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A DN-*mda5* Transgenic Zebrafish 1 Model Demonstrates that Mda5 Plays an Important Role in Snakehead Rhabdovirus Resistance

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Abstract

Melanoma Differentiation-Associated protein 5 (MDA5) is a member of the retinoic acid-inducible gene I (RIG-I)-like receptor (RLR) family, which is a cytosolic pattern recognition receptor that detects viral nucleic acids. Here we show an Mda5-dependent response to rhabdovirus infection *in vivo* using a dominant-negative *mda5* transgenic zebrafish. Dominant-negative *mda5* zebrafish embryos displayed an impaired antiviral immune response compared to wild-type counterparts that can be rescued by recombinant full-length Mda5. To our knowledge, we have generated the first dominant-negative *mda5* transgenic zebrafish and demonstrated a critical role for Mda5 in the antiviral response to rhabdovirus.

Keywords

Zebrafish; *Danio rerio*; *mda5*; rhabdovirus; Type I IFN; antiviral

1. Introduction

The innate immune system is the primary defense against infection and is pivotal in mediating an immediate response to pathogens and activating the adaptive immune response. Innate immunity involves germline-derived pattern recognition receptors (PRRs) to recognize pathogen-associated molecular patterns (PAMPs) (Medzhitov and Janeway, 2000). PRRs permit differentiation of self- and non-self, while retaining the capacity to respond immediately to infection. The retinoic acid-inducible gene I (RIG-I)-like receptors

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(RLRs) are a family of PRRs that sense viral PAMPs and include RIG-I, MDA5 (also known as IFIH1), and laboratory of genetics and physiology 2 (LGP2). RLRs are expressed in the cytoplasm, and can detect a broad range of viruses (Loo and Gale, 2011). The three RLRs exhibit two conserved DExD/H box helicase domains that are connected to the C-terminal regulatory domain (CTD) (Luo et al., 2011). The CTD mediates the interaction of double-stranded RNA (dsRNA) with RIG-I and MDA5 and affords recognition of genomic viral RNA or replication intermediates of target viruses (Baum et al., 2010; Hornung et al., 2006; Peisley et al., 2011). RIG-I and MDA5 possess two N-terminal caspase activation and recruitment domains (CARDs) that are responsible for downstream signal transduction (Kowalinski et al., 2011; Wu et al., 2013). Activation of RIG-I or MDA5 leads to an interaction with the adapter molecule mitochondrial antiviral signaling protein (MAVS; also known as Cardif, IPS-1 or VISA) and culminates in the production of the antiviral protein type I interferon (IFN) (Kawai et al., 2005; Xu et al., 2005). Although RIG-I and MDA5 have conserved functional domains and bind dsRNA, there are differences in the structural basis for target recognition at the CTD that account for the varying modes of non-self RNA sensing. RIG-I engages in high affinity interactions with blunt-ended 5'-triphosphate containing RNA duplexes (Lu et al., 2010; Vela et al., 2012; Wang et al., 2010). Antigen recognition by MDA5 is dependent on the length of accessible RNA duplexes in a range of approximately 1–7 kilobases (Kato et al., 2008; Peisley et al., 2011). These structural differences in dsRNA sensing lead to MDA5 recognition restriction to picornoviruses and some flaviviridae while RIG-I has a broader range of viral species including paramyxoviruses, flaviviruses, influenza viruses and rhabdoviruses (Allen et al., 2007; Gitlin et al., 2006; Kato et al., 2008; Kato et al., 2006; Li et al., 2009; Loo et al., 2008; Takahashi et al., 2008).

