JACS
JOURNAL OF THE AMERICAN CHEMICAL SOCIETY
Article
pubs.acs.org/JACS

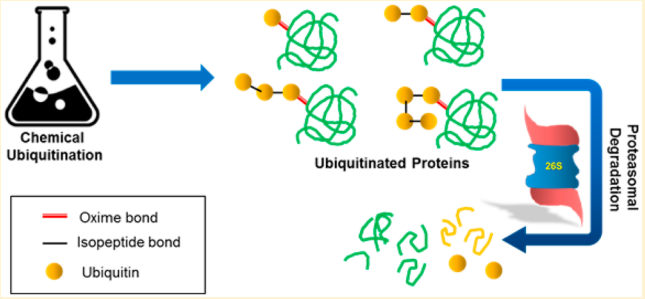
Synthetic Uncleavable Ubiquitinated Proteins Dissect Proteasome Deubiquitination and Degradation, and Highlight Distinctive Fate of Tetraubiquitin

Sumeet K. Singh,[†,#] Indrajit Sahu,[‡,#] Sachitanand M. Mali,[†] Hosahalli P. Hemantha,[†,§] Oded Kleifeld,[‡] Michael H. Glickman,*[,‡] and Ashraf Brik*[,†]

[†]Schulich Faculty of Chemistry, Technion—Israel Institute of Technology, 3200008 Haifa, Israel
[‡]Department of Biology Technion—Israel Institute of Technology, 3200008 Haifa, Israel

Supporting Information

ABSTRACT: Various hypotheses have been proposed regarding how chain length, linkage type, position on substrate, and susceptibility to deubiquitinases (DUBs) affect processing of different substrates by proteasome. Here we report a new strategy for the chemical synthesis of ubiquitinated proteins to generate a set of well-defined conjugates bearing an oxime bond between the chain and the substrate. We confirmed that this isopeptide replacement is resistant to DUBs and to shaving by proteasome. Analyzing products generated by proteasomes ranked how chain length governed degradation outcome. Our results support that (1) the cleavage of the proximal isopeptide bond is not a prerequisite for proteasomal degradation, (2) by overcoming trimming at the proteasome, tetraUb is a fundamentally different signal than shorter chains, and (3) the tetra-ubiquitin chain can be degraded with the substrate. Together these results highlight the usefulness of chemistry to dissect the contribution of proteasome-associated DUBs and the complexity of the degradation process.



INTRODUCTION

Chemical and semisynthesis of ubiquitin conjugates is a rapidly growing field due to its high impact on our understanding of the ubiquitin signal at the molecular level.[1−4] This is also particularly important due to inherent limitations of enzymatic approaches in obtaining highly homogeneous and sufficient amounts of desired ubiquitin conjugates. Hence, several approaches have been developed and applied to understand various biological questions that otherwise are difficult to achieve.[5−13] For example all Lys-linked ubiquitin chains and ubiquitinated proteins have been prepared.[14−19] Linkage-specific conjugates are particularly exciting since they enable studies that dissect the role of the chain on the fate of the substrate.

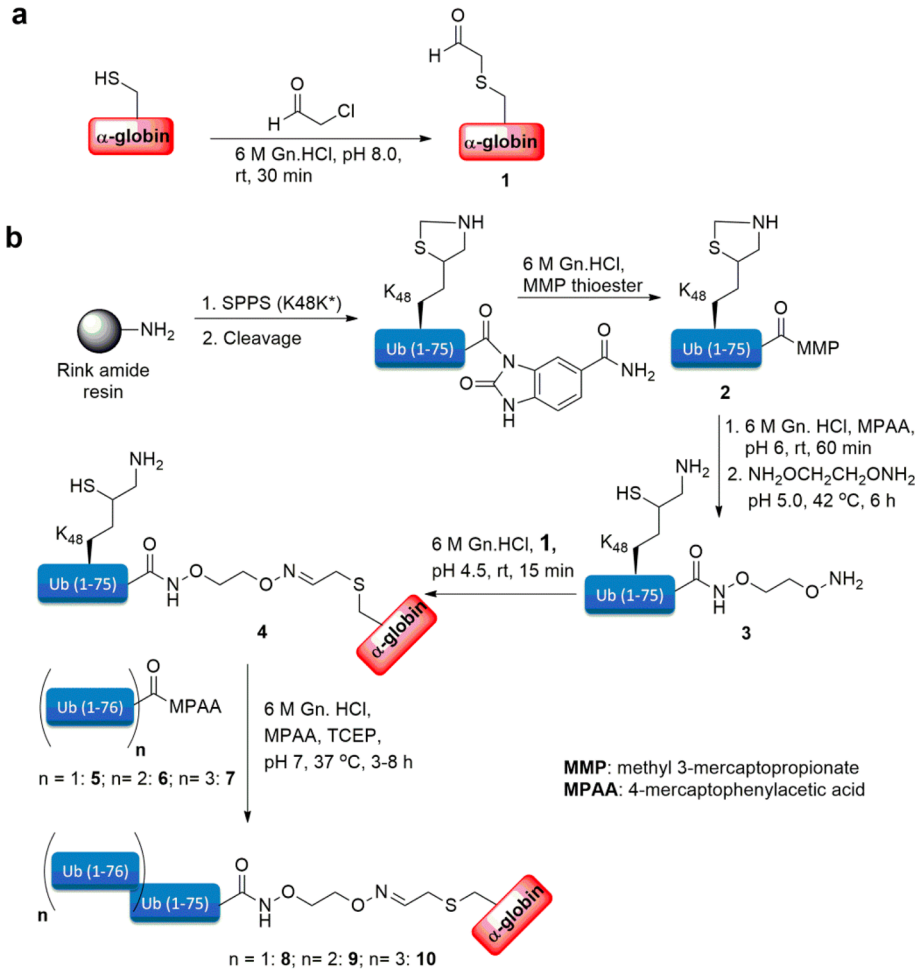
Protein degradation via the ubiquitin proteasome system (UPS) to regulate numerous biological functions is one the most ubiquitous cellular events in eukaryotes.[20] Conjugation of ubiquitin (Ub), a small protein modifier, to the target substrate requires three enzymes known as E1, E2 and E3 to work in a cooperative and sequential manner.[21,22] The product of this cascade is a Ub-conjugate made up of a substrate modified on a Lys side chain via an isopeptide bond to the C-terminus of ubiquitin. Moreover, ubiquitin itself can be extended in a similar fashion to form different types of polyUb chains depending on which of its seven Lys residues (Lys^{63}, Lys^{48}, Lys^{33}, Lys^{29}, Lys^{27}, Lys^{11} and Lys^{6}) or its N-terminus is used to link the consecutive ubiquitin.[23,24] Both the chain length and linkage type affect the topological structure of polyUb chains, which in turn determines the signal and the cellular fate of the modified protein.

The attachment of Lys^{48}-linked polyUb chains to a Lys residue on the target protein is accepted as the most widely employed signal for efficient proteasomal targeting.[25−27] The 26S proteasome is a hybrid of two complexes: the 28-subunit 20S proteolytic core particle (CP) and the ∼20-subunit 19S regulatory particle (RP). Proteolysis of the target in the barrel-shaped 20S CP is regulated by selection, binding, and unfolding at the 19S RP.[28] To carry out such a variety of enzymatic activities, dedicated subunits are present in the 19S RP for polyUb-binding, ATP-dependent unfolding, and chain disassembly, which must act in a highly coordinated manner.[21,29] At least three associated deubiquitinases (DUBs; Usp14/Ubp6, PSMD14/Rpn11, UCH37/UCHL5) present in the mammalian 19S RP contribute to the efficiency of substrates degradation while protecting ubiquitin from a similar destiny.[30,31] While USP14 and UCH37 are Cys-proteases and belong to the USP and UCH families, respectively, Rpn11 is an MPN^{+}/JAMM zinc metalloprotease and is an integral part of a lid subcomplex of the 19S RP.[32,33]

Received: September 13, 2016
Published: December 6, 2016

DOI: 10.1021/jacs.6b09611
J. Am. Chem. Soc. 2016, 138, 16004−16015

Scheme 1. Synthesis of Oxime-Ligated PolyUb-α-globin[a]



[a](a) Selective addition of an acetaldehyde moiety to the Cys[104] of α-globin. (b) Synthesis of Ub^{K*48}(1-75)-Nbz via Fmoc-SPPS (solid phase peptide synthesis). Subsequently, the *N*-acylbenzimida-zolinone (Nbz) was switched to methyl 3-mercaptopropionate thioester (MMP). Next, one-pot switching of MMP thioester and K*48 thiozilodine (thz) ring-opening was achieved by incubation with 1,2-bisaminoxy ethane. Followed by ubiquitin chain elongation through isopeptide ligation to synthesize diUb-, triUb-, or tetraUb-α-globin (**8**, **9**, and **10**, respectively). [Note, K* symbolizes δ-mercaptolysine at position 48].

Once bound to the proteasome, a crucial factor in determining the fate of a ubiquitin-conjugate is deubiquitination by proteasome-associated DUBs. It has been proposed that USP14 and UCH37 trim polyUb chains from the distal-end, gradually reducing the chain length, and consequently the affinity of the conjugate to the proteasome.[31,34] The net effect being that inhibiting these cysteine proteases enhances the rate of proteolysis.[35,36] In contrast, Rpn11 has been proposed to act at later stage, after ATP-dependent engagement of substrate within the translocation channel, to perform polyUb chain amputation from the proximal end.[33,37,38] Guaranteeing that it acts only once substrate is properly aligned, the enzymatic activity of Rpn11 is intimately coupled to conformational changes and interactions with neighboring subunits within the proteasome.[38−43] It has been suggested that this step is essential to proteasome function by keeping the proteolytic channel free from the blocking tetraUb.[33] Consequently, inhibition of Rpn11 slows down proteasomal degradation. Therefore, Rpn11 is a viable target for anticancer therapeutic approaches.[44] Nevertheless, whether the primary role of Rpn11 is to shave the proximal ubiquitin from the aligned substrate, or to assist substrate alignment through these conformational changes for translocation into the 20S CP remains an open question.

