

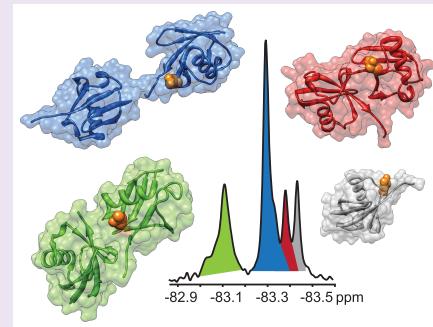
Simultaneous Detection of Distinct Ubiquitin Chain Topologies by ^{19}F NMR

Sujan S. Shekhawat,[†] Grace H. Pham,[†] Jyothiprashanth Prabakaran, and Eric R. Strieter*

Department of Chemistry, University of Wisconsin–Madison, 1101 University Avenue, Madison, Wisconsin 53706, United States

Supporting Information

ABSTRACT: The dynamic interplay between ubiquitin (Ub) chain construction and destruction is critical for the regulation of many cellular pathways. To understand these processes, it would be ideal to simultaneously detect different Ub chains as they are created and destroyed in the cell. This objective cannot be achieved with existing detection strategies. Here, we report on the use of ^{19}F Nuclear Magnetic Resonance (NMR) spectroscopy to detect and characterize conformationally distinct Ub oligomers. By exploiting the environmental sensitivity of the ^{19}F nucleus and the conformational diversity found among Ub chains of different linkage types, we can simultaneously resolve the ^{19}F NMR signals for mono-Ub and three distinct di-Ub oligomers (K6, K48, and K63) in heterogeneous mixtures. The utility of this approach is demonstrated by the ability to interrogate the selectivity of deubiquitinases with multiple Ub substrates in real time. We also demonstrate that ^{19}F NMR can be used to discern Ub linkages that are formed by select E3 ligases found in pathogenic bacteria. Collectively, our results assert the potential of ^{19}F NMR for monitoring Ub signaling in cells to reveal fundamental insights about the associated cellular pathways.



With approximately 5% of the human genome dedicated to the Ub network, Ub modifications play a pervasive role in cellular physiology. Failure to install or remove Ub modifications at the appropriate time can lead to the pathogenesis of a number of human diseases, including many cancers¹ and neurological disorders.² Covalent attachment of Ub to substrate lysine residues—a process termed ubiquitination—occurs through the concerted action of three enzymes: E1s (Ub-activating), E2s (Ub-conjugating), and E3s (Ub-ligases).^{3–7} Once a single Ub has been appended to a target protein, its seven lysine residues (K6, K11, K27, K33, K48, and K63) and N-terminus (M1) can serve as points of attachment to the C-terminus of the next Ub molecule in a growing poly-Ub chain. This process furnishes chains composed of a single linkage (homotypic) or a mixture of linkages (heterotypic). The prevailing view is that each Ub chain type orchestrates distinct biochemical pathways.^{8,9} For example, K48-linked chains that harbor a minimum of four subunits act as proteasome-targeting signals;^{10–12} K63-linked chains promote proteasome-independent events such as kinase activation and lysosomal degradation;^{13–15} M1-linked chains regulate nuclear factor- κB signaling;^{16–18} K6-linked chains respond to UV-induced DNA damage;¹⁹ and K11-linked chains mark proteins for degradation during mitosis.²⁰ To regulate these processes, there are approximately 90 human deubiquitinases (DUBs) that selectively cleave the isopeptide linkages between Ub and its substrate or between individual Ub molecules in a chain.^{21–23} Several open and pressing questions remain regarding (i) how different Ub chains are temporally regulated in the cell, (ii) how chain linkage and topology affect processing by DUBs, and (iii)

how the information imbedded in each type of Ub modification is translated into different biological outcomes. Direct and noninvasive methods that can simultaneously monitor different Ub chain types and their interactions are needed to decipher the intricate Ub signaling networks.

Two approaches are commonly employed to characterize Ub chains: mass spectrometry (MS)²⁴ and mutation of Ub lysine residues to arginine (K-to-R Ub variants). MS is powerful in terms of scope, but this approach requires significant sample preparation and cannot be used to directly identify Ub chains in their cellular context. Analyses with Ub K-to-R variants are useful for elucidating Ub linkages assembled by various E3 ligases. However, the K-to-R substitution strategy only unambiguously examines one linkage type at a time and cannot report on the dynamic regulation of different Ub chain types. To address these shortcomings, we sought to develop an alternative approach that can simultaneously monitor different Ub chains as they are created and destroyed in real time.

Structural studies show that Ub chains reside in two general types of conformation ensembles: compact, in which subunits interact through hydrophobic patches, and open, in which the subunits do not interact. Ub dimers bearing K6-, K11-, and K48-linkages adopt primarily compact conformations,^{20,25–32} whereas M1- and K63-linked dimers are relatively open^{33,34} (Figure 1). We envisioned these conformational differences could be discerned through the use of a probe that is

Received: April 25, 2014

Accepted: August 14, 2014

Published: August 14, 2014

environment-sensitive and detectable in a heterogeneous mixture.

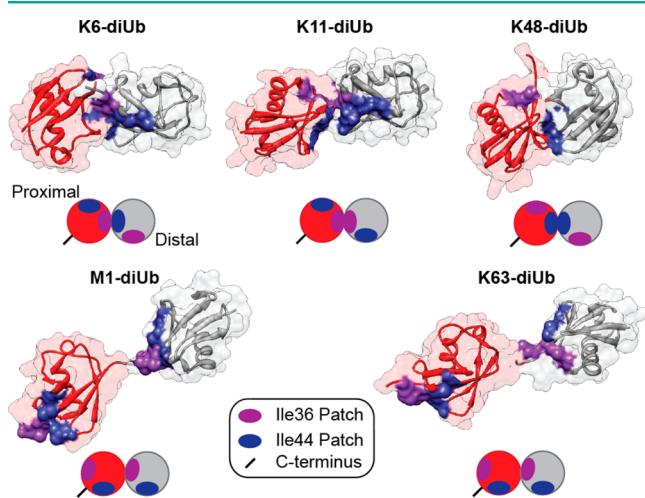
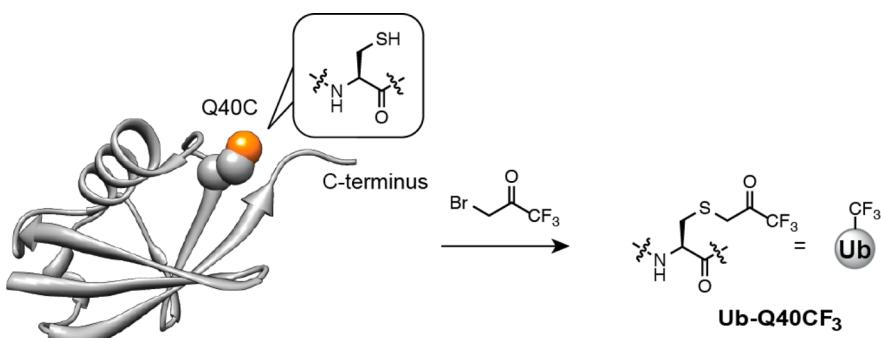


