LGP2 is essential for zebrafish survival through dual regulation of IFN antiviral response

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Highlights

Zebrafish LGP2 is crucial for host survival through initiating IFN response

Zebrafish LGP2 exerts dual regulation of IFN response during SVCV infection

The function switch of zebrafish LGP2 is related to cellular IFN production
LGP2 is essential for zebrafish survival through dual regulation of IFN antiviral response

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SUMMARY
In mammals, LGP2 is the enigmatic RLR family member, being initially believed as an inhibitor of RLR-triggered IFN response but subsequently as an activator of MDA5 signaling and an inhibitor of RIG-I signaling. The contradiction happens to fish LGP2. Here, we generate a lgp2 loss-of-function (lgp2lof/lof) zebrafish mutant, which is highly susceptible to SVCV infection, displaying an initially decreased activation of IFN response and a following increased one. Mechanistically, zebrafish LGP2 functions as the essential activator of IFN response dependent on MDA5 at the early stage of viral infection but as a negative regulator by impairing mRNA levels of tbk1 and ikki at the late stage of viral infection. The function switch of LGP2 is related to cellular IFN production during viral infection. Our data demonstrate that zebrafish LGP2 is a key homeostatic regulator of IFN response and thus essential for zebrafish survival against SVCV infection.

INTRODUCTION
In mammals, innate immunity to virus infection includes the production of type I interferons (IFNs) that provide the first line of antiviral defense for host survival. IFNs restrict viral replication and dissemination through upregulation of hundreds of IFN-stimulated genes (ISGs), as evidenced by the fact that some ISGs encode antiviral effectors directly, facilitating viral infection clearance (Rehwinkel and Gack, 2020). Although essential for controlling virus replication, cellular IFN production is precisely regulated, as less production cannot clear virus infection thoroughly, and instead, overproduction results in host autoimmune diseases and chronic inflammatory responses. Accordingly, some other ISGs play positive or negative roles in fine-tuning IFN signaling cascades (Rehwinkel and Gack, 2020).

IFN response is often initiated through sensing virally derived nucleic acids in the cytosol of infected cells by retinoic acid-inducible gene-I (RIG-I)-like receptors (RLRs), including RIG-I, MDA5 (melanoma differentiation-associated gene 5), and LGP2 (laboratory of genetics and physiology 2, also known as DHX58) (Rehwinkel and Gack, 2020). RIG-I and MDA5 share similar domain organizations, consisting of two N-terminal caspase activation and recruitment domains (CARDs), a central DExD/H-box RNA helicase domain and a C-terminal regulatory domain (CTD). Sensing viral RNA substrates by CTD and helicase domain of RIG-I and MDA5 facilitates structural rearrangements, thus releasing N-terminal CARDs from an autoinhibitory state, which enables oligomerization and activation of MAVS (mitochondrial antiviral signaling protein), an adaptor possessing N-terminal CARDs (Duic et al., 2020; Rehwinkel and Gack, 2020). MAVS recruits protein kinases TBK1 (TANK-binding kinase 1) and IKKε (inhibitor of nuclear factor kappa B kinase subunit epsilon, or IKKi) to phosphorylate and activate IFN regulatory factors 3/7 (IRF3/7), driving the expression of IFN and subsequent ISGs for host antiviral state (Rehwinkel and Gack, 2020).

Similar to RIG-I and MDA5, LGP2 has strong binding affinities to viral dsRNA, although the precise features of RNA are less well defined (Rehwinkel and Gack, 2020). Unlike RIG-I and MDA5, LGP2 lacks the N-terminal CARDs that are required for signaling transmission to MAVS. As a typical ISG, LGP2 is initially believed as a feedback inhibitor of IFN response (Yoneyama et al., 2005). In vitro studies showed that LGP2 downregulates RIG-I signaling, likely through prevention of MAVS from association with RIG-I (Esser-Nobis et al., 2020; Saito et al., 2007) or with IKKi (Komuro and Horvath, 2006), sequestration of viral dsRNA away from RIG-I (Rothenfusser et al., 2005), and inhibition of RIG-I ubiquitination by targeting TRIM25 (Quicke et al., 2019). On the other hand, a series of studies indicated that LGP2 coordinates MDA5 but impairs RIG-I to augment IFN response while a common signaling cascade is shared by MDA5 and RIG-I.

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that zebrafish LGP2 exerts dual function in response to viral infection (Zhang et al., 2018). To better understand the exact role of zebrafish LGP2 in fish IFN response, we generated a zebrafish mutant where LGP2 is a key homeostatic regulator of IFN response during virus infection. In vivo experiments have shown contradictory roles of fish LGP2 in IFN response (Chang et al., 2011; Liu et al., 2013; Iliev et al., 2011; Sun et al., 2010, 2011; Zhang et al., 2014; Zhang and Gui, 2012). Surprisingly, in vitro assays have shown ambivalent roles of LGP2 during innate immune response to virus infection. Teleost fish have orthologs of three RLR receptors and downstream molecules (MAVS, MITA, TBK1, IRF3, and IRF7), all of which exert similar functions in mammals (Bergan et al., 2010; Biacchesi et al., 2012; Chang et al., 2011; Iliev et al., 2011; Sun et al., 2010, 2011; Zhang et al., 2014; Zhang and Gui, 2012). Delineation of the third strain illustrates that LGP2 is a positive regulator of MDA5 signaling, as suggested by in vitro experiments (Childs et al., 2013; Pippig et al., 2009). In addition, two transgenic mouse models that overexpress human LGP2 shows a higher survival rate than WT zebrafish, the zebrafish mutant exhibited attenuated constitutive expression of LGP2 in head kidney, liver, spleen and gill (Figure 1A), and SVCV infection induced a significantly induction of LGP2 mRNA levels. Our results indicate that zebrafish LGP2 exerts dual function in response to viral infection (Zhang et al., 2018). To better understand the exact role of zebrafish LGP2 in fish IFN response, we generated a zebrafish mutant where LGP2 is a positive regulator dependent on MDA5 and a negative one by impairing TBK1 and IKK- mRNA levels. Our results indicate that zebrafish LGP2 is a key homeostatic regulator of IFN response during virus infection.