Despite years of investigations, the parameters governing the optimal ubiquitination pattern for degradation of a particular substrate are still unclear. Various hypotheses have been proposed regarding how chain length, linkage type, position on substrate and susceptibility to DUBs dictate the fate of the Ub-conjugate at the proteasome. For example, it has been proposed that tetra-ubiquitin (tetraUb) is the minimal length for proteasomal degradation.[25] However, other studies have also reported that even monoUb or multiple monoUb could signal for proteasomal degradation.[45−47] Recently, a study of anaphase-promoting complex (APC) substrates concluded that if the same number of total ubiquitin units are present on an identical substrate, short polyUb chains (e.g., diUb) lead to a more efficient signal for degradation than a single polyUb chain (e.g., tetraUb).[48] Interestingly, another study showed that proteasomes degrade APC/C substrates tagged with branched Lys[11]/Lys[48]-linked ubiquitin chains more efficiently than when conjugated to homogeneous Lys[11]-linked or Lys[48]-linked chains.[49] Such studies demonstrate the complexity of the UPS system and explain why there is still some ambiguity

DOI: 10.1021/jacs.6b09611
J. Am. Chem. Soc. 2016, 138, 16004−16015

surrounding what constitutes a preferred ubiquitination signal for proteasome targeting.

Contrasting hypotheses pertaining to complexity of proteasomal degradation results necessitates design of special reagents to dissect the contribution of individual steps. Yet, hurdles enforced by enzymatic approaches limit the ability to generate homogeneous ubiquitinated proteins. Emerging chemical approaches for protein synthesis[50] offer unique opportunities for preparing homogeneous ubiquitinated conjugates with exquisite control on the atomic structure at workable quantities.[2] Harboring two deubiquitinases activities—trimming chains and shaving en bloc—complicates analysis of proteasome mechanism. Fixing one action, while leaving the other to progress unhindered should propel proteasome research. Rather than manipulating the enzyme (mutagenesis; biochemical inhibition), we took advantage of chemical advances to target the substrate.

We have recently reported a strategy of chemical polyubiquitination of expressed proteins relaying on our capabilities to manipulate the ubiquitin chain and the incorporation of various electrophiles at the C-terminus of the proximal ubiquitin for selective modification of a Cys residue in the target protein.[15] A key aspect of our nonenzymatic polyubiquitination approach is the installation of acyl hydrazide functionality using the Liu's approach[51] at the C-terminus of the proximal Ub, which after Ub chain assembly allows the introduction of various reactive electrophiles for protein conjugation. However, the oxidative switching to introduce the desired electrophile for thioether conjugation is not high yielding for di-Ub and negligible for the longer chains.[15] Hence, a new method is needed to obtain polyubiquitinated proteins with a stable bond between the chain the specific substrate. We are particularly interested in using these conjugates to study proteasomal degradation and how each of the different components—e.g., chain length, linkage type, the proteasome itself, and associated deubiquitinases—contribute to the fate of the substrate.

Here we report on a new strategy for the chemical synthesis of ubiquitinated proteins to generate a unique set of well-defined conjugates, each bearing an oxime bond that is resistant to DUBs as an isopeptide replacement between the proximal ubiquitin and substrate. Ubiquitin attached to substrate by a DUB-resistant bond would shed light on similarly fused ubiquitin mutants prevalent in brains of Alzheimer's patients.[52−54] Controlling a single bond within a set of polymeric substrates enabled us to evaluate the contribution of chain amputation to the overall degradation process. We found that chain length governed degradation outcome in unexpected ways, yet in accordance with physicochemical properties of polyUb chains.

RESULTS

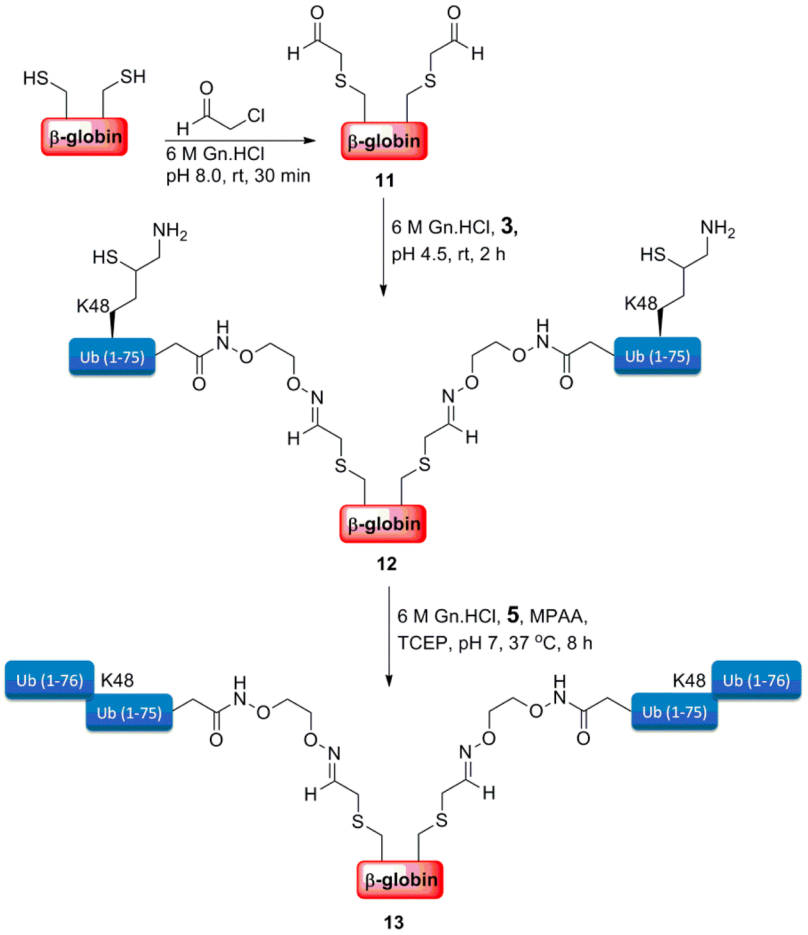
A set of ubiquitinated substrates differing only in length of Lys[48]-linked Ub chains that are linked to the substrate via a DUB-resistant bond could be used to interrogate the role of trimming while eliminating whole chain amputation. In order to achieve a nonhydrolyzable isopeptide bond replacement between a target protein and a polyUb chain, we developed an approach based on selective modification of Cys residue(s) in a target protein. Previously, we were able to attach a polyUb unit to a protein via the disulfide bond.[15] However, as disulfide bonds are reducible in biochemical settings, we aimed to develop a chemical strategy for synthesis of proteins modified with polyUb via a stable bond under reducing conditions, as well as being resistant to DUBs. Initially, we were unsuccessful to perform such a step beyond diUb due to the low efficiency in the incorporation of the desired electrophile on the C-terminus of the proximal ubiquitin.[15] We wondered whether reversing the approach, by switching the Cys residue into an electrophile while modifying the C-terminus of the proximal ubiquitin into nucleophile would overcome the previous limitations. For this purpose, we chose α-globin as a single domain globular substrate, which is known to be degraded via UPS as one of the ways nature restricts β-thalassemia disease.[55,56] In the new strategy, the single Cys[104] of α-globin was converted to an aldehyde, while the ubiquitin C-terminus is modified with oxyamino to enable ligating the two molecules via oxime bond[57,58] (Scheme 1).

Preparation of Building Blocks and Ubiquitination via Oxime Ligation. To test the oxime ligation strategy for synthesis of monoUb-, diUb-, triUb- and tetraUb-α-globin, we first synthesized monoUb-α-globin. Initially, we treated ubiquitin-thioester, with 1,2-bisaminoxy ethane[59] resulting in quantitative switching of the thioester to oxyamino moiety (Ub-ONH_2). No thioester hydrolysis was observed, nor was any measurable amount of oxyamino bridged ubiquitin dimer. In parallel, α-globin was treated with chloroacetaldehyde in 6 M Gn·HCl buffer (pH ∼ 8), which was added quantitatively to the Cys thiol to decorate α-globin with the acetaldehyde moiety (Scheme 1a and Figure S1). To check oxime ligation, α-globin acetaldehyde (**1**) was treated with Ub-ONH_2 in 6 M Gn·HCl buffer (pH ∼ 4.5). Notably, the reaction proceeded smoothly and was complete within 15 min. The reaction was also kept overnight to check the product stability; no product decomposition was observed.

