



Biological significance of structural differences between two highly conserved Ubc variants

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ABSTRACT

Ubiquitin conjugating enzyme variants (Uev) Uev1 and Mms2 share >90% sequence identity but with distinct biological functions. Here, we report the monomeric and heterodimeric crystal structures of Uev1 and comparison with that of Mms2. Uev1 alone or in complex with Ubc13 is nearly identical with the corresponding Mms2 structures, except in one surface area containing 7/14 amino acid variations. To probe the biological significance of this unique region, we raised monoclonal antibodies specifically recognizing this region of Uev1, but not of Mms2. Epitope mapping and site-specific mutagenesis revealed at least two distinct epitopes within this region. These data collectively suggest the existence of cellular proteins capable of distinguishing Uev1 from Mms2 and directing the Ubc13–Uev complex to different pathways.

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Introduction

Ubiquitination, the attachment of ubiquitin (Ub) to a target protein, is utilized by eukaryotes for a number of cellular signaling pathways [1]. The signaling properties of Ub depend on the topology of the linkage in which the chains are constructed. While the Lys48-linked polyubiquitin chains signal for degradation by the 26S proteasome, the noncanonical Lys63-linkage has been shown to regulate target proteins in a non-proteolytic manner [2,3].

Ubc13 is the only Ubc enzyme known to date capable of catalyzing the Lys63-linked polyubiquitination reaction. Ubc13 has a conserved Ubc family catalytic domain and an active site Cys residue for Ub conjugation [3]. However, for Ubc13 to catalyze the Lys63-linked polyubiquitination reaction, a Ubc variant (Uev) cofactor is absolutely required [3,4]. A Uev is defined as a protein that resembles Ubc in structure and amino acid sequence, but does not contain a Cys residue in the active site, rendering the protein catalytically inactive [5]. It is now clear that Uev is able to interact with Ub non-covalently in such a way that it orientates Ub to facilitate the Lys63–Ub chain assembly [4,6]. In addition, structural and mutagenesis analyses determined residues critical for the physical

interaction between Ubc13 and Mms2, and explains why only Ubc13, but not other Ubcs are able to bind Uev [7–9].

All mammals examined to date contain at least two members of the Uev family of genes. For example, human cells contain two yeast *MMS2* homologs, *hMMS2* and *UEV1/CROC1* [10]. The two proteins share >90% sequence identity (Fig. 1) and both are able to form a stable complex with Ubc13, yet they are involved in distinct cellular functions [11]: while the Ubc13–*hMms2* complex is involved in DNA damage response reminiscent of its roles in yeast [12], the Ubc13–Uev1 complex is involved in activating the NF- κ B signaling pathway in response to environmental stresses, including bacterial and viral infections [2,13]. Interestingly, the DNA damage response function is expected to prevent genomic instability and prevent cancer, whereas constitutive NF- κ B activation is anti-apoptotic and has been linked to many types of cancer [14]. Given that both Uev1 and Mms2 are able to form a stable complex with Ubc13 *in vivo* and *in vitro* yet their biological functions are distinct [11], the structural differences between Uev1 and Mms2 are presumed to be responsible for their functionally distinct roles in intracellular signaling. In this study, we addressed this presumption by solving the crystal structure of the Uev1 core domain, which reveals a unique surface area compared to Mms2. We confirmed this hypothesis through the generation of monoclonal antibodies (Mabs) that specifically recognize Uev1, but not Mms2. Our Mab epitope mapping revealed two distinct epitopes

