direct approach for interference in specific protein ubiquitination (Fig. 3). This requires substantial information on the identification of the relevant physiological substrates of the E3, and on the structure of the E3/substrate pair to be targeted. In the past few years, approaches to find E3 inhibitors through disrupting substrate–protein interactions have made considerable progress. This process is aided significantly by the use of structural information and rational design at various stages of the drug discovery program, from assessment of a druggable target, evaluation of the HTS hits, and selection of lead molecules, to medicinal chemistry lead optimization. Examples will be discussed in the following.

#### **4.1 p53-Mdm2**

Mdm2 binds to p53 through a small domain within its first 120 N-terminal residues (Chen et al. 1993), while a 15-residue peptide fragment of p53 contributes to p53 binding to Mdm2 (Picksley et al. 1994). The crystal structure of the p53 peptides in complex with the binding domain of Mdm2 revealed that the p53 peptide adopts a helical structure and inserts three hydrophobic side chains, Phe19, Trp23, and Leu26 into subpockets of the Mdm2 site (Kussie et al. 1996), which made the interface a hot spot possible for small molecule disruption (Fig. 4a).

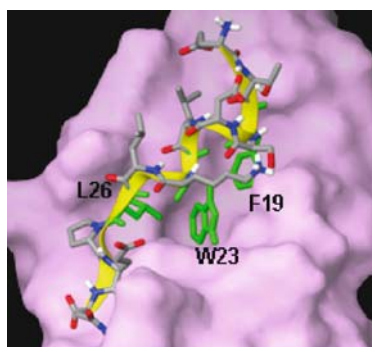
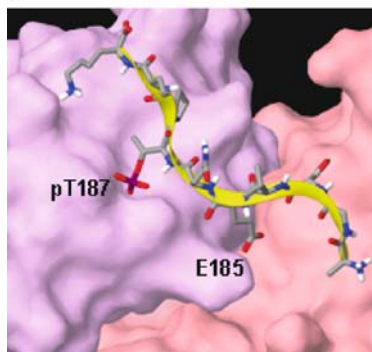
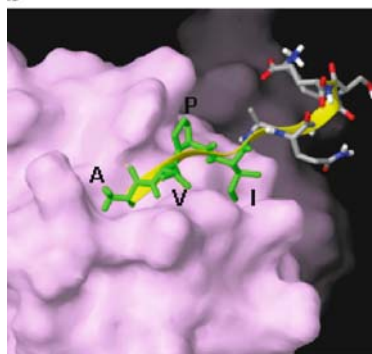
The first potent and selective small molecule inhibitors of this p53–Mdm2 interaction site, Nutlins (cis-imidazoline derivative), have been identified by high-throughput screening followed by structure-based optimization (Vassilev et al. 2004). Nutlins displace recombinant p53 protein from its complex with Mdm2 with IC<sub>50</sub> values in the 100- to 300-nM range. These compounds bind Mdm2 in the p53-binding pocket and activate the p53 pathway in cancer cells, leading to cell cycle arrest, apoptosis, and growth inhibition of human tumor xenografts in nude mice.

Another type of p53 stabilizing small molecule, RITA (2,5-bis(5-hydroxymethyl-2Thienyl)furan), was identified by a cell-based screen of the NCI small molecule library for compounds that specifically arrest growth of a p53-positive cancer cell lines (Issaeva et al. 2004). RITA binds to the N-terminus of p53, prevents p53–Mdm2 interactions, and affects p53 interaction with several negative regulators. Through stabilizing p53, RITA induces apoptosis in various tumor cell lines expressing wild-type p53 and shows substantial p53-dependent anti-tumor effect *in vivo*.

Recently, other small molecule inhibitors of Mdm2-p53 interactions were identified through structure-based virtual screening (Ding et al. 2006; Lu et al. 2006; Bowman et al. 2007). These inhibitors mimic the three critical binding residues of p53, bind to Mdm2 with K<sub>i</sub>s ranging from 3 to 100nM, and are highly effective in the activation of p53 function and inhibition of tumor cell growth.

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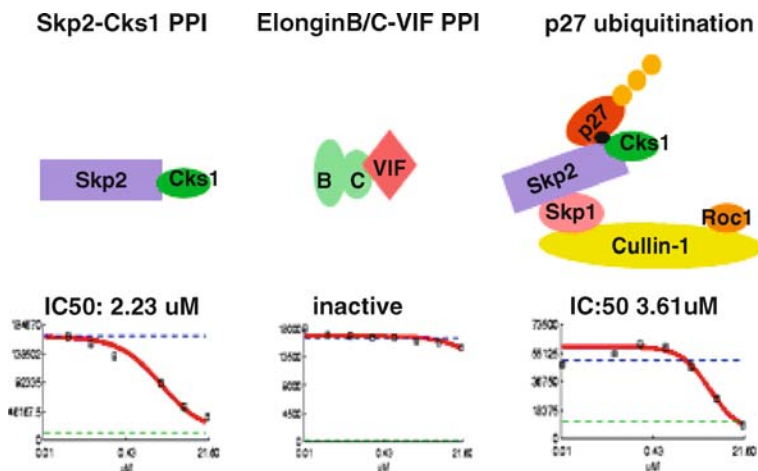
**Fig. 4a–c.** Hotspots on the interaction interface between E3 and its substrate. **a** Mdm2–p53 binding interface. The p53 peptide is represented as a *yellow ribbon*, with three critical side chains of Phe19, Trp23, and Leu26 (*green sticks*) inserted into the Mdm2 hydrophobic cleft. **b** Skp2–Cks1 region involved in the p27 binding. The p27 peptide is shown as a *yellow ribbon*, Cks1 is shaded in *purple*, and Skp2 in *pink*. Cks1 recognizes pThr187 of p27, and the Cks1–Skp2 interface creates the binding pocket for Glu185 of p27. **c** Binding groove on BIR3 for the SMAC N-terminus. The N-terminal four residues of SMAC are shown in *green*

