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Lab Note

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Identification of host protein CBL interacting with *Eimeria acervulina* microneme protein MIC3

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Eimeria acervulina severely damages the intestinal mucosa in infected poultry, resulting in major economic losses [1]. The study of the interaction between *Eimeria* and host cells is the theoretical basis for the prevention and treatment of coccidiosis [2]. However, the specific mechanism of *E. acervulina* invasion is not very clear, so the interaction between the invading protein and the host awaits extensive study.

Previous studies have shown that microneme proteins (MICs) play important roles in host-cell invasion [3]. The majority of MICs is adhesions that bind to host cells during invasion. As one of the most extensively studied MICs, *E. acervulina* microneme protein 3 (EaMIC3) plays an important role in its invasion and colonization. It was reported that EaMIC3 is expressed at the stage of sporozoite and merozoite and is located at the apex of parasite. Immunofluorescence experiments showed that EaMIC3 only binds with the specific sites located in the chicken duodenal epithelial cells. EaMIC3, the major protective antigen, could also significantly protect chickens from *E. acervulina* infection. EaMIC3 has seven microneme adhesive repeat (MAR) domains, and EaMIC3-MAR3 has the strongest binding ability to epithelial cells [4]. These studies suggested that the EaMIC3-MAR3 protein may be the key molecule involved in the invasion process.

Few studies have focused on the receptor of EaMIC3. In order to identify the ligand-receptor molecules that may play an important role in the invasion process of *E. acervulina*, the yeast two-hybrid system was used to screen the associated protein from the yeast complementary DNA (cDNA) library of chicken duodenal epithelium cells using EaMIC3-MAR3 as the bait.

In this experiment, the yeast cDNA library of chicken duodenal epithelium cells in the pGADT7 vector was constructed using Matchmaker Library Construction and Screening kit (Clontech, Palo Alto, USA) as the prey. The preys containing a Gal4 activation domain (AD preys) were transformed into yeast strain Y187. The capacity of yeast cDNA library was 7.4×10^6 cfu and the insert fragment length in the constructed yeast library was 800–2000 bp (**Supplementary Fig. S1**). The efficiency of transformation and the size of the insert fragment satisfied the quality of the yeast library. For yeast two-hybrid screening, EaMIC3-MAR3 was amplified by polymerase chain reaction (PCR) using forward primer 5′-CCC*CATATG*CGCGGATCCATGCATATCCGCCTTGTGCTGT-3′ and reverse primer 5′-CCC*CTGCAG*ACGCGTCGACCGCAATTGCA GGTTCCATAGGAG-3′ from *E. acervulina* cDNA, and then ligated into the *Nde*I-*Pst*I sites of pGBKT7 as the bait (**Fig. 1A**). The recombinant plasmid was transformed into Y2HGOLD yeast cells, and the transformants were separately grown on plates containing the minimal yeast medium without tryptophan (SD/−Trp), SD/−Trp supplemented with 40 μg/ml X-α-Gal (SD/−Trp/X) or SD/−Trp supplemented with 40 μg/ml X-α-Gal and 125 ng/ml Aureobasidin A (SD/−Trp/X/A). Then, the expression of the recombinant plasmid pGBKT7-EaMIC3-MAR3 in the yeast was detected by western blot analysis using anti-myc monoclonal antibody (Epitomics, Burlingame, USA) (**Fig. 1B**). The baits were tested for self-activation and the results showed that EaMIC3-MAR3 protein can be expressed in yeast without self-activation (**Fig. 1C**).

Next, we carried out a yeast two-hybrid screening using EaMIC3-MAR3 as the baits. To search for the host proteins that interact with MIC3, we used Y2HGold cells harboring the pGBKT7-EaMIC3-MAR3 plasmid to mate with Y187 cells containing the chicken duodenal epithelium cell cDNA library. After mating reaction between pGBKT7-EaMIC3-MAR3-transformed Y2HGold and Y2HGOLD containing *E. acervulina* cDNA library, we obtained eight positive clones in selection medium SD/−Leu/−Trp/X/A. The prey plasmids were rescued through transformation of *Escherichia coli* DH5α cells. The specific insert on each prey plasmid was amplified by PCR using primers originated from the vector backbones and analyzed by gel electrophoresis (data not shown). To eliminate false positive hits and retest the specificity of interaction, each of the eight prey plasmids was co-transformed with pGBKT7-EaMIC3-MAR3 into Y2HGold cells and the co-transformants were tested on the SD/−Ade/-His/−Leu/−Trp/X/A plates. The results indicated that one of the eight preys still showed the positive interaction with the MIC3 bait (**Fig. 2A–C**). These results showed that the insert contained the full-length cDNA of Casitas B-lineage lymphoma (CBL) proto-oncogene E3 ubiquitin-ligase enzymes, which have 100% similarity

