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ORIGINAL PAPER

An *Eimeria acervulina* OTU protease exhibits linkage-specific deubiquitinase activity

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Abstract

Ubiquitination is an important post-translational modification process that regulates many cellular processes. Proteins can be modified at single or multiple lysine residues by a single ubiquitin protein or by ubiquitin oligomers. It is important to note that the type of ubiquitin chains determines the functional outcome of the modification. Ubiquitin or ubiquitin chains can be removed by deubiquitinases (DUBs). In our previous study, the *Eimeria tenella* ovarian tumour (Et-OTU) DUB was shown to regulate the telomerase activity of *E. tenella* and affect *E. tenella* proliferation. The amino acid sequences of Et-OTU (GenBank: XP_013229759.1) and *Eimeria acervulina* (*E. acervulina*) ovarian tumour (Ea-OTUD3) DUB (XP_013250378.1) are 74% identical. Although Et-OTU may regulate *E. tenella* telomerase activity, whether Ea-OTUD3 affects *E. acervulina* growth and reproduction remains unclear. We show here that Ea-OTUD3 belongs to the OTU domain class of cysteine protease deubiquitinating enzymes. Ea-OTUD3 is highly linkage-specific, cleaving K48 (Lys48)-, K63-, and K6-linked diubiquitin but not K29-, K33-, and K11-linked diubiquitin. The precise linkage preference of Ea-OTUD3 among these three nonlinear diubiquitin chains is K6 > K48 > K63. Recombinant Ea-OTUD3, but not its catalytic-site mutant Ea-OTUD3 (C247A), exhibits activity against diubiquitin. Ea-OTUD3 removes ubiquitin from the K48-, but to a lesser extent from the K63-linked ubiquitinated *E. acervulina* proteins of the modified target protein, thereby exhibiting the characteristics of deubiquitinase. This study reveals that the Ea-OTUD3 is a novel functional deubiquitinating enzyme. Furthermore, the Ea-OTUD3 protein may regulate the stability of some K48-linked ubiquitinated *E. acervulina* proteins.

Keywords *Eimeria acervulina* · Deubiquitinase · OTU · Ubiquitin

Background

Eimeria parasites induce coccidiosis and cause intestinal bleeding and death in chickens (Ritzi et al. 2014). Seven species are pathogenic to chickens, and *E. acervulina* is well known to be one of the most virulent (Wallach et al. 2008). The application of anti-coccidial drugs and vaccines is the main strategy to control coccidiosis in poultry. However, due

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to limited knowledge of the cellular and molecular biology of the coccidia parasite and the issue of drug resistance, there are no ideal drugs or vaccines to control coccidiosis (Du and Wang 2005; Yin et al. 2014). In our previous study, the down-regulation of *Eimeria tenella* ovarian tumour (Et-OTU) protease expression decreased the telomerase activity of *E. tenella* (Wang et al., 2017). Telomerases can maintain the telomere length, promote cell proliferation, and prolong cell life (Rubtsova et al. 2012). These results implied that Et-OTU may regulate the *E. tenella* proliferation and ageing though the ubiquitin-mediated pathways (Wang et al. 2017). The amino acid sequences of Et-OTU (GenBank: XP_013229759.1) and Ea-OTUD3 (XP_013250378.1) are 74% identical. Although Et-OTU affects the telomerase activity of *E. tenella*, whether Ea-OTUD3 affects *E. acervulina* growth and reproduction remains unclear.

Protein ubiquitination regulates cellular processes by influencing the stability and function of modified proteins (Lim et al. 2013; Yao and Ndoja 2012). The modifier, ubiquitin (Ub) protein, is highly conserved in all eukaryotes and is composed of 76 amino acids (Yu et al. 2010). A single Ub protein or Ub oligomers can modify single or multiple lysine residues of the protein (Habelhah 2010; Komander 2009). Ubiquitin forms covalent chains through each of its seven lysine residues (K6, K11, K27, K29, K33, K48, or K63) or its N terminus. The variations in ubiquitination sites determine different functional outcomes of modification (Staszczak 2017). Modification by a K48-linked chain directs modified proteins to the 26S proteasome for subsequent degradation (Groves et al. 2017), while modification by K63-linked chains modulates the modified protein function, such as its function in DNA damage repair or genome stability (Ali et al. 2018). The K11-linked chain constitutes an alternative degradation signal used during cell cycle progression (Rape 2010). The K6-linked Ub chain modification of parkin facilitates parkin translocation and thus regulates mitophagy and mitochondrial integrity (Durcan et al. 2014). The roles of proteins modified by other types of linked Ub chains remain unclear (Kristariyanto et al. 2015; Yuan et al. 2014). Ub or Ub chains can be removed by deubiquitinases (DUBs) (Richter et al. 2016). DUBs belong to five main subfamilies: the Ub-specific protease (USP) superfamily, the OTU superfamily, the Machado-Josephin domain (MJD) superfamily, the Ub C-terminal hydrolase (UCH) superfamily, and the recently discovered monocyte chemotactic protein-induced protein (MCPIP) superfamily (Farshi et al. 2015). The dynamic modification processes constitute a reversible "switch" for the regulation and control of different substrate protein functions, states and physiological activities such as apoptosis, autophagy, and cell signalling pathways. Some diseases, such as cancer, are also associated with the dysfunction of DUBs (Shanmugham and Ovaa 2008), which are targets for various drugs (Heideker and Wertz 2015). In the present study, the activity of Ea-OTUD3 deubiquitinase is described and its function is predicted. The results pave the way for the development of new anti-coccidial drug targets and understanding of *E. acervulina* growth and reproduction.

