Decoding the assembly of Mixed and Branched heterotypic Ubiquitin chains

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Abstract

In eukaryotes, post-translational modification (PTMs) creates a proteome diversity that is essential for cellular processes. The PTM ubiquitination regulates cell signaling, immune response, protein processing, molecular trafficking, and DNA repair. While molecular trafficking typically relies on substrate monoubiquitination, the other functions require the assembly of polymeric Ubiquitin chains on the substrate. The chains are linked through lysine amino acids of Ubiquitin, and depending on which lysine is linked, the chains could be heterotypic or homotypic. The heterotypic Ubiquitin chains generate myriad cellular signals whose functions are distinct from the homotypic Ubiquitin chains. Heterotypic chains can be mixed, branched, or a combination of both. The molecular rules of heterotypic chain assembly are poorly understood. While several techniques exist to detect these chains, few exist to study their assembly. Here we describe a new technique based on isotopic labeling and mass spectrometry to study the assembly of mixed and branched heterotypic chains. The technique is demonstrated using multiple Ubiquitin enzymes and Ubiquitin chains as substrates and will be instrumental in studying the assembly of large Ubiquitin polymeric chains.

Introduction

The post-translational modification of proteins with Ubiquitin regulates multiple cellular processes, owing to its ability to form various polymeric chains with diverse three-dimensional structures. These structures generate a plethora of signals that can be differentially recognized by various receptor proteins, creating different downstream cellular signals[1–4]. Multiple Ubiquitin monomers can be conjugated to create a multi-monoubiquitinated substrate. Alternately, polymeric Ubiquitin chains can be assembled on a substrate, where a Ubiquitin lysine (or N-terminal Methionine) ε-amino group conjugates to the C-terminal end of another Ubiquitin by an isopeptide bond. The first Ubiquitin is known as the acceptor or proximal Ubiquitin, and the next Ubiquitin linked to it is called the donor or distal Ubiquitin. There are seven lysine amino acids in Ubiquitin; K6, K11, K27, K29, K33, K48, and K63. Consequently, the signals generated by Ubiquitin polymers can be complex with numerous linkages. Homotypic chains are formed by uniformly linking the same proximal lysine amino acid throughout the chain. Heterotypic chains are formed by linking through multiple lysines. The heterotypic chains can be further segregated into two architectures: mixed and branched (Figure 1A). The mixed chains include more than one type of linkage, but each Ubiquitin molecule in the chain is conjugated at only one lysine[5]. Alternately, branched chains consist of at least one proximal Ubiquitin molecule conjugate at two or more different lysines by distal Ubiquitins.

While the functions of homotypic chains are well-studied, those of heterotypic chains are emerging. Nearly 20% of Ubiquitin chains exist as branched chains[6], and the number of mixed heterotypic chains is expected to be higher. Recent studies have uncovered distinct functions of heterotypic chains from homotypic chains. For example, K11/K48 chains are potent signals of proteasomal degradation essential for the clearance of misfolded proteins and ER-associated degradation[7], and K63/M1 chains are critical to assemble signaling molecules in the NF-κB pathway[8]. While the K29/K48 chain specifically targets Ubiquitin fusion degradation substrates in yeast[9], the K48/K63 chain protects its signal from deubiquitination, leading to sustained NF-(B) pathway activation during inflammation[10]. The code of Ub chain formation lies within the E2:E3 pair. A single E3 can recruit multiple E2s with different linkage specificities to create heterotypic chains[3]. Several E3s also can synthesize heterotypic chains with a single E2[11]. Although heterotypic chains can elicit a wide range of novel signals, the molecular rules that regulate their synthesis are poorly understood.

Multiple tools exist to detect and identify heterotypic Ubiquitin chains[4,12], but few exist to monitor the synthesis of such chains. For example, the key molecular features of choosing mixed versus branched chains or selecting appropriate branch points in a branched chain remain unclear. As a chain extends from homotypic to heterotypic, the donor Ubiquitin may be linked to the distal unit to create a

mixed chain or to the proximal unit to create a branched chain (Figure 1A). The molecular interactions between the E2~Ub intermediate, E3, and the substrate polyUb chain determine if a chain expands into mixed or branched. To study the molecular determinants of such chain synthesis, we need techniques to monitor the conjugation site of donor Ubiquitin molecules on a preassembled chain. Here, we propose a new technique based on isotopic labeling and mass-spectrometry to understand the mechanistic basis of synthesizing such heterotypic chains. The technique detects the propensity of a ubiquitination reaction to synthesize mixed or branched heterotypic chains and exclusively measures the synthesis rates of mixed and branched chains. The technique does not require fluorescent tags or substitutions in the proteins/enzymes, causing no perturbation to the biological system during experiments[13].