The zebrafish (*Danio rerio*) model has provided novel insights into pathogenesis (Allen and Neely, 2010; Briolat et al., 2014; Gabor et al., 2014; Ludwig et al., 2011; Meijer and Spaank, 2011; Phelan et al., 2005b; Ramakrishnan; Sullivan and Kim, 2008; Tobin and Ramakrishnan, 2008; Yakoub et al., 2014; Zou et al., 2014b). Recently, a zebrafish MDA5 homolog and splice variant were described as transcriptionally induced upon virus infection and capable of conferring viral resistance when overexpressed *in vitro* (Zou et al., 2014b). The splice variant *mda5b* enhanced *Carassius auratus* interferon promoter activity when co-expressed with full-length *mda5* or *ips-1* *in vitro* (Zou et al., 2014b). The study described herein utilizes zebrafish to further elucidate the antiviral properties of teleost Mda5 *in vivo* over the duration of virus infection. A dominant-negative *mda5* (DN-*mda5*) transgenic zebrafish was generated by removing the signaling CARD domains, while retaining viral RNA binding through the helicase domains. The DN-*mda5* transgene product appears to bind ligands, preventing endogenous Mda5 from mediating a response to snakehead rhabdovirus (SHRV). DN-*mda5* zebrafish were more susceptible to SHRV infection than wild-type, while overexpression of *mda5* conferred resistance to SHRV. The use of DN-*mda5* transgenic zebrafish provides the opportunity to further elucidate the role of RLR pathways in virus resistance.

2. Materials and Methods

2.1 Ethics Statement

Zebrafish used in this study were handled in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Maine (Protocol Number: A2008-06-03). IACUC approved guidelines for zebrafish care were followed using standard procedures (www.zfin.org).

2.2 Constructs

Full-length *mda5* (Accession XM_689032) was isolated using 30 days post fertilization (dpf) zebrafish cDNA libraries and subsequently cloned into pGEM-T Easy (Promega). Constructs encoding full-length *mda5* and CARD*mda5*, where the initial 200 codons were removed from the coding sequence, were subcloned into the expression vector Frm (Sullivan et al., 2009) using gene specific primers *mda5* KpnI fwd ACGACGGGTACCATGGATCCAAACATGAGCAG, CARD*mda5* KpnI fwd ACTACTGGTACCATGCCGTGCGAGGGGGACGA and *mda5* SpeI rev ACGACGACTAGTTCAGTTAGTGTCCATATCTT.

2.3 Generation of Tg(actb:mda5,myl7:EGFP) Zebrafish Line

The Tol2 kit constructs p5E-*bactin2*, p3E-polyA, pDestTol2CG2 (Kwan et al., 2007), and pME- CARD-*mda5* were used to assemble an expression vector by Gateway Recombination Cloning (Invitrogen). To create the middle entry clone pME- CARD-*mda5*, primers GGGGACAAGTTTGTACAAAAAGCAGGCTCACCATGCCGTGCGAGGGGGACGA GG GGAT and GGGGACCACTTTGTACAAGAAAGCTGGGTCCCTAGTTAGTGTCCATATCTTCAT were used to add attB1 and attB2 sites to the 5'- and 3'- ends of CARD-*mda5*, respectively, followed by recombination into pDONR221 using Gateway BP Clonase (Invitrogen). Entry constructs were combined in a MultiSite Gateway recombination reaction using LR Clonase II Plus (Invitrogen) to create the expression vector pDestTol2CG2;actb: CARD*mda5*-polyA,myl7:EGFP. Expression vector (25 ng/μl) combined with Tol2 mRNA (50 pg/embryo) (Kwan et al., 2007) was injected into one-cell-stage AB zebrafish embryos. Individual adult F₀ founders were outcrossed with AB zebrafish and F₁ progeny were screened for EGFP. Positive F₁ adults were intercrossed and embryos positive for EGFP were used.

2.4 Full-length *mda5* RNA injection

Full-length *mda5* was subcloned into pCS2⁺ (Turner and Weintraub, 1994) using primers ACGACGCTCGAGCACCATGGATCCAAACATGAGCAG and ACGACGTCTAGATCAGTTAGTGTCCATATCTTCAT and synthesized *in vitro* using mMESSAGE mMACHINE SP6 transcription according to the manufacturer's recommendations (Life Technologies, Calsbad, CA). One-cell DN-*mda5* zebrafish were

injected with 98.8 ng of full-length *mda5* mRNA or vehicle and subjected to downstream application.