**a****b****c**

## 4.2 SCF E3 Ligases and Their Substrates

The SCF (Skp1-Cullin-F box) complex is a multisubunit ubiquitin ligase that recognizes various substrates involved in different biological pathways through a family of F-box proteins as specific substrate adaptors. Most prominent among these are: SCF<sup>Skp2</sup>-p27 in cell cycle regulation, SCF<sup>βTrCP</sup>-IκB in NFκB activation, SCF-ElonginBC-VHL-HIF1α in hypoxia control, and SCF-Cul5-VIF-A3G in HIV pathogenesis (Carrano et al. 1999; Winston et al. 1999; Kamura et al. 2000; Yu et al. 2003). Due to the structural complexity of these holoenzymes with their substrates, the best approach to obtaining specific and efficient inhibitors of these SCF ligases may be to target specific interaction hot spots between the E3 and its substrate(s).

Ubiquitination of p27 is triggered by Thr187 phosphorylation, which leads to the binding of the SCF<sup>Skp2</sup> ubiquitin ligase complex in the presence of the accessory protein Cks1. The crystal structure of the Skp1-Skp2-Cks1 complex bound to a p27 phosphopeptide provides valuable insight into structural features of the complex that can be employed for development of interaction inhibitors (Hao et al. 2005). Skp2 contains an N-terminal F-box motif for Skp1 interaction, while C-terminal leucine-rich repeats functions in Cks1 and substrate recognition. The phosphorylated Thr187 side chain of p27 is recognized by a Cks1 phosphate binding site, whereas the side chain of an invariant Glu185 inserts into the interface between Skp2 and Cks1 (Fig. 4b). The binding site for Glu-185 is a pocket that would be suitable for small molecule binding, and there are additional nearby clefts at the Cks1-Skp2 interface where an inhibitor may bind. These multiple interactions represent potential sites for small molecule inhibition: phosphorylated p27 binding to Skp2/Cks1 or Cks1 binding to Skp2. In fact, a HTRF assay that allows for the quantification of interaction between Skp2, Cks1, and p27 phosphopeptide has been reported (Xu et al. 2003). High-throughput screening for inhibitors of the Cks1-Skp2 interaction has been performed by others (Huang and Vassilev 2005) and by our group. We have identified a small molecule inhibitor, R276, that selectively inhibits the interaction between Cks1 and Skp2, but does not affect the Elongin BC and VIF binding. Through disrupting the adaptor interaction, this



**Fig. 5.** R276 selectively inhibits the Cks1–Skp2 interaction and p27 ubiquitination by SCF<sup>Skp2</sup> *in vitro*. Activity of the compound was tested in three *in vitro* assays: Cks1–Skp2 interaction assay, ElonginBC–VIF interaction assay, and p27 ubiquitination assay by SCF<sup>Skp2</sup> holoenzyme complex. IC<sub>50</sub>s were determined by eight-point dose ranging from 0.009 to 20 μM

compound also inhibits p27 ubiquitination by the SCF<sup>Skp2</sup> holoenzyme complex with similar potency in the *in vitro* assay (Fig. 5).

Additionally, another compound was identified to prevent incorporation of Skp2 into the SCF<sup>Skp2</sup> complex and interfere with p27 ubiquitination *in vitro* (Chen et al. 2008). This compound accumulated p27 protein in cells, induced G1/S cell cycle arrest, inhibited the growth of a panel of multiple myeloma cells, and also acted synergistically with the proteasome inhibitor Velcade.

Similar approaches can be applied to other SCF and SCF-like E3 ligase complexes, such as inhibitors of βTrCP and phospho-IκBα interaction as anti-inflammatory agent, and interruption of VIF-A3G interaction as novel anti-HIV therapy.

### 4.3 IAPs

IAP (inhibitor of apoptosis proteins) proteins are members of a caspase inhibitor family that blocks a substantial portion of the apoptosis

pathways and are attractive targets for the development of novel cancer therapies (Schimmer et al. 2006). In addition to their signature BIR (baculovirus IAP repeat) domains that bind and inhibit caspases, some IAPs possess carboxyl-terminal RING finger domains that function as E3 ligases to promote ubiquitination and degradation of themselves and several of their binding proteins, such as Caspase-9 and -3.

The anti-apoptotic activity of IAP proteins can be antagonized by the mitochondrial protein SMAC (second mitochondrial activator of caspases). SMAC inhibits IAPs through binding to the BIR domains and antagonizes caspase-BIR interactions. The N-terminal 4 amino acids of SMAC (Ala-Val-Pro-Ile) are necessary and sufficient for binding the BIR pocket of XIAP and preventing XIAP from binding and inhibiting caspase-9 (Wu et al. 2000) (Fig. 5c). Based on the structural studies, several small molecule IAP inhibitors that mimic SMAC in binding to BIR domain and activating caspase have been identified. Some of these molecules are in preclinical development (Schimmer et al. 2006). These IAP antagonists effectively block the interaction between IAP proteins and caspases and show pro-apoptotic activity both *in vitro* and *in vivo*. Interestingly, recent reports suggest a different mechanism of action for the SMAC mimetics (Vince et al. 2007; Varfolomeev et al. 2007; Petersen et al. 2007). These compounds bind to BIR domain, resulting in dramatic induction of autoubiquitination activity and rapid proteasomal degradation of c-IAP, and leading to NF- $\kappa$ B activation and TNF $\alpha$ -dependent apoptosis.

