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# Zebrafish *mavs* Is Essential for Antiviral Innate Immunity

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# Zebrafish *mavs* Is Essential for Antiviral Innate Immunity

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**In mammals, the signaling adaptor mitochondrial antiviral signaling protein (MAVS) is a critical determinant in antiviral innate immunity. However, because of the lack of in vivo data, the physiological function of zebrafish *mavs* in response to viral infection is still not determined. In this study, we demonstrate that the long splicing isoform of zebrafish *mavs* promotes IFN regulatory factor 3 signaling and NF-κB signaling. Overexpression of this isoform of *mavs* enhances cellular antiviral responses. Disruption of *mavs* in zebrafish attenuates survival ratio on challenge with spring viremia of carp virus. Consistently, the antiviral-responsive genes and inflammatory genes are significantly reduced, and the replication of spring viremia of carp virus is increased in *mavs*-null zebrafish. Therefore, we provide in vivo evidence to support that zebrafish *mavs* is essential for antiviral innate immunity, similar to mammalian MAVS.** ***The Journal of Immunology*****, 2023, 210: 1314–1323.**

Innate immunity is the host's first line of defense against viral infection (1, 2). On viral infection, the retinoic acid–inducible gene 1 (RIG-I)-like receptors (RLRs), including RIG-I and melanoma differentiation-associated gene 5, mainly sense viral RNAs, resulting in their subsequent activation and leading to the recruitment of the mitochondrial antiviral signaling protein (MAVS) (also referred to as IPS-1, VISA, or Cardif) to activate the downstream protein kinases TANK-binding kinase 1 and IκB kinase-ε (3–12). These kinases phosphorylate the transcription factor IFN regulatory factor 3 (IRF3) and the inhibitor of NF-κB, leading to activation of IRF3 and NF-κB and eventual induction of type I IFNs, proinflammatory cytokines, and other downstream effector genes (3, 4). As an adaptor protein, MAVS serves as a key hub that links virus recognition to downstream innate antiviral immune responses (4, 13, 14). In mammals, the critical role of MAVS in antiviral innate immunity has been well defined in vitro and in vivo (2, 4, 13, 15–17).

In teleost fish, the RLR signaling pathway and the NF-κB signaling pathway have also been characterized, which play an important function against pathogen infection (18, 19). In fact, fish almost have all orthologous genes of the RLR signaling pathway (20). By different approaches, most of these genes have been identified to be involved in IFN response, similar to their mammalian orthologous genes (21–24). The first fish *mavs* gene is cloned from Atlantic salmon (*Salmo salar*), which induces IFN expression and protects against viral infection in culture cells (25). Lately, Crucian carp (*Carassius auratus*) *mavs* is revealed to be involved in the RLR pathway-mediated IFN response (26). As a model organism, zebrafish have been widely used for pathogen infection studies (27–29). Zebrafish *mavs* was found to play a crucial role in the induction of the innate immune response against RNA and DNA viruses. Subsequently, its different splicing isoforms have been shown to induce IFN responses similarly or oppositely (30, 31). Notably, these assays are mainly achieved by cell culture system (21, 30, 31). Because they are mainly based on in vitro assays, it is not surprising that the conclusions about the role of zebrafish *mavs* are sometimes controversial (21, 30, 31). To date, the physiological role of zebrafish *mavs* is still not thoroughly determined because of the lack of *mavs*-deficient zebrafish even though mavs knockdown experiments have been reported (32).

To further determine the function of zebrafish *mavs*, in this study, we compared the effect of two splicing isoforms of zebrafish *mavs*. Furthermore, we knocked out *mavs* in zebrafish by CRISPR/Cas9 and then compared the antiviral ability of *mavs*-deficient zebrafish with that of their wild-type (WT) siblings after being challenged with spring viremia of carp virus (SVCV). We conclude that zebrafish *mavs* is essential for antiviral innate immunity.

## Materials and Methods

### *Cells, zebrafish, and virus*

Epithelioma papulosum cyprini (EPC) cells (originally obtained from American Type Culture Collection [ATCC]; https://www.atcc.org) were cultured in M-199 medium (Biological Industries) supplemented with 10% FBS and maintained at 28°C in a humidified incubator containing 5% carbon dioxide ($CO_2$). Human embryonic kidney (HEK293T) cells (originally obtained from ATCC) were grown in DMEM (Biological Industries) supplemented with 10% FBS at 37°C in a humidified incubator containing 5% $CO_2$. Zebrafish liver (ZFL) cells (originally obtained from ATCC) were cultured in Ham's F-12 medium supplemented with 1.0 mM L-glutamine and 10% FBS (Biological Industries) and maintained at 28°C in a humidified incubator containing 5% carbon dioxide ($CO_2$).

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The online version of this article contains supplemental material.

Abbreviations used in this article: ATCC, American Type Culture Collection; CPE, cytopathic effect; dpf, days postfertilization; Dr, *Danio rerio*; EPC, epithelioma papulosum cyprini; HA, hemagglutinin; HMA, heteroduplex mobility assay; IRF3, IFN regulatory factor 3; ISRE, IFN-stimulated regulatory element; ISRE-Luc, IFN-stimulated regulatory element luciferase reporter construct; Luc, luciferase; MAVS, mitochondrial antiviral signaling protein; MOI, multiplicity of infection; mpf, months postfertilization; qPCR, quantitative real-time PCR; RIG-I, retinoic acid–inducible gene 1; RLR, retinoic acid–inducible gene 1–like receptor; SVCV, spring viremia of carp virus; $TCID_{50}$, median tissue culture-infective dose; TM, transmembrane; WT, wild-type; ZFL, zebrafish liver.