2.5 Cell culture

EPC (*Epithelioma papulosum cyprini*) cells initially described as originating from common carp (*Cyprinus carpio*) epidermal herpes virus-induced hyperplastic lesions (Fijan, 1983) have subsequently been found to be contaminated with fathead minnow (*Pimephales promelas*) cells (Winton et al., 2010). EPC cells have a broad sensitivity for fish viruses and are commonly used for isolation, propagation, and diagnostic assays for fish viruses. EPC cells were maintained at 28°C, 4% CO₂ in Minimum Essential Medium (MEM) (GIBCO-Invitrogen, Carlsbad, CA) supplemented with 10% heat-inactivated fetal bovine serum (GIBCO-Invitrogen, Carlsbad, CA) and antibiotics. ZFL (zebrafish liver) cells were derived from normal adult zebrafish liver (Ghosh et al., 1994). ZFL cells display an epithelial morphology and it has been demonstrated that the cells exhibit properties in culture that are normally associated with liver cell function *in vivo* (Ghosh et al., 1994). ZFL cells were maintained at 28°C, 0% CO₂ in LDF culture medium (50% Leibovitz's L-15 Medium, 35% Dulbecco's modified Eagle's Medium, and 15% F-12 Medium) supplemented with heat inactivated fetal bovine serum.

2.6 Virus propagation and infection

Snakehead rhabdovirus (SHRV) was propagated in EPC cells as previously described (Phelan et al., 2005b). Briefly, 70–80% confluent EPC cells were infected at a multiplicity of infection (MOI) of 0.1 in MEM without serum for 1 h at 28°C, 4% CO₂ followed by addition of 4 volumes of MEM + 10% FBS. Twenty-four hours after infection, EPC cells were observed to exhibit 80–90% cytopathic effect (CPE). The supernatant was collected following centrifugation and filtered through a 0.22-μm filter to remove cellular debris and obtain virus at a titer of 3.16×10^7 50% tissue culture infectious doses (TCID₅₀)/ml. Wild-type, DN-*mda5* and DN-*mda5*-rescue zebrafish embryos were infected by static immersion 72 hours post fertilization (hpf) for 5 hours with 5×10^6 TCID₅₀/ml SHRV or maintained as uninfected controls. Twenty fish were collected 24–72 h post infection (hpi) for each treatment and homogenized in MEM with 50 μg/ml gentamycin. The homogenate was frozen at –80°C before the TCID₅₀ assay.

2.7 Viral burden and plaque assays

The TCID₅₀ is an endpoint dilution assay that enables the determination of the quantity of a virus suspension that is needed to produce a pathological change (observed as cytopathic effects, or CPE) in 50% of inoculated cells in culture. CPE (i.e. infected cells) was manually observed and recorded for each virus dilution. For our experiments, 80% confluent EPC cells were infected with previously frozen supernatants that were diluted serially in MEM. After a seven day incubation at 28°C, 4% CO₂, cells were monitored for cytopathic effects (CPE) and the TCID₅₀/ml of the virus was calculated according to the Reed-Muench formula (Reed, 1938).

Plaque assays were performed with 80–90% confluent ZFL cells in a 24-well plate 24 h following transfection of empty vector, *mda5* or left as untransfected control as described

below. Cell medium was removed and cells were infected with SHRV at an MOI of 0.01, 0.1 or 1.0 in MEM for 1 h. Virus media were aspirated from each well and replaced with 0.5% methylcellulose in LDF medium. Cells were incubated at 28°C, 0% CO₂ for three days and then stained with 1x crystal violet stain (1% crystal violet, 20% ethanol, 0.5% formalin and 0.675% NaCl) for 5 minutes. After crystal violet stain, each well was rinsed in distilled water to remove methylcellulose and excess crystal violet stain and allowed to dry.

2.8 Mortality

Wild-type and transgenic zebrafish were maintained according to the IACUC guidelines noted above. For the rescued subset of DN-*mda5* zebrafish, one-cell embryos were injected with full-length *mda5* mRNA. Three days post fertilization, wild-type, DN-*mda5*, and DN-*mda5* rescued zebrafish (n=100 embryos/each type) were infected by static immersion with 5x10⁶ TCID₅₀/ml SHRV or maintained as uninfected controls. Water was changed daily and mortality was monitored and recorded from 24–96 hpi.

