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Zebrafish *foxo3b* Negatively Regulates Antiviral Response through Suppressing the Transactivity of *irf3* and *irf7*

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Forkhead box O (FOXO)3, a member of the FOXO family of transcription factors, plays key roles in various cellular processes, including development, longevity, reproduction, and metabolism. Recently, FOXO3 has also been shown to be involved in modulating the immune response. However, how FOXO3 regulates immunity and the underlying mechanisms are still largely unknown. In this study, we show that zebrafish (*Danio rerio*) *foxo3b*, an ortholog of mammalian FOXO3, is induced by polyinosinic-polycytidylic acid stimulation and spring viremia of carp virus (SVCV) infection. We found that foxo3b interacted with irf3 and irf7 to inhibit *ifr3/irf7* transcriptional activity, thus resulting in suppression of SVCV or polyinosinic-polycytidylic acid–induced IFN activation. By suppressing expression of key antiviral genes, *foxo3b* negatively regulated the cellular antiviral response. Furthermore, upon SVCV infection, the expression of the key antiviral genes was significantly enhanced in *foxo3b*-null zebrafish larvae compared with wild-type larvae. Additionally, the replication of SVCV was inhibited in *foxo3b*-null zebrafish larvae, leading to a higher survival rate. Our findings suggest that by suppressing *irf3/irf7* activity, zebrafish *foxo3b* negatively regulates the antiviral response, implicating the vital role of the FOXO gene family in innate immunity. *The Journal of Immunology*, 2016, 197: 4736–4749.

Forkhead box O (FOXO) transcription factors, homologs of DAF-16 in *Caenorhabditis elegans*, play multiple roles in development, longevity, reproduction, and metabolism (1). In mammals, there are four FOXO genes, including FOXO1, FOXO3 (FOXO3a), FOXO4, and FOXO6, which share some common functions but also participate in diverse cellular processes (2). FOXO1 knockout mice exhibit embryonic lethality due to vascular defects, and FOXO3 (FOXO3a) knockout mice have reduced fertility in an age-dependent manner, whereas FOXO4 knockout mice have no obvious phenotype (3). Among the four FOXO genes, FOXO3 has been the most widely investigated. As a transcription factor, FOXO3 regulates expression of several target genes that participate in a series of cellular processes and respond to various cellular stresses (4–8). Alternatively, different cellular stresses cause variant statuses of posttranslational modifications of FOXO3, such as phosphorylation, ubiquitination, acetylation, and methylation, which in turn regulate the stability or activity of FOXO3 (9–14).

In mammals, IFN response is the first line of defense against viral infections and is activated through the recognition of viral Ags by host pattern recognition receptors (15). Two classes of pattern recognition receptors, TLRs and retinoic acid–inducible gene I (RIG-I)–like receptors (RLRs) (primarily RIG-I and melanoma differentiation–associated gene 5), have been well characterized in the process of sensing viral nucleic acids (15). RIG-I and melanoma differentiation–associated gene 5 are the primary RLRs that recognize the viral component in the cytosol upon infection and then trigger mitochondrial antiviral signaling protein (MAVS)–dependent IFN activation. TLRs such as TLR3/7/9 recognize the viral component at the cell surface or within the endosomal compartment in immune cells and then activate Toll/IL-1R domain–containing adaptor-dependent or MyD88-dependent IFN activation (15, 16). These TLR and RLR cascades eventually converge on the activation of transcription factors of the IFN regulatory factor (IRF) family, primarily IRF3 and IRF7, which binds to IFN response elements or IFN-stimulated response elements (ISREs) within the promoters of the type I IFNs to induce IFN expression. Additionally, stimulator of IFN genes (also known as MITA, ERIS, and MYPS), an endoplasmic reticulum–resident transmembrane protein, is identified as an adaptor to link the signaling transduction between MAVS and downstream cytosolic kinase TBK1 (17–19). Stimulator of IFN genes is mainly considered to link cytosolic DNA–mediated signaling to TBK1 and IRF3 activation, leading to initiation of IFN-β (18, 20).

Recently, FOXO3 has been shown to be involved in immunity and inflammation. In addition to its critical role in the cell fate decision of immune-relevant cells, including B and T cells (21), FOXO3 was also identified as a key factor that can control the magnitude of T cell immune response (22). In FOXO3 knockout mice, the expression of the major proinflammatory cytokines IL-6 and TNF-α is increased in response to viral infection (23). Furthermore, the crosstalk between NF-κB activation and FOXO3 has been recognized. On the one hand, FOXO3 functions as a suppressor

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The online version of this article contains supplemental material.

Abbreviations used in this article: CPE, cytopathic effect; DN, dominant-negative; dpf, day postfertilization; EPC, epithelioma papulosum cyprini; FOXO, forkhead box O; foxo3b-MO, *foxo3b* splicing-blocking morpholino; GAL4-DBD, GAL4 DNA binding domain; HA, hemagglutinin; IRF, IFN regulatory factor; ISG, IFN-stimulated gene; ISRE, IFN-stimulated regulatory element; MAVS, mitochondrial antiviral signaling protein; MOI, multiplicity of infection; mt-foxo3b, mutated *foxo3b*; poly(I:C), polyinosinic-polycytidylic acid; RIG-I, retinoic acid–inducible gene I; RLR, retinoic acid–inducible gene I–like receptor; STD-MO, standard morpholino control; SVCV, spring viremia of carp virus; $TCID_{50}$, 50% tissue culture–infective dose; WT, wild-type; ZFL, zebrafish liver.

of NF-κB activation (23); on the other hand, IKK-α and IKK-β, two important kinases involved in NF-κB activation, phosphorylate and inactivate FOXO3 upon TNF-α stimulation (24). Recently, FOXO3 was reported to regulate IFN-β expression in an IKK-ε–controlled manner (25); additionally, the FOXO3–IRF7 gene regulatory circuit was also found to be crucial in antiviral response (26). However, the physiological role and the underlying mechanisms of FOXO3 in immunity are still poorly understood.

Fish have been reported to have the conserved RLR pathway and TLR pathway for IRF3/IRF7 activation (27–30). Moreover, fish IRF3 and IRF7 play a conserved role in IFN activation and antiviral response (31, 32). Thus, this allows the utilization of the zebrafish (*Danio rerio*) animal model in investigating IFN activation and IRF3/IRF7 regulation in vivo. In zebrafish, there are two orthologs of the mammalian FOXO3 gene, termed *foxo3a* and *foxo3b.* Notably, zebrafish *foxo3b*, initially termed zFKHR/*foxo5* (33), is more conserved to human FOXO3a, which shares 55% identity with human FOXO3a (34) and exhibits conserved function of mammalian FOXO3 (34, 35).

In this study, by taking advantage of the zebrafish model, we examined the role of *foxo3b* in response to virus infection. We elucidated that zebrafish *foxo3b* negatively regulates antiviral response through suppressing the transactivity of *irf3* and *irf7*. These data may provide clues for understanding the function and underlying mechanisms of FOXO3 in antiviral innate immunity.

Materials and Methods

Cells, viruses, and fish

Epithelioma papulosum cyprini (EPC) cells (originally obtained from the American Type Culture Collection) were cultured in medium 199 (Invitrogen) supplemented with 10% FBS. Zebrafish liver (ZFL) cells (originally obtained from the American Type Culture Collection) were cultured in 50% L-15 (Invitrogen), 35% DMEM-HG (Invitrogen), and 15% Ham's F12 medium

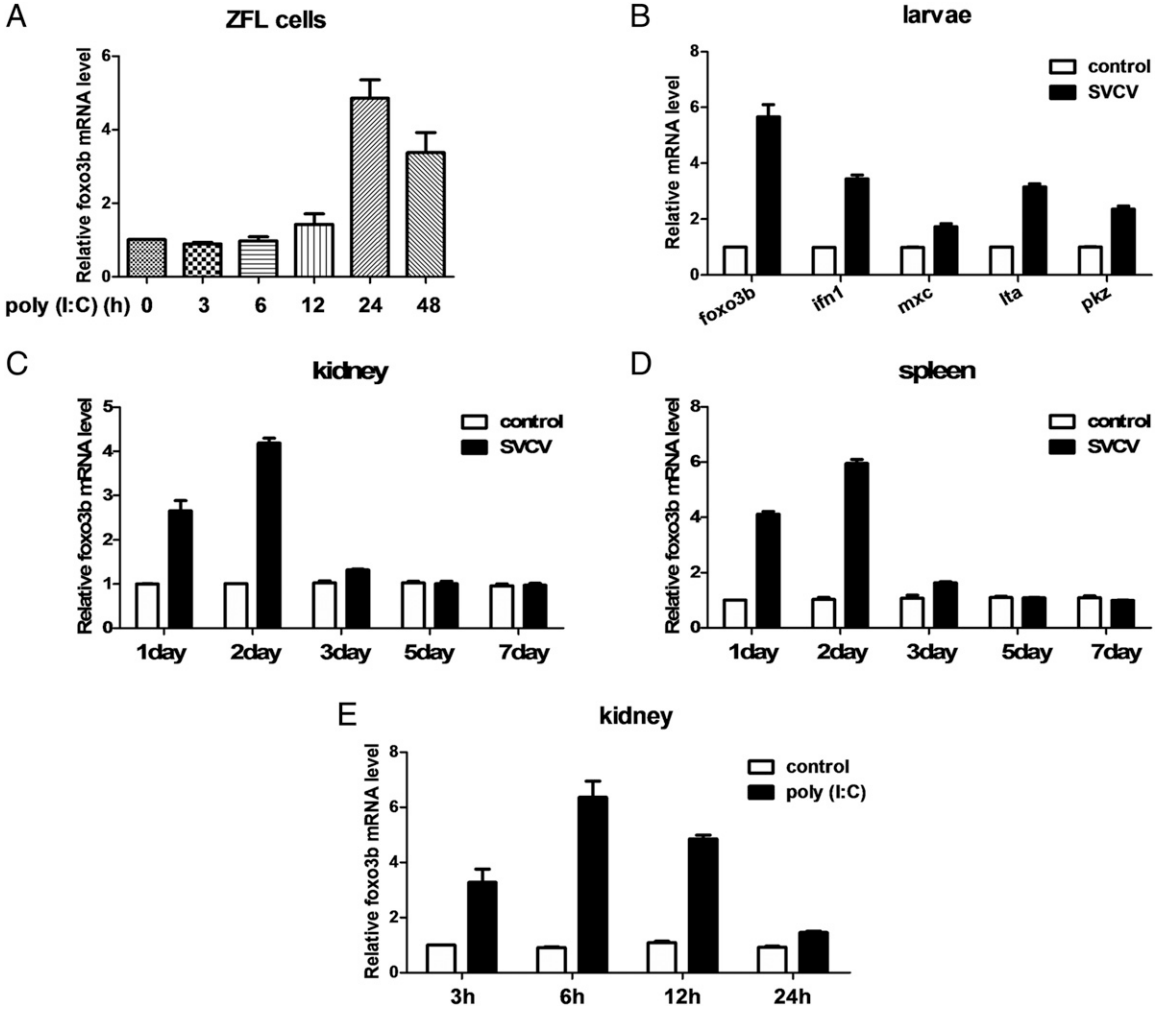

FIGURE 1. *Foxo3b* is stimulated by virus infection. (**A**) *Foxo3b* expression was induced by treatment with poly(I:C) (1 μg/ml) in ZFL cells. ZFL cells were seeded on 60-mm plates overnight and transfected with poly(I:C). At the time points 3, 6, 12, 24, and 48 h, total RNA was extracted for further semiquantitative RT-PCR assays. (**B**) *Foxo3b* expression was induced by SVCV infection (~2×10^8 $TCID_{50}$/ml) in zebrafish larvae. At 3 dpf, SVCV viruses (~2×10^8 $TCID_{50}$/ml) were added into the water containing zebrafish embryos. After incubation for 24 h, total RNA was extracted for semiquantitative RT-PCR assays. The expressions of *inf1*, *mxc*, *lta*, and *pkz* were used as positive controls. (**C** and **D**) *Foxo3b* expression was induced in kidney and spleen of zebrafish i.p. injected with SVCV. Two-month-old zebrafish were i.p. injected with SVCV (~2×10^8 $TCID_{50}$/ml) for 10 μl/individual. At the time points 1, 2, 3, 5, and 7 d, total RNA was extracted from kidney (C) and spleen (D) for further semiquantitative RT-PCR assays; the i.p. injection of cell culture medium was used as a control. (**E**) *Foxo3b* expression was induced in kidney of zebrafish i.p. injected with poly(I:C). Two-month-old zebrafish were i.p. injected with poly(I:C) (50 μg/g). At the time points 3, 6, 12, and 24 h, total RNA was extracted from kidney for further semiquantitative RT-PCR assays; the i.p. injection of PBS was used as a control. Data are presented as means ± SEM of three independent experiments performed in triplicate.

