Structural basis for specific recognition of Lys 63-linked polyubiquitin chains by NZF domains of TAB2 and TAB3

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TAB2 and TAB3 activate the Jun N-terminal kinase and nuclear factor- κ B pathways through the specific recognition of Lys 63-linked polyubiquitin chains by its Npl4 zinc-finger (NZF) domain. Here we report crystal structures of the TAB2 and TAB3 NZF domains in complex with Lys 63-linked diubiquitin at 1.18 and 1.40 Å resolutions, respectively. Both NZF domains bind to the distal ubiquitin through a conserved Thr-Phe dipeptide that has been shown to be important for the interaction of the NZF domain of Npl4 with monoubiquitin. In contrast, a surface specific to TAB2 and TAB3 binds the proximal ubiquitin. Both the distal and proximal binding sites of the TAB2 and TAB3 NZF domains recognize the Ile 44-centred hydrophobic patch on ubiquitin but do not interact with the Lys 63-linked isopeptide bond. Mutagenesis experiments show that both binding sites are required to enable binding of Lys 63-linked diubiquitin. We therefore propose a mechanism for the recognition of Lys 63-linked polyubiquitin chains by TAB2 and TAB3 NZF domains in which diubiquitin units are specifically recognized by a single NZF domain.

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Introduction

Post-translational modification of proteins with ubiquitin regulates a wide variety of biological processes (Hicke, 2001; Glickman and Ciechanover, 2002; Miranda and Sorkin, 2007; Hofmann, 2009; Wickliffe *et al*, 2009). The enzymatic cascade of ubiquitination includes three distinct classes of enzymes: E1 for ubiquitin activation, E2 for ubiquitin conjugation and E3 for ubiquitin transfer to substrate proteins. These three enzymes work together to conjugate the

C-terminal group of ubiquitin with the $\text{N}\epsilon$ group of lysine residues of substrate proteins including ubiquitin itself. All seven lysine residues of ubiquitin (i.e. Lys 6, Lys 11, Lys 27, Lys 29, Lys 33, Lys 48, Lys 63) can act as acceptors for further ubiquitination, producing polyubiquitin chains. Further, recent studies showed that the N-terminal group of ubiquitin can also act as an acceptor for ubiquitination to form linear polyubiquitin chains (Iwai and Tokunaga, 2009; Tokunaga *et al*, 2009). Among these eight types of polyubiquitin chains, the most abundant chain *in vivo* is Lys 48-linked polyubiquitin, which is the primary targeting signal for proteasomal degradation (Glickman and Ciechanover, 2002). Although the functions of the other types of polyubiquitin chains are not fully understood, recent proteomic studies suggest that polyubiquitin chains other than Lys 48-linked chains may also serve as targeting signals for proteasomal degradation and have potentially redundant functions (Meierhofer *et al*, 2008; Xu *et al*, 2009). Lys 63-linked polyubiquitin chains appear to function independent of proteasomal targeting and to instead have crucial functions in cellular processes such as DNA repair, membrane trafficking and inflammatory signalling (Miranda and Sorkin, 2007; Verstrepen *et al*, 2008; Chen and Sun, 2009; Hofmann, 2009; Wickliffe *et al*, 2009).

For instance, assembly of Lys 63-linked polyubiquitin chains is induced in the cytosol by stimulation of cells with cytokines such as tumour necrosis factor- α , interleukin-1 β or Toll-like receptor ligands (Deng *et al*, 2000; Chiu *et al*, 2009; Skaug *et al*, 2009). Lys 63-linked chains recruit and activate a protein kinase complex containing transforming growth factor β -activated kinase 1 (TAK1), TAK1 binding protein 1 (TAB1) and TAB2 or its homologue TAB3. This TAK1 complex has a critical function in the activation of Jun N-terminal kinase (JNK) and nuclear factor- κ B (NF- κ B) signalling pathways (Ninomiya-Tsuji *et al*, 1999; Wang *et al*, 2001). TAK1 is a member of the MAPKKK family, whereas TAB1 is a co-activator of TAK1 (Shibuya *et al*, 1996; Sakurai *et al*, 2000). TAK1 directly phosphorylates both I κ B kinase β and MKK6 to stimulate the NF- κ B and JNK pathways, respectively (Wang *et al*, 2001). TAB2 and TAB3 have redundant functions *in vivo* and act as adaptors to recruit TAK1 and TAB1 to Lys 63-linked polyubiquitin chains for the activation (Ishitani *et al*, 2003; Cheung *et al*, 2004; Kanayama *et al*, 2004; Besse *et al*, 2007; Xia *et al*, 2009). The C-terminal Npl4 zinc-finger (NZF) domain of TAB2 and TAB3 (Supplementary Figure S1) specifically recognizes Lys 63-linked polyubiquitin chains unanchored or anchored to the substrate proteins such as RIP1 (Kanayama *et al*, 2004; Komander *et al*, 2009; Xia *et al*, 2009). This recognition is essential for the TAK1-induced activation of NF- κ B and JNK pathway (Kanayama *et al*, 2004).