Figure 1. Different conformations of Ub dimers. The proximal subunit bearing a free C-terminus is shown in red and the distal subunit is shown in gray. The Ile36 and Ile44 hydrophobic patches are indicated in purple and blue, respectively. The Ile36 patch consists of Ile36, Leu71, and Leu73. The Ile44 patch is comprised of Leu8, Ile44, and Val70. Protein Data Bank (pdb) codes: K6-diUb (2xk5),²⁸ K11-diUb (2mbq),³² K48-diUb (1aar),²⁵ M1-diUb (2w9n),³⁴ and K63-diUb (2jfs).³⁴

We identified fluorine (¹⁹F) nuclear magnetic resonance (NMR) spectroscopy^{35–37} as a promising approach to specifically detect different Ub oligomers. This method has been used to study the structures and dynamics of a number of proteins, including green fluorescent protein,³⁸ α -synuclein,^{39–41} and DNA polymerase.⁴² Since fluorine is virtually absent from biological systems, ¹⁹F NMR spectroscopy can even be used to specifically track fluorinated proteins in cell extracts and *in cellulo*.^{43,44} Fluorine labels can be readily incorporated into proteins using either chemical or biosynthetic methods without affecting structure and function.³⁵ In light of fluorine's unique properties, we hypothesized that a strategically placed ¹⁹F label on Ub oligomers would experience varying electronic environments and exhibit unique ¹⁹F chemical shifts, directed by the type of Ub linkage (Figure 1). Here, we demonstrate a ¹⁹F NMR-based approach to assess heterogeneous protein mixtures for the formation and destruction of select Ub linkages.

Scheme 1. Site-Specific Labeling of Ub-Q40C with 3-Bromo-1,1,1-trifluoroacetone (BFA) to Afford Ub-Q40CF₃



During substrate ubiquitination and Ub chain formation, the C-terminus of Ub undergoes significant chemical changes as it is transformed from a free carboxylate to a thioester and finally to an isopeptide bond. We surmised that a ¹⁹F label incorporated close to the C-terminus of Ub would be sensitive to these changes and exhibit different chemical shifts corresponding to distinct Ub linkages. Previous studies have indicated that the C-terminal region from His68 to Gly76 is intolerant of amino acid substitutions.⁴⁵ Thus, to avoid disrupting Ub structure and function, we looked for a residue that is distant from the substitution-intolerant C-terminus of Ub in terms of sequence but is spatially close to the C-terminus in the folded Ub structure. The X-ray crystal structure of Ub (PDB: 1UBQ) shows glutamine 40 (Q40) oriented toward the C-terminus, and studies in yeast demonstrated that this residue can be substituted without deleterious effects on Ub function.⁴⁶ For our study, we generated a variant of Ub that contains the Q40C substitution; the Cys residue was then modified with 3-bromo-1,1,1-trifluoroacetone (BFA)⁴⁷ to afford a side chain similar in size to the native glutamine residue (Scheme 1).

To assess our experimental approach, we used ¹⁹F NMR to monitor a ubiquitination reaction with a pair of E2 conjugating enzymes, UBE2N-UBE2V1, known to form only K63-linked Ub oligomers.^{13,48} Because solution-phase ¹⁹F NMR measurements are limited to proteins smaller than 100 kDa,³⁶ we anticipated the formation of high molecular weight Ub chains would convolute the analysis of ¹⁹F NMR signals. We therefore limited our studies to the formation of dimers (17 kDa). Two separate Ub variants were used: UbK0-Q40CF₃ and Ub-D77. UbK0-Q40CF₃ lacks all lysine residues found in the native Ub sequence and can only cap a Ub chain (i.e., as a distal Ub) whereas Ub-D77 is blocked at its C-terminus with an aspartate residue and can only serve as the base of a chain (i.e., proximal Ub). As shown in Figure 2A, ¹⁹F NMR analysis can be used to resolve mono-Ub and K63-linked Ub dimers, and continuously monitor dimer formation without additional sample handling. To confirm the new signal represents K63-linked dimers and test whether ¹⁹F NMR can be used to observe hydrolysis of Ub oligomers by DUBs, ATP was removed from the reaction mixture by dialysis and a K63 linkage specific DUB, AMSH (Associated Molecule with the SH3 domains of STAM),⁴⁹ was added. As anticipated, the signal corresponding to the K63-linked dimer disappeared over time as the mono-Ub signal was recovered (Figure 2B). Formation and hydrolysis of K63-linked dimer were confirmed by SDS-PAGE.

Having demonstrated the utility of ¹⁹F NMR for detecting K63-linked Ub dimers, we sought to extend our approach to study other Ub chain types. We focused initial efforts on the