(Invitrogen) supplemented with 0.15 g/l sodium bicarbonate (Sigma-Aldrich), 15 mM HEPES (Sigma-Aldrich), and 10% FBS. All cells were maintained at 28°C in a humidified incubator containing 5% CO_2. Spring viremia of carp virus (SVCV) was propagated in EPC cells until the cytopathic effect (CPE) was complete, and the culture medium was collected and stored at −80°C until use. Viral titers were determined by a 50% tissue culture–infective dose ($TCID_{50}$) assay on EPC cells. Zebrafish strain AB was raised, maintained, reproduced, and staged according to standard protocols. For viral infection, 2-mo-old zebrafish were i.p. injected with SVCV (~2×10^8 $TCID_{50}$/ml) at 10 μl/individual. This amount of injected virus could not cause fish death, but it could induce obvious reddishness in the fish body. At the time points 1, 2, 3, 5, and 7 d, total RNA was extracted from the kidney and spleen for further semiquantitative RT-PCR assays; the i.p. injection of cell culture medium was used as a control. For polyinosinic-polycytidylic acid [poly(I:C)] induction, 2-mo-old zebrafish were i.p. injected with poly(I:C) (50 μg/g). At the time points 3, 6, 12, and 24 h, total RNA was extracted from the kidney for further semiquantitative RT-PCR assays; the i.p. injection of PBS was used as a control.

Plasmid construction and reagents

The plasmids encoding *rig-1*–Nter and *tbk1* in the pcDNA3.1(+) vector (Invitrogen) and the plasmids containing Dr-IFNφ1-luc, Dr-IFNφ2-luc, Dr-IFNφ3-luc, Dr-IFNφ4-luc, and EPC-IFN-luc in the pGL3-Basic vector (Promega) were constructed as described previously (36, 37). The ISRE luciferase reporter construct (ISRE-luc) containing five ISRE motifs in a series and the pFR-luc reporter construct were purchased from Stratagene. Full-length cDNAs of zebrafish *foxo3b*, *mavs*, *irf3*, and *irf7* were subcloned into pCMV-Myc, pCMV-hemagglutinin (HA), pAcGFP-N1, pM-RFP, or pM (all from Clontech) vectors to generate Myc-*foxo3b*, HA-*foxo3b*, Myc-*mavs*, Myc-*irf3*, Myc-*irf7*, GFP-*foxo3b*, RFP-*irf3*,

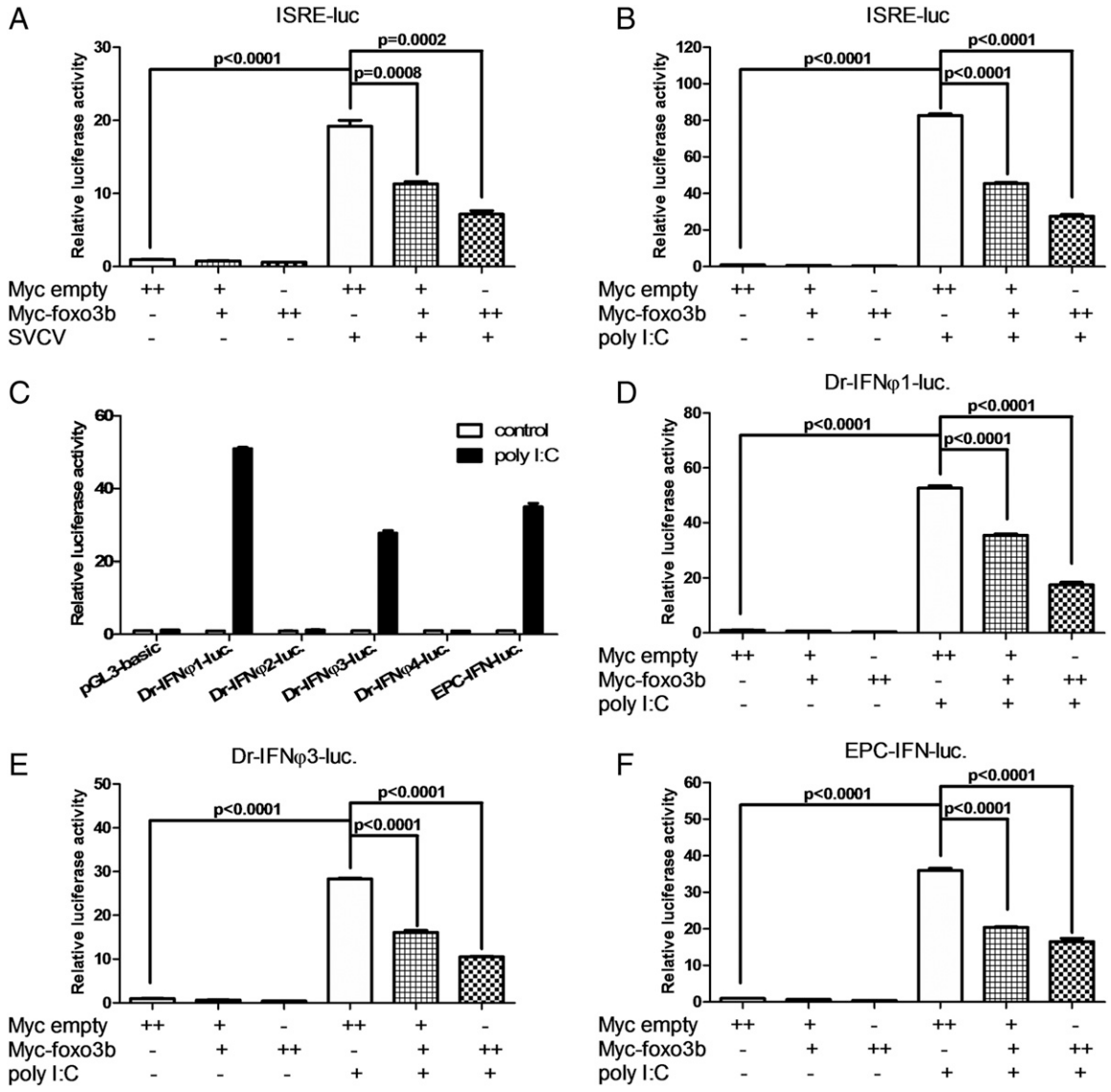

FIGURE 2. Zebrafish *foxo3b* inhibits SVCV- or poly(I:C)-induced IFN activation. (**A**) Overexpression of *foxo3b* suppressed ISRE reporter activity induced by SVCV infection in a dose-dependent manner in EPC cells. EPC cells were transfected with the ISRE luciferase reporter (0.2 μg/well) together with Myc empty vector or an increasing amount of the Myc-*foxo3b* vector (0.1 and 0.2 μg/well). After 24 h, the cells were infected by SVCV (~2×10^8 $TCID_{50}$/ml) for 24 h and then luciferase reporter activity assays were conducted. (**B**) Overexpression of *foxo3b* suppressed ISRE reporter activity induced by poly(I:C) in a dose-dependent manner in EPC cells. EPC cells were transfected with the ISRE luciferase reporter (0.2 μg/well) together with Myc empty vector or an increasing amount of the Myc-*foxo3b* vector (0.1 and 0.2 μg/well). After 24 h, the cells were transfected with poly(I:C) (1 μg/ml) for 24 h and then luciferase reporter activity assays were performed. (**C**) The activity of zebrafish IFNφ1, IFNφ3, and EPC-IFN promoter luciferase reporters (Dr-IFNφ1-luc, Dr-IFNφ3-luc, and EPC-IFN-luc) was activated by poly(I:C) in EPC cells, but the activity of zebrafish IFNφ2 and IFNφ4 promoter luciferase reporters (Dr-IFNφ2-luc and Dr-IFNφ3-luc) was not activated by poly(I:C) in EPC cells. (**D**–**F**) Overexpression of *foxo3b* suppressed the activity of zebrafish IFNφ1, IFNφ3, and EPC-IFN promoter luciferase reporters activated by poly(I:C) in a dose-dependent manner in EPC cells. Data are presented as means ± SEM of three independent experiments performed in triplicate.

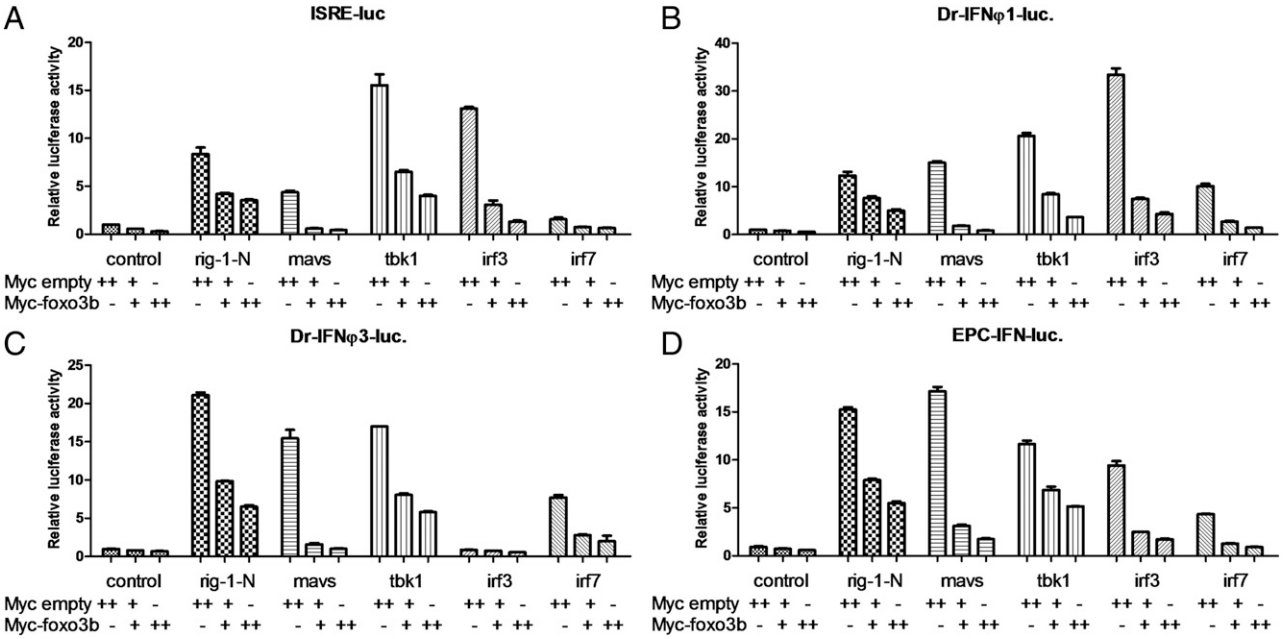

FIGURE 3. Zebrafish *foxo3b* inhibits *irf3*/*irf7*-mediated IFN induction. (**A**) Overexpression of *foxo3b* suppressed *rig-1*–Nter-, *mavs*-, *tbk1*-, *irf3*-, and *irf7*-induced activation of ISRE reporter activity in a dose-dependent manner in EPC cells. (**B**) Overexpression of *foxo3b* inhibited *rig-1*–Nter-, *mavs*-, *tbk1*-, *irf3*-, and *irf7*-induced activation of zebrafish IFNφ1 promoter luciferase reporter activity in a dose-dependent manner in EPC cells. (**C**) Overexpression of *foxo3b* inhibited *rig-1*–Nter-, *mavs*-, *tbk1*-, and *irf7*-induced activation of zebrafish IFNφ3 promoter luciferase reporter activity in a dose-dependent manner in EPC cells. (**D**) Overexpression of *foxo3b* inhibited *rig-1*–Nter-, *mavs*-, *tbk1*-, *irf3*-, and *irf7*-induced activation of EPC-IFN promoter luciferase reporter activity in a dose-dependent manner in EPC cells. Data are presented as means ± SEM of three independent experiments performed in triplicate.

