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Short communication

Grouper (*Epinephelus coioides*) MyD88 adaptor-like (Mal): Molecular cloning, expression, and functionality

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ABSTRACT

Initiation of the innate immune response requires recognition of pathogen-associated molecular patterns by pathogen recognition receptors such as Toll-like receptors (TLRs). MyD88 adaptor-like (Mal) is an adaptor that responds to TLR activation and acts as a bridging adaptor for MyD88. In the present study, the open reading frame of Mal was identified in orange-spotted grouper (*Epinephelus coioides*), and named EcMal. It contained 831 bp encoding 276 aa, and was encoded by a 1299 bp DNA sequence with three exons and two introns. EcMal and the Mal sequence of other species shared different degrees of sequence identity, and clustered into the same group. EcMal was distributed in all tissues tested in healthy grouper, with the highest expression level in the head kidney. After infection with *Cryptocaryon irritans*, the expression level of EcMal was up-regulated in the gill and spleen. In addition, EcMal exhibited global cytosolic and nucleus localization, and could significantly activate NF-κB activity in grouper spleen cells.

1. Introduction

Toll-like receptors (TLRs) are well-studied pattern-recognition receptors responsible for sensing and responding to invading pathogens [1,2]. Twelve TLRs have been found in humans [3]. They recognize various pathogen-associated molecular patterns, such as lipopeptides, lipopolysaccharide, flagellin, ss/dsRNA, unmethylated CpG-ODN, and profilin-like molecule, which are conserved among microbial species. After binding to their respective ligands, TLRs initiate downstream signaling events by interaction with adaptor proteins containing the Toll-interleukin 1 receptor (TIR) domain, and activation of transcription factor NF-κB, MAPKs, and IRFs to induce the expression of pro-inflammatory cytokines, and type I interferon [4]. Five TIR domain-containing proteins have been identified in mammals, including myeloid differentiation primary response gene (88) (MyD88), MyD88 adaptor-like (Mal), TIR domain-containing adaptor protein inducing interferon-β (TRIF), TRIF-related adaptor molecule, and sterile α- and armadillo-motif-containing protein [5]. MyD88 is common to all TLRs, with the exception of TLR3, which uses TRIF as an adaptor protein. Mal is a homologue of MyD88 in TLR2 and TLR4 signaling [6,7]; it acts as a bridging adaptor for MyD88. At the N-terminus, Mal contains a phosphatidylinositol 4, 5-bisphosphate (PIP2) binding domain, which directs Mal to the plasma membrane and interacts with the TIR domain of receptors [8]. Then, Mal recruits MyD88 through its C-terminal TIR domain. Therefore, Mal plays a bridging role for receptors and MyD88.

Although numerous studies report the function of Mal in mammals, the role of Mal in fish is unclear. Liu et al. found that zebrafish Mal could activate NF-κB in CLC cells, but not in HEK293T cells [9]. Xu et al. indicated that miiuy croaker Mal does not induce NF-κB activation, and TLR1 activates NF-κB dependent on both Mal and MyD88 in HEK293T cells [10]. In addition, Shan et al. found that common carp TLR8 used Mal as an adaptor, but not MyD88 or TRIF [11]. To our best knowledge, no other report has focused on fish Mal.

Orange-spotted grouper (*Epinephelus coioides*) is a commercially important fish species reared in south China and is often infected by *Cryptocaryon irritans*. In the present study, we identified the open reading frame (ORF) of Mal from grouper. Mal tissue expression was analyzed in healthy grouper. The expression profile of Mal in local

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infection sites (gill) and immune system organs (spleen) was also analyzed after infection with *C. irritans*. In addition, the function of grouper Mal in NF-κB signaling was analyzed in grouper spleen (GS) cells.