## 5 Concluding Remarks

Many ubiquitin E3 ligases have been validated as therapeutic targets for oncology, inflammation, metabolism, viral infection, and CNS disorders. The success of the proteasome inhibitor Velcade in the treatment of several types of cancers suggests that specific modulation of individual ubiquitin E3 ligase may represent a novel approach with enormous potential for the treatment of a wide range of diseases. Although E3 ligases are not classical enzyme targets with central active sites, the rapid progress in understanding the biochemistry and various drug discovery efforts by both traditional and nontraditional approaches have offered

valuable insights into strategies for interfering with these targets. Despite the challenges in obtaining potent and selective small molecule inhibitors with suitable pharmacological properties, the enormous potential of the target, as well as recent successful cases should encourage and draw much attention from pharmaceutical companies for E3 ligase drug discovery.

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## ***Inhibiting Hdm2 and Ubiquitin-Activating Enzyme: Targeting the Ubiquitin Conjugating System in Cancer***

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A.M. Weissman<sup>(✉)</sup>, Y. Yang, J. Kitagaki, C.A. Sasiela,  
J.A. Beutler, B.R. O’Keefe

Laboratory of Protein Dynamics and Signaling, National Cancer Institute at Frederick,  
Rm 22-103, P.O. Box. Bldg. 560, 21702-1201 Frederick, USA  
email: [amw@nih.gov](mailto:amw@nih.gov)

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**Abstract.** The ubiquitin conjugating system represents a rich source of potential molecular targets for cancer and other diseases. One target of great interest is the RING finger ubiquitin ligase (E3) Hdm2/Mdm2, which is frequently overexpressed in cancer and is a critical E3 for the tumor suppressor p53. For those 50% of tumors that express wild-type p53, agents that inhibit Hdm2 have great potential clinical utility. We summarize our ongoing efforts to identify inhibitors of Hdm2 E3 activity by high-throughput screening of both defined small molecules and natural product extracts. Employing a strategy using both enzymatic and cell-based assays, we have identified inhibitors that block the E3 activity of Hdm2, activate a p53 response, preferentially kill p53-expressing cells, and have the capacity to differentially cause death of transformed cells. Therefore, screening for inhibitors of Hdm2 ubiquitin ligase activity through in vitro assays represents a powerful means of identifying molecules that activate

p53 in cancer cells to induce apoptosis. We also discuss the potential of inhibitors of ubiquitin-activating enzyme (E1) that were discovered during these screens. E1 inhibitors may similarly serve as the basis for novel therapeutics. Additionally, they represent unique tools for providing new insights into the ubiquitin conjugating system.

## 1 Introduction

Ubiquitylation occurs as the result of a multienzyme process involving ubiquitin-activating enzymes (E1s), ubiquitin-conjugating enzymes (E2s), and ubiquitin protein ligases (E3s). It is a potentially reversible process with deubiquitylating enzymes serving to remove ubiquitin from substrates (Hershko and Ciechanover 1998). Among ubiquitin ligases, there are two major classes. There are approximately 50 HECT (homologous to E6-AP carboxyl terminus) domain E3s in the human genome. HECT E3s possess a large catalytic domain of approximately 350 amino acids, the hallmark of which is a cysteine residue to which ubiquitin is transesterified from E2 prior to transfer to heterologous substrates. In contrast, RING finger and RING finger-like E3s, which constitute well over 500 different E3s in humans, are not known to function as catalytic intermediates. Instead, they promote the transfer of ubiquitin from E2 to substrates or to ubiquitin molecules that have already been bound to substrate. The RING finger is a compact structure of approximately 50 amino acids defined by eight cysteines and histidines that coordinate two zinc ions in a cross-braced pattern. This large family includes proteins having a canonical RING finger, those with RING finger variants such as the PHD/LAP finger and also the U-box, which conforms like a RING finger but does so through hydrophilic interactions rather than by coordinating zinc (Fang and Weissman 2004; Lorick et al. 2006).

The ubiquitin conjugating system is involved in virtually all cellular processes in eukaryotes. Numerous proteins that are either components of this system or substrates for ubiquitylation are implicated in cancer and other diseases. For this reason, the ubiquitin conjugating system represents a rich source of clinical molecular targets. The most extensively studied role for ubiquitylation is in the regulated destruction

of proteins by the 26S proteasome. Proof of principle for targeting the ubiquitin-proteasome system (UPS) in cancer has been established with the demonstrated efficacy of the proteasome inhibitor Bortezomib in multiple myeloma (Adams and Kauffman 2004; Leonard et al. 2006). However, as proteasome inhibitors result in the stabilization of many ubiquitylation substrates, it is desirable to target more substrate-specific steps, particularly ubiquitin ligases that are largely responsible for conferring specificity to ubiquitylation.

## 2 Targeting Hdm2

The discovery that RING fingers are, in general, ubiquitin ligase domains, greatly expanded the number of known potential molecular targets (Joazeiro and Weissman 2000; Lorick et al. 1999). Along with our collaborators, we became interested in whether we could identify small molecule inhibitors of the RING finger-dependent ubiquitin ligase activity of Hdm2 (Mdm2 in mouse). Hdm2 is a ubiquitin ligase that leads to proteasomal degradation of the tumor suppressor p53, the guardian of the genome (Aylon and Oren 2007; Levine et al. 2006; Vousden and Lane 2007; Yang et al. 2004). Our logic was that inhibition of the E3 activity of Hdm2 could result in an increase in p53 activity in the roughly 50% of tumors that retain wild-type p53, a substantial number of which have amplified the Hdm2 gene (Momand et al. 1998). An additional premise was that reactivation of p53 would differentially cause apoptosis, as opposed to growth arrest, in tumor cells (Lowe et al. 1993).

