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Characterization of the *Eimeria tenella* rhoptry protein with a nuclear localization sequence (EtROP30)

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Abstract

Rhoptry proteins (ROPs), secreted by specific rhoptry organelles of apicomplexan parasites, are determinants of parasite pathogenesis and sources of vaccine candidates. Twenty-eight ROPs of *Eimeria tenella* have been predicted by genomic approaches, and in the present study, *E. tenella* rhoptry protein 30 (EtROP30) was characterized. Subcellular localizations of EtROP30 in sporozoites and merozoites were in the apical complex and rhoptry-like bulb, suggesting that EtROP30 is a member of ROPs in *E. tenella*. Sequence analysis showed that EtROP30 contained an N-terminal secretory signal, a protein kinase domain with eight *E. tenella*-specific rhoptry kinase 1 subfamily (ROPK-Eten1) motifs, and a C-terminal nuclear localization sequence (NLS), making EtROP30 the only ROP that contains both a secretory signal and an NLS in *E. tenella*. Subsequent experiments showed that EtROP30 was a secreted protein in the sporozoite stage, relying on NLS for migration to the host nucleus. In addition, EtROP30 showed significantly higher expression levels in the parasite merozoite stage, indicating that EtROP30 plays a critical role during parasite reinvasion and development and may be a viable option as a vaccine candidate for anti-parasitic infection. The immunization protection efficacies of EtROP30 were evaluated. Significant improvements in mean body weight gain, reduction of cecum lesion score, and number of oocysts excreted were observed, indicating that EtROP30 has good immunogenicity against *E. tenella*. In the present study, a ROP of *E. tenella* with secretory and nuclear localization characteristics has been identified, and proved to be an effective vaccine candidate against this parasite.

Keywords *Eimeria tenella* · EtROP30 · Host nucleus · Rhoptry proteins · Vaccine candidate

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Introduction

Avian coccidiosis, caused by parasites of the obligate genus *Eimeria* of the phylum Apicomplexa, is a worldwide disease that threatens global poultry production (Blake and Tomley 2014). The global financial cost of coccidiosis in chickens is estimated to be ~£10.4 billion at 2016 prices (Blake et al. 2020), and *E. tenella* is the most virulent and devastating pathogen in the genus *Eimeria* (Rieux et al. 2012).

ROPs secreted by rhoptries have been shown to be the most crucial virulence factors and strongly stimulate the host immune response in Apicomplexa, which play key roles in invasion of host cells, biogenesis of the parasitophorous vacuole, and hijacking and modification of host cells (Dogga et al. 2017; Hakansson et al. 2001; Kemp et al. 2013). ROPs contain protein kinase domains and belong to a specific kinase family of eukaryotic protein kinases (ePKs) (Peixoto et al. 2010). A previous genomic analysis revealed that *E. tenella* contained 90 ePK genes, 28 of which might belong to ROPs (Reid et al. 2014). Several ROPs of *E.*

tenella were identified in proteomic analyses and/or indirect immunofluorescence assays (IFA), but only EtROP1 and Et-ROPK-Eten5-A were studied at that time. EtROP1 induced G0/G1 cell cycle arrest and inhibited host cell apoptosis (Diallo et al. 2019). Et-ROPK-Eten5-A showed good performance as a vaccine candidate (Song et al. 2020).

Rhoptries are club-shaped organelles comprising two distinct substructures, the posterior bulb and the anterior neck, through which ROPs are released (Kats et al. 2006; Preiser et al. 2000). It is now well established that ROPs of *Toxoplasma* are secreted into the host cytosol upon invasion via secretory signals and then trafficked to distinct cellular destinations in response to other signals. For example, many ROPs make their way back to the parasitophorous vacuole membrane (PVM) through an arginine-rich amphipathic helix (RAH) domain (Lim et al. 2012). The TgROP16 and rhoptry protein phosphatase 2 C (PP2C-hn) are migrated into the host cell nucleus via the NLS (Butcher et al. 2011; Gilbert et al. 2007). No ROPs have been reported on the distribution of *Eimeria* parasites in host cells. Our analysis identified EtROP30 as the only ROP of interest among the 28 ROPs of *E. tenella* that contains both secretory signals and NLS.

In the present study, a rhoptry protein of *E. tenella* (EtROP30) was characterized, with sequence features, localization, expression levels at different developmental stages and immunoprotection being investigated.

Materials and methods

Parasites and animals

One-day-old Hy-Line Brown cocks were provided by Dongyue Breeding Poultry Company (Tai'an, China) and reared in a coccidia-free environment. *E. tenella* Shandong strain (SD-01) was maintained and propagated in our laboratory (Liu et al. 2014). Unsporulated oocysts, sporulated oocysts, sporozoites, and merozoites were obtained as previously described (Zhao et al. 2021).

Molecular cloning and EtROP30 analysis

To isolate total RNA and protein, unsporulated and sporulated oocysts were ground with a mortar and pestle while frozen in liquid nitrogen. Total RNA was then extracted from four stages of *E. tenella* (unsporulated oocysts, sporulated oocysts, sporozoites and second-generation merozoites) using an RNA Extraction Kit (Vazyme, Nanjing, China), and cDNA was synthesized using the PrimeScript™ RT reagent Kit with gDNA Eraser (Takara, Tokyo, Japan). Primers were designed based on the mRNA sequence of the putative *E. tenella* rhoptry kinase family protein ROP30 (NCBI

Reference Sequence: XM_013372684.1). The complete coding sequence was amplified from second-generation merozoites cDNA using primers EtROP30-F (5′-ATGAGACTT CTTCTGTTTCTGGCG-3′) and EtROP30-R (5′-CTCGGC CTTGGCTTTTTTCTTG3′). Bioinformatics analyses of the sequences were obtained by the online software ExPASy (http://www.expasy.org/).