Results

We initially studied the chain elongation rates by the Ubiquitin-conjugating enzyme E2-25K, whose structure and activity have been well studied[14–16]. E2-25K is a highly active E2, which can form *in-vitro* polyubiquitin chains without an E3. E2-25K specifically produces K48-linked Ubiquitin (Ub) chains. Taking the purified diUb of seven different linkages as substrates, we measured the rate of triUb formation using E2-25K in a standard ubiquitination reaction, followed by separating the triUb on denaturing gels and then quantifying the triUb band by imaging (Figure S1). The rates of triUb formation by different substrates were distinct (Figure 1B). The K63-diUb is the preferred substrate with a ten-fold higher triUb synthesis rate, observed in previous studies[15,16]. The K48-diUb and K11-diUb have comparable rates of triUb formation. K27-diUb and K33-diUb served as poor substrates for chain elongation by E2-25K. Although these experiments help detect rates of triUb formation, they cannot detect whether the donor Ub is linked to a distal or proximal unit and hence, cannot distinguish between mixed and branch chains.

To study the preference of donor Ub towards distal or proximal Ub in the substrate diUb, we differentially labeled them by nitrogen isotopes (Figure 2A). The proximal unit was ^{14}N labeled, and the distal unit was ^{15}N labeled. The differentially labeled substrates were used in a ubiquitination reaction to form triUb, separated on SDS-PAGE, excised, and trypsinized. The digested peptides were analyzed by MS (details provided in the methods section)(Figure S2). Henceforth, we denote heterotypic chains as Ky/Kx chains, where x and y are the residue numbers of lysines in the Ub sequence, y denotes the homotypic chain, and x denotes the heterotypic extension. K6/K11 chain denotes a K11-linkage heterotypic extension on the K6-linkage homotypic chain.