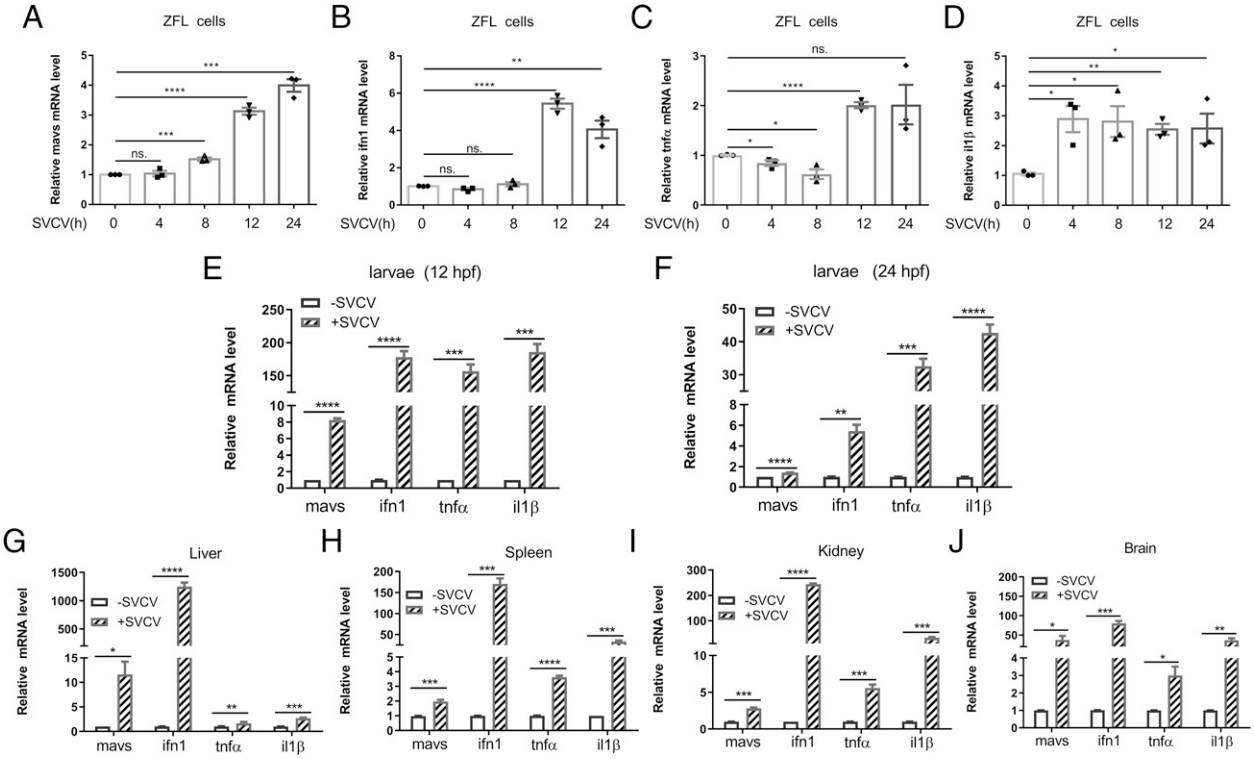


**FIGURE 1.** Zebrafish *mavs* is induced by SVCV infection. (**A**) Quantitative RT-PCR (qRT-PCR) assays for *mavs* expression in ZFL cells on SVCV infection. (**B**) qRT-PCR assays for *ifn1* expression in ZFL cells on SVCV infection. (**C**) qRT-PCR assays for *tnfα* expression in ZFL cells on SVCV infection. (**D**) qRT-PCR assays for *il1β* expression in ZFL cells on SVCV infection. (**E** and **F**) qRT-PCR assays for *mavs*, *ifn1*, *tnfα*, and *il1β* expression in zebrafish larvae (12 and 24 hpf, respectively) with or without SVCV infection. (**G–J**) qRT-PCR assays for *mavs, ifn1*, *tnfα*, and *il1β* expression in zebrafish (3 mpf) liver, spleen, kidney, and brain with or without SVCV infection. ZFL cells were infected with SVCV (~$2.0 \times 10^7$ $TCID_{50}$/ml) (A–D) for the indicated time points; zebrafish larvae were raised in water containing SVCV (~$2.0 \times 10^7$ $TCID_{50}$/ml) for 24 h; adult zebrafish (3 mpf) were injected with 10 μl medium containing SVCV (~$2.0 \times 10^7$ $TCID_{50}$/ml) and raised for 50 h; then total RNA was extracted for qRT-PCR assays. Data based on one representative experiment performed in three biological replicates from at least three independent experiments (mean ± SD) or representative data; the statistical analysis was performed using an unpaired *t* test. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$.

Zebrafish (AB strain) were maintained and raised in a recirculating water system following standard protocols. All experiments with zebrafish were approved by the Institutional Animal Care and Use Committee of the Institute of Hydrobiology, Chinese Academy of Sciences.

SVCV (an ssRNA virus that causes severe diseases affecting cyprinids) (Strain OMG067) was propagated in EPC cells. The culture medium containing SVCV was collected and stored at −80°C until use.

### *Plasmid construction*

The plasmids containing Dr (*Danio rerio*)-IFN-φ1-luciferase (Luc), Dr-IFN-φ3-Luc, and EPC-IFN-Luc in the pGL3-Basic vector (Promega, Madison, WI), as well as the IFN-stimulated regulatory element (ISRE) Luc reporter construct (ISRE-Luc) containing five ISRE motifs, have been previously described (33). The NF-κB-Luc reporter was provided by H.-B. Shu (Wuhan University, Wuhan, China). The zebrafish *tnfα* promoter Luc reporter and *il-1β* promoter Luc reporter were constructed by amplifying from zebrafish genomic DNA and cloned into pGL3-Basic vector. Different splicing isoforms of zebrafish *mavs*, including *mavs*-201 (Ensembl: ENSDART00000086281.6) and *mavs*-202 (Ensembl: ENSDART00000129045.3), were amplified by PCR from zebrafish cDNA and cloned into pCMV vector (Takara Bio). Zebrafish *mavs* mutants, including *mavs*-mutant 1 (M1), *mavs*-mutant 2 (M2), *mavs*-ΔCARD, *mavs*-ΔPRO, and *mavs*-ΔTM, were amplified by PCR from zebrafish cDNA and cloned into pCMV vector (Takara Bio). Zebrafish *mavs*-202 was also cloned into pCMV-hemagglutinin (HA) vector (Takara Bio). Primers are listed in Supplemental Table I.

### *Cytopathic effect assays*

EPC cells were transfected with 1 mg of HA-tagged mavs-202 or HA empty vector. After 24 h, cells were infected with SVCV for 48 h at the dose indicated. Then, the cells were fixed with 4% paraformaldehyde and stained with 1% crystal violet. Visible plaques were used to monitor viral infection. If the cytopathic effect (CPE) appeared later, the anti-SVCV ability in cells was stronger.

### *Virus titer determination*

EPC cells were cultured in 12-well plates overnight and transfected with HA-empty (2 mg) or HA-mavs-202 (2 mg), respectively. After 24 h, the cells were infected with SVCV (multiplicity of infection [MOI], 10) for 14 h, and the culture supernatant (containing virus) was collected.

The culture supernatant (containing virus) was diluted in serial dilutions ($10^0$ to $10^{-10}$) in sterile 1.5-ml tubes using M-199 medium. Subsequently, the diluted viruses were added into EPC cells seeded in 96-well plates. After 4 d, the plates were observed under a microscope, and the detached cells >50% in one well were counted as positive. The titers for SVCV infection were calculated using the Spearman–Kärber method and represented as median tissue culture-infective dose ($TCID_{50}$) per milliliter. The experiments were repeated three times for statistical analysis.

### *Generation of* mavs*-null zebrafish*

The CRISPR/Cas9 technique was used to disrupt zebrafish *mavs*. *Mavs* sgRNA was designed using an online design tool (http://crispr.mit.edu). The primers for amplifying *mavs* sgRNA template were as follows: 5′-GTAATACGACTCACTATAGGACATGTCAGGAGCTGCTTGTT-TTAGAGCTAGAAATAGC-3′ and 5′-AAAAGCACCGACTCGGTG CC-3′.

We initially detected the mutations using heteroduplex mobility assays (HMAs). If the HMA results were positive, the remaining embryos were raised up to adulthood as the F0 generation. The F0 generation was then backcrossed with WT zebrafish (strain AB) to generate the F1 generation, which was initially genotyped using HMAs. F1 genotypes were confirmed by sequencing targeting sites. Heterozygous F1s were backcrossed with the WTs (disallowing offspring–parent mating) to generate the F2 generation. F2 adults carrying the target mutation were intercrossed to generate F3 offspring. The F3 generation contained WT ($^{+/+}$), heterozygous ($^{+/-}$), and homozygous ($^{-/-}$) individuals.

