FTO promotes innate immunity by controlling NOD1 expression via m^6^A-YTHDF2 manner in teleost

Highlights
- The demethylase activity of FTO is required for promoting innate immunity of fish
- NOD1 has abundant methylation modifications
- FTO mediates gene expression of NOD1 in an m^6^A-YTHDF2-dependent manner

Geng et al., iScience 25, 105646 December 22, 2022 © 2022 The Authors. https://doi.org/10.1016/j.isci.2022.105646
FTO promotes innate immunity by controlling NOD1 expression via m\(^6\)A-YTHDF2 manner in teleost

Shang Geng, Weiwei Zheng, Yan Zhao, and Tianjun Xu

SUMMARY

N6-methyladenosine (m\(^6\)A), the most abundant internal mRNA modification in eukaryotes, plays a vital role in regulating innate immunity. However, its underlying mechanism remains largely unknown, especially in lower vertebrates. The results of the present study show that fat-mass- and obesity-associated protein (FTO), also known as a m\(^6\)A demethylase, improved the innate immunity and prevented Siniperca chuatsi rhabdo virus and Vibrio anguillarum infection in miiuy croaker. Significantly, FTO-promoted immunity was dependent on its m\(^6\)A demethylase activity. In terms of mechanism, NOD1 has abundant methylation modification in its CDS and 3'UTR regions, and FTO can reduce its methylation level, thus avoiding its degradation by YTHDF2. In summary, our results indicate that the FTO-mediated m\(^6\)A modification in NOD1 mRNA promotes innate immunity by activating the NOD-like receptor pathway, which provides a molecular mechanism for the regulation of immune response via RNA methylation in teleost.

INTRODUCTION

N6-methyladenosine (m\(^6\)A) is the most abundant internal modification of mRNA and noncoding RNA in eukaryotic cells. Fat-mass- and obesity-associated protein (FTO) has attracted research attention as an obesity- and diabetes-related protein, and FTO works as a nucleic acid demethylase capable of removing methyl groups from single-strand RNA, thus regulating the m\(^6\)A level of RNA. FTO has been widely regarded as an attractive biological target owing to its function on the mRNA modification. However, limited studies have focused on its involvement in the innate immune process. Recent research has shown that the recruitment of FTO to CGAS, IFI16, and STING mRNAs could reduce their m\(^6\)A level and thus inhibit their efficient export, indicating that FTO plays a negative role in innate immunity. By contrast, the ablation of FTO could inhibit NLRP3 inflammasome through FoxO1/NF-\(\kappa\)B signaling in macrophages.

Therefore, the molecular mechanism of m\(^6\)A modification in FTO-mediated immune responses remains elusive.

As the first barrier system for the host to defend against pathogen infection, the innate immune system mainly recognizes the pathogen-associated molecular patterns (PAMPs) of various pathogens through various extracellular or intracellular pattern recognition receptors (PRRs), and starts signal transduction to activate the host immune response. Among these PRRs, nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs) are a kind of cytoplasmic PRRs that play key roles in response to various bacterial infections. In which NOD1 is the first discovered member of NLRs, and it is important for cell signaling in response to \(\gamma\)-D-glutamyl-meso-diaminopimelic acid (iE-DAP), a peptidoglycan component found only in Gram-negative bacteria and certain Gram-positive bacteria. NOD1 can monitor bacterial invasion and activate the nuclear factors-\(\kappa\)B (NF-\(\kappa\)B) and mitogen-activated protein kinase (MAPK) signaling pathway through RIPK2, promote the secretion and transcription of inflammatory factors and trigger antibacterial immune response whether in mammals or fish. Furthermore, NOD1 can induce autophagy to clear pathogens by recruiting ATG16L1 to the plasma membrane at the bacterial entry sites. Moreover, previous studies have reported that NOD1 is widely present in fish, and it can recognize Gram-negative bacteria g-D-glutamyl-meso-diaminopimelic acid and LPS, as well as viruses that invade host cells.

Considering that NOD1 is a significant intracellular receptor that recognizes bacteria and virus, the regulatory mechanism of NOD1 signal transduction pathway is particularly important. Previous studies

1. Laboratory of Fish Molecular Immunology, College of Fisheries and Life Science, Shanghai Ocean University, Shanghai, China
2. Laboratory of Marine Biology and Biotechnology, Qingdao National Laboratory for Marine Science and Technology, Qingdao, China
3. Key Laboratory of Exploration and Utilization of Aquatic Genetic Resources (Shanghai Ocean University), Ministry of Education, Shanghai, China
4. National Pathogen Collection Center for Aquatic Animals, Shanghai Ocean University, Shanghai, China
5. Lead contact
6. Correspondence: tianjunxu@163.com
7. https://doi.org/10.1016/j.isci.2022.105646
8. iScience 25, 105646, December 22, 2022 © 2022 The Authors. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).
Figure 1. FTO promotes host innate immunity

(A) mRNA and protein levels of FTO in MKC cells measured by qRT-PCR and Western blotting at indicated time after SCRV infection.

(B) mRNA and protein levels of FTO in MKC cells measured by qRT-PCR and Western blotting at indicated time after V. anguillarum infection.

(C) FTO knockdown and FTO overexpressing MKC cells seeded in 48-well plates overnight were treated with SCRV at the dose indicated for 48 h. Then, cell monolayers were fixed with 4% paraformaldehyde and stained with 1% crystal violet.

(D) FTO inhibits SCRV replication. MKC cells were transfected with si-Ctrl or si-FTO and pcDNA3.1 vector or FTO expression plasmid for 24 h, then infected with SCRV for 24 h. The qPCR analysis was conducted for intracellular and supernatant SCRV RNA levels.

(E) MKCs were transfected with si-FTO or si-Ctrl and with FTO plasmid or control vector, then infected with FITC-labeled V. anguillarum, and then examined by using a fluorescence microscope. Scale bars, 20 mm; original magnification x400.
have found that some noncoding genes, such as miR-125a, miR-495, and miR-130-3p, can target and negatively modulate NOD1 expression to attenuate NOD1-mediated inflammatory response in fish. In addition, protein regulatory factors CENTB1 and Hsp90 can regulate the NOD1 signaling pathway by directly regulating the expression of NOD1. The molecular chaperone Hsp90 regulates the NOD1 signal pathway by regulating the stability of NOD1. Through our laboratory, we found for the first time that the FTO-m6A-YTHDF2 regulatory network, FTO could target and positively regulate the expression of NOD1 to promote the antibacterial and antiviral innate immune responses of teleost fish.

Teleost is a representative population of lower vertebrates, and as an important part of early vertebrate evolution, fish mainly relies on their innate immune system to deal with the invasion of pathogens. Therefore, it is of great evolutionary and immune significance to study their regulatory network of innate immunity. The invasion of pathogens, especially Gram-negative bacteria and RNA virus (e.g., *Vibrio anguillarum*, *Vibrio harveyi*, and *Siniperca chuatsi* rabdo virus (SCRV)), has resulted in great harm to the aquatic industry. However, limited research has focused on the regulation of fish against pathogen invasion. Therefore, the mechanism that induces fish disease needs to be determined, and the prevention of these fish diseases needs to be realized urgently.

In the present study, an m6A regulatory network that is involved in antibacterial and antivirus responses in teleost fish, *Miichthys miiuy*, was identified. The results demonstrate that NOD1 expression is regulated by FTO-mediated m6A modification and promotes innate immune responses by activating the NLR pathway. The biological function and underlying mechanism of FTO were determined, and the results contributed to the understanding of the m6A network mechanism in the innate immune response of teleost fish.