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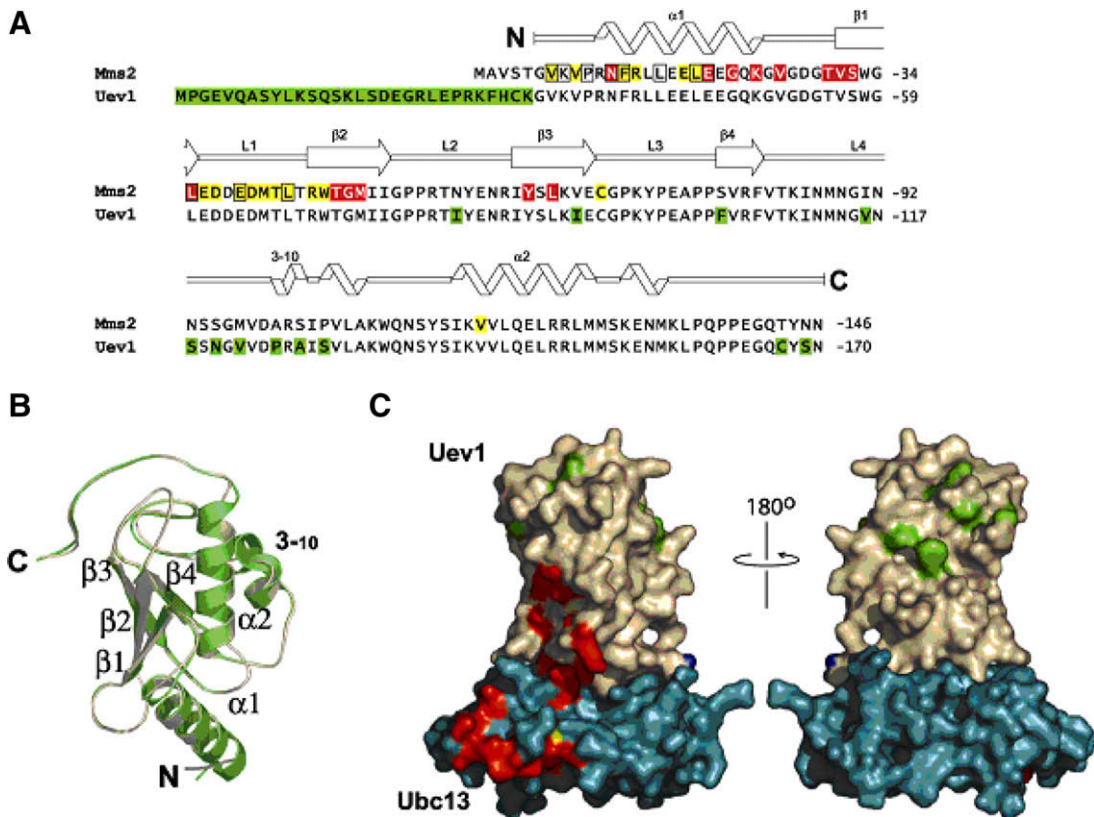


Fig. 1. A structural comparison of Mms2 and Uev1. (A) Structure-based sequence alignment of Mms2 and Uev1. Mms2 residues highlighted in red and yellow have been shown to interact with Ub and Ubc13, respectively. Mms2 residues involved in the Mms2–Ubc13 interface are boxed. Uev1 residues highlighted in green identify the amino acid differences between Uev1 and Mms2. (B) Superimposition of the Uev1 and Mms2 crystal structures. Shown are ribbon representations of Mms2 in green and Uev1 Δ 30 in olive that were aligned using O[21]. The RMSD between these structures was 0.5 Å. (C) Structure of the Uev1 Δ 30–Ubc13 complex. A surface representation of Uev1 Δ 30 bound to Ubc13 is shown in two views rotated 180° relative to one another. Ubc13 is shown in teal and its active site Cys residue is highlighted in yellow. Uev1 is shown in olive and its amino terminus is highlighted in blue. Residues that vary between Mms2 and Uev1, not including their respective amino termini, are highlighted in green. Residues involved in the Uev–Ub non-covalent interaction and in the Ubc13–Ub thioester are highlighted in red on both the Uev1 Δ 30 and Ubc13 structures.

within the Uev1 core that are independent from the surfaces interacting with Ub or Ubc13. This finding demonstrates that the variability of Uev1 and Mms2 within the Uev domain itself contributes to their cellular functions.

Materials and methods

Proteins and structure determination. Recombinant proteins were initially produced as a GST fusion as previously described [4]. The GST fusion protein was purified from soluble cell extracts by GST affinity chromatography and, whenever necessary, the GST fusion partner was cleaved by treatment with the GST-PreScission protease and the GST moiety was removed by affinity or size exclusion chromatography. SDS–PAGE and Western blotting analyses were as previously described [7].

Experimental details for crystal structural analysis are given in the supplemental methods accompanied with Table S1 summarizing the crystallographic statistics.

Mouse immunization, hybridoma fusion, and screening for Mabs. BALB/c mice were initially immunized intraperitoneally with 35 μ g of purified Uev1 and later boosted at day 33, 57, and 100 with 17.5 μ g of Uev1. Enzyme immunoassay (EIA) detection of Uev1-specific antibodies was done as described previously [15]. Production of hybridomas, hybridoma recloning, and ascites production were all performed as previously described [15,16]. Only hybridomas secreting IgG-class Mabs were selected.