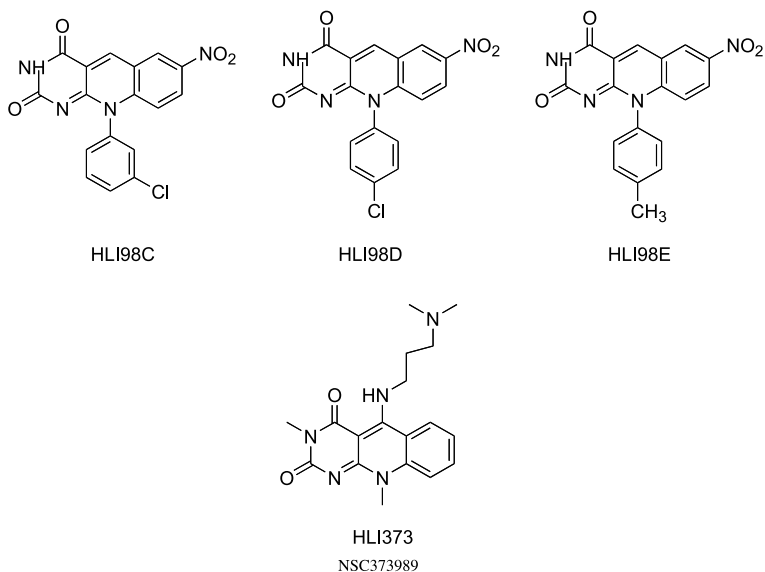
To develop screens for inhibitors of Hdm2 activity, we took advantage of the fact that Hdm2 targets itself as well as p53 for ubiquitin-mediated proteasomal degradation (Fang et al. 2000; Honda and Yasuda 2000). The ability to utilize a decrease in Hdm2 ubiquitylation as a readout for inhibition of its E3 activity simplified assay development by eliminating the need to include p53 in high-throughput screens. Together with our collaborator at NCI (Dr. Karen Vousden, now at the Beatson Institute, Glasgow, UK) and with IGEN (now Bioveris and Meso Scale Discovery), we adapted the gel-based autoubiquitylation assay extensively utilized in our laboratory (Fang et al. 2000; Lorick et al. 1999) to a high-throughput format using GST-Hdm2 bound to mag-

netic beads. Instead of using radioactive ubiquitin or immunoblotting for ubiquitin, an antibody tagged with a ruthenium chelate and IGEN's proprietary ORIGENE technology was employed to carry out our first-generation screens. Inhibition of activity reduced electrochemiluminescence and was scored as a positive hit in this assay (Davydov et al. 2004).

### 3 The Hdm2 Ligase Inhibitor Family of 5-Deazaflavins

Our initial screen of a 10,000 compound small molecule library resulted in approximately 20 hits that showed over 50% inhibition of Hdm2 autoubiquitylation. However, most of these did not show selective inhibition of Hdm2 relative to a HECT domain E3, Nedd4 (neural precursor cell expressed developmentally downregulated 4), when further assessed in our SDS-PAGE-based autoubiquitylation assays. Others appeared to represent nonspecific inhibitors, which we surmised to likely be acting nonspecifically on thiol-active enzymes (i.e., E1 and E2). However, this screen resulted in the isolation of three closely related 7-nitro 5-deazaflavin compounds, referred to as the HLI98s (Hdm2 ligase inhibitors) (Fig. 1), that inhibited Hdm2 autoubiquitylation in a dose-dependent manner *in vitro*. These also caused accumulation of p53 and Hdm2 in cells (Yang et al. 2005).

Since HDM2 is a p53 responsive gene and p53 activity increases in response to genotoxic stress (Aylon and Oren 2007; Levine et al. 2006; Vousden and Lane 2007; Yang et al. 2004), it was important to know that the increases in cellular p53 and Hdm2 observed with the HLI98s were not due to DNA damage. To evaluate this, Hdm2 levels were directly assessed by transfection of Hdm2 under the control of a heterologous promoter into *p53<sup>-/-</sup> mdm2<sup>-/-</sup>* mouse embryonic fibroblasts. Hdm2 accumulated under these conditions, suggesting that the effect observed with the HLI98s could not be accounted for by genotoxic stress. The relative specificity of these compounds was assessed by comparing their inhibition of Hdm2 to effects on other ubiquitin ligases. While there was significant evidence of specificity in cells, this was not complete as there was evidence that the HECT E3, E6-AP, could be partially inhib-



**Fig. 1.** Structure of the HLI compounds. HLI98A-C were identified in an in vitro screen for inhibitors of the Hdm2 ubiquitin ligase activity. HLI373 was evaluated based on its similarity to the HLI98s

ited at higher doses of the HLI98s, as assessed by accumulation of p53 in cells expressing HPV-E6 (Yang et al. 2005).

An additional concern regarding the efficacy of these compounds that stabilize both Hdm2 and p53 relates to the known overlap between the p53 transactivation domain and the site of Hdm2 binding on p53. Thus, the potential existed that stabilized p53 would be functionally inactive as a consequence of being bound to accumulated Hdm2. This concern was alleviated by the finding that there was a clear activation of a p53 response when cells were treated with the HLI98s, although it was not as substantial as that seen with an optimal dose of the DNA damaging agent adriamycin (Yang et al. 2005).

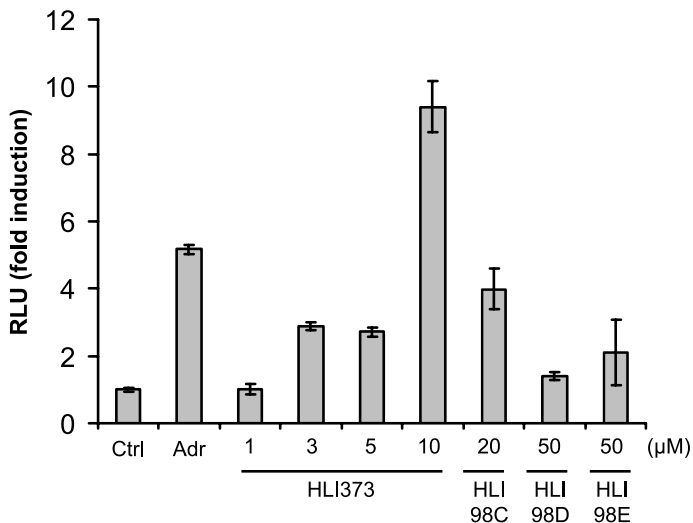
The HLI98s behaved as expected in cell-based assays with differential killing of E1A-transformed tert-immortalized retinal pigment epithelial (RPE) cells as compared to untransformed RPE cells. Further-

more, they showed a differential capacity to kill p53 expressing transformed mouse embryonic fibroblasts compared to untransformed cells (Yang et al. 2005). Together these results established proof of principle for inhibiting the E3 activity of Hdm2 to reactivate p53 in order to induce apoptosis in tumor cells.

