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

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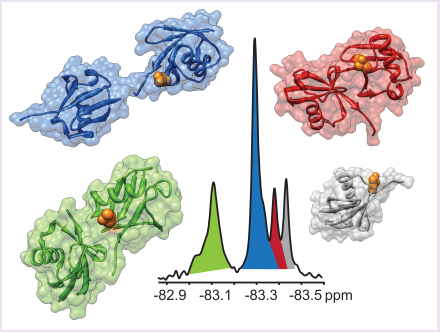
Supporting Information

**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 cancers[1] 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

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environment-sensitive and detectable in a heterogeneous mixture.

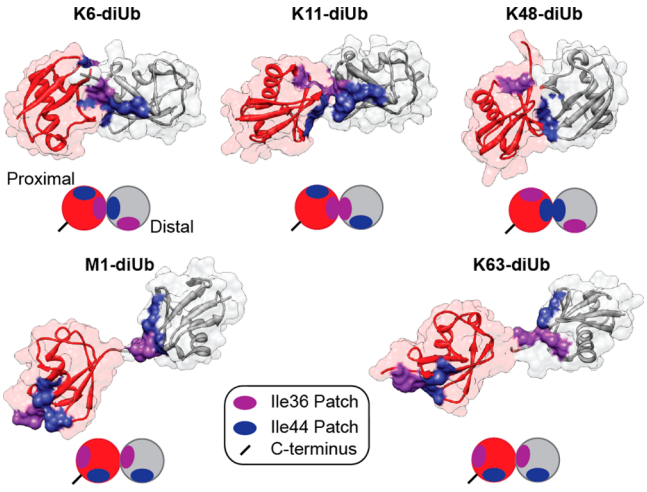



**Figure 1.** Different conformations of Ub dimers. The proximal subunit bearing a free C-terminus is shown in red and the distal subunit is shown in gray. The Ile36 and Ile44 hydrophobic patches are indicated in purple and blue, respectively. The Ile36 patch consists of Ile36, Leu71, and Leu73. The 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 (2jf5).[34]

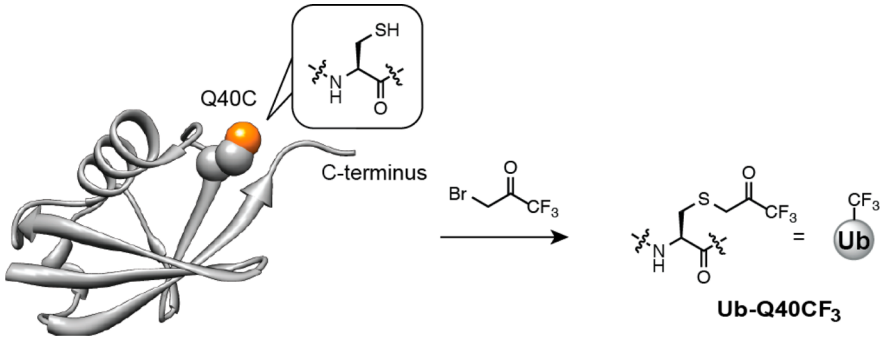
We identified fluorine ($^{19}$F) nuclear magnetic resonance (NMR) spectroscopy[35−37] as a promising approach to specifically detect different Ub oligomers. This method has been used to study the structures and dynamics of a number of proteins, including green fluorescent protein,[38] $\alpha$-synuclein,[39−41] and DNA polymerase.[42] Since fluorine is virtually absent from biological systems, $^{19}$F 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 $^{19}$F label on Ub oligomers would experience varying electronic environments and exhibit unique $^{19}$F chemical shifts, directed by the type of Ub linkage (Figure 1). Here, we demonstrate a $^{19}$F NMR-based approach to assess heterogeneous protein mixtures for the formation and destruction of select Ub linkages.