Materials and methods

Bioinformatic analysis of Ea-OTUs

The full-length Ea-OTUD3 gene (EAH_00001960) was amplified from Changchun Strain of *Eimeria acervulina* cDNA. The Ea-OTUD3 was sequenced by Comate Bioscience Co. Ltd. (Jilin, Changchun, China) and analysed using the Blast-X tool in the NCBI (National Center for Biotechnology Information) database. We identified five putative hits, containing the OTU cysteine protease domain in the *E. acervuline*, using the protein Data Bank (EAH_00037150, EAH_00011890, EAH_00001960, EAH_00001500, EAH_00050270). To further characterise and classify the putative hits, we performed the BLAST alignment of Ea-OUT primary amino acid sequences with the sequence and analysed structural information for human OTUs in the protein Data Bank (Mevissen et al. 2013). The conservation of active site residues was demonstrated by the DNAMAN alignment, and the phylogenetic tree was drawn with the Clustal W building method using the MegAlign Software. The putative hit sequence (EAH_00001960) was aligned and compared with the sequences of TgOTUD3A (*Toxoplasma gondii*; GenBank: EPR62955.1), Otubain 2 (human; SW: Q96DC9), Otubain 1 (human; SW: Q96FW1), A20 (human; SW: P21580), Cezanne (human; SW: Q9NQ53), and VCIP135 (rat; SW: Q8CF97).

Cloning, site-directed mutagenesis, expression, and purification of Ea-OTUD3

The full-length Ea-OTUD3 sequence (1152-bp open reading frame, GenBank: EAH_00001960) was PCR-amplified from *E. acervulina* cDNA using the primers Ea-OTUD3-F: 5′-CGCGGATCCATGGTGCGCACATGTTTTGACTC-3′ and Ea-OTUD3-R: 5′-CCGCTCGAGTTACATGTTCTGAGTTG GGTGTT-3′ and then cloned into the pGEX4T-1 vector fused to the GST-tag. The catalytic core was predicted by sequence alignment with other OTU proteins. The Cys residue in the putative catalytic core was mutated to Ala (C247A) via site-directed mutagenesis using the primers OCM-F: 5′-TTGG AGACGGAAATGCTCAGTTTCGGTCT-3′ and OCM-R: 5′-AGACCGAAACTGAGCATTTCCGTCTCCAA-3′. Both recombinant protein expression plasmids containing the wild-type Ea-OTUD3 (WT) and the mutant Ea-OTUD3 (C247A) genes were transformed into BL-21(DE3) *Escherichia coli*. For protein expression, transformed bacteria were grown in LB-ampicillin medium to an optical density of 600 nm

(OD600) of 0.8 at 37 °C, and expression was induced with 500 μM IPTG (isopropyl-β-D-thiogalactopyranoside) for 16 to 20 h at 20 °C. Bacteria expressing the wild-type and mutant proteins were subsequently harvested and lysed in lysis buffer (10 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, NP-40, and a complete protease inhibitor cocktail from Roche) via sonication, followed by centrifugation at 8000×*g* for 15 min. Next, the supernatants, containing 5 mM dithiothreitol (DTT), were mixed with glutathione-Sepharose 4B at 4 °C for 1 h. The beads were then extensively washed to remove unbound proteins, and the bound proteins were eluted with elution buffer (20 mM reduced glutathione in 50 mM Tris-HCl, pH 8.0). The purified proteins were aliquoted and then either used directly or stored at − 80 °C.

Immunoblotting for detection of linkage-specific ubiquitin modifications

Equivalent amounts of parasites (5×10^6), either sporocyst oocyst or unsporocyst oocyst, or merozoites, were lysed in a tissue grinder. One-fifth of the lysate (10^6) was loaded per gel lane and immunoblotted with a 1:500 dilution of each linkage-specific antibody. The sensitivity and the specificity of the antibodies were determined by loading the same amounts of different ubiquitin dimer (R&D Systems, MN, USA) and treating the parasite extract with an individual linkage-specific antibody of the same dilution.

