An ankyrin-repeat ubiquitin-binding domain determines TRABID’s specificity for atypical ubiquitin chains

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Eight different types of ubiquitin linkages are present in eukaryotic cells that regulate diverse biological processes. Proteins that mediate specific assembly and disassembly of atypical Lys6, Lys27, Lys29 and Lys33 linkages are mainly unknown. We here reveal how the human ovarian tumor (OTU) domain deubiquitinase (DUB) TRABID specifically hydrolyzes both Lys29- and Lys33-linked diubiquitin. A crystal structure of the extended catalytic domain reveals an unpredicted ankyrin repeat domain that precedes an A20-like catalytic core. NMR analysis identifies the ankyrin domain as a new ubiquitin-binding fold, which we have termed AnkUBD, and DUB assays in vitro and in vivo show that this domain is crucial for TRABID efficiency and linkage specificity. Our data are consistent with AnkUBD functioning as an enzymatic S1’ ubiquitin-binding site, which orients a ubiquitin chain so that Lys29 and Lys33 linkages are cleaved preferentially.

Protein ubiquitination is a versatile post-translational modification that regulates an increasingly large number of cellular processes. This versatility originates from the ability of ubiquitin to form eight structurally and functionally distinct polymers, in which ubiquitin molecules are linked through one of seven lysine residues or through the N terminus. It is clear from proteomic analysis that all ubiquitin linkages exist in eukaryotic cells, however the functions of the families are not cleaved, identifying TRABID as the first Lys29-specific OTU domain enzyme. Atypical ubiquitin chains have only recently become available as reagents through chemical biological methods. Ubiquitin linkages in cells are mainly unknown. We here reveal how the human ovarian tumor (OTU) domain deubiquitinase (DUB) TRABID specifically hydrolyzes both Lys29- and Lys33-linked diubiquitin. A crystal structure of the extended catalytic domain reveals a new ubiquitin-binding fold, which we have termed AnkUBD, and DUB assays in vitro and in vivo show that this domain is crucial for TRABID efficiency and linkage specificity. Our data are consistent with AnkUBD functioning as an enzymatic S1’ ubiquitin-binding site, which orients a ubiquitin chain so that Lys29 and Lys33 linkages are cleaved preferentially.

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not yet been identified. Also, TRABID activity toward Lys27 and Lys33 linkages has not yet been tested, as these chain linkages have not been available.

Here, we set out to determine how TRABID DUB activity and specificity are achieved. We tested the DUB activity of TRABID against the complete panel of eight different ubiquitin linkages, and we showed that it cleaves Lys29 and Lys33 linkages with marked preference over Lys63 linkages but cleaves no other chain type. Our crystal structure of the N-terminally extended TRABID OTU domain reveals a catalytic fold similar to that of A20, which is extended by two ankyrin repeats positioned such that they can form a proximal ubiquitin-binding site. Indeed, the isolated ankyrin domain binds to ubiquitin, and NMR experiments map the interaction interfaces to a conserved hydrophobic surface of the ankyrin module and to the hydrophobic Ile44 patch of ubiquitin. We provide evidence that the ankyrin-repeat ubiquitin-binding domain contributes to enzymatic efficiency and linkage specificity in vitro and in vivo.

RESULTS
Specificity of the extended OTU domain of TRABID
The human TRABID protein spans 708 aa and contains three N-terminal Npl4-like zinc-finger (NZF) domains (residues 1–200), and a C-terminal OTU domain (residues 340–700) (Fig. 1a). The C-terminal OTU domain of TRABID is closely related to the previously characterized OTU domain of A20 (refs. 23,36). However, a stretch of ~100 highly conserved residues upstream of the TRABID OTU domain (residues 245–340; see below) indicate an extension of the catalytic fold not present in A20.

We tested the extended fragment of the TRABID OTU domain (residues 245–697) against the complete panel of eight differently linked diubiquitin molecules. In this qualitative analysis, we defined DUB specificity as the enzymatic concentration at which the preferred linkage type was cleaved completely at a defined time point. TRABID cleaved Lys29- and Lys33-linked diubiquitin with higher activity compared to Lys63-linkages (Fig. 1b), consistent with previous quantitative data11. The remaining diubiquitin molecules linked through Lys6, Lys11, Lys27 or Lys48, or linear diubiquitin, were not cleaved (Fig. 1b). Hence, TRABID has a dual specificity against two atypical ubiquitin chains linked through Lys29 and Lys33, in marked preference over Lys63 linkages.