Recombinant EtROP30 protein and antiserum preparation

Recombinant EtROP30 protein was expressed by the pET28a-EtROP30 plasmid in *E. coli* BL21. The bacteria were lysed by sonication and the inclusion bodies were solubilized in binding buffer containing 8 M urea. The protein was purified using His Bind Resin (Merck, Darmstadt, Germany) and its purity was evaluated by SDS-PAGE. Purified protein (200 µg) was emulsified with Freund's adjuvant (Sigma Aldrich, St Louis MO, USA) and Kunming mice were injected subcutaneously four times a week. Antiserum against EtROP30 was obtained one week after the final immunization. Its specificity was evaluated by Western blotting of whole sporozoite lysates according to a previously described method (Liu et al. 2014).

Localization labeling of EtROP30 in *E. tenella* sporozoites and schizozoites

Freshly purified sporozoites and second-merozoites were fixed with 4% (*w/v*) paraformaldehyde on coverslips for 25 min. The permeabilized treatment was performed for 30 min using 0.25% Triton X-100. The coverslips were sealed in 3% BSA at 37 °C for 1 h. Parasites were incubated with mouse anti-EtROP30 serum (1:100) overnight at 4 °C. FITC-labeled second antibodies (Absen, Shanghai, China) were used for labeling following the instructions. DNA was stained blue with 4′6-diamidino-2-phenylindole (DAPI). Images were collected under an oil objective using fluorescence microscopy (Olympus, Tokyo, Japan).

Sporozoite secretion experiment

According to a previous study (Li et al. 2015), fresh sporozoites were incubated in the presence of 400 nM calcium ionophore A23187 (Aladdin, Shanghai, China) at 37℃ for 20 min. The supernatant and whole sporozoite proteins were then analyzed by Western blotting using an antiserum against EtROP30 protein as primary antibodies at 1:1000 and an anti-GAPDH mouse monoclonal antibody (Proteintech, Wuhan, China) as a reference control at 1:6000, and HRP-conjugated anti-mouse IgG (Proteintech, Wuhan, China) as secondary antibodies at 1:6000.

Intracellular location of EtROP30 expression in DF-1 cells

In order to detect the intracellular location of EtROP30 expression in DF-1 cells, open reading frame of EtROP30 was cloned into pEGFP-C1 plasmid with Nhe1 and Age1 restriction sites for construction of recombinant pEGFP-EtROP30 plasmid. The pEGFP-EtROP30 and pEGFP-C1 Plasmids (1 µg/well of 12-well plate) were transfected into DF-1 cells using Lipo8000™ transfection reagent (Beyotime, Shanghai, China), respectively. Nuclear and cytoplasmic proteins were extracted with Nuclear Protein Extraction Kit (Beijing Solarbio Science & Technology Co., Ltd), primary antibodies were anti-GFP mouse monoclonal antibody at 1:6000 (Beijing Solarbio Science & Technology Co., Ltd.), and anti-GAPDH mouse monoclonal antibody and anti-Histone H3 Mouse Monoclonal Antibody (Proteintech, Wuhan, China) were used as reference controls at 1:6000. Secondary antibodies were HRP-conjugated anti-mouse IgG (Proteintech, Wuhan, China) at 1:6000.

Life cycle stages of EtROP30 transcription and expression in *E. tenella*

The transcription of EtROP30 was determined in triplicate at different life cycle stages of *E. tenella* by quantitative real-time PCR (qRT-PCR) using SYBR Green I (Vazyme, Nanjing, China). The 18S rRNA was used as a standard reference gene. The primers were EtROP30-RT-F(5′-CCGCAG CAAGCAGTTGTTGA-3′), EtROP30-RT-R(5′-AAACGT GCTCCAGCTTGTGC-3′), 18S-F (5′-TGTAGTGGAGTC TTGGTGATTC-3′) and 18S-R (5′−CCTGCTGCCTTCCTT AGATG-3′). The expression levels of EtROP30 in the life cycle stages of *E. tenella* were analyzed by Western blotting. Protein lysates from the four stages of *E. tenella* were prepared in RIPA buffer containing protease inhibitor (Beyotime, Shanghai, China). Image J software was used to measure Gray values.

The immune protection of EtROP30 proteins

Sixty chickens were randomly divided into three groups (20 chickens/group). Chickens of the recombinant EtROP30 protein immunized group (EtROP30 group) were injected subcutaneously in the neck with recombinant EtROP30 protein (100 µg/chicken) emulsified with Freund's adjuvant at 7 days of age. Chickens in the challenged control group and the unchallenged control group were injected with PBS emulsified with Freund's adjuvant. One week later, a second immunization was administered at the same dose. After one week, all chickens in the experimental and challenged control groups were orally infested with 8×10^3 fresh sporulated oocysts of *E. tenella* per chicken, while the unchallenged group was given phosphate buffered saline (PBS). Immune protection was evaluated by body weight gain, lesion score, fecal oocyst output, and oocyst reduction rate (%). At 5 day post-challenge, the cecal lesion scores of the chickens ($n = 5$) from each group were recorded, and the grading criteria are consistent with the previous description (Johnson and Reid 1970). Body weight gain, oocyst output and oocyst reduction rate (%) were calculated according to published methods (Song et al. 2020).