2. Materials and methods

2.1. Fish, parasites, and sampling

Healthy orange-spotted grouper (12.3 ± 3.3 g) were purchased from the Marine Fisheries Development Center of Guangdong Province, China. No disease outbreak occurred during the course of breeding. Fish were acclimated for two weeks at 25 °C and 30‰ salinity in a flow-through water system, and fed daily with a commercial grouper feed. Samples of brain, head kidney, gill, heart, spleen, thymus, muscle, skin, liver, trunk kidney, and intestine were sampled from three healthy groupers to analyze the expression profile of Mal.

Cryptocaryon irritans were maintained by serial passage on *Trachinotus ovatus* as described previously [12]. Grouper were infected with *C. irritans* as described previously [13]; samples of gill and spleen were taken at 6 and 12 h, and at 1, 2, 3, 5, and 7 d post challenge from five infected and five control fishes.

2.2. RNA extraction, cDNA synthesis, and DNA isolation

Total RNA was extracted using Trizol reagent (TaKaRa, Japan) according to the manufacturer's protocol. Agarose gel electrophoresis and OD260/280 measurements were used to detect the integrity and concentration of the RNA. CDNA was synthesized from total RNA (1 μg) using ReverTraAce-a reverse transcriptase (Toyobo, Japan). DNA was isolated from grouper fin using the TIANamp Marine Animals DNA Kit (Tiangen, China) following the manufacturer's protocol. DNA and cDNA were stored at −20 °C until use.

2.3. Characteristics and sequence analysis of grouper Mal

Using the Mal sequence of other fish species as bait, we retrieved a unigene sequence from the grouper transcriptome data generated by our lab (unpublished data). Multiple alignment of this unigene showed higher sequence identity with Mal of other fish species, according to the NCBI blast program (https://blast.ncbi.nlm.nih.gov/Blast.cgi). ORF Finder program (http://www.ncbi.nlm.nih.gov/gorf/gorf.html) analysis indicated that this unigene contained the complete open reading frame (ORF) of EcMal. Therefore, primers of (MalFLF/R and DMalF/R, STable 1) were designed to amplify the mRNA and DNA sequence of EcMal ORF.

Isoelectric point (pI) and molecular weight (Mw) were calculated using the Expasy program (http://web.expasy.org/compute_pi/). Multiple sequence alignment was performed with ClustalW2 (http://www.ebi.ac.uk/Tools/clusta-lw2/index.html). A three dimensional model was constructed using SWISS-MODLE software (http://swissmodel.expasy.org/interactive). Protein structure was predicted using the SMART program (http://smart.emblheidelberg.de/). A phylogenetic tree was constructed with the MEGA 5.0 program using the neighbor-joining method and 1000 bootstrap replications.

2.4. Expression analysis

The Roche LightCycler 480 Real-time PCR Detection System (Roche) and SYBR Green Real-time PCR Master Mix (Takara, Japan) were used to detect the expression levels of EcMal. Primers (qMalF/R and β-actinRTF/R, STable 1) were designed to amplify EcMal and β-actin. The PCR conditions were as follows: 95 °C for 3 min, followed by 40 cycles at 94 °C for 5 s and 68 °C for 30 s. The specificity of PCR products was evaluated by melting curve analysis and sequence analysis. The mRNA expression level of target genes was calculated by the $2^{-\Delta\Delta Ct}$ method using reference genes [14].

2.5. Construction of plasmids

The plasmids pEGFP-EcMal-TAA and pEGFP-EcMal were constructed for reporter gene assays and subcellular localization of EcMal. The ORF of EcMal was amplified using primers containing restriction sites EcoRI and BamHI at the 5′ terminal, with (rF1 and rR1, STable 1) or without (rF1 and rR2, STable 1) stop codons. After double digestion, the target sequences were ligated with pEGFP-N1 using T4 DNA ligase (Takara), following the manufacturer instructions. Before use, the plasmids were extracted using an E.Z.N.A.® Endo-free Plasmid Mini Kit (Omega, USA) following the manufacturer instructions.