One of the limiting features of the HLI98s was their poor solubility in aqueous solutions and their relatively low potency, even at concentrations of 20–50  $\mu\text{M}$ . Issues of solubility are a particularly significant problem in development of pharmaceuticals (Dimond 2005). Therefore, we evaluated related 5-deazaflavin compounds for potential activity. A compound referred to as HLI373 was identified (Fig. 1). The characteristics of this compound *in vitro* and in cells have recently been described (Kitagaki et al.). Most striking is its improved solubility, approximately 200 mM in PBS, its increased potency in stabilizing Hdm2 and p53 ( $\text{IC}_{50} = 3 \mu\text{M}$ ), and a level of transactivation of a p53 response element-driven reporter (el-Deiry et al. 1993) that was substantially greater than either the HLI98s or adriamycin (Fig. 2). Also important was its capacity to target transformed cells for degradation without causing DNA damage and to differentially kill tumor cells expressing wild-type p53 (Kitagaki et al.). Thus, HLI373 is a promising lead for further development.

#### 4 Natural Products Screens

To maximize the potential utility of our novel screening system for inhibitors of Hdm2 autoubiquitylation, we adapted the assay for use in screening natural product extracts. Approximately 50% of pharmaceuticals developed for use in cancer over the last 65 years are derived from natural products (Newman and Cragg 2007). Nevertheless, many large pharmaceutical companies no longer screen crude natural product extracts as part of their high-throughput screening programs. Part of the reason for this dichotomy is that natural product extracts contain many nuisance compounds that interfere with commonly used fluorescent and colorimetric assay endpoints. To take advantage of the rich chemical diversity of natural compounds contained within the NCI natural product extract repository, the Hdm2 screen described in Sect. 2 was adapted

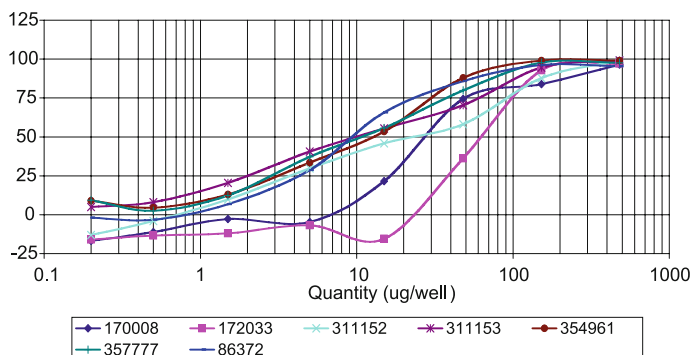


**Fig. 2.** p53 transactivation by HLI compounds. pG13 stably transfected U2OS cells, stably expressing a luciferase reporter under the control of multiple copies of a p53 response element (U2OS-pG13), were incubated with 1  $\mu$ g/ml adriamycin or with the HLI98s, as indicated for 22 h and then evaluated for luciferase activity. Data represent average and standard deviation of three independent experiments. Data previously published in Kitagaki et al. (2008)

for use in a screen of natural products extracts by optimizing a variation of the IGEN ORIGENE technology that had been developed by Meso Scale Discovery. This system utilized plate-based electrochemiluminescent technology rather than the previous bead-based system. Optimizing the plate-based technology required adaptations in the kinetics of the ubiquitylation reactions to allow for efficient 384-well high-throughput screening. This assay was then further modified to the necessities of screening natural product extracts. The changes made to optimize the assay system included: (a) modification of the kinetics of the ubiquitylation reaction by precharging E2 with ubiquitin; (b) prebinding of Hdm2 to the assay plate to prevent identification of extracts that inhibit Hdm2 binding; and (c) the addition of BSA to the assay mixture to prevent false-positive results due to nonspecific protein binding. The assay

already took advantage of electrochemiluminescence as an endpoint, which helped eliminate false-negative results from inherently fluorescent or colored natural compounds (Sasiela et al. 2008).

To test the new assay system, we undertook the screening of the NCI's Structural Diversity Set. This is a group of roughly 1,900 compounds selected to represent the overall chemical diversity present in the NCI Developmental Therapeutics Program's synthetic compound library. Screening of this library resulted in the identification of six compounds that displayed concentration-dependent inhibition of Hdm2 autoubiquitylation (Fig. 3a). The majority of these compounds were alkaloids that demonstrated moderate activity against Hdm2 (Fig. 3b). The fact that the active compounds appeared to be natural products or natural product-derived, validated our overall strategy of screening natural product extracts. These active compounds were further evaluated in a series of cell-based assays that we developed based on studies with the HLI compounds, so as to further prioritize lead compounds and extracts (Fig. 4). This provided the means to differentiate compounds based on their ability to induce desirable cellular responses. As can be seen in Fig. 5a, at a concentration of 5  $\mu$ M, three of these compounds—NSC311152, NSC311153, and NSC354961—resulted in increased levels of endogenous p53 and of Hdm2. As NSC354961 had the most significant effect on p53, it was evaluated further and found to increase both p53 and Hdm2 in a dose-dependent manner (Fig. 5b). This increase was not due to a genotoxic effect resulting in activation of a p53 response, as stabilization of Hdm2 was also observed in *p53<sup>-/-</sup>mdm2<sup>-/-</sup>* mouse embryo fibroblasts (Lowe et al. 1993) (Fig. 5c). To directly evaluate whether p53 ubiquitylation was being inhibited, HCT116 cells expressing p53 were evaluated for accumulation of ubiquitylated p53 (Fig. 5d). While the proteasome inhibitor, ALLN, resulted in the accumulation of p53 as well as ubiquitylated p53 the addition of NSC354961 prevented the proteasome-dependent accumulation of ubiquitylated forms, consistent with inhibition of ubiquitylation. The results presented thus far are all similar to the HLIs in demonstrating clear effects on accumulation of p53 and on inhibition of its ubiquitylation. However, for compounds to have possible clinical utility, they need to differentially kill transformed cells and induce a p53 response. Unfortunately, this agent showed poor induction of a p53-driven reporter