Crystal structure of the extended OTU domain
To understand the molecular basis for the unique DUB specificity of TRABID, we purified the extended OTU domain to homogeneity and crystallized it. Needle crystals diffracted to 2.23 Å on the ID23-2 beamline at the European Synchrotron Radiation Facility (ESRF). We obtained phase information from a SIRAS experiment using crystals derivatized with gold cyanide (AuCN). The resulting

Figure 1 Structure and specificity of an extended TRABID OTU domain. (a) Schematic representation of the functional domains of TRABID (top) and species conservation derived from a multiple sequence alignment (http://www.ensembl.org) (middle). An extended catalytic OTU domain was analyzed (residues 245–697, bottom). (b) Linkage specificity of the extended catalytic OTU domain of TRABID using diubiquitin molecules of all eight linkage types, analyzed as reported before16. TRABID was incubated with diubiquitin for the indicated times, and the reaction mixtures were resolved on an SDS-PAGE gel and silver stained. Ub, ubiquitin. (c) Structure of the extended TRABID OTU domain. The catalytic core is colored in shades of blue, where dark blue indicates the minimal OTU core domain, and the lighter blue indicates additional secondary structure elements found in the A20-like subfamily of OTU DUBs. The ankyrin repeats are shown in two shades of orange. The catalytic triad residues are indicated in ball-and-stick representation. (d) Structure of A20 (green, left, PDB 2VFJ) and superposition with TRABID (blue, right). (e) Catalytic triad residues of TRABID are shown in ball-and-stick representation with yellow sulfur, red oxygen and blue nitrogen atoms. A red sphere indicates a water molecule, and yellow dotted lines indicate hydrogen bonds. A 2Fo – Fo electron density map contoured at 1σ covers relevant residues. (f) The A20 catalytic triad is shown, with atoms colored as in e.
The OTU domain folds of TRABID and A20 superpose well (r.m.s. deviation of 1.5 Å over 275 aa), and all secondary structure elements are conserved (Fig. 1c,d and Supplementary Fig. 1b). The catalytic Cys443 and His585 of TRABID superpose well with the A20 catalytic center (Fig. 1e).

Two ankyrin repeats extend the catalytic domain
Fold analysis of the adjoining α-helical domain (residues 245–340), using Dali37, revealed the presence of two ankyrin repeats (Fig. 2a).

Ankyrin repeats include ~30 aa and consist of two interacting helices connected by short loops. Multiple repeats 'stack' through a conserved ankyrin motif, forming arc-shaped structures38 (Fig. 2b).

In TRABID, the first ankyrin repeat spans residues 260–290 and is connected to the second repeat (residues 313–340) by a long linker that packs against what would correspond to the concave surface in an extended ankyrin-repeat structure (Fig. 2c). However, a conserved N-terminal helix (αA0, residues 245–259) that packs against the first ankyrin repeat, and the C-terminal OTU domain that directly extends from the second repeat, define the boundaries for the two ankyrin repeats in TRABID (Fig. 1c).

The primary sequence of these terminal repeats is divergent from the easily identifiable ankyrin motif in internal repeats, explaining why the ankyrin domain of TRABID had not been annotated.

Ubiquitin-binding sites in TRABID
OTU domain enzymes contain a high-affinity S1 ubiquitin-binding site that binds ubiquitin (termed the 'distal' ubiquitin in a diubiquitin) and positions its C terminus toward the active site independently of the linkage type13. Ovarian tumor–domain complex structures with ubiquitin bound at the S1 site have been reported for yeast Otu1 (yOtu1, Fig. 2d)23 and for a viral OTU domain (vOTU, Supplementary Fig. 1c)26–28. Both OTU domains contain only ~180 aa and define the minimal OTU domain core conserved in TRABID (Figs. 1c and 2d,e). TRABID superposes on yeast Otu1 with an r.m.s. deviation of 2.0 Å over 99 aa (Fig. 2e) and superposes on viral OTU with an r.m.s. deviation of 2.5 Å over 105 aa (Supplementary Fig. 1c).

The structures of the complex indicate the position of the distal ubiquitin bound to the S1 site of the enzyme (Fig. 2e). All residues interacting with the C-terminal tail of the distal ubiquitin are conserved among OTU domains, indicating identical binding modes at the catalytic center itself. However, ubiquitin binds to yeast Otu1 and viral OTU in different orientations, and it interacts with distinct OTU surfaces in the two structures26. Compared to yeast Otu1 and

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Table 1 Data collection statistics

|                | TRABID 245–697 AuCN | TRABID 245–697 |
|----------------|---------------------|---------------|
| **Data collection** |                     |               |
| Space group    | P2₁2₁2₁             | P2₁2₁2₁       |
| Cell dimensions| a, b, c (Å)          |               |
|                | 60.29, 72.24, 132.55 | 60.40, 72.15, 133.01 |
| α, β, γ (°)    | 90, 90, 90          | 90, 90, 90    |
| Resolution (Å) | 72.2–3.50 (3.69–3.50) | 44.7–2.23 (2.35–2.23) |
| Rmerge         | 0.193 (0.513)       | 0.077 (0.494) |
| I / σ         | 10.2 (5.4)          | 12.1 (2.7)    |
| Completeness (%) | 100 (100)          | 100 (100)     |
| Redundancy     | 9.0 (9.2)           | 4.1 (4.1)     |
| **Refinement** |                     |               |
| Resolution (Å) | 44.7–2.23           |               |
| No. reflections| 54,670              |               |
| Rwork / Rfree | 0.202 / 0.246       |               |
| No. atoms      |                     |               |
| Protein        | 3,433               |               |
| Ligand/ion     | 9                   |               |
| Water          | 188                 |               |
| B-factors      |                     |               |
| Protein        | 43.5                |               |
| Ligand/ion     | 61.5                |               |
| Water          | 43.2                |               |
| R.m.s. deviations |                |               |
| Bond lengths (Å) | 0.013              |               |
| Bond angles (°) | 1.38                |               |

Values in parentheses are for the highest resolution shell. A single crystal was used for each dataset.