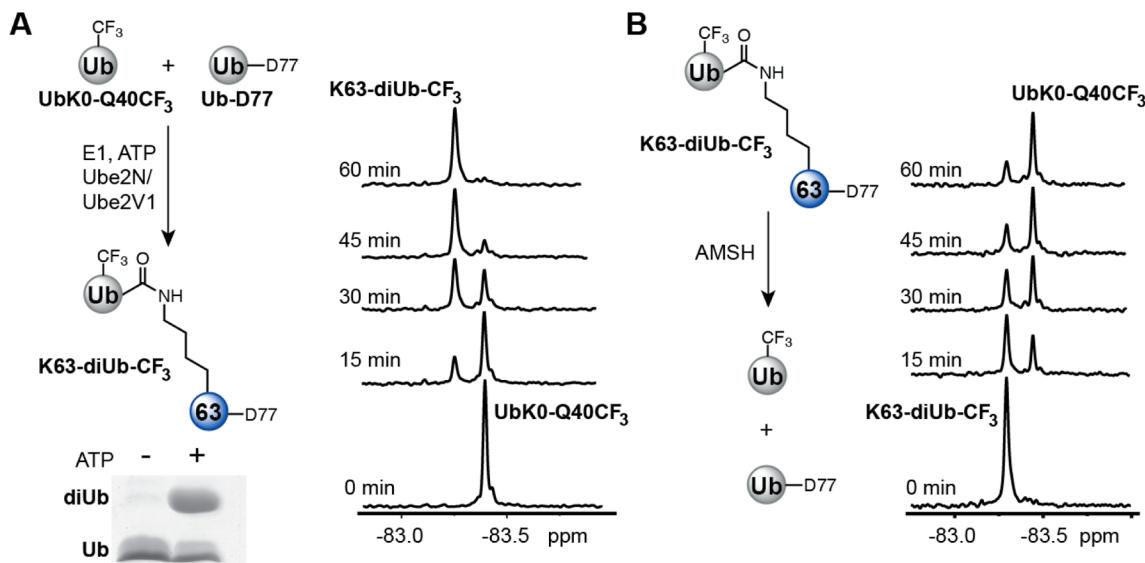


Figure 2. Using ¹⁹F NMR to monitor the formation and disassembly of K63-linked Ub dimers in real-time. (A) Formation of K63-linked diUb-CF₃ using K63 linkage specific enzymes Ube2N/Ube2V1. UbK0-Q40CF₃ (-83.43 ppm) is converted to K63-diUb-CF₃ (-83.25 ppm) within an hour. SDS-PAGE analysis shows formation of dimer in the presence of ATP. B. Cleavage of K63-linked diUb-CF₃ using the K63 linkage specific DUB AMSH.

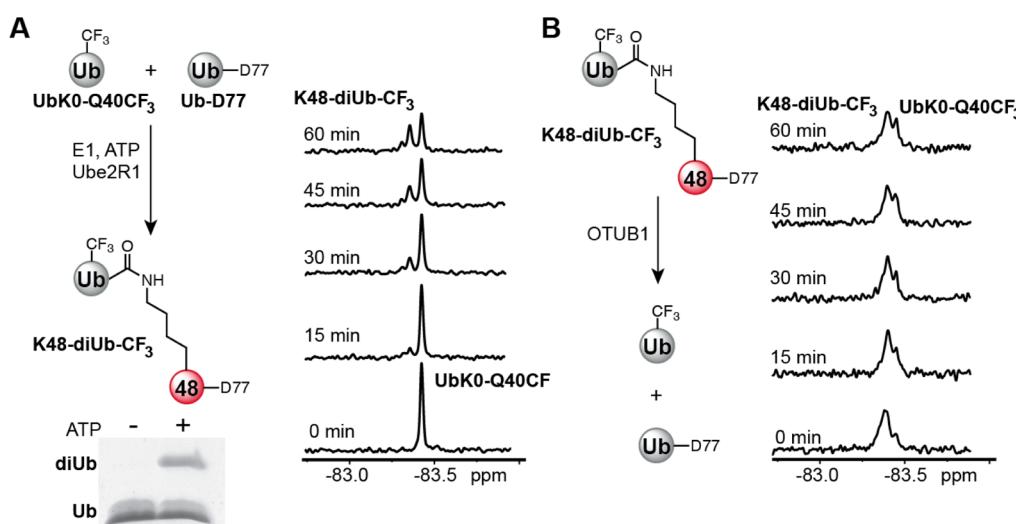


Figure 3. Using ¹⁹F NMR to monitor the formation and disassembly of K48-linked Ub dimers in real-time. A. Formation of K48-linked diUb-CF₃ (-83.36 ppm) using the K48 linkage specific enzyme Ube2R1 and UbK0-Q40CF₃ (-83.43 ppm). SDS-PAGE analysis shows formation of dimer in the presence of ATP. (B) Cleavage of K48-linked diUb-CF₃ using the K48 linkage specific DUB OTUB1.

analysis of K48 linkages due to their high cellular abundance^{50–54} and prominent role as proteasome-targeting signals.^{10,11} The K48 linkage-specific E2 conjugating enzyme Ube2R1^{55–57} was used to assemble Ub dimers from UbK0-Q40CF₃ and Ub-D77. Addition of ATP initiated chain formation, with a new signal (-83.36 ppm) appearing downfield from that of mono-Ub (-83.43 ppm) suggesting the formation of K48-linked dimers (Figure 3A). Addition of OTUB1, a K48 linkage-specific DUB,^{58,59} confirmed this supposition; a time-dependent decrease in the intensity of the new peak was observed along with a concomitant increase in the mono-Ub signal (Figure 3B). Overlaying the spectra of K48- and K63-linked dimers reveals that the two oligomers have distinct chemical shifts (Supporting Information Figure S4). Thus, ¹⁹F NMR can be used to monitor the formation of

the two most abundant forms of Ub oligomers found in cells: those that are linked by K48 and K63.

We next used the E3 ligase activity of a bacterial protein, NleL (non-LEE-encoded ligase), to assess the formation of K6 linkages by ¹⁹F NMR. NleL is produced by the enterohemorrhagic *E. coli* O157:H7⁶⁰ and was recently found to assemble unanchored K6- and K48-linked chains *in vitro*.^{27,61} Addition of NleL to reaction mixtures containing UbK0-Q40CF₃ and Ub-D77 resulted in the appearance of two new peaks, one at -83.10 and another at -83.36 ppm (Figure 4A). OTUB1 triggered the loss of the peak at -83.36 ppm, consistent with the selective removal of K48 linkages (Figure 4B). To determine whether the peak at -83.10 ppm corresponded to K6 linkages, Ub dimers were purified using size exclusion chromatography and analyzed by ¹⁹F NMR and MS. The results of these experiments confirmed the presence of