2.6. Cell culture and transfection

GS cells, kindly provided by Prof. Qiwei Qin (College of Marine Sciences, South China Agricultural University), were cultured in Leibovitz's L-15 Medium (Gibco, USA) with 10% fetal bovine serum at 28 °C, and passaged every three days. Twenty-four hours before transfection, cells were seeded into 24-well (5×10^5 cells/well) plates. Transfection was performed using Lipofectamine 3000 reagent (Invitrogen, USA), following the manufacturer instructions.

2.7. Cellular localization

L-lysine-treated coverslips were put in 24-well plates, and GS cells were seeded according to section 2.6. PEGFP-EcMal or pEGFP-N1 plasmids (500 ng) were transfected into GS cells. Forty-eight hours after transfection, cells were fixed with 4% paraformaldehyde for 10 min, and then 0.5% Triton X-100 was added for 15 min for membrane permeabilization. Finally, 1 μg/mL DAPI (4′,6-diamidino-2-phenylindole) was added into each well for nuclear staining for 5 min. The coverslip was removed and placed on the top of the slide with a drop of anti-fade mounting medium. Localization of EcMal was observed using a Nikon ECLIPSE Ni–U microscope (Tokyo, Japan).

2.8. Reporter gene assay

GS cells were transfected according to methods in section 2.6, with 500 ng of pEGFP-EcMal-TAA or pEGFP-N1, 150 ng of NF-κB reporter plasmids, and 40 ng of pRL-SV40 Renilla (*Renilla reniformis*) luciferase plasmids. Three replicates for each sample were performed. Forty-eight hours after transfection, cells were collected, and luciferase activity was detected with a Dual-Luciferase Reporter Assay System (Promega, USA), according to the manufacturer protocol. Relative luciferase activity was calculated as the firefly luciferase activity relative to the Renilla luciferase activity.

2.9. Statistical analysis

All data were analyzed using one-way analysis of variance and Duncan's test in SPSS (version 22.0). Data are expressed as mean ± SE. The statistical significance threshold was set at $P < 0.05$.

3. Results and discussion

3.1. Characteristics of the EcMal sequence

The ORF of EcMal (GenBank No. MK864108) was 831 bp; it encoded 276 aa (Fig. 1A) with a theoretical pI and Mw of 6.95 and 29.7 kDa, respectively. EcMal ORF was encoded by a 1299 bp DNA sequence, which contained three exons and two introns. The genomic structure of EcMal ORF was similar to that of other piscine species, such as *Larimichthys crocea* and *Danio rerio* (Fig. 1B).

It has been demonstrated that the TIR domain of Mal is crucial for recruiting MyD88 to the activated TLR [8,15], and there are conserved boxes in this domain [16]. SMART analysis and multiple sequence