RFP-*irf7*, PM-*irf3*, and PM-*irf7*. A series of domain mutants of *foxo3b* were subcloned into the pCMV-Myc vector. All constructs were verified by DNA sequencing.

Poly(I:C) was purchased from InvivoGen and used at a final concentration of 1 μg/ml. Anti-Myc (9E10) Ab was purchased from Santa Cruz Biotechnology, and anti-HA Ab was purchased from Covance. Anti–α-tubulin Ab was purchased from Abcam. Anti-irf3 Ab was raised by injecting GST-tagged irf3 (ABclonal) into rabbits. Anti-irf7 Ab was obtained from Shun Li (Institute of Hydrobiolgy, Chinese Academy of Science).

Dominant-negative (DN) forms of *irf3* and *irf7* were cloned into pCMV-Myc (Clontech) and pCMV-HA (Clontech) as described previously and subcloned into the psp64 vector (38). GFP and myc-tagged *foxo3b* in the psp64 vector were described previously (35). The standard morpholino control (STD-MO) and *foxo3b* splicing-blocking morpholino (foxo3b-MO) were described previously (34, 35).

Luciferase reporter assays

EPC cells were grown in 24-well plates and transfected with various amounts of plasmids by VigoFect (Vigorous Biotech, Beijing, China), as well as with pTK-*Renilla* used as an internal control. After the cells were transfected for the indicated time, the luciferase activity was determined by the Dual-Luciferase Reporter Assay System (Promega). Data were normalized to *Renilla* luciferase. Data are reported as means ± SEM from three independent experiments performed in triplicate.

Immunoprecipitation and Western blotting

For immunoprecipitation assays, EPC cells were transfected with different combinations of HA-*foxo3b*, Myc-*irf3*, Myc-*irf7*, or empty vectors (5 μg each). At 24 h posttransfection, the cells were treated with 1 μg/ml poly (I:C). After an additional 24 h, the cells were washed with ice-cold PBS buffer and then lysed in RIPA buffer containing 50 mM Tris (pH 7.4), 1% Nonidet P-40, 0.25% sodium deoxycholate, 1 mM EDTA (pH 8), 150 mM NaCl, 1 mM NaF, 1 mM PMSF, 1 mM Na_3VO_4, and a 1:100 dilution of protease inhibitor mixture (Sigma-Aldrich). After incubation on ice for 1 h, lysates were collected and centrifuged at 10,000 × *g* at 4°C for 15 min. Then the supernatant was transferred into a new tube and incubated with anti-Myc Ab–conjugated agarose beads at 4°C overnight. The immunoprecipitates were washed three times with RIPA buffer. The immunoprecipitate and the total cell lysate were boiled with 1× SDS sample loading buffer, separated on SDS-PAGE, and transferred to a polyvinylidene difluoride membrane (Millipore). Western blot analysis was performed as described previously (39). Anti-Myc Ab–conjugated agarose beads were purchased from Sigma-Aldrich. The Fujifilm LAS4000 mini-luminescent image analyzer was used to image the blots.

Semiquantitative real-time PCR

Total RNA was extracted from EPC and ZFL cells by TRIzol reagent (Invitrogen), and total RNA was extracted from zebrafish embryos by an SV Total RNA Isolation System (Promega). cDNA synthesis was carried out

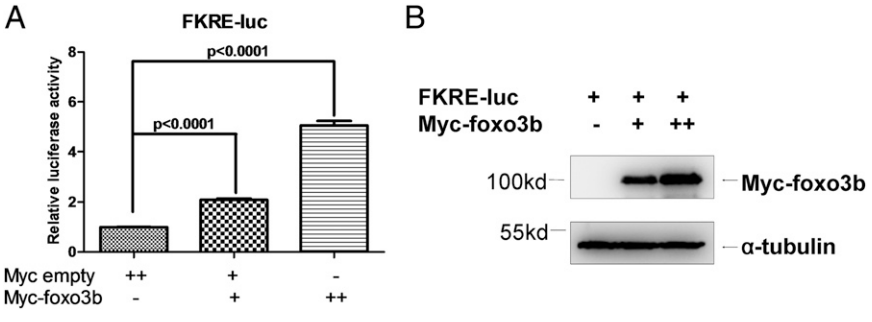

FIGURE 4. Zebrafish *foxo3b* activates FKRE-luciferase reporter activity in a dose-dependent manner in EPC cells. (**A**) EPC cells were transfected with the FKRE-luciferase reporter (0.2 μg/well) together with the Myc empty vector or an increasing amount of the Myc-*foxo3b* vector (0.1 and 0.2 μg/well). After 24 h, the luciferase reporter activity assays were conducted. (**B**) Western blot analysis confirmed expression of the various proteins in (A).

using an All-in-One cDNA Synthesis SuperMix kit (Biotool). Semiquantitative real-time PCR was carried out using SYBR Green Fast quantitative PCR master mix (Biotool). The primers for semiquantitative real-time PCR assays are listed in Supplemental Table I.

Fluorescence microscopy

EPC cells were transfected with different combinations of zebrafish GFP-*foxo3b*, RFP-*irf3*, and RFP-*irf7* vectors. Twenty-four to 30 h after transfection, cells were directly observed and imaged with a Leica SP8 microscope.

Generation of foxo3b-*null zebrafish*

Disruption of the *foxo3b* in zebrafish was accomplished via CRISPR/Cas9 technology. Zebrafish *foxo3b* single-guide RNA was designed using the tools provided in the Web site (http://crispr.mit.edu). The primers for amplifying gRNA template are: 5′-GTAATACGACTCACTATAGGACAACGGCAGCCCAAGCCGTTTTAGAGCTAGAAATAGC-3′ and 5′-AAAAGCACCGACTCGGTGCC-3′. Single-guide RNA was synthesized using a TranscriptAid T7 high-yield transcription kit (Fermentas). After the injected embryos were incubated at 28.5°C for 24 h, the genomic DNA was extracted from 20 to 30 embryos by heating the embryos at 94°C for 40 min in lysis solution (50 mM NaOH) (the reaction was terminated by adding 1 M Tris-HCl [pH 8]). The mutant detection was followed by a heteroduplex mobility assay as described previously (40). When the results were positive, the remaining embryos were raised to adulthood and treated as F_0, which were backcrossed with the wild-type (WT) zebrafish for generating F_1. The F_1 zebrafish were genotyped by a heteroduplex mobility assay initially and confirmed by sequencing target sites. The F_1 zebrafish harboring the mutations were backcrossed with the WT zebrafish to obtain F_2. To exclude the off-targeting effect, the heterozygous zebrafish were backcrossed with the WT zebrafish for at least five generations. The adult zebrafish with the same genotype (+/−) were intercrossed to generate offspring, which should contain WT (+/+), heterozygous (+/−), and homozygous (−/−) zebrafish. The primers for detecting mutants were 5′-TGGACATTGCCATTGATCCAG-3′ (forward) and 5′-CCATGCATTCCTCCTTGAAGA-3′ (reverse).

Statistical analysis

Luciferase assays, RT-PCR assays, and virus titer data are reported as means ± SEM of three independent experiments performed in triplicate. The statistical analysis was performed using GraphPad Prism 5 (unpaired *t* test) (GraphPad Software).

Results

Zebrafish foxo3b *is induced upon poly(I:C) or SVCV stimulation*

To determine the behavior of zebrafish *foxo3b* in response to viral infection, ZFL cells were stimulated with poly(I:C), a mimic of RNA virus (41, 42). As shown in Fig. 1A, *foxo3b* mRNA was increased after treatment with poly(I:C) for 12 h and peaked at 24 h. Subsequently, zebrafish larvae (3 d postfertilization [dpf])