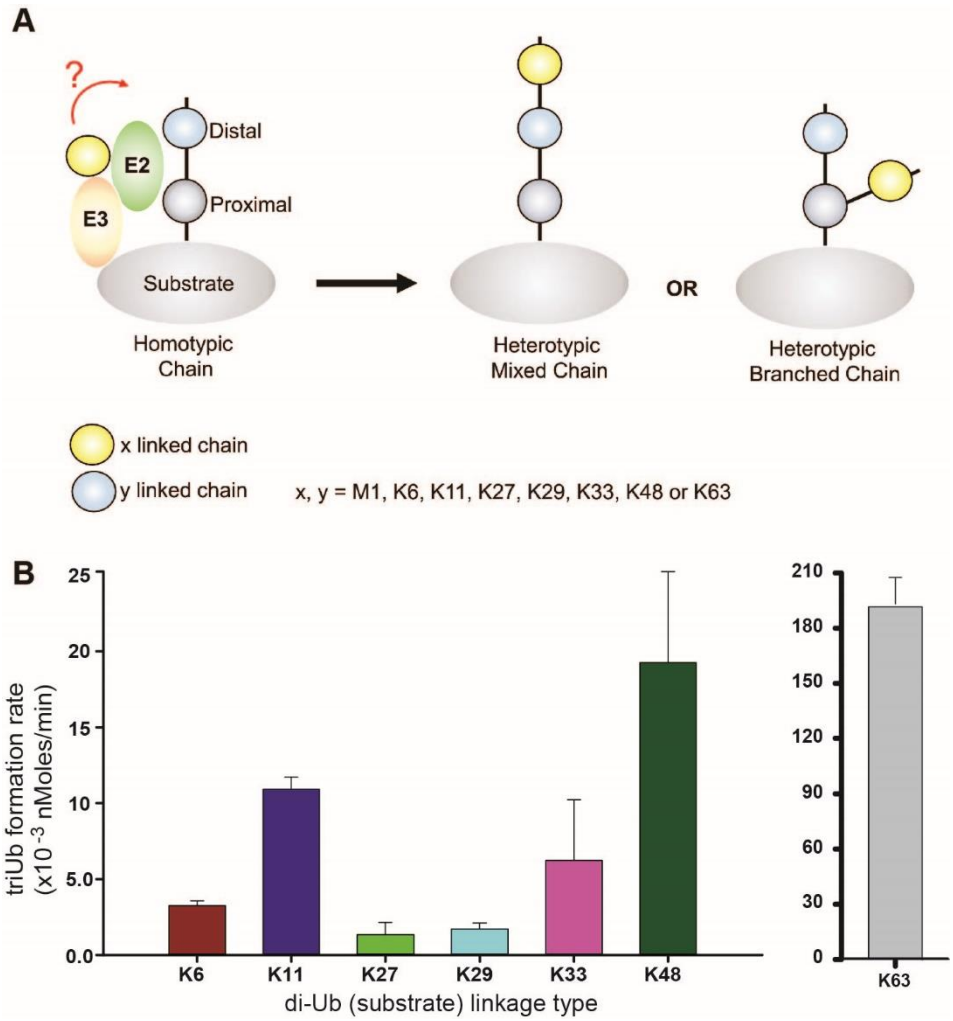

Figure 1. **A**) Synthesis of heterotypic triUb chains. When a homotypic Ubiquitin chain linked by lysine 'y' extends into a heterotypic chain linked by lysine 'x' it can create either a mixed or a branched Ubiquitin chain. The preference for mixed versus branch depends on the three-dimensional conformations of E2, E3, Substrate, and Ub. **B**) The rate of triUb synthesis from diUb by a typical Ubiquitin-conjugating enzyme like E2-25K for different diUb linkages.

Since E2-25K is specific for K48-linkage[13,17], the donor Ub is attached to K48 on either the distal or the proximal Ub. The MS results suggested that for the K63-linked diUb substrate, the donor Ub is exclusively attached to the proximal unit to form branched chains. Hence the K63/K48 heterotypic chains formed by E2-25K were entirely branched, which confirms previous results and benchmarks the assay (Figure 2C)[16]. However, the other substrates produced different amounts of branched and mixed triUb chains. K29-linked diUb exclusively formed mixed K29/K48 chains, a sharp contrast to the K63/K48-linked chains. Interestingly, K27-linked diUb formed equal amounts of branched and mixed K27/K48 chains. The abundance of the mixed chain was higher in all triUb extensions except K63, suggesting that

the distal Ub was a better acceptor than the proximal Ub for all other diUb linkages except K63-linked diUb.

The E2-25K enzyme consists of a conserved catalytic UBC domain present in all eukaryotic E2s. In addition, it includes a Ubiquitin Associated (UBA) domain that binds to Ubiquitin. The UBA domain binds to the distal unit of a K63-linked Ub chain and positions the proximal to serve as the acceptor Ub[15,16]. Hence, in the absence UBA domain, the enzyme should lose preference for the acceptor site of the distal unit. We repeated the assay using the E2-25K-ΔUBA enzyme, where the UBA domain is deleted, but the core UBC domain is retained (Figure S3). The K63-diUb loses the propensity to form exclusively branched chains and forms mixed and branched chains with equal abundance, suggesting that the UBA domain is critical for positioning the K63-linked diUb (Figure 2D). The branched and mixed heterotypic extensions of other diUbs were comparable to full-length E2-25K, except for K27-diUb, which formed higher amounts of K27/K48 mixed chains with E2-25K-ΔUBA than with E2-25K. Specific interactions between the UBA domain and K27-diUb must regulate mixed versus branched chain extension propensity. Intriguingly, the abundance of K29/K63 remained entirely mixed with E2-25K-ΔUBA, suggesting that the K29/K63 extension is dependent on the interactions of the K29-chain with the enzyme active site and not with the accessory UBA domain.