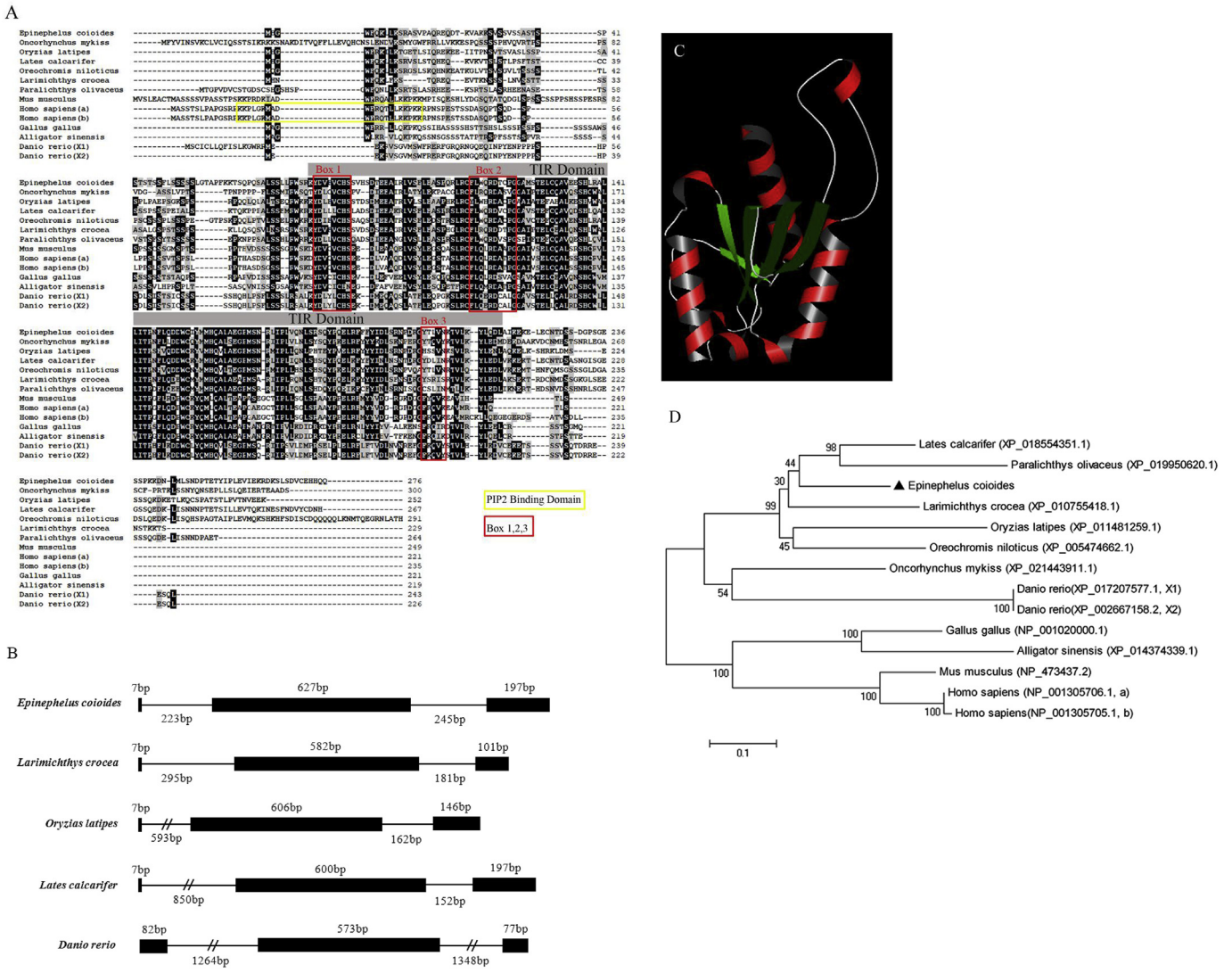

Fig. 1. (A) Multiple alignment of EcMal. The yellow box represents the PIP2 binding domain of human Mal; red boxes represent the three conserved boxes in the TIR domain. (B) Genomic DNA structure of Mal from *Epinephelus coioides*, *Lates calcarifer*, *Larimichthys crocea*, *Oryzias latipes*, and *Danio rerio*. (C) The 3D model of the EcMal TIR domain. The model was constructed using human Mal (2y92.1) as a template. (D) Phylogenetic tree constructed using MEGA5.0. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

alignments of EcMal indicated that Mal from grouper and other fish species also contained a TIR domain with conserved box1 and box2; box3 was not conserved among the fish species (Fig. 1A). Similarly to humans [17] (2y92.1), the 3D structure of the EcMal TIR domain consisted of five β-sheets surrounded by several α-helixes (Fig. 1C), implying that EcMal may perform similar functions. In mammals, Mal has a PIP2 binding motif (KKPLGKMADWFRQTLLKKPKK) at the N-terminus, which mediates the recruitment of Mal to the plasma membrane [8]. However, this motif was not conserved in the Mal of fish, chicken, and alligator (Fig. 1A); zebrafish and grouper Mal activate NF-κB, implying that this motif is not indispensable to its function in fish [9].

