

The circular RNA circBCL2L1 regulates innate immune responses *via* microRNA-mediated downregulation of TRAF6 in teleost fish

Received for publication, June 23, 2021, and in revised form, September 9, 2021 | Published, Papers in Press, September 15, 2021,

<https://doi.org/10.1016/j.jbc.2021.101199>

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Edited by Karin Musier-Forsyth

Growing numbers of studies have shown that circular RNAs (circRNAs) can function as regulatory factors to regulate the innate immune response, cell proliferation, cell migration, and other important processes in mammals. However, the function and regulatory mechanism of circRNAs in lower vertebrates are still unclear. Here, we discovered a novel circRNA derived from the gene encoding Bcl-2-like protein 1 (*BCL2L1*) gene, named circBCL2L1, which was related to the innate immune responses in teleost fish. Results indicated that circBCL2L1 played essential roles in host antiviral immunity and antibacterial immunity. Our study also identified a microRNA, miR-30c-3-3p, which could inhibit the innate immune response by targeting inflammatory mediator TRAF6. And TRAF6 is a key signal transduction factor in innate immune response mediated by TLRs. Moreover, we also found that the antiviral and antibacterial effects inhibited by miR-30c-3-3p could be reversed with the expression of circBCL2L1. Our data revealed that circBCL2L1 functioned as a competing endogenous RNA (ceRNA) of TRAF6 by competing for binding with miR-30c-3-3p, leading to activation of the NF- κ B/IRF3 inflammatory pathway and then enhancing the innate immune responses. Our results suggest that circRNAs can play an important role in the innate immune response of teleost fish.

As the first line of defense against foreign pathogens, the innate immune system mainly relies on various pattern recognition receptors (PRRs) to quickly recognize various pathogen-associated molecular patterns (PAMPs) carried by foreign pathogens and quickly transmit the signal to the key downstream adapter proteins to activate various host immune responses (1). This process can make the host cells produce type I interferon, inflammatory factors, or chemokines in time to remove the invading host pathogens, so as to protect the host from the invasion of pathogenic microorganisms (2). As a major type of PRRs, Toll-like receptors (TLRs) are primarily responsible for the recognition of extracellular pathogens or

pathogens that enter the endosome by endocytosis (3–5). Unlike the TLRs, retinoic-acid-inducible gene-I (RIG-I)-like receptors (RLRs) primarily act as virus sensors, which are responsible for monitoring the virus invading the cytoplasm (3, 4). In the face of the ubiquitous threat of pathogenic microorganisms, all PRRs must cooperate to form a complex immune recognition network, which is the basis for the healthy survival of the host. After these PRRs recognize specific PAMPs, they need specific adaptor molecules to transmit signals to downstream signaling pathways to activate innate immunity. For example, the adaptor molecules of TLRs are myeloid differentiation factor88 (MyD88) and Toll–interleukin 1 receptor domain–containing adaptor molecule (TRIF, also called TICAM-1) (6, 7), while RLRs transmit signals through mitochondrial antiviral signaling protein (MAVS) (8). In the process of these adaptor molecules transmitting signals to downstream signaling pathways, they are often inseparable from the tumor necrosis factor receptor–associated factors (TRAFs), which play an extremely important role in the signaling pathway (9–11).

MicroRNA (miRNA) is a class of highly conserved ncRNAs with a length of only 21 to 24 nucleotides (12, 13). With the development of research in recent decades, miRNAs have been proved to be a kind of very important regulatory factor in organisms. At the same time, a large number of research results show that miRNAs play an irreplaceable role in cell proliferation, growth, apoptosis, and differentiation (13). By binding to the 3'-untranslated region (UTR) of target mRNA, miRNAs inhibit gene expression by inhibiting mRNA translation or promoting mRNA degradation. Recent studies have shown that miRNAs play a key role in the regulation of bacterial or viral induced immune responses among different vertebrates. For example, in mammals, miR-146a can inhibit the induction of interferon by targeting TRAF6 and promote the replication of dengue virus (14); At the same time, miR-146a can negatively regulate the secretion of proinflammatory cytokines and cell activation by inhibiting the TLR4 signaling pathway in LPS-stimulated cells (15). In teleost fish, miR-217 modulates the antibacterial and antiviral

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circRNA regulates innate immune responses in fish

immunity through TAK1-mediated NF- κ B and IRF3 signaling pathways (16). In addition, our previous work also has revealed that miR-217-5p can negatively regulate NOD1, and both suppress the antibacterial and antiviral immune response in miiuy croaker (*Miichthys miiuy*) (17).

TRAFs are cytoplasmic adapter proteins, which are initially discovered as adaptor proteins that couple the tumor necrosis factor receptor family to signaling pathways (18, 19). At present, a total of seven members of the TRAF family have been identified, namely TRAF1-7, and their roles in signal transmission are irreplaceable (20, 21). Most TRAF proteins have an N-terminal ring finger domain and several zinc finger motifs (10, 22–24). TRAF regulates the function of E3 ubiquitination ligase through its ring finger domain mediated multi-ubiquitination of lys-63 (25). As one of the TRAFs, TRAF6 is a key signal transduction factor in innate immune response mediated by TLRs, nucleotide-binding oligomerization domain-like receptors (NLRs), and RLRs (26). In TLRs and NLRs signaling, TRAF6 recruits and activates NF- κ B essential modulator-IKK α / β and TGF- β -activated kinase 1 (TAK1), resulting in the activation of NF- κ B and AP-1, which depend on its E3 ligase activities and Lys-63-linked polyubiquitination (27, 28). In RLRs signaling, TRAF6 can activate downstream IRF3 and NF- κ B by binding to two potential TRAF-interacting motifs (TIMs) (T6BM1, amino acids 153–158; T6BM2, amino acids 455–460) on MAVS (29). As an important cytoplasmic adapter, TRAF6 not only participates in the regulation of inflammation and immune system, but also has many other physiological functions, including proliferation, cell survival, apoptosis, and so on (30–32). The abnormal expression of TRAF6 often causes some very serious autoimmune diseases, so appropriate regulation is very important for TRAF6. In recent decades, the discovery of a new class of regulatory factors miRNA has deepened people's understanding of TRAF6 homeostasis in mammals. For example, miR-146a can inhibit the inflammation of lupus nephritis induced by targeting TRAF6 (33). Moreover, it has been found that miR-144 can attenuate host response to influenza virus by targeting TRAF6-IRF7 signal axis (34). In addition to the regulators that can regulate TRAF6 in mammals, Gao *et al.* have recently found a class of miRNAs that can negatively regulate the TRAF6-mediated NF- κ B and IRF3 signaling pathway in teleost. They have found that miR-2187 and miR-489 can serve as negative regulators of TRAF6 to regulate its mediated immune response and prevent the excessive immune response of the body (35, 36). Although the research of TRAF6 in mammals has been more perfect, we still know little about the role of TRAF6 in innate immune response in lower vertebrates, such as teleost fish; therefore, exploration of the regulatory mechanism of TRAF6-mediated signal transduction is urgently needed.

As a kind of popular noncoding RNAs, the function and significance of circular RNAs (circRNAs) in organisms are gradually being explored. Unlike other traditional noncoding RNAs, circRNA is a covalently closed circular molecule produced by back splicing, and it is first found in the hepatitis

D virus in the 1970s (37). At first, circRNA was considered as a meaningless by-product of genome transcription, but with the deepening of research, it has been proved that circRNA is a very important regulatory factor, which plays an irreplaceable role in the biological process of immunity, promotion, invasion, metastasis, and so on (38–40). Up to now, circRNA has been confirmed to play corresponding functions mainly in three forms, including binding to RNA-binding protein (RBP), involving in protein translation, and can act as sponges of miRNAs to serve as competing endogenous RNAs (ceRNAs) (41–43). Moreover, a large number of studies have reported that circRNA can act as a miRNA sponge to participate in antiviral immune response. For example, CircRNA_0050463 promotes influenza virus replication by sponging miR-33b-5p to regulate EEF1A1 in mammals (44). In addition, our previous studies have shown that circRNA Dtx1 can promote TRIF-mediated antiviral signaling pathway by targeting miR-15a-5p in teleost fish (45). Although there has been evidence that circRNA can regulate antiviral related immune genes through the ceRNA mechanism in lower vertebrates, it is not particularly known whether circRNA can regulate antibacterial immune response by acting as ceRNA in lower vertebrates.

In this study, we identify a ceRNA regulatory network involved in innate immune response in teleost fish, miiuy croaker (*M. miiuy*). Our previous studies have demonstrated the important role of TRAF6 in mediating antiviral and antibacterial immune responses. Here, we have found that miR-30c-3-3p targets TRAF6 and suppresses TRAF6-mediated innate immune responses. Furthermore, our study suggests that a circular RNA, namely circBCL2L1, can serve as a ceRNA for miR-30c-3-3p to facilitate TRAF6 expression, thereby modulating TRAF6-mediated innate responses. Our results not only enrich the biological mechanism of circRNA-miRNA-mRNA axis in fish innate immune response, but also provide new ideas for the study of innate immune regulation in lower vertebrates.

Results

Characterization of CircBCL2L1 involved in innate immunity

In mammals, a large number of circRNAs have been found to participate in the antiviral immune response (46), but little is known about the role of circRNAs in immune response in lower vertebrates. We compared the expression level of circRNA after SCR infection with previous RNA-seq data (GenBank accession number: PRJNA685924) and found that the expression of circBCL2L1 was significantly upregulated after SCR infection (47). We treated miiuy croaker with SCR and LPS to further confirm the reliability of RNA-seq data, sampled tissues at different times to extract RNA, and then quantitatively analyzed the expression level of circBCL2L1 by quantitative real-time polymerase chain reaction (qPCR). In addition, considering that circRNAs were produced by linear RNA splicing, the expression levels of linear Bcl-2-like protein 1 (*BCL2L1*) (GenBank accession

number: MZ164593) and circBCL2L1 were also detected. The qPCR results confirmed that circBCL2L1 was significantly upregulated under SCR and LPS stimulation compared with

linear *BCL2L1* (Fig. 1A). In addition, SCR-treated MKC cells further confirmed the significant expression of circBCL2L1 (Fig. 1B). We then evaluated the expression levels of