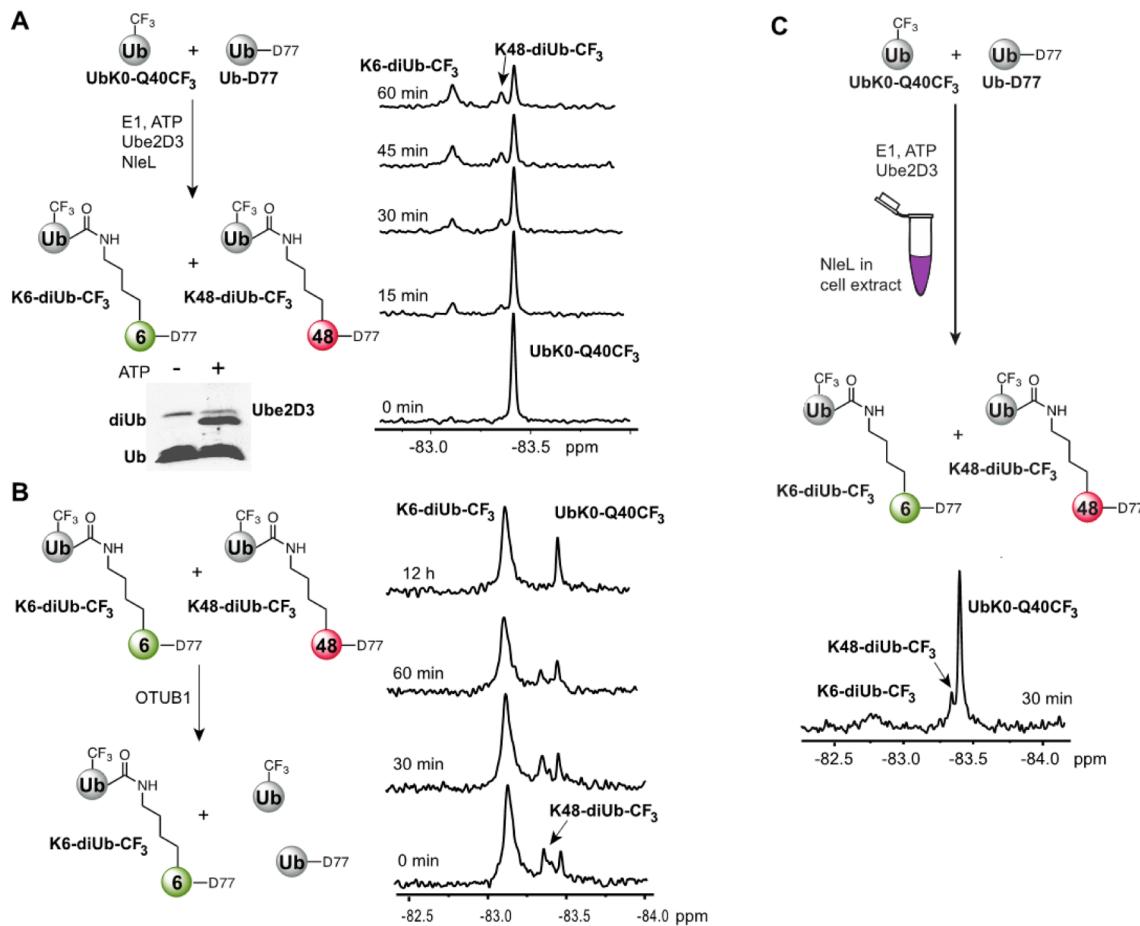


Figure 4. Using ¹⁹F NMR to detect K6 linkages. (A) NleL-catalyzed chain formation. K6-linked diUb-CF₃ (~-83.10 ppm) and K48-linked diUb-CF₃ (~-83.36 ppm) are both formed during the reaction. SDS-PAGE analysis shows formation of dimer in the presence of ATP. (B) OTUB1-catalyzed hydrolysis results in the loss of the signal for K48-linked diUb-CF₃ (~-83.36 ppm) and an increase in the signal for UbK0-Q40CF₃ (~-83.43 ppm). (C) Analysis of dimer formation by NleL translated by *E. coli* S30 cell extract. NleL was added to the reaction without purification and catalyzed formation of K6-linked diUb-CF₃ (~-82.8 ppm) and K48-linked diUb-CF₃ (~-83.35 ppm). The signals for mono-Ub and both dimer species were collectively shifted downfield due to interactions with cell extract components. However, the ubiquitination landscape remains the same as when purified NleL was used for the analysis.

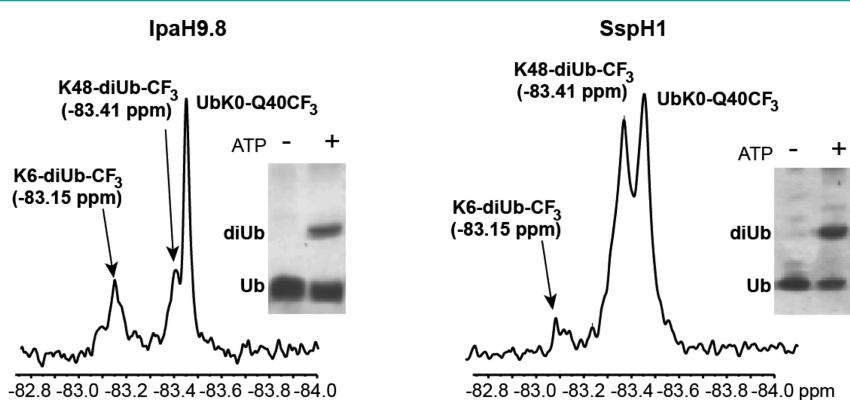


Figure 5. ¹⁹F NMR characterization of the Ub chain linkages assembled by the bacterial E3 ligases IpaH9.8 and SspH1 reveals the presence of K6 and K48 linkages. SDS-PAGE analyses of E3-catalyzed reactions show the formation of Ub dimers.

isopeptide bonds at positions K6 and K48 (Supporting Information Figure S5). In line with previous biochemical studies on NleL, K6 linkages formed to a greater extent than K48, and an intense signal was observed at ~-83.10 ppm along with one of lesser intensity at ~-83.36 ppm. ¹⁹F NMR can therefore simultaneously monitor the formation of three distinct Ub chain topoisomers. In addition to these findings,

we observed that similar results could be obtained with NleL derived from an *in vitro* translation system. More specifically, with no purification step, NleL could facilitate Ub chain formation in an *E. coli* S30 extract (Figure 4C). The complex protein mixture, undetectable by ¹⁹F NMR, did not compromise the signals belonging to different Ub species.