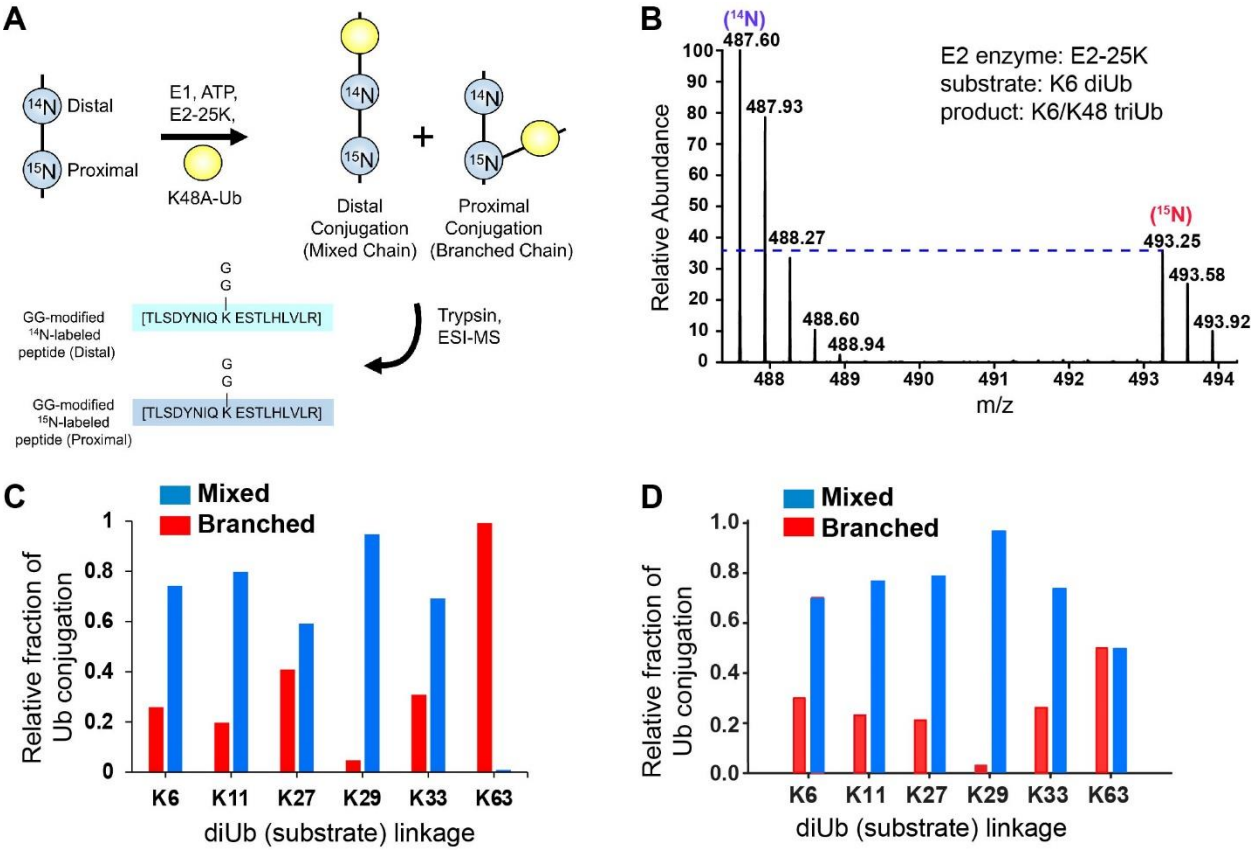

Figure 2. Monitoring the synthesis of mixed and branched Ubiquitin chains. **A**) A schematic of the experiment. E1, ATP, E2 (E2-25K), K48AUb, and diUb were incubated in a reaction buffer for seven hours, quenched by adding EDTA, and further separated on the SDS-PAGE for mass spectroscopy. The two Ubiquitin protomers in the diUb are differentially labeled with ^{15}N and ^{14}N. The triUb band was excised, trypsin digested and detected in MS. The GG motif addition was detected in the ^{15}N-labeled and ^{14}N-labeled peptides. **B**) MS spectra of the GG-modified peptides when K6-diUb was used as the substrate. **C**) The relative fraction of mixed and branched triUb formed for substrate diUb of different linkages. **D**) Same as C, except E2-25K-ΔUBA was used in the reaction.

While E2-25K is a monomeric E2, a few E2s like Ubc13/Mms2 complex are heterodimeric. We applied the technique to investigate heterotypic chain synthesis for the heterodimeric enzyme Ubc13/Mms2. Mms2 is a UBC domain-containing protein, a co-factor of Ubc13[18,19], and binds to acceptor Ub chains and optimally orients K63 to attack the active site of Ubc13 to form K63-specific linkages on Ub chains[20]. When presented with diUb substrates with varied linkages, the enzyme and E3 RNF38 created both mixed and branched triUb chains (Figure 3A, S4). The K11-diUb preferably synthesized branched chains, while the rest of the diUb linkages had a higher propensity to synthesize

mixed chains (Figure 3B). Interestingly, while K27-diUb created an equal population of mixed and branched K27/K48 chains with E2-25K (Figure 2C), it preferably creates mixed K27/K63 chains with the Ubc13/Mms2 enzyme (Figure 3B).