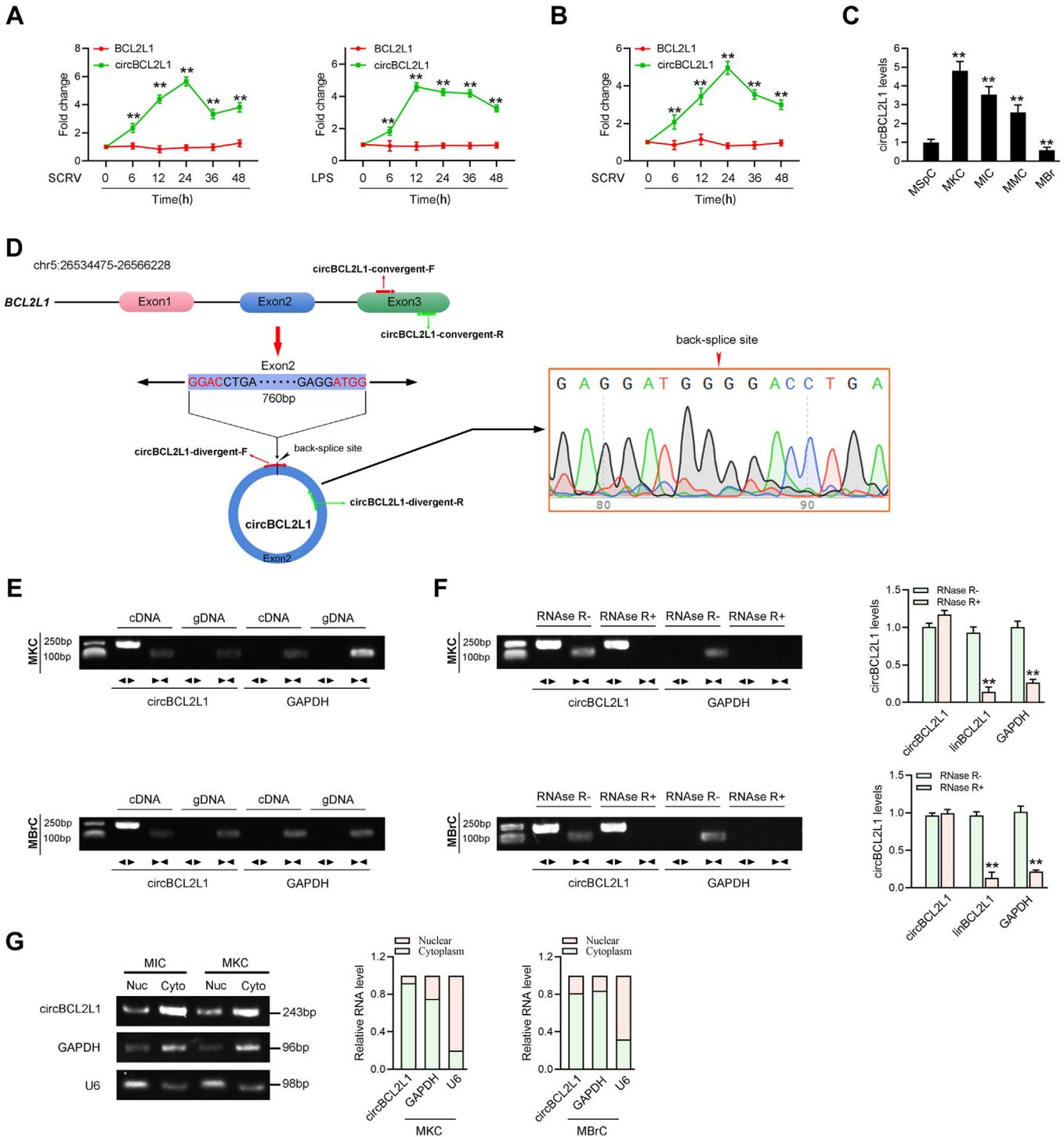


Figure 1. Expression profiles and characterization of circBCL2L1. A, qPCR for the abundance of circBCL2L1 and linear *BCL2L1* (*BCL2L1*) mRNA in spleen tissues treated with SCR (MOI = 5) and LPS at the indicated time points, respectively. B, qPCR analysis of circBCL2L1 and linear *BCL2L1* mRNA in MKC cells treated with SCR (MOI = 5) at the indicated time points. C, relative expression of circBCL2L1 in indicated cell lines was determined by qPCR. D, we confirmed the head-to-tail splicing of circBCL2L1 in the circBCL2L1 RT-PCR product by Sanger sequencing. We labeled the location of circBCL2L1-divergent-F/R and circBCL2L1-convergent-F/R primers. E, RT-PCR validated the existence of circBCL2L1 in MBrC and MKC cell lines. circBCL2L1 was amplified by divergent primers in cDNA but not gDNA. GAPDH was used as a negative control. F, the expression of circBCL2L1 and linear *BCL2L1* mRNA in both MBrC and MKC cell lines was detected by RT-PCR assay followed by nucleic acid electrophoresis or qPCR assay in the presence or absence of RNase R. G, circBCL2L1 was mainly localized in the cytoplasm. RNA isolated from nuclear and cytoplasm was used to analyze the expression of circBCL2L1 by RT-PCR; n = 3. The data represented the mean ± SD from three independent triplicated experiments. **p < 0.01.

circRNA regulates innate immune responses in fish

circBCL2L1 in MSpC, MBrC, MMC, MIC, and MKC cells (Fig. 1C). Among the aforementioned cell lines, MKC and MBrC cells showed the highest and the lowest expression of circBCL2L1, respectively. Therefore, we selected MKC and MBrC to investigate the function and regulatory mechanism of circBCL2L1.

We blasted the *BCL2L1* gene with the whole genome of the miiuy croaker (48, 49) and found that the *BCL2L1* gene was located on chromosome 5. circBCL2L1 was consisted of the head-to-tail splicing of only exon 2, with a spliced mature sequence length of 760 bp. We used several universal circRNAs detection methods to distinguish whether the head-to-tail splicing is the result of *trans*-splicing or the genome rearrangement. We first designed divergent primers to amplify circBCL2L1, and the result of Sanger sequencing confirmed the head-to-tail splicing in the RT-PCR product of circBCL2L1 (Fig. 1D). Then, we used convergent primers to amplify *BCL2L1* gene and divergent primers to amplify circBCL2L1. cDNA and gDNA were extracted separately from MKC and MBrC and subjected to RT-PCR and agarose gel electrophoresis assays. The results shown in Figure 1E indicated that circBCL2L1 was amplified from cDNA by using only divergent primers (an expected 145 bp fragment, and we labeled the location of circBCL2L1-divergent-F/R primers in Fig. 1D.), whereas no amplification product was observed from gDNA. Considering that stability was a crucial characteristic of circRNAs, we thus employed RNase R to confirm the stability of circBCL2L1. The results from the analysis of RT-PCR and agarose gel electrophoresis assay showed that circBCL2L1, rather than linear *BCL2L1* or GAPDH, resisted digestion by RNase R (Fig. 1F). In addition, we detected the distribution of

circBCL2L1 by cytoplasmic nuclear fractionation experiments and found that circBCL2L1 was primarily localized in the cytoplasm (Fig. 1G). Accordingly, these results suggested that circBCL2L1 was a stable circRNA expressed and primarily distributed in the cytoplasm.

circBCL2L1 enhances host innate immunity

The small interfering RNAs (siRNA) against circBCL2L1 and the overexpression plasmid of circBCL2L1 were constructed to detect the biological function of circBCL2L1 (Fig. 2, A and B). Consequently, two siRNAs (si-circBCL2L1-1 and si-circBCL2L1-2) evidently decreased the circBCL2L1 expression level, but such siRNAs did not affect the expression level of linear *BCL2L1* mRNA in MKC cells. As si-circBCL2L1-1 could induce higher inhibitory efficiency, thus we selected si-circBCL2L1-1 (si-circ-1) for the subsequent experiment (left panel of Fig. 2C). Moreover, the circBCL2L1 overexpression plasmid was successfully constructed, as it significantly increased the circBCL2L1 expression levels rather than linear *BCL2L1* mRNA in MBrC cells (right panel of Fig. 2C). Considering that interferon-stimulated genes (ISGs) are important antiviral effectors, we focused on investigating the role of circBCL2L1 in regulating the expression of ISGs and inflammatory cytokines. As shown in Figure 2D, the overexpression of circBCL2L1 could significantly increase the expression levels of antiviral genes such as MX1, ISG15, and Viperin after SCRv infection, and knockdown of circBCL2L1 (oe-circ) could significantly inhibit the expression levels of these genes under SCRv treatment. As shown in Figure 2E, the overexpression of circBCL2L1 could significantly increase the

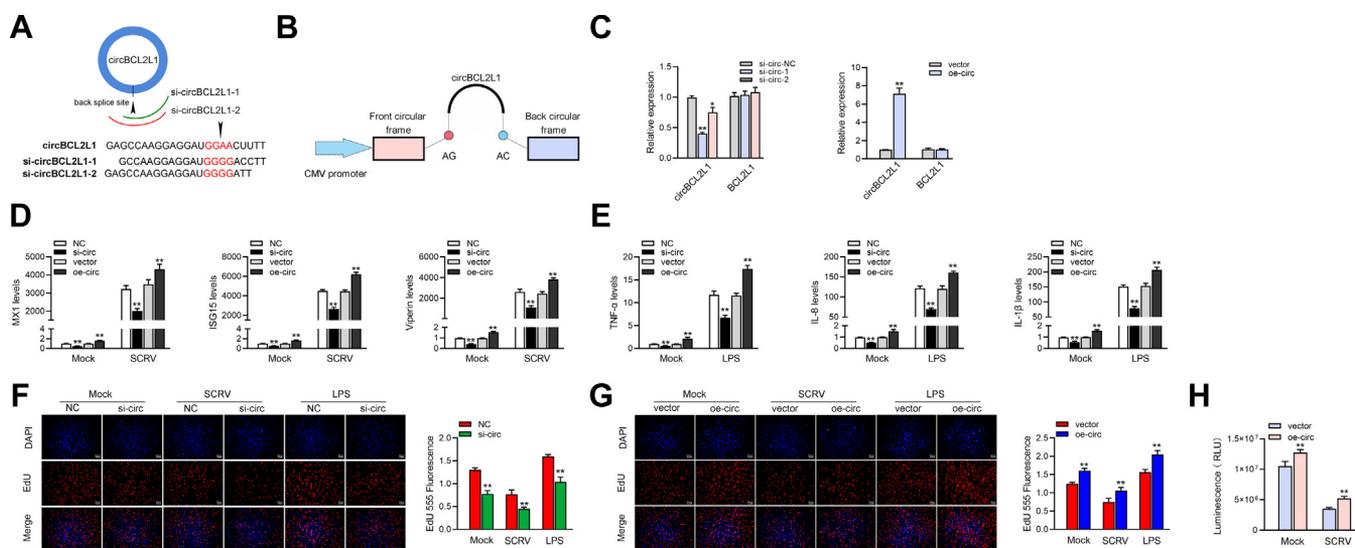


Figure 2. circBCL2L1 promotes the antiviral innate immunity. A and B, the schematic diagram of siRNAs (A) and oe-circ structure (B). C, qPCR analysis of circBCL2L1 and linear *BCL2L1* mRNA in MKC and MBrC cells treated with siRNAs and circBCL2L1 overexpressed plasmid respectively. D, qPCR assays were performed to determine the expression levels of MX1, ISG15, and Viperin in MKC cells transfected with overexpression plasmid (oe-circ) or control vector (pLC5-ciR) and with (si-circBCL2L1-1) si-circ or NC after SCRv infected. E, qPCR assays were performed to determine the expression levels of TNF α , IL-8, and IL-1 β in MKC cells transfected with overexpression plasmid (oe-circ) or control vector (pLC5-ciR) and with si-circ or NC after LPS stimulation. F and G, cell proliferation was assessed by EdU assays in MKC cells transfected with si-circ or NC after SCRv infected 24 h or after LPS stimulation 12 h (F) and MBrC cells transfected with oe-circ or pLC5-ciR vector after SCRv infected 24 h or after LPS stimulation 12 h (G). H, effect of circBCL2L1 on cell viability after SCRv infection. MBrC cells were transfected with pLC5-ciR vector or oe-circ for 24 h, then treated with SCRv. Cell viability assays were measured. All data represented the mean \pm SD from three independent triplicated experiments. * $p < 0.05$; ** $p < 0.01$.