Linkage-specific deubiquitination assay

A linkage-specific deubiquitination assay was performed as follows: Recombinant Et-OTUD3^{WT} or Et-OTUD3^{C247A} were diluted to 5 μM in deubiquitinase (DUB) dilution buffer (25 mM Tris, 150 mM NaCl, and 10 mM DTT, pH 7.5) and incubated at room temperature for 20 min. Then, the solutions of the parasite extract or di-Ub chains (R&D Systems, MN, USA) were diluted to 5 μM in 10 × DUB reaction buffer (500 mM Tris, 500 mM NaCl, and 50 mM DTT, pH 7.5). The reactions were initiated by mixing 10 μl of enzyme and 10 μl of di-Ub solution, followed by incubation at 37 °C for the indicated times. The reactions were stopped by the addition of SDS-PAGE loading buffer, and the reaction mixtures were run in a 4–12% Tris-glycine gradient gel. The proteins were then transferred to a polyvinylidene difluoride (PVDF) membrane, and immunoblotting was performed with a primary antibody against Ub (R&D Systems) at 1:2000 dilution and a goat IgG anti-rabbit HRP secondary antibody (TransGen Biotech) at 1:3000 dilution.

Statistics

Data analysis was performed using Prism 5.0 (GraphPad Software, Inc.) and expressed as the mean ± SEM. To evaluate the differences between two groups, the two-tailed *t* test was used. Significance is shown by $^*P < 0.05$, $^{**}P < 0.01$.

Results

Bioinformatic analysis of Ea-OTUD3

The cDNA sequence of Ea-OTUD3 shared 100% identity with the predicted Ea-OTUD3 (GenBank: EAH_00001960). Sequence alignment analysis revealed that the C-terminus of the predicted Ea-OTUD3 possessed a highly conserved catalytic core containing a Cys box, a His box, and an Asp box (Fig. 1).

We classified and named the *E. acervulina* OTUs (Ea-OTUs) based on structure prediction analysis with respect to the human OTU domain–encoding gene using DNAMAN software. The Ea-OTUs have the highest sequence and structural homology to two human orthologue clades (OTUD and OTUB) but not the human A20-like proteins or OTULIN subfamily members (Fig. 2b). Ea-OTUs (EAH_00001960 and EAH_00050270) possess a C-terminal OTU domain, Ea-OTU (EAH_00011890) contains OTU domains in the middle of the protein sequence, Ea-OTUs (EAH_00037150 and EAH_00001500) possess N-terminal OTU domains, and Ea-OTU (EAH_00006320) has no putative OTU domains. A phylogenetic tree of putative Ea-OTUs domains was

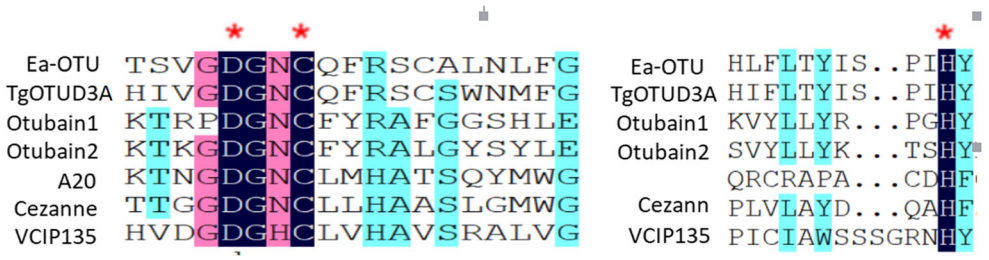

Fig. 1 Conserved sequence alignment of catalytic Cys, Asp, and His residues in the OTU family of DUBs. The amino acid alignment of Ea-OTUD3 (*E. acervulina*; EAH_00001960), TgOTUD3A (*Toxoplasma gondii*; GenBank: EPR62955.1), Otubain 2 (human; SW: Q96DC9), Otubain 1 (human; SW: Q96FW1), A20 (human; SW: P21580), Cezanne (human; SW: Q9NQ53), and VCIP135 (rat; SW: Q8CF97). The critical amino acid residues comprising the catalytic triad (Asp, Cys, and His) are highly conserved across these species (red asterisks) despite their evolutionary distance. The catalytic Cys residue was mutated to generate Ea-OTUD3 (C247A)