2.9 Transfection and Reporter Assays

ZFL cells were grown to 80% confluency prior to transfection. Cells were washed, treated with trypsin, centrifuged at 90×g for 10 min and resuspended to a final concentration of 2×10⁷ cells/mL in SF buffer (Lonza). Using the 96-well transfection module (Lonza), a total of 500ng DNA was added to 4×10⁵ cells in 20μL and the cells were electroporated using the program EW-158. Cells were then resuspended in 180μL of LDF media in quadruplicate and allowed to recover for 24 h. For reporter assays ZFL cells were transiently transfected as described above with expression vector containing *mda5*, *CARDmda5*, or empty vector (250ng) along with 250ng of an IFNφ1 promoter-driven luciferase reporter (pGL3-IFN) (Sullivan et al., 2007) and the Renilla-expressing internal control pRL-CMV (6.25ng) (Promega). After cells were allowed to recover for 24 h, the medium was removed and cells were exposed to SHRV at MOI = 0.1 in MEM for 1 h followed by supplementation with LDF. Dual-luciferase assays (Promega) were performed in triplicate 24 h post infection.

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

Total RNA was extracted after WT, DN-*mda5* and DN-*mda5*-rescue fish were infected with SHRV by static immersion for 5 h. Virus samples were collected by homogenizing 10 fish from each treatment, in TRIzol reagent (Invitrogen, Carlsbad, CA) at 24, 48, and 72 hpi and subsequently storing the samples at –80°C. RNA was extracted according to the manufacturer's protocol. Reverse transcription reactions were performed as previously described (Phelan et al., 2005b) to synthesize cDNA. Quantitation of IFNφ1 was carried out using an I-cycler IQ Detection System (Bio-Rad Laboratories, Hercules, CA) with primers IFNφ1_5'qPCR TCTTAATACACGCAAAGATGAGAACT and IFNφ1_3'qPCR GTCAGGACTAAAACTTCAC and cycling parameters previously described (Levraud et al., 2007). Fluorescence measurements were made at each cycle during the annealing step and the copy number was determined based on a standard curve using the iCycler software. The value for each sample was normalized to the corresponding β-actin value to determine relative copy number as previously described (Phelan et al., 2005a). Fold inductions were

calculated by dividing the copy number in the virus-infected samples by the copy number in the uninfected samples at the same time point.

3. Results

3.1 Role of Zebrafish *mda5* in Response to SHRV

SHRV infection (Gabor et al., 2013; Phelan et al., 2005a) was used to determine the role of *mda5* in the antiviral response. Zebrafish liver cells (ZFL) overexpressing *mda5* were infected with SHRV at an MOI of 0.01, 0.1 and 1.0 for 24 h prior to methylcellulose overlay and observed for cytopathic effects (CPE) 5 days later. These cells exhibited reduced CPE, suggesting protection against infection (Fig. 1A). To determine if the reduction in CPE correlated with reduced viral load, supernatants were removed 24 h post infection (hpi) from untransfected, empty vector, and *mda5* treatments and used in viral burden assays (Phelan et al., 2005b). After seven days, CPE was evaluated and TCID₅₀/ml was calculated (Reed, 1938). Results corroborated observations in the plaque assay, as overexpressing *mda5* cells had a 2.5×10^{-6} -fold reduction in viral burden (Fig. 1B). Taken together, these results demonstrate that *mda5* overexpression increases resistance to SHRV.

3.2 Development of Tg(actb:*mda5*,myl7:EGFP) DN-*mda5* zebrafish

To analyze the specific antiviral function of Mda5 *in vivo* we sought to develop a dominant negative *mda5* transgenic zebrafish. The CARD domains of MDA5 facilitate the interaction with IPS-1 and downstream signal transduction (Kawai et al., 2005). Transgenic expression of CARD*mda5* is predicted to function as a dominant negative by binding available ligand and oligomerizing with endogenous Mda5, where the mixed oligomer fails to signal through IPS-1 by forming a constitutive repressor domain trans inhibitory complex (Saito et al., 2007). Utilizing the Tol2 kit (Kwan et al., 2007) and Gateway Recombination Cloning (Invitrogen) we created the expression vector pDestTol2CG2:actb: CARD*mda5*-polyA,myl7:EGFP (Figure 2C). The bicistronic cassette is flanked by Tol2 autonomous transposons (Kawakami et al., 2000) and contains the ubiquitously expressed CARD*mda5* transgene controlled by the β -actin promoter and an EGFP marker that becomes localized in the myocardium facilitating easy screening of the offspring using a fluorescent dissection microscope (Figure 2A, B).