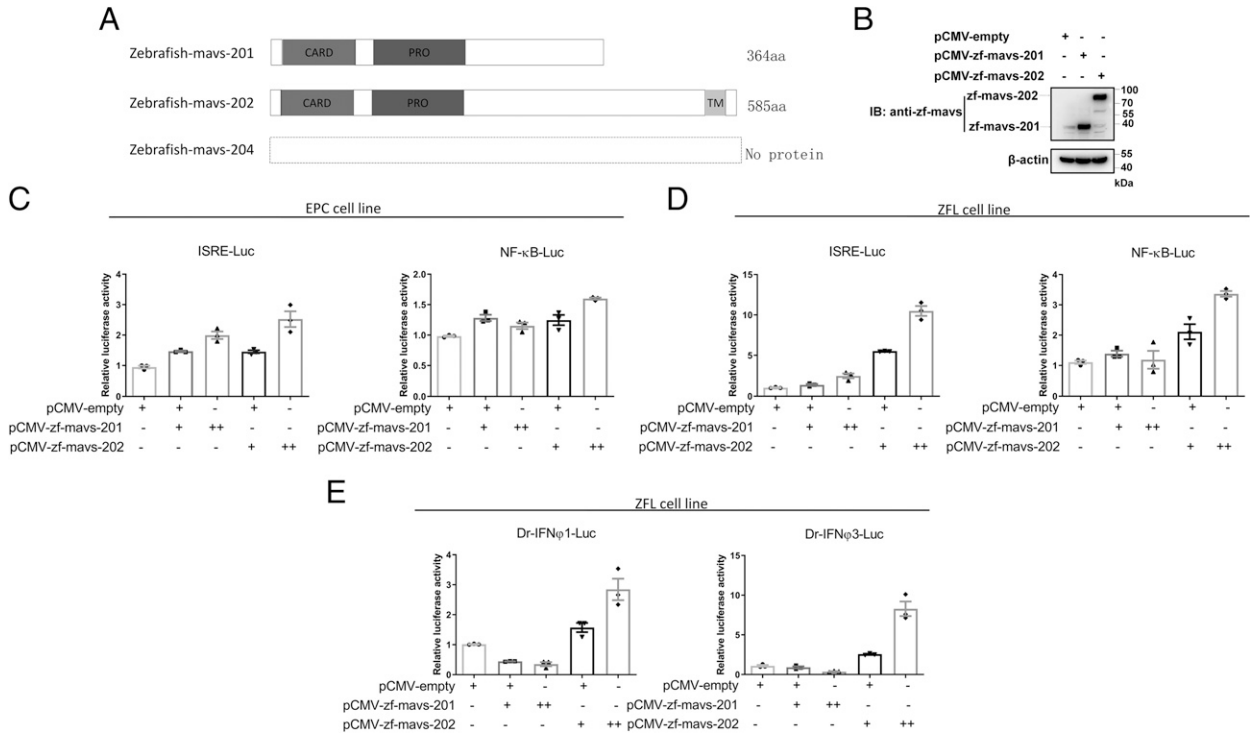


**FIGURE 2.** Comparison of the effect of zebrafish *mavs* splicing isoforms on the induction of IRF3 signaling and NF-κB signaling. (**A**) Schematic of zebrafish *mavs* splicing isoforms obtained from Ensemble (http://www.ensembl.org): *mavs*-201 (ENSDART00000086281.6), *mavs*-202 (ENSDART00000129045.3), and *mavs*-204 (ENSDART00000135100.3). (**B**) Validation of overexpressed *mavs*-201 and *mavs*-202 by immunoblotting. (**C**) Luc activity of ISRE reporter or NF-κB reporter in EPC cells with overexpression of empty vector control (pCMV-empty) or two *mavs* transcript isoforms. (**D**) Luc activity of ISRE reporter or NF-κB reporter in ZFL cells with overexpression of empty vector control (pCMV-empty) or two *mavs* transcript isoforms. (**E**) Luc activity of Dr-IFN-φ1-Luc reporter or Dr-IFN-φ3-Luc reporter in ZFL cells with overexpression of empty vector control (pCMV-empty) or two *mavs* transcript isoforms. Each experiment was performed in triplicate.

Two *mavs* mutants were obtained: mavs$^{ihboygms1}$ (https://zfin.org/ZDB-ALT-220120-4) (M1) and mavs$^{ihboygms2}$ (https://zfin.org/ZDB-ALT-220120-5) (M2), both named following zebrafish nomenclature guidelines (http://www.wiki.zn.org/display/general/ZFIN+Zebrafish+Nomenclature+Guidelines).

### *Viral infection*

For viral infection of EPC cells, the cells were grown overnight and transfected with the indicated plasmids. After 24 h, the cells were infected with SVCV (~2.0 × $10^7$ $TCID_{50}$/ml) for the indicated times, and then the assays were conducted.

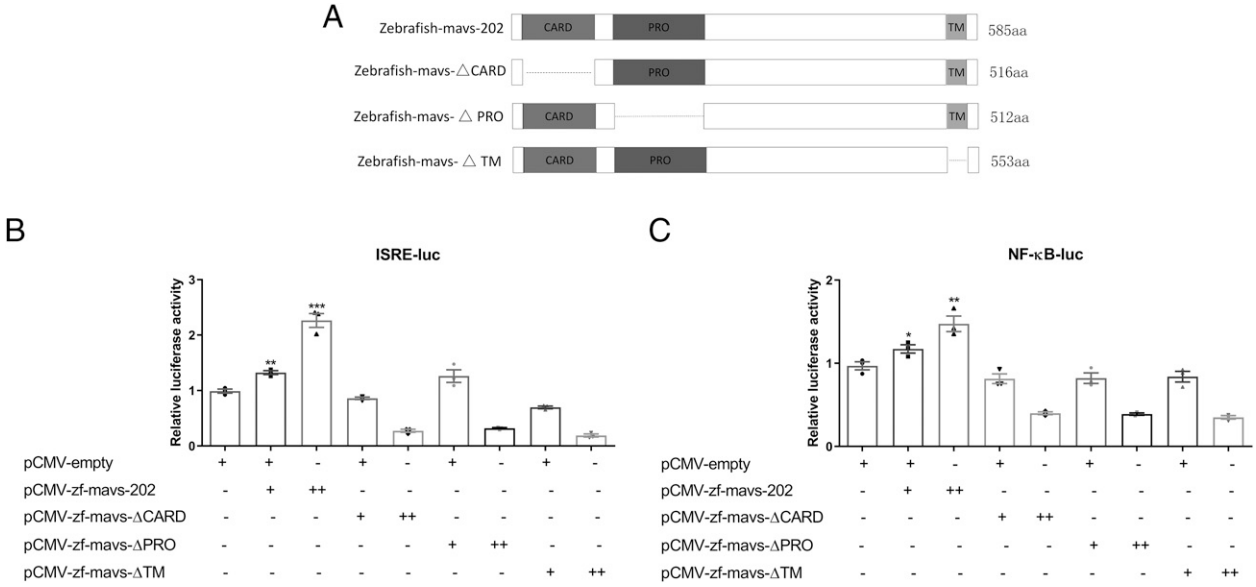


**FIGURE 3.** The effect of *mavs*-202 mutants on the induction of IRF3 signaling and NF-κB signaling. (**A**) Schematic of zebrafish *mavs*-202 mutants: *mavs*-△CARD (CARD domain–deleted mutant), *mavs*-△PRO (PRO domain–deleted mutant), and *mavs*-△TM (TM domain–deleted mutant). (**B** and **C**) Luc activity of ISRE reporter or NF-κB reporter in EPC cells with overexpression of empty vector control (pCMV-empty) or *mavs*-202 mutants. Data were based on one representative experiment performed in three biological replicates from at least three independent experiments (mean ± SD) or representative data; the statistical analysis was performed using an unpaired *t* test. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

