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# Functional Role of Zebrafish TLR Proteins

Carol H. Kim

*Principal Investigator; University of Maine, Orono, carolkim@maine.edu*

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PI: Carol Kim

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Title: Functional Role of Zebrafish TLR Proteins

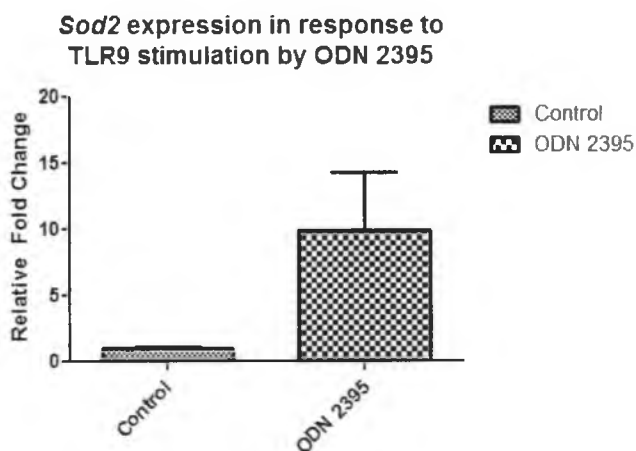
Influenza virus infections lead to significant illness, mortality, and social disruption worldwide. Herein, the first studies establishing the zebrafish as a model for human influenza infection are presented and it is shown that influenza infection proceeds and can be resolved through similar mechanisms in zebrafish and humans (Gabor, et al.). Our laboratory has previously characterized a fish rhabdovirus infection model in the zebrafish (Phelan, et al.).

Perhaps one of the most important pattern recognition receptors (PRR) is the Toll-like receptor (TLR) gene family. Our findings suggest that TLR9, a PRR for unmethylated CpG DNA, plays a protective role against influenza infection. Stimulation of the TLR pathway leads to downregulation of *tumor necrosis factor alpha inducible protein 8-like 1 (TNFAIP8L1)* and upregulation of *superoxide dismutase 2 (SOD2)*. A novel negative regulator role in antiviral immunity is proposed for the *speckle-type POZ protein (SPOP)*. Our studies demonstrate that the cytosolic sensor, *Ifih1*, is crucial for combating fish rhabdovirus infection.

Since a robust innate immune response potentiates a subsequent heightened adaptive immune response, zebrafish embryos provide an opportunity to study the initial infection events to which the innate immune system responds, independent of adaptive immunity. New targets for antivirals or adjuvant therapies could come from zebrafish studies of the host's innate immune response to influenza and fish rhabdovirus infection.

## 1. Discover novel zebrafish TLR signaling pathway components

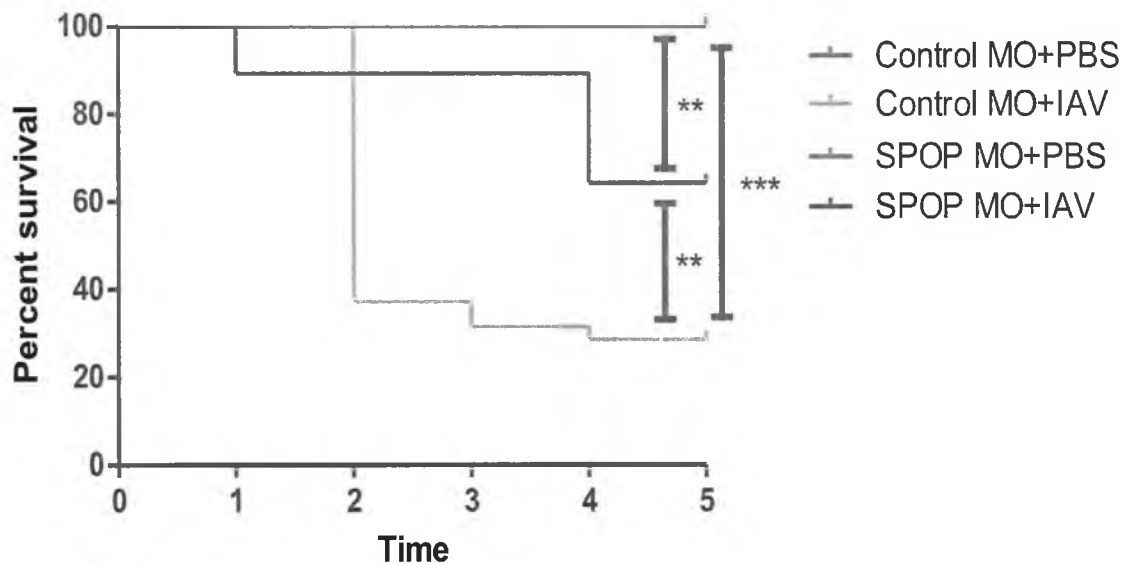
### a. Identify the zebrafish TLR receptors that recognize viral ligands