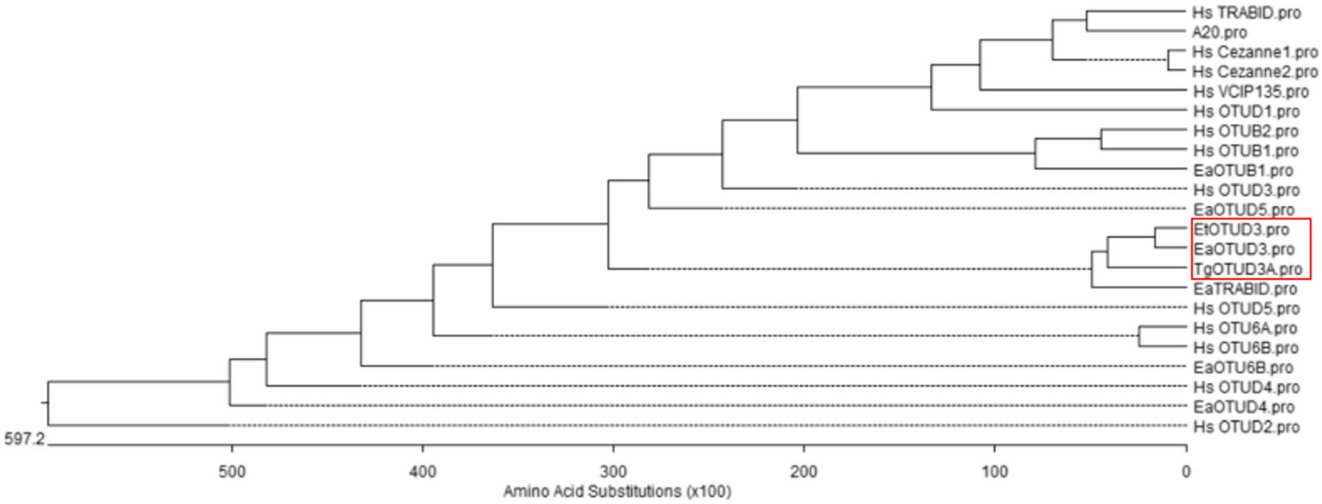

Fig. 2 Phylogenetic analysis of putative *E. acervulina* OTUs. The phylogenetic tree of putative *E. acervulina* OTU domains based on the homology of amino acid sequences with the human OTU domains. The Ea-OTUs were designated based on Clustal W alignment with the closest human OTU domains. The nucleotide sequence accession numbers for *E. acervulina* genes were obtained from the NCBI Pubmed

constructed based on the amino acid sequences homology of the human OTU domains. The Ea-OTUs were classified into five groups with respect to sequence and structural homology to the human OTU domain–encoding genes (Table 1).

Investigation of Ea-OTUD3 deubiquitinase (DUB) activity and linkage preference in vitro

Ea-OTUD3 was purified using glutathione-Sepharose 4B beads, and the purified target band accounted for 95% total protein as quantified using ImageJ software (Fig. 3a). In vitro activity experiments confirmed that Ea-OTUD3 belonged to the DUB family and hydrolysed K48-, K63-, and K6-linked Ub chains, but not other Ub chains under the same reaction conditions (Fig. 3b). All K6-linked diubiquitins were digested into ubiquitin monomers within 15 min, whereas the accumulation of ubiquitin monomers from K48-linked ubiquitin dimers gradually increased within 30 min. After 30 min, a significant amount of ubiquitin monomers was present in the digestion reaction of K63-linked chains (Fig. 3c), and significant amounts of digested dimers were present after 40 min. However, the accumulation of ubiquitin monomers from the K63-linked ubiquitin dimers gradually increased from 40 min to 70 min, as analysed using image J software (Supplement 1). At a 1:2 dilution (0.8 μM), Ea-OTUD3 digested all K6-linked ubiquitin dimers to monomers within 20 min. In the same dilution range, the reactions containing K48- and K63-linked chains still had significant amounts of undigested dimers (Fig. 3d). The accumulation of ubiquitin monomers from K48-linked ubiquitin dimers gradually increased from 0.4 μM to 6.4 μM, as analysed using image J software (Supplement 2). As shown in the second panel of Fig. 3d, Ea-OTUD3 DUBs were unable to hydrolyse all the K48-linked diubiquitin chains after 20 min but were able to hydrolyse all the K48-linked diubiquitin chains within 30 min (Fig. 3c). The hydrolysis efficiency of Ea-OTUD3 DUBs for K48-linked

Table 1 A catalogue of *E. acervulina* OTUs based on amino acid sequence homology and predicted structural similarity with characterised human OTUs. These may not reflect the true linkage specificities determined by biochemical characterisation. Such a functional deviation is evident for Ea-OTUD3 (in italic). Some Ea-OTU domains lack sequence-based structural homology to human Hs-OTUs, noted as ND (not determined). Predicted linkage specificity is based on Hs-OTU activity as reported by Mevissen et al. (2013)

Ea-OTU	Gene identifier	Closest human orthologue	% identity to Hs-OTU domain	Predicted linkage specificity based on Hs-OTU activity
Ea-OTUD6B	EAH_00037150	Hs-OTU6B	32	ND
Ea-OTUD5	EAH_00011890	Hs-OTUD5	29	K48, K63
Ea-OTUD3	*EAH_00001960*	*Hs-OTUD3*	*28*	*K6, K11*
Ea-OTUD4	EAH_00001500	Hs-OTUD4	40	K48
Ea-OTUB1	EAH_00050270	Hs-OTUB1	39	K48