To evaluate the reaction feasibility for attaching polyUb chains on α-globin, the same conditions were applied, this time for Lys[48]-linked diUb-ONH_2 to substrate **1**. However, the oxyamino moiety suffered considerable amounts of cleavage of the N−O bond during ligation step,[60] which we were unable to minimize even with modified reaction conditions. To overcome this cleavage problem we explored an alternate synthetic sequence by using isopeptide sequential ligation after attaching the proximal ubiquitin to the substrate. For this, we first performed one pot oxyamino switching and thiozilodine (Thz) ring opening of the δ-mercaptolysine in Ub^{K*48}-thioester (**2**) with 1,2-bisaminoxy ethane (Scheme 1b). Such dual transformation was completed within 6 h to give the desired product **3** as the sole peak according to the HPLC analysis (Figure S2). Next, we wondered how such ubiquitin bearing two reactive functionalities—i.e., 1,2-amino thiol and the oxyamino—will behave toward the aldehyde present in α-globin. For this, modified ubiquitin (**3**) was mixed with α-globin acetaldehyde (**1**), which rapidly gave ubiquitinated globin linked via oxime bond (**4**; Scheme 1b and Figure S3). Following the conjugation of **1** and **3** under 1:1 stoichiometry of the reactants we calculated a high rate constant (72.53 ± 0.58 M^{-1} s^{-1}) for this reaction (Figure S14). To confirm that oxime formation was preferred over thiozilodine formation (due to possible reaction between the aldehyde and free 1,2-amino thiol), we reacted the conjugate with *N*-(2-aminoethyl)-2-bromoacetamide. Under these conditions, quantitative addition of the acetamide was observed supporting oxime bond formation while leaving 1,2-amino thiol on the ubiquitin unit unreacted (Figure S4). Further support came from the next step where the obtained conjugate with the opened mercaptolysine (**4**) ligated with

DOI: 10.1021/jacs.6b09611
J. Am. Chem. Soc. 2016, 138, 16004−16015

Scheme 2. Synthesis of Oxime-Ligated PolyUb-β-globin[a]



[a]Selective addition of acetaldehyde moiety to the Cys^{94} and Cys^{113} thiol of the β-globin followed by oxime ligation with Ub^{K*48}(1-75)-ONH$_2$ (**3**). Next, ubiquitin chain elongation via isopeptide ligation to obtain bis(diUb)-β-globin (**13**). [Note, K* symbolizes δ-mercaptolysine at position 48].

another ubiquitin-thioester (**5**) to afford the Lys^{48}-linked diUb-α-globin (**8**; Scheme 1b). For such a step we measured a rate constant of 1.82 ± 0.01 M^{-1} s^{-1}, which is similar to published rates of conjugating peptides via native chemical ligation[61] (Figure S15). In a similar manner, Lys^{48}-linked diubiquitin-thioester or triUb-thioester (**6** and **7**, respectively), were reacted with **4** to obtain the corresponding triUb- or tetraUb-α-globin (**9** and **10**, respectively; Scheme 1b), completing the synthesis of the set of homogeneously ubiquitinated α-globin. Notably, in all these conjugates the ubiquitin units were linked via an isopeptide bond bearing the thiol handle. Efforts to desulfurize the thiol handle failed due to instability that we observed for the oxime bond. Our previous study on such an isopeptide bond with the thiol handle in unanchored ubiquitin chains showed no interference with deubiquitinases or with binding to ubiquitin-binding domains.[8]

Having successfully prepared a set of substrates modified by a single chain, we then synthesized a substrate modified with ubiquitin at two sites in order to examine how multiple ubiquitination sites influence outcome with proteasome.[48] For this purpose, we choose β-globin, which is the second subunit of hemoglobin, and is similar in sequence to α-globin. The two are obtained from same HPLC purification[15] and are nearly identical in size and fold as indicated by the superimposed tertiary structure obtained by protein data bank (Figure S25). To further examine the stability of both proteins, we measured the melting temperature by circular dichroism (CD), which suggested similar thermal stability ($\Delta T_m \sim 2.9$ °C, Figure S24). Notably, β-globin contains two Cys residues (Cys^{94} and Cys^{113}) in the sequence, located in same region as the single Cys^{104} in α-globin. As described above, we introduced aldehyde moieties on each of the two Cys residues of β-globin via its two Cys residues to give β-globin diacetaldehyde (**11**; Scheme 2). One has to note that our approach might be demanding when multiple Cys residues exist in the substrate and specificity is required. Next, purified **11** was treated with **3** to afford bis(monoUb)-β-globin (**12**). Later, ubiquitin-thioester **5** was reacted with **12** to achieve bis(diUb)-β-globin (**13**; Scheme 2).

Each member of our set of purified synthesized bioconjugates was analyzed by HPLC and mass spectrometry to confirm their molecular weight (example in Figure 1a, and Figure S5−S12) and was also resolved by SDS-PAGE gel electrophoresis to further confirm homogeneity (Figure 1b). Furthermore, all these synthesized bioconjugates were analyzed by CD confirming the expected secondary structure content of the ubiquitin and globin domains of the hybrid protein conjugate (Figure 1c,d,e). To confirm that the linkage between the proximal ubiquitin and globin is resistant to DUBs, we treated our bioconjugates with USP2. Indeed, monoUb-α-globin was not processed by the potent broad specificity DUB, USP2, whereas diUb-α-globin was trimmed within 30 min to generate monoUb-α-globin and free ubiquitin (Figure 1f). Although the

DOI: 10.1021/jacs.6b09611
J. Am. Chem. Soc. 2016, 138, 16004−16015

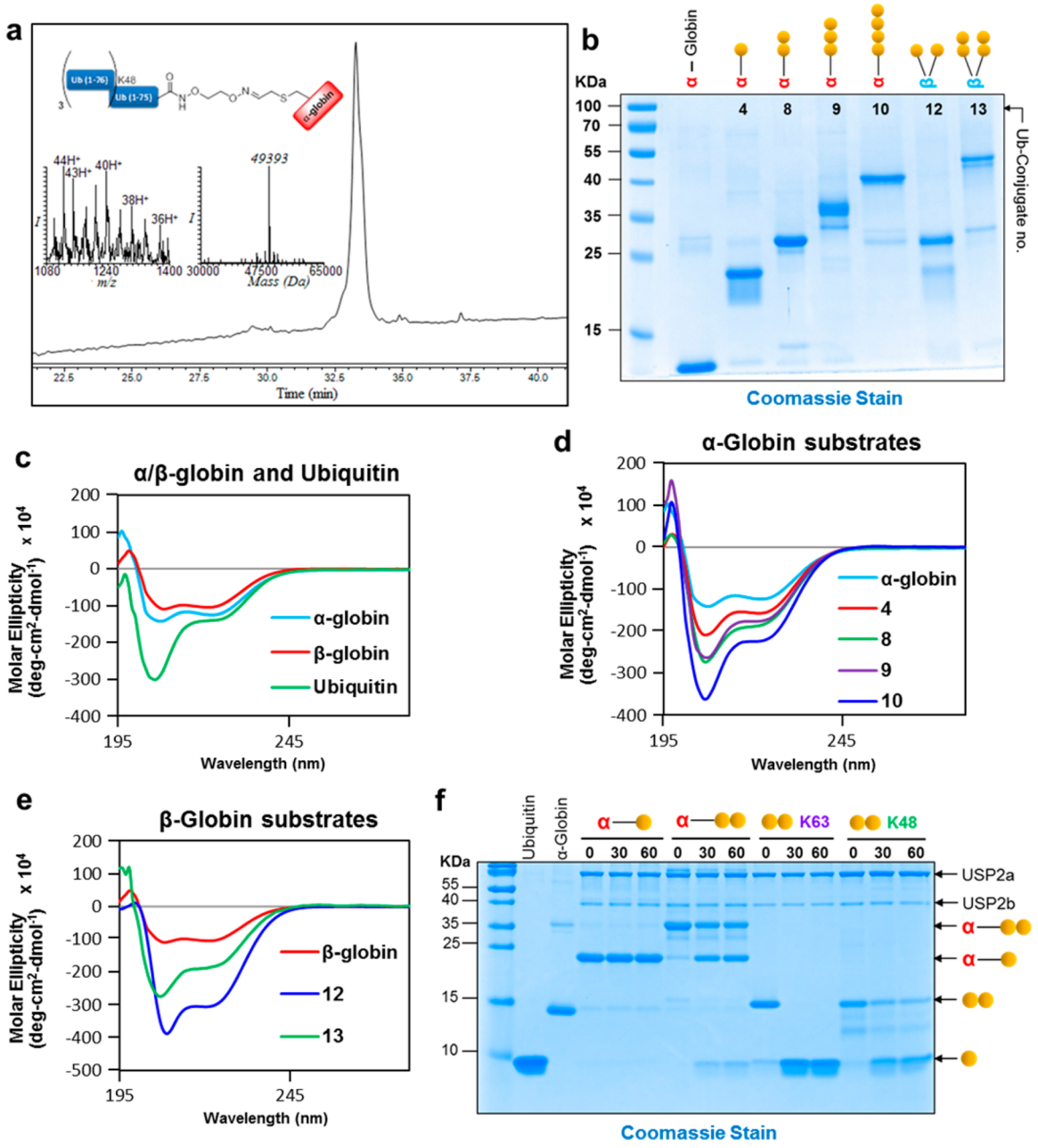


Figure 1. Characterization of synthesized ubiquitin-conjugates. (a) Purified synthetic tetraUb-α-globin migrates as a single peak by HPLC with an experimental mass of 49 393 Da (calculated 49 398) as determined by ESI-MS. (b) Ubiquitinated α-globin and β-globin substrates resolved by Tris-Glycine 12% SDS-PAGE and stained by Coomassie blue. (c,d,e) Circular dichroism (CD) analyses of free ubiquitin, naked α-globin and β-globin, and all synthetic ubiquitin-conjugates shows the additive effect of increasing ubiquitin units on the expected overall secondary structure of the conjugate. (f) monoUb-α-globin, diUb-α-globin, Lys^{63}-diUb, and Lys^{48}-diUb were treated with USP2 at a 50:1 Molar ratio for up to 60 min. Reaction products were resolved by Tris-Tricine 14% SDS-PAGE and stained with Coomassie blue. Migration pattern of free ubiquitin and α-globin is shown on left.

distal ubiquitin-ubiquitin isopeptide bonds were susceptible to USP2, the proximal bond between ubiquitin and substrate was stable, supporting our proposed design (even after 16 h incubation; Figure S16). Notably, Lys^{48}-linked isopeptide bond processing rates were comparable regardless of whether diUb was chemically conjugated to the α-globin target or as an unanchored chain (Figure 1f).