**Figure 1.** *Sod2* is induced in RAW264.7 cells upon exposure to a TLR9 agonist. RAW264.7 cells were exposed to a TLR9 agonist, and RNA was collected 6 hours post-exposure. Quantitative PCR was performed to assess the levels of *Sod2* transcript, and it was found that *Sod2* is upregulated in response to TLR9 activation, possibly indicating that *Sod2* is downstream of *TLR9* and could be playing a role in the antiviral state. Data was normalized using GAPDH. Error bars are represented by SEM.

SOD2 is a mitochondrial antioxidant that is responsible for the neutralization of mitochondrial reactive oxygen species. Previously, we have examined *sod2* in the context of bacterial infection, and found that zebrafish *sod2* morphants were more susceptible to *Pseudomonas aeruginosa* (Peterman et al, *Neutralization of mitochondrial superoxide by superoxide dismutase 2 promotes bacterial clearance and regulates phagocyte numbers in zebrafish*, Infection and Immunity, under review). While *Sod2* had been characterized in the adaptive immune response to influenza infection, no such characterization has been performed on *Sod2* in the innate immune system. Here, we begin this characterization *in vitro* using RAW264.7 macrophages. TLR9 stimulation resulted in increased levels of *Sod2*, possibly indicating a role for *Sod2* in TLR9-dependent response to virus infection. Further studies need to be performed *in vivo* to assess the role of *sod2* in response to a viral infection.

*b. Identify novel interactors of the zebrafish TLR pathways via a yeast two-hybrid screen*



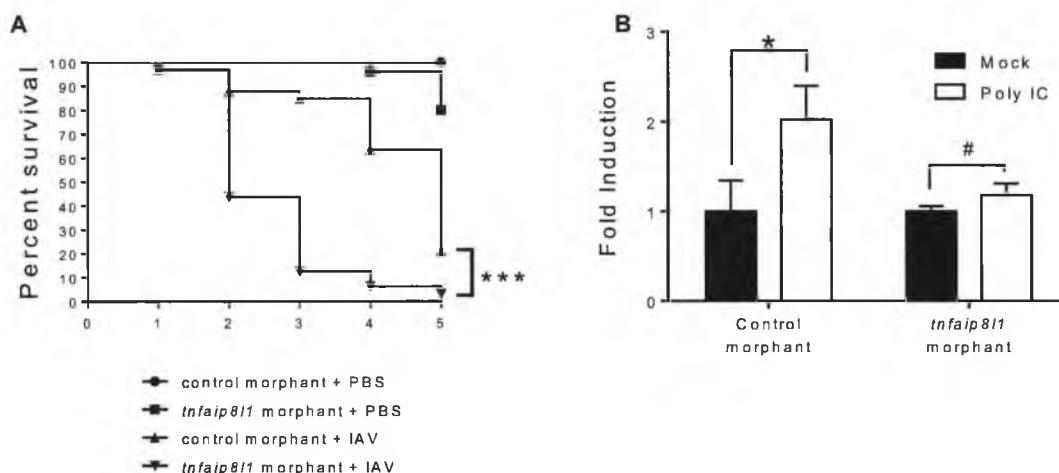
**Figure 2.** *Spop* may function as a negative regulator of the antiviral immune response. At the 1-2 cell stage, zebrafish were injected with SPOP or control morpholino (GeneTools, LLC, Philomath, OR). At 48 hpf, *Spop* morphants and control zebrafish were injected via the Duct of Cuvier with 2.0 nL influenza strain A/PR/8/34 (H1N1, EID50  $10^{10.7}$ ) (Charles River, North Franklin, CT). There was a decreased mortality in *Spop* morphants exposed to influenza when compared to controls. \*\* indicates a statistically significant difference with a p value of  $<0.0025$ . \*\*\* indicates a statistically significant difference with a p value of  $<0.0001$ .