Epitope mapping. Purification of Mabs from ascites fluid, conjugation with horse-radish peroxidase (HRP), and titration of HRP–Mab conjugates was done as described [15]. To determine whether an unconjugated Mab was able to inhibit the binding of an HRP–conjugated Mab, two EIAs were performed using the same Mab dilutions [15]. Because Mabs in ascites fluid were used, the dilution of each Mab able to inhibit 50% of the binding of a given dilution of HRP–conjugated Mab in a competition EIA was related to the Mab dilution that gave an assay background corrected OD₄₅₀ of 1.0 in the direct Mab binding EIA. This was done through the calculation of a competition index [15]. The competition index for each Mab was then divided by the competition index for the Mab that had been used to create the HRP–Mab conjugate used in a given competition EIA, giving a relative competition index.

Results

Structures of Uev1 Δ 30 and the Uev1 Δ 30–Ubc13 heterodimer

We hypothesized that the structural differences between Uev1A and Mms2 are responsible for directing the Lys63–linked Ub chains synthesized with Ubc13 into distinct cellular pathways. The protein sequence alignment of Uev1 and Mms2 shown in Fig. 1A indicates that they differ in their core Uev domain by merely 12 amino acids. We reasoned that determining the structure of the Uev1 core domain lacking the first 30 amino acids (Fig. 1A, highlighted), here-

in called Uev1 Δ 30, would provide insight into whether these subtle differences might come together in three dimensions to constitute a sufficiently distinct surface.

The structure of Uev1 Δ 30 was solved by molecular replacement using a poly-alanine model of Mms2 (PDB accession number: 1J74). The density for the core 140 residues of Uev1 Δ 30 was well ordered (Fig. 1B) and adopted a structure very similar to Mms2 [8] with an RMSD of 0.5 Å. This was expected given the high degree of protein sequence homology between the two Uevs (Fig. 1A). Like Mms2, Uev1 Δ 30 possesses a noncanonical Ubc fold that consists of an amino-terminal α -helix followed by four anti-parallel β -strands, a 3_{-10} helix and a supporting α -helix, but lacks the active site cysteine and the two carboxy terminal helices found in the core domain of Ubc (Fig. 1B) [8,17]. Based on a primary structure alignment, the residues important for binding Ub (Mms2-Ser32 and -Ile62) [18] and Ubc13 (Mms2-Phe13) [7] are identical between Uev1 Δ 30 and Mms2 (Fig. 1A). Interestingly, the 14 amino-acid stretch that contains 7 out of 12 total variable residues between Uev1 Δ 30 and Mms2 clusters on a surface of each protein and does not overlap with the Uev1 and Mms2 surfaces used in Ub and Ubc13 interactions (Fig. 1C).

The secondary structure similarities observed for both Uev1 Δ 30 and Mms2 indicated that they would exhibit similar modes of interaction with Ubc13. This assumption was tested directly by solving the Uev1 Δ 30–Ubc13 heterodimeric crystal structure and comparing it to that of Mms2–Ubc13 [8]. When superimposed, these structures were virtually identical with an RMSD of 0.39 Å. Comparison of the structures of Uev1 Δ 30 to Uev1 Δ 30–Ubc13 shows that upon binding to Ubc13 the amino terminal helix of Uev1 Δ 30 unravels by one turn similar to that observed for Mms2. This observation suggests that the respective amino termini of Uev1 and Mms2 become repositioned upon binding to Ubc13. Taken together, the Uev1 Δ 30 structural data reveal that the major cluster of amino acids differing from the corresponding Mms2 core domain is positioned on “available” protein interaction surfaces, namely, those that do not participate in binding Ub or Ubc13.

Generation of antibodies against Uev1

The above Uev1–Ubc13 and Mms2–Ubc13 crystal structure analysis revealed that Uev1 contains a unique surface as compared to Mms2, supporting our previous report that the two proteins confer distinct cellular functions [11]. Based on this knowledge, we hypothesized that Uev1 and Mms2 have distinct cellular interaction partners that bind via the above unique surface. In the absence of identity of such binding partners, we decided to test this hypothesis through the generation of antibodies with affinity for Uev1 Δ 30, but not for Mms2. Successful generation of such antibodies would confirm the possibility of a novel protein interaction surface within the Uev1 core, as well as provide an invaluable antibody that discriminates between the two Uevs.