The crystallized fragment of TRABID (Fig. 1c) contains an OTU domain (residues 339–693) with a characteristic triangular shape similar to that of A20 (Fig. 1c,d)23,24 that is preceded by an α-helical domain of 96 aa (see below). The two independent domains are connected by the last helix of the helical domain (αB2) projecting away from the OTU fold (Fig. 1c).

The existence of a flexible linker between the domains and the lack of notable additional interactions at the small interface (313 Å²) indicate potential conformational freedom of the α-helical domain with respect to the OTU domain.
viral OTU, TRABID contains additional secondary structure elements that would require the distal ubiquitin to rotate and/or shift in order to bind the S1 site (Fig. 2e). Hence, although the position of the S1 ubiquitin-binding site can be confidently assigned, the orientation of the S1 ubiquitin is difficult to predict without knowing the structure of the complex.

By contrast, the existence or location of an S1’ ubiquitin-binding site (coordinating the ‘proximal’ ubiquitin in diubiquitin, which provides the lysine residue to the isopeptide bond) is not known for any OTU domain. We find it interesting that in TRABID, the adjacent ankyrin module preceding the OTU fold is located such that it could serve as an S1’ ubiquitin-binding site (Fig. 2e). Ankyrin-repeat domains mediate protein interactions through a variety of surfaces\(^3\), but ubiquitin has not been reported to bind ankyrin repeats.

**TRABID ankyrin domain mediates ubiquitin interactions**

To understand whether the ankyrin domain interacts with ubiquitin, and how, we conducted NMR studies using a $^{13}$C,$^{15}$N-labeled ankyrin domain. We obtained full sequence-specific backbone resonance assignments from triple resonance experiments (Fig. 3a and Supplementary Fig. 2a). The addition of 250-µM unlabeled ubiquitin resulted in marked perturbations of a subset of ankyrin domain resonances in $^{1}$H-$^{15}$N HSQC spectra. A further increase of ubiquitin concentration (1 mM) allowed us to unambiguously assign the perturbed resonances (Fig. 3b and Supplementary Fig. 2a). This confirmed that the ankyrin domain of TRABID interacts with ubiquitin.

To our knowledge, this is the first description of an ankyrin repeat as a ubiquitin-binding fold, which we refer to as the ankyrin ubiquitin-binding domain, or AnkUBD.

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**Figure 3** A conserved hydrophobic surface on AnkUBD binds ubiquitin. (a) $^{1}$H-$^{15}$N HSQC spectrum of $^{13}$C,$^{15}$N–labeled TRABID ankyrin domain. (b) Closeup of the region within the red box in a, showing resonances of the doubly labeled ankyrin domain (blue) and their shifts upon addition of 250-µM (yellow) or 1 mM (red) unlabeled ubiquitin (Ub). Arrows indicate the shift of individual resonances. (c) Weighted chemical shift perturbation map of the AnkUBD binding to ubiquitin. (d) AnkUBD residues are colored according to the degree of perturbation from blue (unperturbed) to red (strongly perturbed), and crucial residues are shown in stick representation. (e) The AnkUBD surface is shown colored as in d and key residues are labeled. (f) Invariant residues derived from a species sequence alignment (Supplementary Fig. 2b) are shown in red on a white AnkUBD surface.
The resulting chemical shift map shows that the entire second ankyrin repeat (residues 313–340) and an additional small loop (residues 291–295) experienced strong perturbations upon addition of ubiquitin (Fig. 3c). The four residues that had the strongest perturbation—Ala292, His317, Ile320 and Leu332—form a hydrophobic patch on the AnkUBD (Fig. 3d,e). Furthermore, Thr314 and Leu332 showed exchange broadening upon addition of ubiquitin, indicating that they may also be involved in ubiquitin interactions (Supplementary Fig. 2a).

The AnkUBD extension of the TRABID OTU domain is an evolutionarily conserved feature. A sequence alignment derived from all TRABID sequences annotated in the Ensembl database (http://www.ensembl.org/) reveals that the ubiquitin-interacting residues on the AnkUBD are evolutionarily invariant among TRABID orthologs, indicating their functional importance (Fig. 3f and Supplementary Fig. 2b).

Ankyrin UBD interacts with ubiquitin hydrophobic patch
We next confirmed the interaction of the isolated AnkUBD with 15N-labeled ubiquitin using chemical shift analysis of 1H-15N HSQC spectra. Ubiquitin resonances that were perturbed upon addition of increasing AnkUBD concentrations (from 100 to 535 µM, Fig. 4a) were found in three regions of ubiquitin, surrounding Leu8, Ile44 and Val70, which constitute the most common ubiquitin-binding site for protein interactions, the Ile44 hydrophobic patch (Fig. 4b–d). NMR-based affinity measurements indicated a dissociation constant $K_d$ of $134 \pm 19$ µM for the AnkUBD-ubiquitin interaction (Fig. 4a), which is comparable to other UBD-ubiquitin interactions. Hence, the hydrophobic surface of the AnkUBD interacts with the hydrophobic Ile44 patch of ubiquitin.