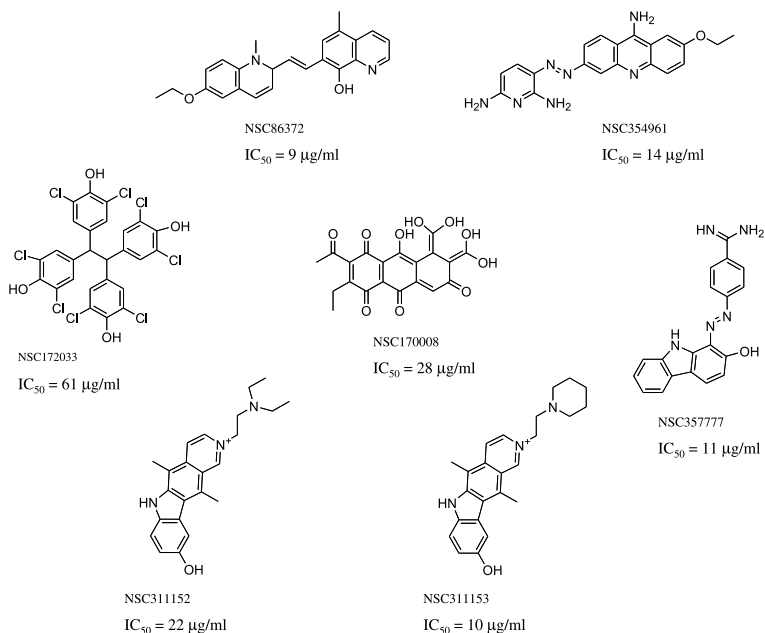


**Fig. 3a.** In vitro inhibition of Hdm2 E3 ligase activity by select pure compounds. Compounds from the NCI Developmental Therapeutics Program's Structural Diversity Set were tested for their ability to inhibit Hdm2 autoubiquitylation in an electrochemiluminescent assay system. Concentration response curves were determined for seven active compounds

and significant toxicity was found in nontransformed cells at concentrations necessary to obtain over 50% killing of transformed cells (data not shown). Thus, while NSC354961 has the potential to stabilize p53, it is less than ideal for further development.

Initial validation of the Hdm2 screen with the Structural Diversity Set and the identification of natural compound inhibitors encouraged further screening of natural product extracts to identify additional compounds with more favorable characteristics. We proceeded to screen more than 140,000 natural product extracts. This resulted in the identification of over 2,800 extracts that inhibited autoubiquitylation of Hdm2 in the primary screen and were scored as positive hits. Greater than 2,200 of these extracts were confirmed by subsequent screening (~80% confirmation rate).

As more than 2,200 extracts were initially identified as inhibiting Hdm2, additional assays were designed, utilizing other ubiquitin ligases, to help prioritize extracts with the ability to selectively inhibit Hdm2. For these secondary assays, the RING finger E3 ligases X-linked inhibitor of apoptosis (XIAP) (Salvesen and Duckett 2002) and muscle ring finger 1 (MuRF1) (Glass 2003) along with the HECT domain E3



**Fig. 3b.** In vitro inhibition of Hdm2 E3 ligase activity by select pure compounds. Chemical structures and calculated IC<sub>50</sub> values for the seven Hdm2 inhibitory compounds identified from the Diversity Set

Nedd4 (Kumar et al. 1997) were selected. Of the more than 2,200 confirmed hits, 472 were found to selectively inhibit only the RING finger ligases; having little or no activity against Nedd4. Furthermore, of those 472 RING finger-selective extracts, 94 displayed selectivity for Hdm2 (Sasiela et al. 2008). Finally, in order to enhance the opportunity to find selective E3 inhibitors, these extracts were evaluated for inhibition of E2. As shown in Fig. 6, many of the extracts showed the desired selectivity for Hdm2 while showing little or no activity against XIAP, MuRF1, Nedd4, or E2. An additional advantage of the strategy of prioritizing extracts is that this also leads to the identification of extracts that show activity against these other E3 ligases. As XIAP, MuRF1, and Nedd4 are all interesting targets in their own right, the identifica-

### Cell-based Metric for Evaluation of Hdm2 Inhibitors

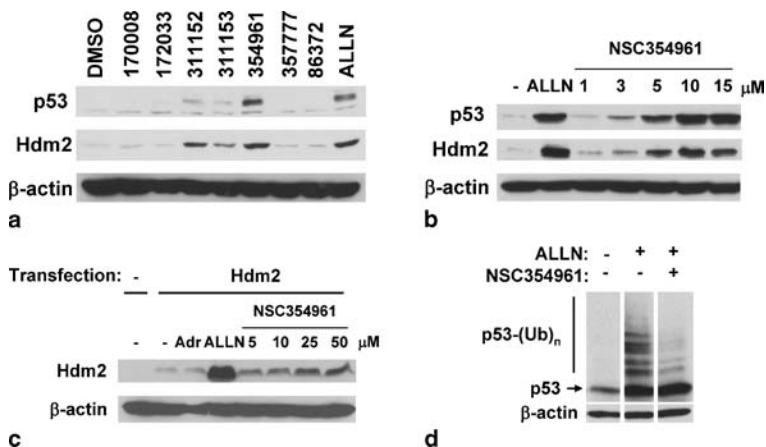
- Stabilization of cellular p53
- Stabilization of Hdm2 independent of p53
- Inhibition of p53 ubiquitylation
- Induction of p53 transcriptional activity
- Relative specificity for Hdm2
- Selective killing of transformed cells
- Selective killing of p53<sup>+</sup> cells