RESULTS

LGP2 deficiency impairs zebrafish survival against SVCV infection

TALEN technique was used to target a 15-bp spacer DNA within the exon 10 of zebrafish lgp2 gene (Figure S1A). Sequencing confirmed a mutant line with seven base mutations and a single base deletion at the TALEN site, thus generating a truncated protein, LGP2-talen (Figure S1B). LGP2-talen is 530aa in length due to lack of the C-terminal RD domain of wild-type (WT) LGP2 (Figure S1C). RT-PCR showed that compared with WT zebrafish, the zebrafish mutant exhibited attenuated constitutive expression of lgp2 in head kidney, liver, spleen and gill (Figure 1A), and SVCV infection induced a significantly induction of lgp2-wt in WT zebrafish but a marginal one of lgp2-talen in the zebrafish mutant (Figure 1B). Using a LGP2-specific Ab that was made by a fragment (192–417aa) of zebrafish LGP2 protein (Figures S1D–S1F), western blots revealed a detectable level of LGP2 protein in gill of lgp2lof homozygous mutant (lgp2−/−), and no detection in homozygous mutant (lgp2−/−/−) mice have different phenotypes (Satoh et al., 2010; Suthar et al., 2012; Venkataraman et al., 2007). The first knockout mice exhibit resistance to VSV infection (recognized by RIG-I) and sensitivity to EMCV infection (recognized by MDAS) (Venkataraman et al., 2007), but the second deficient mice are highly vulnerable to RNA viruses recognized by both RIG-I and MDA5 (Satoh et al., 2010). Delineation of the third strain illustrates that LGP2 is not essential for IFN response but rather regulates T cell function during viral infection (Suthar et al., 2012). Despite these differences, the former two lgp2−/− mice are susceptible to EMCV infection, indicating that LGP2 is a positive regulator of MDAS signaling, as suggested by in vitro experiments (Childs et al., 2013; Pippig et al., 2009). In addition, two transgenic mouse models that overexpress human LGP2 exhibits a diminished IFN response and a reduced viral load (Chopy et al.; Si-Tahar et al., 2014), still showing ambivalent roles of LGP2 during innate immune response to virus infection.

Tealeost fish have orthologs of three RLR receptors and downstream molecules (MAVS, MITA, TBK1, IRF3, and IRF7), all of which exert similar functions in mammals (Bergan et al., 2010; Biacchesi et al., 2012; Chang et al., 2011; Iliev et al., 2011; Sun et al., 2010, 2011; Zhang et al., 2014; Zhang and Gui, 2012). Surprisingly, in vitro assays have shown contradictory roles of fish LGP2 in IFN response (Chang et al., 2011; Liu et al., 2017a; Ohtani et al., 2010; Rao et al., 2017; Sun et al., 2011; Yu et al., 2016). Our in vitro data suggested that zebrafish LGP2 exerts dual function in response to viral infection (Zhang et al., 2018). To better understand the exact role of zebrafish LGP2 in fish IFN response, we generated a zebrafish mutant where LGP2 is a positive regulator dependent on MDA5 and a negative one by impairing TBK1 and IKK- mRNA levels. Our results indicate that zebrafish LGP2 is a key homeostatic regulator of IFN response during virus infection.
zebrafish exhibits a reduced induction of IFN response at the early phase of SVCV infection but an increased one at the late phase.

To evaluate the change of IFN response by LGP2 deficiency, we compared mRNA expression of two *ifn* genes (*ifn* 4.1 and *ifn* 4.3) and three ISGs (*mxb*, *irf3*, and *irf7*) between *lgp2lof/lof* and WT larvae following SVCV infection. All genes had a basal expression and were induced by SVCV infection (Figure 2A).

**Figure 1.** LGP2 deficiency impairs zebrafish survival against SVCV infection

(A and B) RT-PCR detection of *lgp2* (or *lgp2-talen*) mRNA in the indicated tissues from *lgp2*+/+ and *lgp2lof/lof* zebrafish adults (60 dpf) in healthy state (A) or following i.p. injection with SVCV (10^8 TCID50/mL) (B).

(C) Western blot analysis of LGP2 protein in the gill from *lgp2*+/+ and *lgp2lof/lof* zebrafish (120 dpf) by with LGP2 antibody. * indicated the LGP2 protein band. The bands below * were nonspecific proteins.

(D) Representative imaging of LGP2 protein in the gill from *lgp2*+/+ and *lgp2lof/lof* zebrafish (120 dpf) by with LGP2 antibody. * indicated the LGP2 protein band. The bands below * were nonspecific proteins.

(E and F) Mortality analysis of *lgp2lof/lof* larvae (E) and adults (F) together with WT zebrafish following SVCV infection.

(F) *lgp2*+/+ and *lgp2lof/lof* zebrafish adults (60 dpf) were injected i.p. with SVCV (10^8 TCID50/mL), at 20 μL per fish.

(G and H) LGP2 deficiency exacerbated SVCV replication in zebrafish larvae (G) and adults (H). *lgp2lof/lof* larvae (6 dpf) and adults (60 dpf) together with WT zebrafish were infected with SVCV as in (E) and (F), respectively. The expression of five SVCV genes, including nucleoprotein (N), phosphoprotein (P), matrix protein (M), glycoprotein (G), and RNA polymerase (L) was detected by RT-PCR. Data were shown as mean ± SD (N = 3). *P values were calculated using Student's t test. *p < 0.05, **p < 0.01. See also Figure S1, and Table S1.

*lgp2lof/lof* zebrafish exhibits a reduced induction of IFN response at the early phase of SVCV infection but an increased one at the late phase.

To evaluate the change of IFN response by LGP2 deficiency, we compared mRNA expression of two *ifn* genes (*ifn* 1 and *ifn* 3) and three ISGs (*mxb*, *irf3*, and *irf7*) between *lgp2lof/lof* and WT larvae following SVCV infection. All genes had a basal expression and were induced by SVCV infection (Figure 2A, left...
panels). Generally, <5-fold induction was detectable until 24 h postinfection (the early phase of SVCV infection), in contrast to >40-fold from 24 to 60 h postinfection (the late phase of SVCV infection), with an exception of ifn43 gene that showed <6-fold induction throughout the infection. Further comparison revealed an attenuated induction in lgp2lof/lof zebrafish larvae compared with WT larvae until 24 h postinfection and an enhanced induction from 24 to 60 h postinfection (Figure 2A, left panels).