To investigate the interaction between AnkUBD and ubiquitin further, three AnkUBD mutants (H317A, I320D and L332E) were analyzed for their ability to interact with 15N-labeled ubiquitin. The correct folding of all mutants was confirmed by NMR and circular dichroism spectroscopy (data not shown). We observed no chemical shift perturbation in 15N-labeled ubiquitin with AnkUBD L332E, and we detected fewer and greatly attenuated shift perturbations with H317A and I320D AnkUBD mutants, indicating some residual ubiquitin binding (Fig. 4e and Supplementary Fig. 3). $K_d$ measurements confirmed this and indicated an affinity that was at least ten times weaker (>1,200 µM) for the AnkUBD H317A mutant than for the others (no $K_d$ values could be determined for the I320D or L332E mutants). Our mutagenesis analysis therefore confirms the presence of a single ubiquitin-binding surface on the AnkUBD of TRABID.

Ankyrin UBD contributes to linkage-specificity of TRABID
The presence of the AnkUBD as a ubiquitin-binding fold in close proximity to the catalytic site suggests that it may serve as an enzymatic S1’ ubiquitin-binding site, directly affecting TRABID’s DUB efficiency. Moreover, the S1’ site may affect TRABID specificity, as it may preferentially present a subset of ubiquitin lysine residues to the catalytic site.
Indeed, the crystallized fragment that included the AnkUBD and the AnkOTU domain showed higher DUB activity compared to the isolated OTU domain (residues 339–697), which was less active at similar concentration (Supplementary Fig. 4a). The concentration of the isolated OTU domain was thus increased by six-fold to allow us to detect enzymatic activity (Fig. 5a). The OTU fragment hydrolyzed Lys29, Lys33 and Lys63 with similar efficiency and also hydrolyzed Lys48 linkages. In addition, Lys6- and Lys11-linked diubiquitin was also cleaved but with low efficiency (Fig. 5a). This contrasted with the AnkOTU fragment, which cleaved Lys29-linked and Lys33-linked ubiquitin and—less efficiently—Lys63-linked diubiquitin (Fig. 5a).

Figure 5 Analysis of TRABID DUB activity. (a) Bacterial TRABID variants were incubated with polyubiquitin substrates for indicated times and visualized by silver staining. Comparison of activity and specificity of the isolated OTU domain (above, [E] 1.2 µM) with TRABID AnkOTU (below, [E] 0.2 µM, reproduced from Fig. 1b to allow comparison). Input enzyme levels are shown in OTU panel (see also Supplementary Fig. 4a). Ub, ubiquitin. (b–e) Mammalian TRABID variants were incubated with polyubiquitin substrates for indicated times and visualized by silver staining. Flag-tagged TRABID variants were purified from HEK293 cells and used in DUB assays. (b) Specificity of mammalian full-length (FL), AnkOTU and OTU TRABID against the diubiquitin panel after overnight (O/N, 16 h) incubation. (c) Time-course analysis of mammalian TRABID variants against its substrate linkages. FL ΔAnk means full-length, lacking AnkUBD. Input (Inp) controls highlight the stability of ubiquitin substrates in the absence of enzyme in the reaction mixture. (d) Activity of full-length TRABID with point mutations in the AnkUBD against its preferred diubiquitin substrates. Full-length C443S, catalytic mutant. DUB activity assays carried out with material obtained from Flag-empty vector (ev) immunoprecipitation showed no activity. (e) Time-course activity of TRABID variants against Lys63-linked hexaubiquitin.
This result was reflected in a second analysis, in which TRABID variants were purified by using an N-terminal 3× Flag-tag from HEK293 cells and used in endpoint DUB assays. Full-length TRABID, similarly to AnkOTU, only cleaved Lys29-, Lys33- and Lys63-linked diubiquitin when left overnight for 16 h, whereas an OTU construct was more promiscuous, cleaving Lys48-linked diubiquitin (as well as weakly cleaving Lys6 and Lys11 linkages) (Fig. 5b). We confirmed the specificity of HEK293-expressed TRABID variants for Lys29 and Lys33 linkages by time-course analysis, in which these linkages were cleaved within 60 min by full-length and AnkOTU TRABID, whereas Lys63-linked diubiquitin was not cleaved during the first hour but only after incubation overnight for 16 h (Fig. 5c, panels 1 and 3 from the top). Although this was not a quantitative analysis, there was slightly higher activity of TRABID against Lys29 linkages compared to Lys33 linkages in our qualitative time-course assays (Fig. 5a,c). Together, these data show that the AnkUBD restricts activity of the OTU domain, which cleaves (at least) four linkage types, to make TRABID most efficient for hydrolyzing Lys29 and Lys33 linkages.