Detection of serum antibody level

At 0 day and 7 day post-challenge, the serum antibodies of five chickens per group were detected by ELISA as described previously (Zhao et al. 2020). Briefly, the IgY levels were estimated using ELISA plates coated with purified recombinant EtROP30 protein (0.5 µg/well), along with horseradish peroxidase-conjugated (HRP) rabbit anti-chicken IgY (Sigma) as secondary antibody. All the samples were analyzed in triplicate, and the optical density at 450 nm (OD_{450}) was determined using an automated microplate reader (Biotek, USA).

Statistical analysis

Statistical analysis was performed by GraphPad Prism 8.2.1 (GraphPad software, Inc., La Jolla, USA) and plotted as bar graphs. Data were analyzed using a *t*-test. $p < 0.05$ was considered a statistically significant difference.

Results

Cloning and sequence analysis of EtROP30

The cDNA sequence of *E. tenella* rhoptry protein 30 (EtROP30) was amplified to a length of 1578 bp, with 100% identity to the sequence deposited in the NCBI database (XM_013372684.1). Sequence analysis revealed that EtROP30 contains an 18 aa N-terminal secretory signal sequence with a cleavage site between residues 18 and 19 ($p = 0.9969$), a protein kinase domain (175–510 aa) and a C-terminal nuclear localization sequence (516–525 aa) (Fig. 1a). A previous study divided deductive ROPs of *E. tenella* into 10 subfamilies of the rhoptry protein kinase (ROPK) family by evolutionary adaptation and separated them into active ROPK and inactive ROPK based on the presence or absence of a "catalytic triad" of residues essential for the kinase enzymatic activity (Talevich and Kannan 2013). According to this criterion, the protein kinase domain of EtROP30 contains eight conserved motifs of the *E. tenella*-specific rhoptry kinase 1 subfamily (ROPK-Eten1) and three key residues (K^{211}, D^{360}, D^{378}) required

Fig. 1 Molecular characterization of EtROP30. **a** Primary structural analysis of EtROP30; **(b)** Eight conserved motifs of the *E. tenella*-specific rhoptry kinase 1 subfamily; **(c)** Alignment analysis of the amino acid sequences of EtROP30 homologous protein in *Eimeria* species. Kinase conserved motifs (I-VIII) were framed out with dotted lines. The active sites of protein kinases were marked with asterisks (*); **(d)** Evolutionary phylogenetic relationship analysis of EtROP30 by MegAlign
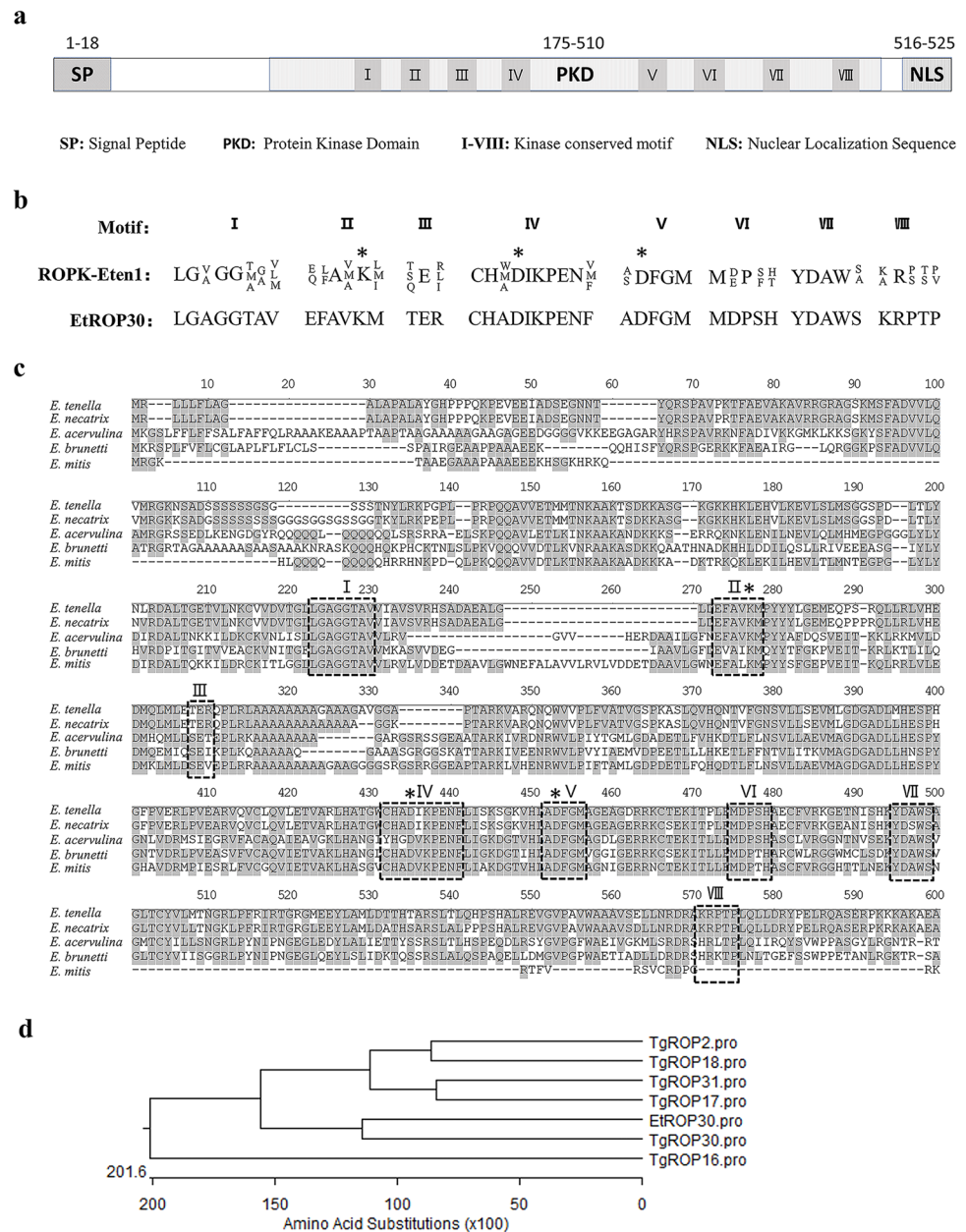