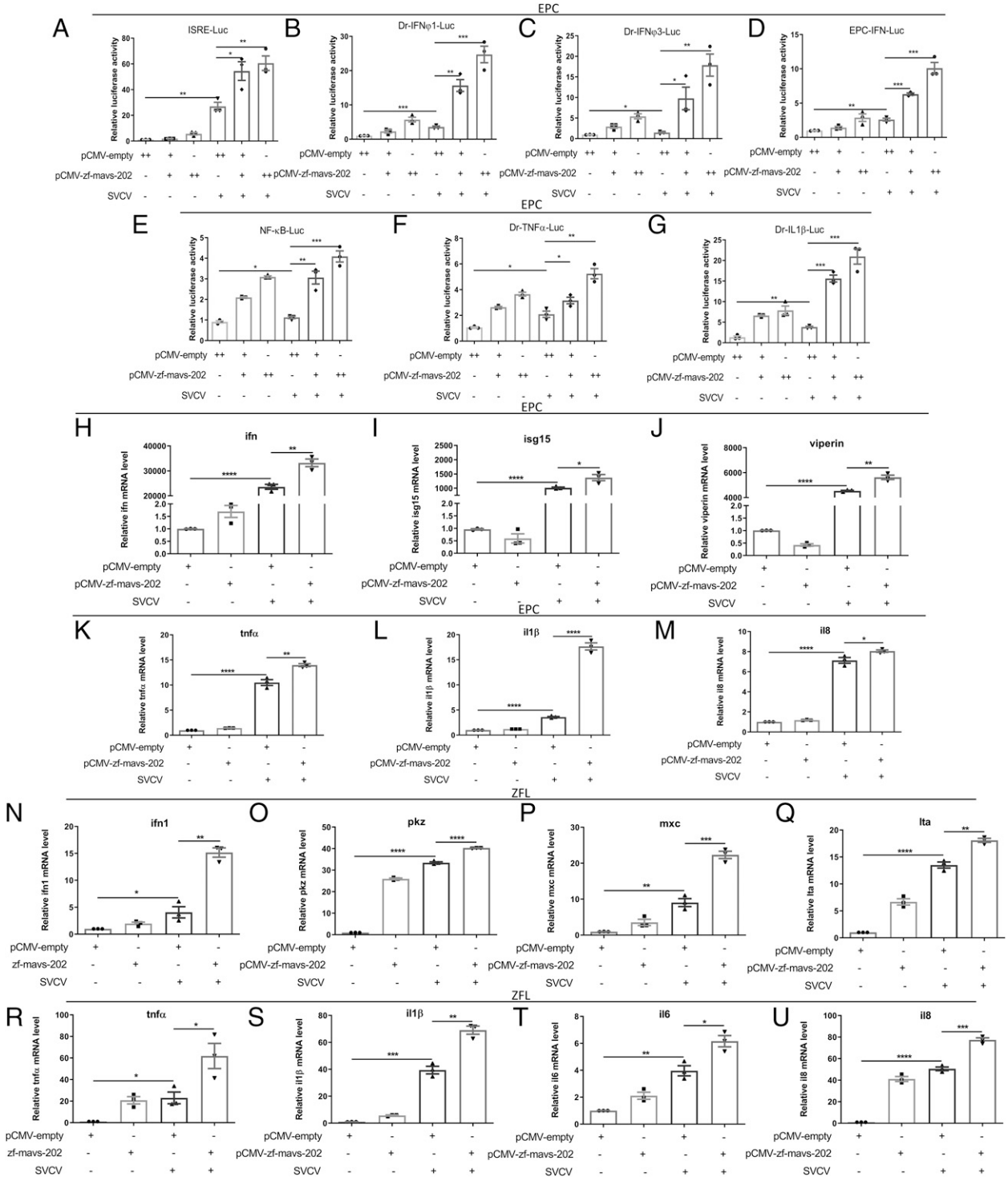


**FIGURE 4.** Zebrafish *mavs*-202 enhances SVCV-activated IRF3 signaling and NF-κB signaling. (**A**–**D**) Overexpression of *mavs*-202 increased ISRE (A), zebrafish IFN-φ1 (B), zebrafish IFN-φ3 (C), and EPC-IFN (D) reporter activity induced by SVCV infection in a dose-dependent manner. EPC cells were transfected with each Luc reporter (0.3 mg/well) together with pCMV-empty vector or an increasing amount of the pCMV-zf-mavs-202 vector (0.2 and 0.4 mg/well), respectively. After 12 h, the cells were infected with SVCV (~$2.0 \times 10^7$ $TCID_{50}$/ml) for 12 h, and then Luc reporter activity assays were performed. (**E**–**G**) Overexpression of *mavs*-202 increased NF-κB (E), zebrafish *tnfα* promoter (Dr-TNF-α-Luc) (F), and zebrafish *il-1β* promoter (Dr-IL-1β-Luc) (G) reporter activity in EPC cells induced by SVCV infection in a dose-dependent manner. EPC cells were transfected with each Luc reporter (0.2 mg/well) together with pCMV-empty vector or an increasing amount of the pCMV-zf-mavs-202 vector (0.2 and 0.4 mg/well), respectively. After 12 h, the cells were infected with SVCV (~$2.0 \times 10^7$ $TCID_{50}$/ml) for 12 h, and then Luc reporter activity assays were conducted. (**H**–**J**) Overexpression of *mavs*-202 increased expression of *ifn* (H), *isg15* (I), and *viperin* (J) induced by SVCV (~$2.0 \times 10^7$ $TCID_{50}$/ml) infection in EPC cells. (**K**–**M**) Overexpression of *mavs*-202 increased expression of *tnfα* (K), *il1β* (L), and *il8* (M) induced by SVCV (~$2.0 \times 10^8$ $TCID_{50}$/ml) infection in EPC cells. (**N**–**Q**) Overexpression of *mavs*-202 increased expression of *ifn1* (N), *pkz* (O), *mxc* (P), and *lta* (Q) induced by SVCV (~$2.0 \times 10^7$ $TCID_{50}$/ml) infection in ZFL cells. (**R**–**U**) Overexpression of *mavs*-202 increased expression of *tnfα* (R), *il1β* (S), *il6* (T), and *il8* (U) induced by SVCV (~$2.0 \times 10^7$ $TCID_{50}$/ml) infection in ZFL cells. Data were based on one representative experiment performed in three biological replicates from at least three independent experiments (mean ± SD) or representative data; the statistical analysis was performed using an unpaired *t* test. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$.