expression levels of inflammatory cytokines such as TNF- α , IL-8, and IL-1 β after LPS stimulation, and knockdown of circBCL2L1 (oe-circ) could significantly inhibit the expression levels of these genes under LPS stimulation. We conducted EdU assays to examine the cell proliferation in MKC and MBrC and explore the function of circBCL2L1 in antiviral innate immunity. The results showed that knockdown of circBCL2L1 considerably decreased the percentages of EdU-positive cells (Fig. 2F) but greatly increased at overexpression of circBCL2L1, suggesting that circBCL2L1 promoted the proliferation of miuiy croaker cell (Fig. 2G). This result shows that the circBCL2L1 can positively regulate the antiviral responses and upregulate the expression of inflammatory cytokines and antiviral genes, reducing the attack of the virus to cells and promoting cell proliferation. Moreover, as a process of normal healthy cells, cell proliferation can often reflect the state of cells. Therefore, we can understand the state of cells from the situation of cell proliferation. When we investigated the effect of circBCL2L1 on the cell viability of MBrC, we found that overexpression of circBCL2L1 significantly increased cell viability compared with the control group after 24 h of SCR V infection (Fig. 2H). In summary, these data indicate that circBCL2L1, as a positive regulator, is involved in the regulation of innate immunity, and the data of the cell proliferation and viability suggestion that the circBCL2L1 can positively regulate the antiviral responses and upregulate the expression of inflammatory cytokines and antiviral genes,

reducing the attack of the pathogen to cells and promoting cell proliferation and increasing cell viability.

circBCL2L1 is able to regulate miR-30c-3-3p expression and activity

We examined the ability of circBCL2L1 to bind to miRNAs to explore whether circBCL2L1 can function as a miRNA sponge. To this end, we transfected Ago2-flag or pcDNA3.1-flag into MKC cells to conduct RNA immunoprecipitation (RIP) for Argonaute (Ago2). The results showed that endogenous circBCL2L1 could be pulled down by Ago2-flag (Fig. 3A), indicating that circBCL2L1 might have a binding site with miRNAs. Next, to find miRNAs combined with circBCL2L1, we first used miRNA target prediction tools including TargetScan, miRanda, and RNAhybrid for prediction and selected five candidate miRNAs for further verification (Fig. 3, B and C). Afterward, we compared the expression levels of these candidate miRNAs in MKC cells transfected with si-circ or negative control and MBrC cells transfected with overexpression plasmid (oe-circ) or control vector (vector). Among the five candidate miRNAs, miR-30c-3-3p expression was significantly reduced when circBCL2L1 was overexpressed (Fig. 3D), whereas miR-30c-3-3p expression was significantly enhanced in response to circBCL2L1 inhibition compared with other candidate miRNAs (Fig. 3E). We constructed the miR-30c-3-3p sensor to detect whether circBCL2L1 affects the

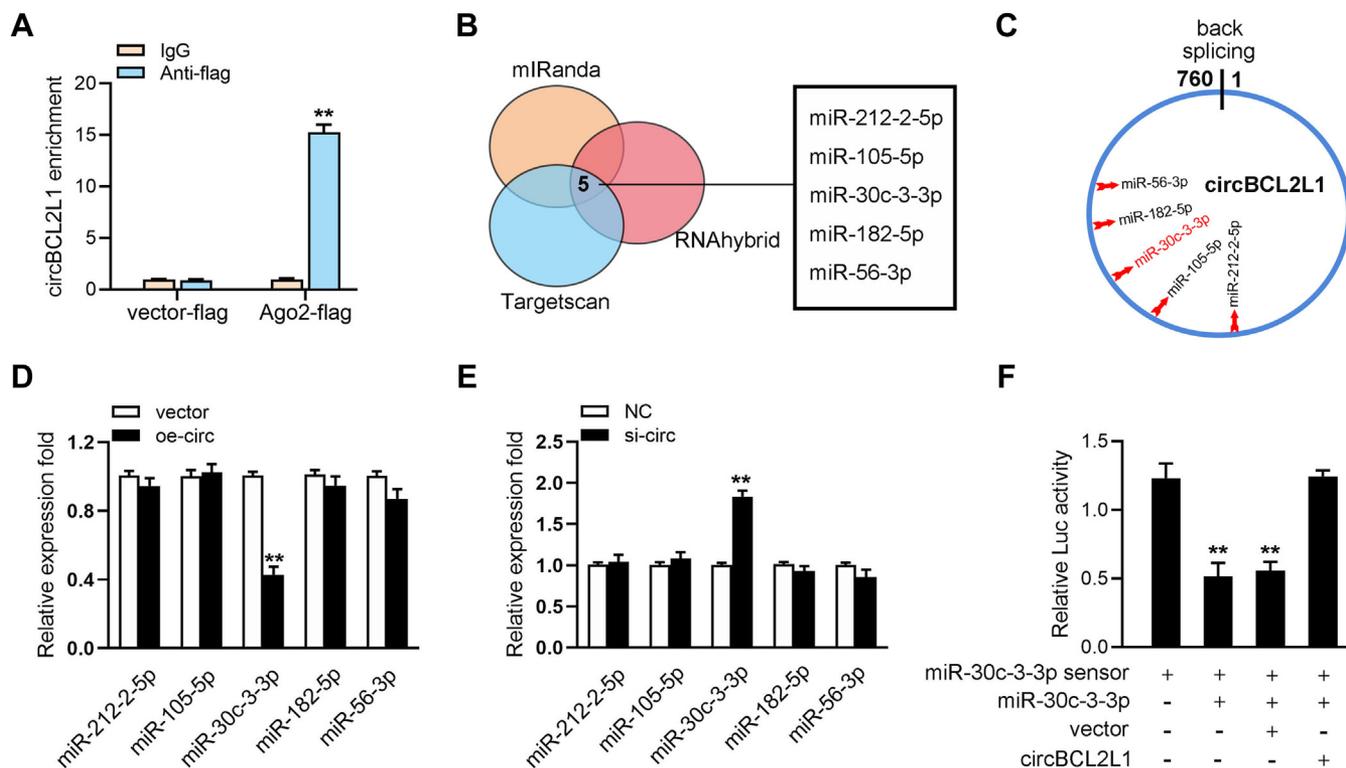


Figure 3. circBCL2L1 regulates miR-30c-3-3p expression and activity. A, the Ago2-RIP assay for the amount of circBCL2L1 in MKC cells transfected Ago2-flag or pcDNA3.1-flag. B, a schematic illustration showing overlapping of the target miRNAs of circBCL2L1 predicted by TargetScan, miRanda, and RNAhybrid. C, schematic drawing showing the putative binding sites of the miRNAs associated with circBCL2L1. D and E, relative expression of candidate miRNAs in MBrC and MKC cells transfected with oe-circ (D) and si-circ (E), respectively. F, circBCL2L1 reduces miR-30c-3-3p activity. The relative luciferase activity was analyzed in MBrC cells cotransfected with mimics, circBCL2L1 overexpression plasmid, and control vector, together with miR-30c-3-3p sensor. All data represented the mean \pm SD from three independent triplicated experiments. ** $p < 0.01$.

circRNA regulates innate immune responses in fish

activity of miR-30c-3-3p and consolidate the direct binding of miR-30c-3-3p and circBCL2L1. Then, we transfected the miR-30c-3-3p sensor with miR-30c-3-3p, pLC5-ciR vector, or circBCL2L1 overexpression plasmid. The decreased luciferase activity induced by miR-30c-3-3p was recovered when cotransfected with circBCL2L1 overexpression plasmid, suggesting that circBCL2L1 specifically sponged miR-30c-3-3p, thereby preventing it from inhibiting luciferase activity (Fig. 3F). Collectively, circBCL2L1 could regulate miR-30c-3-3p expression and activity, and circBCL2L1 might function as a sponge of miR-30c-3-3p.

circBCL2L1 functions as a miRNA sponge of miR-30c-3-3p

We analyzed the sequences of circBCL2L1 to investigate whether circBCL2L1 could interact with miR-30c-3-3p and

found that circBCL2L1 contained a binding site of miR-30c-3-3p (Fig. 4A). Next, we constructed a luciferase plasmid of circBCL2L1 and the mutated form of miR-30c-3-3p binding sites mutated (Fig. 4A). Luciferase assays revealed that miR-30c-3-3p could suppress the luciferase activity of the wild form of circBCL2L1 luciferase plasmid, but it had no effect on the mutated form (Fig. 4B). In addition, miR-30c-3-3p mimics inhibited luciferase activity in time-dependent and dose-dependent manner (Fig. 4, C and D). Moreover, we inserted a wild or a mutated form of circBCL2L1 into the mVenus-C1 vector and examined whether cotransfecting with miR-30c-3-3p could suppress the levels of green fluorescent protein (GFP). As shown in Figure 4, E and F, the results revealed that miR-30c-3-3p could significantly inhibit the levels of GFP, which suggested that a direct interaction might exist between circBCL2L1 and miR-30c-3-3p.