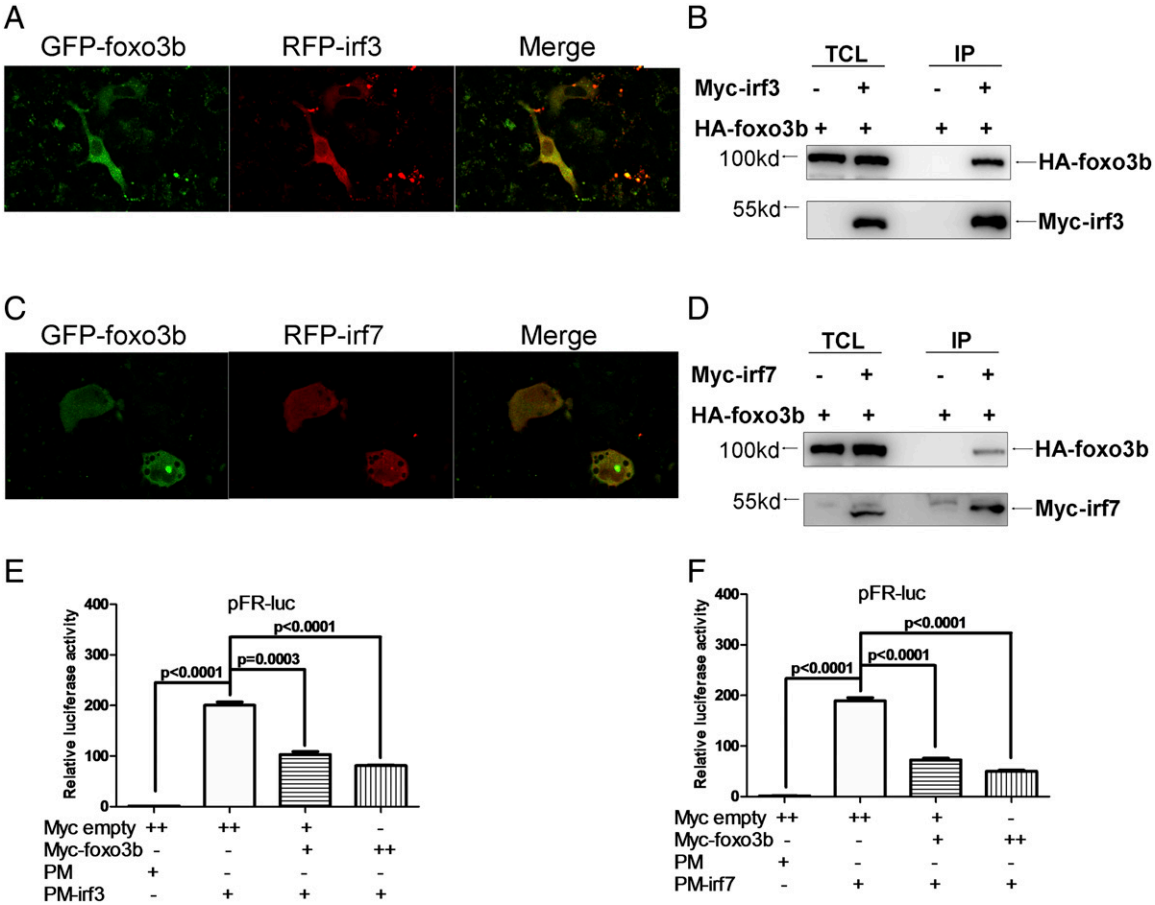

FIGURE 5. Zebrafish *foxo3b* represses the transcriptional activity of *irf3* and *irf7* through protein–protein interaction. (**A**) GFP-tagged *foxo3b* (GFP-*foxo3b*) colocalized with RFP-tagged *irf3* (RFP-*irf3*) in EPC cells. Original magnification ×400. (**B**) *Foxo3b* interacted with *irf3* in EPC cells as revealed by coimmunoprecipitation assays. (**C**) GFP-tagged *foxo3b* (GFP-*foxo3b*) colocalized with RFP-tagged *irf7* (RFP-*irf7*) in EPC cells. Original magnification ×400. (**D**) *Foxo3b* interacted with *irf7* in EPC cells as revealed by coimmunoprecipitation assays. (**E**) *Irf3* transcriptional activity was significantly inhibited by overexpression of *foxo3b* in a dose-dependent manner in EPC cells. (**F**) *Irf7* transcriptional activity was significantly inhibited by overexpression of *foxo3b* in a dose-dependent manner in EPC cells. EPC cells were transfected with the pFR-luciferase reporter (0.2 μg/well) together with PM empty vector or PM-*irf3* or PM-*irf7* as well as the Myc empty vector or an increasing amount of the Myc-*foxo3b* vector (0.1 and 0.2 μg/well). After 24 h, a luciferase assay was performed. pRL-TK was used as an internal control. Data are presented as means ± SEM of three independent experiments performed in triplicate.

were infected with SVCV, and *foxo3b* mRNA was detected at 24 h postinfection. Similar to that of other key antiviral genes, including *ifn1*, *mxc*, *lta*, and *pkz* (43, 44), *foxo3b* mRNA was greatly induced (Fig. 1B). Moreover, the i.p. injection of SVCV into zebrafish (2 mo old) resulted in the induced expression of *foxo3b* in kidney and spleen at day 1 and day 2 of injection (Fig. 1C, 1D). Additionally, i.p. injection of poly(I:C) into zebrafish could also upregulate the expression of *foxo3b* in kidney (Fig. 1E). These data suggest that zebrafish *foxo3b* was induced upon virus infection, and thus may be involved in the antiviral response.

Zebrafish foxo3b *inhibits SVCV or poly(I:C)-induced IFN activation*

To elucidate the functional importance of *foxo3b* induction in response to poly(I:C) or SVCV stimulation, we examined the role of *foxo3b* in SVCV or poly(I:C)-induced IFN activation. Initially, we performed reporter assays by taking advantage of the ISRE reporter, a commonly used reporter for monitoring viral infection or poly(I:C) stimulation (37). As shown in Fig. 2A and 2B, either SVCV infection or poly(I:C) treatment activated ISRE reporter activity, whereas *foxo3b* overexpression suppressed these inductions in a dose-dependent manner in EPC cells. Of note, four type I IFN promoter luciferase reporters (IFNφ1-luc, IFNφ2-luc, IFNφ3-luc, and IFNφ4-luc) of zebrafish and the EPC IFN promoter luciferase reporter have previously been used for detecting poly(I:C) stimulation in EPC cells (36). Zebrafish IFNφ1 and IFNφ3 promoter luciferase reporters and the EPC IFN promoter luciferase reporter could be induced by poly(I:C), whereas zebrafish IFNφ2 and IFNφ4 promoter luciferase reporters could not be induced by poly(I:C) (36). These inductions are validated in Fig. 2C. However, overexpression of *foxo3b* inhibited IFNφ1 (Fig. 2D), IFNφ3 (Fig. 2E), and EPC IFN (Fig. 2F) promoter luciferase reporter activity induced by poly(I:C) stimulation in a dose-dependent manner. Expressions of transfected foxo3b were confirmed by Western blot analysis (Supplemental Fig. 1). These data suggest that IFN activation induced by SVCV or poly(I:C) is inhibited by overexpression of *foxo3b*.

Foxo3b *suppresses IFN activation via* irf3 *and* irf7

It has been reported that similar to that of mammalian species, the fish RLR signaling cascade can activate IFN expression (36). To determine whether zebrafish *foxo3b* also inhibits IFN expression

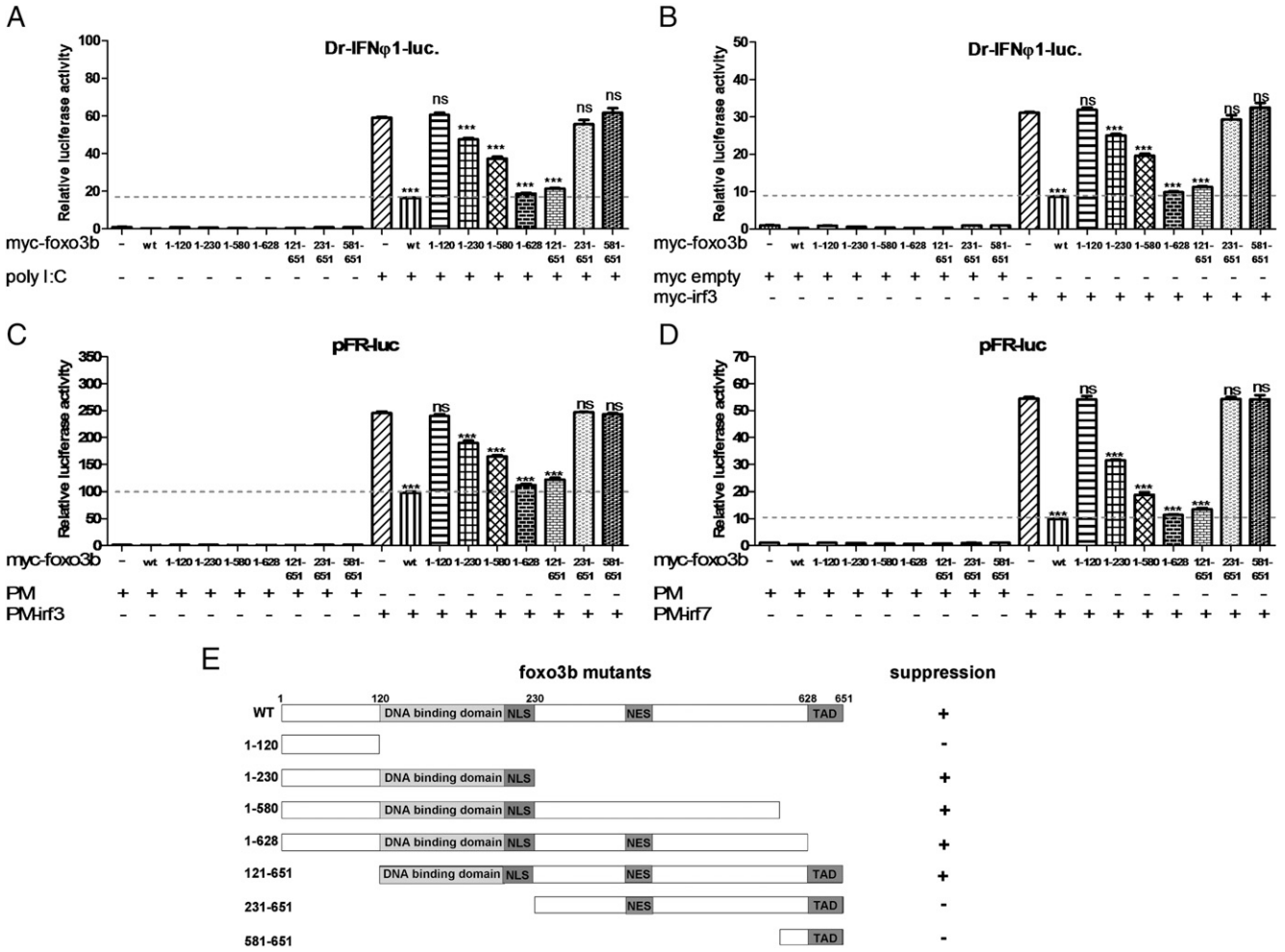

FIGURE 6. The N-terminal of *foxo3b* (1–120 aa) is not required for the suppressive function of *foxo3b* on *irf3*/*irf7* activity, and the C-terminal containing the transactivation domain of *foxo3b* (628–651 aa) is not sufficient to suppress *irf3*/*irf7* activity. (**A**) The effect of different truncated mutants of *foxo3b* on zebrafish IFNφ1 promoter luciferase reporter activity induced by poly (I:C) in EPC cells. (**B**) The effect of different truncated mutants of *foxo3b* on zebrafish IFNφ1 promoter luciferase reporter activity activated by overexpression of *irf3*. (**C** and **D**) The effect of different truncated mutants of *foxo3b* on the transcriptional activity of *irf3* (C) and *irf7* (D). (**E**) Scheme of *foxo3b* truncated mutants and their suppressive effect on *irf3*/*irf7* transcriptional activity. Data are presented as means ± SEM of three independent experiments performed in triplicate. ***$p < 0.0001$. ns, not significant.

through its effect on RLR signaling pathway, we examined the activity of ISRE-luc, IFNφ1-luc, IFNφ3-luc, and EPC-IFN-luc stimulated by overexpression of cascaded factors in the RLR signaling pathway, including the zebrafish N-terminal domain of *rig-1* (*rig-1*-Nter), *mavs*, *tbk1*, *irf3*, or *irf7* together with empty Myc vector or Myc-tagged *foxo3b*. As shown in Fig. 3A, overexpression of the cascaded factors in the RLR signaling pathway activated the ISRE luciferase reporter activity dramatically. However, cotransfection of *foxo3b* inhibited the activity of the ISRE reporter activated by the cascaded factors in a dose-dependent manner. Similar results were obtained by using IFNφ1-luc and EPC-IFN-luc reporters (Fig. 3B, 3D). It has been reported that zebrafish IFNφ3 could be activated by *irf7* but not *irf3* (38). As shown in Fig. 3C, the activity of IFNφ3 was induced by overexpression of *rig-1*–Nter, *mavs*, *tbk1*, and *irf7*, but not *irf3*. This induction was also suppressed by *foxo3b* overexpression in a dose-dependent manner. Expressions of various proteins were confirmed by Western blot analysis (Supplemental Fig. 2). Taken together, these data suggest that *foxo3b* might directly inhibit the activity of both *irf3* and *irf7*.

It seemed in all of the above experiments that overexpression of *foxo3b* resulted in decreased inductions. Thus, it raised a possibility that *foxo3b* might have a general suppressive role in transcriptional induction in the assay system that we employed for this study. To rule out this possibility, we examined the effect of *foxo3b* overexpression on activity of the FKRE luciferase reporter, a well-defined reporter for monitoring FOXO3 transactivity (10). Fig. 4 shows that overexpression of *foxo3b* in EPC cells could induce activity of FKRE luciferase reporter dramatically in a dose-dependant manner. These data suggest that the suppressive role of *foxo3b* on activity of *irf3* and *irf7* is specific.