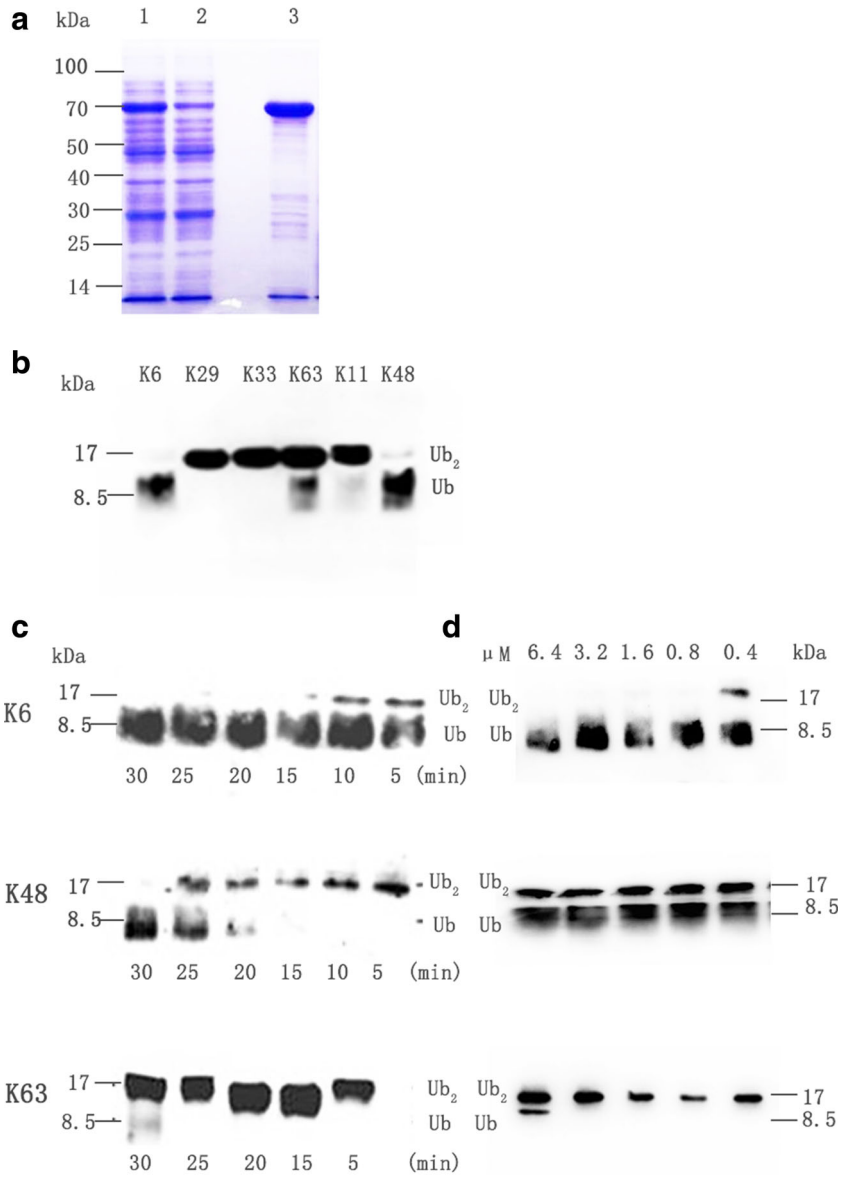

Fig. 3 Ea-OTUD3 exhibits linkage specificity for different K-linked polyubiquitin chains in vitro. **a** Ea-OTUD3 was expressed in *Escherichia coli* and purified using glutathione-Sepharose 4B beads. 1. The induced whole-cell proteins; 2. The uninduced whole-cell proteins; 3. The purified protein. **b** Cleavage assays of purified Ea-OTUD3 on di-Ub WT substrates in vitro. The purified Ea-OTUD3 DUB was incubated with the K48-, K63-, K6-, K33-, K29-, or K11-linked di-Ub chains for 30 min and detected by western blotting. **c** The purified Ea-OTUD3 DUB was incubated individually with the K48-, K6-, and K63-linked di-Ub chains for the indicated times. **d** Ea-OTUD3 DUB at different concentrations was incubated individually with the K48-, K6-, and K63-linked di-Ub chains for 30 min

diubiquitin was increased, with the molar concentration going up in 20 min (Fig. 3d). These results agreed with those in Fig. 3c. This activity was selective for polyubiquitin chains, with a preference for specific lysine linkages (K6 > K48 > K63). This Ea-OTUD3 activity was predictable based on structural analogy with human and *Eimeria tenella* orthologues (Table 1).