**RESULTS**

**FTO protects the host from *Siniperca chuatsi* rabdo virus and *V. anguillarum* infection**

Only a few studies have reported that mammalian FTO involve in regulating innate immunity, and no study has yet investigated the functional role of FTO in teleost fish in response to viral or bacterial infection. In view of the special biological role of FTO, we explored the expression pattern of FTO after *miiuy* croaker was infected by SCRV and *V. anguillarum*. As shown in Figure S1A, after being stimulated by SCRV, the expression of FTO all showed a trend of first slightly down-regulated and then gradually up in kidney and spleen. Consistent with that in tissues, after SCRV stimulation, the mRNA and protein expression levels of FTO in MKC cells showed a trend of first decreasing and then increasing (Figure 1A). Similarly, we also found that after being infected by *V. anguillarum*, the expression level of FTO decreased first and then increased in kidney and spleen (Figure S1B). We explored the expression pattern of IFN-1, ISG15, and inflammatory cytokines after *miiuy* croaker was infected by SCRV and *V. anguillarum*. As shown in Figure S1C, after being stimulated by SCRV, the expression of IFN-1 and ISG15 all showed a trend of increasing rapidly and gradually decreasing in the later period in kidney and spleen. Similarly, we also found that after being infected by *V. anguillarum*, the expression level of TNF-α and IL-8 all showed a trend of increasing rapidly and gradually decreasing in the later period in kidney and spleen (Figure S1D). We further detect the mRNA and protein levels in *V. anguillarum*-infected MKC cells. As shown in Figure 1B, the results showed that the mRNA and protein levels of FTO peaked at 5 h after *V. anguillarum* infection and gradually decreased after that. To summarize, these data suggested that FTO may play an important role during the process of pathogenic infection, and the different expression of FTO between the early stage and middle to late stage of pathogen infection means that it may play different roles in different infection periods.

We further explore the role of FTO on the innate immune response induced by SCRV or *V. anguillarum* in *miiuy* croaker. Firstly, we designed the two-small interfering RNAs (siRNA) against FTO and transfected...
these two siRNAs (si-FTO-1 and si-FTO-2) and si-Ctrl into MKC cells. Consequently, two siRNAs evidently decreased the expression levels of mRNA and protein of FTO, and si-FTO-2 could induce higher inhibitory efficiency. Thus, si-FTO-2 (termed si-FTO) was selected for the subsequent experiment (Figure S1E). In addition, we constructed FTO overexpression plasmid and carried out the following functional experiments with si-FTO. As expected, the FTO plasmid could remarkably increase the mRNA and protein levels of FTO (Figure S1F). To investigate the biological significance of FTO in SCRV-induced host cells, we used the virus plaque method to explore whether fish FTO could affect viral replication. As shown in Figure 1C, the knockdown of FTO can significantly increase the virus plaque, whereas the overexpression of FTO significantly decreased the viral plaque. In addition, SCRV replication was monitored by measuring the SCRV TCID50 levels in the intracellular and supernatant of SCRV-infected MKC cells. The qPCR results show that the inhibition of FTO facilitated SCRV replication, whereas the overexpression of FTO inhibited SCRV replication (Figure 1D). We further investigated the effect of FTO on the cell viability of MKC cells and found that FTO knockdown significantly decreased cell viability compared with the control group, and overexpression FTO significantly increased cell viability (Figure S1G). Then, by labeling *V. anguillarum* with FITC-D-Lys, the results show that FTO knockdown could significantly enhance the invasion of *V. anguillarum* to MKCs, and FTO overexpression reduced the invasion ability of *V. anguillarum* (Figure 1E). To further confirm the effect of FTO on *V. anguillarum* invading cells, we used a plate counting method to confirm the invasion or intracellular replication ability of *V. anguillarum*. The results showed that knockdown or overexpression of FTO could significantly enhance or weaken the invasion or intracellular replication ability of *V. anguillarum* (Figures 1F and 1G). In addition, considering that inflammatory cytokines and IFN-stimulated genes (ISGs) are important effectors, the role of FTO in regulating the expression of IFN, ISGs, and inflammatory cytokines was investigated. As shown in Figures 1H and 1I, knockdown or overexpression FTO can significantly inhibit or promote the expression levels of TNF-α, Mx1, and ISG15 upon SCRV infection. Then, knockdown or overexpression of FTO can significantly inhibit or promote the expression levels of TNF-α, IL-6, and IL-8 upon *V. anguillarum* stimulation. In addition, fish type I interferon has antibacterial function,27 so the expression of IFN-1 stimulated by *V. anguillarum* was detected, and the results showed that *V. anguillarum* could also stimulate the expression of IFN-1 and was promoted by FTO. Therefore, FTO can positively regulate the antiviral and antibacterial responses of the host.

### The RNA demethylase activity of FTO is required for promoting innate immunity

Considering that FTO is a demethylase of m^6^A in RNA, we next collected data to assess whether FTO-mediated m^6^A demethylation in mRNA is required for resisting pathogen invasion. As shown in Figure 2A, according to the results of FTO protein sequence alignment between different species, the R at position 95 of the fish is necessary for FTO to exercise demethylase activity. Accordingly, we further constructed the point mutation plasmid of FTO (FTO-mut, R95Q). Next, we investigated the effect of FTO on the overall methylation level of MKC cells. As expected, the knockdown of FTO significantly increased the global m^6^A levels of MKCs, while overexpression of FTO significantly decreased the overall methylation level of MK cells, and the expression of FTO-mut had no effect (Figure 2B). We then examined the effect of FTO-mut on the expression of IFN-1 and TNF-α, and the results showed that FTO-mut counteracted the promotion effect of FTO on IFN-1 and TNF-α (Figure 2C). In addition, compared with FTO-mut or control cells, overexpression of FTO significantly decreased the viral plaque and inhibited viral replication (Figures 2D and 2E). Lastly, the ability of *V. anguillarum* to infect MKC cells was only decreased in cells expressing FTO wild-type plasmid, rather than FTO-mut (Figure 2F). Therefore, the m^6^A demethylation activity of FTO is required for its positive effect on the host immune system.

### NOD1 has abundant methylation modifications and high expression changes

The global landscape of m^6^A modification was described, and the potential mechanism by which FTO promotes innate immunity was explored by mining the MeRIP-seq data (GenBank: PRJNA819945 and PRJNA8464999). As shown in Figure 3A, 15,725 m^6^A peaks were detected in untreated spleen tissues of miuy croaker, indicating that m^6^A modification was widespread in teleost fish. In addition, KEGG enrichment analysis indicated that many transcripts participating in the NLR pathway were perturbed by m^6^A modification whether in SCRV or *V. anguillarum*-infected spleen (Figure 3B). Next, with the use of cross-analysis of the m6A-seq and RNA-seq data, we found that compared with the control group, hypo-methylated m^6^A peaks in the NOD1 mRNA transcripts were found to be significant in both SCRV and *V. anguillarum* infection groups (fold change >1.5, p < 0.05), and the expression level of NOD1 in the
infection group was higher than that in the control group, indicating an opposite relationship with the m6A peak of NOD1 (Figure 3C). Further analysis of the m6A-seq data showed that NOD1 contains m6A sites in the 3' UTR and CDS region, in which the m6A levels at 3' UTR were markedly lower in the infection group than that in the control group (Untreat VS SCRV: p = 0.02, Untreat VS V. anguillarum: p = 0.03). To verify the m6A peaks in MeRI analysis of the -seq data, we designed four specific primers on both sides of the m6A peak, and m6A quantitative real-time PCR was performed. In line with the m6A-seq data, the results further confirmed that m6A sites exactly exist in NOD1 3' UTR and CDS (Figure 3E). The mRNA level of NOD1 was further detected after MKC cells were exposed to V. anguillarum and SCRV, and the results showed that the expression of NOD1 was significantly up-regulated (Figure 3F). All in all, these results suggest that NOD1 is an m6A-modified gene with the function of regulating innate immunity.