Given that *foxo3b* might suppress *irf3*- and *irf7*-induced IFN activation directly, we speculated that *foxo3b* might interact with *irf3* and *irf7* to function in its suppressive role. To test this possibility, we initially performed colocalization assays. Cotransfection of GFP-tagged *foxo3b* and RFP-tagged *irf3* into EPC cells led to their colocalization (Fig. 5A). Similarly, GFP-tagged *foxo3b* also colocalized with RFP-tagged *irf7* in EPC cells (Fig. 5C). Subsequently, we conducted coimmunoprecipitation assays to further evaluate the potential interaction between *foxo3b* and *irf3*/*irf7*. The HA-*foxo3b* vector was cotransfected with Myc-*irf3* or Myc-*irf7* into EPC cells. As shown in Fig. 5B, HA-*foxo3b* could be coimmunoprecipitated by Myc-*irf3* using anti-Myc agarose beads. Similarly, HA-*foxo3b* could also be coimmunoprecipitated by Myc-*irf7* (Fig. 5D).

Because *foxo3b* could interact with *irf3*/*irf7*, we further speculated that *foxo3b* might inhibit the transactivity of *irf3* and *irf7*. To test this possibility, we constructed artificial transcription factors by cloning zebrafish *irf3* and *irf7* into the PM vector (Clontech), which contains the GAL4 DNA binding domain (GAL4-DBD). The luciferase reporter pFR-luc (Stratagene) harboring five repeats of the GAL4-DBD binding site in its promoter was used to monitor the transactivation activity of *irf3* or *irf7* fused with GAL4-DBD. Luciferase assays in EPC cells showed that overexpression of *foxo3b* inhibited the transcriptional activity of both *irf3* and *irf7* significantly (Fig. 5E, 5F). Expressions of transfected foxo3b, irf3, and irf7 were confirmed by Western blot analysis (Supplemental Fig. 3A, 3B).

Taken together, these observations suggest that *foxo3b* may serve as a suppressor of IFN activation by dampening the transcriptional activity of *irf3* and *irf7* through protein–protein interaction. Thus, zebrafish *foxo3b* might negatively regulate antiviral response.

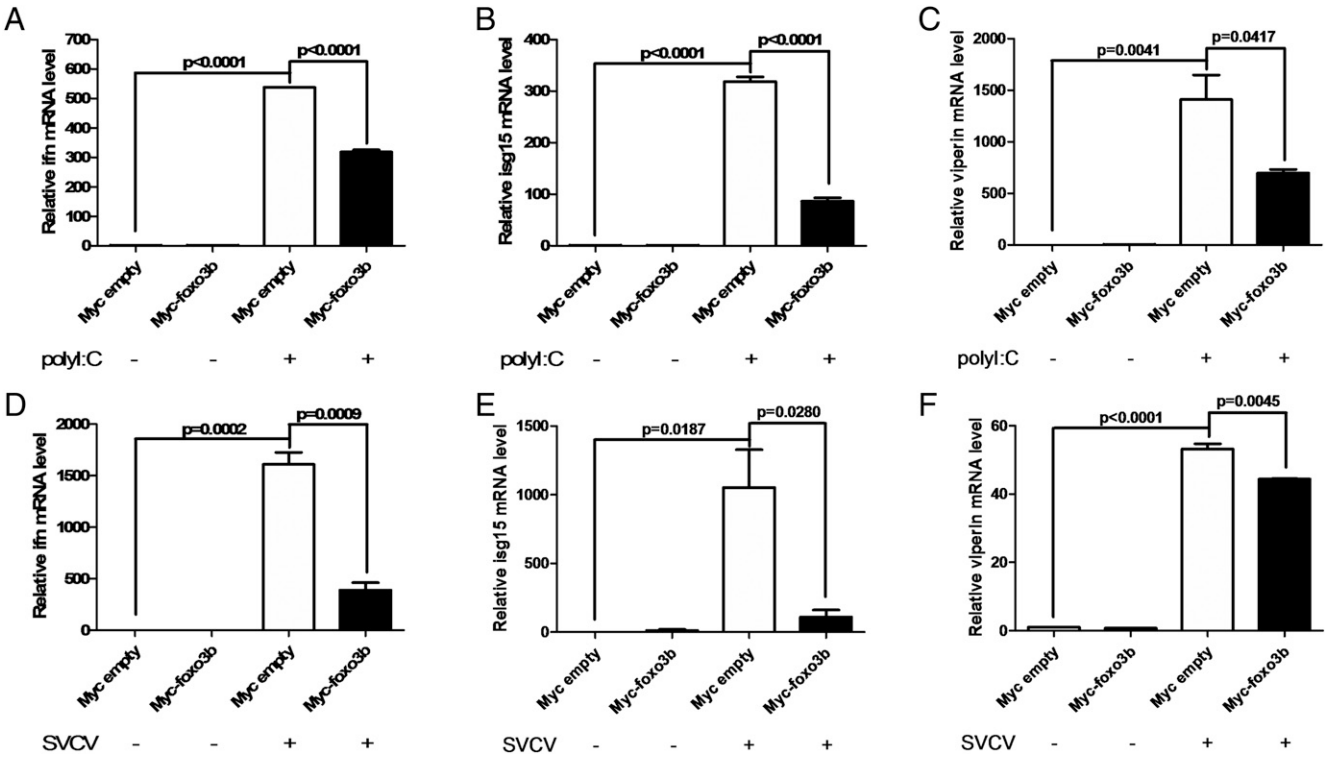

FIGURE 7. Zebrafish *foxo3b* suppresses expression of IFN and ISGs induced by poly(I:C) stimulation and SVCV infection. (**A**–**C**) Overexpression of *foxo3b* suppressed expression of *ifn* (A), *isg15* (B), and *viperin* (C) induced by poly(I:C) in EPC cells. (**D**–**F**) Overexpression of *foxo3b* suppressed expression of *ifn* (D), *isg15* (E), and *viperin* (F) induced by SVCV infection in EPC cells. Data are presented as means ± SEM of three independent experiments performed in triplicate.

The N-terminal of foxo3b *(1–120 aa) is not required for the suppressive function of* foxo3b *on* irf3/irf7 *activity, and the C-terminal containing the transactivation domain of* foxo3b *(628–651 aa) is not sufficient to suppress* irf3/irf7 *activity*

To determine which domain of *foxo3b* is required or sufficient for suppressing *irf3/irf7* activity, we conducted domain mapping via promoter assays and transactivity assays. A series of truncated mutants of *foxo3b* were initially constructed and their effect on IFN activation was assessed. As shown in Fig. 6A–D, overexpression of the truncated *foxo3b* mutants (1–120, 231–651, and 581–651 aa) failed to inhibit IFN activation, and overexpression of the mutants (1–230 and 1–580 aa) had a slight inhibitory effect on IFN activation. However, overexpression of the mutants (1–628 and 121–651 aa) had an obvious inhibitory effect on IFN activation, similar to that of the full length of *foxo3b*. A scheme of foxo3b truncated mutants and their effect on *irf3/irf7* transcriptional activity are summarized in Fig. 6E. Of note, the mutant (581–651 aa) contains the entire transactivation domain of *foxo3b*, corresponding to the region 650–673 aa of human FOXO3a (45, 46). Thus, these data suggest that the N-terminal of *foxo3b* (1–120 aa) is not required for the suppressive function of *foxo3b* on *irf3/irf7* activity, and the C-terminal containing the transactivation domain of *foxo3b* (628–651 aa) is not sufficient to suppress *irf3/irf7* activity.

Zebrafish foxo3b *negatively regulates cellular antiviral response*

To further elucidate the role of zebrafish *foxo3b* in the antiviral response, we examined the expression of *ifn*, a typical *irf3/irf7* downstream gene, as well as two typical IFN-stimulated genes (ISGs; *isg15* and *viperin*) upon poly(I:C) stimulation and SVCV infection (38). As shown in Fig. 7A–C, treatment with poly(I:C) greatly induced the expression of *ifn*, *isg15*, and *viperin* in EPC cells. However, overexpression of *foxo3b* significantly attenuated this induction. Similarly, overexpression of *foxo3b* in EPC cells also resulted in reduction of *ifn*, *isg15*, and *viperin* induced by SVCV infection (Fig. 7D–F). Expressions of transfected foxo3b were confirmed by Western blot analysis (Supplemental Fig. 3C, 3D).

To validate the effect of zebrafish *foxo3b* on antiviral response, we further performed CPE assays. As shown in Fig. 8A, overexpression of *foxo3b* in EPC cells resulted in enhanced CPE compared with the empty vector control after EPC cells infected with different titers of SVCV (from a multiplicity of infection [MOI] of 1–1000). Consistently, the titer of SVCV was significantly increased (720-fold) in the supernatant of *foxo3b*-overexpressed EPC cells as determined by plaque assays (Fig. 8B). Furthermore, the copy numbers of SVCV-related genes, including the *P* gene (Fig. 8C), *G* gene (Fig. 8D), and *N* gene (Fig. 8E), were significantly