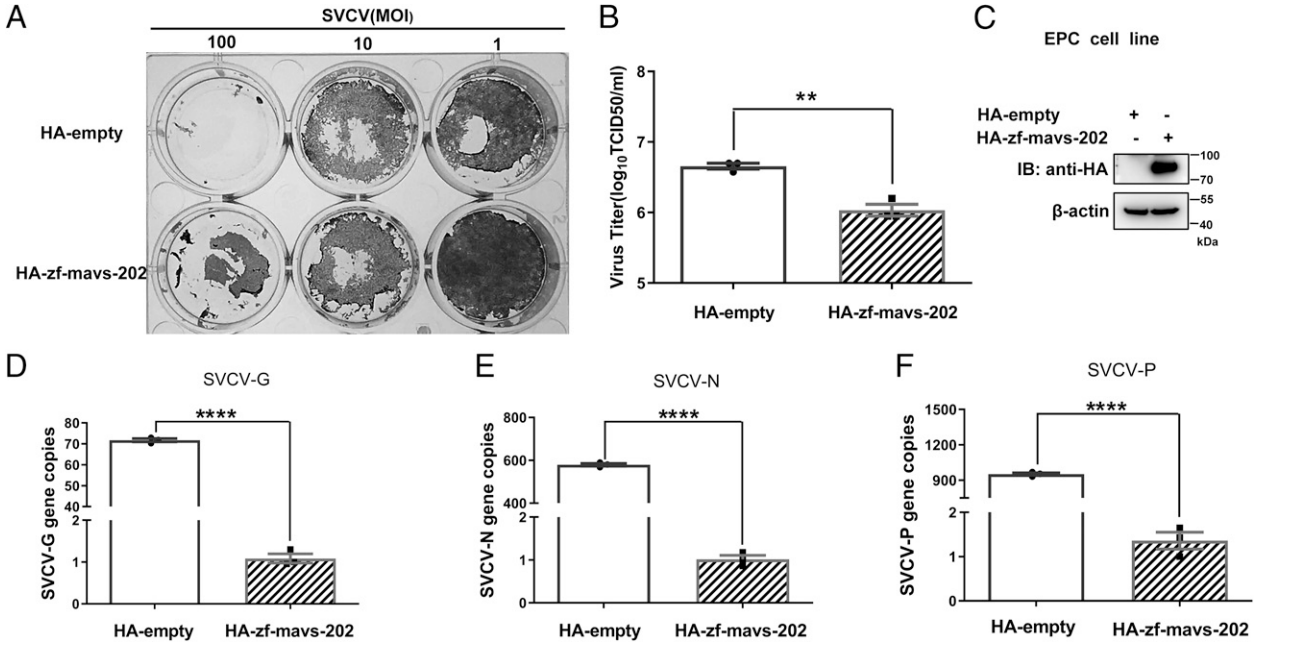


**FIGURE 5.** Overexpression of zebrafish *mavs*-202 enhances cellular antiviral responses. (**A**) Overexpression of *mavs*-202 increased cell survival after SVCV infection in EPC cells. EPC cells were seeded into 12-well plates, and then CPE assays were performed. (**B**) Overexpression of *mavs*-202 reduced virus titer after SVCV infection in EPC cells. Culture supernatant was collected from EPC cells infected with SVCV (MOI, 10), and the viral titer was determined by plaque assay. (**C**) Validation of overexpressed *mavs*-202 in EPC cells by immunoblotting. (**D**–**F**) Overexpression of *mavs*-202 reduced the copy number of SVCV-related genes in SVCV-infected EPC cells. EPC cells were transfected with HA-empty (2 mg) or HA-zf-*mavs*-202 (2 mg) vector. After 12 h, the cells were infected with SVCV (MOI, 10) for 12 h, and then total RNAs were extracted to examine the mRNA levels of the G protein (D), N protein (E), and P protein (F) of SVCV by qPCR analysis. Data were based on one representative experiment performed in three biological replicates from at least three independent experiments (mean ± SD) or representative data; the statistical analysis was performed using an unpaired *t* test. $^{**}p < 0.01$, $^{****}p < 0.0001$.

For viral infection of zebrafish larvae, zebrafish larvae (3 d postfertilization [dpf]; $n = 40$) were placed in a disposable 60-mm cell culture dish filled with 5 ml egg water and 2 ml SVCV (~$2.0 \times 10^7$ $TCID_{50}$/ml) culture medium. After incubation at 28°C for 24 h, the larvae were photographed. For gene expression assays, after the larvae were incubated for the indicated times, total RNAs were extracted from the larvae, and quantitative real-time PCR (qPCR) was performed. For survival ratio assays, zebrafish larvae were placed in a 96-well plate individually, and then 100 μl egg water containing SVCV (6 ml egg water plus 4 ml SVCV [~$2.0 \times 10^7$ $TCID_{50}$/ml] culture medium) was added into each well. The mortality was monitored every 2 h over a 24-h period.

For viral injection of adult zebrafish, zebrafish (3 mo postfertilization [3 mpf]) were i.p. injected with SVCV (~$2.0 \times 10^7$ $TCID_{50}$/ml) at 10 μl per individual. An i.p. injection with cell culture medium was used as the control.

### *Semiquantitative real-time PCR*

Equivalent amounts of total RNA (2000 ng) were used for cDNA synthesis with the One-Step gDNA Removal and cDNA Synthesis SuperMix (TransGen BioTech Co., Beijing, China) in a 20-μl reaction volume. The synthesized cDNA was used as a template for qRT-PCR analysis. qRT-PCR was performed using the CFX Connect Real-Time PCR System (Bio-Rad Laboratories) with MonAmp SYBR Green qPCR Mix (High Rox; Monad Biotech Co., Wuhan, China) under the following conditions: 95°C for 5 min, followed by 50 cycles at 95°C for 3 s and at 60°C for 15 s. The instrument's default dissolution curve acquisition program was used to draw the cycle threshold value (34). The changes of gene expression were calculated as the relative fold changes by the comparative cycle threshold method, and the β-actin of each species was used as an internal control gene for normalization. The results were obtained from three independent experiments, and each was performed in triplicate. The primers used are listed in Supplemental Table I.

### *Luciferase reporter assay*

We grew EPC cells in 24-well plates and transfected them with the indicated amounts of vectors, including *Renilla* as an internal control, by VigoFect (Vigorous Biotech, Beijing, China). Luc activity was assayed 24 h after transfection. The Luc activity in cell extracts was determined by a dual-Luc reporter assay system (Promega) according to the protocol supplied by the manufacturer. The relative light units were measured using a luminometer (Sirius; Zylux Corp., Oak Ridge, TN). Data were normalized to *Renilla* Luc. Data are reported as means ± SD of three repeated experiments.

### *Statistical analysis*

The statistical analysis was performed using an unpaired *t* test (where two groups of data were compared) or two-way ANOVA (where more than two groups of data were compared) in GraphPad Prism 7.0 (GraphPad Software). Data are based on one representative experiment performed in three biological replicates from at least three independent experiments (mean ± SD) or representative data. A $p$ value $<0.05$ was considered significant. Statistical significance is represented as follows: $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, and $^{****}p < 0.0001$.

## Results

### *Zebrafish* mavs *was induced by viral infection*

To validate whether zebrafish *mavs* is involved in antiviral responses, similar to mammalian *MAVS* (11, 15, 17), we initially examined whether zebrafish *mavs* was induced by viral infection. On SVCV infection in ZFL cells, *mavs* was steadily increased, similar to antiviral type I IFN gene *ifn1* and inflammatory genes *tnfα* and *il1β* (Fig. 1A–D). Furthermore, in zebrafish larvae, *mavs*, as well as *ifn1*, *tnfα*, and *il1β*, was induced by SVCV infection (Fig. 1E, 1F). In adult zebrafish, SVCV infection also enhanced expression of *mavs*, as well as *ifn1*, *tnfα*, and *il1β*, dramatically in liver, spleen, kidney, and brain (Fig. 1G–I). These data indicate that zebrafish *mavs* is induced by viral infection, which might also be involved in antiviral responses.

### *The long isoform of zebrafish mavs has the strong ability to induce antiviral responses*

After searching in the Ensemble database (http://ensemblgenomes.org), we found that zebrafish have six potential splicing isoforms of *mavs*, including *mavs*-201 (Ensembl: ENSDART00000086281.6),