Processing of Ubiquitinated Globin by Human 26S Proteasome. 26S proteasome was purified from human erythrocytes (h26S proteasome; Figure S17), and was confirmed to possess inherent deubiquitinases activity attributed to both metalloproteases and Cys-based DUBs (Figure S18). We then analyzed the products generated from incubating each of the substrates in the set (**4**, **8**, **9**, **10**, **12** and **13**) at 100:1 molar ratio with purified h26S proteasome. Unmodified α-globin or monoUb-α-globin remained stable with no evidence for degradation (Figure 2a). Furthermore, no deubiquitination of monoUb-α-globin by the 26S proteasome was measured, validating the resistance of the bond between the proximal ubiquitin and α-globin to proteasome-associated DUBs (Figure 2a). In contrast, diUb-α-globin (**8**) was trimmed from the distal end of the polyUb chain generating monoUb-α-

DOI: 10.1021/jacs.6b09611
J. Am. Chem. Soc. 2016, 138, 16004−16015

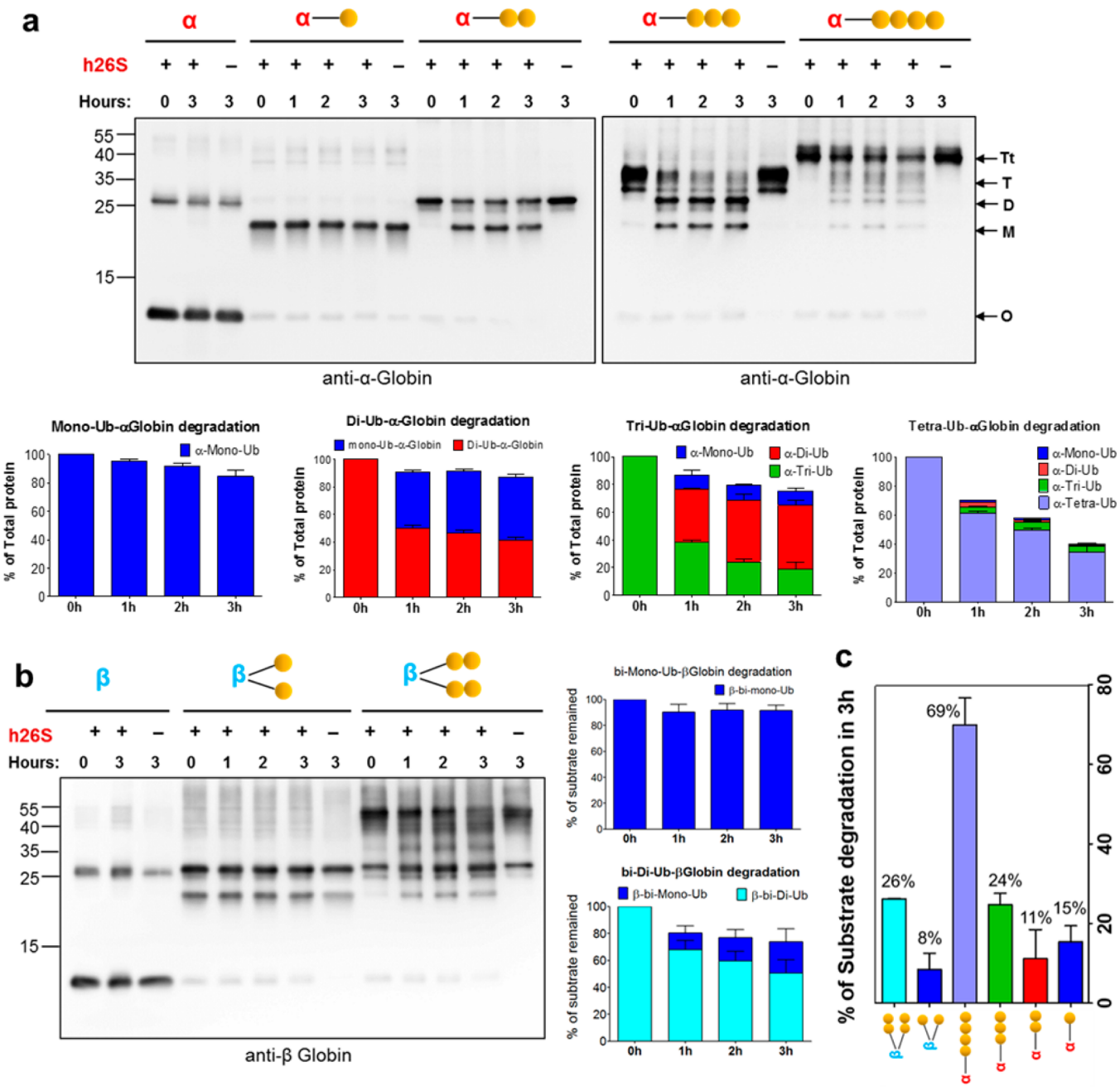


Figure 2. Product analysis of 26S proteasome reaction with a set of ubiquitinated globins: Purified human 26S proteasome was incubated with substrates from a set of ubiquitinated α- or β-globin at 1:100 ratio for up to 3 h. (a) Reaction mix of α-globin substrates were resolved by Tris-Glycine 15% SDS-PAGE and immunoblotted with anti-α-globin. Abundance of each specie (labeled on right; Tt: tetraUb-α-globin, T: triUb-α-globin, D: diUb-α-globin, M: monoUb-α-globin, O: naked α-globin) was quantified at each time point with ImageJ. For each substrate, product distribution as percentage of starting material is displayed (error bars from triplicate experiments) and color-coded according to specie. (b) Reaction same as in panel a, but with β-globin substrates, probed with anti-β-globin antibody. (c) Summary of substrate degradation, quantified by subtracting all products generated by 26S proteasome from initial substrates in panels a and b.

globin (~50% of initial substrate), whereas triUb-α-globin (**9**) generated both mono and diUb conjugates of α-globin (~10% and ~50%) (Figure 2a). In the case of triUb-α-globin, the sum of the different species present after 3 h indicates that ~25% of initial substrate was degraded (Figure 2a). TetraUb-α-globin (**10**) behaved considerably different, exhibiting degradation as the primary outcome of incubation with 26S proteasomes (~70%) with only trace deubiquitination detectable (Figure 2a).

The results obtained with tetraUb-α-globin prompted us to test how dividing the tetra-Ub signal into two diUb modifications would affect the reaction outcome. As shown in Figure 2b, β-globin modified with two diUb chains in close vicinity (bis(diUb)-β-globin, **13**) was deubiquitinated at one or both sites resulting in multiple products. The sum of these products pointed to ~25% degradation, similar to triUb-α-globin, but significantly less than was obtained by a single tetraUb chain modifying a protein. Even though both substrates were modified by a total of four ubiquitin units we observed