3.3 DN-*mda5* Diminishes IFN ϕ 1 Response

IFN ϕ 1 was measured *in vitro* and *in vivo* to assess Mda5 function in the antiviral immune response. Using Nucleofector Technology, ZFL cells were nucleofected with an empty frn vector to serve as control, *mda5*, or DN-*mda5*, and IFN ϕ 1-promoter luciferase reporter (pGL3-IFN) and an internal control (pRL-CMV). Cells were allowed to recover for 24 hours prior to SHRV infection. Overexpression of *mda5* alone was capable of promoting IFN ϕ 1 luciferase activity while empty frn expression vector and DN-*mda5* expression exhibited minimal induction ($265,026 \pm 45,075$ relative luminescence units (RLU) compared to $37,373 \pm 1,346$ and $39,545 \pm 1,807$ for empty frn and DN-*mda5* expression respectively. $p < 0.05$). Upon SHRV infection IFN ϕ 1 luciferase activity significantly increases over uninfected treatment in the vector control and *mda5* overexpressing groups but not in the DN-*mda5* group. In SHRV infected-*mda5* overexpressing cells, IFN ϕ 1

luciferase activity is dramatically increased compared to vector control and DN-*mda5* infected groups ($1,052,592 \text{ RLU} \pm 198,362$ compared to $167,182 \text{ RLU} \pm 25,180$ and $76,615 \text{ RLU} \pm 12,237$ for empty frn and DN-*mda5* expression respectively. $p < 0.05$). (Fig. 3A). These results indicate that ZFL cells are able to respond to SHRV infection by inducing IFN ϕ 1 activity. Overexpression of *mda5* induces IFN ϕ 1 activity that is further enhanced during SHRV infection and is completely abrogated when DN-*mda5* is expressed.

To determine if a reduction in IFN ϕ 1 response would also be observed *in vivo* and if this reduction could be rescued, DN-*mda5* zebrafish were injected with full-length *mda5* RNA or vehicle and compared to wild-type zebrafish. Fish were infected with SHRV (1×10^6 TCID₅₀/ml) and collected for RNA isolation at 48 and 72 hpi. Total RNA was extracted from 10 fish/treatment and purified. Reverse transcription reactions were performed as described (Phelan et al., 2005b) and quantitative PCR was performed. Copy number was determined based on the standard curve method and normalized to β -actin. Results demonstrated a strong Mda5- dependent IFN ϕ 1 response (13.14-fold ± 1.94) to SHRV infection at 48 hpi, which was diminished in DN-*mda5* fish (3.48-fold ± 0.80), but could be rescued with *mda5* overexpression (9.86-fold ± 4.37). Similarly, at 72 hpi WT fish displayed a 25.41-fold ± 6.54 IFN ϕ 1 induction to SHRV infection while DN-*mda5* fish had a 3.69 ± 0.41 induction that could be rescued by *mda5* overexpression (36.45 ± 7.36) (Fig. 3B).

3.4 Mda5 is important for Survival from SHRV Infection *in vivo*

To address the importance of *Mda5* function on zebrafish survival during SHRV infection DN-*mda5* zebrafish were injected with full-length *mda5* RNA or vehicle and compared with wild-type controls. Each treatment group was left uninfected or infected with 1×10^6 TCID₅₀/ml SHRV and observed daily for mortality (Fig. 4A). Following SHRV infection, DN-*mda5* embryos showed increased mortality ($p < 0.0001$). Mortality in DN-*mda5* embryos injected with full length *mda5* RNA was reduced and comparable to controls ($p < 0.01$). For viral burden studies, 20 fish per treatment were sampled daily. Viral burden (Fig. 4B) was greatest in DN-*mda5* fish (6.05×10^7 , 1.35×10^7 , and 3.23×10^5 TCID₅₀/ml at 24, 48 and 72 hpi, respectively). Lower viral burden was observed in WT fish (3.34×10^6 , 7.48×10^5 , and 1.95×10^4 TCID₅₀/ml at 24, 48, and 72 hpi, respectively). Rescued DN-*mda5* fish had viral burden comparable to controls (5.86×10^6 , 6.51×10^5 , and 5.04×10^3 TCID₅₀/ml at 24, 48, and 72 hpi, respectively). Viral burden was at least 10-fold greater in DN-*mda5* fish. These data demonstrated that mortality (Fig. 4A) and viral burden (Fig. 4B) were greater in DN-*mda5* zebrafish and could be rescued. DN-*mda5* fish were not completely susceptible to SHRV infection, suggesting other viral sensing pathways such as *rig-i* or the anti-viral Toll-like receptors are likely to be involved in mediating SHRV resistance.