Immunoblotting to detect linkage-specific ubiquitin modifications

The purified merozoites, unsporulated oocysts, and sporulated oocysts of *E. acervulina* were analysed in Fig. 4a. The intensities of the GAPDH and K48 bands were measured using Image J software and plotted as the ratio of K48 to GAPDH. The ratio of K48 to GAPDH in sporulated *E. acervulina* oocysts was significantly higher than that in merozoites and unsporulated oocysts (Fig. 4b). The ratio of K63 to GAPDH was similarly determined (Fig. 4c). The diversity of K63-linked proteins in the parasite extracts, which appear in gels as discrete bands, was considerably lower than that of K48-linked proteins. The ratio of K63 to GAPDH in unsporulated *E. acervulina* oocysts was significantly higher than that in merozoites and sporulated oocysts (Fig. 4c). The ratio of Ea-OTUD3 to GAPDH in sporulated *E. acervulina* oocysts was higher than that in merozoites and unsporulated oocysts (Supplement 3) and was the same as that for K48-linked ubiquitin-modified proteins in *E. acervulina*. The ratio of K48-linked ubiquitin-modified proteins to GAPDH in *E. acervulina* gradually increased from merozoites to sporulated oocysts. During the 30-min incubation, wild-type Ea-OTUD3 digested the most K48-linked ubiquitin

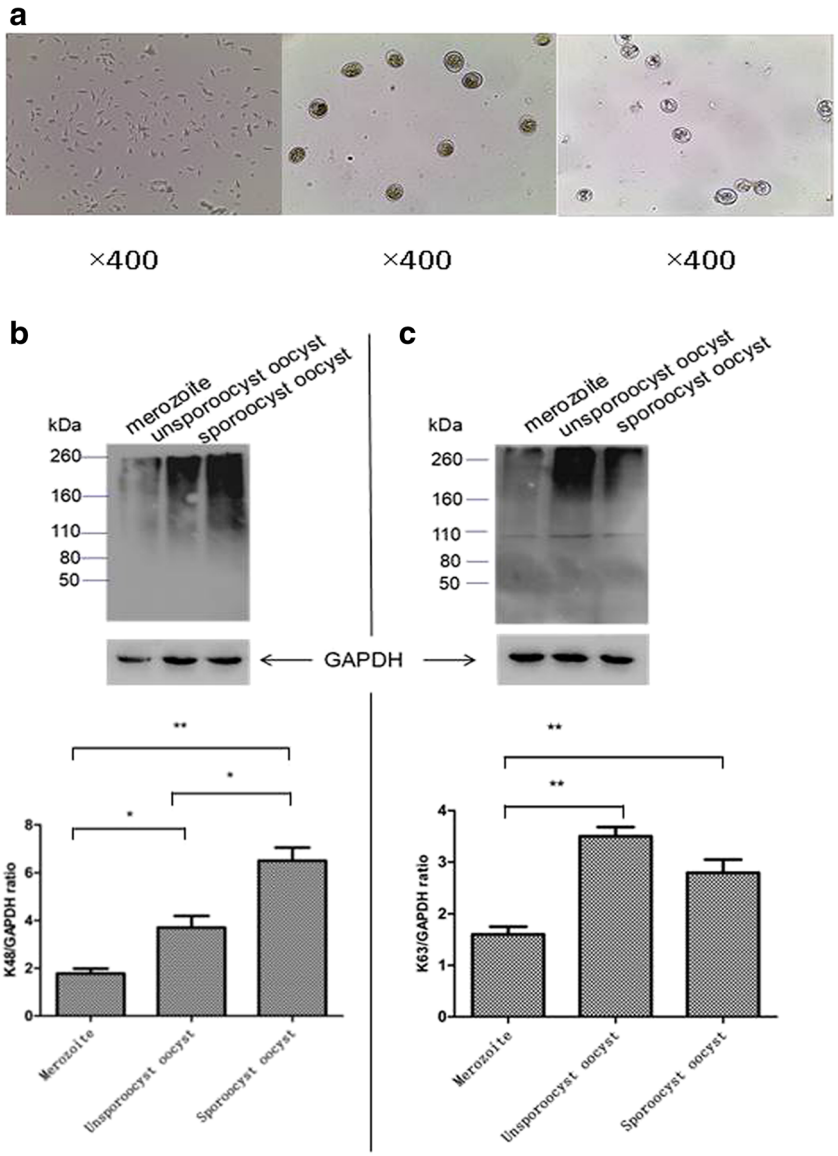

Fig. 4 The DUB activity of WT Ea-OTUD3 to K48- and K63-linked ubiquitin-modified proteins in *E. acervulina*. **a** The microscopic images of purified merozoites, unsporulated oocyst and sporulated oocysts of *E. acervuline*. **b** The merozoites, unsporulated oocyst, and sporulated oocysts of *E. acervulina* (10^6) were subjected to immunoblot analysis using anti-K48 linkage-specific antibodies. The immunoreactivity signals for the K48 linkage-specific ubiquitin antibodies and anti-GAPDH antibody were measured by using the ImageJ (NIH) gel lane analysis tool in arbitrary units (AU) for the three lanes. To determine the relative levels of K48-linked ubiquitin-modified proteins, the total signal per lane and the K48 signal relative to GAPDH were established for the merozoites, unsporulated oocyst, and sporulated oocysts of *E. acervulina*. **c** The merozoites, unsporulated oocyst, and sporulated oocysts of *E. acervulina* (10^6) were subjected to immunoblot analysis using anti-K63 linkage-specific antibodies. The steps are in accordance with the panel B. The data are representative of three independent experiments and presented as the mean ± SEM (*$P < 0.05$; **$P < 0.01$)

modifications of diverse parasite proteins but digested no K63-linked modifications in *E. acervulina* (Fig. 5a, b). In addition, although the C247A catalytically inactive enzyme deubiquitinated some modified proteins from *E. acervulina*, regardless of linkage specificity, this effect was not statistically significant (Fig. 5a, b). The C247A mutation eliminated the DUB activity of Ea-OTUD3 directed against K48- and K63-linked diubiquitin in vitro (Fig. 5c, d).