**Fig. 4.** Cell-based assays used to evaluate potential inhibitors of Hdm2. Cell-based secondary assays were utilized to evaluate the ability of compounds, identified in cell-free primary assays, to recapitulate Hdm2 inhibitory activity in cells. Direction of *arrow* indicates increasing stringency in the selection process for desirable compound attributes

tion of pure compounds that were found to target these ligases is potentially significant. Isolation of active compounds from these extracts that targets each of these ligases, as well as those that specifically inhibit Hdm2, is ongoing.

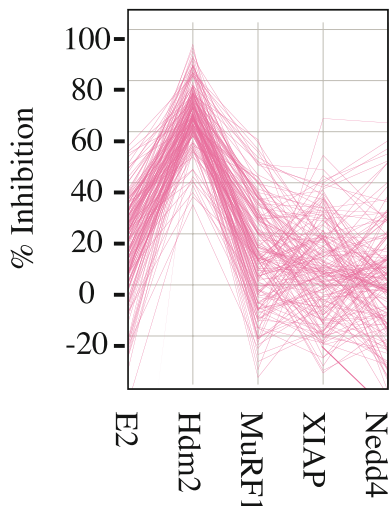
One of the results of our screen of natural products was the identification of a pure natural product with inhibitory activity against Hdm2. This compound, sempervirine (Fig. 7A), inhibited Hdm2 autoubiquitylation with an  $IC_{50}$  of 8  $\mu\text{g/ml}$ . Sempervirine was evaluated more thoroughly employing the aforementioned algorithm of cell-based assays (Fig. 4) to determine if its activity in a cellular context was consistent with its ability to inhibit Hdm2 autoubiquitylation. The results, as recently reported (Sasiela et al. 2008), clearly showed that sempervirine leads to the accumulation of Hdm2 in a manner that is independent of genotoxic stress-mediated activation of p53 and that, in accord with its predicted function, it leads to accumulation of p53 and an inhibition of p53 ubiquitylation in cells. In addition, increasing doses of this plant-derived compound induces a p53 response and selectively induces apoptosis in transformed cells in a p53-dependent manner.



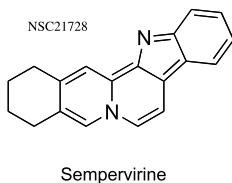
**Fig. 5a–d.** NSC354961 inhibits Hdm2 activity in cells. **a** RPE cells were incubated with vehicle control, 5  $\mu\text{M}$  of each of seven compounds assessed in Fig. 3, or 50  $\mu\text{M}$  of the proteasome inhibitor ALLN for 8 h. This was followed by lysis of cells, resolution by SDS-PAGE and immunoblotting for p53, Hdm2 and  $\beta$ -actin as a loading control. **b** RPE cells were incubated as in **a** and accumulation of p53 and Hdm2 assessed. **c**,  $p53^{-/-}mdm2^{-/-}$  mouse embryo fibroblasts (MEFs) were transfected with plasmid encoding Hdm2 for 48 h prior to incubation with 1  $\mu\text{g}/\text{ml}$  adriamycin (Adr), 50  $\mu\text{M}$  ALLN or 5–50  $\mu\text{M}$  NSC354961 for 8 h. Hdm2 was analyzed by immunoblotting. **d** Human colon cancer HCT116-p53<sup>+</sup> cells were pretreated with 15  $\mu\text{M}$  NSC354961 for 1 h and then for an additional 7 h with 50  $\mu\text{M}$  ALLN. p53 was assessed by immunoblotting

## 5 Inhibiting Ubiquitin-Activating Enzyme, Identification of PYR-41

While our initial screening of a 10,000-compound small molecule library resulted in the identification of the HLI98s and led to the identification of HLI373, a subsequent screen of a 100,000-member-small-molecule library from Chembridge resulted in hits that appeared promising in the high-throughput assay but did not specifically inhibit Hdm2 when evaluated further. However, among these compounds was one that demonstrated inhibition of loading of the ubiquitin E1 (UBA1) but not of E2s. Further assessment led to the conclusion that this pyrazone-

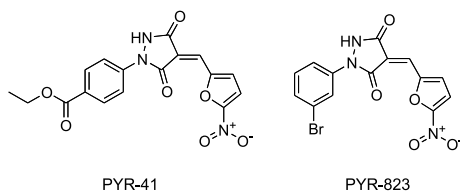


**Fig. 6.** Specificity of selected natural product extracts for inhibition of Hdm2. Organic and aqueous extracts (68.5  $\mu\text{g/ml}$ ) were tested in a cell-free electrochemiluminescent assay system that measured E3 ligase activity via quantification of autoubiquitylation of four ubiquitin ligases. Additional control assays determined the ability of the same extracts to inhibit E2 (UbcH5B) activity utilizing a similar electrochemiluminescent assay system



**Fig. 7.** The chemical structure of sempervirine

derived compound, PYR-41 (pyrazone-41) (Fig. 8) modified E1 in an irreversible manner. This inhibition was prevented by co-incubation with reducing agent (Yang et al. 2007). This suggests that PYR-41 may be acting on the active site cysteine of E1 rather than on the ATP binding site required for initial activation of ubiquitin (Haas and Rose 1982;



**Fig. 8.** The chemical structures of E1 inhibitory pyrazole derivatives

Haas et al. 1982). Similar results were obtained with a related compound, PYR-823 (Fig. 8) (Yang et al. 2007).