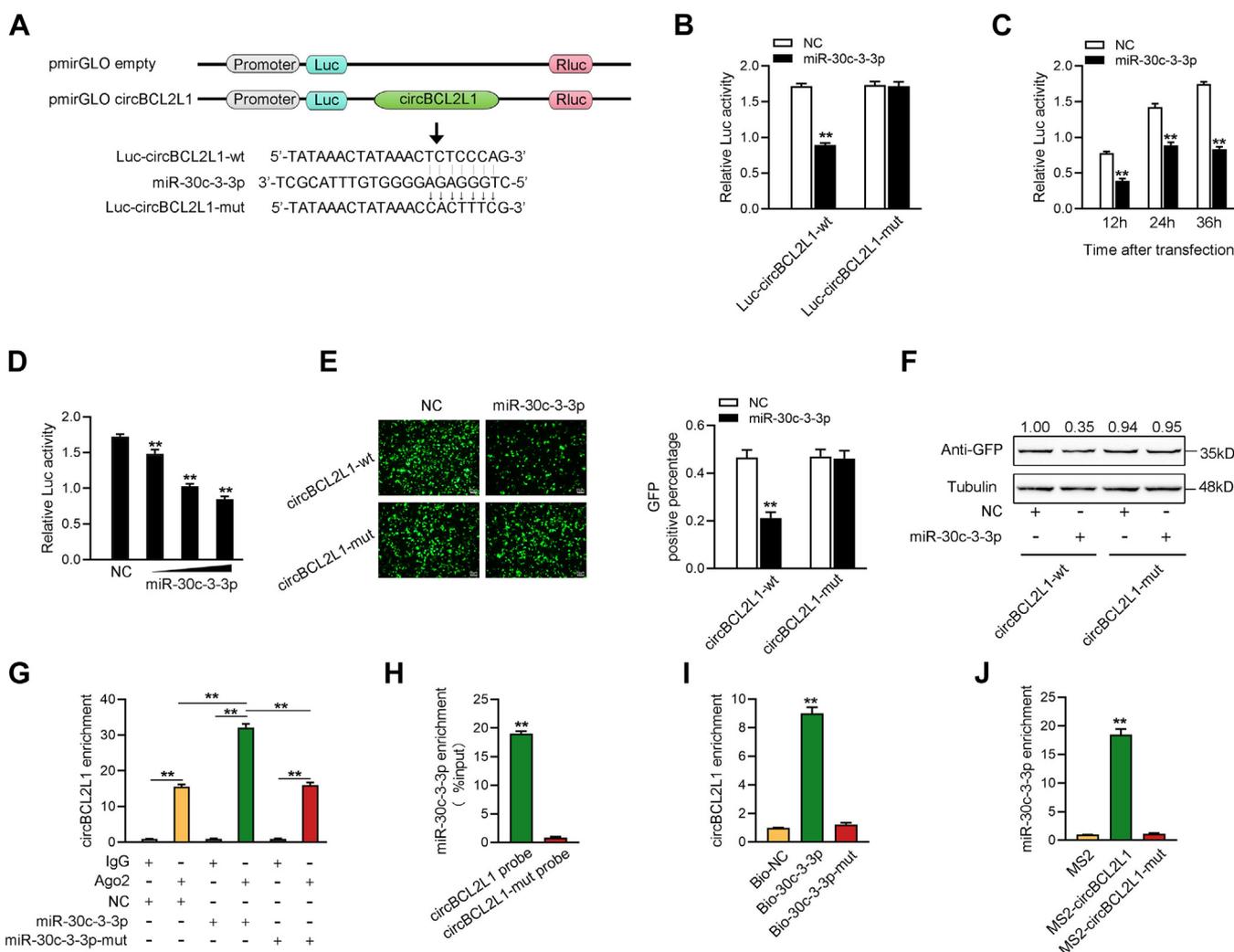


Figure 4. circBCL2L1 functions as a miRNA sponge of miR-30c-3-3p. A, schematic illustration of circBCL2L1-wt and circBCL2L1-mut luciferase reporter vectors. B, the relative luciferase activities were detected in EPC cells after cotransfection with circBCL2L1-wt or circBCL2L1-mut and mimics or NC. C and D, the time gradient (C) and concentration gradient (D) experiment of miR-30c-3-3p mimics was conducted. E and F, circBCL2L1 downregulated GFP expression. EPC cells were cotransfected with circBCL2L1-wt or circBCL2L1-mut and mimics or NC. The fluorescence intensity and the GFP expression were evaluated by enzyme-labeled instrument and western blotting, respectively. G, the Ago2-RIP assay was executed in MKC cells after transfection with miR-30c-3-3p, miR-30c-3-3p-mut, and NC, followed by qPCR to detect circBCL2L1 expression levels. H and I, RNA pull-down assay was executed in MKC cells, followed by qPCR to detect the enrichment of circBCL2L1 and miR-30c-3-3p. J, the MS2-RIP assay was executed in MKC cells after transfection with pLC5-circ-MS2, pLC5-MS2-circBCL2L1, pLC5-MS2-circBCL2L1-mut, followed by qPCR to detect the enrichment of miR-30c-3-3p. All data represented the mean \pm SD from three independent triplicated experiments. * $p < 0.05$; ** $p < 0.01$.

Given that miRNAs regulated target gene expression by binding to Ago2, we further tested the ability of circBCL2L1 to bind to miR-30c-3-3p. To this end, RIP assays were performed in MKC cells by cotransfecting Ago2-flag, miR-30c-3-3p, and miR-30c-3-3p-mut. The results from qPCR analysis indicated that circBCL2L1 and miR-30c-3-3p were efficiently pulled down by Ago2-flag, but not miR-30c-3-3p-mut (Fig. 4G). We conducted RNA pull-down assays with biotin-labeled circBCL2L1 probe or biotin-labeled miR-30c-3-3p to further confirm the direct interaction between circBCL2L1 and miR-30c-3-3p. The results from the qPCR analysis revealed that miR-30c-3-3p could be pulled down by biotin-labeled circBCL2L1 but not circBCL2L1-mut (Fig. 4H). Additionally, biotin-labeled miR-30c-3-3p captured more circBCL2L1 compared with the negative control and biotin-labeled miR-30c-3-3p-mut (Fig. 4I). Furthermore, RIP assays were applied to test the direct interaction between circBCL2L1 and miR-30c-3-3p. We cloned an MS2 fragment into pLC5-ciR, pLC5-circBCL2L1, and pLC5-circBCL2L1-mut plasmids to construct plasmids that could produce circBCL2L1 identified by the MS2 protein. We also constructed a GFP and MS2 gene fusion expression vector to produce a GFP-MS2 fusion protein

that could bind with the MS2 fragment and be identified using an anti-GFP antibody. Hence, miRNAs that interacted with circBCL2L1 could be pulled down by the GFP-MS2-circBCL2L1 compounds. The results from qPCR assays showed that the pLC5-circBCL2L1 RIP was significantly enriched for miR-30c-3-3p compared with pLC5-circBCL2L1-mut or empty vector (Fig. 4J). Collectively, these data demonstrated that circBCL2L1 could directly bind to miR-30c-3-3p, and circBCL2L1 served as a sponge of miR-30c-3-3p.

miR-30c-3-3p inhibits innate immune response by targeting TRAF6

miRNAs could posttranscriptionally regulate the expression of target mRNAs by binding to their 3'UTR. To this end, we predicted the potential target genes of miR-30c-3-3p using miRNA prediction programs. Among those candidate target genes, the innate immune-related gene TRAF6, which we are interested, contained in it. Thus, we selected TRAF6 as a target gene of miR-30c-3-3p for further investigation. We first analyzed the sequence of TRAF6 3'UTR and found that

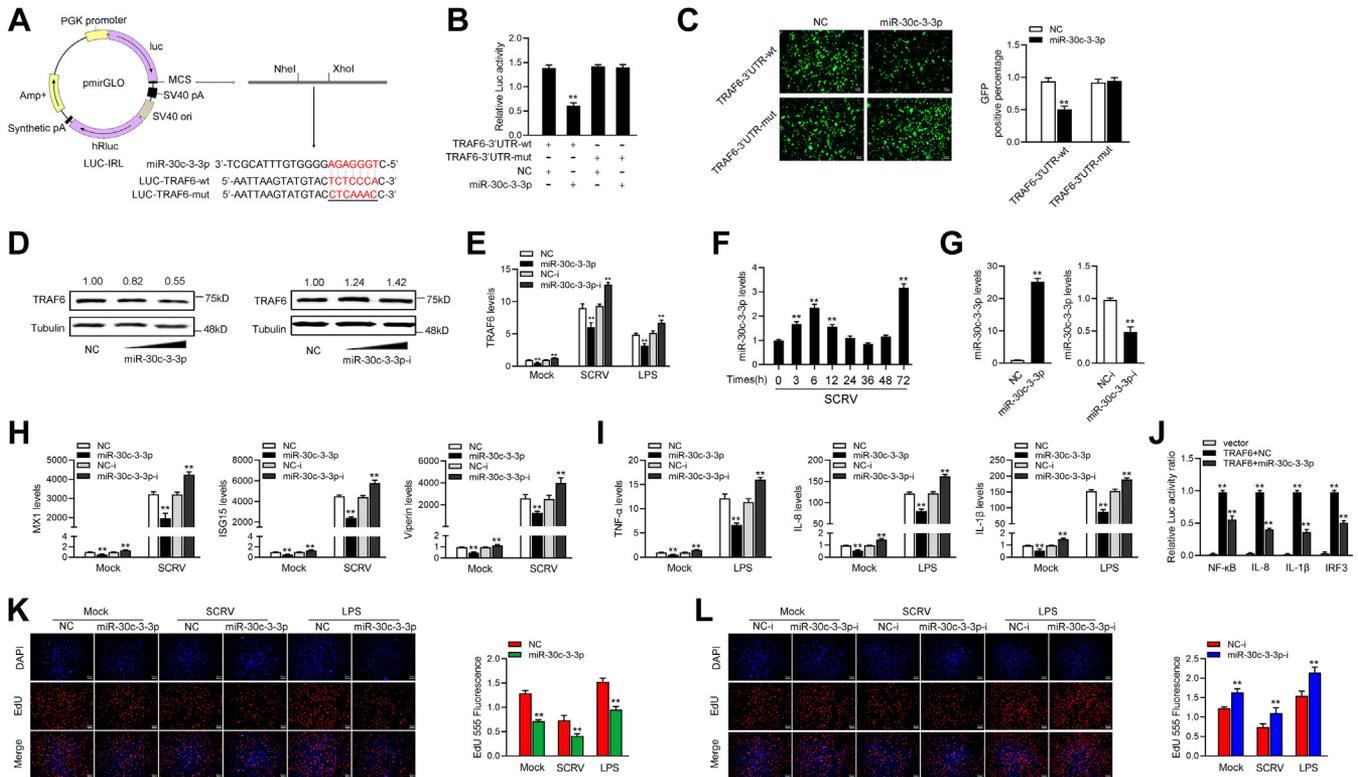


Figure 5. miR-30c-3-3p inhibits innate responses by targeting TRAF6. *A*, schematic illustration of TRAF6-wt and TRAF6-mut luciferase reporter vectors. *B*, the relative luciferase activities were detected in EPC cells after cotransfection with TRAF6-wt or TRAF6-mut and mimics or NC. *C*, miR-30c-3-3p down-regulated GFP expression. EPC cells were cotransfected with TRAF6-3'UTR-wt or TRAF6-3'UTR-mut and mimics or NC. The fluorescence intensity and the GFP expression were evaluated by enzyme-labeled instrument. *D* and *E*, relative protein and mRNA levels of TRAF6 were evaluated by western blot (*D*) and qRT-PCR (*E*) in MKC cells after cotransfected with the miR-30c-3-3p mimics or inhibitors. *F*, qPCR analysis of miR-30c-3-3p in MKC cells treated with SCRv (MOI = 5) at the indicated time points. *G*, the miR-30c-3-3p expression levels were detected in MKC cells after cotransfection with NC or mimics (*left panel*) and NC-i or inhibitors (*right panel*). *H*, qRT-PCR assays were performed to determine the expression levels of MX1, ISG15, and Viperin in MKC cells after cotransfected with NC or mimics and NC-i or inhibitors after SCRv infected 24 h. *I*, qRT-PCR assays were performed to determine the expression levels of TNF α , IL-8, and IL-1 β in MKC cells after cotransfected with NC or mimics and NC-i or inhibitors after LPS stimulation 12 h. *J*, the relative luciferase activities were detected in MKC cells after cotransfection with TRAF6 expression plasmid, phRL-TK Renilla luciferase plasmid, luciferase reporter genes, NC, or mimics. *K* and *L*, cell proliferation was assessed by EdU assays in MKC cells after cotransfected with NC or mimics (*K*) and MBRc cells after cotransfected with NC-i or inhibitors (*L*). Scale bar, 20 μ m. All data represented the mean \pm SD from three independent triplicated experiments. **p* < 0.05; ***p* < 0.01.

circRNA regulates innate immune responses in fish

miR-30c-3-3p had a complementary sequence with TRAF6 3'UTR (Fig. 5A). Then, the wild-type and the mutant-type of TRAF6 3'UTR were cloned into luciferase reporter vector pmirGLO and then cotransfected with miR-30c-3-3p mimics or control mimics to determine the interaction between miR-30c-3-3p and TRAF6. The results showed that miR-30c-3-3p mimics markedly inhibited the luciferase activity when the wild-type 3'UTR was transfected, whereas the mutated form had no response to miR-30c-3-3p mimics (Fig. 5B). Moreover, we inserted a wild or a mutated form of TRAF6 3'UTR into the mVenus-C1 vector and examined whether cotransfecting with miR-30c-3-3p could suppress the levels of GFP. As shown in Figure 5C, the results revealed that miR-30c-3-3p could significantly inhibit the levels of GFP, which suggested that a direct interaction might exist between TRAF6 mRNA and miR-30c-3-3p. We transfected miR-30c-3-3p mimics and inhibitors into MKC cells to test whether miR-30c-3-3p participates in the regulation of TRAF6 expression. The results from Western blotting and qRT-PCR assays displayed that transfection of miR-30c-3-3p mimics suppressed the expression levels of TRAF6, whereas miR-30c-3-3p inhibitors markedly enhanced the expression levels of TRAF6 (Fig. 5D).