Zinc fingers are generally used as recognition modules that have divergent functions and zinc ligation topologies (Gamsjaeger *et al*, 2007). The NZF domain contains approximately

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30 amino-acid residues that coordinate a single zinc ion with four conserved Cys residues (Wang *et al*, 2003; Alam *et al*, 2004). The NZF domain is frequently found in proteins involved in ubiquitin-dependent pathways, such as TAB2, TAB3 (Kanayama *et al*, 2004), Npl4 (Meyer *et al*, 2002), TRABID (Tran *et al*, 2008) and yeast Vps36 (Alam *et al*, 2004). Some of the isolated NZF domains bind with ubiquitin with K_d values of 100 μ M or higher, whereas some others do not (Meyer *et al*, 2002; Wang *et al*, 2003; Alam *et al*, 2004). The ubiquitin-binding NZF domain comprises a highly conserved TF/ Φ motif, where Φ represents a hydrophobic residue and 10 amino-acid residues are inserted between the -Thr-Phe- sequence and Φ (Wang *et al*, 2003). Most of the ubiquitin-binding NZF domains bind polyubiquitin chains without apparent specificity, whereas the NZF domains of TAB2 and TAB3 specifically bind Lys 63-linked polyubiquitin chains (Kanayama *et al*, 2004; Komander *et al*, 2009). Although structures of the NZF domain of Npl4 in isolation and in complex with ubiquitin have been reported (Wang *et al*, 2003; Alam *et al*, 2004), it has remained unclear how the NZF domains of TAB2 and TAB3 specifically recognize Lys 63-linked polyubiquitin chains. Here we report the crystal structures of the NZF domains of TAB2 and TAB3 in complex with Lys 63-linked diubiquitin (K63-Ub₂) at 1.18 and 1.40 Å resolutions, respectively. The complex structures and structure-based mutagenesis analyses reveal the mechanism for specific binding of Lys 63-linked polyubiquitin chains by TAB2 and TAB3 NZF domains.

Results and discussion

Overall structures of the TAB2 and TAB3 NZF domains in complex with K63-Ub₂

The NZF domains of mouse TAB2 (residues 665–693) and mouse TAB3 (residues 688–716) are sufficient for the specific binding with Lys 63-linkage polyubiquitin chains (Figure 1A). These regions of TAB2 and TAB3 are hereafter referred to as TAB2-NZF and TAB3-NZF, respectively. The crystal structure of the TAB2-NZF·K63-Ub₂ complex was solved by the single-wavelength anomalous dispersion (SAD) method using the zinc edge (Supplementary Table S1) and the crystal structure of the TAB3-NZF·K63-Ub₂ complex was solved by molecular replacement using the structure of the TAB2-NZF·K63-Ub₂ complex as a search model. The TAB2- and TAB3-NZFs are 79.3% identical and, as expected, the TAB2-NZF·K63-Ub₂ complex structure is nearly identical to the TAB3-NZF·K63-Ub₂ complex structure (Figures 1B, C, 2 and Supplementary Figure S2). The C α atoms of the TAB2-NZF·K63-Ub₂ complex can be superposed onto those of the TAB3-NZF·K63-Ub₂ complex with a root mean squared deviation (r.m.s.d.) value of 0.37 Å (177 residues in total, except for the structurally variable C-terminal tail of the proximal ubiquitin). We will therefore describe the structure of the TAB2-NZF·K63-Ub₂ complex below and generally refer to Supplementary Data for discussion of the TAB3 complex. The TAB2-NZF consists of a pair of anti-parallel β -sheets and a following long loop. A zinc ion is coordinated by Cys 670, Cys 673, Cys 684 and Cys 687 of the TAB2-NZF, in a manner similar to those of the Npl4 NZF domain (Npl4-NZF) (Supplementary Figure S3; Wang *et al*, 2003; Alam *et al*, 2004). The C α atoms of the TAB2-NZF can be superposed onto the Npl4-NZF with an r.m.s.d. value of 0.79 Å over 28 amino-acid residues. TAB2-NZF crystallized