FTO-mediated m6A demethylation of NOD1 mRNA affects its stability and expression level

In view of the above-mentioned negative correlation between NOD1 expression and methylation level, we further investigate the effect of FTO on NOD1 expression and methylation level. As shown in Figure 4A, the m6A levels in NOD1 mRNA rose from 1.5- to 2.5-fold in the FTO-knockdown MKC cells, according to gene-specific m6A quantitative real-time PCR assays. In FTO-overexpressed MKC cells, the level of NOD1 methylation decreased to 0.2- to 0.7-fold that of the control group (Figure 4B). To further investigate the effect of m6A modification on NOD1 expression, quantitative real-time PCR and Western blot assays were conducted. The results showed that FTO knockdown or overexpression could inhibit or promote the expression of NOD1, while FTO-mut plasmid without demethylase activity had no effect on the
NOD1 expression (Figures 4C and 4D). The regulation of NOD1 was further examined by FTO during pathogen infection, we explored the effects of FTO knockdown and overexpression on NOD1 under the stimulation of SCRV and V. anguillarum. Results showed that FTO knockdown and overexpression respectively downregulated and upregulated the mRNA and protein levels of NOD1 dramatically, respectively (Figures 4E and 4F). Next, RNA stability assays were used to measure the effect of m^6^A levels on the stability of NOD1. A shortened half-life of NOD1 mRNA was observed in FTO-knockdown cells treated with actinomycin D compared with control MKC cells, in contrast, whereas the overexpression of FTO wild-type but not FTO-mut plasmids increased the half-life of NOD1 (Figure 4G). In view of the abundant methylation modifications in the CDS and 3'UTR of NOD1 (Figure 3D), two reporter plasmids, namely NOD1-CDS and NOD1-3'UTR, were constructed respectively. As expected, results showed that the luciferase activity of NOD1-CDS and NOD1-3'UTR reporter was markedly reduced or enhanced upon FTO knockdown or overexpression, and FTO-mut did not affect the fluorescence activity of the two reporter plasmids (Figure 4H). Then, potential sites on NOD1-3'UTR were further investigated, as shown in Figure 4I, two potential m^6^A sites were found in the 3'UTR of NOD1. Then we constructed mutant NOD1 3'UTR reporter. For the mutant form of NOD1-3'UTR reporter genes, the adenosine bases in m^6^A consensus sequences (DRACH) were replaced by uracil, thus canceling m^6^A decoration (Figure 4I). As expected, our results showed that the luciferase activity of wild-type pmirGLO-NOD1-3'UTR reporter, but not of mutant pmirGLO-NOD1-3'UTR reporter, was markedly changed upon FTO knockdown or overexpression (Figure 4J). We further used other M. miiuy cell lines (M. miiuy liver cells, MLCs) and mammalian cell lines (HEK293 cells) to explore the regulatory effect of FTO on NOD1. Results showed that knockdown or overexpression of FTO can decrease or increase the mRNA, protein, and stability levels of NOD1 in MLC cells.
FTO mediates gene expression of NOD1 in an m6A-YTHDF2-dependent manner

The major mechanism by which m6A modification influences mRNA fate is by recruiting m6A-binding proteins. Current data indicate that YTHDF2 and YTHDF3 have the function of recognizing m6A sites and accelerating mRNA decay. Therefore, m6A-modified NOD1 might be recognized and destabilized by YTHDF2.
or YTHDF3. We first examined the binding ability between YTHDF2 and YTHDF3 protein and NOD1 mRNA, and the results showed that both of them bound to NOD1, while the binding ability between NOD1 and YTHDF2 was stronger than that between YTHDF3 (Figure 5A). The mechanism in which they regulate NOD1 was determined by further designing two siRNAs that target YTHDF2 and YTHDF3, which both have good silencing effects (Figure 5B). Next, their effects on NOD1 expression were investigated. Results showed that the knockdown and overexpression of YTHDF2 influenced the mRNA and protein levels of NOD1, while the knockdown and overexpression of YTHDF3 had no significant effect on the mRNA and protein expression of NOD1 (Figures 5C and 5D). Next, the effect of YTHDF2 on NOD1 stability was examined. As shown in Figure 5E, the knockdown of YTHDF2 significantly increased the stability of NOD1 mRNA, and in contrast, overexpression of YTHDF2 can significantly decrease the half-life of NOD1 mRNA. To this end, we co-transfected YTHDF2 and FTO expression plasmids into MKC cells and found that FTO can reverse the negative effect of YTHDF2 overexpression on the mRNA and protein expression of NOD1 (Figure 5F). The knockdown of YTHDF2 could significantly counteract the effects of the knockdown of FTO on NOD1 (Figure 5G). Overall, these results indicate that the FTO-mediated demethylation of NOD1 may upregulate its expression in m6A-YTHDF2-dependent manner.

FTO-mediated upregulation of NOD1 promotes innate immunity

Considering the important role of FTO-mediated m6A in regulating immunity and NOD1 expression, we investigated whether the immune function of FTO depends on the NOD1-mediated NLR pathway. The forced expression of NOD1 alone rescued the inhibition of NOD1 expression caused by FTO knockdown (Figures 6A and 6B), and subsequently restored the reduced expression levels of IFN-1 and TNF-α in FTO-depleted MKC cells (Figure 6B). Moreover, NOD1 overexpression restored the reduced ability of resisting SCRV viral infection in FTO-depleted cells (Figures 6C and 6D). Consistently, NOD1 overexpression achieved a similar restoration effect in terms of antibacterial defense in FTO-depleted conditions.
DISCUSSION

Various diseases caused by pathogens, such as viruses and bacteria, are the biggest threat to the aquaculture industry. Among these viruses, Rhabdoviruses are a group of enveloped, single-stranded, and negative-sense RNA viruses which are among the most significant viral pathogens in teleost fish and cause severe hemorrhagic septicemia in freshwater and marine fish. V. anguillarum is the most lethal and widely spread pathogenic bacterium in the aquatic industry. It causes huge economic losses to the aquatic industry every year. However, limited research has focused on the regulation of viral and bacterial infections, thus requiring in-depth and urgent research. As an immune-related intracellular receptor, NOD1 plays an important role in sensing or resisting the invasion of pathogens, such as viruses or bacteria. Fish NOD1 also has similar functions. For example, miuy croaker NOD1 can recognize g-D-glutamyl-meso-diaminopimelic acid and SCRV virus, thereby activating the NF-κB or IRF3 signaling pathway, promoting the production of inflammatory factors and antiviral factors, and resisting bacterial and viral invasion. In addition, the innate immune response initiated by the NOD1 often requires the participation of a series of regulatory factors, including some coding genes and non-coding RNA.
noncoding genes. Based on the present study, we found that two RNA methylation-related genes, namely FTO and YTHDF2, can co-regulate the expression of NOD1, thus affecting the immune response of host.