In addition, we investigated whether miR-30c-3-3p regulated TRAF6 at the posttranscriptional level. As shown in Figure 5E, the miR-30c-3-3p mimics significantly reduced the expression level of TRAF6. On the contrary, the miR-30c-3-3p inhibitor significantly increased the expression level of TRAF6 and then detected the miR-30c-3-3p levels in MKC cells by SCR infection. And the result shows that miR-30c-3-3p levels were significantly increasing after SCR infection 6 h and 72 h (Fig. 5F). Meanwhile, we first investigated the role of miR-30c-3-3p in regulating MX1, ISG15, Viperin, TNF- α , IL-8, and IL-1 β to explore the biological function of miR-30c-3-3p. To this end, we measured the effects of synthetic miR-30c-3-3p mimics and inhibitors on the expression of miR-30c-3-3p, and the results indicated that miR-30c-3-3p mimics enhanced miR-30c-3-3p expression sharply, whereas miR-30c-3-3p inhibitors decreased miR-30c-3-3p expression (Fig. 5G). Moreover, the results showed that MX1, ISG15, and Viperin were significantly decreased by the introduction of miR-30c-3-3p mimics upon SCR infection, and the inhibition of endogenous miR-30c-3-3p significantly increased these gene expressions compared with transfection of control inhibitors (Fig. 5H). It is similar to that of the results showing that TNF- α , IL-8, and IL-1 β were significantly decreased by the introduction of miR-30c-3-3p mimics upon SCR infection, and the inhibition of endogenous miR-30c-3-3p significantly increased this, elevating these gene expressions compared with transfection of control inhibitors (Fig. 5I). Next, given that miR-30c-3-3p targeted TRAF6 and regulated its expression, we aimed to test whether miR-30c-3-3p affected TRAF6-mediated activation of NF- κ B and IRF3. The results from dual-luciferase reporter assays showed that after cotransfection of TRAF6 overexpression plasmid, miR-30c-3-3p mimics suppressed the activity of NF- κ B, IL-8, IL-1 β , and IRF3 luciferase reporters compared with control mimics (Fig. 5J). Furthermore, we attempted to investigate whether miR-30c-3-3p could regulate

cell proliferation after SCR or LPS stimulation. As shown in Figure 6, K and L, overexpression of miR-30c-3-3p decreased cell proliferation, whereas the inhibition of miR-30c-3-3p led to an efficiently increased cell proliferation. Collectively, these data demonstrated that miR-30c-3-3p could inhibit innate immune responses. The result may indicate that the miR-30c-3-3p can negatively regulate the antiviral responses and downregulate the expression of inflammatory cytokines and antiviral genes, increasing the attack of virus to cells and reducing cell proliferation.

circBCL2L1 serves as a sponge of miR-30c-3-3p to enhance TRAF6 expression

Given that circBCL2L1 could interact with miR-30c-3-3p and miR-30c-3-3p targets TRAF6 and regulates its expression, we tested whether circBCL2L1 could regulate TRAF6. As shown in Figure 6A, overexpression of circBCL2L1 increased the expression of TRAF6 protein, while knockdown of circBCL2L1 significantly reduced the protein level of TRAF6. In addition, the qPCR results showed that knockdown of circBCL2L1 led to reducing the expression levels of TRAF6 in cells treated with SCR and LPS (right panel of Fig. 6B). By contrast, overexpression of circBCL2L1 increased the expression of TRAF6 and its mRNA levels (left panel of Fig. 6B). Then, we tested whether circBCL2L1 regulated TRAF6 expression through miR-30c-3-3p. To this end, we cotransfected cells with TRAF6 3'UTR, together with miR-30c-3-3p, circBCL2L1 overexpression plasmid, and circBCL2L1 mutant plasmid. The results showed that circBCL2L1 could counteract the inhibitory effect of miR-30c-3-3p on TRAF6 3'UTR (Fig. 6C). Strikingly, circBCL2L1 could also counteract the effect of miR-30c-3-3p on TRAF6 expression levels (Fig. 6D). These results demonstrated that circBCL2L1 regulated TRAF6 expression through miR-30c-3-3p. Given that miR-30c-3-3p and TRAF6 participated in the regulation of NF- κ B, IL-8, IL-1 β , and ISRE luciferase reporters, we examined the functional role of circBCL2L1 in regulating these reporters. The results showed that circBCL2L1 could counteract the negative effect of miR-30c-3-3p on the luciferase activities of NF- κ B, IL-8, IL-1 β , and ISRE luciferase reporters (Fig. 6E). Moreover, we attempted to explore the effect of the circBCL2L1/miR-30c-3-3p regulatory loop on cell proliferation. The results indicated that overexpression of circBCL2L1 could counteract the negative effect of miR-30c-3-3p on cell proliferation upon SCR infection or LPS stimulation (Fig. 6F). Meanwhile, we attempted to explore the effect of the circBCL2L1/miR-30c-3-3p regulatory loop on cell viability. The results showed that overexpression of circBCL2L1 could counteract the negative effect of miR-30c-3-3p on cell viability upon SCR infection (Fig. 6G). Collectively, these data demonstrated that circBCL2L1 served as a ceRNA for miR-30c-3-3p to regulate TRAF6 expression.

ceRNA network that regulates TRAF6 is widely found in teleost fish

We performed sequence alignment of circBCL2L1 from different teleost fish to illustrate the universality of our

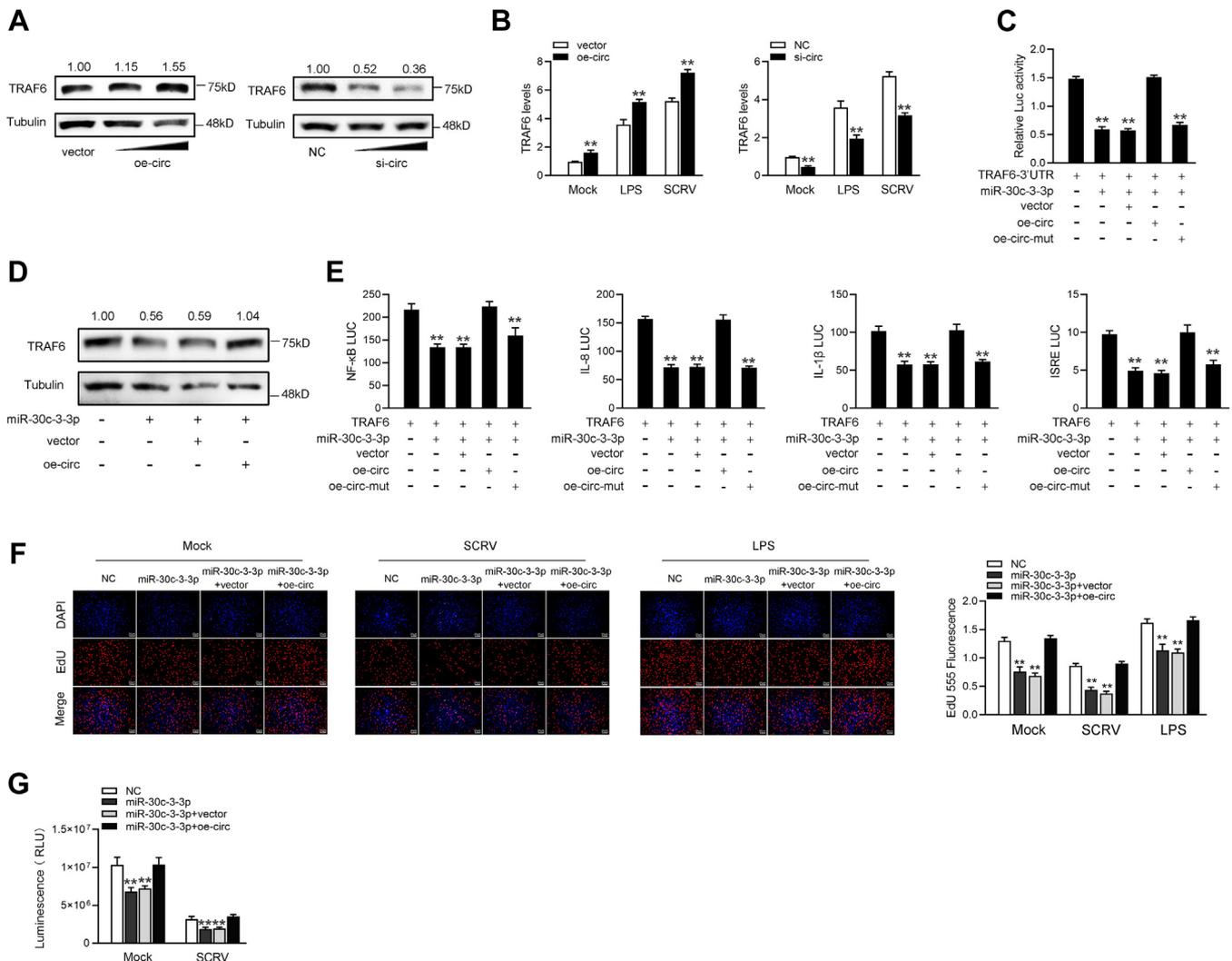


Figure 6. circBCL2L1 acts as a sponge of miR-30c-3-3p to enhance TRAF6 expression. *A*, relative mRNA and protein levels of TRAF6 in MKC or MBrC cells after cotransfected with NC, si-circ, vector, or oe-circ by western blot. *B*, relative mRNA levels of TRAF6 in MBrC or MKC cells after cotransfected with si-circ, NC, si-circ, vector, or oe-circ by qPCR assay. *C*, the relative luciferase activities were detected in EPC cells after cotransfection with TRAF6 3'UTR luciferase reporter vector, NC, mimics, oe-circ, or oe-circ-mut. *D*, western blot assays were detected in MBrC cells after cotransfection with TRAF6 overexpression plasmid, NC, mimics, or oe-circ. *E*, oe-circ counteracts the negative effect of miR-30c-3-3p. The relative luciferase activities were detected in EPC cells after cotransfection with TRAF6 expression plasmid, pRL-TK Renilla luciferase plasmid, luciferase reporters, NC, mimics, or oe-circ. *F*, cell proliferation was assessed by EdU assays in MKC cells after cotransfected with NC, mimics, or oe-circ. *G*, cell viability was assessed by ATP viability assays in MKC cells after cotransfected with NC, mimics, or oe-circ. All data represented the mean \pm SD from three independent triplicated experiments. * $p < 0.05$; ** $p < 0.01$.