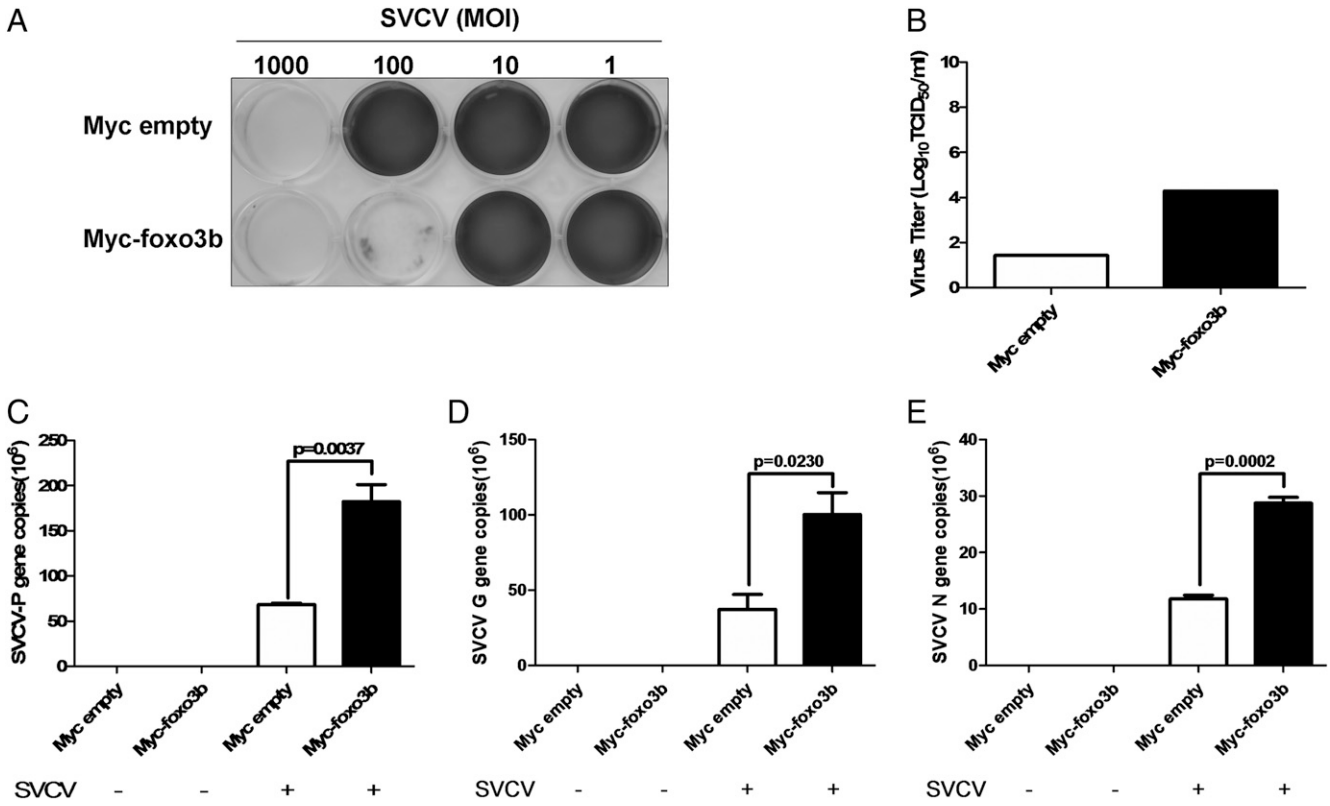

FIGURE 8. Zebrafish *foxo3b* enhances virus replication in SVCV-infected EPC cells. (**A** and **B**) Virus replication was enhanced by overexpression of *foxo3b*. (A) Overexpression of *foxo3b* reduced cell survival after SVCV infection in EPC cells. EPC cells were transfected with 0.5 μg of myc-tagged *foxo3b* or empty vector. At 24 h posttransfection, cells were infected with SVCV at the dose indicated for 2 d. Then, cells were fixed with 4% paraformaldehyde and stained with 1% crystal violet. (B) Overexpression of *foxo3b* increased virus titer after SVCV infection in EPC cells. Culture supernatant was collected from EPC cells infected with SVCV (MOI of 100), and the viral titer was measured by plaque assay. The results are representative of three independent experiments. (**C**–**E**) Overexpression of *foxo3b* increased copy number of SVCV-related genes after SVCV infection in EPC cells. EPC cells were transfected with myc-tagged *foxo3b* or empty vector and infected with SVCV (MOI of 10) at 24 h posttransfection. After 24 h, total RNAs were extracted to examine the mRNA levels of the P, G, and N transcripts of SVCV by semiquantitative RT-PCR analysis. Data are presented as means ± SEM of three independent experiments performed in triplicate.

increased in *foxo3b*-overexpressed EPC cells as revealed by semi-quantitative RT-PCR assays.

Taken together, these data suggest that zebrafish *foxo3b* plays an important role in cellular antiviral response by attenuating expression of the key antiviral genes and facilitating the replication of SVCV.

Knockout of foxo3b *in zebrafish enhances antiviral response*

To determine the physiological role of *foxo3b* in response to viral infection, we knocked out *foxo3b* in zebrafish via CRISPR/Cas9 technology (Fig. 9A). In this mutated line, 5-bp nucleotides (5′-CGGCA-3′) were inserted into exon 2 of the DNA sequence of *foxo3b*, resulting in reading frame shift, which introduced seven missense mutations followed by a nonsense mutation at the aa 85. To exclude off-targeting effects, we backcrossed $foxo3b^{+/-}$ to WT zebrafish ($foxo3b^{+/+}$) for at least five generations, and then $foxo3b^{+/-}$ zebrafish were intercrossed for further assays. The intercrossing between $foxo3b^{+/-}$ and $foxo3b^{+/-}$ generated offspring with $foxo3b^{+/+}$, $foxo3b^{+/-}$, and $foxo3b^{-/-}$ genetic backgrounds at a Mendelian ratio of 1:2:1. Overall, no obvious phenotypes were observed in $foxo3b^{-/-}$ zebrafish, and $foxo3b^{-/-}$ zebrafish were indistinguishable from their WT siblings under normal conditions.

To determine whether the mutated *foxo3b* (mt-*foxo3b*) could partially restore the suppressive function of WT *foxo3b* upon IFN activation, a series of luciferase assays were performed. As shown in Fig. 9B and 9C, mt-*foxo3b* failed to suppress zebrafish IFNφ1 promoter activity induced by poly(I:C) or *irf3* overexpression. Additionally, mt-*foxo3b* had no effect on the transcriptional activity

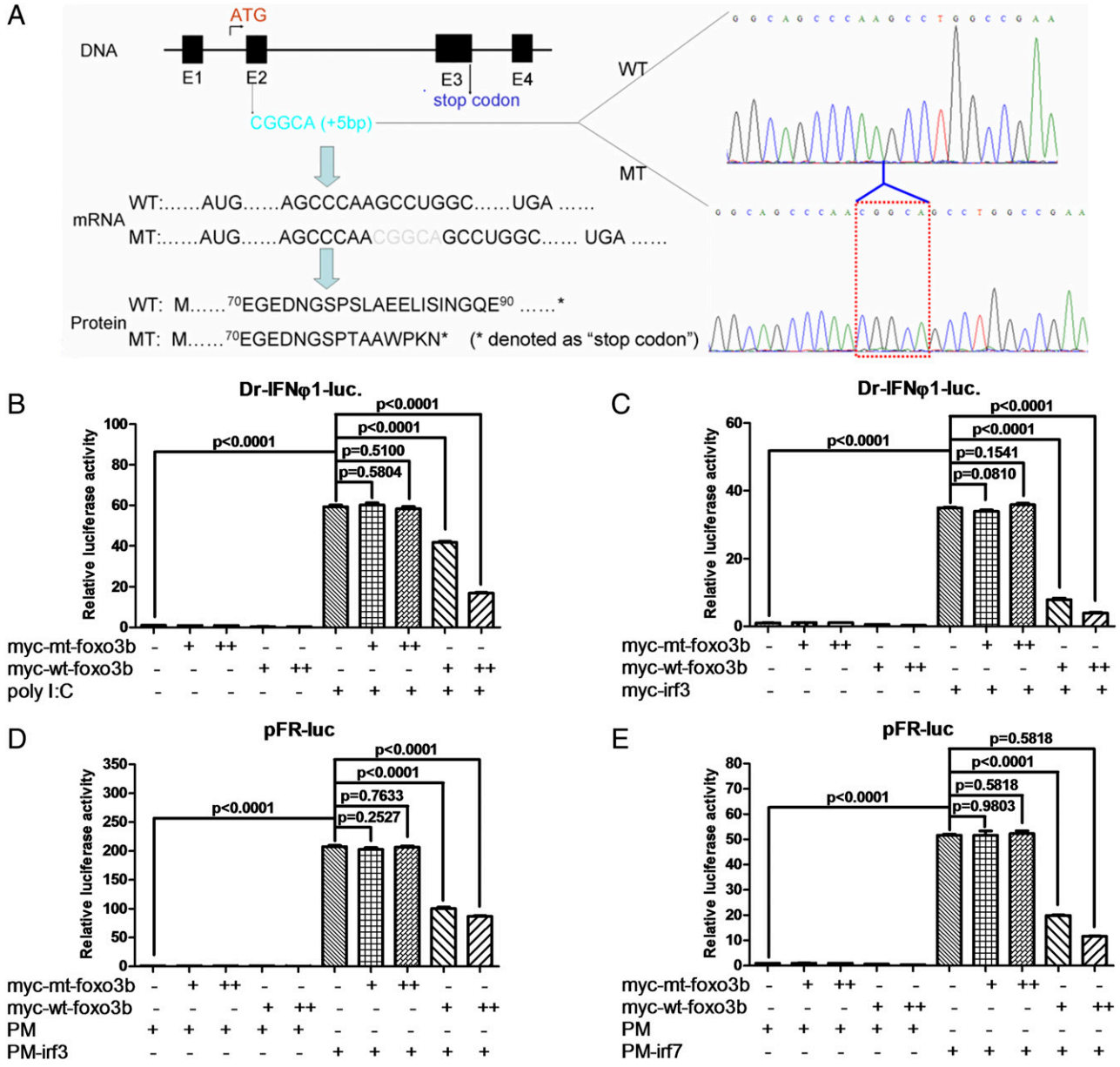

FIGURE 9. Generation of *foxo3b*-null zebrafish via CRISPR/Cas9 technology. (**A**) Scheme of targeting sites and the sequence information in *foxo3b*-null zebrafish. Five–base pair nucleotides (5′-CGGCA-3′) were inserted into exon 2 of *foxo3b* in the mutant, resulting in a reading frame shift and generating a truncated protein with 85 aa (termed mt-*foxo3b*). (**B**–**E**)The truncated protein (mt-foxo3b) had no suppressive role on *irf3*/*irf7* activity. (B) Overexpression of mt-*foxo3b* did not suppress zebrafish IFNφ1 promoter luciferase activity induced by poly(I:C) in EPC cells. (C) Overexpression of mt-*foxo3b* did not suppress zebrafish IFNφ1 promoter luciferase reporter activity induced by *irf3* overexpression in EPC cells. (D and E) Overexpression of mt-*foxo3b* did not suppress the transcriptional activity of *irf3* (D) and *irf7* (E) in EPC cells. Data are presented as means ± SEM of three independent experiments performed in triplicate.

of *irf3* (Fig. 9D) and *irf7* (Fig. 9E). Expressions of various proteins were confirmed by Western blot analysis (Supplemental Fig. 3E–H). Therefore, this *foxo3b*-mutated zebrafish line could be used as a *foxo3b*-deficient model to investigate the role of *foxo3b* in response to viral infection.

To determine the role of *foxo3b* in response to viral infection, we examined the expressions of *ifn1*, *pkz*, *mxc*, and *lta* (four well-defined *irf3/irf7* downstream genes of zebrafish) in response to viral infection (43, 44). As shown in Fig. 10, SVCV infection resulted in the increased expression of *ifn1*, *pkz*, *mxc*, and *lta* in *foxo3b*-deficient zebrafish larvae (*foxo3b*$^{-/-}$) compared with the WT larvae.

Subsequently, *foxo3b*-null larvae (n = 90) and the WT larvae (n = 90) were infected with high-titer SVCV and the numbers of dead larvae were counted at different time points. As shown in Fig. 11A and 11B, *foxo3b*-null larvae had a higher survival rate compared with the WT larvae after SVCV infection. Consistently, the copy numbers of the *P*, *G*, and *N* genes of SVCV indicated by mRNA level were significantly reduced in *foxo3b*-null larvae compared with the WT larvae (Fig. 11C–E). Thus, knockout of *foxo3b* could enhance zebrafish antiviral capability.

To further evaluate *foxo3b* loss of function on zebrafish antiviral response, we knocked down *foxo3b* in zebrafish embryos by injection of foxo3b-MO (34, 35). Similar to those observed in *foxo3b*-null larvae, expressions of *ifn1*, *mxc*, and *pkz* were upregulated in the embryos with foxo3b-MO injection compared with the embryos with the STD-MO injection (Fig. 12A–C). Consistently, the copy numbers of *P*, *G*, and *N* genes of SVCV indicated by mRNA level were significantly reduced in the embryos with foxo3b-MO injection compared with the embryos with the STD-MO injection (Fig. 12D–F). These data further validate that *foxo3b* loss of function enhances zebrafish antiviral capability.

Moreover, we examined the effect of *foxo3b* gain of function on the zebrafish antiviral response. Ectopic expression of *foxo3b* by mRNA injections in embryos suppressed the induction of key antiviral genes by SVCV infection (Fig. 13A–C). On the contrary, the copy numbers of *P*, *G*, and *N* genes of SVCV indicated by mRNA level were significantly upregulated in the embryos with *foxo3b* mRNA injection compared with the embryos with control GFP mRNA injection (Fig. 13D–F). These data further suggest the inhibitory role of *foxo3b* on zebrafish antiviral response in vivo.