Figure 1 Overall structures of the TAB2- and TAB3-NZFs in complex with K63-Ub₂. (A) Pull-down assays using GST-fused TAB2-, TAB3-, Npl4- and Vps36-NZFs to assess their interaction with K63-, K48- or linear Ub₂. The bound proteins were analysed by SDS-PAGE and stained with Coomassie Brilliant Blue. (B) The TAB2-NZF·K63-Ub₂ complex. The NZF is coloured grey. The proximal and distal ubiquitin moieties are coloured pink and cyan, respectively. Ile 44 in each ubiquitin moiety is shown as green spheres. The N ϵ atoms of lysine residues and the nitrogen atom of the N-terminal Met in the proximal ubiquitin are shown as blue spheres. The K63R mutation in the distal ubiquitin is indicated as sticks. (C) The TAB3-NZF·K63-Ub₂ complex. The drawing schemes are the same as in B.

as a stoichiometric complex with K63-Ub₂ (Figure 1B). The TAB2-NZF binds to the distal and the proximal ubiquitin moieties with buried surface areas of 333 and 302 Å², respectively (Figures 1B and 2). Relative orientations between the distal and proximal ubiquitin moieties bound to TAB2- and TAB3-NZFs are considerably different from those of the free K63-Ub₂ and Lys 63-linked tetraubiquitin chains (Komander *et al*, 2009; Weeks *et al*, 2009; Supplementary Figure S4). The TAB2-NZF interacts with the Ile 44-centred hydrophobic patches of both proximal and distal ubiquitin moieties, but does not directly recognize the Lys 63-linked isopeptide bond of K63-Ub₂ (Supplementary Figure S3C).

Figure 2 Recognition of the distal and proximal ubiquitin moieties by TAB2. Stereo view of the detailed TAB2-NZF·K63-Ub₂ binding interface. The colouring schemes are the same as in Figure 1. Hydrogen bonds are indicated as dashed orange lines. The labels of the NZF and the proximal and distal ubiquitin moieties are coloured black, pink and cyan, respectively. (A) The interface between the proximal ubiquitin and the TAB2-NZF. (B) The interface between the distal ubiquitin and the TAB2-NZF.

Proximal ubiquitin recognition

The TAB2-NZF domain recognizes the Ile 44-centred hydrophobic patch of the proximal ubiquitin moiety that is formed by the side chains of Ile 44, Ala 46 and Val 70 and aliphatic portions of the His 68 side chain. The side chains of Phe 675, Ala 680 and Leu 681 and aliphatic portions of the His 678 and Glu 685 side chains in the TAB2-NZF form a hydrophobic surface that interacts with the Ile 44-centred hydrophobic patch of the proximal ubiquitin (Figure 2A). Further, the two O ϵ atoms of Glu 685 in the TAB2-NZF hydrogen bond with the main-chain NH atoms of Ala 46 and Gly 47 in the proximal ubiquitin, respectively (Figure 2A). The functional importance of these interactions was confirmed by GST pull-down analyses using TAB2-NZF mutants (Figure 3). H678Q, L681T and E685V mutants completely lost the ability to bind K63-Ub₂, whereas A680G and A680K mutants exhibit decreased binding to K63-Ub₂. The proximal ubiquitin binding residues of TAB2 and TAB3 are well conserved among five organisms (Supplementary Figure S5), whereas those of the TAB2 and TAB3 are not fully conserved among the other ubiquitin-binding NZFs (Supplementary Figure S6). These facts suggest that the proximal binding residues of the TAB2- and TAB3-NZFs that we found in our complex structure are likely to have key functions in the specific recognition of Lys 63-linked polyubiquitin chains.

Figure 3 Pull-down assays assessing the K63-Ub₂ binding by proximal and distal ubiquitin binding site mutants of the TAB2-NZF. The bound K63-Ub₂ molecules were analysed by SDS-PAGE and stained with Coomassie Brilliant Blue.