Multiple alignments of EcMal revealed that full-length EcMal shares different degrees of sequence identity with Mal of other species (STable 2). In brief, full-length EcMal shared 43%–65% sequence identity with that of other fish species; the highest sequence identity was with *Lates calcarifer* (65%), and the lowest with other vertebrates (42%–46%). Phylogenetic analysis revealed that Mal from grouper and from other fish species clustered into one group, and Mal from amphibians, chickens and mammals clustered into another group (Fig. 1D). In the fish group, EcMal had the closest relationship with that of Mal from *L. calcarifer* and *Paralichthys olivaceus*.

3.2. Expression analysis of EcMal

EcMal was detected in all tissues tested in healthy *E. coioides*, with the highest expression in head kidney, followed by thymus and trunk kidney, and with the lowest expression in the muscle and intestine (Fig. 2A). Both kidney and thymus are important immune organs; therefore, higher expression of EcMal in these tissues may imply a vital role of EcMal in host immune responses.

It has been demonstrated that Mal is required against *Leishmania* and *Trypanosoma cruzi* infection in humans and mice [18–23]. In zebrafish, the expression of Mal increased after *Mycobacterium* infection [24]. However, the role of Mal in anti-parasitic infections response in fish is unclear. After infection with *C. irritans*, the expression of EcMal was significantly up-regulated in the gill at day 2 and day 5, with the highest level at day 2 (Fig. 2B). In the spleen, however, the expression of EcMal was up-regulated from 12 h to day 5, with the highest expression occurring on day 5 (Fig. 2C). At day 7 post infection, the expression of EcMal was significantly down-regulated (Fig. 2C). These results imply that EcMal is involved in the host *anti-C. irritans* infection