for catalysis (Fig. 1b), which suggests that EtROP30 is an active ROPK of the ROPK-Eten1 subfamily. A search of EtROP30 sequences in NCBI identified five homologous genes of chicken *Eimeria spp.* Comparative sequence analysis showed that homologous rhoptry protein sequences of *Eimeria* spp. were conserved, especially the key sites of protein kinases and eight conserved motifs, while motif VIII was absent in *E. mitis* (Fig. 1c). EtROP30 exhibited 91.91% identity to *E. necatrix* (XP_013432728.1), 55.46% identity to *E. mitis* (XP_013355441.1), 52.50% identity to *E. brunetti* (CDJ51611.1), and 48.13% identity to *E. acervulina* (XP_013248738.1). Evolutionary phylogenetic relationship analysis showed that EtROP30 was closest to

TgROP30 in homologous proteins of *T. gondii*, as a closely related species in the phylum *Apicomplexa* (Fig. 1d).

The localization of EtROP30

Identification of the anti-EtROP30 polyclonal antibody prepared from purified recombinant EtROP30 (Fig. 2a) showed that the prepared polyclonal antibody specifically recognized the recombinant EtROP30 protein (Fig. 2b) and a unique band was obtained in the entire sporozoite protein mixture, conforming to the theoretical molecular weight (Fig. 2c). The molecular mass of the recombinant protein was greater than that of the native protein, due to the presence of the

Fig. 2 The localization and secretion of EtROP30. **a** SDS-PAGE analysis of the purified recombinant EtROP30; (**b**) Western blot analysis of the recombinant EtROP30 recognized by the anti-EtROP30 polyclonal antibody (1:1000); (**c**) Western blot analysis of the whole sporozoite protein by the anti-EtROP30 polyclonal antibody (1:1000); (**d**) EtROP30 localization visualized by IFA. Sporozoites and merozoites were labeled with the anti-EtROP30 primary antibody (1:100). Scale bar, 20 μm; (**e**) Western blot analysis of the whole sporozoite protein and sporozoite secretory protein by the anti-EtROP30 polyclonal antibody (1:1000) and the anti-GAPDH mouse monoclonal antibody (1:6000). GAPDH was used as a reference
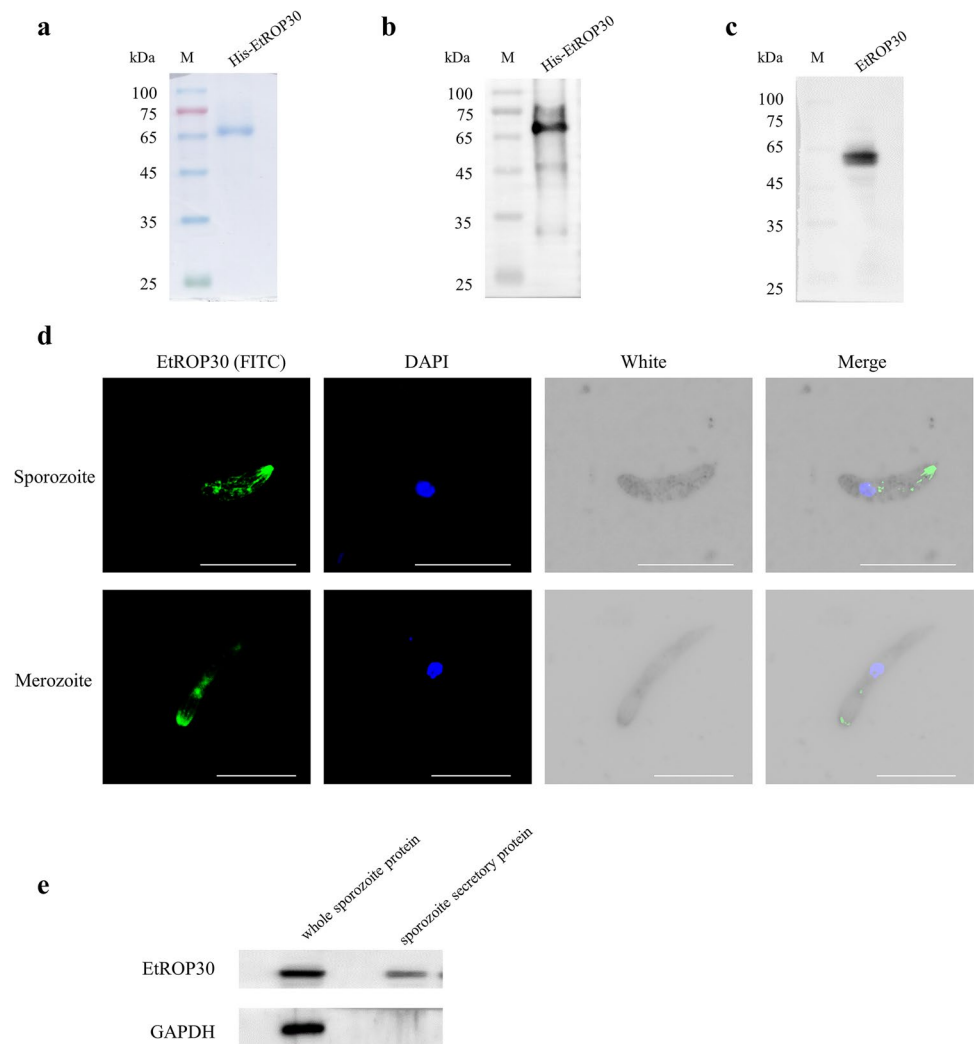
signal peptide and His-tag. Immunolocalization tests of EtROP30 in sporozoites and merozoites were carried out. The results revealed that EtROP30 is mainly localized at the apical end (apical complex of the *Apicomplexa* parasite) and in the interior of the rhoptry-like bulb (Fig. 2d). The sporozoite secretion experiment was carried out to detect whether EtROP30 is a secreted protein. Western blotting showed that it could be detected from the supernatant in which the sporozoites were incubated, while the cytoplasmic reference GAPDH could not be detected (Fig. 2e), indicating that the EtROP30 detected is derived from sporozoites secreting protein rather than parasite lysis. The results suggest that EtROP30 is a secretory protein.