Discussion

The bioinformatic analysis of Ea-OTUDs showed that Ea-OTUD3 contains a C-terminal OTU domain. Our experiments confirmed that Ea-OTUD3 belongs to the DUB family and primarily hydrolyses K48-, K63-, and K6-linked ubiquitin chains in vitro. Because no K6-linked specific ubiquitin antibody is available, we can only detect K48- and K63-linked specific ubiquitinated *E. acervulina* proteins. Although *E. acervulina* OTU can hydrolyse K48-, K6-, and K63- diubiquitin in vitro, *E. acervulina* OTU was only able to hydrolyse K48-linked ubiquitin, and to a lesser extent the K63-linked ubiquitin, in ubiquitin-modified protein purified from *E. acervulina*. Proteins modified with K48-linked ubiquitin chains are directed to the 26S proteasome for subsequent degradation (Groves et al. 2017). Because Ea-OTUD3 can deubiquitinate K48-linked diubiquitin chains, Ea-OTUD3 may be associated with bulk protein turnover. Currently, K6-linked polyubiquitin chains are thought to affect the concentration of repair factors near DNA lesion, act in signal amplification or enable DNA decondensation and/or nucleosome and chromatin assembly (Morris and Solomon 2004). DNA damage is dependent on K6-linked ubiquitin chains (Heidelberger et al. 2018). Because Ea-OTUD3 can