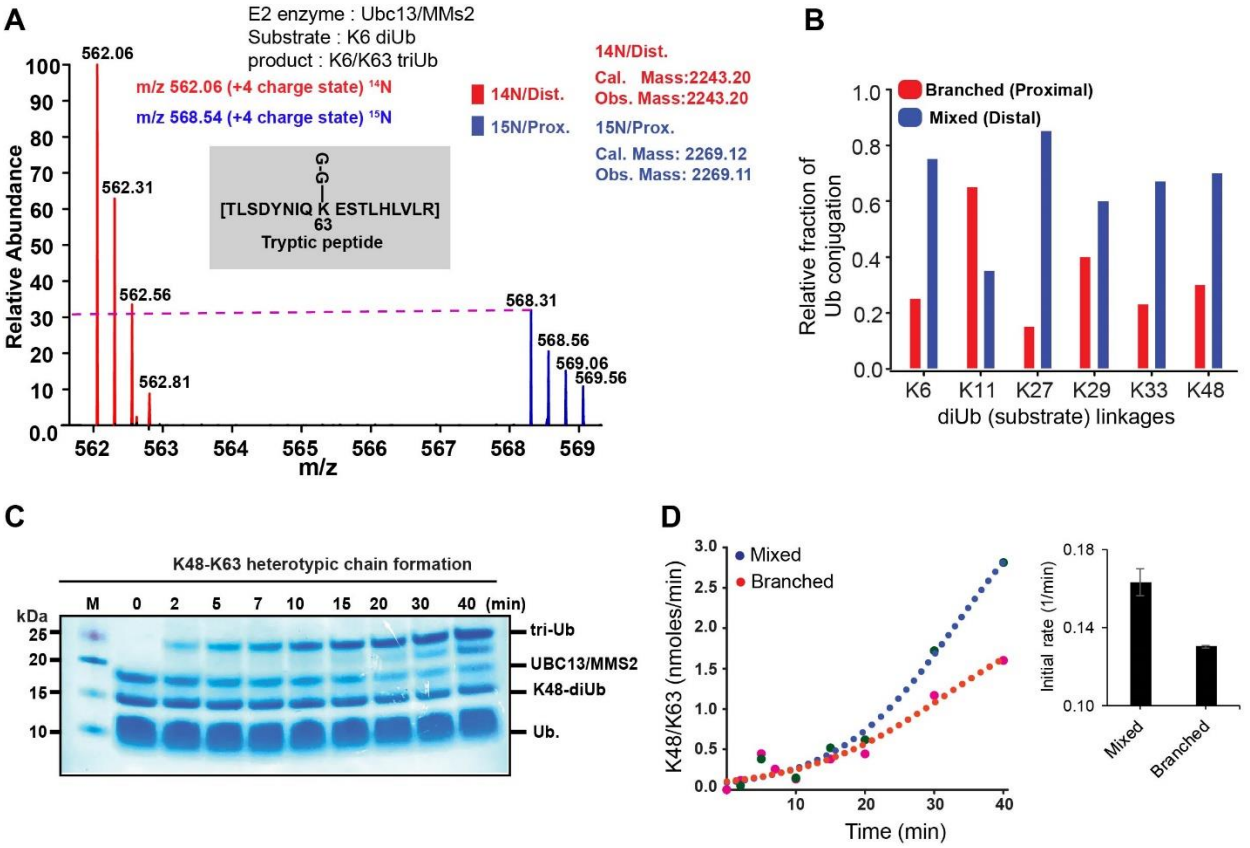

Figure 3. The reaction kinetics of mixed/branched chain synthesis by the Ubc13/Mms2 heterodimeric Ubiquitin E2 enzyme. **A**) MS spectra of the GG-modified peptides when K6-diUb was used as a substrate. **B**) The relative fraction of mixed and branched triUb formed for substrate diUb of different linkages. **C**) A SDS-PAGE showing the triUb synthesis at various time points. **D**) The triUb band in C) is quantified by MS to yield the rate of mixed and branched chains.

The assay can also monitor mixed and branched chain synthesis rates exclusively. Aliquots of the reaction were taken at different time intervals and analyzed as described above (Figure 3C). An internal control peptide of similar mass was used as a control in these experiments. We used Ubc13/Mms2 as the enzyme and K48-diUb as the substrate. The amount of mixed and branched triUb increased over time, albeit at different rates (Figure 3D, S5, and S6). Interestingly, the synthesis of both mixed and branched triUb showed a sigmoidal pattern over time, suggesting that the product triUb accelerates the catalytic

rate. While we detect autocatalysis by triUb in E2:RING-E3 ligase complexes here, a similar catalytic effect of Ub has been recently observed in E2:RBR-E3 ligases[21].