Protection against harmful pathogens depends on the activation of the immune system, which relies on the strict regulation of gene expression. Recently, m6A plays an important role in this regulation, while its role in immunity is two-sided. Two studies of the Cao group showed that m6A plays a crucial role in amplifying the antiviral responses by enhancing the export of different components in the type I IFN pathway. By contrast, other groups demonstrated that m6A modification inhibits immune responses. For example, the mRNAs of IFN-β and IFN-α, genes encoding the main cytokines that drive the type I interferon response, are m6A-modified and are stabilized following the depletion of METTL3 or METTL14, leading to increases in type I IFN expression and ISG induction. Consequently, another study illustrated that the m6A reader YTHDF3 also restrains the type I IFN response and ISG expression in an m6A-independent way. In addition, some results suggested that m6A modification interrupts the RIG-I and TLR innate immune activation pathway, and that nucleotide modifications with similar chemical structures may be organized into classes, which contribute to avoiding innate immune response. In the present study, we focused on FTO, a demethylase, and found that it can inhibit viral replication and bacterial infection and play an active role in the immune process of the body. In addition, after constructing FTO mut mutant plasmid to remove its demethylase activity, the immune function of FTO disappeared, indicating that the function of FTO to improve immunity depends on its demethylase activity.

We further explored the potential immune regulatory mechanism of FTO. Our findings provide a immunoregulatory mechanism of m6A, in which FTO activates the NLR pathway via NOD1 mRNA m6A methylation, promotes the host innate immunity, and improves resistance to viruses and bacteria. The function of FTO in promoting innate immunity depends on its demethylase activity. However, we cannot rule out other signaling pathways that could be altered directly or indirectly by FTO in immune response. Moreover, it is worth noting that previous studies have shown that the regulatory network of m6A on NOD1 exists in mouse macrophage cell lines, their findings suggested that METTL3 depletion inhibits the degradation of NOD1 mRNA mediated by YTHDF1 and YTHDF2, which upregulate the NOD1 pathway and subsequently promote the inflammatory response. Our results confirmed with each other that the FTO-mediated demethylation of NOD1 improves the immune capacity of the miyu croaker, and then promotes the immune response induced by SCRV and V. anguillarum. However, our further study showed that FTO seemed to have no regulatory effect on NOD1 in HEK293 cells, which may be due to the species specificity of m6A. The m6A is emerging as a wide-spread regulatory mechanism that is specific in different species and even in various tissue and cell types. In addition, their studies did not reveal the regulatory effect of methylase on the methylation level of NOD1 itself. This limitation can be attributed to the large number of methylation sites and wide regional distribution of NOD1, which brings difficulties in the primer design in MeRIP-qPCR. In the present study, we designed four pairs of m6A-specific primers for the m6A peak in the control group, SCRV-infected group, and V. anguillarum infected group, and successfully verified the presence of m6A sites in these four regions. Moreover, we verified two specific sites on NOD1-3’UTR by double fluorescence experiments, one of which is just on the stop codon of NOD1 mRNA, according to the law of the previous studies. NOD1 signaling is linked by RIPK2 kinase in fish and mammals. However, it is not clear at present whether FTO affects RIPK2 kinase in fish like METTL3 in mouse macrophages, which is undoubtedly a point of great concern.

Generally, m6A modification regulates mRNA fate and functions mainly through recruiting proteins. Considering the negative correlation between FTO-mediated mRNA expression of NOD1 and m6A level, and the ability of YTHDF2 and YTHDF3 to destabilize the mRNA containing the m6A site, we further examined the binding ability and expression effect of YTHDF2 and YTHDF3 on NOD1. Our results showed that both YTHDF2 and YTHDF3 can bind to NOD1, while it is worth noting that the binding ability of YTHDF2 to NOD1 is notably much higher than that of YTHDF3 and the knockdown and overexpression of YTHDF3 have no obvious effect on the expression of NOD1. Hence, YTHDF3 may only play an assisting role in the degradation of NOD1 by YTHDF2. Additionally, previous studies showed that although the depletion of single YTHDF paralogs may lead to mild or no effects on mRNA abundance and stability, the depletion of all three DF proteins led to robust stabilization of m6A-mRNAs. Thus, the function of YTHDF3 combined with NOD1 may need to be further explored. Moreover, based on the m6A peak map of MeRIP-seq data.
and online prediction of the m6A site of NOD1, NOD1 has abundant methylation sites, and there is evidence that all m6A-modified mRNAs are subjected to the combined action of YTHDF proteins in proportion to the number of m6A sites. Therefore, YTHDF2 obtains a more obvious degradation effect by binding more NOD1 m6A sites than YTHDF3.

In summary, we found that FTO, an m6A demethylase, plays an active role in the inflammation and antibacterial response of teleost fish. Specifically, FTO and YTHDF2 cooperatively regulate the expression of NOD1 via m6A modification, thus influencing the NLR pathway. Our results not only highlight the importance of the m6A modification-mediated regulation of gene expression during innate immunity but also provide profound insights into the molecular mechanisms of m6A modifications in the process by which the host resists viral and bacterial invasion.

Limitations of the study
Our study evidenced that FTO-mediated demethylation modification in NOD1 mRNA promotes innate immunity by activating the NLR pathway, which provides a molecular mechanism for the regulation of immune response via RNA methylation in lower vertebrates. However, the role of m6A in the regulation of innate immune response is still worthy of further study. On the one hand, whether FTO is involved in regulating other genes in the immune pathway remains to be studied. On the other hand, the roles of many other m6A-related genes in the immune process, even the regulation effects to NOD1, are still unclear. As a very important antiviral and antibacterial immune gene, NOD1 has still not been reported to be modified by m6A in lower vertebrates, NOD1 as a target gene of FTO may explain why FTO promoted host innate immune system in miuy croaker.

STAR METHODS
Detailed methods are provided in the online version of this paper and include the following:

- KEY RESOURCES TABLE
- RESOURCE AVAILABILITY
  - Lead contact
  - Materials availability
  - Data and code availability
- EXPERIMENTAL MODEL AND SUBJECT DETAILS
  - Ethics and animals
  - Siniperca chuatsi rhabdovirus
  - Cell line and bacterial
- METHOD DETAILS
  - MeRIP-seq assays and sequencing data analysis
  - MeRIP-qPCR
  - Virus yield quantification
  - Bacteria invasion
  - Colony formation assay
  - Plasmids construction
  - RNA interference
  - Cells transfection
  - RNA extraction and quantitative real-time PCR
  - RNA binding protein immunoprecipitation (RIP)
  - Total m6A quantification assay
  - RNA stability assays
  - Dual-luciferase reporter assays
  - Western blotting
  - Cell viability
  - Bioinformatic analysis
- QUANTIFICATION AND STATISTICAL ANALYSIS

SUPPLEMENTAL INFORMATION
Supplemental information can be found online at https://doi.org/10.1016/j.isci.2022.105646.
ACKNOWLEDGMENTS
This study was supported by the National Natural Science Foundation of China (31822057).