DOI: 10.1021/jacs.6b09611
J. Am. Chem. Soc. 2016, 138, 16004−16015

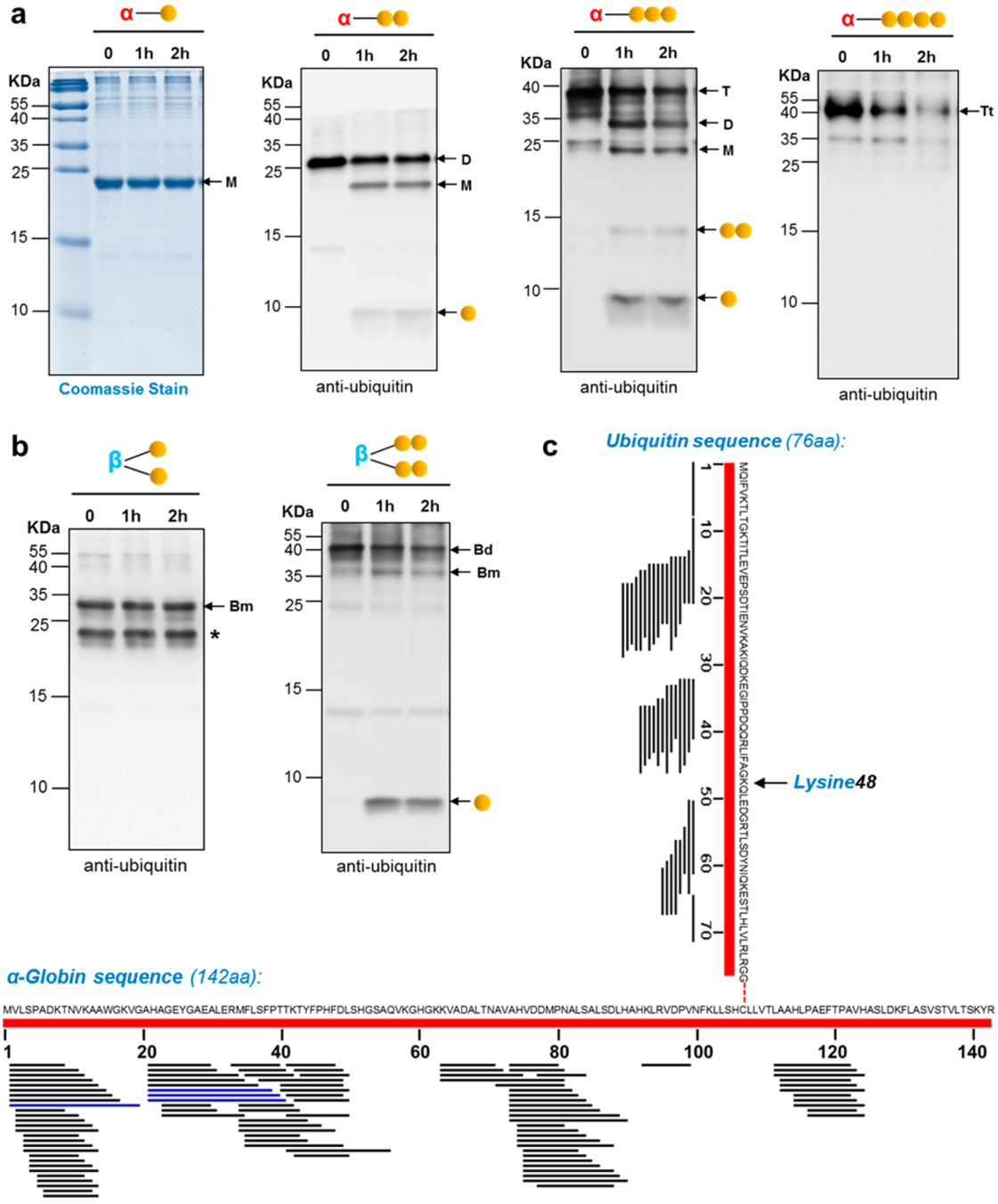


Figure 3. Fate of ubiquitin in 26S proteasome reaction with a set of ubiquitinated globins: Purified h26S proteasome was incubated with a set of ubiquitinated globin conjugates at 1:100 ratio for up to 3 h. (a) Samples were resolved by Tris-Tricine 16% SDS-PAGE and immunoblotted with antiubiquitin. Migration of initial substrates (monoUb-α-globin, diUb-α-globin, triUb-α-globin, tetraUb-α-globin), ubiquitin-containing intermediates, and liberated free ubiquitin are marked on right side of each gel. (b) Migration of initial substrates (bis(monoUb)-β-globin and bis(diUb)-β-globin), ubiquitin-containing intermediates, and liberated free ubiquitin are marked on right side of each gel (Tt: tetraUb-α-globin, T: triUb-α-globin, D: diUb-α-globin, M: monoUb-α-globin, Bd: bis(diUb)-β-globin, Bm: bis(monoUb)-β-globin). (c) Peptide products generated from tetraUb-α-globin after 3 h incubation with h26S were separated and subjected to LC−MS/MS. Sequence coverage of MS/MS identified peptides are marked next to ubiquitin and globin sequences. Peptides longer than 20 aa are marked in blue.

significant degradation for a single tetra-ubiquitin chain (Figure 2a), while we did not measure efficient degradation of bis(diUb)-β-globin by h26S proteasome (Figure 2b). The primary product in the latter case was bis(monoUb)-βglobin, a result of efficient trimming of both chains rather than degradation. As expected, bis(monoUb)-β-globin (**12**) did not undergo deubiquitination on account of the stable linkage between the proximal ubiquitin unit and the substrate (Figure 2b). Likewise, no significant degradation of naked β-globin or bis(monoUb)-β-globin was detected. One has to note that although the proteins have high structure similarity and to certain extent of similar thermal stability, one cannot draw strong correlation between these aspects and proteasomal stability, since the effect of the Ub attachment site on the overall stability of the conjugates cannot be excluded.[62] Taken together, Lys^{48}-linked chains shorter than tetraUb were not an

DOI: 10.1021/jacs.6b09611
J. Am. Chem. Soc. 2016, 138, 16004−16015

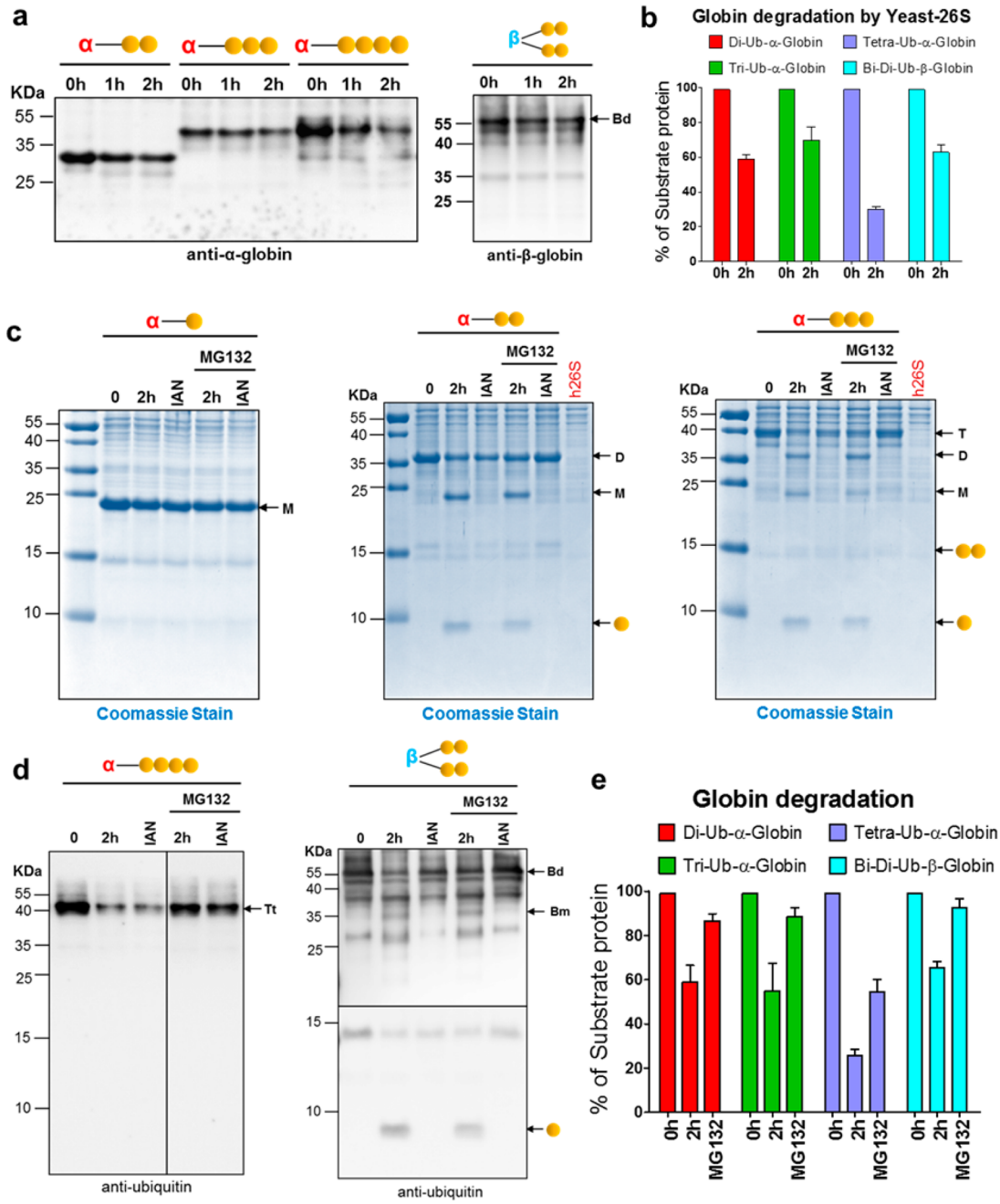


Figure 4. Blocking Ubiquitin chain trimming from Ub-conjugates accelerates substrate degradation by proteasome: (a) Purified yeast 26S proteasome was incubated at 1:100 ratio for up to 2 h with substrates from a set of ubiquitinated α- or β-globin. Samples were resolved by Tris-Glycine SDS-PAGE 15% and immunoblotted with anti-α-globin. (b) At each time point, abundance of residual substrate in panel A was quantified with ImageJ. (c) Reaction of h26S proteasome on monoUb-α-globin, diUb-α-globin or triUb-α-globin was carried out as in Figure 2 and 3 but in the presence of 2.5 μM iodoacetamide and/or 10 μM MG132; reaction products visualized by Coomassie staining after resolving by Tris-Tricine 16% SDS PAGE. (d) Reaction on tetraUb-α-globin, or bis(diUb)-β-globin as in C but reaction products immunoblotted with anti-Ub. (Tt: tetraUb-α-globin, T: triUb-α-globin, D: diUb-α-globin, M: monoUb-α-globin, Bd: bis(diUb)-β-globin, Bm: bis(monoUb)-β-globin). (e) The rate of degradation were measured by quantifying above Coomassie stain/WB band by ImageJ software and represents in graphs.

efficient signal for degradation by h26S proteasome of either α-globin or β-globin substrates (Figure 2c).

TetraUb Chain Is Degraded along with the Conjugated Substrate. Having observed significantly fewer reaction intermediates with a substrate modified by tetraUb compared to shorter chains, we followed the fate of ubiquitin during reaction progression. Free ubiquitin was generated concomitantly with trimming of the distal ubiquitin unit from diUb-α-globin and from triUb-α-globin (Figure 3a). Seeing as free ubiquitin was the primary product even from a triUb modification, trimming appears to progress primarily from the distal end. In stark contrast, neither free ubiquitin nor any ubiquitinated intermediates were detected from tetraUb-α-globin incubated with h26S (Figure 3a; right panel). Quantification of substrate depletion points to degradation of the complete conjugate—tetraUb along with globin (Figure 2a). Release of free ubiquitin from bis(diUb)-β-globin along with generation of ubiquitinated intermediates confirmed deubiqui-

DOI: 10.1021/jacs.6b09611
J. Am. Chem. Soc. 2016, 138, 16004−16015

tination as the primary outcome of proteasome action on this substrate (Figure 3b right panel).

In order to gain additional information on proteolysis of a tetraUb chain was along with conjugated α-globin, peptide products were separated from residual proteins following a 3 h incubation with proteasome. These peptides were analyzed directly by MS/MS and sequences determined by MaxQuant software. Almost the entire sequences of ubiquitin and of globin were covered by multiple peptides of various lengths (Figure 3c). Many of these peptides contain overlapping sequences, and some were identified repeatedly (SI section 19 and accompanying SI Spreadsheet S1).