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 $^{19}$F 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 $^{19}$F 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 $^{19}$F 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 $^{19}$F NMR signals. We therefore limited our studies to the formation of dimers (17 kDa). Two separate Ub variants were used: UbK0-Q40CF$_3$ and Ub-D77. UbK0-Q40CF$_3$ 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, $^{19}$F 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 $^{19}$F 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 $^{19}$F 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

**Scheme 1. Site-Specific Labeling of Ub-Q40C with 3-Bromo-1,1,1-trifluoroacetone (BFA) to Afford Ub-Q40CF$_3$**





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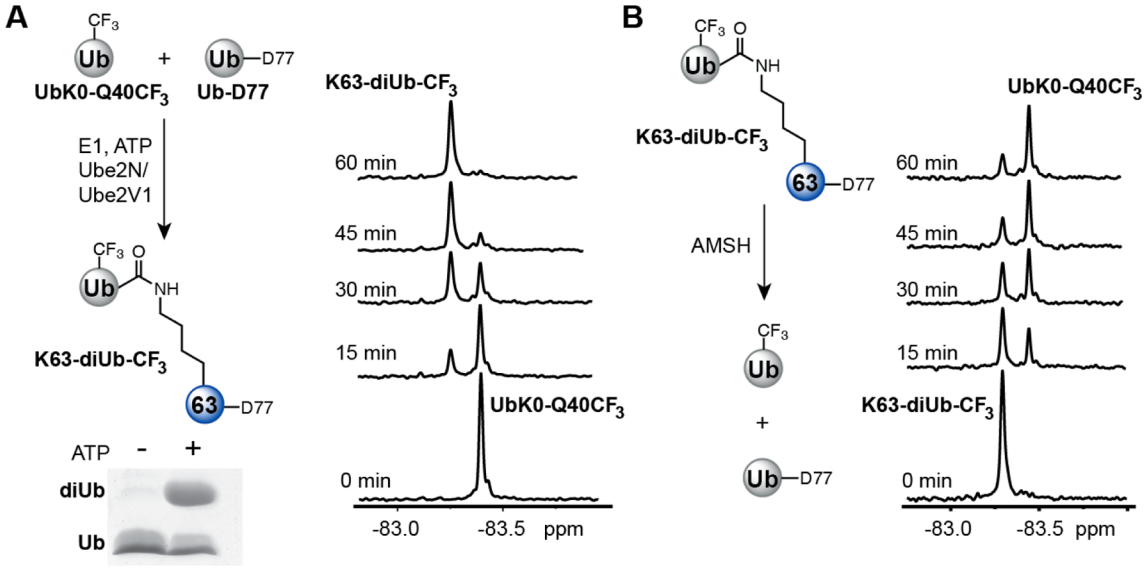


**Figure 2.** Using $^{19}F$ NMR to monitor the formation and disassembly of K63-linked Ub dimers in real-time. (A) Formation of K63-linked diUb-$CF_3$ using K63 linkage specific enzymes Ube2N/Ube2V1. UbK0-Q40$CF_3$ (−83.43 ppm) is converted to K63-diUb-$CF_3$ (−83.25 ppm) within an hour. SDS-PAGE analysis shows formation of dimer in the presence of ATP. B. Cleavage of K63-linked diUb-$CF_3$ using the K63 linkage specific DUB AMSH.