AUTHOR CONTRIBUTIONS
S.G. designed and performed experiments, analyzed the data, performed bioinformatics analysis, and wrote the article. W.Z. performed experiments and provided advice on experimental plans. Y.Z. performed experiments. T.X. directed all the experiments and participated in experimental design, data analyses, interpretation, and article writing.

DECLARATION OF INTERESTS
The authors declare no competing interests.

Received: August 18, 2022
Revised: October 10, 2022
Accepted: November 18, 2022
Published: December 22, 2022

REFERENCES
1. Shulman, Z., and Stern-Ginossar, N. (2020). The RNA modification N-6-methyladenosine as a novel regulator of the immune system. Nat. Immunol. 21, 501–512.
2. Dina, C., Meyre, D., Gallina, S., Durand, E., Körner, A., Jacobsson, P., Carlsson, L.M.S., Kress, W., Vatin, V., Lecocq, C., et al. (2007). Variation in FTO contributes to childhood obesity and severe adult obesity. Nat. Genet. 39, 724–726.
3. Scuteri, A., Sanna, S., Chen, W.M., Uda, M., Albai, G., Strait, J., Najjar, S., Nagaraja, R., Orrú, M., Usala, G., et al. (2007). Genomewide association scan shows genetic variants in the FTO gene are associated with obesity-related traits. PLoS Genet. 3, e115.
4. Jia, G., Fu, Y., Zhao, X., Dai, Q., Zheng, G., Yang, Y., Yi, C., Lindahl, T., Pan, T., Yang, Y.G., and He, C. (2011). N6-Methyladenosine in nuclear RNA is a major substrate of the obesity-associated FTO. Nat. Chem. Biol. 7, 885–887.
5. Wang, L., Wen, M., and Cao, X. (2019). Nuclear hnRNPA2B1 initiates and amplifies the innate immune response to DNA viruses. Science 365, eaax0756.
6. Luo, J., Wang, F., Sun, F., Yue, T., Zhou, Q., Yang, C., Rong, S., Yang, P., Xiong, F., Yu, Q., et al. (2021). Targeted inhibition of FTO demethylase protects mice against LPS-induced septic shock by suppressing NLRP3 inflammasome. Front. Immunol. 12, 663295.
7. Takeda, K., and Akira, S. (2005). Toll-like receptors in innate immunity. Int. Immunol. 17, 1–14.
8. Ellis, A.E. (2001). Innate host defense mechanisms of fish against viruses and bacteria. Dev. Comp. Immunol. 25, 827–839. https://doi.org/10.1016/s0145-305x(01)00038-6.
9. Matzinger, P. (2002). An innate sense of danger. In Reparative Medicine: Growing Tissues and Organs, J. D. Sipe, C.A. Kelley, and L.A. McNicol, eds., pp. 341–342.
10. Kim, Y.K., Shin, J.S., and Nahm, M.H. (2016). NOD-like receptors in infection, immunity, and diseases. Yonsei Med. J. 57, 5–14.
11. Roy, A.C., Chang, G., Ma, N., Wang, Y., Roy, S., Liu, J., Aabdin, Z.U., and Shen, X. (2019). Sodium butyrate suppresses NOD1-mediated inflammatory molecules expressed in bovine hepatocytes during LPS treatment. J. Cell. Physiol. 234, 1966–1970.
12. Chamaillard, M., Hashimoto, M., Horie, Y., Masumoto, J., Qiu, S., Saab, L., Oguro, Y., Kawasaki, A., Fukase, K., Kusumoto, S., et al. (2003). An essential role for NOD1 in host recognition of bacterial peptidoglycan containing diaminopimelic acid. Nat. Immunol. 4, 702–707.
13. Hasegawa, M., Fujimoto, Y., Lucas, P.C., Nakano, H., Fukase, K., Nüñez, G., and Inohara, N. (2003). Critical role of RICK/RIP2 polyubiquitination in Nod-induced NF-kappa B activation. EMBO J. 22, 373–383.
14. Park, J.H., Kim, Y.G., McDonald, C., Kanneganti, T.D., Hasegawa, M., Body-Malapel, M., Inohara, N., and Nüñez, G. (2007). RICK/RIP2 mediates innate immune responses induced through Nod1 and Nod2 but not TLRs. J. Immunol. 178, 2380–2386.
15. Bi, D., Wang, Y., Gao, Y., Li, X., Chu, Q., Cui, J., and Xu, T. (2018). Recognition of lipopolysaccharide and activation of NF-kappa B by cytokotic sensor NOD1 in teleost fish. Front. Immunol. 9, 1413.
16. Cao, M., Yan, X., Li, Q., Fu, Q., Yang, N., Song, L., and Li, C. (2021). Genome-wide identification and analysis of NOD-like receptors and their potential roles in response to Edwardsiella tarda infection in black rockfish (Sebastes schlegelii). Aquaculture 541, 736803.
17. Travassos, L.H., Carneiro, L.A.M., Ramjeet, M., Hussey, S., Kim, Y.G., Magalhães, J.G., Yuan, L., Soares, F., Chea, E., Le Bourhis, L., et al. (2010). Nod1 and Nod2 direct autophagy by recruiting ATG16L1 to the plasma membrane at the site of bacterial entry. Nat. Immunol. 11, 55–62.
18. Wu, X.M., Zhang, J., Li, P.W., Hu, Y.W., Cao, L., Ouyang, S., Bi, Y.H., Nie, P., and Chang, M.X. (2020). NOD1 promotes antiviral signaling by binding viral RNA and regulating the interaction of MDAS and MAVS. J. Immunol. 204, 2216–2231.
19. Zheng, W., Su, H., Lu, X., Xin, S., and Xu, T. (2022). Exon-intron circular RNA circRNF217 promotes innate immunity and antibacterial activity in teleost fish by reducing miR-130-3p function. J. Immunol. 208, 1099–1114.
20. Wang, X., Jin, H., Jiang, S., and Xu, Y. (2018). MicroRNA-495 inhibits the high glucose-induced inflammation, differentiation and extracellular matrix accumulation of cardiac fibroblasts through downregulation of NOD1. Cell. Mol. Biol. Lett. 23, 23.
21. Kang, H., Park, Y., Lee, A., Seo, H., Kim, M.J., Choi, J., Ho, H.N., Jeong, H.N., Cho, J.G., Chang, W., et al. (2017). Negative regulation of NOD1 mediated angiogenesis by PPAR gamma-regulated miR-125a. Biochem. Biophys. Res. Commun. 482, 28–34.
22. Hahn, J.S. (2005). Regulation of Nod1 by Hsp90 chaperone complex. FEBS Lett. 579, 4513–4519.
23. Yamamoto-Furusho, J.K., Barnich, N., Xavier, R., Hisamatsu, T., and Podolsky, D.K. (2006). Centaurin beta 1 down-regulates nucleotide-binding oligomerization domains 1- and 2-dependent NF-kappa B activation. J. Biol. Chem. 281, 36603–36607.
24. Frans, I., Michels, C.W., Bossier, P., Willems, K.A., Lievens, B., and Rediers, H. (2011). Vibrio anguillarum as a fish pathogen: virulence factors, diagnosis and prevention. J. Fish. Dis. 34, 643–667.
25. Defoirdt, T. (2014). Virulence mechanisms of bacterial aquaculture pathogens and antivirulence therapy for aquaculture. Rev. Aquac. 6, 100–114. https://doi.org/10.1111/raq.12030.

26. Zhang, Q., and Gui, J.F. (2015). Virus genomes and virus-host interactions in aquaculture animals. Sci. China Life Sci. 58, 156–169.