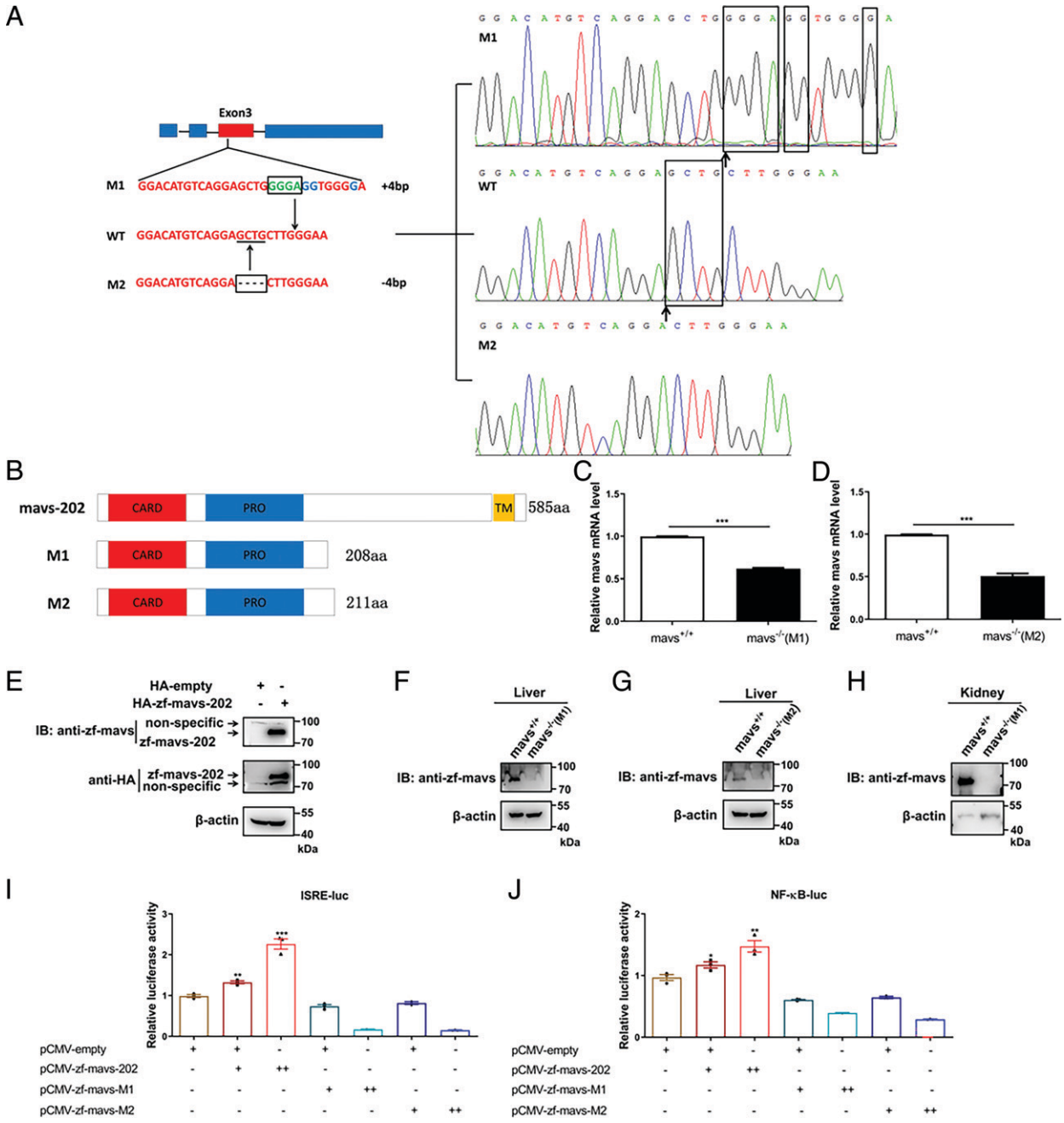


**FIGURE 6.** Generation of *mavs*-null zebrafish. (**A**) Schematic of targeting site in *mavs*. The different nucleotides between WT and M1 or M2 are labeled by green font (left panel) or surrounded by rectangle boxes (right panel); the arrows show where the insertion and deletion are located. (**B**) The predicted protein products of *mavs* in mutant 1 (M1) and mutant 2 (M2). (**C**) qRT-PCR assays for mavs in M1. (**D**) qRT-PCR assays for *mavs* in mutant 2 (M2). (**E**) Validation of the specificity of anti-zebrafish mavs antibody. (**F**) Detection of mavs in $mavs^{+/+}$ and $mavs^{-/-}$ (M1) livers. (**G**) Detection of mavs in $mavs^{+/+}$ and $mavs^{-/-}$ (M2) livers. (**H**) Detection of mavs in $mavs^{+/+}$ and $mavs^{-/-}$ (M1) kidneys. (**I** and **J**) Comparison of the effect of mavs-202, M1, and M2 in the activity induction of ISRE-Luc reporter and NF-κB-Luc reporter. Data were based on one representative experiment performed in three biological replicates from at least three independent experiments (mean ± SD) or representative data; the statistical analysis was performed using an unpaired *t* test. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

*mavs*-202 (Ensembl: ENSDART00000129045.3), *mavs*-203 (Ensembl: ENSDART00000134421.3), *mavs*-204 (Ensembl: ENSDART00000135100.3), *mavs*-205 (Ensembl: ENSDART00000142240.2), and *mavs*-206 (Ensembl: ENSDART00000193644.1). *mavs*-203 and *mavs*-205 contain only a partial sequence of *mavs*-202. *mavs*-204 cannot be translated into protein. *mavs*-202 and *mavs*-206 are identical, which encode the same protein with 585 aa (Fig. 2A, Supplemental Fig. 1A). The two predicted proteins of zebrafish mavs contain the CARD-like domain and Proline-rich domain, but the long peptide (mavs-202/mavs-206) contains the transmembrane (TM) domain (Supplemental Fig. 1A). In fact, mavs-202 is evolutionarily conserved among human, mouse, rat, and zebrafish, which contain all functional domains displayed in mammalian MAVS (Supplemental Fig. 1B). These data implicate that zebrafish mavs-202 might have a similar function as that of mammalian MAVS in antiviral innate immunity.

By promoter assays, we further found that zebrafish mavs-202 appeared to trend toward inducibility on the reporter promoter activity, including ISRE-Luc reporter, NF-κB-Luc reporter, Dr-IFN-φ1-Luc reporter, and Dr-IFN-φ3-Luc reporter (Fig. 2B–E).