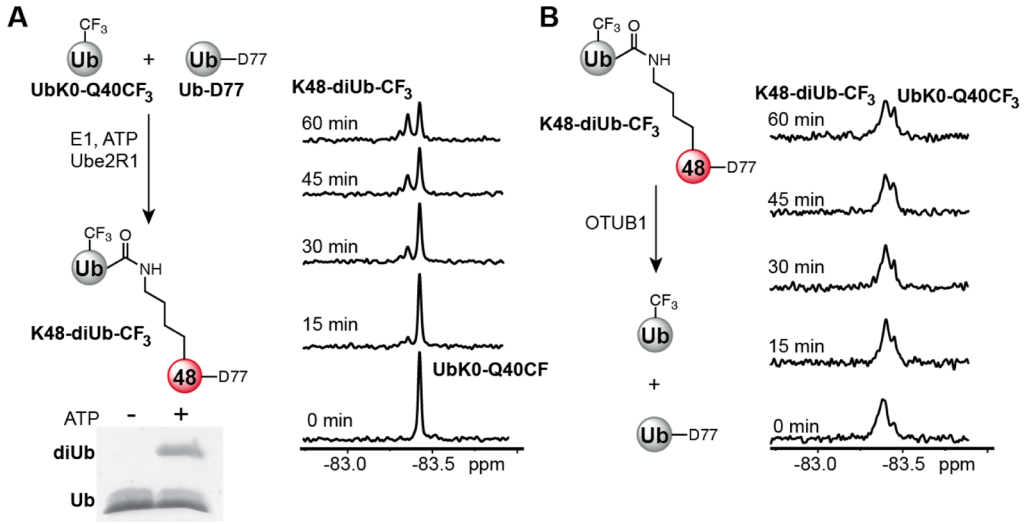


**Figure 3.** Using $^{19}F$ NMR to monitor the formation and disassembly of K48-linked Ub dimers in real-time. A. Formation of K48-linked diUb-$CF_3$ (−83.36 ppm) using the K48 linkage specific enzyme Ube2R1 and UbK0-Q40$CF_3$ (−83.43 ppm). SDS-PAGE analysis shows formation of dimer in the presence of ATP. (B) Cleavage of K48-linked diUb-$CF_3$ 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-Q40$CF_3$ 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, $^{19}F$ 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 $^{19}F$ NMR. NleL is produced by the enterohemorrhagic *E. coli* O157:H7[60] and was recently found to assemble unanchored K6- and K48-linked chains *in vitro*.[27,61] Addition of NleL to reaction mixtures containing UbK0-Q40$CF_3$ 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 $^{19}F$ NMR and MS. The results of these experiments confirmed the presence of


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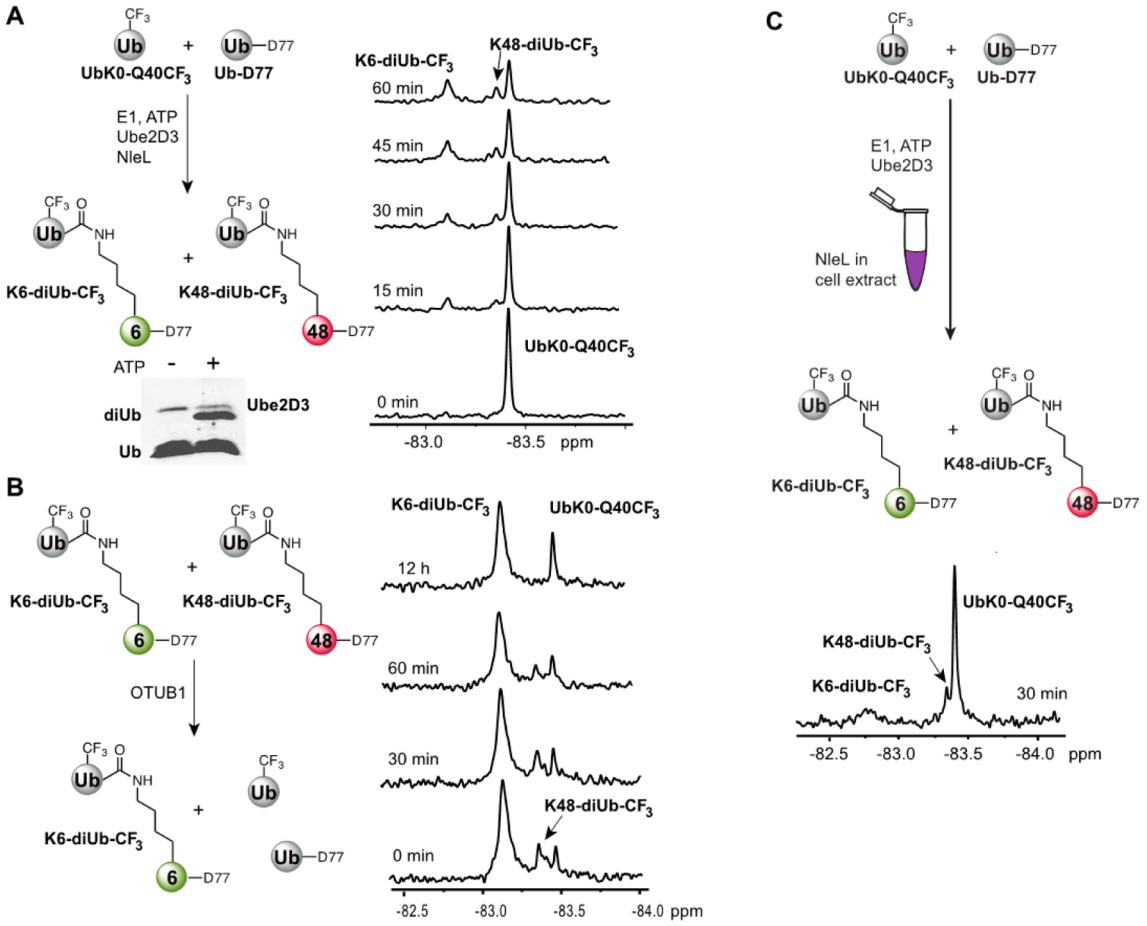