Figure 2. LGP2 promotes IFN response at the early phase of SVCV infection but attenuates at the late phase
(A) mRNA expression comparison of two ifn genes and three ISGs between lgp2+/+ and lgp2lof/lof zebrafish larvae following SVCV infection. Left panels: lgp2+/+ and lgp2lof/lof zebrafish larvae (6 dpf) were immersed with SVCV (5 × 10^5 TCID50/mL) for the indicated time points, followed by RT-PCR detection of the mRNA levels of ifn1, ifn3, mxb, irf3, and irf7. The expression values were expressed as fold induction relative to that in mock-infected larvae following normalization to β-actin. Right panels: the induction ratio of each gene between lgp2lof/lof and WT larvae was calculated based on the fold induction at the same time point, followed by normalization to the ratio at 0 h postinfection, which was set to 1, indicating no change.

(B) mRNA expression comparison of two ifn genes and four ISGs in spleens between lgp2+/+ and lgp2lof/lof zebrafish adults following SVCV infection. Left panels: lgp2+/+ and lgp2lof/lof zebrafish adults (60 dpf) were injected i.p. with SVCV (10^8 TCID50/mL) for the indicated time points, followed by RT-PCR detection. Right panels: the fold induction ratios of each gene in spleen between lgp2+/+ and lgp2lof/lof zebrafish adults. Data were shown as mean ± SD (N = 3). P values were calculated using Student’s t test. *p < 0.05, **p < 0.01; n.s., not significant. See also Figure S2, and Table S1.
The expression alteration of five genes in response to SVCV infection was quantified as the ratio of fold induction at the same time points between \( \text{lgp2}^{\text{lof/lof}} \) and WT larvae, followed by normalization to the ratio at 0 h postinfection, which was set to 1, indicating no change (Figure 2A, right panels). It clearly showed that, compared with WT larvae, \( \text{ifn} \) expression was significantly attenuated in \( \text{lgp2}^{\text{lof/lof}} \) larvae during the early phase of viral infection, particularly at 6 h postinfection (5-fold lower than WT), then started to increase from 12 h postinfection, and peaked at 36 h postinfection (10-fold higher than WT), indicating that \( \text{lgp2} \) deficiency resulted in a first impaired and a subsequent promoted expression induction of \( \text{ifn} \). Similar results were seen in \( \text{ifn1}, \text{mxb}, \text{irf3}, \text{and irf7} \) (Figure 2A, right panels).

Similar comparison was next performed between \( \text{lgp2}^{\text{lof/lof}} \) and WT adults following SVCV infection. All five gene expressions were significantly induced in spleen of \( \text{lgp2}^{\text{lof/lof}} \) and WT adults (Figure 2B, left panels); however, \( \text{lgp2} \) deficiency caused an obviously weakened induction during the early phase of infection, particularly at 12 h postinfection, but from 24 to 72 h postinfection, an enhanced induction was generally detected compared with WT adults (Figure 2B, right panels). Most of these genes, particularly \( \text{ifn1} \), displayed a similar expression pattern in gill and head kidney (Figures S2A and S2B). These data together indicated that LGP2 promotes IFN antiviral response during the early phase of viral infection but attenuates IFN response during the late phase of viral infection.

**LGP2-talen is not functional in fish IFN response**

To exclude a possible role of LGP2-talen in \( \text{lgp2}^{\text{lof/lof}} \) zebrafish, we first compared the stimulatory ability of LGP2-wt, LGP2-talen, and LGP2-DH that is devoid of RD and is similar to LGP2-talen in size (Figure 3A). Neither LGP2-talen nor LGP2-DH showed any stimulatory effects, but overexpression of LGP2-wt stimulated a robust luciferase activity of crucian carp IFN promoter-driven luciferase construct (CaIFNpro-luc) (Figure 3B). Notably, LGP2-wt exhibited a strongest potential at a relatively low dose (10 ng in 24-well plates) but a dose-dependent weakened induction at relatively high doses (50 and 200 ng in 24-well plates) (Figure 3B). RT-PCR showed that LGP2-wt, but not LGP2-talen nor LGP2-DH, significantly upregulated the mRNA expression of cellular \( \text{ifn} \) gene (Figure 3C) and four ISGs (\( \text{mx}, \text{viperin}, \text{irf3}, \text{and irf7} \)) (Figure S3), also with a strongest induction at a relatively low dose (40 ng in 6-well plates, corresponding to 10 ng in 24-well plates) and a dose-dependent weakened one at relatively high doses (200 and 800 ng). In addition, overexpression of LGP2-talen and LGP2-DH lost the ability to inhibit poly(I:C)-induced activation of CaIFNpro-luc, which was significantly inhibited by LGP2-wt in a dose-dependent manner (Figure 3D). These results indicated that LGP2-talen is not functional in fish IFN response despite a marginal expression in \( \text{lgp2}^{\text{lof/lof}} \) zebrafish.

**LGP2 functions as a dual regulator in itself- and virus-induced IFN response**

The finding that zebrafish LGP2 provokes a strong IFN response at low doses but a weak one at high doses indicated that an inhibition should happen to LGP2 itself. To this end, titration assays showed that low doses of LGP2 (\( \leq 10 \) ng in 24-well plates) stimulated fish IFN promoters obviously in a dose-dependent manner, up to a peak when 10 ng of LGP2 was transfected; thereafter, the promoter activation was gradually reduced along with LGP2 doses increasing (>10 ng), indicating that the highest promoter activation at 10 ng of LGP2 was indeed inhibited by the extra amount of LGP2, also in a dose-dependent manner (Figure 4A). Similarly, low dose of LGP2 (40 ng in 6-well plates) upregulated cellular PKR and IRF3 proteins, which were diminished when LGP2 doses were increased to 200 and 800 ng (Figure 4B).