Attenuation of PolyUb Trimming Enhances Substrate Degradation by h26S Proteasome. Having observed that Ub-chains of less than 4 units were trimmed by h26S, we wondered whether interfering with proteasome-associated DUB activity would enhance degradation of their conjugated substrate. Since Ubp6, the ortholog of USP14, detaches during purification of 26S proteasome from yeast,[63,64] this enzyme was employed to examine the contribution of DUBs to the degradation process. Similar to the reaction catalyzed by h26S proteasome, degradation of tetraUb-α-globin by y26S proteasome proceeded to ∼70% (Figure 4b). However, no detectable deubiquitination of any of our substrates by y26S proteasomes was observed (Figure 4a). On the contrary, degradation was observed even for chains as short as diUb. However, even without the contribution of Ubp6, two diUb modifications did not render the globin a better substrate for proteasome action than a substrate with a single tetraUb modification (Figure 4a,b).

The results obtained with y26S proteasome indicated that it might possible to enhance proteolysis of a conjugated substrate by inhibition of Cys-based DUBs on proteasomes. Indeed, h26S proteasome pretreated with 2.5 μM iodoacetamide[46] lost the ability to trim the distal ubiquitin unit from diUb-, triUb-, or bis(diUb)- modifications (Figure 4c,d) without affecting proteasome stability or proteolytic activity (Figure S19). As a result, about 40% of the intact conjugate was degraded, once again globin along with the covalently attached ubiquitin (Figure 4c). This degradation was arrested in the presence of 10 μM MG132, a 20S inhibitor (Figure 4c). Treatment with MG132 completely inhibited basal peptidase activity attributed to the β5 20S core particle subunit without any deleterious effects on proteasome stability (Figure S20). Since tetraUb-α-globin was only minimally susceptible to trimming by h26S proteasomes, inhibition with iodoacetamide resulted in no discernible effects on proteolysis (Figure 4d). Here too, MG132 arrested degradation, regardless of chain length or trimming (Figure 4d). Quantification of these results highlighted the competition between chain disassembly and proteolysis of the conjugated protein at the proteasome (Figure 4e).

DISCUSSION

Proteasomal degradation involves multiple steps each driven by a subset of proteasome-associated subunits including shuttles, binders, ATPase unfoldases, proteases and DUBs. Understanding the inner working of the proteasome as a holistic machine would benefit from focusing on a particular step by limiting the degrees of freedom. Great advances to this objective have been made by tinkering with the proteasome, for example, by eliminating or mutating specific subunits. While this approach has been instrumental in ascribing roles to individual subunits and thus exposing the key players, it could lead to changes in overall complex structure or stability. Alternatively, chemical inhibition has also been effective in dissecting the contribution of resident enzymes such as DUBs. However, multiple roles played by proteasome-associated DUBs—both Rpn11 and USP14/Ubp6 have catalytic as well as noncatalytic roles—limit applications of inhibition studies. Here, we took a complementary chemical approach by focusing on the substrate. We designed and synthesized a unique set of modified substrates to illuminate a specific feature of proteasome action. Specifically, we stabilized the isopeptide bond between a given substrate and the proximal ubiquitin to prevent removal of the chain during the degradation process. For this, we developed a chemical approach based on oxime bond formation between the proximal ubiquitin bearing an oxyamino at the C-terminus and a selectively modified protein with an aldehyde functionality at a controlled site. With this parameter fixed, a set of substrates was synthesized that varied only in chain length.

Examining the reaction of our set of substrates with purified proteasome demonstrated, unequivocally, that cleavage of the proximal isopeptide bond is not a prerequisite for proteasomal degradation. It has been proposed that removal of a polyUb chain by Rpn11 is an essential step in the degradation process, since failure to remove would block the entry port.[33,41] Our results show that despite the size and rigidity of the chain, and each unit within, h26S proteasome can proteolyze a Lys^{48}-linked tetraUb chain conjugated to a substrate. Under certain conditions ubiquitin can be degraded.[47,65−68] Nevertheless, degradation of a complete Lys^{48}-linked tetraUb chain is remarkable as it requires overcoming several structural hurdles: stretching the closed form of tetraUb held together via hydrophobic patches between units,[69] unfolding the highly thermostable ubiquitin unit,[70] and processing the unique covalent branched structure of the isopeptide linkage. Degradation of an intact polyUb-conjugate highlights the powerful unfolding mechanism of the proteasome machine. It would be interesting to measure the mechanical force exerted by proteasome to achieve such an outcome.[71]

Comparison of reaction outcome accounting for both chain trimming and proteolysis, enabled dissection of the importance of chain length. Shorter chains did engage proteasomes, but were trimmed, resulting in a nonproteolytic event. TetraUb stood out by far from monoUb, diUb, or triUb modifications as an efficient signal for proteolysis by 26S proteasomes. That tetraUb was relatively resistant to trimming by proteasome-associated DUBs is likely a feature of its closed compact conformation.[64,69,72−74] In the current work, splitting the tetraUb chain into two closely positioned diUb modifications on a single globular protein, globin in this case, did not serve as an efficient signal for degradation, as each of the two dimers were susceptible to trimming. For other, loosely folded substrates, shorter chains or even multiple modifications by monoUb may be sufficient for proteasomes to degrade a conjugate.[47,48] Taken together, the results point to residency time as a key parameter of 26S proteasome-dependent degradation. Longer polyUb chains provide longer residency time with tetraUb providing a particularly potent signal, probably due to its resistance to disassembly by proteasome resident DUBs. Eliminating chain trimming by removing or inhibiting Cys-based DUBs associated with proteasomes converted a protein tagged with diUb, triUb or bis(diUb) into a substrate for proteolysis due to increased residency time,

DOI: 10.1021/jacs.6b09611
J. Am. Chem. Soc. 2016, 138, 16004−16015

or/and to better positioning and alignment for translocation.[39,41,75] Under circumstances in which the proximal Ub is not shaved from the substrate before the extended polypeptide enters the internal cavity of the 26S proteasome, ubiquitin was degraded—along with substrate. Furthermore, ubiquitin was proteolyzed to completion at both sides of the isopeptide bond on Lys48, indicating that branched polypeptides do not pose a hindrance for substrate translocation or proteasome mechanism. This observation can explain how ubiquitin fusion degradation (UFD) substrates are degraded. This is particularly pertinent as a noncleavable ubiquitin fusion termed UBB+1 is found in correlation with neurodegeneration pathologies neurons.[66,76−78]

SUMMARY

Controlling substrate features at the atomic level by applying innovative chemistry to lower degrees of freedom provides unprecedented opportunities to dissect a complicated enzymatic process, such as in the proteasome. In the current study, replacing the proximal isopeptide bond with an oxime bond provided new information about the contribution of the shaving step (attributed to Rpn11) to the overall degradation process. Rpn11 is a drug target mainly because of its catalytic activity.[33,44,79,80] However, recent structural studies point to a dynamic repositioning of Rpn11 within the 26S proteasome during the catalytic cycle.[38,42,81] New drug discovery efforts could take into account, for example, noncatalytic features of Rpn11 such as repositioning or alignment of the substrate. The finding that tetraUb can be degraded with the substrate triggers a fresh look at natural conjugates for which no known DUBs are identified for the shaving step, specifically ubiquitin chains linked not through isopeptide bond to a Lys residue, but via an ester bond to a Ser or Thr residues.[82,83]

METHODS

Synthesis of Globin Aldehyde (1). To a solution of purified α-globin[15] (7 mg, 0.46 mmol) in Gn·HCl buffer (460 μL, 1 mM, pH 8.0), chloroacetaldehyde (50 wt % solution in water, 3 μL, 50 equiv) was added and the reaction mixture was kept at RT for 30 min. The product **1** was analyzed using analytical HPLC and later isolated via semipreparative HPLC using 5−70% B in 40 min gradient on C4 column.

*Synthesis of K48-monoUb-ONH$_2$ (**3**).* K48*-monoUb75-MMP[15] (8 mg, 0.92 mmol) was dissolved in a solution of MPAA (25 equiv) in 6 M Gn·HCl (231 μL, pH 6). After 60 min at RT, a solution of bis-aminoxyethane (100 equiv, 150 μL) in Gn·HCl was added and the pH was adjusted to 5 and kept for 60 min at RT to complete the aminoxy switching, then reaction mixture was incubated at 42 °C for 6 h to unmask the thiazolidine. The reaction was followed using analytical HPLC (C18 column) and a gradient of 5−55% B in 40 min. For semipreparative HPLC, the same gradient was used to afford the purified product.

*Conjugation of **3** with **1** via Oxime Linkage.* **3** (4.5 mg, 0.52 mmol) and **1** (6.35 mg, 0.8 equiv) were dissolved together in 6 M Gn·HCl (145 μL, pH 4.5) and kept at RT for 15 min the conjugated product **4** was analyzed using analytical HPLC/mass and isolated via semipreparative HPLC using 5−70% B in 40 min gradient on a C4 column.

*Ligation of Ub-MPAA with **4**.* Ub-MPAA[15] (**5**) (0.95 mg, 1.3 equiv) and **4** (2 mg, 0.08 mmol) were dissolved in 6 M Gn·HCl buffer (85 μL, 1 mM). To this solution, 30 equiv each of MPAA and TCEP were added, the pH was adjusted to 7 and kept at 37 °C for 3 h. The reaction was followed using analytical HPLC (C4 column) and a gradient 5−70% B over 40 min. For semipreparative HPLC, same gradient was used to afford diUb-α-globin **8**.

Deubiquitination Assay. 100 nM of USP2 (Boston Biochem) and 5 μM of synthetic Ub-conjugates or ubiquitin dimers[64] were used for deubiquitination assay in a molar ratio of 1:50 at 37 °C. The assay buffer contains 50 mM TRIS (pH 7.4), 50 mM NaCl, 100 μM EDTA and 5 mM DTT.