Distal ubiquitin recognition

The TAB2-NZF also recognizes the Ile 44-centred hydrophobic patch of the distal ubiquitin moiety that is formed by the side chains of Leu 8, Ile 44 and Val 70 and aliphatic portions of the His 68 side chain. The side chain of Phe 675 and aliphatic portions of the Cys 673, Thr 674, Gln 686 and Cys 687 side chains in the TAB2-NZF form a hydrophobic surface to interact with the Ile 44-centred hydrophobic patch of the distal ubiquitin (Figure 2B). Thr 674 and Phe 675 of the TAB2-NZF are included in the aforementioned TF/ Φ motif for the ubiquitin binding, whereas Cys 673 and Cys 687 are also involved in the Zn²⁺ coordination. Further, the main-chain CO of Ala 672, the main-chain NH of Leu 676 and the O γ atom of Thr 674 in the TAB2-NZF hydrogen bond with the N δ atom of His 68, the main-chain CO and the main-chain NH of Gly 47 in the distal ubiquitin, respectively (Figure 2B). We sought to confirm the functional importance of these interactions by GST pull-down analyses using TAB2-NZF mutants (Figure 3). As expected from our structure, T674A and F675A mutants completely lost their binding activities. However, the Q686A mutant can bind to K63-Ub₂ as efficiently as the wild type, indicating that Gln 686 of the TAB2-NZF contributes little to the binding affinity with the distal ubiquitin. It is important to note that Thr 674 and Phe 675 of the TAB2-NZF are completely conserved among five organisms, whereas Gln 686 of the TAB2-NZF is replaced by Ala or Glu in fruit fly or zebra fish, respectively (Supplementary Figure S5A). Intriguingly, Gln 686 of the TAB2-NZF is located in the position corresponding to the hydrophobic residue (Φ) in the TF/ Φ motif. The role of this Φ -Gln replacement will be discussed below, in the context of the linkage specificity.

Amino-acid sequence alignment of the ubiquitin-binding NZFs of TAB2, TAB3, Npl4, TRABID and Vps36 shows that the amino-acid residues that are required for the distal ubiquitin binding by TAB2 are almost completely conserved (Supplementary Figure S6A). Only Phe 675 of the TAB2-NZF is replaced by Tyr in TRABID. The near-perfect sequence conservation of the distal ubiquitin binding residues suggests that all ubiquitin-binding NZFs share a common strategy for the ubiquitin binding. In fact, the physical basis for distal ubiquitin binding by the TAB2- and TAB3-NZFs is similar to that for monoubiquitin binding by the Npl4-NZF (Supplementary Figure S3). The C α atoms of the TAB2-NZF and the distal ubiquitin moiety (100 residues in total) in the TAB2-NZF·K63-Ub₂ complex can be superposed onto those of

the Npl4-NZF-ubiquitin complex with an r.m.s.d. value of 1.63 Å (Alam *et al*, 2004). Their ubiquitin binding modes are essentially identical. However, the relative orientations between ubiquitin and NZF are slightly different and the Thr rotamers in the TF/Φ motif are different between the Npl4-NZF and TAB2-NZF. The dihedral angle around the Cα–Cβ bond of Thr 558 in the Npl4 rotates 120 deg, relative to that of Thr 674 in the TAB2-NZF, lacking a hydrogen bond with ubiquitin. Thr 674 of the TAB2-NZF in our high-resolution structure is highly ordered with low temperature factors (~11 Å²) and clear electron density. Further, the proximal ubiquitin does not interact with Thr 674 of the TAB2-NZF, and thus is unlikely to affect the side-chain rotamer of Thr 674 in the TAB2-NZF. Therefore, we suggest that the Thr rotamer in the TF/Φ motif of the Npl4-NZF should likely be the same as that of the TAB2-NZF observed in our structures, consistent with the perfect sequence conservation of Thr in the TF/Φ motif among the ubiquitin-binding NZFs.

Linkage specificity

For linkage-specific ubiquitin chain recognition, binding proteins must exclusively bind to ubiquitin chains but not to monoubiquitin, as we observe for the TAB2- and TAB3-NZFs (Figures 1A and 4A). In contrast, the Npl4-NZF and the second NZF domain of *Saccharomyces cerevisiae* Vps36 (Vps36-NZF) bind to both monoubiquitin and all diubiquitins tested (Figures 1A and 4A).

Chain-selective binding of the TAB2- and TAB3-NZFs seems to be related to the hydrophilic Gln that is located in the position of Φ in the TF/Φ motif for ubiquitin binding. The Npl4- and Vps36-NZF domains contain the complete TF/Φ motif (Φ is Met 570 in the Npl4-NZF or Ile 199 in the Vps36-NZF) where Phe and Φ form a hydrophobic surface to interact with the Ile 44-centred hydrophobic patch of ubiquitin. In contrast, Φ is replaced by the hydrophilic Gln 686/709 in TAB2/TAB3 (Supplementary Figure S5A), which contributes little to the distal ubiquitin binding, as mentioned above (Figure 3).