Speckle-type POZ protein (SPOP) is a protein that has been characterized as an important regulator of certain cancers and cellular process, including transcriptional regulation and ubiquitination. But little research has been conducted to implicate a role for SPOP in antiviral immunity. Previous research conducted by Rual, et al. discovered a protein-protein interaction between SPOP and myeloid differentiation primary response gene 88 (MyD88). The Carol Kim laboratory has recently characterized human influenza viral infection in the zebrafish model (Gabor, et al.). Using a reverse genetics approach known as gene knockdown via morpholino oligonucleotides targeting the zebrafish *Spop* gene, we were able to examine the effects of SPOP deficiency on the host's ability to mount an immune response to influenza through mortality studies. Findings show that SPOP morphant zebrafish were less susceptible to influenza compared to control zebrafish, suggesting that SPOP may function as a negative regulator of the antiviral immune response.

*c. Determine if the interactions identified in the zebrafish also occur in the human TLR pathways*

The tumor necrosis factor alpha inducible protein 8 (*tnfaip8*) gene family is require for immune homeostasis. We recently submitted our findings to the *Journal of Immunology* describing a role for *tnfaip811* gene in antibacterial immunity and demonstrated that its expression is downregulated upon Toll-like receptor stimulation. We have begun *in vivo* studies aimed at understanding the role *tnfaip811* plays in the host response to viral infection. We have found that morpholino-mediated knockdown of *tnfaip811* expression disrupts the ability of zebrafish to mount anti-viral responses, as evidenced by the diminished survival of *tnfaip811* morphants relative to control morphants upon influenza infection (A). Zebrafish *tnfaip811* morphants also failed

to trigger transcription of *mx*a antiviral gene upon stimulation with the IFIH1 agonist poly IC (LyoVec) (Invivogen) (B).