Subsequent assays were performed to determine the antithetic role of LGP2 during SVCV infection. IRF3 dimerization was not easily detectable in CAB cells transfected with LGP2 alone; however, a low titer of SVCV stimulated IRF3 dimerization, which was more effectively enhanced by LGP2 at 40 ng than at 800 ng (Figure 4C). A high titer of SVCV yielded a robust IRF3 dimerization, which was markedly diminished by LGP2 at either 40 ng or 1000 ng (Figure 4D). Time titration showed that IRF3 dimerization was not detectable until 12 h postinfection, which was gradually obvious along with LGP2 doses; from 24 to 36 h postinfection, transfection of LGP2 at 40 ng or 1000 ng resulted in reduced IRF3 dimerization relative to SVCV infection alone (Figure 4E). Compared with the control without LGP2 transfection, SVCV-induced expression of cellular genes (\( \text{ifn}, \text{viperin}, \text{and irf3} \)) was enhanced in the presence of LGP2 until 24 h postinfection, being a strong promotion by 40 ng of LGP2 and a weak one by 1000 ng; however, at 36 h postinfection, the promotional effect was undetectable (Figure 4F). These results indicated that LGP2 firstly promoted and subsequently attenuated IFN response during viral infection.
We hypothesized that zebrafish LGP2 switches its function roles as a result of the cellular IFN expression levels. Transfection of dominant negative mutant plasmids of RLR signaling factors, including RIG-I-DN, MDA5-DN, MAVS-DN, MITA-CT, TBK1-K38M, IRF3-DN, and IRF7-DN, could efficiently block SVCV infection, which triggers IFN response in fish cells (Figure S4A). Next, we compared fish IFN promoter activation by titration of LGP2 from 0 to 200 ng in the absence or presence of these dominant negative mutants (Figure 5A). Similarly, overexpression of LGP2 induced a dose-dependently positive regulation at low doses (<10 ng) and a dose-dependently negative regulation at high doses (>10 ng). In the presence of MITA-CT, TBK1-K38M, IRF3-DN, or IRF7-DN, the positive regulatory trend triggered by low doses of LGP2 was reversed.

**Function switch of LGP2 is related to IFN production but not to IFN signaling**

We hypothesized that zebrafish LGP2 switches its function roles as a result of the cellular IFN expression levels. Transfection of dominant negative mutant plasmids of RLR signaling factors, including RIG-I-DN, MDA5-DN, MAVS-DN, MITA-CT, TBK1-K38M, IRF3-DN, and IRF7-DN, could efficiently block SVCV infection, which triggers IFN response in fish cells (Figure S4A). Next, we compared fish IFN promoter activation by titration of LGP2 from 0 to 200 ng in the absence or presence of these dominant negative mutants (Figure 5A). Similarly, overexpression of LGP2 induced a dose-dependently positive regulation at low doses (<10 ng) and a dose-dependently negative regulation at high doses (>10 ng). In the presence of MITA-CT, TBK1-K38M, IRF3-DN, or IRF7-DN, the positive regulatory trend triggered by low doses of LGP2 was reversed.
LG2 (≤ 10 ng) was still seen, albeit to a low degree of induction values, but the negative regulatory trend by high dose of LG2 alone (≥10 ng) was undetectable or delayed and even changed to a positive regulatory trend in the presence of TBK1-K38M, IRF3-DN, or IRF7-DN (Figure 5A). Under the same conditions, LG2 inhibited itself-triggered expression of cellular genes (ifn, mx, viperin, irf3, and irf7) in a dose-dependent manner; however, this inhibitory trend was abolished and even changed to a promoting trend when RLR-IFN signaling was functionally blocked by these dominant negative mutants (Figures 5B and S4B). Notably, overexpression of MDA5-DN completely blocked LG2-triggered fish promoter activation.
the function switch of LGP2 during viral infection. MDA5 (Figure 5F). These results together indicated that cellular IFN expression is a key factor to determine the switch of LGP2 from cytoplasm into nucleus. These results together indicated that LGP2 triggers fish IFN signaling dependently of MDA5.

Forms of IRF3 proteins were detected in cytoplasm (Figure 6C), and only the largest one, representing a functional mutant of IRF9 (Yu et al., 2010), could not rescue LGP2-mediated negative regulatory trend, which was still observed in the presence of IRF3-DN or IRF7-DN (Figure 5C, right panel). Moreover, functional blockade of IFN-triggered JAK-STAT signaling by overexpression of IRF9-DN, a dominant negative mutant of IRF9 (Yu et al., 2010), could not rescue LGP2-mediated negative regulatory trend, which always occurred in the presence of high doses of LGP2 itself (Figure 5D), poly(I:C) (Figure 5E), and MDA5 (Figure 5F). These results together indicated that cellular IFN expression is a key factor to determine the function switch of zebrafish LGP2 during viral infection.

**LGP2 positively regulates fish IFN response dependently of MDA5**

To corroborate the finding that MDA5 is essential for LGP2 regulation of IFN response (Figures 5A and 5B), we compared the effects of different dominant negative mutants of RLR molecules on cellular IRF3 and IRF7 expression. Western blots showed that IRF3 and IRF7 proteins were significantly upregulated in fish cells by transfection of LGP2 alone at a low (250 ng) or high dose (5 μg), as compared with the control (lane 6 versus lane 1 in Figure 6A); however, this upregulation was suppressed to different extents by individual cotransfection of RIG-I-DN, MDA5-DN, MAVS-ΔTM, and MITA-CT, with the most severe suppression by MDA5-DN (lane 8 versus lanes 6–7 and 9–10 in Figure 6A), highlighting the relevance of MDA5 to LGP2-directed IFN signaling.

Further fractionation of nucleus and cytoplasm showed that overexpression of LGP2 alone, regardless of the doses transfected, resulted in a robust nuclear accumulation of IRF3 and IRF7, which was always attenuated by simultaneous overexpression of MDA5-DN (Figure 6B). As an IFN-inducible protein, cellular LGP2 expression was also inhibited particularly by overexpression of MDA5 (Figures 6A–6C). Notably, three forms of IRF3 proteins were detected in cytoplasm (Figure 6C), and only the largest one, representing a phosphorylated form of IRF3 (Bergan et al., 2010; Feng et al., 2016; Iliev et al., 2011; Sun et al., 2016), was detected in nucleus (Figure 6B), implying that the phosphorylated IRF3 and IRF7 were transported from cytoplasm into nucleus. These results together indicated that LGP2 triggers fish IFN signaling dependently of MDA5.