27. Xiao, X., Zhu, W., Zhang, Y., Liao, Z., Wu, C., Yang, C., Zhang, Y., Xiao, S., and Su, J. (2021). Broad-spectrum robust direct bactericidal activity of fish IFN phi 1 reveals an antimicrobial peptide-like function for type I IFNs in vertebrates. J. Immunol. 206, 1337–1347.

28. Zhou, Y., Zeng, P., Li, Y.-H., Zhang, Z., and Cui, Q. (2016). SRAMP: prediction of mammalian N-6-methyladenosine (m6A) sites based on sequence-derived features. Nucleic Acids Res. 44, e91.

29. Caruso, R., Warner, N., Inohara, N., and Núñez, G. (2014). SARM: prediction of mammalian N-6-methyladenosine (m6A) sites based on sequence-derived features. Nucleic Acids Res. 44, e91.

30. Vegna, S., Gregoire, D., Moreau, M., Lassus, P., Duranet, D., Assenat, E., Hibner, U., and Simonin, Y. (2016). NOD1 participates in the innate immune response triggered by Hepatitis C virus polymerase. J. Virol. 90, 6022–6035.

31. Qin, F., Cai, B., Zhao, J., Zhang, L., Zheng, Y., Liu, B., and Gao, C. (2021). Methyltransferase-like protein 14 attenuates mitochondrial antiviral signaling protein expression to negatively regulate antiviral immunity via N-6-methyladenosine modification. Adv. Sci. 8, e2100636.

32. Zhang, Y., Wang, X., Zhang, X., Wang, J., Ma, Y., Zhang, L., and Cao, X. (2019). RNA-binding protein YTHDF3 suppresses interferon-dependent antiviral responses by promoting FOXO3 translation. Proc. Natl. Acad. Sci. USA 116, 976–981.

33. Durbin, A.F., Wang, C., Marcoatrigiano, J., and Gehrke, L. (2016). RNAs containing modified nucleotides fail to trigger RIG-I conformational changes for innate immune signaling. mBio 7, e00833-00833-16.

34. Kanka, K., Buckstein, M., Ni, H., and Weissman, D. (2005). Suppression of RNA recognition by Toll-like receptors: the impact of nucleoside modification and the evolutionary origin of RNA. Immunity 23, 165–175.

35. Cai, Y., Yu, R., Kong, Y., Feng, Z., and Xu, Q. (2022). METTL3 regulates LPS-induced inflammatory response via the NOD1 signaling pathway. Cell. Signal. 93, 110283.

36. Fang, X., Li, M., Yu, T., Liu, G., and Wang, J. (2020). Reversible N6-methyladenosine of RNA: the regulatory mechanisms on gene expression and implications in physiology and pathology. Genes Dis. 7, 585–597.

37. Chen, C., Chen, H., Zhang, Y., Thomas, H.R., Frank, M.H., He, Y., and Xia, R. (2020). TBtools: an integrative toolkit developed for interactive analyses of big biological data. Mol. Plant 13, 1194–1202.

38. Zheng, W., Chu, Q., Yang, L., Sun, L., and Xu, T. (2021). Circular RNA circDtx1 regulates IRF3-mediated antiviral immune responses through suppression of miR-15a-5p-dependent TRIF downregulation in teleost fish. PLoS Pathog. 17, e1009438.

39. Chu, Q., Xu, T., Zheng, W., Chang, R., and Zhang, L. (2020). Long noncoding RNA MARL regulates antiviral responses through suppression of miR-122-dependent MAVS downregulation in lower vertebrates. PLoS Pathog. 16, e1008670.

40. Edwards, D.J., Grodevant, N.W., Lee, P.J., and Peralta, J.B.; IEEE (2007). DNA-MAN: Dynamic Natural Attributes for Synthetic Military Forces 27 (IEEE), pp. 246–250.

41. Livak, K.J., and Schmittgen, T.D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2(T)(-Delta Delta C) method. Methods 25, 402–408.
## STAR METHODS

### KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| **Antibodies**      |        |            |
| Rabbit anti-Miiuy croaker NOD1 | This paper | N/A |
| Rabbit anti-human NOD1 | Absin | Cat# abs115515 |
| Rabbit anti-FTO | Beyotime | Cat# AF6936 |
| mouse anti-Flag | Beyotime | Cat# AF519 |
| mouse anti-Tubulin | Beyotime | Cat# AT819 |
| HRP-conjugated anti-rabbit IgG | Abbkine | Cat# A25022; RRID: AB_2893334 |
| HRP-conjugated anti-mouse IgG | Abbkine | Cat# A25012; RRID: AB_2737290 |
| **Bacterial and virus strains** | | |
| Vibrio anguillarum | This paper | N/A |
| Siniperca chuatsi rhabdovirus | This paper | N/A |
| **Chemicals, peptides, and recombinant proteins** | | |
| DAPI | Beyotime | Cat# C1002 |
| FITC-D-lys | Bioluminor | Cat# I0201 |
| **Critical commercial assays** | | |
| EpiQuikTM CUT&RUN m6A RNA Enrichment Kit | Epigentek | Cat# A-P-9018 |
| Lipofectamine RNAiMAX | Invitrogen | Cat# 13778150 |
| Lipofectamine 3000 | Invitrogen | Cat# L3000015 |
| FastQuant RT Kit | Tiangen | Cat# KR106-03 |
| Magna RIP RNA-Binding Protein Immunoprecipitation Kit | Millipore | Cat# 17-700 |
| EpiQuikm6A RNA Methylation Quantification Kit | Colorimetric | Cat# P-9005-48 |
| BCA Protein Assay kit | Beyotime | Cat# P00125 |
| CellTiter-Glo Luminescent Cell Viability assays Kit | Promega | Cat# G7570 |
| Mut Express II Fast Mutagenesis Kit V2 | Vazyme | Cat# C214 |
| Endotoxin-Free Plasmid DNA Minprep Kit | Tiangen | Cat# DP118 |
| SYBR Premix Ex Taq™ | Takara | Cat# DRR0415 |
| **Deposited data** | | |
| Methylated RNA immunoprecipitation sequencing data | This paper | GenBank: PRJNA819945 and PRJNA846999 |
| **Experimental models: Cell lines** | | |
| HEK293 | ATCC | Cat# CRL-1573 |
| MKC | This paper | N/A |
| MLC | This paper | N/A |
| **Experimental models: Organisms** | | |
| Miiuy croaker (~50 g, five-months-old, male or female) | This paper | N/A |
| **Oligonucleotides** | | |
| Primers are listed in Table S1 | This paper | N/A |
| **Recombinant DNA** | | |
| pcDNA3.1-Flag (or the mutant) | This paper | N/A |
| PmirGLO (or the mutant) | This paper | N/A |

(Continued on next page)
RESOURCE AVAILABILITY

Lead contact
Further information and requests for resources should be directed to and will be fulfilled by the lead contact, Tianjun Xu (tianjunxu@163.com).

Materials availability
All materials generated in this study are available from the lead contact without restriction.

Data and code availability
The Methylated RNA immunoprecipitation sequencing data reported in this paper have been submitted to GenBank: PRJNA819945 and PRJNA846999.

This paper does not report original code.

Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Ethics and animals
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). Miuy croaker (~50 g, five-months-old, sex-randomized) was obtained from Zoushan Fisheries Research Institute, Zhejiang Province, China. Fish was acclimated in aerated seawater tanks at 25°C for six weeks before experiments. Animals were then randomly selected for study.

Siniperca chuatsi rhabdovirus
The virus strain used in this study was a gift from the Aquatic Pathogen Collection Centre of Ministry of Agriculture, China. In the SCRV injection group, fish was challenged with 200 μL SCRV at a multiplicity of infection (MOI) of 5 through intraperitoneal. As a comparison, 200 μL of physiological saline was used to challenge the individuals.

Cell line and bacterial
M. miuy kindey cells (MKCs), M. miuy liver cells (MLCs) were cultured in L-15 medium (HyClone) supplemented with 15% fetal bovine serum (FBS; Gibco), 100 U/mL penicillin, and 100 μg/mL streptomycin at 26°C. The above two cell lines were prepared from the corresponding tissues of the miuy croaker. Tissues were minced thoroughly with scissors and pushed carefully through a 100-μm nylon mesh in L-15 medium containing penicillin (100 IU/mL), streptomycin (100 mg/mL), 2% fetal bovine serum (FBS), and heparin (20 units/mL) to give a single cell suspension. The filtered cell suspension was loaded onto 34/51% Percoll (Pharmacia, USA) density gradient, and then centrifuged at 400 x g for 40 min at 4°C. Subsequently, the supernatant was removed and the cells at the interfaces were obtained with care and washed twice in L-15 medium at 300 x g for 10 min at 4°C. Cells were cultured in L-15 containing 0.1% FBS at 26°C, 4% CO₂. The next day, the cell pellet was re-suspended in fresh complete L-15 medium supplemented with 20% FBS. HEK293 cells were cultured in Dulbecco’s modified Eagle medium (GE, USA) supplemented with 10% fetal bovine serum (FBS) (Thermo Fisher Scientific, USA), 100 U/mL of penicillin, and 100 μg/mL of streptomycin at 37°C in 5% CO₂. In the V. anguillarum

Continued
injection group, fish were injected intraperitoneally with 1 mL bacteria suspension, which was made after *V. anguillarum* (Aquatic Pathogen Collection Centre of Ministry of Agriculture, China) centrifuge to approximately 3.0 x 10^7 colony forming units (CFU)/mL in phosphate-buffered saline while the control fish were challenged with 1 mL phosphate-buffered saline and were maintained in separate tanks. Afterwards, fishes were respectively sacrificed at different time point and the kidney and spleen tissues were collected for RNA extraction.

**METHOD DETAILS**

**MeRIP-seq assays and sequencing data analysis**

Total RNA was isolated using TRIzol reagent (Invitrogen; 15596018). m^6^A immunoprecipitation and library preparation were performed following the manufacturer’s protocol. Poly (A) RNA is purified from 50 µg total RNA using Dynabeads Oligo (dT) 25-61005 (Thermo Fisher, CA, USA) using two rounds of purification. Then the poly(A) RNA was fragmented into small pieces using Magnesium RNA Fragmentation Module (NEB, USA; e6150) under 86°C for 7 min. Then the cleaved RNA fragments were incubated for 2 h at 4°C with m^6^A-specific antibody (No. 202003, Synaptic Systems, Germany) in IP buffer (50 mM Tris-HCl, 750 mM NaCl and 0.5% Igepal CA-630). Then the IP RNA was reverse-transcribed to create the cDNA by SuperScript™ II Reverse Transcriptase (Invitrogen, USA, 1896649). The average insert size for the final cDNA library was 300 ± 50 bp. For sequencing data analysis, we used HISAT2 to map reads to the reference genome of *M. miiuy*. Mapped reads of IP and input libraries were provided for R package exomePeak, which identifies m^6^A peaks with bed or bigwig format that can be adapted for visualization on the IGV software. MEME and HOMER were used for de novo and known motif finding followed by localization of the motif with respect to peak summit. Called peaks were annotated by intersection with gene architecture using R package ChIPseeker. The differentially expressed mRNAs were selected with log2 (fold change) >1 and p value <0.05 by R package edgeR. The circus map of m^6^A peaks in untreated spleen tissues of miiuy croaker was showed by TBtools.37

**MeRIP-qPCR**

Total RNA was extracted using TRIzol reagent (Invitrogen; 15596018). Methylated m^6^A RNA immunoprecipitation (Me-RIP) was performed according to the protocol of EpiQuik™ CUT&RUN m^6^A RNA Enrichment Kit (Epigentek; A-P-9018). qPCR analysis of the methylated RNA was performed to detect methylated NOD1 mRNA levels.

**Virus yield quantification**

For stimulation experiments, Cells were challenged with SCRV at a multiplicity of infection (MOI) of 5 and harvested at different times for RNA extraction. SCRV virus was isolated as described previously, and the replication of SCRV was detected by quantitative PCR (qPCR). For virus plaque assay, a volume of 0.1 mL of the cultural supernatant was then serially diluted on the monolayer of MKCs, and MKCs were seeded into 48-well plates 1 day before measurement. The cell monolayer was washed with PBS, fixed with 4% paraformaldehyde, and stained with 1% crystal violet.

**Bacteria invasion**

*V. anguillarum* was washed with fresh medium three times, centrifuged, and diluted with fresh medium containing 100 mM FITC-D-Lys (Xiamen Bioluminor Bio-Technology). After 30 min of incubation at 37°C, the cells were centrifuged, washed with fresh medium three times, and then resuspended in PBS. MKCs (1 x 10^5 cells/well) were distributed in a 48-well plate and cultured in L-15 medium (HyClone; LA9510) supplemented with 15% FBS (Life Technologies; 10099-141), 100 U/mL penicillin, and 100 mg/mL streptomycin at 26°C. Cells were grown to 60-70% confluence and then synchronized by serum starvation overnight. Then the cells were washed three times with PBS. FITC-labeled *V. anguillarum* (1 x 10^6 cells/mL) was added to the dishes in 200 mL of serum-free L-15 medium, which was allowed to infect MKCs at different times for invasion. The infected cells were washed three times with PBS, and 200 mL of L-15 medium supplemented with 15% FBS (Life Technologies; 10099-141), 100 U/mL penicillin, and 100 mg/mL streptomycin was added at 26°C for 1 h. Then the infected cells were washed three times with PBS and immobilized by incubating with 0.2% Triton X-100 in 4% paraformaldehyde for 30 min at room temperature. After DAPI staining (Beyotime; C1002), the cells were photographed and counted under a Leica DMLB fluorescence microscope and evaluated by using a Thermo Scientific Varioskan LUX. These experiments were repeated three times.
Colony formation assay
MKC (1 x 10^5 cells/well) were distributed in a 48-well plate and cultured in L-15 medium (HyClone; LA9510) supplemented with 15% FBS (Life Technologies; 10099-141), 100 U/mL penicillin, and 100 mg/mL streptomycin at 26°C. Cells were grown to 60-70% confluence and then synchronized by serum starvation overnight. Then the cells were washed three times with PBS. V. anguillarum (1 x 10^6 cells/mL) was added to the dishes in 200 mL serum-free L-15 medium, which was allowed to infect MKCs at different times for invasion. The infected cells were washed three times with PBS, and 200 mL of L-15 medium supplemented with 15% FBS (Life Technologies; 10099-141), 100 U/mL penicillin, and 100 mg/mL streptomycin was added at 26°C for 1 h. Then the infected cells were washed three times with PBS, 1 mL sterile ddH2O was added, and the cells were lysed at 4°C for 30 min. Then the above cell lysate was diluted 1000 times and coated on the agar plates. The plates were incubated at 37°C for 12 h, and the colonies that emerged on the plates were counted.