**Figure 4.** Using $^{19}$F NMR to detect K6 linkages. (A) NleL-catalyzed chain formation. K6-linked diUb-CF$_3$ (−83.10 ppm) and K48-linked diUb-CF$_3$ (−83.36 ppm) are both formed during the reaction. SDS-PAGE analysis shows formation of dimer in the presence of ATP. (B) OTUB1-catalyzed hydrolysis results in the loss of the signal for K48-linked diUb-CF$_3$ (−83.36 ppm) and an increase in the signal for UbK0-Q40CF$_3$ (−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-CF$_3$ (−82.8 ppm) and K48-linked diUb-CF$_3$ (−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.

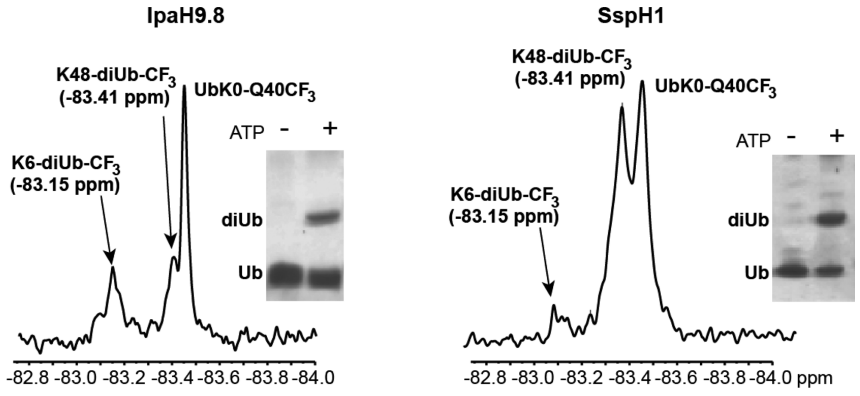


**Figure 5.** $^{19}$F NMR characterization of the Ub chain linkages assembled by the bacterial E3 ligases IpaH9.8 and SspH1 reveals the presence of K6 and K48 linkages. SDS-PAGE analyses of E3-catalyzed reactions show the formation of Ub dimers.

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. $^{19}$F 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 $^{19}$F NMR, did not compromise the signals belonging to different Ub species.