Transfer of EtROP30 into nucleus by NLS

PEGFP-EtROP30 and PEGFP-ΔNLS-EtROP30 fusion proteins (Fig. 3a) were constructed to detect comparable intracellular localizations under conditions of overexpression in DF1 cells. The results showed that the majority of the

EtROP30 protein in DF1 cells was localized in the nucleus, while the mutated EtROP30 (ΔNLS-EtROP30) absence of NLS was expressed in the cytoplasm (Fig. 3b). The results were further confirmed by western blot analysis of the different cellular fractions (cytoplasmic and nuclear) that were separated. EtROP30 appeared in both nuclear and cytoplasmic fractions, and ΔNLS-EtROP30 was observed only in the cytoplasmic fraction (Fig. 3c). These results suggest that EtROP30 can be transferred to the nucleus via NLS in DF1 cells.

Transcription and expression levels of EtROP30 in different life cycle stages of *E. tenella*

The transcription levels of EtROP30 at different life cycle stages of *E. tenella* were detected by qRT-PCR (Fig. 4a). Taking the transcription of EtROP30 at the unsporulated oocyst stage as a reference, the transcription levels of EtROP30 in merozoites were 5.13-fold higher and significantly different ($p < 0.01$). The transcription levels of

Fig. 3 The localizations of EtROP30 and ΔNLS-EtROP30 in DF-1 cells. **a** Schemes of PEGFP-EtROP30 and PEGFP-ΔNLS-EtROP30; (**b**) Intracellular localization of EtROP30 and ΔNLS-EtROP30 analyzed by confocal microscopy in DF-1 cells. EGFP fusion proteins were expressed in DF-1 cells and nuclei were labeled by DAPI; (**c**) Western blot analysis of the cytoplasm and fractions. GAPDH was used as a cytoplasmic reference and Histone H3 as a nuclear reference
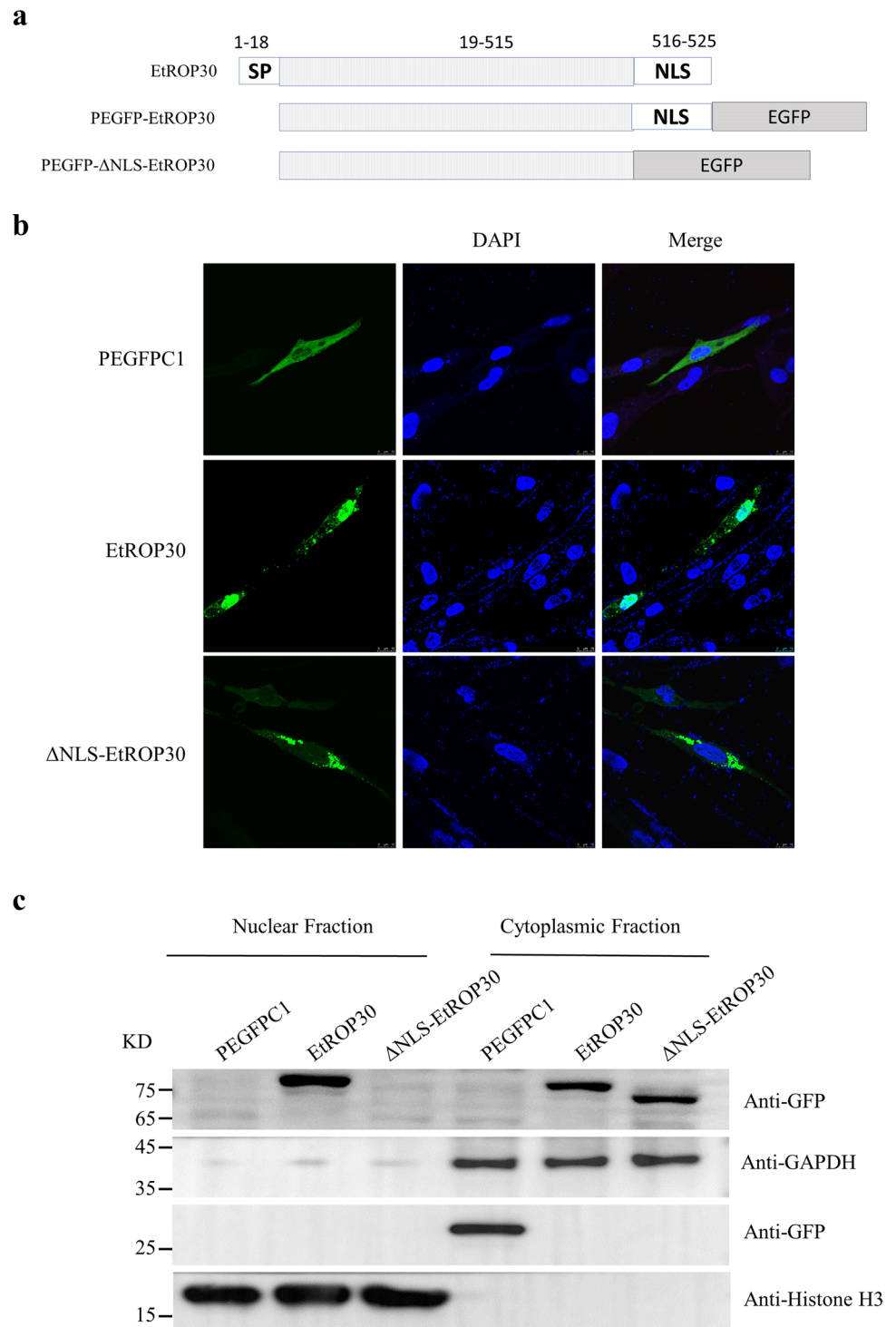
EtROP30 in sporozoites were 1.81-fold higher and significantly different ($p < 0.01$). The transcription levels of EtROP30 were not significantly different between sporulated and unsporulated oocysts ($p > 0.05$). In particular, the highest transcription levels of EtROP30 were found in merozoites, which were 2.93-fold higher than in sporozoites ($p < 0.01$).