**LGP2 negatively regulates IFN response by attenuating tbk1 and ikki mRNA levels**

Luciferase assays showed that overexpression of LGP2 significantly blocked IFN promoter activation by each of the RLR signaling molecules except for IRF3 and IRF7 (Figure 7A). The same is true for mRNA induction of cellular genes (ifn, mx, and viperin) (Figure 7B). Western blot analysis showed that overexpression of LGP2 resulted in reduced protein levels of TBK1 and IKKi, in a dose-dependent manner (Figure 5A) and also in a time-dependent manner (Figure 5B). However, the protein reduction was abolished by the protein synthesis inhibitor cycloheximide (CHX) (Figure 7C), but not by proteasome inhibitor MG132 or lysosome inhibitors NH4Cl and chloroquine (Figure 7C), indicating that LGP2 cannot downregulate the expression of TBK1 and IKKi at protein levels.
Further, we evaluated LGP2-mediated expression changes of TBK1 and IKKi at mRNA levels. As shown in Figure 7D, whereas overexpression of LGP2 alone did not nearly change cellular tbk1 transcript level, overexpression of LGP2 and TBK1 together reduced tbk1 transcript level, in LGP2’s dose-dependent manner, by either vector primers or universal primers, both of which respectively amplified the mRNAs only from the

Figure 6. LGP2 positively regulates fish IFN response dependently of MDA5

(A–C) LGP2-directed nuclear accumulation of IRF3 and IRF7 was significantly inhibited by functional blockade of MDA5 signaling. EPC cells seeded in 10-cm dishes were cotransfected with LGP2-HA at a low dose of 250 ng (left panels) or a high dose of 5 μg (right panels), together with the indicated dominant negative mutants (5 μg each). Forty-eight hours later, the transfected cells were harvested for fractionation of the nucleus and cytoplasm. IRF3, IRF7, and LGP2 proteins were detected by western blot analysis of whole-cell lysates as input (A), nuclear extracts (B), and cytoplasmic extracts (C), with antibodies specific to fish IRF3, IRF7, and LGP2, respectively. The red boxes highlighted the essential effects of MDA5-DN on LGP2-triggered signaling.

Further, we evaluated LGP2-mediated expression changes of TBK1 and IKKi at mRNA levels. As shown in Figure 7D, whereas overexpression of LGP2 alone did not nearly change cellular tbk1 transcript level, overexpression of LGP2 and TBK1 together reduced tbk1 transcript level, in LGP2’s dose-dependent manner, by either vector primers or universal primers, both of which respectively amplified the mRNAs only from the
Figure 7. LGP2 negatively regulates IFN response by attenuating tbk1 and ikki mRNA levels

(A and B) LGP2 inhibited fish IFN promoter activation (A) and ifn expression (B) induced by RLR signaling molecules upstream of IRF3/7. EPC cells seeded in 24-well plates were transfected with DrIFNpro-luc, LGP2, and each of the indicated RLR signaling molecules (200 ng each) for 48 h, followed by luciferase assays (A) or by RT-PCR detection of cellular ifn, mx, and viperin mRNA (B). Western blots in (A) showed the expression of LGP2 protein in (A and B) by western blots using anti-LGP2 Ab.

(C) LGP2-mediated protein reduction of TBK1 and IKKi was abolished by CHX. EPC cells seeded in 6-well plates overnight were transfected for 24 h with TBK1 or IKKi (0.6 μg each), together with LGP2 at increasing doses, followed by addition of CHX (8 μg/mL) or DMSO as control. Another 2 h later, cells were collected for western blotting analysis of TBK1 and IKKi by anti-HA Ab and LGP2 by anti-LGP2 Ab. The numbers show the densitometric quantification of TBK1 or IKKi protein expression normalized to β-actin.

(D and E) LGP2 attenuated mRNA levels of the transfected TBK1 (D) and IKKi (E). CO cells seeded in 6-well plates were transfected with LGP2 at increasing doses (0, 0.1, 0.2, 0.4, 0.8, 1 μg), together with TBK1 (300 ng, D) or IKKi (300 ng, E) for 48 h, followed by RT-PCR detection of lgp2, tbk1, and ikki mRNA, respectively. Universal primers were designed against the ORF sequences of tbk1 and ikki for detection of mRNA from cellular genes and the transfected
transfected constructs expressing tbk1 or from the cellular tbk1 and transfected tbk1 together. The same happened to iki mRNA (Figure 7E). Moreover, the reduction of tbk1 and iki mRNA levels was not impaired by the transcription inhibitor actinomycin D (ActD) (Figure 7F). These results together implied that LGP2 downregulates TBK1 and IKKi expression via attenuating their mRNA levels.

RT-PCR analysis of wild-type zebrafish larvae showed that tbk1 and iki mRNA levels during the late phase of SVCV infection (Figure 7G). These results indicated that LGP2 is a key homeostatic regulator of tbk1 and iki expression and that it attenuates tbk1 and iki mRNA levels to downregulate IFN response during the late stage of viral infection.

**LGP2 is essential for IFN response at the early stage of viral infection**

To further determine the biological relevance of low doses of LGP2 in fish IFN response, we compared the patterns of IFN induction by LGP2, RIG-I, and MDA5. Unlike zebrafish LGP2, zebrafish RIG-I and MDA5 activated fish IFN promoters exclusively in a dose-dependent manner (Figure 8A). At low doses (5 and 10 ng in 24-well plates), either RIG-I or MDA5 did not show any effects on fish IFN promoter activation; however, LGP2 exhibited dose-dependent stimulatory potential to fish IFN promoters (14- and 60-fold induction over the control) (Figure 8A). At a high dose (200 ng), LGP2 was a poorest stimulator among three RLR members (Figure 8A). Consistently, LGP2 triggered a stronger expression of cellular ifn gene than either MDA5 or RIG-I at low doses (20 and 40 ng in 6-well plates) but a weaker one at a high dose (800 ng) (Figure 8B). Combined with the fact that during the early phase of viral infection (<24 h postinfection), RIG-I and MDA5 were expressed at very low levels in zebrafish larvae and tissues (Figure 8C), similar to LGP2 (Figure 1B), these data indicated that zebrafish LGP2 exerts more potential to IFN response than RIG-I or MDA5 at the early stage of viral infection.

**DISCUSSION**

It is documented that a fully functional adaptive immunity is not well developed in zebrafish until 4 weeks postfertilization (Lam et al., 2004; Traver et al., 2003). This peculiar characteristic makes zebrafish as a useful animal model to study innate antiviral response (Varela et al., 2017), after the first in vivo infection mode with SVCV was established (Sanders et al., 2003). LGP2 knockout exacerbates the mortality of zebrafish larvae (6 dpf) following SVCV infection, highlighting that the consequent mortality is ascribed to an impaired zebrafish innate immunity. Similar phenotypes are replicated in zebrafish adults (60 dpf), demonstrating that LGP2 is indispensable for zebrafish resistance to SVCV infection.

Detection of IFN response reveals that zebrafish LGP2 exerts bilateral function during virus infection in vivo and in vitro. In the early phase of SVCV infection (<24 h postinfection), function loss of LGP2 resulted in a decreased induction of ifn and ISGs in zebrafish larvae and adults and instead, an increased one in the late phase of SVCV infection (>24 h postinfection) (Figure 2). Similar results were replicated in cultured fish cells, when infected with SVCV (Figure 4). Obviously, the enhanced IFN response during the late phase of viral infection in lgp2lof/lof zebrafish seemed not helpful for viral infection clearance, as zebrafish mutants actually had higher virus loads than WT zebrafish. Therefore, only the IFN-stimulatory potential of zebrafish LGP2 at the early infection is essential for zebrafish survival advantages.