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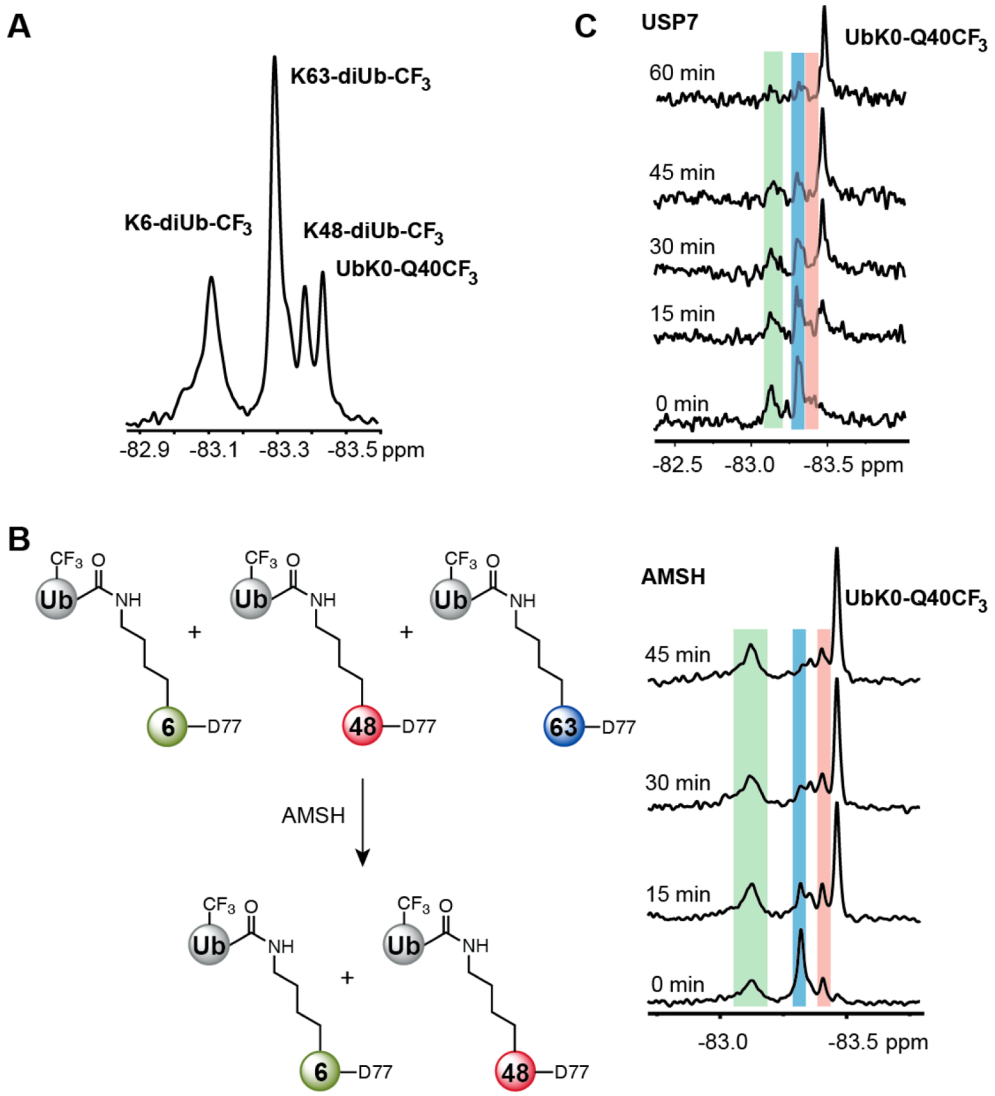


**Figure 6.** Examining the selectivity of DUBs using complex mixtures of substrates. (A) $^{19}$F NMR spectrum showing the resolution of K6, K48, and K63 linkages along with mono-Ub. (B) AMSH-catalyzed hydrolysis of K63-linked chains in the presence of other chain types. (C) USP7-catalyzed cleavage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


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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 $^{19}$F-labeled Ub dimers. As shown in Figure 6A, all three topoisomers (K6, K48, and K63) and mono-Ub are distinguishable by $^{19}$F 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 $^{19}$F NMR in studying the activity of DUBs.

In conclusion, $^{19}$F 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 $^{19}$F 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 $^{19}$F 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 $^{19}$F NMR experiments. This material is available free of charge via the Internet at http://pubs.acs.org.

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### Notes

The authors declare no competing financial interest.

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