The translation levels of EtROP30 at different life cycle stages of *E. tenella* were detected by western blotting. The results showed that EtROP30 could be specifically recognized by the polyclonal antibody and expressed in unsporulated oocysts, sporulated oocysts, sporozoites and merozoites of *E. tenella* (Fig. 4b). EtROP30 expression levels were significantly higher in sporozoites and merozoites than in
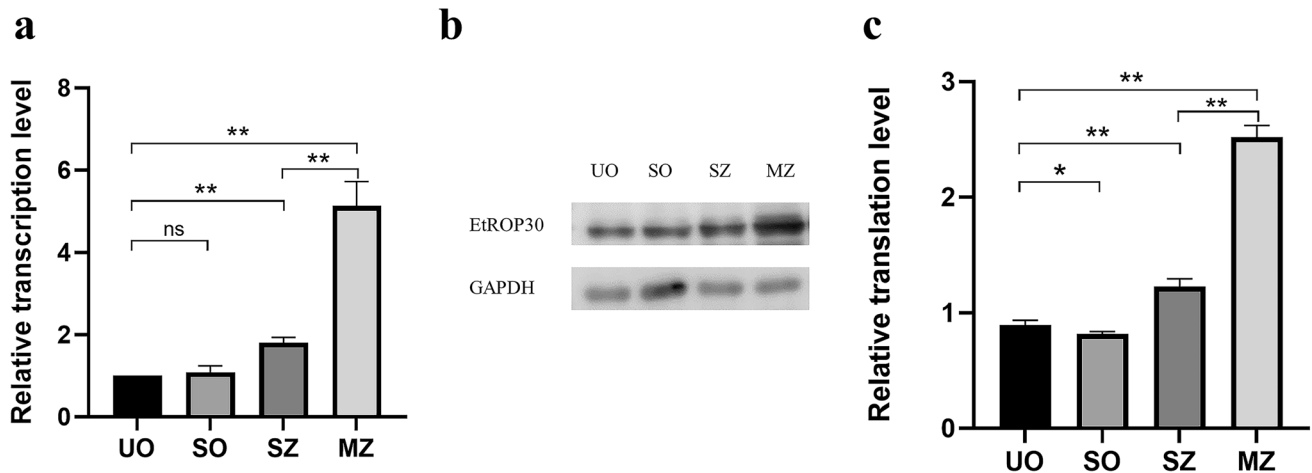
a

b

c

Fig. 4 Dynamic expression levels of EtROP30 at different developmental stages of *E. tenella*. Unsporulated oocysts (UO); sporulated oocysts (SO); sporozoites (SZ); merozoites (MZ) **(a)** Analysis of mRNA transcription levels of EtROP30 at different developmental stages by qRT-PCR; **(b)** Western blot analysis of EtROP30 with

unsporulated oocysts ($p < 0.01$) (Fig. 4c). EtROP30 expression levels were lower in sporulated oocysts than in unsporulated oocysts ($p < 0.05$) (Fig. 4c). Similar to transcription trends, the expression levels of EtROP30 were highest and significantly higher in merozoites than in the other three stages ($p < 0.01$).