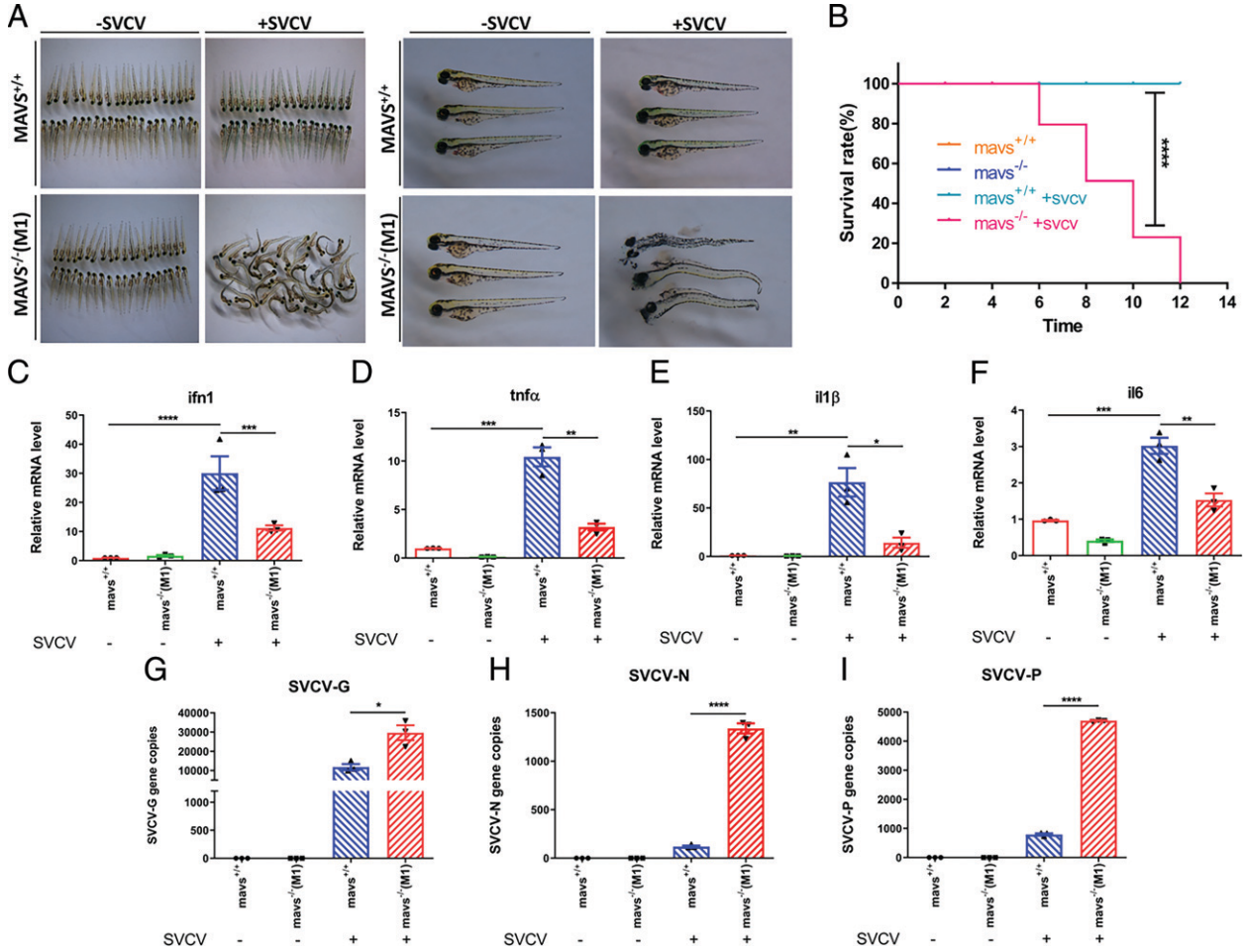


**FIGURE 7.** *Mavs*-null zebrafish larvae are more susceptible to SVCV infection. (**A**) *mavs*-null zebrafish ($mavs^{-/-}$) (3 dpf) were more susceptible to SVCV infection than the WT zebrafish ($mavs^{+/+}$) (3 dpf). The representative images were obtained when *mavs*-null zebrafish larvae and WT were infected with (+) or without (−) SVCV for 18 h. The dead larvae exhibited no movement, no blood circulation, and a degenerated body. (**B**) *mavs*-null zebrafish ($n = 40$ for each experiment; $n = 120$ in total) were more susceptible to SVCV infection than WT zebrafish ($n = 40$ for each experiment; $n = 120$ in total), based on the survival ratio. (**C–F**) The induction of key antiviral genes and key inflammatory genes, including *ifn1* (C), *tnfα* (D), *il1β* (E), and il6 (F), on SVCV infection was more significant in the WT larvae ($mavs^{+/+}$) than in *mavs*-null larvae ($mavs^{-/-}$). (**G–I**) The virus replication number indicated by the expression of the G (G), N (H), and P (I) genes of SVCV was lower in the WT larvae compared with the *mavs*-null infected with SVCV. Data were based on one representative experiment performed in three biological replicates from at least three independent experiments (mean ± SD) or representative data; the statistical analysis was performed using an unpaired *t* test (C–I) or two-way ANOVA (B). $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$.

These data suggest that zebrafish *mavs*-202 behaves similar to that of mammalian MAVS in antiviral innate immunity. In subsequent assays, we used *mavs*-202 to investigate the function of zebrafish *mavs*.

### *The long isoform of zebrafish mavs exhibits similar function as that of mammalian MAVS in antiviral innate immunity*

To further determine whether zebrafish mavs-202 behaves similarly to mammalian MAVS, we constructed three mutants of mavs-202 corresponding to human MAVS (11), including mavs-△CARD (in which the CARD domain is deleted), mavs-△PRO (in which the Proline-rich domain is deleted), and mavs-△TM (in which the TM domain is deleted) (Fig. 3A). Similar to human MAVS mutants (11), overexpression of these three mutants could not activate ISRE-Luc reporter and NF-κB-Luc reporter (Fig. 3B, 3C).

Subsequently, we examined the effect of zebrafish *mavs*-202 on virus-induced IRF3 signaling and NF-κB signaling by promoter assays. Overexpression of *mavs*-202 activated SVCV-induced all-promoter reporter activity significantly in a dose-dependent manner, including ISRE-Luc, Dr-IFN-φ1-Luc, Dr-IFN-φ3-Luc, EPC-IFN-Luc, NF-κB-Luc, Dr-TNF-α-Luc, and Dr-IL-1β-Luc (Fig. 4A–G). These promoters are widely used for monitoring IRF3 signaling and NF-κB signaling (33, 35). Next, we examined the effect of zebrafish *mavs*-202 on virus-induced expression of key antiviral-responsive genes and inflammatory-responsive genes. Overexpression of *mavs*-202 dramatically promoted SVCV-induced expression of *ifn*, *isg15*, *viperin*, *tnfα*, *il1β*, and *il8* in EPC cells and *ifn1*, *pkz*, *mxc*, *lta*, *tnfα*, *il1β*, *il6*, and *il8* in ZFL cells (Fig. 4H–U).

To determine the role of zebrafish *mavs* in cellular antiviral responses, we performed CPE assay. Overexpression of zebrafish *mavs*-202 significantly enhanced cell survival after SVCV infection (Fig. 5A). Consistently, the virus title was reduced in *mavs*-202–overexpressed EPC cells after SVCV infection (Fig. 5B). The expression of transfected HA-mavs-202 was confirmed by Western blot analysis (Fig. 5C). As expected, the reduction of SVCV replication rate indicated by expression of G, N, and P genes of SVCV was overt in *mavs*-202–overexpressed EPC cells compared with the control cells with HA-empty vector transfection (Fig. 5D–F).

Taken together, these data suggest that the long isoform of zebrafish *mavs*, mavs-202, functions similar to mammalian MAVS in activating IRF3 and NF-κB signaling (11).