Our data might provide valuable clues to resolve the controversial understanding of LGP2’s biological roles in fish and mammals. Supposing the function of LGP2 is largely conserved across vertebrates, it is helpful to understand why LGP2-deficient mice are more susceptible to virus infection than WT mice, because these
mice actually have less IFN production at the early phase of virus infection (within 24 h postinfection) (Satoh et al., 2010); at this time, LGP2 should be expressed at a low level in wild-type mice, thus potentiating IFN antiviral response as the best IFN stimulator of RLRs. It is also easy to understand why two LGP2-transgenic mice have better survival advantages than WT mice, but they display a diminished IFN response and a reduced viral load (Chopy et al., 2011; Si-Tahar et al., 2014). In the latter two studies, endogenous IFN expression was detected during a late phase of virus infection, one from 4 to 8 d postinfection (Si-Tahar et al., 2014) and the other from 8 to 11 d postinfection (Chopy et al.), in which LGP2 functions as a negative regulator of IFN response in mice. Therefore, it is relevant to investigate whether the ectopically expressed LGP2 in the two LGP2-transgenic mice have more IFN production during the early phase of viral infection, a finding as in the current study.

These data reveal that at the beginning of viral infection, it is LGP2 rather than either MDA5 or RIG-I that is responsible for the rapid onset of host IFN response. Fish RIG-I, MDA5, and LGP2 are constitutively expressed at very low levels in tissues of zebrafish at the early phase of SVCV infection. Importantly, LGP2 at low expression levels harbors the best potential to activate IFN antiviral response, and on the contrary, either RIG-I or MDA5 at low expression levels does not nearly exhibit stimulatory effects on fish IFN promoter. It is documented that a rapid initiation of IFN response during the early phase of virus infection is beneficial for host survival (Feng et al., 2021; Gao et al., 2021; Gough et al., 2012). Accordingly, the severe mortality of lgp2<sub>lof/lof</sub> zebrafish is ascribed to the loss of the initial positive regulation of LGP2 during the early phase of infection, and the physiological relevance of LGP2's negative regulation at the late phase of infection might contribute to brake IFN response naturally in wild-type zebrafish, thus ensuring homeostatic regulation of host innate antiviral responses.

Despite no in vivo evidence for function switch of LGP2 in mammals, several in vitro studies have revealed that lower levels of LGP2 synergize with MDA5 to augment IFN signaling, and higher levels of LGP2 act as...
inhibitors of RIG-I and MDA5 signaling (Bruns et al., 2013, 2014; Childs et al., 2013). Correlating with the expression characteristics of LGP2, these data suggested that cellular LGP2 expression level might determine its function switch in RLR signaling (Bruns et al., 2013, 2014; Childs et al., 2013; Pippig et al., 2009). However, we provide solid evidence that zebrafish LGP2, either at lower levels or at higher levels, shows dual effects on the IFN response triggered by SVCV (Figure 4). These results mean that whether zebrafish LGP2 functions as a positive or a negative regulator should be tightly associated with the exact doses or titers of IFN stimuli, rather than the exact expression levels of LGP2 at that time.

Because the exact doses or titers of IFN stimuli, including LGP2 itself at lower levels (<10ng in 24-well plates), are generally proportional to the resultant amounts of IFN products, we hypothesized that a threshold level of cellular IFN production drives the functional switch of zebrafish LGP2 from a positive regulator to a negative one. Evidences come from the LGP2 self-inhibitory model (Figure 5). When RLR signaling was functionally blocked, cellular ifn and ISGs expression was inhibited to a low level, and higher doses of LGP2-mediated negative regulation trend was abolished and even changed to a successively positive regulation trend; however, similar phenomenon did not happen when IFN-triggered JAK-STAT signaling was blocked. This means that the function switch of LGP2 is related to IFN production but not to IFN signaling. Therefore, at the early stage of viral infection, SVCV replicates in cells at lower levels, stimulating a lower level of cellular IFN expression; at this time, LGP2, RIG-I, and MDA5 are expressed at lower levels, and LGP2 is most essential for the initiation of IFN response likely dependently of MDA5. At the late stage of infection, SVCV replicates to higher levels, thus inducing cellular IFNs to a threshold level, which switches LGP2 to the negative role.

Although overexpression of LGP2 in human cells cannot activate IFN expression, in vitro depletion of PUM1 in HEp-2 cells significantly upregulates the constitutive expression of LGP2, which in turn upregulates the constitutive expression of ifnb and some ISGs for host cell survival against virus infection (Liu et al., 2017b), and the constitutively expressed LGP2 might induce IFN response through sensing self RNA and dependently of MDA5 (Stok et al., 2023). These data indicate that human LGP2 at constitutive expression is indeed an essential stimulator of IFN response. Similar to human LGP2, zebrafish LGP2 triggers IFN response with the requirement of MDA5. It is well documented that mammalian LGP2 coordinates fiber formation and subsequent activation of MDA5 (Bruns et al., 2013, 2014; Duic et al., 2020; Esser-Nobis et al., 2020). These results suggest that during the early stage of viral infection, zebrafish LGP2 might facilitate MDA5 activation for the onset of host IFN response.

Limitations of the study
We think that future studies need to further investigate in vitro and in vivo mechanisms underlying function switch of zebrafish LGP2 using knockout technologies. Recent studies have revealed that human LGP2 enhances apoptosis as an antiviral defense mechanism (Takahashi et al., 2020), and LGP2 downregulates innate immune signaling by inhibiting TRAF ubiquitin ligase (Parisien et al., 2018), blocking the interaction between RIG-I and MAVS (Esser-Nobis et al., 2020), and the K63 conjugating enzyme Ubc13 (Lenoir et al., 2021). However, our results showed that zebrafish LGP2 negatively regulates IFN response likely through attenuating tbk1 and ikki mRNA levels. The transcript levels of tbk1 and ikki are constant in WT zebrafish but significantly upregulated in lgp2−/−/− zebrafish, highlighting that zebrafish LGP2 is a key homeostatic regulator to shape IFN signaling toward viral infection. Although the underlying mechanisms of how LGP2 synergizes with MDA5 or impairs mRNA levels of tbk1 and ikki to regulate IFN response are waiting for further clarification using knockout technologies, our results have revealed the in vivo bilateral function of zebrafish LGP2 during viral infection, thus providing a clue that similar mechanisms might be involved in mammals.