Effective immune protection induced by recombinant EtROP30 against *E. tenella*

Recombinant EtROP30 protein was analyzed by western blot using serum isolated from chicken infected with *E. tenella*, and uninfected chicken serum as control. It reacted with chicken serum from the challenged control group, and chicken serum from the unchallenged control group was used as a reference (Fig. 5a). The results demonstrated that EtROP30 could stimulate the immune response in chickens under naturally infected conditions. The immunization efficacies of recombinant EtROP30 were evaluated by body weight gain, relative body weight gain ratio, cecal lesion score, number of oocysts excreted, and oocyst excretion reduction ratio. The mean body weight gains in the EtROP30-immunized group (82.20 ± 11.29 g, $p < 0.01$) were significantly higher than those in the challenged control group (56.28 ± 4.58 g) (Fig. 5b), corresponding to the relative weight gain ratios of 75.92% (EtROP30-immunized group) and 52.33% (challenged control group) (Fig. 5c). The mean lesion score was significantly lower in the EtROP30-immunized group chickens compared to the challenged control group ($p < 0.001$) (Fig. 5d). The number of oocysts excreted was significantly reduced between chickens in the EtROP30-immunized group and the challenged control

GAPDH being used as a reference; **(c)** Quantification of Western blot bands using Image J software. Data represent the mean of triplicate determinations. No significant difference (ns) $p > 0.05$, significant difference * $p < 0.05$, ** $p < 0.01$

group ($p < 0.0001$) (Fig. 5e), with an oocyst excretion reduction ratio of 61.43%. The survival rate of chickens in all three groups was 100%. The results suggest that recombinant EtROP30 has a significant immune protection effect on *E. tenella*-infected chickens.

Serum antibody levels

Serum IgY levels against the EtROP30 at 0 and 7 day post-challenge were shown in Fig. 5f. Levels of anti-EtROP30 serum antibody in EtROP30-immunized group were significantly higher than control groups at 0 day post-challenge (7 day post the second immunization) ($p < 0.0001$). The serum antibodies levels were significantly increased at 7 day post-challenge than 0 day post-challenge in EtROP30-immunized group ($p < 0.05$) and challenged control group ($p < 0.001$). These results indicated that EtROP30 could induce a high level of humoral immunity response for against of *E. tenella* challenge.

Discussion

In this study, EtROP30 was characterized and showed consistency with the common features of ROPs. Interestingly, it was defined to enter host nucleus by NLS, which is the first report on the entry of an *Eimeria* parasite protein into the host nucleus. Moreover, it showed high expression levels in the merozoite stage and is a promising vaccine candidate against *E. tenella* infection.

ROPs, secreted from unique rhoptries organelles of apicomplexan parasites, play critical roles in parasite invasion
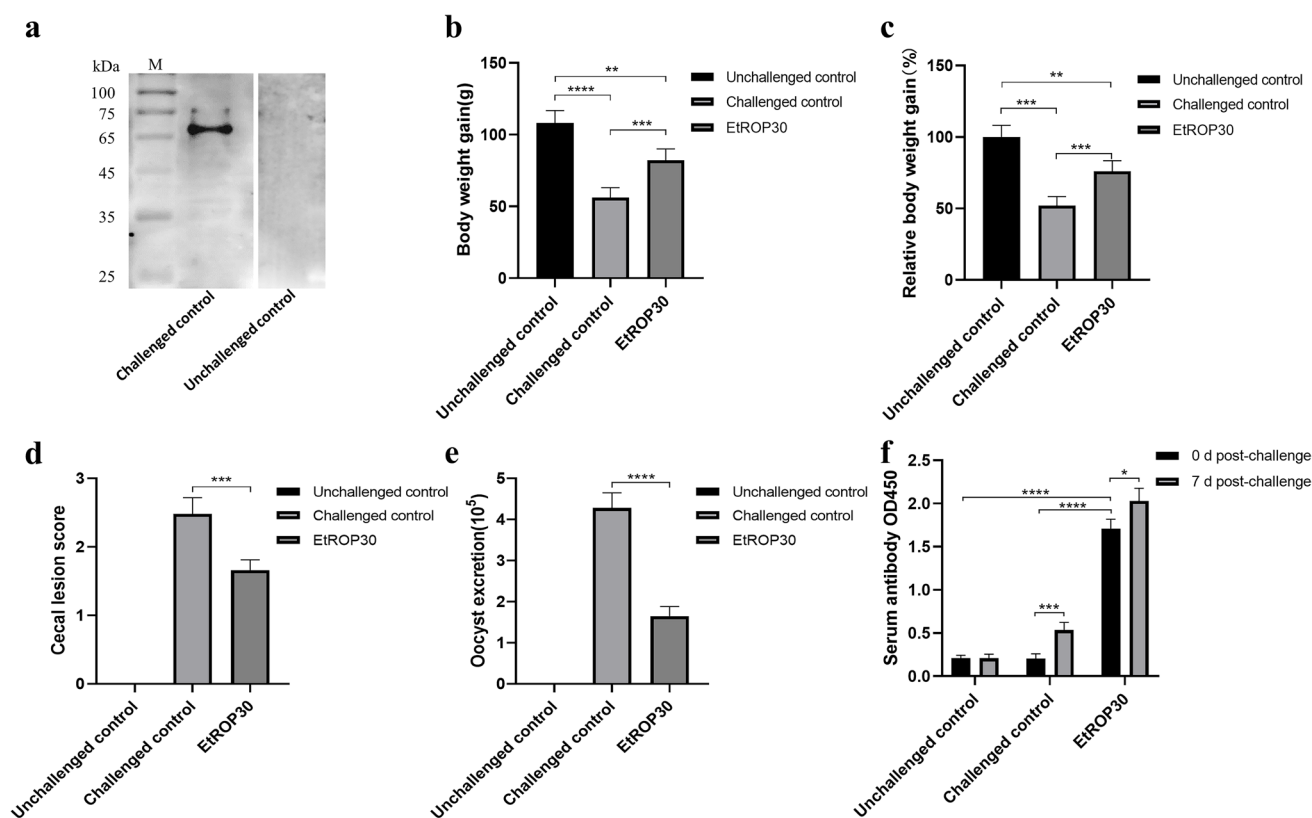
Fig. 5 Effective immune protection induced by recombinant EtROP30 against *E. tenella*. **a** Western blot analysis of the recombinant EtROP30 recognized by the serum of the challenged control group and the unchallenged control group; **(b)** Effective immune protection on body weight gain; **(c)** Relative body weight gain; **(d)** Cecum lesion score; **(e)** Number of oocyst excretion; **(f)** Serum IgY levels. Data are expressed as mean $\pm SD$ (error bars) of three independent experiments. All data were analyzed using t-tests. *p*-values are represented by asterisks: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$