Removal of the AnkUBD from either construct (that is, from full-length ΔAnk (FL ΔAnk) or OTU) also resulted in a less active protein that lacked noticeable activity in the first 60 min (Fig. 5c, panels 2 and 4 from the top), confirming the role of the AnkUBD in TRABID efficiency.

We next analyzed HEK293-expressed TRABID variants with point mutations in the AnkUBD that affect ubiquitin binding (Fig. 3). Mutation of H317A, L332E and I320D in the AnkUBD reduced TRABID activity against diubiquitin substrates, either in the context of the full-length protein (Fig. 5d) or in context of bacterially produced AnkOTU (Supplementary Fig. 4b). The observed reduced activity of TRABID point mutants (in particular H317A and L332E) was similar to that resulting from removal of the AnkUBD from either construct.

Although Lys29 and Lys33 linkages are available only as diubiquitin, Lys63-linked ubiquitin chains are available as longer polymers, so we next studied the activity of TRABID variants against Lys63-linked hexa-ubiquitin (Fig. 5e). Full-length TRABID cleaved hexa-ubiquitin efficiently, hydrolyzing most input material within 60 min. Consistent with our diubiquitin assays, removal or mutation of the AnkUBD decreased activity against K63-linked hexa-ubiquitin (Fig. 5e), suggesting that the AnkUBD is also essential for hydrolysis of longer polyubiquitin chains.

**Figure 6** Role of the NZF domains in cleaving longer ubiquitin chains. Mammalian TRABID variants were incubated with polyubiquitin substrates for indicated times and visualized by silver staining. (a) Activity of TRABID variants against Lys29, Lys33 and Lys63-linked diubiquitin (Ub2) at indicated time point. Full-length C443S, catalytic mutant; full-length NZFmut, full-length with mutations in all three NZF domains; FL ΔAnk, full-length, lacking AnkUBD; AnkOTU, crystallized fragment; OTU, OTU domain. (b) Time-course analysis of mammalian full-length TRABID, full-length NZFmut and AnkOTU activity toward Lys63-linked hexa-ubiquitin. (c) Model for the role of the AnkUBD as an S1′ ubiquitin-binding site in TRABID. (d) Model for the additional contribution of the NZF domains in cleaving longer polyubiquitin chains.

NZF domains contribute to hydrolysis of longer ubiquitin chains

TRABID contains three N-terminal NZF domains, which may also affect TRABID activity and specificity. A TRABID variant with mutations in all three NZF domains (changing the Thr-Tyr motif to Leu-Val for the full-length NZF mutant (FL NZFmut)) was as active against diubiquitin as full-length TRABID or AnkOTU (Fig. 6a). However full-length NZFmut TRABID was less active than full-length TRABID, when longer Lys63 chains were used as a substrate (Fig. 6b), suggesting that the NZF domains contribute to cleaving longer ubiquitin chains by providing additional binding sites. The combined S1−S1′ site in TRABID may constitute the highest affinity diubiquitin-binding module (Fig. 6c), otherwise the NZF domains would compete for binding to the hydrophobic patches in diubiquitin and render TRABID less active toward diubiquitin substrates, which was not the case (Fig. 5e). The NZF domains, however, provide increased affinity for longer TRABID substrates that can bind simultaneously to AnkOTU and NZF domains (Fig. 6d).

Overall, our in vitro analysis showed that (i) the TRABID OTU domain cleaves four out of eight linkage types, (ii) addition of the AnkUBD increases activity of TRABID for Lys29 and Lys33 linkages, effectively making it specific for these linkages (Fig. 6c) and (iii) the N-terminal NZF domain does not affect TRABID specificity against diubiquitin but may increase the efficiency of the enzyme against longer polymers (Fig. 6d). This confirms the importance of the AnkUBD as a crucial determinant of TRABID DUB efficiency and specificity in vitro.

Inactive TRABID forms puncta enriched in atypical polyubiquitin

We next set out to provide evidence for the mechanistic models shown in Figure 6 in a physiological setting, for which we had to establish DUB assays in vivo. It has been reported that hemagglutinin (HA)-tagged, catalytically inactive, full-length TRABID C443S (that is, mutation of the catalytic Cys443 to serine) forms distinct cytoplasmic puncta upon overexpression in various human cell lines, whereas wild-type (WT) TRABID is distributed diffusely throughout the cytoplasm and nucleus. An equivalent localization was observed with green fluorescent protein (GFP)-tagged TRABID, for full-length WT and full-length C443S (Fig. 7a,b). We used the latter for fluorescence recovery after photobleaching (FRAP) experiments, revealing that the full-length C443S puncta are highly dynamic (that is, 50% of puncta fluorescence recovers within 30 s) and in rapid equilibrium exchange with the diffuse protein (Fig. 7c). Thus, inactive TRABID forms dynamic protein assemblies rather than stable
aggregates. An equivalent catalytically inactive mutant of the closely related DUB Cezanne does not form puncta upon overexpression (Supplementary Fig. 5a), suggesting that the propensity to form dynamic protein assemblies is not a general property of catalytically inactive OTU family proteins.