STAR+METHODS
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Supplemental information can be found online at https://doi.org/10.1016/j.isci.2022.104821.

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AUTHOR CONTRIBUTIONS
Y.B.Z. conceived the project, and Y.B.Z. and X.Y.G. designed the experiments. X.Y.G. performed the majority of the experiments. Q.M.Z. and Z.L. performed lgp2 knockout zebrafish. Y.B.Z., X.Y.G., X.Z., Y.L.L., Z.L.Q., and C.D. analyzed the data. J.F.G. provided useful insights and reagents. Y.B.Z. and X.Y.G. wrote the manuscript. All authors have read and approved this manuscript.

DECLARATION OF INTERESTS
The authors declare no competing interests.

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# STAR METHODS

## KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| **Antibodies**      |        |            |
| HA-Tag Rabbit mAb   | Cell Signaling Technology | Cat# 3724; RRID:AB_1549585 |
| FLAG Tag Rabbit mAb | Cell Signaling Technology | Cat# 70,569; RRID:AB_2799786 |
| Myc-tag Rabbit mAb  | Cell Signaling Technology | Cat# 2040; RRID:AB_2148465 |
| α-Tubulin Rabbit mAb| Cell Signaling Technology | Cat# 2125; RRID:AB_2619646 |
| Lamin A/C Rabbit mAb| Cell Signaling Technology | Cat# 2032; RRID:AB_2136278 |
| CalRF3 Rabbit mAb   | (Sun et al., 2010) | N/A |
| CalRF7 Rabbit mAb   | (An et al., 2022) | N/A |
| CaPKR Rabbit mAb    | (Liu et al., 2011) | N/A |
| Zebrafish LGP2 Rabbit mAb | In this study | N/A |
| **Bacterial and virus strains** |        |            |
| Spring viraemia of carp virus (SVCV) | (Gao et al., 2009) | N/A |
| **Chemicals, peptides, and recombinant proteins** |        |            |
| Protease Inhibitor Cocktail | Bimake | Cat# B14002 |
| Phosphatase Inhibitor Cocktail | Bimake | Cat# B15001 |
| Cycloheximide | KH | Cat# A1410 |
| Actinomycin D | KH | Cat# C7698; CAS: 50-76-0 |
| poly(I:C) | SIGMA | Cat# P0913; CAS: 42,424-50-0 |
| **Critical commercial assays** |        |            |
| Endo-free Plasmid Mini Kit II | OMEGA | Cat# D6950-02 |
| SV Total RNA Isolation | Promega | Cat# Z3100 |
| GoScript™ Reverse Transcription System | Promega | Cat# A5001 |
| Universal Blue qPCR SYBR Green Master Mix | YEASEN, China | Cat# 11201ES08 |
| Dual-Luciferase® Reporter Assay System | Promega | Cat# P0913 |
| Nuclear and Cytoplasmic Protein Extraction Kit | YEASEN, China | Cat# 20126ES0 |
| ANTI-FLAG(R) M2 Affinity Gel | Sigma | Cat# A2220 |
| **Deposited data** |        |            |
| Raw and analyzed data | This paper | Lead Contact, Yibing Zhang (ybzhang@ihb.ac.cn) |
| **Experimental models: Cell lines** |        |            |
| HEK293T | ATCC (CRL-3216) | N/A |
| Epithelioma papulosum cyprini cells (EPC) | ATCC (CRL-2872) | N/A |
| Ovary cells of grass carp (CO) | Kept in IHB,CAS | N/A |
| Crucian carp (C. auratus L.) blastulae embryonic cells (CAB) | (Chen et al., 1985) | N/A |
| Zebrafish liver cells (ZFL) | ATCC, CRL2643 | N/A |
| **Experimental models: Organisms/strains** |        |            |
| Zebrafish (Danio rerio) strain AB | China Zebrafish Resource Center | N/A |

(Continued on next page)
RESOURCE AVAILABILITY

Lead contact
Further information and requests for resources and reagents should be directed to the Lead Contact, Yibing Zhang (ybzhang@ihb.ac.cn).

Materials availability
The lgp2-deficient zebrafish mutant line and plasmids generated in this study are available from the Lead Contact without restriction.

Data and code availability
Raw and analyzed data reported in this paper will be shared by the lead contact upon request.

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

Cell lines and cell culture
Epithelioma papulosum cyprini cells (EPC), zebrafish liver cells (ZFL) and HEK293T cells are from ATCC. Grass carp (C. idellus) ovary cells (CO), and crucian carp (C. auratus) blastulae embryonic cells (CAB) are kept in our Institute of Hydrobiology, Chinese Academy of Sciences. EPC, CAB and CO cells were grown in medium 199 (Gibco) supplemented with 10% fetal bovine serum (FBS) at 28°C, and HEK293T cells were cultured in DMEM (Gibco) with 10% FBS at 37°C.

Virus strains and experimental models
Spring viraemia of carp virus (SVCV), a negative-sense single-stranded RNA virus of the family Rhabdoviridae (Gao et al., 2009), was propagated and tittered in EPC cells by a 50% tissue culture-infective dose (TCID50) assay. Zebrafish (Danio rerio) strain AB (from China Zebrafish Resource Center) were raised, maintained, reproduced, and staged according to standard protocols, which were approved by the Animal Care and Use Committee of Institute of Hydrobiology, Chinese Academy of Sciences. For in vivo infection, zebrafish larvae were immersed with SVCV, and zebrafish adults injected i.p. (intraperitoneally injection) with SVCV.