4. Discussion

Many zebrafish models for virus infection and immunity have been recently described (Gabor et al., 2014) and comprehensively reviewed (Levraud et al., 2014). These valuable models can be used to investigate the functions of genes in different pathogenic interactions, either through the use of morpholino knockdown in embryos, stable knockout lines, or transgenic lines, such as the dominant-negative model described herein. Targeted genetic

modifications using zinc finger nuclease (ZFN) (Meng et al., 2008), clustered regularly interspaced short palindromic repeats (CRISPR) (Hwang et al., 2013) or transcription activator-like effector nuclease (TALEN) technology (Huang et al., 2011) may also be used to assess gene function and the mechanisms of the host response to infection. The use of zebrafish to investigate host-pathogen interactions and mechanisms of resistance of targeted genes demonstrates the utility of this model system to elucidate critical antiviral pathways.

Dominant negative-*mda5* zebrafish displayed an increased susceptibility to SHRV challenge exhibiting greater viral burden, increased mortality and reduced antiviral gene expression. However, DN-*mda5* fish did not experience complete susceptibility to SHRV infection suggesting other pathways are likely involved. The existence of components of RLR signaling in teleosts has been demonstrated (Aoki et al., 2013; Biacchesi et al., 2009; Biacchesi et al., 2012; Chang et al., 2011; Nie et al., 2014; Rajendran et al., 2012; Seth et al., 2005; Su et al., 2010; Wan et al., 2013; Zou et al., 2014a; Zou et al., 2014b) and suggests that a zebrafish *rig-i* orthologue may also be involved in SHRV recognition and resistance. The development of this dominant negative *mda5* zebrafish model presents researchers with new opportunities to explore the role of antiviral signaling cascades that coordinate with or work in conjunction with RLR pathways during virus infection. Here we report the Mda5-specific response to the rhabdovirus SHRV. Interestingly, mammalian recognition of rhabdoviruses has been shown to occur through RIG-I and not MDA5 (Furr et al., 2008; Kato et al., 2005). Phylogenetic analysis of RIG-I and MDA5 across invertebrate and vertebrate species reveals distinct clusters for each gene and suggests that RIG-I evolved from a common invertebrate ancestor while MDA5 likely evolved later on from a gene duplication event (Zou et al., 2009). The zebrafish *rig-i* pathway during SHRV infection has not been investigated, however, given the evolutionary evidence one can hypothesize that *rig-i* would be involved in rhabdovirus resistance. With the use of the DN-*mda5* zebrafish model and exploiting the genetic tools available in zebrafish mentioned above, one would be able to ascertain the full extent of RLR involvement in SHRV resistance. Recently, diversifying selection in all functional domains of MDA5 and RIG-I were identified in mammals, providing evidence for the evolution of these viral recognition receptors to have non-redundant roles in virus recognition (Cagliani et al., 2014). The adaptations identified within the RLRs are suspected to be due to positive selection from host-virus interactions. Given the varying habitats and types of pathogens that afflict vertebrates, one hypothesis for the diversity of virus recognition seen between teleost and mammalian MDA5 is that the differences in host-virus interactions and host adaption lead to a diverse teleost Mda5 virus recognition repertoire. The use of the zebrafish model provides us with an evolutionarily more primitive system that can be related to mammalian systems and may provide clues to how the immune system has evolved to cope with changes to pathogens and in the environment.