Full-length Uev1 was used for immunization of BALB/c female mice. Polyclonal serum obtained approximately a month after the third booster immunization and directly tested by EIA against Uev1 and Mms2 revealed a 3-fold higher antibody response to Uev1A, thus providing confidence in the likelihood of producing Mabs specific to Uev1. Consequently, two hybridoma fusions with two Uev1A-intravenously boosted BALB/c mice were performed.

Characterization of three Types of Mabs

Analysis of Mabs secreted in the supernatant of hybridoma cell growth medium was performed using direct antibody binding EIAs. Almost a thousand hybridomas were tested for secretion of a Mab able to bind to Uev1 and Mms2. The majority of the hybridomas secreted a Mab which reacted with both Uev1 and Mms2,

suggesting that they recognize the surface region shared by the two proteins. This is not surprising given that the two proteins share >90% sequence identity in the core domain and adapt very similar folding in their secondary structures. As expected, Mabs that are positive for Uev1, but negative for Mms2 were also identified. Overall, we expected to obtain and did obtain three different types of Mabs. Type 1 Mabs (represented by Mab LN1) will react with the N-terminal 30 amino acid unique region of Uev1 not shared with Mms2 (Fig. 1A). Type 2 Mabs (represented by Mabs LN2, LN2A, and LN2B) will react with the Uev1 core domain, but not Mms2. Finally, Type 3 Mabs (represented by Mab LN3) will react with both Uev1 and Mms2. For the purpose of testing our hypothesis, we focus on the three Type 2 Mabs in this report.

In order to distinguish the three Mab Types, we performed Western blot analysis using three purified proteins, namely, Uev1, Uev1 Δ 30, and Mms2. As shown in Fig. 2, the three Type 2 Mabs bind to Uev1 and Uev1 Δ 30, but not to Mms2, indicating that they recognize core region(s) unique to Uev1. In contrast, Western blot analysis showed that Mab LN1 binds to Uev1, but not Mms2 or Uev1 Δ 30, while Mab LN3 binds to all three antigens and therefore does not distinguish between Uev1 and Mms2.

All three Type 2 Mabs recognize the unique Uev1 core region

In order to determine whether all three Type 2 Mabs recognize the same Uev1 unique region, we first constructed a deletion of residues 110–130 from Uev1 Δ 30, which represents the segment of Uev1 with the majority (7 out of 12) of residues differing from Mms2 (Fig. 1A). This deletion eliminates a large loop region after the fourth β -strand and encroaches slightly into the short 3_{-10} helix (Fig. 1). Importantly, this deletion does not eliminate any major secondary structure elements; hence, we expected the resulting Uev1 Δ 30(Δ 110–130) protein to otherwise fold properly. Indeed, we observed no significant solubility or expression problems with this deletion protein, and it is recognized in a direct binding EIA by Mab LN3 (data not shown). In contrast, as seen in Fig. 3, all three Type 2 Mabs did not bind to Uev1 Δ 30(Δ 110–130). This result confirms our anticipation that all three Type 2 Mabs recognize the Uev1 region (a.a. 116–129) most divergent from Mms2 in sequence.

The Uev1 unique region contains at least two epitopes

Within the unique region of Uev1 recognized by the three Type 2 Mabs (Fig. 1A), we were interested in determining whether each of the three Mabs recognizes the same or distinct binding sites. This question was approached through Mab epitope mapping, which investigated the ability of each Mab to compete with the HRP-conjugated homologous Mab and the other HRP-Mabs to bind Uev1 in an EIA. The calculated relative competition index reflects the ability of one Mab to compete with another given Mab–HRP relative to competing with itself (i.e., the homologous Mab–HRP). We found that Mabs LN2 and LN2B reciprocally inhibited each other essentially as efficiently as each Mab inhibited the binding of its own HRP-conjugate (relative competition indices of 0.5–2.1). In contrast, neither Mab LN2 nor LN2B showed any reciprocal inhibition with Mab LN2A (relative competition indices were all >50). As expected, all three Type 2 Mabs did not show any reciprocal inhibition of binding with Mabs LN1 or LN3 (relative competition indices were all >50). These results show that there are at least two epitopes within the Uev1 unique domain that are recognized by Mabs LN2 and LN2B, and Mab LN2A.