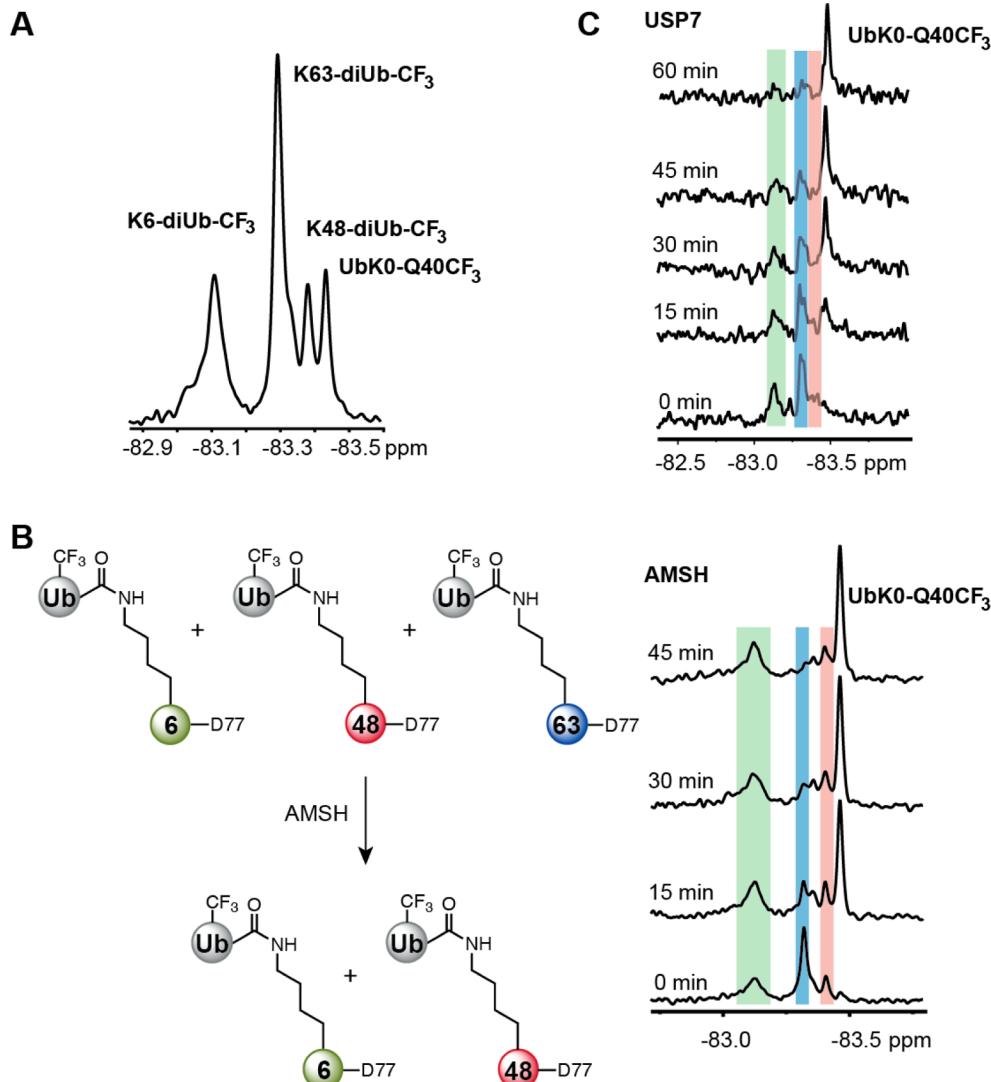


Figure 6. Examining the selectivity of DUBs using complex mixtures of substrates. (A) ^{19}F NMR spectrum showing the resolution of K6, K48, and K63 linkages along with mono-Ub. (B) AMSH-catalyzed hydrolysis of K63-linked chains in the presence of other chain types. (C) USP7-catalyzed cleavage of K6, K48, and K63 linkages.

This result suggests our strategy may be extended to analyze Ub chains in mammalian cell extract or *in cellulo*.

Encouraged by our findings with NleL, we decided to use ^{19}F NMR to study the linkage selectivity of E3 ligases that are poorly characterized. We focused on the bacterial E3 ligases IpaH9.8 and SspH1 from *Shigella* and *Salmonella*, respectively.^{62–64} These proteins share no sequence or structural similarity with eukaryotic E3 ligases, yet subvert defense responses by attaching Ub chains to proteins in the host cell. Although the precise nature of chains constructed by SspH1 is unknown, there is evidence suggesting IpaH9.8 builds conjugates bearing K27 linkages.⁶⁴ To examine the types of chains assembled by these bacterial ligases, we recombinantly expressed IpaH9.8 and SspH1 in *E. coli*, and each protein was added to a sample with eukaryotic E1, E2 and Ub monomers for ^{19}F NMR analysis. Both IpaH9.8 and SspH1 catalyzed the formation of Ub dimers with chemical shifts similar to that of K6 and K48 linkages (Figure 5). MS analysis of the dimer products indicated that isopeptide modifications predominantly occurred at K6 and K48 (Supporting Information Figures S7 and S8). Because we did not detect a major peak that could be

assigned to K27-linked Ub dimers as suggested by Ashida et al., we sought to test whether the use of UbK0 and Ub-D77 could bias chain formation with IpaH9.8. Reactions were therefore performed with wild type Ub (WT-Ub) and analyzed by MS. Results from these experiments show that once again K6 and K48 are the predominant linkages, indicating the use of UbK0 and Ub-D77 does not affect chain linkage selectivity of IpaH9.8. It is plausible that K27 is important for Ub recognition by IpaH9.8 or its substrate the NF- κ B essential modulator (NEMO), and other types of linkages cannot form when K27 has been mutated to arginine. However, upon replacing Ub-D77 with UbK27C-D77, ^{19}F NMR analysis did not show a discernible attenuation in Ub dimer formation. Hence the intrinsic ligase activity of IpaH9.8 does not require K27 (Supporting Information Figure S9). Collectively, these findings suggest that once injected into a host cell, IpaH9.8 and SspH1 can subvert the host defense response by promoting proteasomal degradation of target proteins with K48-linked Ub chains or proteasome-independent events with K6-linked Ub chains. Moreover, the aforementioned experiments show ^{19}F

NMR can be used to rapidly assess the types of Ub chains forged by E3 ligases.

Ub chains bearing multiple linkage types coexist in the cell, and the level of each chain type is regulated by DUBs that selectively hydrolyze Ub linkages. Understanding the linkage-selectivity of DUBs is necessary to elucidate their biochemical functions, and analyses of DUB activity with purified Ub substrates have already yielded valuable insights. This approach is limited, however, because the cellular environment contains an assortment of Ub chains that can simultaneously affect the enzymatic activity of DUBs. With our ability to simultaneously monitor different Ub linkage types, we sought to evaluate, in real time, the disassembly of a heterogeneous population of ¹⁹F-labeled Ub dimers. As shown in Figure 6A, all three topoisomers (K6, K48, and K63) and mono-Ub are distinguishable by ¹⁹F NMR when present in the same solution. Treatment of this mixture with AMSH diminished the signal corresponding to K63-linked dimers and left the other peaks intact, thereby confirming the known linkage specificity of this enzyme (Figure 6B). Using this same strategy with USP7, a representative member of the Ub specific protease family of DUBs, we observed a similar decrease in abundance of each topoisomer along with formation of mono-Ub (Figure 6C). These results, which are congruent with previously reported kinetic data, demonstrate that USP7 acts indiscriminately toward different Ub dimers.⁶⁵ Together, these experiments highlight the utility of ¹⁹F NMR in studying the activity of DUBs.