To determine whether the inhibitory role of *foxo3b* on the zebrafish antiviral response in vivo is mediated by suppressing the activity of irf3 and irf7, we took advantage of DN forms of irf3 and irf7 (38). As shown in Fig. 14A, upon SVCV infection, foxo3b-MO injection caused *mxc* expression to be dramatically enhanced; however, when the DN forms of irf3 and irf7 were coinjected, this enhancement disappeared. Consistently, the copy numbers of the *N* gene of SVCV indicated by mRNA level was reduced when only foxo3b-MO was injected. However, when injected in combination with the DN forms of irf3 and irf7, this reduction was fully recovered (Fig. 14B). These data suggest that

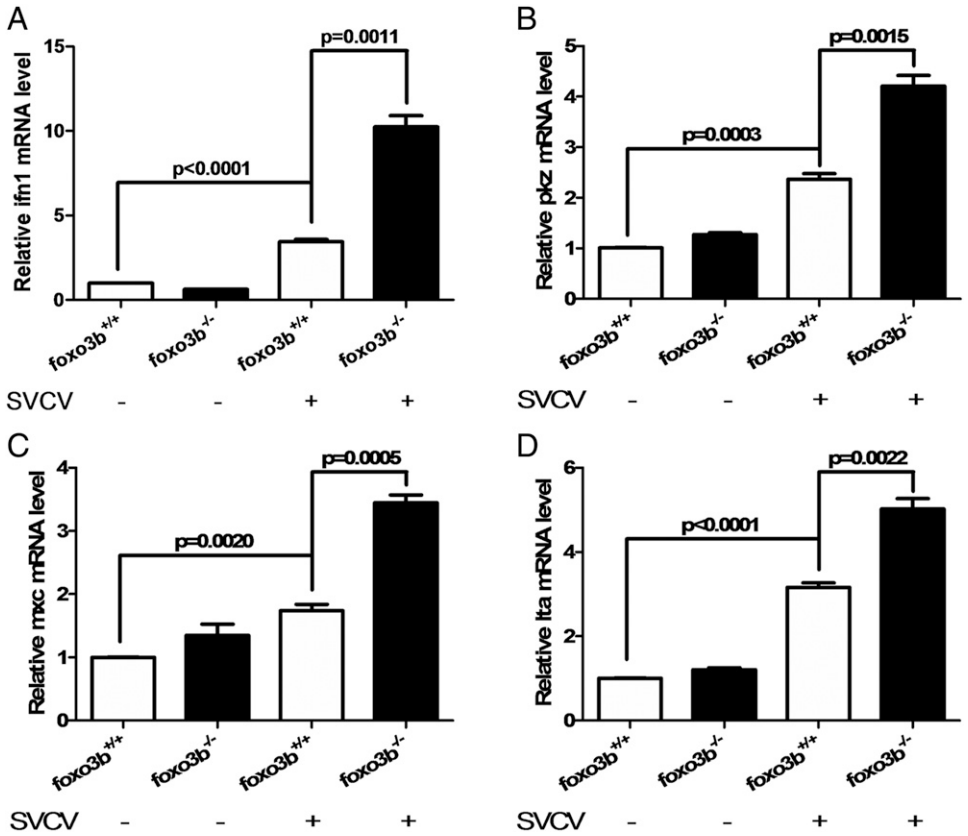

FIGURE 10. Induction of key antiviral genes by SVCV infection is more dramatic in *foxo3b*-null larvae compared with WT larvae. (**A**) Induction of *ifn1* by SVCV (~2 × 10^8 $TCID_{50}$/ml) infection was more dramatic in *foxo3b*-null larvae (*foxo3b*$^{-/-}$) compared with the WT larvae (*foxo3b*$^{+/+}$). (**B**) Induction of *pkz* by SVCV (~2 × 10^8 $TCID_{50}$/ml) infection was more dramatic in *foxo3b*-null larvae (*foxo3b*$^{-/-}$) compared with WT larvae (*foxo3b*$^{+/+}$). (**C**) Induction of *mxc* by SVCV (~2 × 10^8 $TCID_{50}$/ml) infection was more dramatic in *foxo3b*-null larvae (*foxo3b*$^{-/-}$) compared with WT larvae (*foxo3b*$^{+/+}$). (**D**) Induction of *lta* by SVCV (~2 × 10^8 $TCID_{50}$/ml) infection was more dramatic in *foxo3b*-null larvae (*foxo3b*$^{-/-}$) compared with WT larvae (*foxo3b*$^{+/+}$). *Foxo3b*-null larvae (*foxo3b*$^{-/-}$) and WT larvae (*foxo3b*$^{+/+}$) are offspring of siblings. At 3 dpf, SVCV viruses (~2 × 10^8 $TCID_{50}$/ml) were added to the water containing zebrafish larvae. After incubation for 24 h, total RNA was extracted from larvae and semiquantitative RT-PCR analysis was conducted for detecting expression levels of *inf1*, *pkz*, *mxc*, and *lta*. Data are presented as means ± SEM of three independent experiments performed in triplicate.

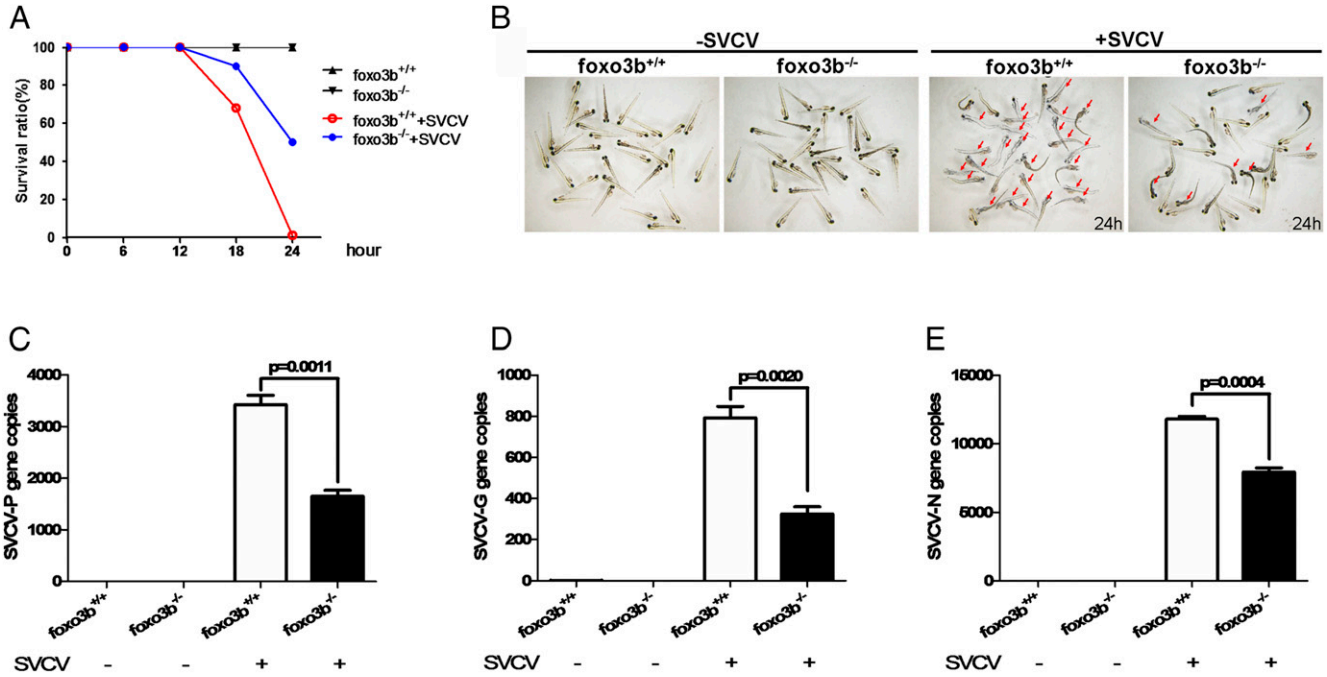

FIGURE 11. *Foxo3b*-null zebrafish display higher antiviral capability. (**A**) *Foxo3b*-null zebrafish were more resistant to SVCV infection compared with the WT based on the survival ratio. (**B**) Representative images of *foxo3b*-null zebrafish larvae and the WT (3 dpf) treated with or without SVCV for 24 h. The dead larvae (marked by red arrows) exhibited no movement, no blood circulation, and a degenerated body. SVCV viruses (~6 × 10^8 $TCID_{50}$/ml) in water were added to *foxo3b*-null larvae (n = 90, 3 dpf) and the WT (n = 90, 3 dpf), and then the numbers of dead larvae were counted at time points 6, 12, 18, and 24 h. (**C**–**E**) The virus replication number was lower in *foxo3b*-null zebrafish larvae compared with that of the WT after infection by SVCV. *Foxo3b*-null larvae (*foxo3b*$^{-/-}$) and the WT larvae (*foxo3b*$^{+/+}$) are offspring of siblings. SVCV viruses (~2 × 10^8 $TCID_{50}$/ml) in water were added to *foxo3b*-null larvae (3 dpf) and the WT (3 dpf). After incubation for 24 h, the expression levels of P protein (C), G protein (D), and N protein of SVCV (E) were detected by semiquantitative RT-PCR analysis. Data are presented as means ± SEM of three independent experiments performed in triplicate.

the reduced SVCV-mediated induction of antiviral genes and enhanced SVCV replication by *foxo3b* might be *irf3*- and *irf7*-dependent.

Taken together, these data suggest that zebrafish *foxo3b* could indeed negatively regulate the antiviral response in vivo.

Discussion

As a transcription factor, FOXO3 is involved in multiple processes via regulation of gene expression (2, 47, 48). Recently, FOXO3 has been suggested to be a determinant of B and T cell fate and to control the magnitude of the T cell immune response (21, 22). Moreover, FOXO3 negatively regulates NF-κB activation (23), and FOXO3 is controlled by IKK-ε for regulating IFN-β expression (25). Intriguingly, FOXO3 has also been identified as a negative regulator of IRF7 transcription to participate in the antiviral response (26). Therefore, the function of FOXO3 in negatively regulating innate immunity response is well recognized. However, whether FOXO3 could affect TLR and RLR pathways through direct interaction with the components of these pathways is still largely unclear. In this study, we took advantage of a zebrafish in vivo model and demonstrated that *foxo3b*, an ortholog gene of mammalian FOXO3, negatively regulated antiviral responses via suppression of *irf3*/*irf7* transactivity. Further elucidation of whether mammalian FOXO3 has a similar functional capacity as zebrafish *foxo3b* will give insight into the physiological role of FOXO3 in mammalian antiviral response.

Of note, expression of *foxo3b* was induced by SVCV infection or poly((I:C) stimulation, which not only suggests that *foxo3b* might be involved in antiviral response, but it also implicates that *foxo3b* might be an ISG. Future studies that will determine whether the promoter of *foxo3b* contains ISRE and clarify whether *foxo3b* is indeed an ISG will help us fully understand the function of *foxo3b* in the antiviral response. Additionally, that *foxo3b* induced by antiviral response served to suppress the same response implicates that *foxo3b* might mediate a negative feedback loop to inhibit the antiviral response, resulting in enhancement of virus infection.

As a typical transcription factor, FOXO3, as well as its zebrafish homolog *foxo3b*, regulates the activity of other transcription factors through protein–protein interactions (34, 35). In the present study, we found that *foxo3b* suppressed *irf3*/*irf7* activity through interaction with *irf3*/*irf7*. However, the detailed mechanisms underlying this suppression are still unclear. To further define the process of *foxo3b* (FOXO3) functioning in its inhibitory role will open a new window for understanding the physiological role of FOXO3 in gene suppression.