and host cell development (Dlugonska 2008). Since apicomplexan protozoa share relatively conserved protein transport and secretion mechanisms in evolutionary terms (Kemp et al. 2013), some common features of ROPs, such as their location in rhoptry organelles, secreted characteristics, and the presence of protein kinase domains, are usually retained. In this study, sequence analysis revealed that EtROP30 contains a protein kinase domain with eight conserved motifs of the ROPK-Eten1 subfamily and three key catalytic residues, which suggests that EtROP30 is the active ROPK of the ROPK-Eten1 subfamily. In addition, ROPs were considered to be secretory proteins, whereas ePKs are typically cytosolic (Peixoto et al. 2010). EtROP30 contained a secretory signal peptide that was verified as a secreted protein in the present study, which is consistent with the secreted characteristic of ROPs. ROPs were secreted from those in the posterior "bulb," traversing the anterior rhoptry "neck", and are shown in positions one to three of "bulb," "neck," and apical end of the parasite as observed by immunofluorescence (Barylyuk et al. 2020; Dogga et al. 2017; Lehmann et al. 2018). In the present study, specific antibodies labeled most of EtROP30 on the apical end of sporozoites and

merozoites, and also appeared inside, such as the rhoptry bulb. The locations of EtROP30 are consistent with known subcellular localization features of previously characterized ROPs (Song et al. 2020). These features match those common to the ROPs family of proteins, which indicate that EtROP30 is a novel ROP for *E. tenella*.

Notably, EtROP30 contains an NLS, which is rare in the ROPK family. We found in this study that it can enter the host cell nucleus via the NLS. The specific subcellular localization of pathogenic secretory proteins in host cells is usually closely related to their functions (Sangare et al. 2019). Proteins secreted from parasites in the nucleus of the host cell usually play important roles in the regulation of host cells. TgROP16 enters the host nucleus via the NLS and phosphorylates STAT3 and STAT6 transcription factors to downregulate the host cell immune response, which is critical for virulence regulation and survival of parasites (Jensen et al. 2013; Peixoto et al. 2010). In addition to ROPs, TgGRA16 forms a complex with HAUSP and PP2A-B55, which is translocated to the host cell nucleus via the NLS and controls p53 protein levels to promote host cell survival under stress conditions (Bougdour

et al. 2013). TgGRA24 translocates to the host nucleus and induces upregulation of the transcription factors Fos and EGR1 (Braun et al. 2013; Hakimi et al. 2017). In another study, we found a 14-fold upregulation of Fos transcription levels under conditions of EtROP30 overexpression in HEK293 cells (unpublished date). Fos is required for intracellular development of *T. gondii* and *Eimeria falciformis* (Ren et al. 2021), whereas the role of *E. tenella* is unknown. Most studies on parasite proteins that modify host cells have been conducted through genetic manipulation. It is possible that this aspect of *E. tenella* research has been delayed due to the difficulty of continuous culture in vitro and genetic operation of *E. tenella*. There have not yet been reports on *E. tenella* proteins located in the nucleus of host cells. Whether EtROP30 plays an important role in the regulation of host cells and parasite infection needs to be further studied.

Expression levels at different developmental stages are usually related to the roles of proteins in the parasitic life cycle. In this study, EtROP30 showed significantly higher expression levels in the merozoite stage. The merozoite stage is crucial for parasite reinvasion and development and is a vulnerable stage in immunology (Rafiqi et al. 2018). Therefore, proteins highly expressed in the merozoite stage are conducive to be ideal vaccine candidates (Rezaei et al. 2019). Animal experiments have shown that EtROP30 can induce effective immune protection against *E. tenella* in chickens. In studies on anti-*T. gondii*, ROPs have been targeted as promising vaccine candidates with achievement of remarkable results (Faridnia et al. 2018). Nevertheless, studies on vaccine development with ROPs of *E. tenella* are rare. Et-ROPK-Eten5-A, the only ROP vaccine for *E. tenella* to date, showed significant effects as a vaccine and was superior to Et-GRA12, Et-SAG and Et-SAG13 (Song et al. 2020). The relative weight gain of EtROP30-immunized chickens (75.92%) was better than that of Et-ROPK-Eten5-A immunized chickens (47%). The oocyst reduction rate was lower in EtROP30 (61.43%) than Et-ROPK-Eten5-A (82.75%). The large gap in mean lesion scores between the two unimmunized control groups in the protective evaluation of EtROP30 and Et-ROPK-Eten5-A could be caused by different strains and different challenge doses. Therefore, we cannot tell which vaccine is more effective in reducing intestinal injury.

Declarations

Ethics approval The study protocol and all associated animal studies were approved by the Animal Care and Use Committee of Shandong Agricultural University (approval number: SDAUA-2021–043).

Conflict of interest The authors declare no competing interests.

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