5. Conclusion

This work demonstrates an influential role that an Mda5-dependent pathway plays in zebrafish immunity. The ability of Mda5 to respond to rhabdovirus suggests the capability to recognize a wide array of viruses early in evolution. Studies of the Mda5 signaling cascade in non-mammalian species may provide insight into both the evolution of viral immune

evasion strategies and vertebrate detection of pathogens. The development of a DN-*mda5* zebrafish provides new opportunities for exploration of the role of *mda5* and will help elucidate the antiviral signaling networks in lower vertebrates.

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Highlights

- Overexpression of *mda5* induced zebrafish IFN promoter activity and conferred protection against SHR V infection in vitro.
- A dominant negative *mda5* transgenic zebrafish was generated to assess the *in vivo* function of *mda5*
- DN-*mda5* zebrafish resistance to SHR V infection is significantly reduced and leads to higher viral burden and mortality.
- Recombinant *mda5* confers protection against SHR V infection in DN-*mda5* zebrafish.

Figure 1. Over-expression of *mda5* Protects Against Virus Infection

ZFL cells were nucleofected with empty vector or *mda5* or left as a no transfection control (NTC). 24 h post transfection (hpt), cells were exposed to the indicated amounts of SHRV and incubated for an additional 72 h. Supernatants were removed and viral burden was measured by TCID50 assay (B) (*, $p < 0.05$). The remaining cells were fixed and stained with crystal violet (A).

Figure 2. Development of Tg(actb:mda5,myl7:EGFP) DN-*mda5* zebrafish

Anesthetized three day post fertilization DN-*mda5* F₂ zebrafish were screened for the presence of the transgene EGFP marker and imaged with an Olympus IX-81 inverted epifluorescence microscope equipped with a Hamamatsu ORCA-ER charge-coupled-device (CCD) camera and IPLab software (BD Biosciences, Rockville, MD). The *myl7* promoter driven EGFP expression is restricted to the heart as seen at 4× (A) and 20× (B). (C) A schematic of the bicistronic expression vector with flanking *Tol2* transposable elements. Black arrows indicate direction of transcription.

Figure 3. Rescue of DN-*mda5* demonstrates critical role for *mda5* in IFN̐1 response both *in vitro* and *in vivo*

A) ZFL cells were transfected with empty vector (frm) or the expression vector containing full length *mda5* or the dominant negative CARD *mda5* (250ng) along with reporter construct pGL3-IFN (250ng) and the internal control pRL-CMV (6.25ng). Twenty four hpt cells were exposed to SHRV at MOI=0.1. After an additional 24 h cells were lysed and reporter activity was measured. Brackets indicate significant difference between uninfected and infected treatments for vector and *mda5* groups. Individual * over each treatment

indicates a significant difference between *mda5* and vector and DN-*mda5*. A two-tailed heteroscedastic T-test was performed to calculate significance (*, $p < 0.05$). B) DN-*mda5* zebrafish were injected at the 1-cell stage with *mda5* RNA or pCS2+ empty vector. Wild-type AB lines were kept as controls. Fish were infected with 1×10^6 TCID₅₀/ml SHR V and collected for RNA isolation at 48 and 72 hpi. RNA was purified and IFN ϕ 1 was subsequently measured by qPCR (*, $p < 0.05$).

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Figure 4. *mda5* is critical for immune response to SHR in vivo

A) Control, DN-*mda5*, and rescued DN-*mda5* zebrafish embryos were treated as uninfected (UI) or infected by static immersion 48 hpf with 1×10^6 TCID₅₀/ml SHR and monitored daily for mortality. Results are representative of three separate experiments. Statistical analysis (Wilcoxon test) of the Kaplan-Meier curve was performed (**, $p < 0.01$; ***, $p < 0.001$) where ** is the comparison between DN-*mda5* SHR versus DN-*mda5* rescue SHR while *** is the comparison between control SHR and DN-*mda5* SHR. B) Control, DN-*mda5*, and rescued DN-*mda5* zebrafish embryos were infected 48 hpf 1×10^6

TCID₅₀/ml virus and harvested for viral burden analysis. The graph indicates that at 24, 48, and 72 h post infection there is an increased viral burden in the DN-*mda5* embryos, while the control and rescued DN-*mda5* embryos had comparable viral burden. Figure is representative of three experiments; error bars are standard error of the mean (*, $p < 0.05$; **, $p < 0.01$).

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