Similar to mammals, fish IFNs, including zebrafish IFNs, also play important roles in the antiviral response, which activate expression of numerous IFN-stimulated genes to affect viral replication, assembly, and release (49). Through inducing the expression of *viperin*, zebrafish IFN participates in the antiviral response (50). Two typical IFN-inducible genes, *pkr* and *pkz*, can suppress replication of grass carp reovirus (51, 52). To date, zebrafish have been widely employed to investigate the function of genes involved in innate immunity (43, 53–55). In this study, we showed that infection with high titers of SVCV can result in body degeneration of zebrafish larvae and eventual death. Thus, the death rate of zebrafish larvae could be used to monitor viral infection and the antiviral response. Along with the improvement and maturity of gene-targeting technologies in zebrafish, the advantages of zebrafish as an in vivo model for innate immunity studies will receive more attention due to their convenience and ease in manipulation (55).

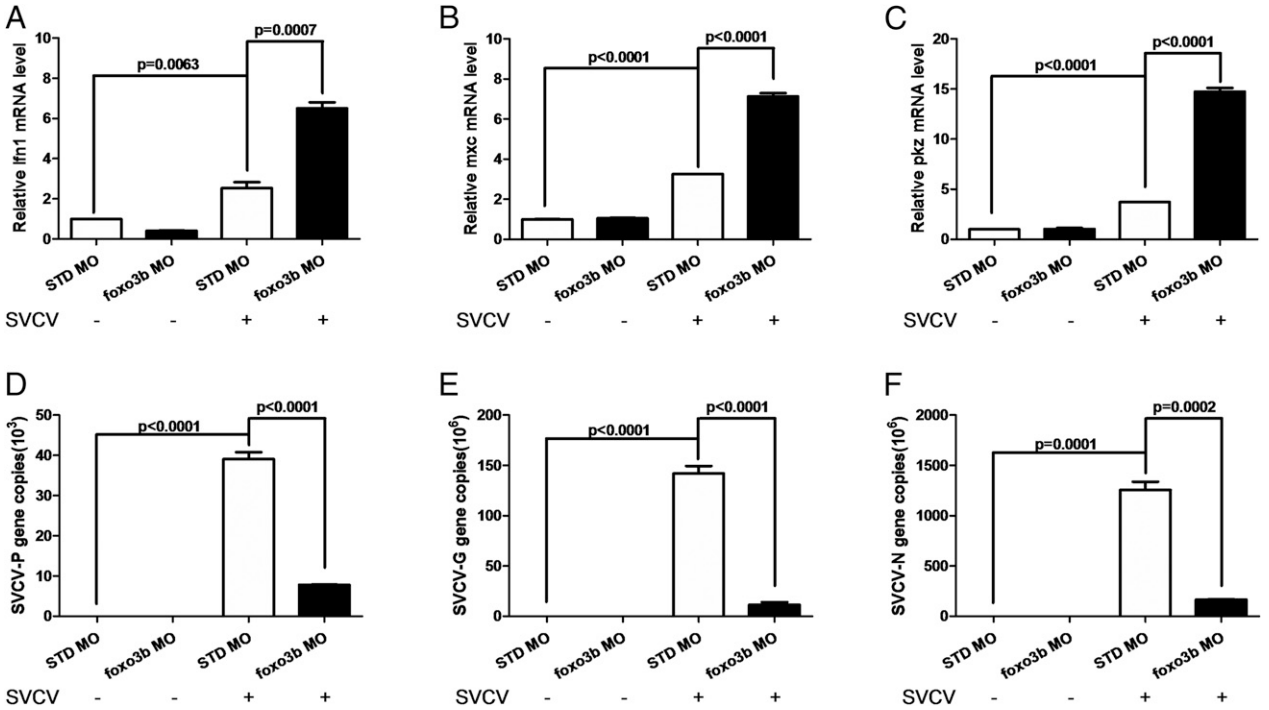

FIGURE 12. Knockdown of *foxo3b* in embryos by morpholino injection activates the induction of key antiviral genes by SVCV infection and suppresses virus replication in vivo. (**A**–**C**) Knockdown of *foxo3b* in embryos by morpholino injection activates the induction of key antiviral genes by SVCV infection in embryos. (**D**–**F**) Knockdown of *foxo3b* in embryos by morpholino injection decreased copy number of SVCV-related genes after SVCV infection in embryos. The STD-MO (8 ng/individual embryo) and foxo3b-MO (8 ng/individual embryo) were injected into one-cell embryos and STD-MO was used as a control. At 3 dpf, SVCV viruses (~2×10^8 $TCID_{50}$/ml) were added to the water containing zebrafish larvae. After incubation for 24 h, the expression levels of *ifn1* (A), *mxc* (B), and *pkz* (C) and P protein (D), G protein (E), and N protein (F) of SVCV were detected by semiquantitative RT-PCR analysis. Data are presented as means ± SEM of three independent experiments performed in triplicate.

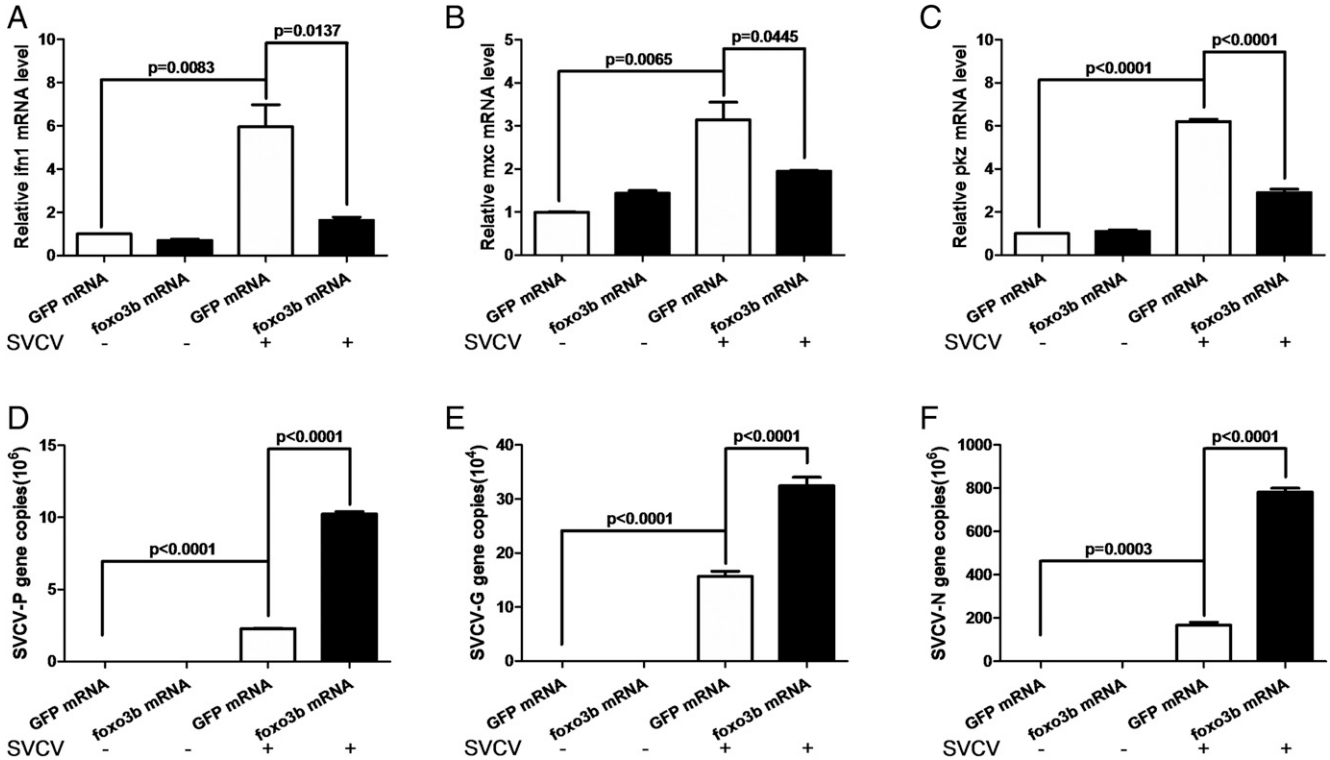

FIGURE 13. Overexpression of *foxo3b* suppresses the induction of key antiviral genes by SVCV infection and enhances virus replication in vivo. (**A**–**C**) Ectopic expression of *foxo3b* by mRNA injections suppressed the induction of key antiviral genes by SVCV infection in embryos. (**D**–**F**) Ectopic expression of *foxo3b* by mRNA injections increased copy number of SVCV-related genes after SVCV infection in embryos. mRNA encoding Myc-tagged *foxo3b* was injected into one-cell embryos and *GFP* mRNA was used as a control. At 3 dpf, SVCV viruses (~2×10^8 $TCID_{50}$/ml) were added to the water containing zebrafish larvae. After incubation for 24 h, the expression levels of *ifn1* (A), *mxc* (B), and *pkz* (C) and P protein (D), G protein (E), and N protein (F) of SVCV were detected by semiquantitative RT-PCR analysis. Data are presented as means ± SEM of three independent experiments performed in triplicate.

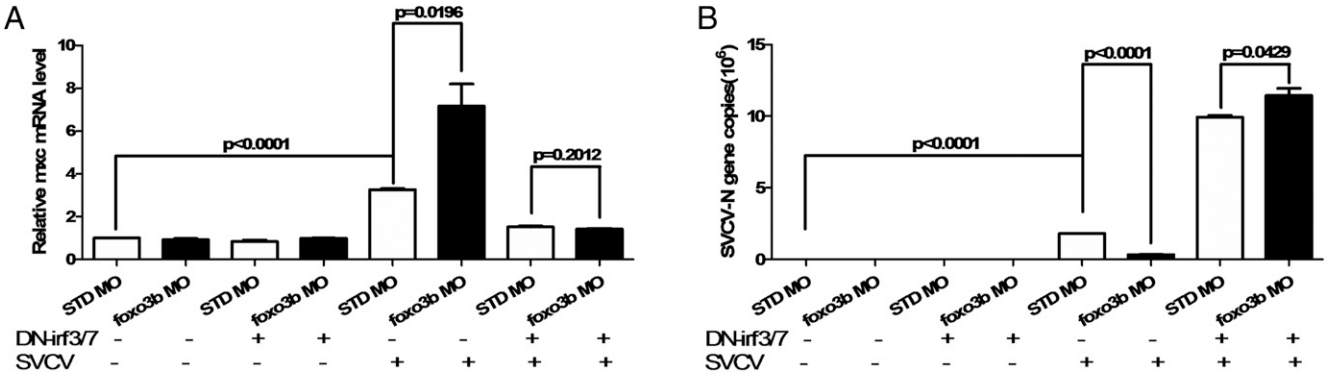

FIGURE 14. DN *irf3* and *irf7* (DN-*irf3*/*irf7*) suppress the effect of *foxo3b* knockdown in embryos upon SVCV infection. The STD-MO (8 ng/individual embryo) or foxo3b-MO (8 ng/individual embryo) combined with or without DN *irf3*/*irf7* was injected into one-cell embryos. At 3 dpf, SVCV viruses ($\sim 2 \times 10^8$ $TCID_{50}$/ml) were added to the water containing zebrafish larvae. After incubation for 24 h, the expression levels of *mxc* (**A**) and N protein of SVCV (**B**) were detected by semiquantitative RT-PCR analysis. Data are presented as means ± SEM of three independent experiments performed in triplicate.

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Disclosures

The authors have no financial conflicts of interest.

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