In conclusion, ¹⁹F NMR can be used to simultaneously monitor the formation and destruction of different Ub modifications in complex and dense proteinaceous environments. Our method can be adopted for high throughput screening of E3 ligases and DUBs to determine activity and link these enzymes to conformationally distinct Ub chains responsible for cellular signaling. We have characterized the Ub linkages formed by bacterial E3 ligases IpaH9.8 and SspH1. Although both enzymes are injected into a host cell to facilitate infection, there is limited insight into how these E3 ligases operate to subvert the host defense response. We found that IpaH9.8 and SspH1 form K48 and K6 linkages, similar to the E3 ligase NleL from *E. coli*. Our finding suggests these bacterial E3 ligases can perturb proteasome-dependent Ub signaling as directed by K48-linked Ub chains and noncanonical signaling pathways as directed by K6-linked Ub chains. To expand the utility of ¹⁹F NMR for studying the vast Ub signaling network, we are currently working on employing unnatural amino acid incorporation methods to observe the formation of distinct Ub chains in eukaryotic cells. This strategy, along with the rapid advancement of solution-phase ¹⁹F NMR to enhance sensitivity, can more completely inform on how distinct Ub conjugates regulate myriad cellular pathways.

■ ASSOCIATED CONTENT

Supporting Information

Detailed experimental procedure, MS characterization of Ub linkages, and additional ¹⁹F NMR experiments. This material is available free of charge via the Internet at <http://pubs.acs.org>.

■ AUTHOR INFORMATION

Corresponding Author

*Email: strieter@chem.wisc.edu.

Author Contributions

[†]S.S.S. and G.H.P. contributed equally to this work.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

The authors gratefully acknowledge Dr. Charles Fry and Dr. Martha Vestling for instrumentation guidance. NMR and MALDI-TOF MS analyses were performed at the Paul Bender Chemical Instrument Center, UW–Madison. Financial support was provided by NIGMS (RO1GM110543), UW–Madison, the Greater Milwaukee Shaw Scientist Program, Susan G. Komen for the Cure (KG110081), the Wisconsin Alumni Research Foundation, and NCRR (1S10RR024601-01; UW–Madison Department of Chemistry).

■ REFERENCES

- (1) Hoeller, D., and Dikic, I. (2009) Targeting the ubiquitin system in cancer therapy. *Nature* 458, 438–444.
- (2) Tai, H. C., and Schuman, E. M. (2008) Ubiquitin, the proteasome and protein degradation in neuronal function and dysfunction. *Nat. Rev. Neurosci.* 9, 826–838.
- (3) Deshaies, R. J., and Joazeiro, C. A. (2009) RING domain E3 ubiquitin ligases. *Annu. Rev. Biochem.* 78, 399–434.
- (4) Pickart, C. M. (2004) Back to the future with ubiquitin. *Cell.* 116, 181–190.
- (5) Pickart, C. M. (2001) Mechanisms underlying ubiquitination. *Annu. Rev. Biochem.* 70, 503–533.
- (6) Dye, B. T., and Schulman, B. A. (2007) Structural mechanisms underlying posttranslational modification by ubiquitin-like proteins. *Annu. Rev. Biophys. Biomol. Struct.* 36, 131–150.
- (7) Komander, D., and Rape, M. (2012) The ubiquitin code. *Annu. Rev. Biochem.* 81, 203–229.
- (8) Kulathu, Y., and Komander, D. (2012) Atypical ubiquitination—the unexplored world of polyubiquitin beyond Lys48 and Lys63 linkages. *Nat. Rev. Mol. Cell Biol.* 13, 508–523.
- (9) Chau, V., Tobias, J. W., Bachmair, A., Marriott, D., Ecker, D. J., Gonda, D. K., and Varshavsky, A. (1989) A multiubiquitin chain is confined to specific lysine in a targeted short-lived protein. *Science* 243, 1576–1583.
- (10) Finley, D., Sadis, S., Monia, B. P., Boucher, P., Ecker, D. J., Crooke, S. T., and Chau, V. (1994) Inhibition of proteolysis and cell cycle progression in a multiubiquitination-deficient yeast mutant. *Mol. Cell. Biol.* 14, 5501–5509.
- (11) Thrower, J. S., Hoffman, L., Rechsteiner, M., and Pickart, C. M. (2000) Recognition of the polyubiquitin proteolytic signal. *EMBO J.* 19, 94–102.
- (12) Spence, J., Sadis, S., Haas, A. L., and Finley, D. (1995) A ubiquitin mutant with specific defects in DNA repair and multiubiquitination. *Mol. Cell. Biol.* 15, 1265–1273.
- (13) Hofmann, R. M., and Pickart, C. M. (1999) Noncanonical MMS2-encoded ubiquitin-conjugating enzyme functions in assembly of novel polyubiquitin chains for DNA repair. *Cell.* 96, 645–653.
- (14) Chen, Z. J., and Sun, L. J. (2009) Nonproteolytic functions of ubiquitin in cell signaling. *Mol. Cell.* 33, 275–286.
- (15) Clague, M. J., and Urbe, S. (2010) Ubiquitin: same molecule, different degradation pathways. *Cell.* 143, 682–685.
- (16) Ikeda, F., Deribe, Y. L., Skanland, S. S., Stieglitz, B., Grabbe, C., Franz-Wachtel, M., van Wijk, S. J., Goswami, P., Nagy, V., Terzic, J., Tokunaga, F., Androulidaki, A., Nakagawa, T., Pasparakis, M., Iwai, K., Sundberg, J. P., Schaefer, L., Rittinger, K., Macek, B., and Dikic, I. (2011) SHARPIN forms a linear ubiquitin ligase complex regulating NF- κ B activity and apoptosis. *Nature* 471, 637–641.
- (17) Tokunaga, F., Nakagawa, T., Nakahara, M., Saeki, Y., Taniguchi, M., Sakata, S., Tanaka, K., Nakano, H., and Iwai, K. (2011) SHARPIN is a component of the NF- κ B-activating linear ubiquitin chain assembly complex. *Nature* 471, 633–636.