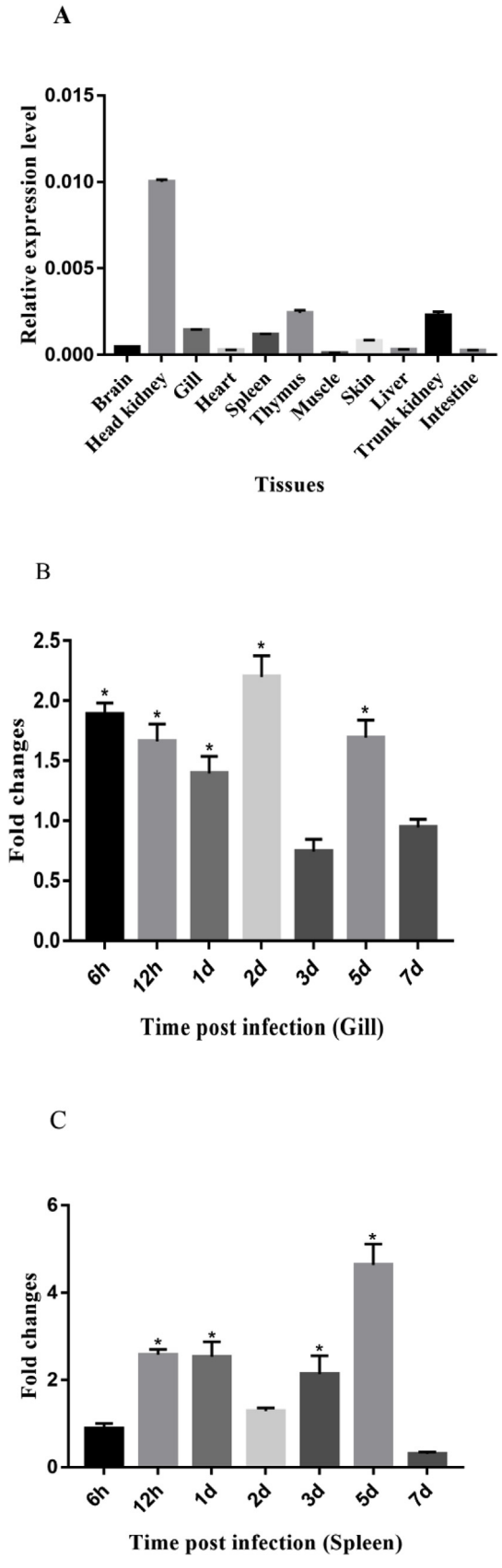

Fig. 2. (A) Tissue expression of EcMal in healthy grouper. The mRNA expression levels of EcMal were examined by real-time PCR, after normalizing to β-actin transcripts. Data are shown as mean ± SE (N = 3). (B) and (C) show the expression analysis of EcMal in the gill and spleen, respectively, at various time points after *C. irritans* infection. The mRNA expression level of EcMal was first normalized to β-actin; the graph shows the ratio of the treatment group to the control group. The data are shown as mean ± SE (N = 5), and significant differences ($P < 0.05$) are indicated by asterisks (*).

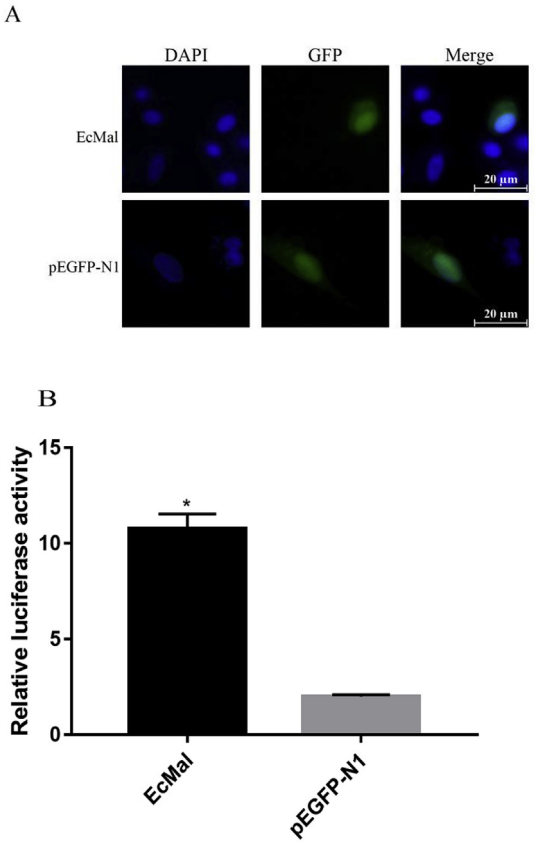

Fig. 3. (A) Subcellular localization of EcMal in grouper spleen (GS) cells. Green fluorescence shows the position of the proteins; blue DAPI staining shows the position of the nucleus. (B) Over-expression of EcMal activates NF-κB in GS cells. GS cells were seeded in 24-well plates and then co-transfected with the NF-κB-dependent firefly luciferase reporter gene, and the Renilla luciferase gene. Forty-eight hours after transfection, cells were collected for detecting luciferase activity. Data represent three replicates; bars are mean ± SE. Significant differences ($P < 0.05$) are indicated by asterisks (*). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

response.

3.3. Subcellular localization and luciferase reporter assay of EcMal

We first examined the subcellular localization of EcMal in GS cells. Similar to the localization of Mal in mammals and zebrafish [8,9], EcMal is distributed throughout the nucleus and cytoplasm (Fig. 3A). To explore the role of EcMal, a luciferase reporter assay was used to analyze NF-κB activity induced by EcMal. Overexpression of EcMal significantly activated NF-κB in GS cells (Fig. 3B), as in zebrafish and humans, suggesting that the role of Mal is conserved between mammals and fish [9,25].

In summary, the ORF of grouper Mal was identified and detected in many tissues in healthy grouper. After infection with *C. irritans*, Mal expression was up-regulated in the gill and spleen. EcMal exhibits

global cytosolic and nucleus localization, significantly enhancing NF-κB activity, demonstrating that EcMal has a positive effect on TLR signaling.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fsi.2019.07.065.

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