In order to further characterize the two epitopes within the unique Uev1 domain, two sets of Uev1 residues in this region were mutated to the corresponding Mms2 sequences based on sequence and structural analysis (Figs. 1 and 4). We reasoned that these sub-

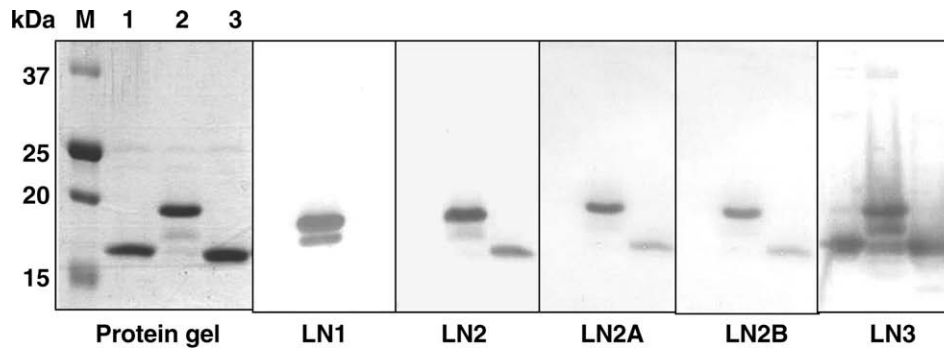


Fig. 2. Mab binding analyzed by Western blotting. Purified Mms2 (lane 1), Uev1 (lane 2) and Uev1 Δ 30 (lane 3) were run on SDS–PAGE gels, transferred to a PVDF membrane and reacted with Mabs in the form of ascites fluid (diluted 1:2000).

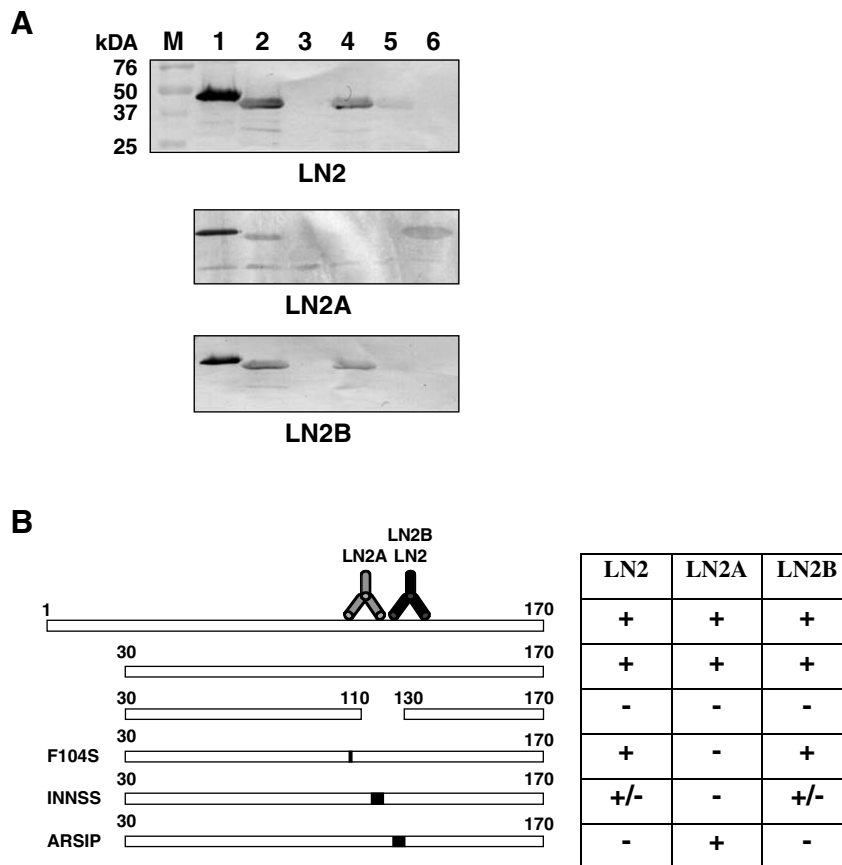


Fig. 3. Characterization of the three Type 2 Mabs. (A) Western blot analysis of the Type 2 Mabs against a series of Uev1 derivatives. Lane 1, the full-length GST-Uev1; lane 2, GST-Uev1 Δ 30; lane 3, GST-Uev1 Δ 30(Δ 110–130); lane 4, GST-Uev1 Δ 30-F104S; lane 5, GST-Uev1 Δ 30-INNSS; and lane 6, GST-Uev1 Δ 30-ARSIP. (B) A diagram illustrating the sequences of Uev1 derivatives and the summary of binding data as obtained from Fig. 3A.