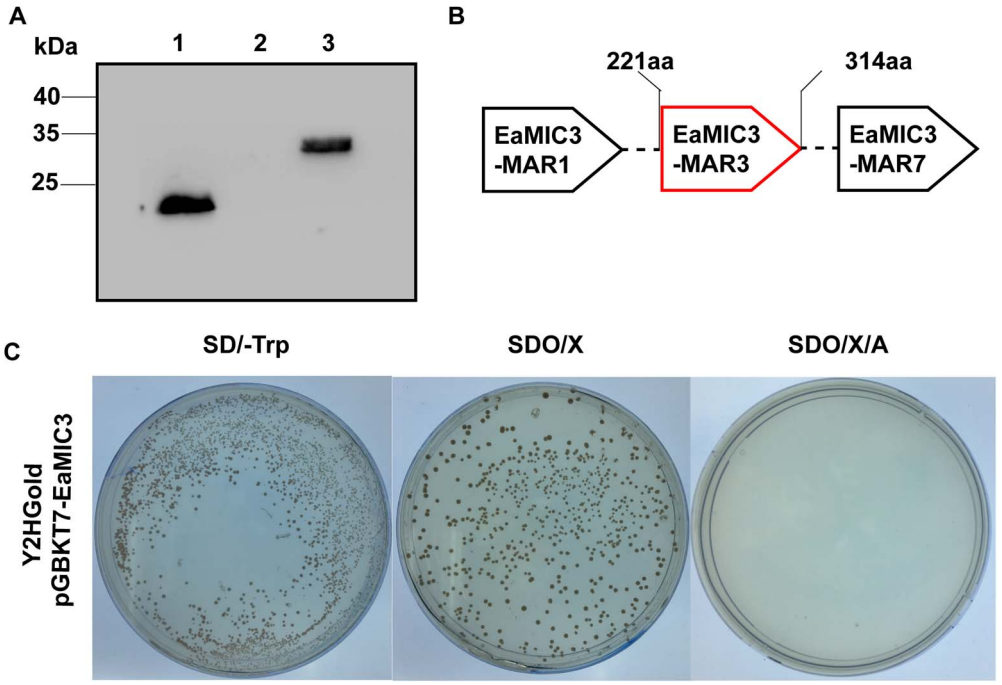

Figure 1. Expression and auto-activation of EaMIC3-MAR3 baits in yeast cells (A) Western blot analysis on lysate of the yeast strain Y2HGold containing the following bait plasmids. 1, pGBKT7-EaMIC3-MAR3; 2, untransfected yeast strain Y2HGold; 3, pGBKT7. (B) Schematic illustration of EaMIC3-MAR3 domain used as the bait in the yeast-two-hybrid screen. (C) Determination of the auto-activation activity of EaMIC3-MAR3 baits in yeast cells.