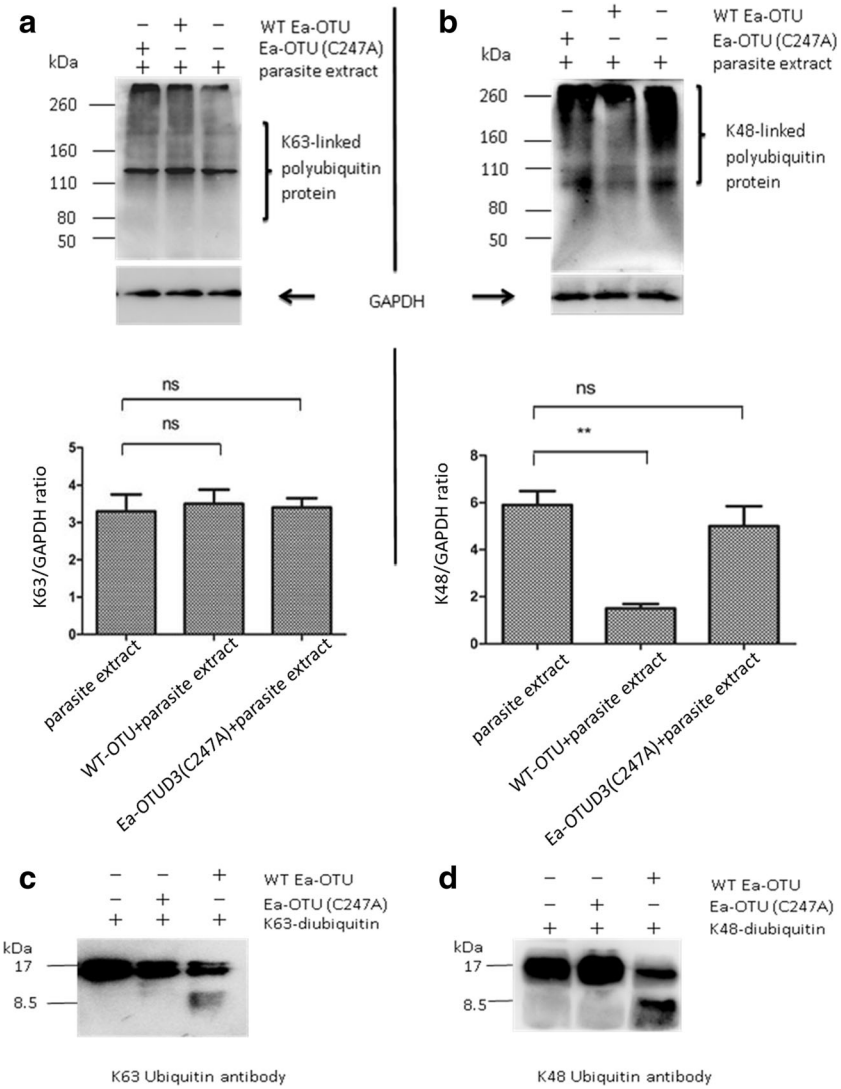

Fig. 5 Ea-OTUD3 selectively deubiquitinates K48-linked *E. acervulina* proteins. The parasites were mechanically lysed and incubated in the DUB reaction buffer without (control) or with either recombinant wild-type *E. acervulina* or mutant *E. acervulina* (C247A) enzyme for 30 min, resolved using SDS-PAGE, transferred to the membrane, and probed with linkage-specific antibodies. **a** The same parasite extract and the enzyme used in panel A were resolved and probed with a K63 linkage-specific antibody. The wild-type or mutant enzyme did not digest any *E. acervulina* K63-linked ubiquitinated proteins, confirming that the K63-linked polyubiquitin is a poor substrate for Ea-OTUD3. **b** The K48 linkage-specific antibody shows the obvious removal of K48-linked ubiquitin *E. acervulina* proteins in the presence of the wild-type Ea-OTUD3, but not its cysteine (C247A) mutant. The data are representative of three independent experiments and presented as the mean ± SEM. (*$P < 0.05$; **$P < 0.01$). **c** The enzyme mix used in panel A was tested for the synthetic K63 polyubiquitin chains, confirming activity of the wild-type enzyme and inactivity of the C247A mutant. **d** The enzyme mix used in panel B was tested for the synthetic K48 polyubiquitin chains, confirming activity of the wild-type enzyme and inactivity of the C247A mutant

deubiquitinate K6-linked diubiquitin chains, Ea-OTUD3 may be associated with the stability and structural integrity of *E. acervulina* chromosomal DNA through DNA damage repair.

Recently, ubiquitin-mediated cell cycle regulation has been observed in *Toxoplasma gondii*. TgOTUD3A hydrolyses K48-linked ubiquitin and, to a lesser extent, K63-linked ubiquitin in ubiquitin-modified proteins from *Toxoplasma* (Dhara and Sinai 2016). Ea-OTUD3 can also hydrolyse K48-linked ubiquitin and, to a lesser extent, K63-linked ubiquitin in ubiquitin-modified proteins from *E. acervulina*. The cysteine active site of Ea-OTUD3 has been identified. We analysed the conserved regions of TgOTUD3A (GenBank: TGGT1_258780) and found 61% amino acid identity between Ea-OTUD3 and TgOTUD3A. TgOTUD3A-knockout (TgOTUD3A-KO) parasites exhibit a complex phenotype associated with the fidelity of parasite replication. Otherwise, recent work has shown that knocking down OTU domain–containing cysteine proteases of *Plasmodium falciparum* (PfOTU) blocked parasite development beyond the early-schizont stage (Datta et al. 2017). However, whether Ea-

OTUD3 affects parasite replication in *E. acervulina* remains unclear.

In *Toxoplasma* OTU family members, TgOTUD1B and TgOTUD1C are most closely related to TgOTUD3A. In TgOTUD3A-KO, TgOTUD1B and TgOTUD1C expression levels were increased (Dhara et al. 2017). Homology analysis showed that Ea-OTUs contain six OTU sequences. Ea-OTUs exhibit the highest structural homology to two human orthologue clades (OTUD and OTUB). The presence of multiple OTUs (six in number) may indicate some "redundant activity" based on their phylogenetic clustering.

The ratio of Ea-OTUD3 to GAPDH in *E. acervulina* increased gradually from merozoites to sporulated oocysts and was the same as the level of K48-linked ubiquitin-modified proteins, suggesting that increased Ea-OTUD3 from merozoites to sporulated oocysts may dynamically balance the levels of K48-linked polyubiquitin chains in *E. acervulina* and help maintain homeostasis. Although the C247A catalytically inactive enzyme deubiquitinated some modified proteins, the effect was not statistically significant. Otherwise, residual activity from an enzyme containing a "catalytic cysteine" mutation may indicate that the catalytic cysteine is not critically important for DUB activity, which is not typical among OTU family proteases.

In this study, we identified a novel Ea-OTUD3 DUB and demonstrated Ea-OTUD3 DUB activity against both synthetic substrates and parasite proteins. Ubiquitin-based post-translational modification plays a role not only in protein degradation but also in other diverse cellular processes. Indeed, cell cycle progression may require a large number of OTU DUBs and Ub-ligases, which are related to diverse linkages (Natalia et al. 2018). Our results confirmed that Ea-OTUD3 can hydrolyse K6-, K48-, and K63-linked diubiquitin and that these three ubiquitin chains participate in different aspects of the *E. acervulina* cell cycle. The present study paves the way for identifying new drug targets and for understanding how Ea-OTUD3 impacts highly specialised parasitic processes.

Conclusions

Ea-OTUD3 efficiently deconjugates the K48-, K63-, and K6-linked di-Ub chains in vitro. Ea-OTUD3 removed ubiquitin from the K48- but to a lesser extent from the K63-linked ubiquitinated *E. acervulina* proteins of the modified target protein. This discovery is a promising start for further studies on the effect of Ea-OTUD3 on the molecular biological characteristics of *E. acervulina*.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflicts of interest.

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