findings. Interestingly, as shown in Figure 7A, circBCL2L1 showed high conservation in different fish species. In addition, the binding site of miR-30c-3-3p in circBCL2L1 also showed high conservation. In addition, we analyzed the binding sites of miR-30c-3-3p and TRAF6 in other species, and the TRAF6 showed high conservation at the site of miR-30c-3-3p in different fish species (Fig. 7B). First, we hypothesized that miR-30c-3-3p might interact with TRAF6 across different fish species. In verifying this hypothesis, the TRAF6 3'UTR sequences of *Larimichthys croceas* and *N. albiflora* were cloned into the pmirGLO vector, and their mutated forms with miR-30c-3-3p binding sites mutated. Significantly, luciferase assays revealed that miR-30c-3-3p could suppress luciferase activity of the wild form of TRAF6 3'UTR luciferase plasmid in both fish species, but it had no effect on mutated forms (Fig. 7C). Furthermore, we hypothesized that miR-30c-3-3p might

interact with circBCL2L1 across different fish species. In verifying this hypothesis, the circBCL2L1 sequences of *L. croceas* and *Sciaenops ocellatus* were cloned into the pmirGLO vector, and their mutated forms with miR-30c-3-3p binding sites mutated. Significantly, luciferase assays revealed that miR-30c-3-3p could suppress luciferase activity of the wild form of circBCL2L1 luciferase plasmid in both fish species, but it had no effect on mutated forms (Fig. 7D). In addition, we conducted luciferase assays to test whether *Larimichthys croceas* and *N. albiflora* circBCL2L1 can affect the miR-30c-3-3p activity and found that both *L. croceas* and *N. albiflora* circBCL2L1 could counteract the inhibitory effect of miR-30c-3-3p on TRAF6 3'UTR (Fig. 7E). Collectively, these results showed that circBCL2L1 could act as endogenous sponge RNA to interact with miR-30c-3-3p among different teleost fish, which suggested that circBCL2L1 contains

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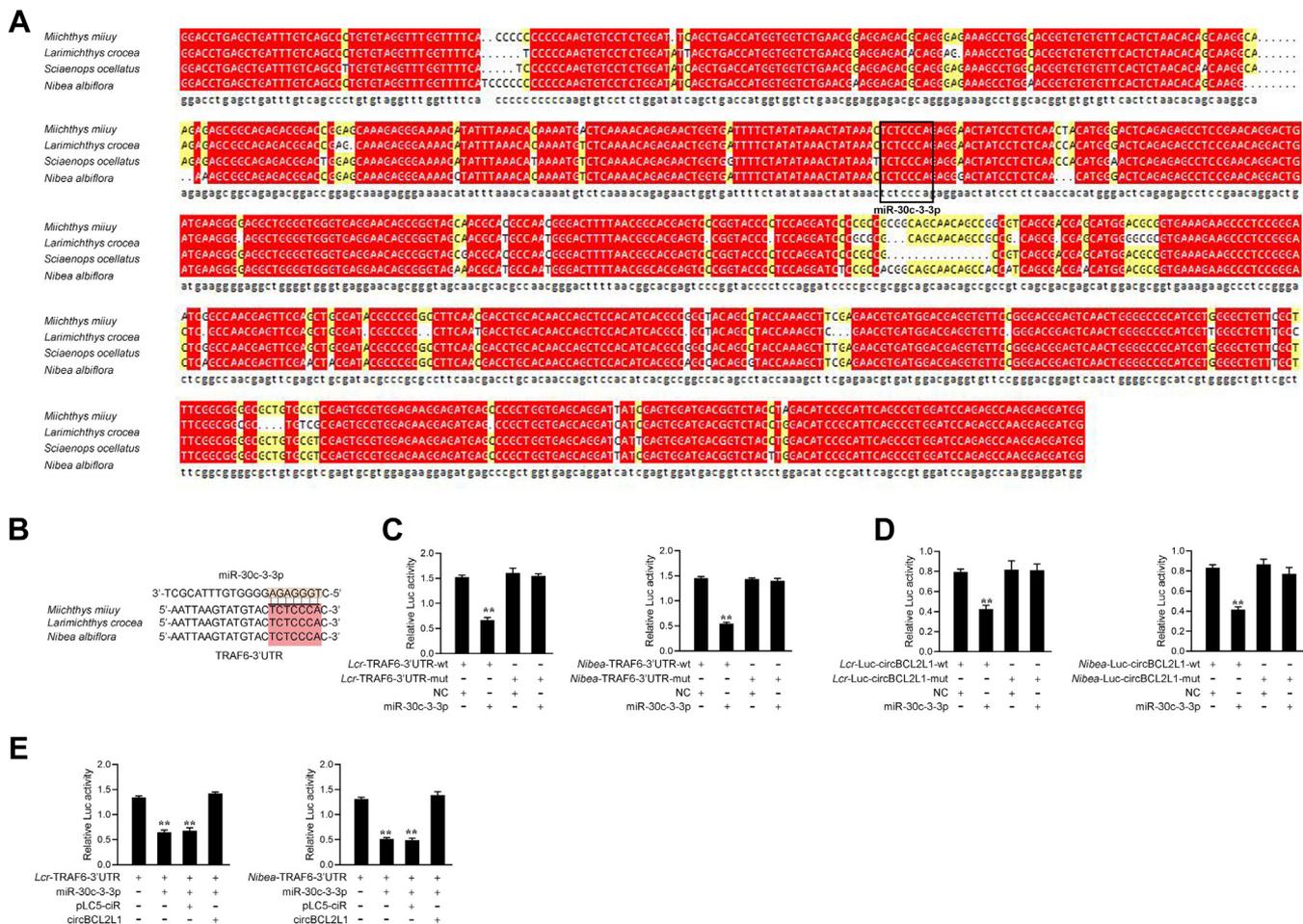


Figure 7. The ceRNA network of regulating TRAF6 is widely found in teleost fish. A, sequence alignment of circBCL2L1 from various teleost fish species. B, sequence alignment of TRAF6 containing miR-30c-3-3p binding site among different teleost species. C, miR-30c-3-3p regulating the luciferase activity of TRAF6-3'UTR is examined in *L. crocea* and *N. albiflora*. D, the relative luciferase activities were detected in EPC cells after cotransfected with *LrcircBCL2L1*-wt or *LrcircBCL2L1*-mut and mimics or NC (left panel) and cells after cotransfected with *SoccircBCL2L1*-wt or *SoccircBCL2L1*-mut and mimics or NC (right panel). E, *LrcircBCL2L1* and *NibeacircBCL2L1* reduce miR-30c-3-3p activity. EPC cells were transfected with *LrcircBCL2L1* or *NibeacircBCL2L1* expression plasmid, mimics, control vector, together with TRAF6-3'UTR, the luciferase activity was analyzed and normalized to renilla luciferase activity. All data represented the mean \pm SD from three independent triplicated experiments. * $p < 0.05$.

strongly conserved elements among species, which is very important for preserving its function.

Discussion

Viral diseases and bacterial diseases are two of the most serious threats to the teleost fish. Once the host recognizes the PAMPs carried by invading bacteria or viruses, it will trigger a series of signaling cascades, mediate the activation of downstream signaling pathways, leading to the production of inflammatory cytokines and antiviral factors such as interferon, and trigger the innate immune response to protect the host. However, abnormal immune response not only cannot protect the host, but also may cause serious damage to the host, so the appropriate intensity of immune response is very critical. For example, although excessive immune response can remove the invading pathogens, it is very likely to damage the host due to excessive immunity. If the immune response intensity is too weak, it is not conducive to the elimination of the invading pathogens. Therefore, it is very important to maintain the

homeostasis of the innate immune system. Herein, we reported an interaction network regulating teleost TRAF6-mediated innate immune signaling pathways. We found that fish TRAF6 served as a crucial signaling molecule during SCR infection or LPS stimulation, which mediated NF- κ B and IRF3 activation and led to type I IFNs and inflammatory cytokine production. miR-30c-3-3p can reduce the expression of TRAF6 and suppress TRAF6-mediated innate immune response, which may help pathogens evade host immune responses. We further proved that circRNA circBCL2L1 served as endogenous sponge RNA to interact with miR-30c-3-3p and facilitate TRAF6 expression, thereby enhancing the antiviral and antibacterial signaling pathways (Fig. 8). Therefore, circBCL2L1 restored the weakened immune response caused by miR-30c-3-3p, thereby maintaining the stability of immune responses and ensuring appropriate inflammatory responses.

After recognizing PAMPs, almost all TLRs (except TLR3) can recruit downstream key adaptor molecule MyD88 to form TLR/MyD88 complex, and then TLR/MyD88 complex recruits Interleukin-1 (IL-1) receptor associated kinase (IRAK) family