Ubiquitin binding through the N-terminal NZF domains of TRABID is crucial for puncta formation (because AnkOTU C443S or full-length NZFmut C443S do not form puncta35, see Fig. 7d), indicating that full-length C443S puncta contain ubiquitin. Indeed, coexpression of GFP-tagged full-length C443S with Flag-ubiquitin revealed that the full-length C443S puncta were positive for Flag-ubiquitin (Fig. 7e). We then tested the panel of seven Flag-ubiquitin ‘K-only’ mutants (in which six out of seven lysine residues were mutated to arginine) for colocalization with TRABID puncta. We found that the full-length C443S puncta were positive for K29only, K33only, K27only and K63only ubiquitin mutants, whereas there was no detectable colocalization with K6only, K1only or K48only ubiquitin mutants (Fig. 7e and Supplementary Fig. 6a). This indicated that protein assemblies containing inactive TRABID have the defined set of atypical ubiquitin chains that TRABID preferentially hydrolyzes (with the exception of Lys27 linkages; see above). All ubiquitin mutants were expressed at similar levels and did not form punctate patterns when transfected alone, either in the absence or presence of the proteasome inhibitor MG132 (Supplementary Fig. 5b). Notably, the number or size of TRABID puncta was not affected when cells were pretreated with MG132 (Supplementary Fig. 5c). It is possible that the puncta reflect TRABID self-assembly mediated by NZF domain binding to ubiquitin chains on TRABID itself. Coimmunoprecipitation of Flag-tagged TRABID C443S with HA-tagged ubiquitin after stringent washing under denaturing conditions revealed that TRABID is indeed ubiquitinated (data not shown). However, the puncta may also contain TRABID substrates bearing Lys29-, Lys33- and/or Lys63-linked ubiquitin chains.

**In vivo deubiquitinase assay based on TRABID puncta**

Given that atypical ubiquitin chains are enriched in TRABID puncta, we explored whether we could exploit these as substrates for *in vivo* DUB assays to validate the mechanistic models derived from our structural analysis (Fig. 7f–i and Supplementary Fig. 6b). Coexpression of puncta forming GFP-tagged full-length C443S with full-length Flag-tagged TRABID results in the disappearance of GFP puncta, instead producing a diffuse GFP fluorescence throughout the cell (Fig. 7g); the levels of GFP-tagged full-length C443S did not change under these conditions, as judged by western blot analysis (data not shown). By contrast, coexpression with full-length NZFmut (Fig. 7h) or with full-length C443S (data not shown) did not alter the punctate pattern. High expression of AnkOTU resulted in complete loss of puncta formation but moderate expression did not (Supplementary Fig. 6b).
Similarly, high overexpression of the TRABID OTU domain did not result in loss of TRABID puncta (Supplementary Fig. 6b).

Notably, the punctate pattern was also unchanged upon coexpression of full-length AAnk TRABID, even after high levels of overexpression (Fig. 7i). This construct still contains functional NZF domains, and it localizes to full-length C443S puncta (Supplementary Fig. 5d). This highlights the important functional role of the AnkUBD in TRABID activity and efficiency, which is essential to antagonize C443S puncta in vivo.

DISCUSSION

Availability of all ubiquitin chain types now allows a comprehensive linkage-specificity analysis of DUBs, and in the case of TRABID, reveals its dual specificity for Lys29- and Lys33-linked ubiquitin chains, two atypical ubiquitin chain types whose cellular roles are unclear. We achieved a molecular understanding of this unique specificity profile and identified a previously unknown UBD based on an ankyrin-repetition fold, which serves as a S1’ ubiquitin-binding site to position a subset of ubiquitin chains across the active site of the OTU domain.

The TRABID OTU domain is similar in sequence and structure to that of A20; however, both domains show distinct specificities. Whereas A20 preferentially cleaves Lys48 linkages (ref. 11; T.E.T.M. and D.K., unpublished data), the TRABID OTU domain cleaves Lys29, Lys33, Lys63 and Lys48 chains, but not the remaining chain types. Other OTU family members also have distinct specificity profiles21,26. This indicates that OTU domain folds have intrinsic specificity; however, the molecular basis for this is currently unknown. Linkage specificity in DUBs is so far only understood for the Lys63-specific JAMM-family DUB AMSH-LP, which forms specific interactions with Lys63-flanking sequences39. However, the sequences flanking Lys29, Lys33, Lys48 and Lys63 are different15, so this is not likely to explain TRABID OTU domain specificity.

A previously unknown ubiquitin-binding fold, the AnkUBD, further restricts the linkage specificity of the TRABID OTU domain and makes TRABID most efficient for hydrolyzing Lys29 and Lys48 linkages. Our data are consistent with the AnkUBD serving as an enzymatic S1’ site that positions proximal ubiquitin moieties such that Lys29 and Lys33 are juxtaposed to the catalytic triad (Fig. 6c). The AnkUBD binds directly to Lys48 of the proximal ubiquitin, suggesting that a Lys48-linked chain cannot use the AnkUBD. This is consistent with our in vitro data showing that residual Lys48 cleavage is independent of the AnkUBD. Therefore, the AnkUBD adjacent to the catalytic domain explains TRABID’s DUB specificity profile.