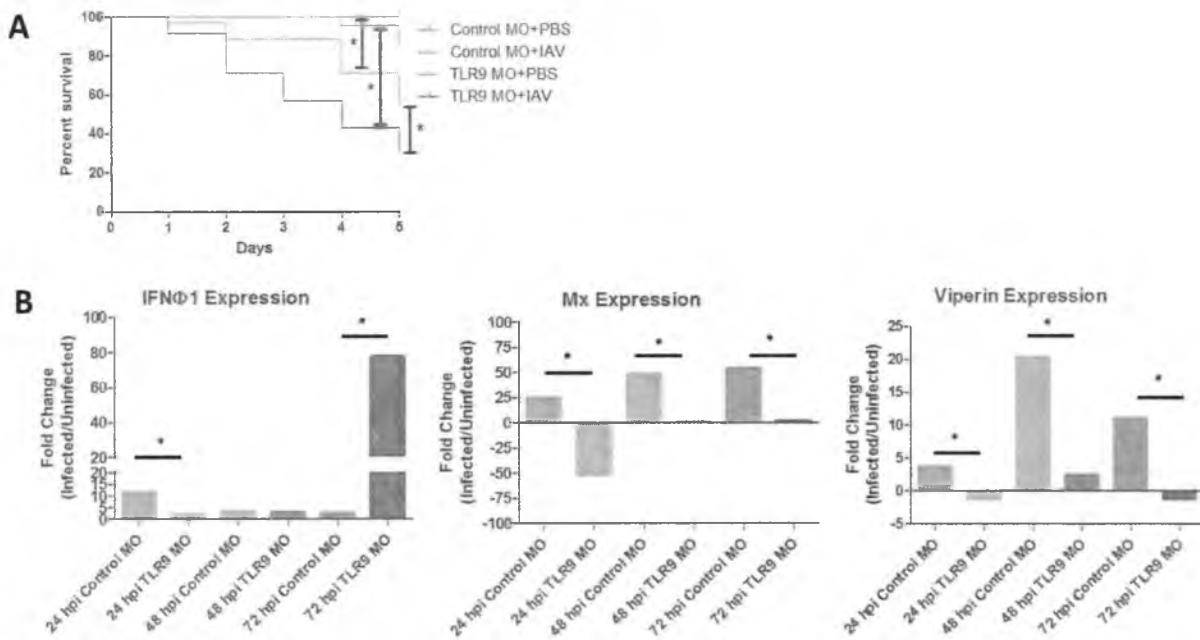


**Figure 3.** *tnfai811* is required for the antiviral immune response. At the 1-2 cell stage, zebrafish were injected with *tnfai811* or control morpholinos. At 48 hpf, *tnfai811* and control morphants were injected via the tail vein with 1.5 nL influenza strain A/PR/8/34 (H1N1, EID50  $10^{10.7}$ ) (Charles River, North Franklin, CT). (A) There was an increased mortality in *tnfai811* morphants exposed to influenza when compared to controls. \*\*\* indicates a statistically significant difference with a p value of <0.0001. (B) At 1 h post-infection, total RNA was collected from zebrafish and converted into first-strand cDNA. RT-qPCR analysis reveals that unlike the control morphant, there was no significant change in the expression of *mx*a transcripts upon exposure to 75 pg of poly I:C (LyoVec), a known agonist of IFIH1 signaling. \* indicates a statistically significant difference with a p value <0.05. # indicates no statistically significant difference.

## 2. Determine the role of the zebrafish antiviral pathway components in protecting the host from infection *in vivo*

### Role of Toll-Like Receptor Signaling During Influenza Virus Infection in the Zebrafish Model

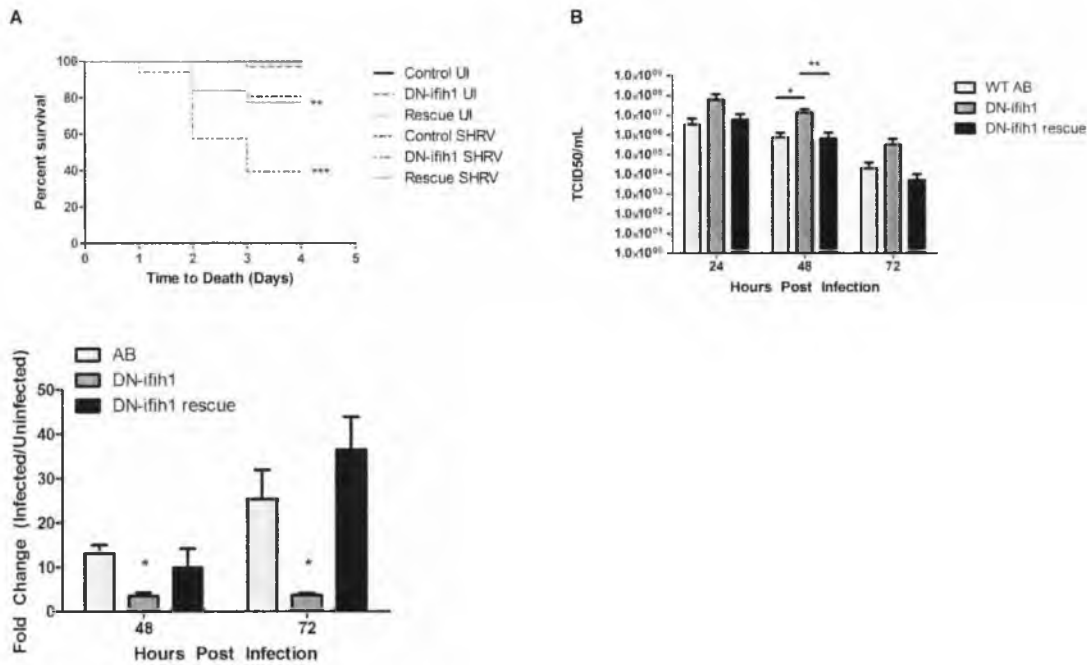
The Toll-like receptor gene family is an important group of host pattern recognition receptors (PRR) that recognize a multitude of pathogen-associated molecular patterns (PAMP), such as lipopolysaccharide (LPS)/TLR4 or unmethylated CpG DNA/TLR9. But little research has been conducted to implicate a role for TLR9 in viral infections of RNA origin, such as influenza. The Carol Kim laboratory has recently characterized human influenza viral infection in the zebrafish model (Gabor, et al.). Previous research in the mouse model shows that priming TLR9 with exposure to unmethylated CpG DNA prior to influenza challenge results in improved survival compared to controls. In addition, *Tlr9* expression in humans infected with influenza is upregulated compared to controls. Using a reverse genetics approach known as gene knockdown via morpholino oligonucleotides targeting the zebrafish *Tlr9* gene, we were able to examine the effects of TLR9 deficiency on the host's ability to mount an immune response to influenza through (A) mortality studies and (B) antiviral gene expression. Findings suggest that TLR9 morphant zebrafish were more susceptible to influenza compared to control zebrafish, implicating a protective role for TLR9 in antiviral immunity to influenza.