To clarify the relationship between the monoubiquitin binding and Φ in the TF/Φ motif, we mutated Met 570 of the Npl4-NZF and Ile 199 of the Vps36-NZF to Gln, and also mutated Gln 686 of the TAB2-NZF to Ile or Met for GST pull-down analyses. Strikingly, the Q686M and Q686I mutations enabled the TAB2-NZF to bind to monoubiquitin and resulted

in linkage-independent binding to K63-, K48- and linear diubiquitins (Figure 4). In contrast, M570Q and I199Q mutations disrupt the abilities of the Npl4- and Vps36-NZF domains to bind to monoubiquitin, respectively. These results clearly show that Φ in the TF/Φ motif is a key residue that regulates the ability of the NZF domain to bind to monoubiquitin. The presence of a non-hydrophobic residue in the Φ position of the TF/Φ motif of the TAB2- and TAB3-NZFs diminishes binding to monoubiquitin and enables linkage-specific binding when combined with a proximal ubiquitin binding surface.

The Φ–Gln replacement in the TF/Φ motif of the TAB2- and TAB3-NZFs is essential but not sufficient for Lys 63-linkage-specific polyubiquitin binding. Because mutations in both the distal and proximal ubiquitin binding sites of the TAB2-NZF disrupt binding of K63-Ub₂, simultaneous binding of the distal and proximal ubiquitin moieties to the TAB2-NZF is essential for Lys 63-linkage-specific binding (Figure 3). The distal and proximal ubiquitin binding sites of the TAB2-NZF are organized to optimize simultaneous binding to each ubiquitin moiety in a configuration characteristic to Lys 63-linked ubiquitin chains. Among all the lysine residues and the N-terminal methionine residue of the proximal ubiquitin, Lys 63 is the closest to the C-terminal tail of the distal ubiquitin in the present complex structure (Figure 1 and Supplementary Figure S7A). Because of this proximity of Lys 63 of the proximal ubiquitin to the C-terminal tail of the distal ubiquitin, the distal and proximal ubiquitin moieties of K63-Ub₂ bind simultaneously to the TAB2-NZF. In contrast, in the orientations of the distal and proximal ubiquitin moieties bound to TAB2- and TAB3-NZFs, the C-terminal tail of the distal ubiquitin cannot reach either the Nε group of Lys 48 or the N-terminal group in the proximal ubiquitin, even if considering the structurally flexible C-terminal tail of the distal ubiquitin. One of the most elongated forms of the ubiquitin C-terminal tail has been observed in the complex with the de-ubiquitinating enzyme (DUB) AMSH-LP (Sato *et al*, 2008), where the distance between the Cα atom of Leu 71 (positioned in the end of the last β-sheet) and that of Gly 76 in the distal ubiquitin is 19 Å (Supplementary Figure S7B). This is shorter than the distance between the Cα atom of Leu 71 in the distal ubiquitin and the Lys 48 Nε group (23 Å) or the N-terminal group in the proximal ubiquitin (24 Å) in the TAB2-NZF·K63-Ub₂ complex (Supplementary Figure S7A).

Figure 4 Pull-down assays using NZF variants containing point mutations in the Φ position of the TF/Φ motif to assess their mono- or diubiquitin binding. **(A)** Pull-down assays using the GST-fused TAB2-, Npl4- and Vps36-NZF mutants to assess their monoubiquitin binding. The bound monoubiquitin molecules were analysed by tricine SDS-PAGE and stained with Coomassie Brilliant Blue. **(B)** Pull-down assays using GST-fused TAB2-, Npl4- and Vps36-NZF mutants to assess their binding with K63-Ub₂, K48-Ub₂ or linear Ub₂. The bound diubiquitin molecules were analysed by SDS-PAGE and stained with Coomassie Brilliant Blue.

Unexpectedly, the distance between the C α atom of Leu 71 and the N ϵ group of Lys 6 is 14 Å, and the ubiquitin C-terminal tail conformation observed in the Npl4-NZF-ubiquitin complex appears likely to enable the bonding between Gly 76 in the distal ubiquitin and Lys 6 in the proximal ubiquitin (Supplementary Figures S3 and S7A). From a structural point of view, we cannot exclude the possibility that TAB2- and TAB3-NZFs might be able to bind to Lys 6-linked chains.

Strategies for the linkage specificity

Our crystal structures of the TAB2- and TAB3-NZFs in complex with K63-Ub₂ and structure-based mutagenesis analyses revealed that a single zinc-finger domain contains both distal and proximal ubiquitin binding sites that enable Lys 63-linked chains to simultaneously approach both sites in the specific geometry required for binding. The TAB2- and TAB3-NZFs effectively measure the length between Ile 44-centred hydrophobic patches and Lys 63-linked chains have the correct spacing to bind both the proximal and distal ubiquitin binding sites simultaneously. It is important to note that direct recognition of the isopeptide linkage is not required for this mode of linkage-specific binding. This strategy seems to be typical for linkage-specific ubiquitin chain binding by ubiquitin binding domains (UBDs), as illustrated by crystal structures of the RAP80 tandem UIMs (ubiquitin-interacting motifs) in complex with K63-Ub₂ (Sato *et al*, 2009) and the UBAN (ubiquitin binding domains of ABINs and NEMO) of NEMO in complex with linear Ub₂ (Rahighi *et al*, 2009) and the NMR-based structural model of the second UBA (ubiquitin-associated motif) of hHR23A in complex with K48-Ub₂ (Varadan *et al*, 2005).