Proteasome Purification. Human 26S proteasomes were purified following an adapted protocol that we used to isolate proteasomes from yeast,[64] with the modification that the source was human erythrocytes and the first separation step was on a DEAE column. LC−MS/MS assessment of final preparation confirmed presence of the canonical subunits viz., PSMA1−7, PSMB1−7, PSMC1−6 and PSMD1−14. Yeast proteasomes were purified as published.[64]

Proteasomal Degradation Assay. Twenty nM of human or yeast 26S proteasome and 2 μM of α/β-globin and ubiquitinated α/β-globin proteins were used for the degradation assay in a molar ratio of 1:100. Assay buffer contain 25 mM TRIS (pH 7.4), 10 mM $MgCl_2$, 10% Glycerol, 1 mM ATP and 1 mM DTT. For DUBs inhibition experiments 2.5 mM Iodoacetamide (IAN) was added to h26S proteasome mixture, incubated for 30 min at RT, and then the substrates were added to mixture followed by incubation at 37 °C as per mentioned. For proteasome inhibition assay 25 mM TRIS (pH 7.4), 10 mM EDTA, 20 μM MG132, 10% Glycerol, and 1 mM DTT were used in the assay buffer. All degradation reaction were carried out at 37 °C.

Western Blot Analysis. All the sample were heated at 90 °C with SDS-Loading dye contain 20 mM DTT for 5 min. The samples were run in TRIS-Tricine or TRIS-Glycine SDS-PAGE. The antibodies anti-Ubiquitin (Mouse monoclonal in 1:8000, Rabbit polyclonal in 1:4000), anti-α-globin (Rabbit polyclonal in 1:5000) and anti-β-globin (Mouse monoclonal in 1:5000) were used for immunoblot assay.

In-Gel Proteasome Activity Assay. Two μg of purified of human and yeast proteasome were run in 4% Native-PAGE in TRIS-boric acid Buffer for 2 h at 130 V and 400 mA. The gel was incubated in assay buffer containing 25 mM TRIS (pH 7.4), 10 mM $MgCl_2$, 10% Glycerol, 1 mM ATP, 1 mM DTT, 50 μM Suc-LLVY-AMC and/or 0.02% SDS for 10 min at 30 °C.

Mass Spectroscopy. After 3 h incubation of tetraUb-α-globin with purified h26S proteasome, the reaction mixture was processed for LC−MS/MS analysis as described in Supporting Information section 19.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/jacs.6b09611.

Experimental methods and HPLC and mass spectrometry analyses of the synthetic monomers and ligation products; Kinetics data for oxime and isopeptide ligations; Western blots for biochemical studies; Thermal denaturation analysis for proteins (PDF)

Spreadsheet S1 (PDF)

AUTHOR INFORMATION

Corresponding Authors

*glickman@tx.technion.ac.il

*abrik@technion.ac.il

ORCID

Ashraf Brik: 0000-0001-8745-2250

Present Address

§Synthetic R&D, Sami Laboratories Ltd., 560058 Bangalore, India.

Author Contributions

#S.K.S. and I.S. contributed equally.

Notes

The authors declare no competing financial interest.

DOI: 10.1021/jacs.6b09611
J. Am. Chem. Soc. 2016, 138, 16004−16015

■ ACKNOWLEDGMENTS

We thank the Israel Science Foundation for financial support (to AB and to MHG) and NIH GM095755 to MHG. A. Brik is a Neubauer Professor and a Taub Fellow-Supported by the Taub Foundations; MH Glickman is an Israel Isaac and Natalia Kudish Academic Chair. We thank Ortal Hayat for aiding in enzymatic synthesis of K63-linked ubiquitin dimers. Zanlin Yu and Amnon Golan thanked for aiding in 26S proteasome purifications.

■ REFERENCES

(1) Bondalapati, S.; Jbara, M.; Brik, A. *Nat. Chem.* **2016**, *8*, 407.
(2) Spasser, L.; Brik, A. *Angew. Chem., Int. Ed.* **2012**, *51*, 6840.
(3) Strieter, E. R.; Korasick, D. A. *ACS Chem. Biol.* **2012**, *7*, 52.
(4) Weller, C. E.; Pilkerton, M. E.; Chatterjee, C. *Biopolymers* **2014**, *101*, 144.
(5) Castaneda, C. A.; Dixon, E. K.; Walker, O.; Chaturvedi, A.; Nakasone, M. A.; Curtis, J. E.; Reed, M. R.; Krueger, S.; Cropp, T. A.; Fushman, D. *Structure* **2016**, *24*, 423.
(6) Chen, J. J.; Ai, Y. X.; Wang, J. L.; Haracska, L.; Zhuang, Z. H. *Nat. Chem. Biol.* **2010**, *6*, 270.
(7) Eger, S.; Castrec, B.; Hubscher, U.; Scheffner, M.; Rubini, M.; Marx, A. *ChemBioChem* **2011**, *12*, 2807.
(8) Haj-Yahya, N.; Haj-Yahya, M.; Castaneda, C. A.; Spasser, L.; Hemantha, H. P.; Jbara, M.; Penner, M.; Ciechanover, A.; Fushman, D.; Brik, A. *Angew. Chem., Int. Ed.* **2013**, *52*, 11149.
(9) McGinty, R. K.; Kim, J.; Chatterjee, C.; Roeder, R. G.; Muir, T. W. *Nature* **2008**, *453*, 812.
(10) Morgan, M. T.; Haj-Yahya, M.; Ringel, A. E.; Bandi, P.; Brik, A.; Wolberger, C. *Science* **2016**, *351*, 725.
(11) Pan, M.; Gao, S.; Zheng, Y.; Tan, X. D.; Lan, H.; Tan, X. L.; Sun, D. M.; Lu, L. N.; Wang, T.; Zheng, Q. Y.; Huang, Y. C.; Wang, J. W.; Liu, L. *J. Am. Chem. Soc.* **2016**, *138*, 7429.
(12) Valkevich, E. M.; Guenette, R. G.; Sanchez, N. A.; Chen, Y. C.; Ge, Y.; Strieter, E. R. *J. Am. Chem. Soc.* **2012**, *134*, 6916.
(13) Yang, K.; Gong, P.; Gokhale, P.; Zhuang, Z. H. *ACS Chem. Biol.* **2014**, *9*, 1685.
(14) El Oualid, F.; Merkx, R.; Ekkebus, R.; Hameed, D. S.; Smit, J. J.; de Jong, A.; Hilkmann, H.; Sixma, T. K.; Ovaa, H. *Angew. Chem., Int. Ed.* **2010**, *49*, 10149.
(15) Hemantha, H. P.; Bavikar, S. N.; Herman-Bachinsky, Y.; Haj-Yahya, N.; Bondalapati, S.; Ciechanover, A.; Brik, A. *J. Am. Chem. Soc.* **2014**, *136*, 2665.
(16) Kumar, K. S. A.; Bavikar, S. N.; Spasser, L.; Moyal, T.; Ohayon, S.; Brik, A. *Angew. Chem., Int. Ed.* **2011**, *50*, 6137.
(17) Sommer, S.; Weikart, N. D.; Brockmeyer, A.; Janning, P.; Mootz, H. D. *Angew. Chem., Int. Ed.* **2011**, *50*, 9888.
(18) Virdee, S.; Kapadnis, P. B.; Elliott, T.; Lang, K.; Madrzak, J.; Nguyen, D. P.; Riechmann, L.; Chin, J. W. *J. Am. Chem. Soc.* **2011**, *133*, 10708.
(19) Weller, C. E.; Dhall, A.; Ding, F.; Linares, E.; Whedon, S. D.; Senger, N. A.; Tyson, E. L.; Bagert, J. D.; Li, X.; Augusto, O.; Chatterjee, C. *Nat. Commun.* **2016**, *7*, 12979.
(20) Glickman, M. H.; Ciechanover, A. *Physiol. Rev.* **2002**, *82*, 373.
(21) Finley, D. *Annu. Rev. Biochem.* **2009**, *78*, 477.
(22) Pickart, C. M. *Annu. Rev. Biochem.* **2001**, *70*, 503.
(23) Ikeda, F.; Dikic, I. *EMBO Rep.* **2008**, *9*, 536.
(24) Yau, R.; Rape, M. *Nat. Cell Biol.* **2016**, *18*, 579.
(25) Thrower, J. S.; Hoffman, L.; Rechsteiner, M.; Pickart, C. M. *EMBO J.* **2000**, *19*, 94.
(26) Chau, V.; Tobias, J. W.; Bachmair, A.; Marriott, D.; Ecker, D. J.; Gonda, D. K.; Varshavsky, A. *Science* **1989**, *243*, 1576.
(27) Gregori, L.; Poosch, M. S.; Cousins, G.; Chau, V. *J. Biol. Chem.* **1990**, *265*, 8354.
(28) Tanaka, K.; Mizushima, T.; Saeki, Y. *Biol. Chem.* **2012**, *393*, 217.
(29) Bhattacharyya, S.; Yu, H.; Mim, C.; Matouschek, A. *Nat. Rev. Mol. Cell Biol.* **2014**, *15*, 122.
(30) Guterman, A.; Glickman, M. H. *Curr. Protein Pept. Sci.* **2004**, *5*, 201.
(31) Lee, M. J.; Lee, B. H.; Hanna, J.; King, R. W.; Finley, D. *Mol. Cell. Proteomics* **2011**, *10*, R110.003871.
(32) Maytal-Kivity, V.; Reis, N.; Hofmann, K.; Glickman, M. H. *BMC Biochem* **2002**, *3*, 28.
(33) Verma, R.; Aravind, L.; Oania, R.; McDonald, W. H.; Yates, J. R., 3rd; Koonin, E. V.; Deshaies, R. J. *Science* **2002**, *298*, 611.
(34) Hu, M.; Li, P.; Song, L.; Jeffrey, P. D.; Chenova, T. A.; Wilkinson, K. D.; Cohen, R. E.; Shi, Y. *EMBO J.* **2005**, *24*, 3747.
(35) Lee, B. H.; Lee, M. J.; Park, S.; Oh, D. C.; Elsasser, S.; Chen, P. C.; Gartner, C.; Dimova, N.; Hanna, J.; Gygi, S. P.; Wilson, S. M.; King, R. W.; Finley, D. *Nature* **2010**, *467*, 179.
(36) Peth, A.; Besche, H. C.; Goldberg, A. L. *Mol. Cell* **2009**, *36*, 794.
(37) Yao, T.; Cohen, R. E. *Nature* **2002**, *419*, 403.
(38) Pathare, G. R.; Nagy, I.; Sledz, P.; Anderson, D. J.; Zhou, H. J.; Pardon, E.; Steyaert, J.; Forster, F.; Bracher, A.; Baumeister, W. *Proc. Natl. Acad. Sci. U. S. A.* **2014**, *111*, 2984.
(39) Aufderheide, A.; Beck, F.; Stengel, F.; Hartwig, M.; Schweitzer, A.; Pfeifer, G.; Goldberg, A. L.; Sakata, E.; Baumeister, W.; Forster, F. *Proc. Natl. Acad. Sci. U. S. A.* **2015**, *112*, 8626.
(40) Bashore, C.; Dambacher, C. M.; Goodall, E. A.; Matyskiela, M. E.; Lander, G. C.; Martin, A. *Nat. Struct. Mol. Biol.* **2015**, *22*, 712.
(41) Dambacher, C. M.; Worden, E. J.; Herzik, M. A.; Martin, A.; Lander, G. C. *eLife* **2016**, *5*, e13027.
(42) Worden, E. J.; Padovani, C.; Martin, A. *Nat. Struct. Mol. Biol.* **2014**, *21*, 220.
(43) Yu, Z.; Livnat-Levanon, N.; Kleifeld, O.; Mansour, W.; Nakasone, M. A.; Castaneda, C. A.; Dixon, E. K.; Fushman, D.; Reis, N.; Pick, E.; Glickman, M. H. *Biosci. Rep.* **2015**, *35*, 1.
(44) Spataro, V.; Simmen, K.; Realini, C. A. *Anticancer Res.* **2002**, *22*, 3905.
(45) Boutet, S. C.; Disatnik, M. H.; Chan, L. S.; Iori, K.; Rando, T. A. *Cell* **2007**, *130*, 349.
(46) Guterman, A.; Glickman, M. H. *J. Biol. Chem.* **2004**, *279*, 1729.
(47) Shabek, N.; Herman-Bachinsky, Y.; Buchsbaum, S.; Lewinson, O.; Haj-Yahya, M.; Hejjaoui, M.; Lashuel, H. A.; Sommer, T.; Brik, A.; Ciechanover, A. *Mol. Cell* **2012**, *48*, 87.
(48) Lu, Y.; Lee, B. H.; King, R. W.; Finley, D.; Kirschner, M. W. *Science* **2015**, *348*, 1250834.
(49) Meyer, H. J.; Rape, M. *Cell* **2014**, *157*, 910.
(50) Jasnin, M.; Ecke, M.; Baumeister, W.; Gerisch, G. *Structure* **2016**, *24*, 1031.
(51) Zheng, J. S.; Tang, S.; Qi, Y. K.; Wang, Z. P.; Liu, L. *Nat. Protoc.* **2013**, *8*, 2483.
(52) Dantuma, N. P.; Lindsten, K.; Glas, R.; Jellne, M.; Masucci, M. G. *Nat. Biotechnol.* **2000**, *18*, 538.
(53) Johnson, E. S.; Ma, P. C.; Ota, I. M.; Varshavsky, A. *J. Biol. Chem.* **1995**, *270*, 17442.
(54) Varshavsky, A. *Methods Enzymol.* **2005**, *399*, 777.
(55) Bank, A.; O'Donnell, J. V. *Nature* **1969**, *222*, 295.
(56) Shaeffer, J. R. *J. Biol. Chem.* **1983**, *258*, 13172.
(57) Shanmugham, A.; Fish, A.; Luna-Vargas, M. P.; Faesen, A. C.; El Oualid, F.; Sixma, T. K.; Ovaa, H. *J. Am. Chem. Soc.* **2010**, *132*, 8834.
(58) Stanley, M.; Virdee, S. *ChemBioChem* **2016**, *17*, 1472.
(59) Yi, L.; Sun, H.; Wu, Y. W.; Triola, G.; Waldmann, H.; Goody, R. S. *Angew. Chem., Int. Ed.* **2010**, *49*, 9417.
(60) Moyal, T.; Hemantha, H. P.; Siman, P.; Refua, M.; Brik, A. *Chem. Sci.* **2013**, *4*, 2496.
(61) Saito, F.; Noda, H.; Bode, J. W. *ACS Chem. Biol.* **2015**, *10*, 1026.
(62) Hagai, T.; Levy, Y. *Proc. Natl. Acad. Sci. U. S. A.* **2010**, *107*, 2001.
(63) Leggett, D. S.; Hanna, J.; Borodovsky, A.; Crosas, B.; Schmidt, M.; Baker, R. T.; Walz, T.; Ploegh, H.; Finley, D. *Mol. Cell* **2002**, *10*, 495.
(64) Mansour, W.; Nakasone, M. A.; von Delbruck, M.; Yu, Z.; Krutauz, D.; Reis, N.; Kleifeld, O.; Sommer, T.; Fushman, D.; Glickman, M. H. *J. Biol. Chem.* **2015**, *290*, 4688.
(65) Shabek, N.; Herman-Bachinsky, Y.; Ciechanover, A. *Proc. Natl. Acad. Sci. U. S. A.* **2009**, *106*, 11907.