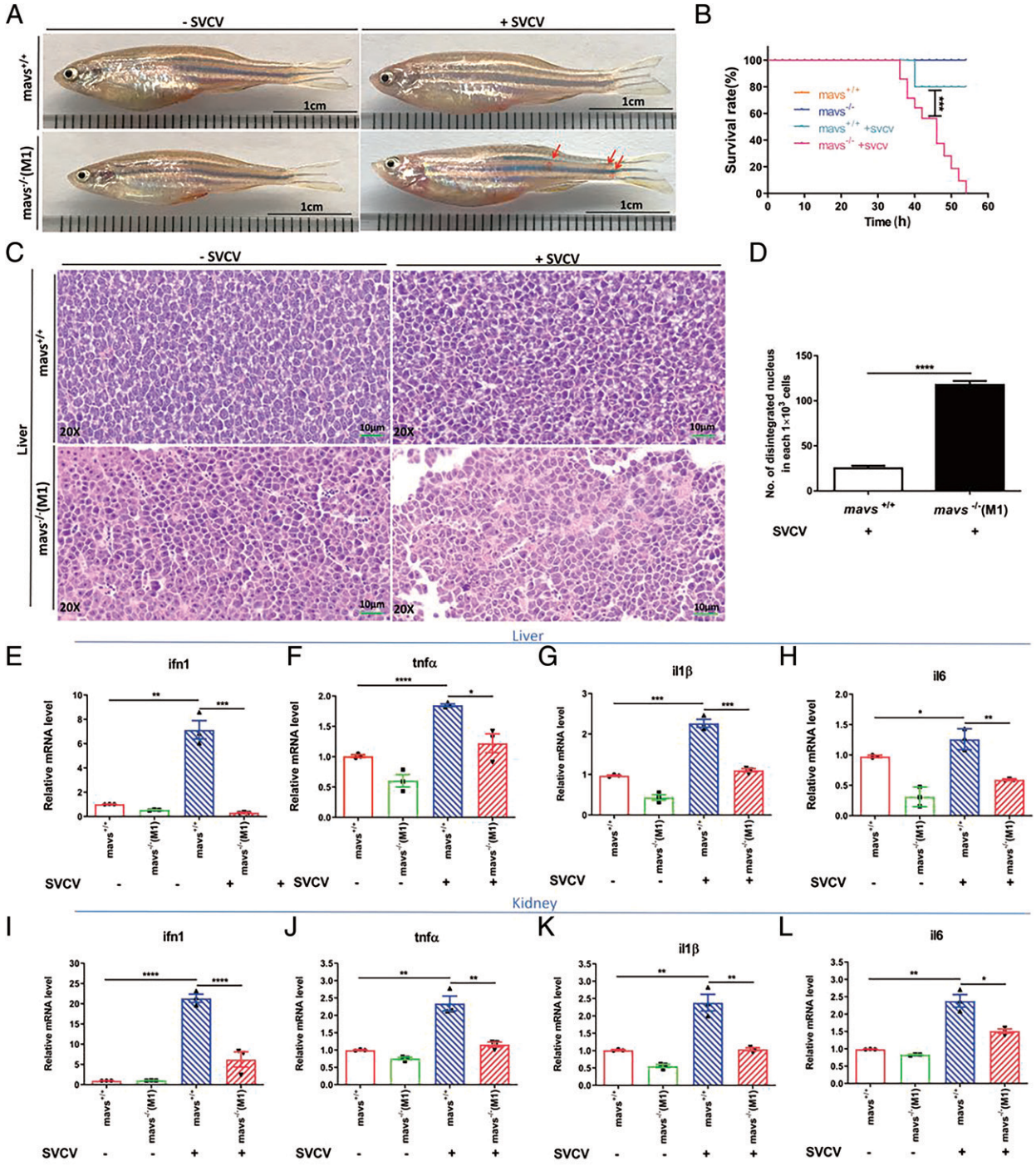


**FIGURE 8.** *Mavs*-null adult zebrafish are more susceptible to SVCV infection. (**A**) Representative images of *mavs*-null zebrafish (3 mpf) and their WT siblings (3 mpf) i.p. injected with or without SVCV at 10 μl per individual for 50 h. Red arrows indicate hemorrhage. (**B**) *mavs*-null zebrafish (3 mpf) ($n = 12$) were more resistant to SVCV infection compared with the WT zebrafish (3 mpf) ($n = 12$) based on the survival ratio. (**C**) H&E-stained liver sections from *mavs*-null zebrafish and the WT zebrafish after i.p. injection with 10 μl cell culture medium or 10 μl SVCV (~2–3 × $10^7$ $TCID_{50}$/ml). Compared with the WT zebrafish, *mavs*-null zebrafish displayed more nuclei in liver after SVCV infection. (**D**) Quantification of disintegrated nuclei in each of the 1000 cells of liver sections from WT or *mavs*-null zebrafish. (**E**–**H**) qRT-PCR assays for *ifn1* (E), *tnfα* (F), *il1β* (G), and *il6* (H) in SVCV-infected livers of *mavs*-null or WT zebrafish. (**I**–**L**) qRT-PCR assays for *ifn1* (I), *tnfα* (J), *il1β* (K), and *il6* (L) in SVCV-infected kidneys of *mavs*-null or WT zebrafish. Data were based on one representative experiment performed in three biological replicates from at least three independent experiments (mean ± SD) or representative data; the statistical analysis was performed using an unpaired *t* test (D–L) or two-way ANOVA (B). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.

### *Disruption of* mavs *in zebrafish abolishes the activation of IRF3 and NF-κB by virus*

To determine the function of *mavs* in vivo, we disrupted *mavs* in zebrafish via CRISPR/Cas9 and obtained two mutant lines (Fig. 6A). By crossing $mavs^{+/-}$ (♀) × $mavs^{+/-}$ (♂), the offspring with $mavs^{+/+}$, $mavs^{+/-}$, and $mavs^{-/-}$ genetic backgrounds were born at a Mendelian ratio (1:2:1), and no obvious defects in growth rate and reproduction capability were detected in $mavs^{-/-}$ zebrafish under normal conditions. Notably, the death rate of $mavs^{-/-}$ zebrafish was much higher than other zebrafish with a different genetic background raised in the

same circulated water system. By prediction, two short peptides might be translated in the two mutant lines (Fig. 6B). qPCR assays indicated that *mavs* was disrupted efficiently in the two mutant lines (M1 and M2) (Fig. 6C, 6D). We developed an Ab against zebrafish mavs-202. Its specificity was validated by Western blot analysis at first (Fig. 6E). Immunoblotting further confirmed that *mavs* was disrupted efficiently in the two mutant lines (M1 and M2) (Fig. 6F–H). Promoter assays showed that these two predicted peptides lost their ability on the induction of IRF3 and NF-κB signaling (Fig. 6I, 6J). These data suggest that the function of *mavs* is completely disrupted in the two mutant lines.

Subsequently, we challenged $mavs^{-/-}$ larvae (3 dpf) and $mavs^{+/+}$ larvae (3 dpf) (having WT allele of *mavs*) (WT) with SVCV and photographed the larvae after 18 h (Fig. 7A). The dead larvae exhibited no movement, no blood circulation, and a degenerated body (Fig. 7A). The survival rate of WT larvae was higher than that of $mavs^{-/-}$ larvae after SVCV infection (Fig. 7B). Consistently, expression of the key antiviral genes and inflammatory genes, including *ifn1*, *tnfα*, *il1β*, and *il6*, was lower in $mavs^{-/-}$ larvae compared with $mavs^{+/+}$ larvae (Fig. 7C–F). In agreement, the replication rate of SVCV indicated by expression of G, N, and P genes of SVCV was higher in $mavs^{-/-}$ larvae compared with those in $mavs^{+/+}$ larvae (Fig. 7G–I). Similar results were obtained in the mutant line 2 (Supplemental Fig. 2A–M).

Next, we i.p. injected SVCV, as well as using cell culture medium as control, into $mavs^{+/+}$ and $mavs^{-/-}$ adult zebrafish (3 mpf, M1) and then observed their phenotype. Compared with SVCV-injected $mavs^{+/+}$ adult zebrafish, SVCV-injected $mavs^{-/-}$ adult zebrafish exhibited much more swelling and hemorrhagic symptoms in the abdomen (Fig. 8A). At different time points after SVCV injection, we counted dead zebrafish and made a survival curve. As shown in Fig. 8B, after challenge with SVCV, $mavs^{-/-}$ zebrafish displayed lower survival rate compared with $mavs^{+/+}$ zebrafish. Histopathological assays showed that $mavs^{-/-}$ zebrafish displayed more nuclei in the liver compared with the WT zebrafish after SVCV infection (Fig. 8C, 8D). Then, we examined gene expression in different tissues of these infected zebrafish, including liver, kidney, and brain. The key antiviral genes, including *ifn1*, *pkz*, *mxc*, and *lta*, and inflammatory genes, including *tnfα*, *il1β*, *il6*, and *il8*, were significantly reduced in $mavs^{-/-}$ zebrafish compared with those of $mavs^{+/+}$ zebrafish (Fig. 8E–L, Supplemental Fig. 3A–H).

Taken together, these data suggest that zebrafish *mavs* is essential for the activation of IRF3 and NF-κB in response to viral infection.

## Discussion

As a model organism, zebrafish has been widely used for developing human disease models, including infection disease models (28, 36). Even though the innate immunity system is evolutionarily conserved between mammals and zebrafish (37), divergent functions or even complete opposite functions in some genes have also been revealed between mammals and zebrafish (35, 38, 39). Well characterizing the in vivo function of zebrafish *mavs* will benefit the development of infection disease models using zebrafish. Notably, different from mammalian MAVS, zebrafish *mavs* has multiple splicing isoforms. Due to the lack of clear in vivo data, some confusion about the role of zebrafish *mavs* in innate immunity exists so far (30, 31). In this study, we not only thoroughly compare the effect of two *mavs* splicing isoforms in the induction of IRF3 signaling and NF-κB signaling but also provide in vivo data to show that zebrafish *mavs* is essential for antiviral innate immunity. These data reveal that zebrafish *mavs* functions similar to mammalian MAVS, supporting that zebrafish can be used as a model to investigate the diseases related to antiviral innate immunity.

Notably, it has been reported that WT zebrafish larvae do not mount a proper IFN response when infected with SVCV (40). However, we and others have provided multiple evidence to show that zebrafish larvae do have a proper IFN response when challenged with SVCV (33–35, 41). This discrepancy might result from different strains of SVCV used in the studies for zebrafish larvae infection (42). In addition, the inducibility of zebrafish mavs expression by viral infection is inconsistent with previous reports, which might be because of different viruses used in the studies or other unknown reasons (43, 44).

Given that aggregation, phosphorylation, and ubiquitination are not only successively connected events during the process of MAVS activation but also prerequired for MAVS activation (4, 10, 15, 16), zebrafish *mavs* might also behave similarly to mammalian MAVS, even though we have no data to show these aspects. Further determining the structural changes and modification of zebrafish mavs in response to viral infection will give us a full picture of the function and the underlying mechanisms of *mavs* in antiviral innate immunity.

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## Disclosures

The authors have no financial conflicts of interest.

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