It is not clear how the AnkUBD orients a proximal ubiquitin toward the active site. Docking of ubiquitin onto the AnkUBD using HADDOCK40 indicated several potential binding modes (data not shown); however, all models indicated that the AnkUBD has to shift and rotate for any diubiquitin to bind across the active site. The flexible linker between the AnkUBD and the OTU domain probably permits this required plasticity, but future crystallographic studies of TRABID–diubiquitin complexes will be required to confirm this.

Almost 20,000 ankyrin repeats have been annotated in >3,500 human proteins, making this domain one of the most common protein folds38. Ankyrin repeats serve as protein binding modules, and our work adds ubiquitin to the list of ankyrin-interacting proteins. Ubiquitin binding by the AnkUBD is mediated by both helices of the C-terminal ankyrin repeat, through a hydrophobic surface that is mediated by residues that are conserved among ankyrin repeats. Indeed, these residues are reminiscent of the TPLH motif that is characteristic of internal ankyrin repeats41, and it is possible that other ankyrin repeat–containing proteins may bind ubiquitin similarly to TRABID. However, terminal ankyrin repeats suited to bind ubiquitin are not easily identified by sequence analysis alone.

Although the AnkUBD is essential for TRABID efficiency and specificity, our analysis has also indicated a functional contribution of the NZF domains (Fig. 6), but their relative position with respect to the catalytic site is unclear. A linker of ~50 nonconserved and flexible residues separates the NZF domains and the AnkUBD, and individual NZF domains are themselves connected by nonconserved, flexible linkers. Hence, the NZF module could provide additional ubiquitin-binding sites toward the distal (S2, S3, S4) or proximal (S2’, S3’, S4’) end (Fig. 6d shows the distal ubiquitin-binding sites). The number of ubiquitin-binding sites within the three NZF domains is also not clear, as they may consist of two ubiquitin-binding interfaces each42,43. Alternatively, the NZF domains may target TRABID to its polyubiquitinated substrates, increasing DUB efficiency. This is suggested by our finding that the NZF domains are essential for recognition of TRABID puncta (Supplementary Fig. 5d).

Additional UBDs are found in many DUBs, and seven of the 14 human OTU DUBs contain known UBD folds15. A ubiquitin-interacting motif (UIM) in the deubiquitinase DUBA was shown to be functionally important for its role in interferon signaling31, and the A20 ZnF domains bind ubiquitin molecules through several interfaces44. The USP enzyme USP5 (IsoT) contains three UBDs that constitute S1’, S2 and S3 sites respectively45. However, for most DUBs, it is not known whether their additional UBDs constitute enzymatic ubiquitin-binding sites (for example, S1’ or S2) or whether they serve as targeting domains. Our work highlights the potential of UBDs to contribute additional levels of linkage specificity to DUBs, and presumably also to other aspecific enzymes of the ubiquitin cascade, such as E2s and E3s.

Recent proteomic data in unstimulated HEK293 cells suggests a relatively high abundance of Lys63 and Lys29 linkages, whereas Lys33 linkages are less abundant4. Although the cellular functions for the atypical ubiquitin chain types cleaved by TRABID remain to be determined, others have suggested that β-catenin itself is modified by the HECT domain ligase EDD, using Lys29 linkages (Lys33 chains were not analyzed). However, whether this leads to β-catenin stabilization3 or destabilization46 is not clear. We think it will be interesting to identify the functionally relevant TRABID substrates within the Wnt pathway that carry ubiquitin chains linked through Lys29 or Lys33, and to determine their functional relevance in signal transduction. This may shed light on the cellular function of these unusual and rare atypical ubiquitin linkages.

METHODS

Methods and any associated references are available in the online version of the paper at http://www.nature.com/nsmb/

Accession codes. Coordinates and structure factors have been deposited in the Protein Data Bank under accession number 3ZRH.

Note: Supplementary information is available on the Nature Structural & Molecular Biology website.

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AUTHOR CONTRIBUTIONS

D.K., M.B. and J.D.F.L. designed the research. J.D.F.L., D.K., J.M., T.E.T.M., T.J.R. and M.A. conducted the experiments. F.E., H.O., S.V. and J.W.C. contributed reagents. D.K. wrote the manuscript, with help from all authors.