- (18) Gerlach, B., Cordier, S. M., Schmukle, A. C., Emmerich, C. H., Rieser, E., Haas, T. L., Webb, A. I., Rickard, J. A., Anderton, H., Wong, W. W., Nachbur, U., Gangoda, L., Warnken, U., Purcell, A. W., Silke, J., and Walczak, H. (2011) Linear ubiquitination prevents inflammation and regulates immune signalling. *Nature*. 471, 591–596.
- (19) Povlsen, L. K., Beli, P., Wagner, S. A., Poulsen, S. L., Sylvestersen, K. B., Poulsen, J. W., Nielsen, M. L., Bekker-Jensen, S., Mailand, N., and Choudhary, C. (2012) Systems-wide analysis of ubiquitination dynamics reveals a key role for PAF15 ubiquitination in DNA-damage bypass. *Nat. Cell Biol.* 14, 1089–1098.
- (20) Matsumoto, M. L., Wickliffe, K. E., Dong, K. C., Yu, C., Bosanac, I., Bustos, D., Phu, L., Kirkpatrick, D. S., Hymowitz, S. G., Rape, M., Kelley, R. F., and Dixit, V. M. (2010) K11-linked polyubiquitination in cell cycle control revealed by a K11 linkage-specific antibody. *Mol. Cell* 39, 477–484.
- (21) Nijman, S. M., Luna-Vargas, M. P., Velds, A., Brummelkamp, T. R., Dirac, A. M., Sixma, T. K., and Bernards, R. (2005) A genomic and functional inventory of deubiquitinating enzymes. *Cell*. 123, 773–786.
- (22) Komander, D., Clague, M. J., and Urbe, S. (2009) Breaking the chains: Structure and function of the deubiquitinases. *Nat. Rev. Mol. Cell Biol.* 10, 550–563.
- (23) Reyes-Turcu, F. E., Ventii, K. H., and Wilkinson, K. D. (2009) Regulation and cellular roles of ubiquitin-specific deubiquitinating enzymes. *Annu. Rev. Biochem.* 78, 363–397.
- (24) Xu, P., and Peng, J. (2006) Dissecting the ubiquitin pathway by mass spectrometry. *Biochim. Biophys. Acta* 1764, 1940–1947.
- (25) Cook, W. J., Jeffrey, L. C., Carson, M., Chen, Z., and Pickart, C. M. (1992) Structure of a diubiquitin conjugate and a model for interaction with ubiquitin conjugating enzyme (E2). *J. Biol. Chem.* 267, 16467–16471.
- (26) Eddins, M. J., Varadan, R., Fushman, D., Pickart, C. M., and Wolberger, C. (2007) Crystal structure and solution NMR studies of Lys48-linked tetraubiquitin at neutral pH. *J. Mol. Biol.* 367, 204–211.
- (27) Hospenthal, M. K., Freund, S. M., and Komander, D. (2013) Assembly, analysis, and architecture of atypical ubiquitin chains. *Nat. Struct. Mol. Biol.* 20, 555–565.
- (28) Virdee, S., Ye, Y., Nguyen, D. P., Komander, D., and Chin, J. W. (2010) Engineered diubiquitin synthesis reveals Lys29-isopeptide specificity of an OTU deubiquitinase. *Nat. Chem. Biol.* 6, 750–757.
- (29) Bremm, A., Freund, S. M., and Komander, D. (2010) Lys11-linked ubiquitin chains adopt compact conformations and are preferentially hydrolyzed by the deubiquitinase Cezanne. *Nat. Struct. Mol. Biol.* 17, 939–947.
- (30) Ye, Y., Blaser, G., Horrocks, M. H., Ruedas-Rama, M. J., Ibrahim, S., Zhukov, A. A., Orte, A., Klenerman, D., Jackson, S. E., and Komander, D. (2012) Ubiquitin chain conformation regulates recognition and activity of interacting proteins. *Nature*. 492, 266–270.
- (31) Castaneda, C. A., Kashyap, T. R., Nakasone, M. A., Krueger, S., and Fushman, D. (2013) Unique structural, dynamical, and functional properties of k11-linked polyubiquitin chains. *Structure*. 21, 1168–1181.
- (32) Varadan, R., Walker, O., Pickart, C., and Fushman, D. (2002) Structural properties of polyubiquitin chains in solution. *J. Mol. Biol.* 324, 637–647.
- (33) Datta, A. B., Hura, G. L., and Wolberger, C. (2009) The structure and conformation of Lys63-linked tetraubiquitin. *J. Mol. Biol.* 392, 1117–1124.
- (34) Komander, D., Reyes-Turcu, F., Licchesi, J. D., Odenwaelder, P., Wilkinson, K. D., and Barford, D. (2009) Molecular discrimination of structurally equivalent Lys 63-linked and linear polyubiquitin chains. *EMBO Rep.* 10, 466–473.
- (35) Danielson, M. A., and Falke, J. J. (1996) Use of ¹⁹F NMR to probe protein structure and conformational changes. *Annu. Rev. Biophys. Biomol. Struct.* 25, 163–195.
- (36) Kitevski-LeBlanc, J. L., and Prosser, R. S. (2012) Current applications of ¹⁹F NMR to studies of protein structure and dynamics. *Prog. Nucl. Magn. Reson. Spectrosc.* 62, 1–33.
- (37) Chen, H., Viel, S., Ziarelli, F., and Peng, L. (2013) ¹⁹F NMR: A valuable tool for studying biological events. *Chem. Soc. Rev.* 42, 7971–7982.
- (38) Khan, F., Kuprov, I., Craggs, T. D., Hore, P. J., and Jackson, S. E. (2006) ¹⁹F NMR studies of the native and denatured states of green fluorescent protein. *J. Am. Chem. Soc.* 128, 10729–10737.
- (39) Li, C., Lutz, E. A., Slade, K. M., Ruf, R. A., Wang, G. F., and Pielak, G. (2009) ¹⁹F NMR studies of α -synuclein conformation and fibrillation. *J. Biochem.* 48, 8578–8584.
- (40) Wang, G. F., Li, C., and Pielak, G. J. (2010) Probing the micelle-bound aggregation-prone state of α -synuclein with ¹⁹F NMR spectroscopy. *ChemBioChem* 11, 1993–1996.
- (41) Wang, G. F., Li, C., and Pielak, G. J. (2010) ¹⁹F NMR studies of α -synuclein–membrane interactions. *Protein Sci.* 19, 1686–1691.
- (42) Holzberger, B., Rubini, M., M?ller, H. M., and Marx, A. (2010) A highly active DNA polymerase with a fluorous core. *Angew. Chem., Int. Ed. Engl.* 49, 1324–1327.
- (43) Sakakibara, D., Sasaki, A., Ikeya, T., Hamatsu, J., Hanashima, T., Mishima, M., Yoshimasu, M., Hayashi, N., Mikawa, T., Walchli, M., Smith, B. O., Shirakawa, M., Guntert, P., and Ito, Y. (2009) Protein structure determination in living cells by in-cell NMR spectroscopy. *Nature* 458, 102–105.
- (44) Li, C., Wang, G. F., Wang, Y., Creager-Allen, R., Lutz, E. A., Scronce, H., Slade, K. M., Ruf, R. A., Mehl, R. A., and Pielak, G. J. (2010) Protein (¹⁹F) NMR in *Escherichia coli*. *J. Am. Chem. Soc.* 132, 321–327.
- (45) Sloper-Mould, K. E., Jemc, J. C., Pickart, C. M., and Hicke, L. (2001) Distinct functional surface regions on ubiquitin. *J. Biol. Chem.* 276, 30483–30489.
- (46) Roscoe, B. P., Thayer, K. M., Zeldovich, K. B., Fushman, D., and Bolon, D. N. (2013) Analyses of the effects of all ubiquitin point mutants on yeast growth rate. *J. Mol. Biol.* 425, 1363–1377.
- (47) Brown, W. E., and Seamon, K. B. (1978) Quantitation and characterization of the trifluoroacetyl derivative of cysteine: A useful NMR probe. *Anal. Biochem.* 87, 211–222.
- (48) Hofmann, R. M., and Pickart, C. M. (2001) *In vitro* assembly and recognition of Lys-63 polyubiquitin chains. *J. Biol. Chem.* 276, 27936–27943.
- (49) McCullough, J., Clague, M. J., and Urbe, S. (2004) AMSH is an endosome-associated ubiquitin isopeptidase. *J. Cell Biol.* 166, 487–492.
- (50) Xu, P., Duong, D. M., Seyfried, N. T., Cheng, D., Xie, Y., Robert, J., Rush, J., Hochstrasser, M., Finley, D., and Peng, J. (2009) Quantitative proteomics reveals the function of unconventional ubiquitin chains in proteasomal degradation. *Cell*. 137, 133–143.
- (51) Dammer, E. B., Na, C. H., Xu, P., Seyfried, N. T., Duong, D. M., Cheng, D., Gearing, M., Rees, H., Lah, J. J., Levey, A. I., Rush, J., and Peng, J. (2011) Polyubiquitin linkage profiles in three models of proteolytic stress suggest the etiology of Alzheimer disease. *J. Biol. Chem.* 286, 10457–10465.
- (52) Kim, W., Bennett, E. J., Huttlin, E. L., Guo, A., Li, J., Possemato, A., Sowa, M. E., Rad, R., Rush, J., Comb, M. J., Harper, J. W., and Gygi, S. P. (2011) Systematic and quantitative assessment of the ubiquitin-modified proteome. *Mol. Cell* 44, 325–340.
- (53) Wagner, S. A., Beli, P., Weinert, B. T., Nielsen, M. L., Cox, J., Mann, M., and Choudhary, C. (2011) A proteome-wide, quantitative survey of *in vivo* ubiquitination sites reveals widespread regulatory roles. *Mol. Cell. Proteomics* 10, M111 013284.
- (54) Ziv, I., Matiuhin, Y., Kirkpatrick, D. S., Erpazoglou, Z., Leon, S., Pantazopoulou, M., Kim, W., Gygi, S. P., Haguenauer-Tsapis, R., Reis, N., Glickman, M. H., and Kleifeld, O. (2011) A perturbed ubiquitin landscape distinguishes between ubiquitin in trafficking and in proteolysis. *Mol. Cell. Proteomics* 10, M111 009753.
- (55) Dong, K. C., Helgason, E., Yu, C., Phu, L., Arnott, D. P., Bosanac, I., Compaan, D. M., Huang, O. W., Fedorova, A. V., Kirkpatrick, D. S., Hymowitz, S. G., and Dueber, E. C. (2011) Preparation of distinct ubiquitin chain reagents of high purity and yield. *Structure* 19, 1053–1063.