PYR-41 is active in cells, resulting in a marked decrease in E1~Ub thiolester formation as well as inhibition of a number of proteasomal and nonproteasomal ubiquitin-mediated processes. The potential for this reagent to be of therapeutic efficacy is underscored by its inhibition of NF- $\kappa$ B activation, which correlated with inhibition of both K63 ubiquitylation of TRAF6 and inhibition of K48 I $\kappa$ B ubiquitylation. Further, much like the Hdm2 inhibitors, PYR-41 resulted in stabilization of p53 and a p53 response, as assessed by transactivation of a reporter gene. This response was substantially greater than that seen with the HLI98s and sempervirine, although not as striking as that seen with HLI373. This activation correlated with other findings observed with Hdm2 inhibitors, including differential killing of transformed cells and particularly transformed cells expressing p53 (Yang et al. 2007).

A striking finding with PYR-41 was a marked increase in total cellular sumoylation. While this could represent an off-target effect of this compound by, for example, inhibiting desumoylating enzymes, a similar increase in sumoylation was observed using two different well-characterized cells expressing a temperature-sensitive form of the ubiquitin E1, UBA1 (Yang et al. 2007). The relationship between ubiquitylation and sumoylation uncovered in these studies now becomes an interesting area for future study. One possible explanation relates to the known competition between ubiquitylation and sumoylation on targets such as I $\kappa$ B and p53 (Ulrich 2005); our findings might suggest that this relationship is more general than previously appreciated. However, there is another highly intriguing non-mutually exclusive possibility. At least one ubiquitin ligase, RNF4 (SNURF) (Hakli et al. 2004) has re-

cently been found to recognize and ubiquitylate certain sumoylated proteins (Prudden et al. 2007; Sun et al. 2007). Thus, part of the explanation for the findings observed could be a consequence of failure to degrade sumoylated species. There are clearly many issues to be explored with inhibitors of the ubiquitin E1 both with regard to basic scientific issues and potential clinical utility.

## 6 Discussion

The ubiquitin conjugating system is a rich source of potential molecular targets in cancer (Fang et al. 2003; Nalepa et al. 2006). The general disruption of the system through the use of proteasome inhibitors represents a relatively nonspecific means of disrupting this system. Nevertheless, proteasome inhibitors are proving to have a significant therapeutic index in at least some malignancies (Adams and Kauffman 2004; Leonard et al. 2006).

p53 plays a central role in response to genotoxic stress, has clear importance in inducing cell growth arrest and apoptosis in cancer cells, and is regulated to a great degree by ubiquitylation and proteasomal degradation. As Hdm2 has consistently proven to be a major and essential ubiquitin ligase for p53, there is significant interest in preventing Hdm2 from targeting p53 for degradation. One approach has been to reactivate p53 by disrupting its interaction with Hdm2 (Issaeva et al. 2004; Li et al. 2005; Vassilev et al. 2004). Indeed the identification of the nutlins, which block the interaction between the two proteins, suggests that this approach may be efficacious (Vassilev 2007). Another approach utilized by us and others has focused on identifying small molecules that might decrease the ubiquitin ligase activity of Hdm2 and thereby increase p53 levels and increase its cellular activity (Davydov et al. 2004; Kitagaki et al.; Lai et al. 2002; Sasiela et al. 2008; Wilson et al. 2007; Yang et al. 2005).

In our studies, we have identified multiple members of the 5-deaza-flavin family as inhibitors of Hdm2 that activate p53. Active members of this family now include a potent water-soluble version. Thus, there is great potential for this compound to serve as a lead for development of additional reagents.

As natural products provide the opportunity to explore an unparalleled range of chemical space and represent a rich source of reagents either for direct use or to serve as the basis for generating synthetic compounds, we have expanded our screening to include the more than 140,000 natural product extracts maintained by the NCI. Purification and characterization of hits from screening of natural products extracts is ongoing at NCI and it is evident that, using this approach, there is significant potential to identify new potent inhibitors of Hdm2 that will reactive p53. Proof of principle for the potential utility of natural products is borne out with results achieved with sempervirine and with the demonstration that at least one alkaloid contained within the NCI diversity set has the potential to stabilize Hdm2 and p53. We believe, based on our results to date, that there is great potential for natural products to yield novel ubiquitin ligase inhibitors with therapeutic potential in cancer and other diseases.

As conjugation of proteins with ubiquitin is a multienzyme process, screens for inhibitors of substrate-specific ubiquitin ligases always have the potential to result in the identification of inhibitors of E1 or E2, the proximal enzymes in this cascade. We have isolated at least one family of compounds that include PYR-41 and PYR-823 that show relative selectivity in inhibiting E1. These represent the first members of a potential new set of tools to explore the ubiquitin system and its relationship to sumoylation. However, what is also apparent from our *in vitro* studies is that PYR-41 can have desirable effects that may be useful in cancer treatment. These include inhibition of the pro-survival NF- $\kappa$ B family of transcription factors and reactivation of p53. Studies to determine how PYR-41 and related compounds function to inhibit E1 are currently ongoing; however, data accumulated to date suggest that it is acting on the active site of E1 (Yang et al. 2007).

Our efforts began to provide proof of principle for inhibiting RING finger E3s. We now find that we have agents in hand with real potential for reactivating p53 in cancer that may serve as leads for new therapeutics. At the same time, we have come full circle in showing that we can nonspecifically inhibit the ubiquitin system by blocking E1 and that this too has the potential to serve as a basis for therapeutics. These are still early days and further advancement will await additional structure–activity relationship studies, purification of natural products from the

extracts that have now been identified, and the testing of these inhibitors in the appropriate *in vivo* models.

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