Both E2-25K and Ubc13/Mms2 synthesize specific linkage chains; hence, the heterotypic extension is limited to specific linkages. The method can be further exploited for an E2 enzyme like Ube2d2 synthesizing chains of multiple linkages[22]. When presented with diUb of different linkages as substrates in individual reactions, each substrate's propensity to extend to branched and mixed heterotypic linkage could be quantified (Figure S7-S12). The Ube2d2 enzyme with RNF38 E3 ligase synthesizes typically K6, K11, K48 and K63 linked chains[23]. When the relative abundance was quantified, the mixed chain was greater than the branched chains for K11/K48 and K11/K63 heterotypic chains (Figure 4A). Alternately, branched and mixed chains were equally abundant for K48/K11 and K48/K63 heterotypic chains (Figure 4B). The K6/K27 heterotypic triUb is exclusively branched (Figure 4C). In contrast, K27/K6 chain is mixed. K27-linked diUb has a propensity to form mixed triUb, irrespective of the type of linkage extension. On the other hand, K63-linked formed roughly similar amounts of branched and mixed chains (Figure 4C). Overall, the technique quantifies the relative formation of mixed versus branched chains for all heterotypic chains.

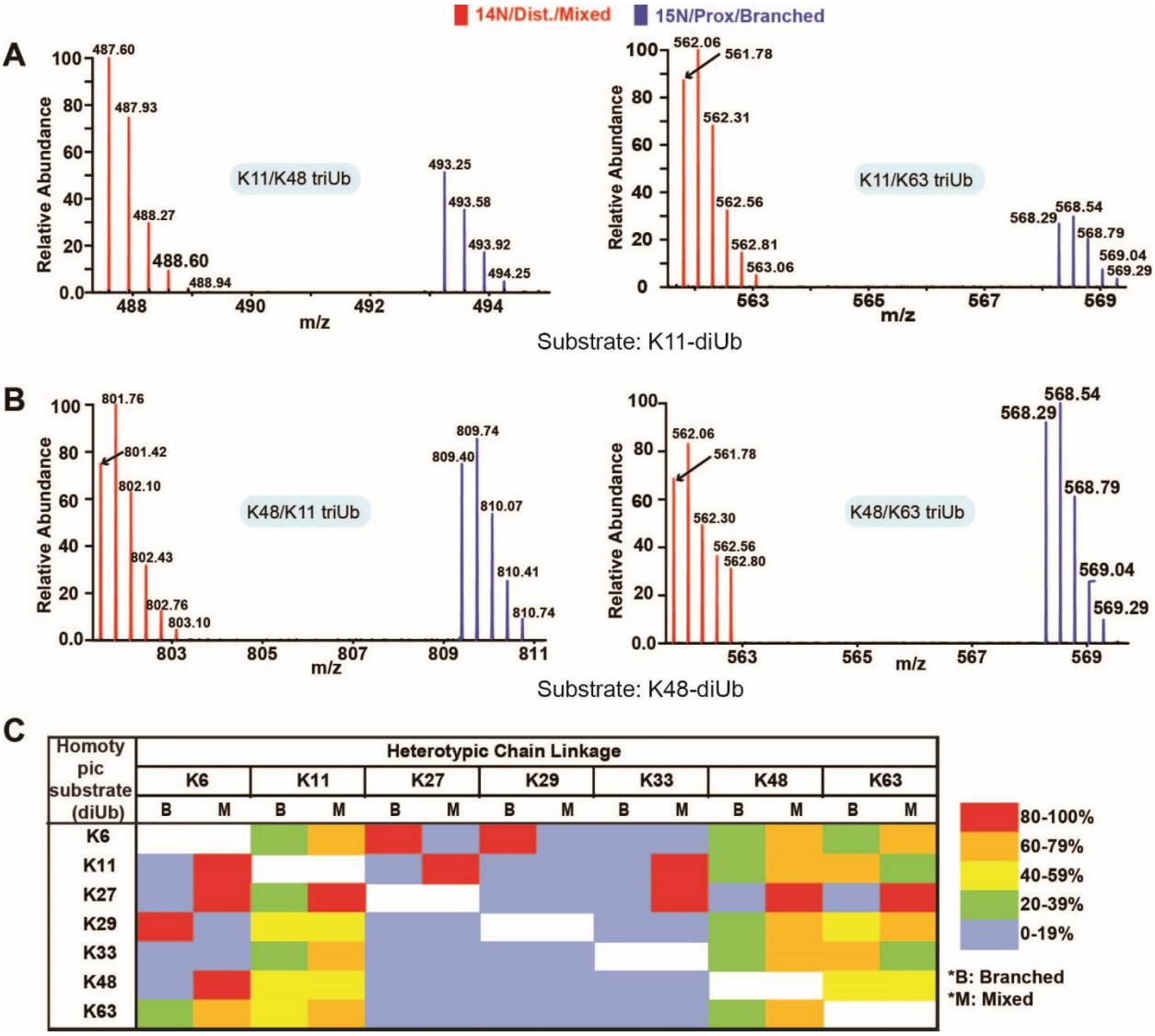

Figure 4. Mass-spectrometry-based analysis of the mixed and branched Ubiquitin chains formed by Ube2d2. **A**) Mass spectra showing the relative abundance of mixed and branched chains in K48-linked (or K63-linked on the right side) donor Ub on a K11-diUb substrate. **B**) Similar to A) for K48-diUb substrate. **C**) A heatmap showing the relative abundance of mixed and branched chains for each linkage when different diUb were used as substrates.