DOI: 10.1021/jacs.6b09611
J. Am. Chem. Soc. 2016, 138, 16004−16015

(66) Krutauz, D.; Reis, N.; Nakasone, M. A.; Siman, P.; Zhang, D.; Kirkpatrick, D. S.; Gygi, S. P.; Brik, A.; Fushman, D.; Glickman, M. H. *Nat. Chem. Biol.* **2014**, *10*, 664.
(67) Hanna, J.; Leggett, D. S.; Finley, D. *Mol. Cell. Biol.* **2003**, *23*, 9251.
(68) Verhoef, L. G.; Heinen, C.; Selivanova, A.; Halff, E. F.; Salomons, F. A.; Dantuma, N. P. *FASEB J.* **2009**, *23*, 123.
(69) Eddins, M. J.; Varadan, R.; Fushman, D.; Pickart, C. M.; Wolberger, C. *J. Mol. Biol.* **2007**, *367*, 204.
(70) Ciechanover, A.; Elias, S.; Heller, H.; Ferber, S.; Hershko, A. *J. Biol. Chem.* **1980**, *255*, 7525.
(71) Luan, B.; Huynh, T.; Li, J.; Zhou, R. *ACS Nano* **2016**, *10*, 317.
(72) Haj-Yahya, M.; Fauvet, B.; Herman-Bachinsky, Y.; Hejjaoui, M.; Bavikar, S. N.; Karthikeyan, S. V.; Ciechanover, A.; Lashuel, H. A.; Brik, A. *Proc. Natl. Acad. Sci. U. S. A.* **2013**, *110*, 17726.
(73) Schaefer, J. B.; Morgan, D. O. *J. Biol. Chem.* **2011**, *286*, 45186.
(74) Jacobson, A. D.; Zhang, N. Y.; Xu, P.; Han, K. J.; Noone, S.; Peng, J.; Liu, C. W. *J. Biol. Chem.* **2009**, *284*, 35485.
(75) Glickman, M. H.; Adir, N. *PLoS Biol.* **2004**, *2*, E13.
(76) De Vrij, F. M.; Sluijs, J. A.; Gregori, L.; Fischer, D. F.; Hermens, W. T.; Goldgaber, D.; Verhaagen, J.; Van Leeuwen, F. W.; Hol, E. M. *FASEB J.* **2001**, *15*, 2680.
(77) Dennissen, F. J.; Kholod, N.; Hermes, D. J.; Kemmerling, N.; Steinbusch, H. W.; Dantuma, N. P.; van Leeuwen, F. W. *FEBS Lett.* **2011**, *585*, 2568.
(78) van Leeuwen, F. W.; de Kleijn, D. P.; van den Hurk, H. H.; Neubauer, A.; Sonnemans, M. A.; Sluijs, J. A.; Koycu, S.; Ramdjielal, R. D.; Salehi, A.; Martens, G. J.; Grosveld, F. G.; Peter, J.; Burbach, H.; Hol, E. M. *Science* **1998**, *279*, 242.
(79) Gallery, M.; Blank, J. L.; Lin, Y.; Gutierrez, J. A.; Pulido, J. C.; Rappoli, D.; Badola, S.; Rolfe, M.; Macbeth, K. J. *Mol. Cancer Ther.* **2007**, *6*, 262.
(80) Buckley, D. L.; Crews, C. M. *Angew. Chem., Int. Ed.* **2014**, *53*, 2312.
(81) Matyskiela, M. E.; Lander, G. C.; Martin, A. *Nat. Struct. Mol. Biol.* **2013**, *20*, 781.
(82) Tait, S. W.; de Vries, E.; Maas, C.; Keller, A. M.; D'Santos, C. S.; Borst, J. *J. Cell Biol.* **2007**, *179*, 1453.
(83) Vosper, J. M.; McDowell, G. S.; Hindley, C. J.; Fiore-Heriche, C. S.; Kucerova, R.; Horan, I.; Philpott, A. *J. Biol. Chem.* **2009**, *284*, 15458.

DOI: 10.1021/jacs.6b09611
J. Am. Chem. Soc. 2016, 138, 16004−16015