In the crystal structure of the RAP80 tandem UIMs, UIM1-UIM2, in complex with K63-Ub₂, the RAP80 UIM1 and UIM2 recognize the Ile 44-centred hydrophobic patch on the proximal and the distal ubiquitin moieties, respectively, without any interactions with the Lys 63-linked isopeptide bond (Sato *et al*, 2009). The affinity between each UIM of RAP80 and monoubiquitin is too weak (K_d value is $\sim 500 \mu\text{M}$) to form a stable complex (Sims and Cohen, 2009). Therefore, a robust interaction between RAP80 and Lys 63-linked ubiquitin chains is only achieved by simultaneous interactions between multiple UIMs and multiple ubiquitin moieties in polyubiquitin chains, termed avidity. The key determinant of the Lys 63-linkage-specific avidity is the length of the inter-UIM region (Sato *et al*, 2009; Sims and Cohen, 2009). The appropriate length of the inter-UIM region organizes the UIM1 and UIM2 to optimize the spatial relationship between UIM1 and UIM2 for simultaneous binding to both ubiquitin moieties of K63-Ub₂. The inter-UIM region therefore measures the intermolecular distance between the proximal and distal ubiquitin moieties of K63-Ub₂. Similarly, the structures of the hHR23A-K48-Ub₂ and NEMO-linear Ub₂ complexes show that hHR23A and NEMO optimize the spatial relationship between multiple ubiquitin binding sites to enable simultaneous binding to the multiple ubiquitin moieties of specific polyubiquitin chains (Varadan *et al*, 2005; Rahighi *et al*, 2009). Intriguingly, this strategy is also observed in anti-Lys 63-linked ubiquitin chain antibodies in complex with K63-Ub₂ (Newton *et al*, 2008).

In contrast, the strategy for the linkage specificity of UBDs differs from that of the linkage-specific DUBs. To date, the

crystal structure of the AMSH-LP DUB domain in complex with K63-Ub₂ is the only reported structure of a linkage-specific DUB in complex with an isopeptide-linked ubiquitin chain (Sato *et al*, 2008). AMSH-LP is a zinc-dependent DUB that specifically cleaves Lys 63-linked polyubiquitin chains. A single AMSH-LP DUB domain interacts with both proximal and distal ubiquitin moieties. Compared with the extensive binding site for distal ubiquitin, the binding site for proximal ubiquitin makes few contacts with AMSH-LP and contributes little to the binding affinity. However, AMSH-LP directly interacts with the isopeptide-linked Lys 63 and neighbouring residues in the proximal ubiquitin. This interaction facilitates the correct orientation and accurate positioning of the isopeptide bond for efficient linkage-specific de-ubiquitination. A similar mechanism has been proposed for other linkage-specific DUBs, CYLD (Komander *et al*, 2008) and OTU1 (Wang *et al*, 2009). Therefore, linkage specificity of DUBs seems to be predominantly achieved in the hydrolysis step rather than in the binding step. Further, the linkage-specific DUBs directly recognize the (iso)peptide linkage in striking contrast to the linkage-specific UBDs.

Triple ubiquitin-binding NZF domains of TRABID

TRABID contains three NZFs (TRABID-NZF1–3), which specifically bind to Lys 63-linked and linear polyubiquitin chains (Tran *et al*, 2008; Komander *et al*, 2009). Individually, each TRABID-NZF domain fails to bind monoubiquitin by GST pull-down (data not shown). Chain-selective binding by the TRABID-NZF1–3 can be partially understood on the basis of its amino-acid sequence and our complex structure. In the TF/ Φ motifs of the TRABID-NZF1–3, Φ in the TRABID-NZF2 is replaced by the hydrophilic Gln, similar to the TAB2- and TAB3-NZFs (Supplementary Figure S6A). In addition, as mentioned above, the phenylalanine residue of each TRABID-NZF1–3 is replaced by Tyr, which may also diminish binding to monoubiquitin. However, the TRABID-NZF1–3 domains seem to lack proximal ubiquitin interacting sites, suggesting that the structural basis for linkage-specific recognition by TRABID-NZF1–3 differs from that of the TAB2- and TAB3-NZFs. The most plausible mechanism for the linkage specificity of the TRABID-NZF1–3 is that the multiple NZF domains bind a single ubiquitin chain and linkage-specific binding is accomplished by an avidity-based mechanism (Komander *et al*, 2009; Sims and Cohen, 2009). Because Lys 63-linked and linear polyubiquitin chains have similar distances between Ile 44 centred hydrophobic patches, it is possible that the multiple ubiquitin moieties of Lys 63-linked and linear polyubiquitin chains can simultaneously bind to the multiple NZFs of TRABID whereas those of Lys 48-linked chains cannot. Elucidation of the basis for linkage-specific binding by the TRABID-NZF1–3 awaits the structural analysis of it in complex with a Lys 63-linked or linear ubiquitin chain.