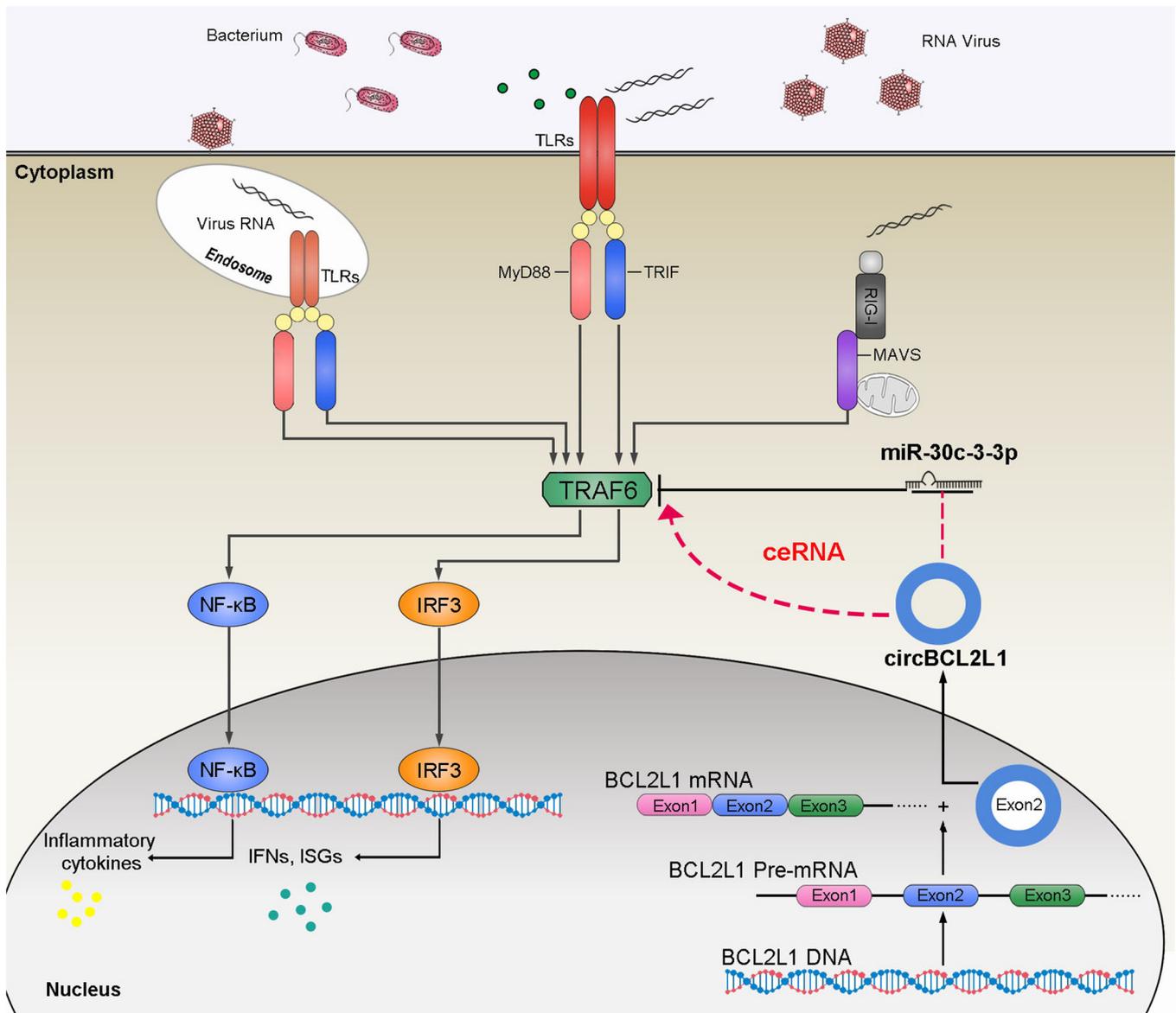


Figure 8. The schematic diagram shows the mechanism underlying circBCL2L1 as a ceRNA for miR-30c-3-3p to regulate TRAF6 expression. miR-30c-3-3p targets TRAF6 and represses TRAF6-mediated innate immune responses. circBCL2L1 acts as a molecular sponge regulating miR-30c-3-3p to enhance TRAF6 expression, thereby maintaining the stable of innate immune responses and ensuring appropriate inflammatory responses.

(50). TRAF6 forms ubiquitin-binding enzyme complex and recruits transforming growth factor activated kinase 1 (TAK1) and TAK1-binding protein (TAB), resulting in phosphorylate I kappa B kinase (IKK) complex. Finally, including nuclear factor- κ B (NF- κ B), interferon regulatory factor 3 (IRF3), and IRF7 entered the nucleus from the cytoplasm (51). In teleost, after TLR3 and TLR22 (endemic to fish) specifically recognize viral RNA, they will recruit another key adaptor molecule TRIF, and then the activated TRIF will continue to recruit TRAF3 and TRAF6, eventually leading to the activation of IRF3 and NF- κ B signaling pathways (52). Moreover, studies have shown that when RIG-I and MDA5 recognize virus invasion, they will quickly recruit downstream adaptor protein MAVS, and then recruit TRAF6 to bind to T6BM1 and T6BM2 motifs of MAVS, and finally activate IRF3 and NF- κ B signaling pathways (23). Here, we further confirm the important role of fish

TRAF6 in antibacterial and antiviral immune response and provide evidence similar to mammalian TRAF6; fish-TRAF6 mediates the activation of NF- κ B and IRF3 in response to the invasion of pathogens. Further studies show that ncRNAs including miR-30c-3-3p and circBCL2L1 play an important regulatory role in TRAF6-mediated signal transduction pathway.

With the deepening of research, people's understanding and cognition of ncRNA are constantly improving, but most of the current research is only limited to mammals, but there are few studies on ncRNA in lower vertebrates. However, after decades of research, the research system of ncRNA in a few lower vertebrates has preliminarily formed. Particularly, the complex ncRNA regulatory network in teleost has made some progress in regulating innate immunity. For example, it has been reported that small ncRNA miR-144 and miR-217 could regulate

circRNA regulates innate immune responses in fish

inflammatory responses through NOD1-induced NF- κ B signaling pathways (53). Moreover, it has been reported that the regulation mechanism that long ncRNA NARL can competitively adsorption miR-217-5p to regulate the miR-217-5p/NOD1 axis is widespread in teleost fish (36). lncRNA IRL can competitively adsorb miRNA to regulate the miR-27c-3p/IRAK4 axis that is widespread in teleost fish (54). In addition, we found an lncRNA MARL in teleost fish, which can target and regulate MAVS-mediated antiviral immune response through competitive adsorption of miR-122 (55). Although many studies have explored the regulatory effect of ncRNAs on mammalian TRAF6 gene, there are few studies on the regulation of TRAF6 gene by ncRNAs in teleost. In this study, small ncRNA miR-30c-3-3p was proved to be a miRNA-targeting TRAF6 in miiuy croaker. miR-30c-3-3p negatively regulates TRAF6 expression and suppresses TRAF6-mediated immune responses. In view of the important role of TRAF6 mediated signaling pathway in innate immune response, finding more ncRNAs that can regulate immune related genes will be an interesting and important work in the future.

Growing number of studies have shown that circRNAs can be used as ceRNAs to regulate mammalian protein-coding genes. For example, the circRNA hsa_circ_100395 regulates lung cancer cell proliferation, migration, and invasion through modulating miR-1228/TCF21 pathway (56). Moreover, a novel identified circular RNA, circRNA_010567, promotes myocardial fibrosis *via* suppressing miR-141 by targeting TGF- β 1 (57). Additionally, CircRNA-5692 inhibits the progression of hepatocellular carcinoma by sponging miR-328-5p to enhance DAB2IP expression (58). Although a large number of studies have been reported on the function of circRNA through ceRNA mechanism in mammals, there are few reports in lower vertebrates, especially in teleost fish. Here, we showed that a new circRNA circBCL2L1, which was upregulated upon SCR V infection and LPS stimulation respectively, can modulate TRAF6 expression through competitively sponging miR-30c-3-3p in lower vertebrate, miiuy croaker. We found that the expression of circBCL2L1 increased significantly through the stimulation of SCR V or LPS, but its *BCL2L1* did not, and we think that this may be because the circBCL2L1 plays a very important role in innate immunity, and *BCL2L1* may not respond to the innate immune response induced by SCR V or LPS. These results further demonstrate that circRNAs are involved in the regulation of immune response as a new immune regulatory molecule in lower vertebrates.

In this study, we found a novel circRNA, circBCL2L1, which was involved in the regulation of innate antiviral immune responses in teleost fish. Among them, miR-30c-3-3p was also a newly discovered miRNA that could negatively regulate TRAF6-mediated innate immune responses. On the contrary, circBCL2L1 played a positive role in TRAF6-mediated innate immune responses. We confirmed that the mechanism of the two ncRNAs played a role in regulating the innate immune responses. circBCL2L1 could be used as the ceRNA of miR-30c-3-3p to reduce its inhibitory effect on TRAF6 expression, thereby inhibiting the replication of the virus. In addition, we also found that the structure and function of circBCL2L1

were highly conserved in different teleost fish. In conclusion, our study revealed that circRNA is involved in the host pathogen interaction mechanism and confirmed the important role of circRNA in innate immunity.

Experimental procedures

Ethics statement

All animal experimental procedures were performed in accordance with the National Institutes of Health's Guide for the Care and Use of Laboratory Animals, and the experimental protocols were approved by the Research Ethics Committee of Shanghai Ocean University (No. SHOU-DW-2018-047).

Sample and challenge

Miiuy croaker (~50 g) was obtained from Zhoushan Fisheries Research Institute, Zhejiang Province, China. Fish was acclimated in aerated seawater tanks at 25 °C for 6 weeks before experiments. Experimental procedures and *Siniperca chuatsi rhabdovirus* (SCR V) infect were performed as described (55).

Cell culture and treatment

M. miiuy spleen cells (MSPc), *M. miiuy* kidney cells (MKC), *M. miiuy* muscle cells (MMC), *M. miiuy* brain cells (MBrc), and *M. miiuy* intestine cells (MIC) were cultured in L-15 medium (HyClone) supplemented with 15% fetal bovine serum (FBS; Gibco), 100 U/ml penicillin, and 100 μ g/ml streptomycin at 26 °C. And the above five cell lines were prepared from the corresponding tissues of the miiuy croaker, and the specific preparation process was as described previously (59). Fish epithelioma papulosum cyprini cells (EPCs) were maintained in medium 199 (Invitrogen) supplemented with 10% FBS, 100 U/ml penicillin, and 100 mg/ml streptomycin at 28 °C in 5% CO₂. For SCR V infection experiments, MKC and MBrc cells were challenged with SCR V at a multiplicity of infection (MOI) of 5 and harvested at different times for RNA extraction.

Plasmids construction

To construct the TRAF6 3'UTR reporter vector, the 3'UTR region of *M. miiuy* TRAF6 gene, as well as *L. crocea* and *S. ocellatus* TRAF6 3'UTR, was amplified using PCR and cloned into pmirGLO luciferase reporter vector (Promega). To construct the TRAF6 expression plasmid of *M. miiuy*, the TRAF6 cDNA was amplified by specific primer pairs and cloned into pcDNA3.1 (Invitrogen) vector. Meanwhile, the TRAF6 3'UTR sequences of *M. miiuy* were inserted into mVenus-C1 vector (Invitrogen), which included the sequence of enhanced GFP. To construct circBCL2L1 overexpression vector, the full-length circBCL2L1 cDNA was amplified by specific primer pairs and cloned into pLC5-ciR vector (Geneseed Biotech), which contained a front and back circular frame to promote RNA circularization. Also, the circBCL2L1 overexpression vectors of *L. crocea* and *S. ocellatus* were constructed by synthesizing the full-length circBCL2L1 cDNA

of *L. crocea* and *S. ocellatus*, respectively. The empty vector with no circBCL2L1 sequence was used as negative control. The mutated forms with point mutations in the miR-30c-3-3p binding site were synthesized using Mut Express II Fast Mutagenesis Kit V2 with specific primers. A miR-30c-3-3p sensor was created by inserting two consecutive miR-30c-3-3p complementary sequences into the psiCHECK vector (Promega). The correct construction of the plasmids was verified by Sanger sequencing and extracted through EndoFree Plasmid DNA Miniprep Kit (Tiangen). To build pLC5-ciR-MS2, the MS2 fragment was inserted into the pLC5-ciR vector, and then the MS2 sequence was inserted into any position in the circBCL2L1 sequence in the pLC5-ciR-BCL2L1 vector, except for the binding site of miR-30c-3-3p. To build pcDNA3.1-MS2, the MS2-12X fragment was inserted into the pcDNA3.1 vector, and then the TRAF6-3'UTR was amplified and cloned into pcDNA3.1-MS2. The mutated forms with point mutations in the miR-30c-3-3p binding site were synthesized using Mut Express II Fast Mutagenesis Kit V2 with specific primers (Table S1).

RNA oligoribonucleotides

The miR-30c-3-3p mimics are synthetic double-stranded RNAs (dsRNAs) with stimulating naturally occurring mature miRNAs. The miR-30c-3-3p mimics sequence was, 5'-CUGGGAGAGGGGUGUUUACGCU-3'. The miR-30c-3-3p mimics mutant sequence was 5'-CAUUGUGGGGGU-GUUUACGCU-3'. The negative control mimics sequence was 5'-UUCUCCGAACGUGUCACGUTT-3'. miRNA inhibitors are synthetic single-stranded RNAs (ssRNAs) that sequester intracellular miRNAs and block their activity in the RNA interfering pathway. The miR-30c-3-3p inhibitors sequence was 5'-ACAAACCAUCCGUGUCGCUA-3'. The negative control inhibitor sequence was 5'-CAGUACUUUUGUGUA-GUACAA-3'. The RNA interferences for circBCL2L1 are as follows: si-circBCL2L1-1 sequence was 5'-GCCAAGGAG-GAUGGGGACCTT-3'; si-circBCL2L1-2 sequence was 5'-GA-GCCAAGGAGGAUGGGGATT-3'. The scrambled control RNA sequences were 5'-GCCAAGGAGGAUGGAACUU-TT-3'. The RNA interference for TRAF6 is as follows: si-TRAF6 sequence was 5'-ACACCAUGCAAGGAGCUUUTT-3'. The negative control mimics sequence was 5'-UUCUCC-GAACGUGUCACGUTT-3'.