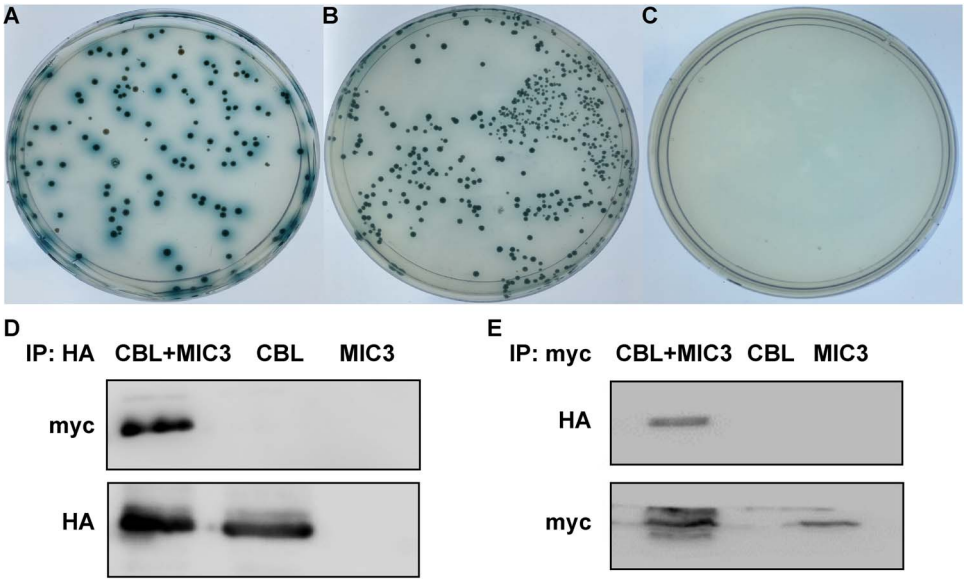

Figure 2. Confirmation of the interactions between EaMIC3 and CBL protein Y2HGOLD strains were transformed with (A) pGBKT7-EaMIC3+pGADT7-CBL, (B) pGBKT7–53+pGADT7-T used as positive control, and (C) pGBKT7-Lam+pGADT7-T used as the negative control. Interaction of EaMIC3 with CBL in mammalian cells. 293T cells were transfected with the expressing plasmids pcDNA-Myc-EaMIC3 only, pcDNA-HA-CBL only, and pcDNA-Myc-EaMIC3+pcDNA-HA-CBL. (D) Immunoprecipitation was performed using anti-HA antibodies and detected by western blot analysis using anti-Myc antibodies. (E) Immunoprecipitation was performed using anti-Myc antibodies and detected by western blot analysis using anti-HA antibodies.

with the known gene of CBL (NM_204208.1). The putative functions of CBL are shown in Table 1. Thus, the host protein CBL was identified to interact with MIC3 and was further analyzed by co-immunoprecipitation.

The full length of EaMIC3 was cloned into pcDNA3.1-Myc, and the full length of CBL was cloned into pcDNA3.1-HA. 293T cells were transfected with recombinant plasmids pcDNA-Myc-EaMIC3 and pcDNA-HA-CBL, pcDNA-Myc-CBL only, or pcDNA-HA-EaMIC3 only, using Lipofectamine 2000 (Invitrogen, Carlsbad, USA) according to the manufacturer's instruction. Twenty-four hours after transfection, protein extracts were harvested using lysis buffer, then pre-cleared with protein A beads. The pre-cleared extracts were

Table 1. BLAST results and putative functions of the positive hit from the yeast two-hybrid screen

GenBank ID	Homologs in GenBank	Putative function
NM_204208.1	E3 ubiquitin-ligase enzymes CBL	Regulation of receptor recycling Ubiquitination of target protein Apoptosis Cell proliferation Cellular protein localization

incubated with protein A beads and anti-HA (Proteintech, Chicago, USA) or anti-Myc (Epitomics, Burlingame, USA) antibodies. The beads were washed sequentially with lysis buffer, and finally the bound proteins were eluted for western blot analysis [5]. The result showed that Myc-tagged EaMIC3 was co-precipitated with HA-tagged CBL (Fig. 2D). Subsequently, we conducted co-IP with an anti-Myc antibody followed by western blot analysis with an anti-HA antibody. The results showed that EaMIC3 bound with CBL but not with the control (Fig. 2E). These results confirmed that MIC3 interacts with CBL. CBL has not been reported to interact with MIC3 before. CBL E3 ubiquitin-ligase enzymes may play an important role in the process of *E. acervulina* invasion during the ubiquitin–proteasome pathway of EaMIC3. Taken together, we discovered the interaction between MIC3 and CBL, which may help to understand the cellular functions of EaMIC3 in the invasion process of *E. acervulina*.

Supplementary Data

Supplementary data is available at *Acta Biochimica et Biophysica Sinica* online.

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