stitutions would provide the most discerning test of Mab binding, while also minimizing perturbations to the overall Uev1 secondary structure, given the nearly identical backbone conformation of Mms2 and Uev1 (Fig. 1B). As seen in Fig. 3, Uev1 Δ 30-INNSS (a.a. 116–120) caused a complete loss in binding by LN2A, but only partially reduced binding by LN2 or LN2B. In sharp contrast, the other substitution construct, Uev1 Δ 30-ARSIP (a.a. 125–129) completely abolished binding by LN2A, but did not affect binding by LN2 and LN2B. To refine the epitopes further, we mutated Uev1 Δ 30-Phe104 to the corresponding Ser residue found in Mms2 (Fig. 1A), because it lies in proximity to a.a. 116–129 in the Uev1 molecular structure (Fig. 1C). The Uev1 Δ 30-F104S single amino acid substitution abrogated binding by LN2A, but did not affect

binding by LN2 or LN2B (Fig. 3). These results clearly define two separate epitopes within the unique Uev1 core domain (Fig. 4B), strongly suggesting that different proteins may selectively bind Uev1 or Mms2 in this region.

Discussion

The different biological pathways in which Uev1 and Mms2 participate [11] must be accounted for by the limited structural differences that exist between them. The structural comparison of each of these UeVs alone or complexed with Ubc13 illustrate that they differ only in their amino termini and at 12 amino acid positions that cluster to a surface within the core region of the proteins.