Discussion

The molecular mechanisms underlying the synthesis of heterotypic polymeric chains provide a valuable handle to enhance or inhibit their synthesis in the cell and regulate the cellular processes. To dissect the molecular interactions that dictate chain synthesis requires monitoring the conjugation of the next Ubiquitin as the chain grows. Here, we describe a method to detect the site of donor Ubiquitin

conjugation as a di-Ubiquitin substrate grows to tri-Ubiquitin, and allows quantifying the ratio of mixed versus branched Ubiquitin chains. The method can be used to study the activity of multiple E2-E3 pairs toward synthesizing or regulating Ubiquitin chain architecture.

In a branched Ubiquitin chain, the initiation of branching requires the selection of appropriate branch point linkage and location[5]. The branching point needs to engage with the enzyme's active site, which depends on the conformational dynamics of the enzyme, ligase, and ubiquitinated substrate. Our method could effectively uncover the branching point in a substrate Ubiquitin chain. Although the method was demonstrated here for extension from a diUbiquitin to a triUbiquitin, it can also detect modifications of larger Ubiquitin chains, provided each Ubiquitin in the chain is exclusively labeled. We have used ^{15}N isotopic labeling here to distinguish between distal and proximal Ubiquitin. Similarly, the ^{13}C and ^{2}H isotopic labeling of Ubiquitin is straightforward. Ubiquitins can be produced with specific isotopic labeling of lysine amino acids or their specific unlabeling[24]. These labeling techniques can distinguish each Ubiquitin molecule in a polymeric chain of more than $2^5 = 64$ Ubiquitins. Such sized polymeric chains are sufficient to provide a detailed understanding of Ubiquitin branching.

The activity of E2 enzymes for creating branched versus mixed chains depending on the substrate linkage type is intriguing. For example, the promiscuous E2 Ube2d2 prefers to synthesize mixed chains for K27-linked diUb substrate irrespective of the extension linkage (Figure 4D). However, for the K29-linked diUb substrate, mixed and branched chains are synthesized with equal abundance (Figure 4D). Although K27 and K29 are separated by one amino acid in the Ubiquitin sequence, they are located on the central α-helix, and their sidechains are oriented in opposite directions. Consequently, the proximal units of their diUbs are conformationally different (Figure S13), which could explain the difference in their chain extension activity. Further structural studies and molecular modeling can provide insights into the atomistic interactions that control the synthesis of these chains, which can be substantiated by the assay described here.

The assembly of ubiquitin chains is a complex process. Several E2 and E3 enzymes include Ubiquitin binding domains that can promote or regulate the architecture of these enzymes[14,25]. In addition, the role of Deubiquitinating enzymes (DUBs) in regulating heterotypic chains remains unappreciated. DUBs may edit larger polymeric chains into smaller branched/mixed chains to regulate their function. Moreover, the heterotypic polymer chains are not limited to Ubiquitin but extend to other Ubiquitin-like family proteins, all of which have multiple lysines. Finally, hybrid chains of SUMO with Ubiquitin, FAT10 with Ubiquitin, and Nedd8 with Ubiquitin are common[26]. The method described here will be vital to study the key features of the bio-macromolecular assembly of these diverse chains.

Conflicts of interest

There are no conflicts to declare.

Acknowledgment

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Material and Methods

Cloning and mutagenesis: Site-directed mutagenesis for the Ubiquitin and other proteins was performed using standard approaches. Sequences of the Ubiquitin and other protein mutants were confirmed by DNA sequencing.

Protein expression and purification: Ubiquitin was expressed in BL-21 DE3 cells and purified described earlier[27]. Ubiquitin E1, E2 (E2-25K, E2-25K-ΔUBA, UBC13, MMS2, E2D2, and RNF-38 enzymes, were purified as described previously[19,28]. For the expression of ^{15}N-labeled Ubiquitin (lysine to cysteine) mutants, cells were resuspended in lysis buffer (50 mM Sodium acetate, pH 4.5). Cells were lysed by sonication, and cell lysate was subjected to centrifugation. The supernatant was passed through the SP-FF column (GE Healthcare), and the protein was eluted using a linear gradient of NaCl. Eluted protein was purified by gel filtration chromatography on Superdex 75 (16/600 column, GE Healthcare) in PBS buffer pH 7.4.