Plasmids construction
To construct flag-tagged FTO, NOD1, YTHDF2, and YTHDF3 expression plasmid, the FTO (no. OP168916), NOD1 (no. KP715094.1), YTHDF2 (no. OM095375), and YTHDF3 (no. OP219797) sequences of M. miiu were amplified by PCR and then ligated into a pcDNA3.1 vector (Invitrogen), respectively. Then, FTO-mut plasmid (R95Q) was acquired by point mutation of FTO plasmid. To construct NOD1-3'UTR and NOD1-CDS reporter vectors, the 3'UTR and CDS region of M. miiu NOD1 cDNA were amplified using PCR with the gene-specific primers and ligated into a pmir-GLO luciferase reporter vector (Promega). For mutant reporter vectors (NOD1-3'UTR-mut), adenosine (A) in the predicted m^6A motif was replaced by an uracil (U) by using the Mut Express II Fast Mutagenesis Kit V2 (Vazyme; C214) with specific primers 1). The correct construction of the recombinant plasmids was verified through Sanger sequencing and extracted using an Endotoxin-Free Plasmid DNA Miniprep Kit (Tiangen, China; DP118) before using plasmids. The primers were listed in Table S1.

RNA interference
The FTO-specific small interfering RNA (si-FTO-1 and si-FTO-2) sequences were 5'- GGAGACCGG CUAACAAUUUTT-3' and 5'-GGUUAAACCAAGUGAAAUATT-3', respectively. The YTHDF2 and YTHDF3-specific small interfering RNA (si-YTHDF2 and si-YTHDF3) sequences were 5'-CUAUGCUCCACGUCA AUUTT-3' and 5'-UCUACGUAACAGCUAUGT-3' respectively. The human FTO-specific small interfering RNA (si-hsFTO-1 and si-hsFTO-2) sequences were 5'- UUCACAACCUCGGUUAGTUTT-3' and 5'-UGAAAUUCUACCUAACAUUTT-3', respectively. The scrambled control RNA sequences were 5'-UUCU CCGAAGUGUCACGUTT-3'. They were purchased from GenePharma (Shanghai, China).

Cells transfection
Transient transfection of cells with siRNA was performed in 24-well plates using Lipofectamine RNAiMAX (Invitrogen; 13778150), and cells were transfected with DNA plasmids was performed using Lipofectamine 3000 (Invitrogen; L3000015) according to the manufacturer’s instructions. For functional analyses, the overexpression plasmid (500 ng per well) or control vector (500 ng per well) and siRNA (100 nM) were transfeeted in cells in culture medium and then harvested for further detection.

RNA extraction and quantitative real-time PCR
Total RNA was extracted using the TRizol Reagent (Invitrogen; 15596018) and the cDNA was synthesized using the FastQuant RT Kit (Tiangen; KR106-03) which contains DNase treatment of RNA to eliminate genomic contamination, following the manufacturer’s instructions. The expression profiles of each gene were conducted by using the SYBR Premix Ex Taq™ (Takara; DRR041S), as previously described. The quantitative real-time PCR was conducted in an Applied Biosystems® QuantStudio 3 (Thermo Fisher Scientific). β-actin was used as internal controls for mRNA respectively. Primer sequences are listed in Table S1.

RNA binding protein immunoprecipitation (RIP)
To identify that YTHDF2 and YTHDF3 whether has the ability to directly bind to NOD1, MKC cells (~2.0 x 10^5) were cotransfected with pcDNA3.1-Flag, pcDNA3.1-YTHDF2-Flag and pcDNA3.1-YTHDF3-Flag for RNA immunoprecipitation (RIP) assays. In brief, MKC cells were harvested after 48 h transfection and RIP assays were carried out with Magna RIP RNA-Binding Protein Immunoprecipitation Kit.
and anti-Flag antibody (Beyotime; AF519) following the manufacturer’s protocol. Then, the expression of target gene NOD1 was detected by quantitative real-time qRT-PCR analysis.

**Total m^6^A quantification assay**
Total RNA was extracted by the TRIzol Reagent. Then, an EpiQuik™ A RNA Methylation Quantification Kit (Colorimetric; EpiGentek, USA; P-9005-48) was used to detect the total m^6^A level according to the manufacturer’s protocol. Briefly, positive control (PC), negative control (NC), and 200 ng isolated mRNA were added to each well with the capture antibody. Next, the detection antibody was added. After several incubations, the m^6^A level was quantified colorimetrically at a wavelength of 450 nm.

**RNA stability assays**
Cells were treated with actinomycin D (5 µg/mL) and then collected at different time points. RNA was extracted by TRIzol reagent, and the mRNA levels were measured using qRT-PCR.

**Dual-luciferase reporter assays**
For FTO target identification, the wild NOD1-3’UTR and NOD1-CDS or mutant of NOD1-3’UTR luciferase reporter was cotransfected with FTO expression plasmid or pcDNA3.1 vector and si-Ctrl or si-FTO into MKC cells. After 48 h transfection, the cells were collected and lysed for the reporter luciferase activities measured by using a dual-luciferase reporter assay system (Promega). All the luciferase activity values were gained against the Renilla luciferase control. For each experiment, three independent experiments were conducted, and each experiment was done in triplicate.

**Western blotting**
Cellular lysates were generated by using 1×SDS-PAGE loading buffer. Proteins were extracted from cells and measured with the BCA Protein Assay kit (Beyotime; P0012S), then subjected to SDS-PAGE (8%) gel and transferred to PVDF (Millipore; ISEQ00010) membranes by semidyblotting (Bio-Rad Trans Blot Turbo System). The membranes were blocked with 5% BSA. Protein was blotted with different antibodies. The antibody against FTO was diluted at 1: 500 (Beyotime; AF6936); The antibody against NOD1 was diluted at 1: 1000 (Customized by Genscript); The antibody against NOD1 of human was diluted at 1: 1000 (Absin; abs115515); Anti-Flag (Beyotime; AF519) and anti-Tubulin (Beyotime; AT819) monoclonal antibody were diluted at 1: 2,000; and HRP-conjugated anti-rabbit IgG (Abbkine; A25022) or anti-mouse IgG (Abbkine; A25012) at 1: 5,000. The results were the 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.

**Cell viability**
Cell viability was measured at 48 h after transfection in MKC cells with CellTiter-Glo Luminescent Cell Viability assays Kit (Promega; G7570) according to the manufacturer’s instructions.

**Bioinformatic analysis**
In order to find out the site that can affect the activity of FTO demethylase in the miuy croaker, DNAMAN program was used to perform multiple sequence alignment from *Homo sapiens*, *Mus musculus*, *Gallus gallus*, *Xenopus laevis*, *Danio rerio*, *Miichthys miuy* and *Callorhinchus milii*.40

**QUANTIFICATION AND STATISTICAL ANALYSIS**
Data are expressed as the mean ± SE 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^(-ΔΔCT) method, and comparisons between groups were analyzed by one-way analysis of variance (ANOVA) followed by Duncan’s multiple comparison tests.41 A value of p < 0.05, *, 0.01 < p < 0.05, **, was considered significant.