METHOD DETAILS

Generation of LGP2 mutants by TALEN technique
TALEN design software TAL Effector Nucleotide Targeter 2.0 (https://talent.cac.cornell.edu/) and E-TALEN (http://www.etalen.org/E-TALEN/) were used to design the LGP2 target sites and TALEs recognition sequence. The TALEN pair and spacer sequences were targeted to the exon 10 of zebrafish LGP2 gene as below: TALEN-left: TGTTTGAGCTCCAGAA; TALEN-right: TCGCCTCCATCGA; TALEN-spacer: AACAGCTGTGGTGAT, with a site of Pvu II. TALEN plasmids were constructed using the “unit assembly method” through assembling the correct sequences into pCS2-FokI, for in vitro synthesis of mRNAs.
using an SP6 mMessage mMachine kit (Ambion), which were co-injected into the embryos at the one-cell stage at a dose of 500–600 pg per embryo. 24–48 h later, DNA spanning the LGP2 targeting sites were amplified by PCR to evaluate mutation (Yang et al., 2017). LGP2 mutants is resistant to _Pvu_ II digestion due to mutations in spacer, which was further confirmed by sequencing. After the F1 of LGP2 mutant was obtained, the mutant male and female fish with the same phenotype were bred to obtain the F2 mutant. The _lgp2<sup>2fl/fl</sup>_ mutants are viable, albeit slightly low fertilization rates and slightly small sizes when grown up.

**Plasmids**

For Co-IP assays, plasmids including zebrafish LGP2-HA, MDA5-HA, RIG-I-HA, TBK1-HA and IKKα-HA were generated by inserting the open reading frames (ORFs) into _EcoR_ I site of pcDNA3.1(+) vector (Invitrogen) that had preexisted an HA coding sequence into _Not_ I site. Zebrafish LGP2-HA, MDA5-Myc, MITA-Myc, TBK1-Myc, IRF3-Myc and IRF7-Myc were made by insertion of the corresponding ORFs into _EcoR_ I/ _BamH_ I site of pcDNA3.1/myc-His(−) vector (Invitrogen). The dominant negative mutants of RLR signaling factors, including zebrafish RIG-I-DN, MDA5-DN, MAVS-ΔTM, MITA-CT, TBK1-K38M, IRF3-DN, IRF7-DN, IRF9-ΔC were made previously in our lab (Feng et al., 2015, 2016; Sun et al., 2010, 2011; Wu et al., 2019; Zhang et al., 2018). CaliFNpro-luc was made by insertion of 5’ flanking sequence (−233 to +34) of crucian carp _C. auratus_ IFN (GenBank accession no. HM187723) into _Kpn_ I/ _Xho_ I sites of pGL3 (Sun et al., 2010). Zebrafish IFNα1pro-luc was made by insertion of 5’ flanking sequence (−586 to +38) of zebrafish IFNα1 (GenBank accession no. NM_207640) into pGL3 (Sun et al., 2011). For overexpression assays, all plasmids, unless indicated, were expressed as non-tagged proteins.

**Transfection and luciferase activity assays**

Transfection assays were performed according to our previous reports (Gong et al., 2019; Zhang et al., 2018). Typically, fish cells were seeded overnight in plates, transfected for 24h with various constructs at a ratio of 10:10:1 (promoter-driven luciferase plasmid/expression plasmid/Renilla luciferase plasmid _pRL-TK_) using polyethylenimine, linear (PEI, MW25000; Aldrich, 1 mg/mL of storage concentration) according to the manufacturer’s instructions. Generally, cells seeded overnight were replenished with the transfection mixture. The transfection mixture was made by diluting the indicated plasmids with Opti-mem (200, 50 and 25 μL for each well in 6-, 24- or 48-well plates, respectively), followed by addition of PEI with a ratio of 1:3 [plasmid (μg)/PEI (μL)] for fish cells and 1:5 for HEK293T cells. The doses of transfected plasmids were proportional to the number of fish cells seeded in different plates or dishes. For example, 4-fold doses of the plasmids were transfected in 6-well plates compared to that in 24-well plates (10 ng for low dose of LGP2 and 200 ng for high dose). If necessary, the cells were transfected again with poly(I:C) (SIGMA) or infected with SVCV infection. Luciferase activities were measured by a Junior LB9509 luminometer (Berthold, Pforzheim, Germany), according to the Dual-Luciferase Reporter Assay System (Promega, USA). All results were shown as a representative of at least three independent experiments, each performed in triplicate.

**RNA extraction, cDNA synthesis, and quantitative real-time PCR**

Total RNA was extracted by TRIZOL Reagent (Promega), followed by removing genomic DNA by RNase-free DNase I. First-strand cDNA was synthesized using random primers or Oligo(dT) <sub>20</sub> VN (Promega). RT-qPCR was performed with Universal Blue qPCR SYBR Green Master Mmix (YEASEN, China) in a DNA Engine Chromo four real-time system (BioRad, USA). Gene expression was normalized to β-actin in a given sample, indicated as relative expression values of mRNA, or further normalized to the control as a fold induction of mRNA. The primers used in this study were listed in Table S1.

**Fractionation of nuclear and cytoplasmic proteins**

Nuclear and cytoplasmic proteins were extracted according to the manufacturer’s protocol (YEASEN, China) (Sun et al., 2010). Cells were collected with cell scrapers, followed by centrifugation. Briefly, cell pellets were added with reagent A containing PMSF, vortexed thoroughly, incubated on ice for 10–15 min, and added with cytoplasmic protein extraction reagent B. After vortex and centrifugation, the supernatants were collected as cytoplasmic proteins. The remained pellets were further incubated with reagent C containing PMSF for 30 min on ice, vortexed thoroughly, and finally centrifuged to obtain nuclear proteins.
Coimmunoprecipitation and western blotting

Coimmunoprecipitation (Co-IP) assays and western blotting were performed as previously described (Feng et al., 2015; Sun et al., 2010, 2011; Zhang et al., 2018). For Coimmunoprecipitation (Co-IP) assays, cells were lysed with NP-40 lysis buffer (Beyotime, China), followed by centrifugation. Cell lysates were incubated with anti-Tag beads (Sigma, USA) at 4°C overnight. The beads were washed with Co-IP wash buffer (50mM Tris-HCl, pH7.5, 150mM NaCl, 1mM DTT, 1% NP-40), resolved in SDS loading buffer (Biosharp, China), followed by western blotting with the indicated antibodies. Tag-specific Abs were purchased from Cell Signaling Technology and ABclonal. Antibodies for CaIRF3, CaIRF7 and CaPKR were described previously (Sun et al., 2010; Zhang et al., 2018). Zebrafish LGP2-specific Ab was generated by immunization of rabbits with a purified peptide corresponding to 192–417 aa of zebrafish LGP2.

QUANTIFICATION AND STATISTICAL ANALYSIS

All quantitative experiments were performed with at least three independent biological repeats unless otherwise indicated. The results were analyzed and graphed using the GraphPad Prism 8 software. Data were shown as mean ± SD (N = 3). P values were calculated using Student’s t test. *p < 0.05, **p < 0.01; n.s., not significant.