Enzymatic synthesis of diUb: For the synthesis of selectively ^{15}N labeled (proximal unit) K48 and K63 diUbs, the reaction was carried out in re-cycling buffer having 0.5 μM human E1, 20 μM E2 (E2-25Kor UBC13/MMS2), 200 μM Ub (K48A or K63A), 100 μM ^{15}N labeled D77 Ub, 10 mM ATP in 40 mM Tris pH 7.4, 10 mM $MgCl_2$. The reaction was carried out for 7-9 h at 37 °C.

Chemical synthesis of diUb: Synthesis of diUbs of K6, K11, K27, K29, and K33 linkages was carried out using DTNB mediated disulfide conjugation method[29]. The G76C Ub was reacted with 10 molar excess of DTNB (dissolved in pure DMSO to a final concentration of 100 mM) to form the Ub-TNB. The excess of the TNB was removed by dialysis, and the protein was purified using size exclusion chromatography. Purified protein was concentrated and stored at -80°C. To form diUb, cysteine to lysine mutants of Ubiquitin (K6C, K11C, K27C, K29C, and K33C) were mixed with Ub-TNB in equimolar ratio and purified by ion exchange and size exclusion chromatography.

Ubiquitin chain assembly (Ub3) reactions using E2-25K: Assays were performed by mixing 0.5 μM human E1, 20 μM E2-25K, 1 μM K48A^{Alexa}, 2020 μM diUb in 10 mM ATP in 40 mM Tris pH 7.4, 10 mM $MgCl_2$ buffer. TriUb formation was monitored as a function of time. All the reaction images were taken using a Gel documentation system (Uvitec). The intensity of gel images was measured by the ImageJ software[30] and further analyzed by the sigma plot.

Mass spectrometric analysis of ubiquitin chain assembly reactions: The reaction was carried out using by mixing 0.5 μM human E1, 20 μM E2-25K, 100 μM k48A, 50 μM diUb in the reaction buffer 10 mM ATP in 40 mM Tris pH 7.4, 10 mM $MgCl_2$. Reactions were incubated at 37 °C for seven hours and quenched with SDS buffer. The reaction samples were loaded on NuPAGE 4-12% gradient Bis-Tris gel. The triUb band was excised from the gel and subjected to in-gel trypsin digestion. The same protocol was followed for reactions using Ubc13/Mms2 as the E2 complex.

Kinetic study of heterotypic chain formation by Ubc13/Mms2: The reaction was assembled with 0.5 μM human E1, 20 μM E2-25K, 100 μM K63A, 50 μM K48 diUb in the reaction buffer ten mM ATP in 40 mM Tris pH 7.4, 10 mM $MgCl_2$. The proximal unit of diUb was labeled with ^{15}N. The reaction was carried out at 37 °C. At indicated time points (0, 2, 5, 7, 10, 15, 20, 30, 40 minutes), 30 μl samples were mixed with 6 μL 5xSDS loading buffer and resolved on 4-12% gradient SDS PAGE gels in MES buffer (Invitrogen). The triUb band was excised and subjected to trypsin digestion before MS studies.

Analysis of heterotypic chains by using mass spectrometry: The ESI-MS data reported in this study were carried out on an orbitrap mass spectrometer (Orbitrap Fusion Tribrid Mass Spectrometer, Thermo Scientific) equipped with a nano-LC (Easy-nLC 1200, Thermo Scientific) The Ubiquitin tryptic peptides, were reconstituted in 0.1% formic acid and loaded onto an Acclaim PepMap 100 C18 analytical column (particle size 3 μm; length 2 cm; diameter 75 μm; pore size 100 Å). The samples were eluted using MS-grade water, acetonitrile, and 0.1% formic acid as a mobile phase, with a flow rate of 300 nL min^{-1}. The mass spectral acquisition parameters were: spray voltage 2100 V, ion transfer tube temperature was 275 °C, scan range 100−1000 *m/z*, resolution 1.20000, AGC target 4×10^5, RF lens (%) 60, dynamic exclusion 30 s. MS^2 was done under HCD conditions, in positive ion mode, using normalized collision energy (NCE) of 32%. For MS^2, the AGC target value was kept at $1 \times 10^{4,}$ and the detector's resolution was set to 30000. The data were viewed and analyzed in the Thermo-Xcaliber qual browser.

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