In summary, linkage-independent ubiquitin-binding NZFs (i.e. Npl4 and Vps36) contain a complete TF/ Φ motif and bind tightly to monoubiquitin, whereas linkage-specific ubiquitin-binding NZFs (i.e. TAB2, TAB3 and TRABID) contain an incomplete TF/ Φ motif (i.e. TF/Q for TAB2 and TAB3, and TY/ Φ or Q for TRABID) and bind weakly to monoubiquitin. Linkage-specific binding further requires simultaneous interaction with both the proximal and distal ubiquitin moieties for a specific and robust interaction, which is achieved by the

additional, proximal binding site for the TAB2 and TAB3 or likely by an avidity-based mechanism involving binding by the multiple NZFs for TRABID. These variations enable NZF domains to show a wide range of affinities and specificities that allow them to perform their specific functions in ubiquitin-dependent signalling pathways.

Materials and methods

Preparation of the TAB2K63-Ub₂ and TAB3K63-Ub₂ complex

The genes encoding NZF domains of mouse TAB2 (residues 665–693) and TAB3 (residues 688–716) were PCR amplified from a mouse cDNA library. The amplified genes were cloned into the pCold-GST expression vector (Hayashi and Kojima, 2008) with *Nde*I and *Xho*I sites to produce N-terminal GST fusion protein and were confirmed by DNA sequencing. *Escherichia coli* strain RosettaTM (DE3) cells (Invitrogen) were transformed with the expression vector, and were cultured in LB containing 100 mg/l ampicillin at 37°C. When the optical density at 600 nm of the culture reached ~0.5, the culture was incubated for 30 min at 15°C. Thereafter, isopropyl-β-D-thiogalactopyranoside was added to a final concentration of 0.3 mM to induce protein expression for 24 h at 15°C. The cells were collected by centrifugation at 8000 g for 15 min and were disrupted by sonication in phosphate-buffered saline (PBS), containing 1 mM dithiothreitol (DTT), 1 mM phenylmethylsulphonyl fluoride and 0.5% Triton X-100. The lysates were centrifuged at 30 000 g for 60 min and the supernatants were then loaded onto a Glutathione Sepharose FF column (GE Healthcare) that had been pre-equilibrated with PBS containing 1 mM DTT and 0.5% Triton X-100. The column was washed with PBS containing 1 mM DTT and 0.5% Triton X-100 and then with PBS containing 1 mM DTT. GST fusion proteins were eluted with 50 mM Tris-HCl buffer (pH 8.0), containing 200 mM NaCl, 1 mM DTT and 15 mM reduced glutathione. The GST tags were cleaved by Turbo3C (HRV3C) protease (Accelagen), and the samples were dialysed against 50 mM Tris-HCl buffer (pH 8.0), containing 40 mM NaCl and 1 mM DTT. TAB2 was loaded onto a ResourceQ anion exchange column (GE Healthcare) pre-equilibrated with 50 mM Tris-HCl buffer (pH 8.0) containing 40 mM NaCl and 1 mM DTT, and were eluted with a linear gradient of 0–1 M NaCl. TAB3 was passed over a ResourceQ anion exchange column (GE Healthcare) pre-equilibrated with 50 mM Tris-HCl buffer (pH 8.0) containing 40 mM NaCl and 1 mM DTT. The samples were loaded onto a Superdex 75 16/60 (prep grade) column (GE Healthcare) pre-equilibrated with 10 mM Tris-HCl buffer (pH 7.2) containing 50 mM NaCl and 5 mM β-mercaptoethanol. The fractions rich in the purified proteins were collected for crystallization.