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ONLINE METHODS

Purification and structure determination of TRABID ankyrin OTU. TRABID AnkOTU was expressed as a GST-fusion protein in Escherichia coli and purified by affinity chromatography. After removal of the GST tag by PreScission protease, anion-exchange chromatography and gel filtration produced homogeneous protein that was crystallized at 3.5 mg ml⁻¹. AnkOTU crystals grew from 150 mM NaCl, 100 mM NaOAc, 5 mM MgCl₂, 50 mM MES, pH 5.9. Crystals were soaked in mother liquor containing 27.5% (v/v) ethylene glycol before freezing in liquid nitrogen. To obtain phase information, crystals were soaked in 1 mM KAu(CN)₂ for 1 h before cryoprotection. Diffraction data of the AnkOTU crystals were collected in the ESRF beamline ID23-2 to 2.23 Å (native) and 3.0 Å resolution at the peak wavelength for gold. Phases were obtained by SIRAS, from a site set of obtained with the SHELX/hkl2map suite. Site refinement was carried out in SHARP, and subsequent density modification resulted in a high-quality map, which was interpreted by WarpNTrace and manually rebuilt in Coot. NMR analysis. 13C,15N-labeled TRABID AnkUBD (residues 245–339) was expressed in TopSpin 2.1 (Bruker) and analyzed in Sparky (http://www.cgl.ucsf.edu/home/sparky/). NMR experiments were conducted on Bruker DRX 600 MHz and AV2+ 700 MHz spectrometers equipped with cryogenic triple resonance (Thermo Scientific). NMR experiments were conducted on Bruker DRX 600 MHz and AV2+ 700 MHz spectrometers equipped with cryogenic triple resonance (Thermo Scientific).

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Confocal image analysis and in vivo DUB assays. GFP-TRABID constructs (500 ng) were transfected in COS-7 cells. After 18 h the cells were rinsed in PBS and fixed using 4% (w/v) paraformaldehyde, permeabilized in 0.1% (v/v) Triton X-100, blocked for 24 h in 3% (w/v) BSA, stained using a mouse monoclonal IgG (Invitrogen) (1:1,000 dilution). Slides were finally washed and mounted as described above. Images were obtained using a Zeiss LSM510 (Jena) confocal microscope. Double-label images of Cy3-GFP conjugates were detected with standard filter sets and laser lines.

Titration experiments and K₅ estimation. Titration experiments and K₅ estimation are described in Supplementary Methods online.

Production of full-length TRABID from mammalian cells. Five-hundred nanograms of plasmid was transfected using Lipofectamine 2000 (Invitrogen) in each well of a 6-well plate containing HEK293 cells. Forty-eight hours after transfection, cells from the six wells were combined and lysed in 1 ml of lysis buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 1% Triton X-100, complete protease inhibitor (Roche)), sonicated and centrifuged at 15,000g for 20 min. The entire lysate was incubated with 80 µL of a 50% (v/v) slurry of anti-Flag M2 affinity gel (Sigma) for 2 h at 4 °C. Beads were then washed five times in 50 mM Tris, pH 7.4, 500 mM NaCl, 0.5 mM EDTA, 1% (v/v) Triton X-100, 1 mM DTT, complete protease inhibitor; five times in 50 mM Tris, pH 7.4, 150 mM NaCl, 0.5 mM EDTA, 1% (v/v) Triton X-100, 1 mM DTT, complete protease inhibitor; and twice in 50 mM Tris, pH 7.4, 150 mM NaCl, 1 mM DTT. Immunoprecipitated TRABID was finally eluted in two consecutive steps of 100-µl elution buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 1 mM DTT) with a final concentration of 100 µg ml⁻¹ 3x Flag peptide (Sigma). The expression level of all TRABID variants was similar (see Figs. 5e and 6b).

Deubiquitinase assays. Deubiquitination assays using purified bacterial enzymes were done as reported. DUBs were diluted to 2× final concentration in 150 mM NaCl, 25 mM Tris, pH 7.5, and 10 mM DTT and activated at 23 °C for 10 min. Subsequently, 10 µl of diluted enzyme was mixed with 1–2 µg diubiquitin and 2 µl of 1× DUB buffer (500 mM NaCl, 500 mM Tris, pH 7.4, and 50 mM DTT) in 20 µl of reaction mixture. For DUB assays with TRABID produced in mammalian cells, 6 µl of eluted enzyme was used in 12 µl of reaction mixture in DUB buffer containing 200 ng di- or hexaubiquitin. Reactions were stopped by addition of 4 µl LDS sample buffer (containing 100 mM DTT) after 5, 30, 60 and 360 min or after overnight incubation for 16 h at 37 °C. Ubiquitin cleavage was detected by silver staining using the Silver Stain Plus kit (BioRad).

Confocal image analysis and in vivo DUB assays. GFP-TRABID constructs (500 ng) were transfected in COS-7 cells. After 18 h the cells were rinsed in PBS and fixed using 4% (w/v) paraformaldehyde, permeabilized in 0.1% (v/v) Triton X-100 and mounted using Vectashield mounting medium with DAPI (Vector Laboratories).

For in vivo DUB assays, cells were cotransfected with 500 ng of GFP-TRABID C443S and 500 ng of various 3× Flag-TRABID constructs, permeabilized, blocked in PBS supplemented with 3% (w/v) BSA, stained using a mouse monoclonal anti-Flag M2 antibody (Sigma) (1:500 dilution) followed by Cy3 goat anti-mouse IgG (Invitrogen) (1:1,000 dilution). Slides were finally washed and mounted as described above. Images were obtained using a Zeiss LSM510 (Jena) confocal microscope. Double-label images of Cy3-GFP conjugates were detected with standard filter sets and laser lines.

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