- (56) Ptak, C., Varelas, X., Moraes, T., McKenna, S., and Ellison, M. J. (2005) Purification and properties of the ubiquitin-conjugating enzymes Cdc34 and Ubc13.Mms2. *Meth. Enzymol.* 398, 43–54.
- (57) Sadowski, M., Suryadinata, R., Lai, X., Heierhorst, J., and Sarcevic, B. (2010) Molecular basis for lysine specificity in the yeast ubiquitin-conjugating enzyme Cdc34. *Mol. Cell. Biol.* 30, 2316–2329.
- (58) Edelmann, M. J., Iphofer, A., Akutsu, M., Altun, M., di Gleria, K., Kramer, H. B., Fiebiger, E., Dhe-Paganon, S., and Kessler, B. M. (2009) Structural basis and specificity of human otubain 1-mediated deubiquitination. *Biochem. J.* 418, 379–390.
- (59) Wiener, R., DiBello, A. T., Lombardi, P. M., Guzzo, C. M., Zhang, X., Matunis, M. J., and Wolberger, C. (2013) E2 ubiquitin-conjugating enzymes regulate the deubiquitinating activity of OTUB1. *Nat. Struct. Mol. Biol.* 20, 1033–1039.
- (60) Piscatelli, H., Kotkar, S. A., McBee, M. E., Muthupalani, S., Schauer, D. B., Mandrell, R. E., Leong, J. M., and Zhou, D. (2011) The EHEC type III effector NleL is an E3 ubiquitin ligase that modulates pedestal formation. *PloS One* 6, e19331.
- (61) Lin, D. Y., Diao, J., Zhou, D., and Chen, J. (2011) Biochemical and structural studies of a HECT-like ubiquitin ligase from *Escherichia coli* O157:H7. *J. Biol. Chem.* 286, 441–449.
- (62) Rohde, J. R., Breitkreutz, A., Chenal, A., Sansonetti, P. J., and Parsot, C. (2007) Type III secretion effectors of the IpaH family are E3 ubiquitin ligases. *Cell Host Microbe.* 1, 77–83.
- (63) Keszei, A. F., Tang, X., McCormick, C., Zeqiraj, E., Rohde, J. R., Tyers, M., and Sicheri, F. (2014) Structure of an SspH1-PKN1 complex reveals the basis for host substrate recognition and mechanism of activation for a bacterial E3 ubiquitin ligase. *Mol. Cell. Biol.* 34, 362–733.
- (64) Ashida, H., Kim, M., Schmidt-Suprian, M., Ma, A., Ogawa, M., and Sasakawa, C. (2010) A bacterial E3 ubiquitin ligase IpaH9.8 targets NEMO/IKK γ to dampen the host NF- κ B-mediated inflammatory response. *Nat. Cell Biol.* 12, 66–73.
- (65) Faesen, A. C., Luna-Vargas, M. P., Geurink, P. P., Clerici, M., Merkx, R., van Dijk, W. J., Hameed, D. S., El Oualid, F., Ovaas, H., and Sixma, T. K. (2011) The differential modulation of USP activity by internal regulatory domains, interactors, and eight ubiquitin chain types. *Chem. Biol.* 18, 1550–1561.