Crystallization and data collection

K63-Ub₂ was prepared as described previously (Sato *et al*, 2008). The purified mouse TAB2 (665–693) and TAB3 (688–716) were concentrated to 10 g/l by using Amicon Ultra-15 3000 MWCO filter (Millipore), following the manufacturer's instructions. The TAB2 (665–694) or TAB3 (688–716) was mixed with K63-Ub₂ in a 2:1 molar ratio for crystallization. Initial crystallization screening was performed using the sitting drop vapour diffusion method at 20°C, with a Mosquito[®] liquid-handling robot (TTP Lab Tech). We tested about 500 conditions, using crystallization reagent kits supplied by Hampton Research, and initial hits were further optimized. The best crystals of the TAB2-K63-Ub₂ complex were obtained at 20°C with the sitting drop vapour diffusion method by mixing 0.5 µl of protein solution with an equal amount of precipitant solution containing 90 mM Bis-Tris-HCl buffer (pH 6.5), 180 mM ammonium acetate, 21% PEG3350 and 4% pentaerythritol ethoxylate, and equilibrating against 500 µl of reservoir solution containing 100 mM Bis-Tris-HCl buffer (pH 6.5), 200 mM ammonium acetate and 27% PEG3350. The best crystals of the TAB3-K63-Ub₂ complex were obtained at 20°C with the sitting drop vapour diffusion method by mixing 0.5 µl of protein solution with an equal amount of precipitant solution containing 100 mM Bis-Tris-HCl buffer (pH 6.5) and 21% PEG3350, and equilibrating against 500 µl of reservoir solution containing 100 mM Bis-Tris-HCl buffer (pH 6.5) and 21% PEG3350. The crystal of the TAB2K63-Ub₂ complex belonged to space group

*P*₂₁₂₁, with unit cell dimensions *a* = 30.1 Å, *b* = 71.5 Å and *c* = 72.0 Å. The crystal of the TAB3-K63-Ub₂ complex belonged to space group *P*₂₁₂₁, with unit cell dimensions *a* = 29.9 Å, *b* = 71.0 Å and *c* = 71.6 Å.

Structural determination and refinement

Diffraction data sets were collected at the beamline BL41XU in SPring-8 (Hyogo, Japan), and were processed with the program HKL2000 (Otwinowski and Minor, 1997) and the CCP4 program suite (Collaborative Computational Project, 1994). To solve the structure of the TAB2-K63-Ub₂ complex from the SAD data set, we used SHELX97 (George and Schneider, 1997), SHARP (Fortelle and Bricogne, 1997), SOLOMON/DM (Cowtan and Main, 1993; Abrahams and Leslie, 1996) and ARP/warp (Morris *et al*, 2003) for heavy-atom site search, phase calculation, density improvement and automatic model-building, respectively. These programs were controlled by autoSHARP (Vonrhein *et al*, 2007). MOLREP (Vagin and Teplyakov, 1997) was used to solve the structure of the TAB3-K63-Ub₂ complex by molecular replacement. The crystal structure of the TAB2-K63-Ub₂ complex was used as a search model. Atomic models were corrected by using COOT (Emsley and Cowtan, 2004) with careful inspection. Refinement was carried out by using Refmac5 (Murshudov *et al*, 1997) with iterative correction and refinement of the atomic models. The final models have excellent stereochemistry and *R*_{free} values of 0.191 for the TAB2-K63-Ub₂ complex and 0.218 for the TAB3-K63-Ub₂ complex at 1.18 and 1.40 Å resolution, respectively. Data collection, phasing and refinement statistics are shown in Supplementary Table S1. All molecular graphics were prepared with PyMOL (DeLano Scientific; <http://www.pymol.org>).

GST pull-down assays

For GST pull-down assays, GST-fused NZFs of TAB2, TAB3, Npl4 and Vps36 were overproduced in *E. coli* strain RosettaTM (DE3) (Invitrogen), and purified by Glutathione Sepharose FF and Resource Q anion exchange columns (GE Healthcare). Mutations were generated by PCR. 100 µg of the GST-fused wild-type and mutants of NZFs were immobilized on Glutathione Sepharose FF beads (GE Healthcare), and then incubated with 55 µg of K63-Ub₂, K48-Ub₂, linear Ub₂ or monoubiquitin for 15 min at 4°C in 25 mM Tris-HCl buffer (pH 7.2) containing 20 µM zinc chloride, 1 mM DTT and 0.1% Triton X-100. The beads were washed with the same buffer three times. The diubiquitin or monoubiquitin molecules bound to the beads were released by boiling in SDS loading buffer and analysed by SDS-PAGE or tricine SDS-PAGE, respectively. The gels were stained with Coomassie Brilliant Blue.

Accession codes

The coordinates and structure factors of the TAB2- and TAB3-NZFs in complex with K63-Ub₂ have been deposited in the Protein Data Bank with accession codes 3A9J and 3A9K, respectively.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (<http://www.embojournal.org>).

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Conflict of interest

The authors declare that they have no conflict of interest.

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