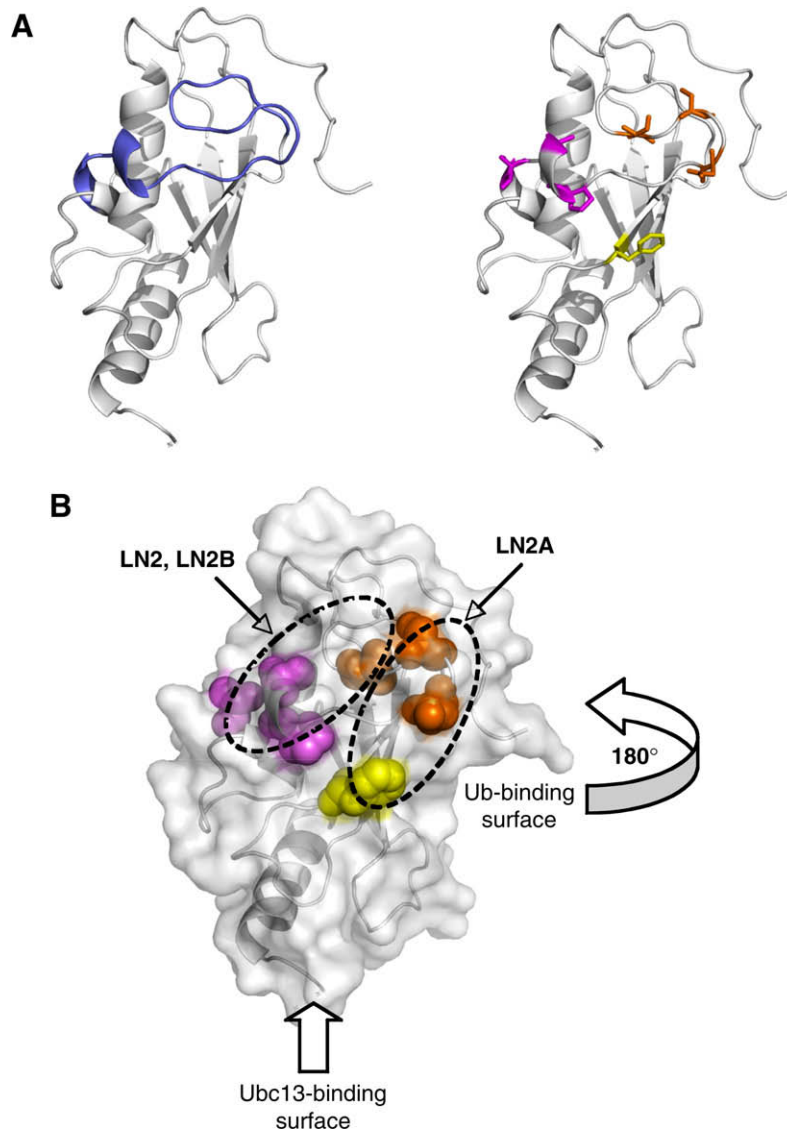


Fig. 4. Structural illustration of Uev1 mutant derivatives and Mab-epitope mapping. (A) Molecular structure depiction of the Uev1 Δ 30 derivatives used for epitope mapping. The region deleted in the Uev1 Δ 30(Δ 110–130) construct is indicated in blue (left panel). Residues mutated for substitution to the corresponding Mms2 residues are shown in orange for Uev1 Δ 30-INNSS(116–119), magenta for Uev1 Δ 30-ARSIP(125–130), and yellow for Uev1 Δ 30-F104S. (B) An approximate area was mapped for the epitopes recognized by Mabs LN2 and LN2B, and Mab LN2A. Relative locations of Ub- and Ubc13-binding sites are indicated. The Ub-binding region is on the backside of the structure. Color representation is as in (A).

Notably, this cluster does not overlap with the Uev surfaces defining the Ub or Ubc13 binding sites, consistent with the notion that they play no direct role in ubiquitination or poly-Ub chain catalysis. The amino acid differences between these UeVs raise the possibility that the surface cluster serves as a binding site for interacting partners specific to each Uev, while other regions of the two proteins perform similar biochemical activities, namely, to interact with Ub and Ubc13 and to facilitate Lys63-linked poly-Ub chain assembly. Hence, Uev1 and Mms2 not only promote Ubc13-mediated Lys63-linked polyubiquitination of target proteins, they also serve as regulatory subunits. The hypothesis that the sequence difference between this region of Uev1 and Mms2 serves as a functional determinant through binding to different protein(s) is further supported by a highly non-conservative amino acid substitution in the same surface area that occurs at Uev1 position 104, in which a structurally well-ordered Phe residue is replaced by Ser in Mms2, whereas among other scattered variable residues, they are either conserved or embedded within the folded structure, or located in the C-terminal unstructured region.

In order to experimentally test the above hypothesis, we took an unusual approach by raising Mabs specifically recognizing the core region of Uev1, but not Mms2. Obtaining this type of antibody not only gives us invaluable reagents for distinguishing between cellular Uev1 and Mms2, but also provides supporting evidence that potential proteins may exist that specifically bind to Uev1 or Mms2 on this surface and direct the two Uev-Ubc13 complexes to distinct cellular functions. In the present study, we have characterized three *bona fide* Mabs that recognize the core region of Uev1, but not Mms2. The differential binding of these three Mabs to Uev1 Δ 30 and Uev1 Δ 30(Δ 110–130) confirms that all three Mabs recognize the amino acid cluster of Uev1 with the majority of sequence variation compared to Mms2, indicating that this region is indeed the surface receptor for binding partners to distinguish between Uev1 and Mms2. In our view, this finding is significant in at least three ways. Firstly, it provides experimental evidence that the very limited sequence variation between Uev1 and Mms2 is sufficient to confer distinct biological functions. Secondly, it suggests a potential means by which one can screen total cellular proteins for those differen-

tially binding Mms2 or Uev1 as tentative candidates that confer the distinct function mediated by each Uev. Thirdly, Uev1 and Mms2 not only confer distinct functions, but the two known functions may be opposite [11], with one (Mms2) preventing mutagenesis [19] and possibly cancer, and the other (Uev1) promoting cancer [20]. Hence, Mabs capable of distinguishing the two proteins are of great interest for future diagnosis and possibly therapeutics.

To our surprise, we were able to detect two epitopes with the three available Type 2 Mabs and precisely map their binding sites within the unique Uev1 region. In addition, it remains possible that the Uev1–Phe104 residue represents a third distinct Mab epitope (Fig. 4B). This observation suggests more than one protein recognition site within this region and presents a possibility that either multiple proteins interact with a single Uev, or an interacting protein contains more than one binding motif to enhance its affinity. Given the fact that an increasing number of proteins have been reported in recent years to be ubiquitinated by Lys63-linked chains or Ub ligases (E3s) involved in this process, our detection of multiple epitopes within Uev1 is consistent with the notion that different Uev-binding proteins may adopt a variety of binding motifs to recruit this ubiquitination complex for target protein modification and signaling.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at [doi:10.1016/j.bbrc.2008.11.089](https://doi.org/10.1016/j.bbrc.2008.11.089).

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