Cell transfection

Transient transfection of cells with miRNA mimic, miRNA inhibitor, or siRNA was performed in 24-well plates using Lipofectamine RNAiMAX (Invitrogen), and cells were transfected with DNA plasmids, which was performed using Lipofectamine 3000 (Invitrogen) according to the manufacturer's instructions. For functional analyses, the overexpression plasmid (500 ng per well) or control vector (500 ng per well) and miRNA mimics (100 nM), miRNA inhibitor (100 nM), or siRNA (100 nM) were transfected into cells in culture medium and then harvested for further detection. For luciferase experiments, miRNA mimics (100 nM) or miRNA inhibitor

(100 nM) and pmirGLO (500 ng per well) containing the wild or mutated plasmid of TRAF6 3'UTR were transfected into cells.

RNA extract and quantitative real-time PCR

For the isolation and purification of both cytoplasmic and nuclear RNA from MBrC and MKC cells, the Cytoplasmic & Nuclear RNA Purification Kit has been used according to the manufacturer's instructions (Norgen Biotek). Total RNA was isolated with TRIzol Reagent (Invitrogen) and the cDNA was synthesized using the FastQuant RT Kit (Tiangen), which includes DNase treatment of RNA to eliminate genomic contamination. The expression patterns of each gene were performed by using SYBR Premix Ex Taq (Takara). The small RNA was extracted by using miRcute miRNA Isolation Kit (Tiangen), and miRcute miRNA FirstStrand cDNA Synthesis Kit (Tiangen) was applied to reverse transcription of miRNAs. The expression analysis of miR-30c-3-3p was executed by using the miRcute miRNA qPCR Detection Kit (Tiangen). Real-time PCR was performed in an Applied Biosystems QuantStudio 3 (Thermo Fisher Scientific). GAPDH and 5.8S rRNA were employed as endogenous controls for mRNA and miRNA, respectively (47). Primer sequences are displayed in Table S1.

Luciferase report assay

The wild-type of circBCL2L1 and the mutant devoid of the miR-30c-3-3p binding site were cotransfected with miR-30c-3-3p mimics into EPC cells. At 48 h posttransfection, reporter luciferase activities were measured using the dual-luciferase reporter assay system (Promega). To determine the functional regulation of circBCL2L1, cells were cotransfected TRAF6 overexpression plasmid or circBCL2L1 overexpression plasmid, together with NF- κ B, IL-8, IL-1 β , and IRF3 luciferase reporter gene plasmids (60), pRL-TK Renilla luciferase plasmid, either miR-30c-3-3p mimics or negative controls. At 48 h posttransfection, the cells were lysed for reporter activity using the dual-luciferase reporter assay system (Promega). The miR-30c-3-3p sensor was cotransfected with miR-30c-3-3p mimics or circBCL2L1 overexpression plasmid. At 48 h posttransfection, the cells were lysed for reporter activity. All the luciferase activity values were achieved against the renilla luciferase control. Transfection of each construct was performed in triplicate in each assay. Ratios of renilla luciferase readings to firefly luciferase readings were taken for each experiment, and triplicates were averaged.

Western blotting

Cellular lysates were generated by using 1 \times SDS-PAGE loading buffer. Proteins were extracted from cells and measured with the BCA Protein Assay kit (Vazyme), then subjected to SDS-PAGE (8%) gel and transferred to PVDF (Millipore) membranes by semidry blotting (Bio-Rad Trans Blot Turbo System). The membranes were blocked with 5% BSA. Protein was blotted with different antibodies. The antibody against TRAF6 was diluted at 1: 500 (Abcam); anti-Flag

circRNA regulates innate immune responses in fish

and anti-Tubulin monoclonal antibody were diluted at 1: 2000 (Sigma); and HRP-conjugated anti-rabbit IgG or anti-mouse IgG (Abbkine) at 1: 5000. The results were representative of three independent experiments. The immunoreactive proteins were detected by using WesternBright ECL (Advansta). The digital imaging was performed with a cold CCD camera.

RNase R treatment

The RNAs (10 μ g) from MBrC and MKC cells were treated with RNase R (3 U/ μ g, Epicenter) and incubated for 30 min at 37 °C. Then, the treated RNAs were reverse transcribed with a divergent primer or convergent primer and detected by qPCR and RT-PCR assay followed by nucleic acid electrophoresis (61).

Nucleic acid electrophoresis

The cDNA and gDNA PCR products were investigated using 2% agarose gel electrophoresis with TAE running buffer. DNA was separated by electrophoresis at 100 V for 30 min. The DNA marker was Super DNA Marker (100–10,000 bp) (CWBI). The bands were examined by UV irradiation.

RNA pull-down assay

circBCL2L1 and circBCL2L1-mut with miR-30c-3-3p binding sites mutated were transcribed *in vitro*. The two transcripts were biotin-labeled with the T7 RNA polymerase and Biotin RNA Labeling Mix (Roche), treated with RNase-free DNase I, and purified with an RNeasy Mini Kit (Qiagen). The whole-cell lysates from MKC cells ($\sim 1.0 \times 10^7$) were incubated with purified biotinylated transcripts for 1 h at 25 °C. The complexes were isolated by streptavidin agarose beads (Invitrogen). RNA was extracted from the remaining beads, and qPCR was used to evaluate the expression levels of miRNAs.

To conduct pull-down assay with biotinylated miRNA, MKC cells were harvested at 48 h after transfection, then incubated on ice for 30 min in lysis buffer (20 mM Tris, pH 7.5, 200 mM NaCl, 2.5 mM MgCl₂, 1 mM DTT, 60 U/ml Superase-In, 0.05% Igepal, protease inhibitors). The lysates were precleared by centrifugation for 5 min, and 50 μ l of the sample was aliquoted for input. The remaining lysates were incubated with M-280 streptavidin magnetic beads (Sigma). To prevent nonspecific binding of RNA and protein complexes, the beads were coated with RNase-free BSA and yeast tRNA (Sigma). The beads were incubated for 4 h at 4 °C, washed twice with ice-cold lysis buffer, three times with the low salt buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl pH 8.0, and 150 mM NaCl) and once with the high salt buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl pH 8.0 and 500 mM NaCl). RNA was extracted from the remaining beads with TRIzol Reagent (Invitrogen) and evaluated by qPCR.

RNA immunoprecipitation assay (RIP)

RIP experiments were performed by using the Magna RIP RNA-Binding Protein Immunoprecipitation Kit (Millipore)

following the manufacturer's protocol. The Ago-RIP assay was conducted in MKC cells ($\sim 2.0 \times 10^7$) transfected Ago2-flag or pcDNA3.1-flag and miR-30c-3-3p mimics or control mimics. After 48 h transfection, the cells were extracted and incubated with magnetic beads conjugated with IgG and anti-Flag antibody (Sigma). RNA was extracted from the remaining beads, and qPCR was used to evaluate the expression levels of circBCL2L1.

The MS2-RIP assay was also conducted in MKC cells ($\sim 2.0 \times 10^7$) transfected with pLC5-ciR-MS2, pLC5-ciR-MS2-circBCL2L1, pLC5-ciR-MS2-circBCL2L1-mut, or pMS2-GFP (Addgene). To construct plasmids that could produce circBCL2L1 identified by the MS2 protein, an MS2 fragment was cloned into pLC5-ciR, pLC5-ciR-circBCL2L1, and mutated type of circBCL2L1 plasmid. Furthermore, a GFP and MS2 gene fusion expression plasmid was also constructed to produce a GFP-MS2 fusion protein that could bind with the MS2 fragment and be identified using an anti-GFP antibody (Abcam). After 48 h transfection, the MKC cells were used in RIP assays *via* the Magna RIP RNA-Binding Protein Immunoprecipitation Kit (Millipore) and an anti-GFP antibody following the manufacturer's protocol. RNA was extracted from the remaining beads, and qPCR was used to evaluate the expression levels of miRNAs.

Cell viability and proliferation assay

Cell viability was measured 48 h after transfection in SCRVTreated MKC with Celltiter-Glo Luminescent Cell Viability assays (Promega) according to the manufacturer's instructions. The EdU assay was performed to assess the proliferation of cells by using BeyoClick EdU cell Proliferation Kit with Alexa Fluor 555 (Beyotime) following the manufacturer's instructions. The EdU cell lines were photographed and counted under a Leica DMiL8 fluorescence microscope and evaluated by Thermo Scientific Varioskan LUX. These experiments were repeated three times.

Statistical analysis

Data are expressed as the mean \pm SD from at least three independent triplicated experiments. Student's *t* test was used to evaluate the data. The relative gene expression data was acquired using the $2^{-\Delta\Delta CT}$ method, and comparisons between groups were analyzed by one-way analysis of variance (ANOVA) followed by Duncan's multiple comparison tests (62). A value of $p < 0.05$ was considered significant.

Data availability

All data are contained within the manuscript.

Supporting information—This article contains [supporting information](#).

Acknowledgments—This study was supported by National Natural Science Foundation of China (31822057) and National Key Research and Development Project (2018YFD0900503).

Author contributions—T. X. conceptualization; W. Z. data curation; W. Z., L. Y., and T. X. formal analysis; T. X. funding acquisition; W. Z. investigation; W. Z., L. S., and L. Y. methodology; T. X. project administration; T. X. resources; W. Z., L. S., and L. Y. software; T. X. supervision; L. S., L. Y., and T. X. validation; W. Z. visualization; W. Z. writing—original draft; T. X. writing—review and editing.

Conflict of interest—The authors declare that they have no conflicts of interest with the contents of this article.

Abbreviations—The abbreviations used are: ceRNA, competing endogenous RNA; circRNA, circular RNA; EPC, epithelioma papulosum cyprini cell; FBS, fetal bovine serum; GFP, green fluorescent protein; IL-1, interleukin-1; IRF3, interferon regulatory factor 3; MAVS, mitochondrial antiviral signaling; MBrC, *M. miiuy* brain cell; miRNA, microRNA; MKC, *M. miiuy* kidney cell; MSpC, *M. miiuy* spleen cell; NF- κ B, nuclear factor- κ B; NLR, nucleotide-binding oligomerization domain-like receptor; PAMP, pathogen-associated molecular pattern; PRR, pattern recognition receptor; qPCR, quantitative real-time polymerase chain reaction; RLR, retinoic-acid-inducible gene-I (RIG-I)-like receptor; RPI, RNA immunoprecipitation assay; siRNA, small interfering RNA; TAK1, TGF- β -activated kinase 1; TIM, TRAF-interacting motif; TLR, Toll-like receptor; TRAF, tumor necrosis factor receptor-associated factor.

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circRNA regulates innate immune responses in fish

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