**Figure 4.** *Tlr9* is required for the antiviral immune response. At the 1-2 cell stage, zebrafish were injected with TLR9 or control morpholino (GeneTools, LLC, Philomath, OR). At 48 hpf, *Tlr9* morphants and control zebrafish were injected via the Duct of Cuvier with 2.0 nL influenza strain A/PR/8/34 (H1N1, EID50  $10^{10.7}$ ) (Charles River, North Franklin, CT). (A) There was an increased mortality in *Tlr9* morphants exposed to influenza when compared to controls. \* indicates a statistically significant difference with a p value of  $<0.05$ . (B) At 24, 48, and 72 h post-infection, total RNA was collected from zebrafish and converted into first-strand cDNA. RT-PCR analysis reveals that there was a significant change in the expression of *ifnΦ1*, *mxa*, and *viperin* transcripts upon exposure to influenza. Data was normalized using 18S.

#### **Ifih1-dependent response to rhabdovirus infection**

Interferon induced with helicase C domain 1 (IFIH1) is a cytosolic pattern recognition receptor that detects viral nucleic acids. We recently submitted our findings to the *Journal of Virology* describing an Ifih1-dependent response to rhabdovirus infection *in vivo* using a dominant-negative *ifih1* transgenic zebrafish. These *in vivo* studies helped to elucidate a role for *ifih1* in the host response to viral infection. We demonstrated that zebrafish embryos lacking *ifih1* protein (e.g. DN-*ifih1*) are unable to mount an antiviral immune response, evidenced by the increased mortality of DN-*ifih1* embryos relative to controls upon SHRV infection, an effect that could be rescued with overexpression of *ifih1* (A). Corroborating the survival results, DN-*ifih1* zebrafish embryos failed to clear the virus and sustained higher viral titers after SHRV challenge (B). DN-*ifih1* zebrafish also displayed diminished transcription of interferon upon viral infection relative to controls, which could be rescued with *ifih1* overexpression (bottom figure).



**Figure 5.** *Ifih1* is critical for immune response to SHRV *in vivo*. At the 1 cell stage, DN-*ifih1* zebrafish were injected with *ifih1* RNA or pCS2+ empty vector. A) Control, DN-*ifih1* and rescued DN-*ifih1* zebrafish embryos were left as uninfected controls or infected by static immersion 48 hpf with  $1 \times 10^6$  TCID<sub>50</sub>/mL virus and monitored daily for mortality. Results are representative of three separate experiments. Statistical analysis (Wilcoxon test) of the Kaplan-Meier curve was performed (\*\*,  $p < 0.01$ ; \*\*\*,  $p < 0.001$ ). B) Control, DN-*ifih1*, and rescued DN-*ifih1* zebrafish embryos were infected 48 hpf  $1 \times 10^6$  TCID<sub>50</sub>/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-*ifih1* embryos, while the control and rescued DN-*ifih1* embryos had comparable viral burden. Figure is representative of three experiments; error bars are standard error of the mean (\*,  $p < 0.05$ ; \*\*,  $p < 0.01$ ). Rescue of DN-*ifih1* demonstrates critical role for *ifih1* in IFN response. DN-*ifih1* zebrafish were injected at the 1-cell stage with *ifih1* RNA or pCS2+ empty vector. Wild-type AB lines were kept as controls. Fish were infected with  $1 \times 10^6$  TCID<sub>50</sub>/mL SHRV and collected for RNA isolation at 48 and 72 hpi. RNA was purified and IFN was subsequently measured by qPCR (\*,  $p < 0.05$ ).

## Publications

Sullivan, C., M.F. Goody, E. Peterman, I. Rodriguez-Nunez, C.R. Lage, J.A. Yoder, J.H. Postlethwait, **C.H. Kim**. 2014. Functional evolution of the *tnf-alpha* inducible protein 8-like 1 gene in vertebrate immune homeostasis. *J Immunol. Submitted*.

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Gabor, K.A., J.R. Charette, M.J. Pietraszewski, D. Wingfield, J.S. Shim, P.J. Millard, **C.H. Kim**. 2014. A DN-*ifih1* transgenic zebrafish model demonstrates that *ifih1* is essential to snakehead rhabdovirus resistance. *J Virol. Submitted*.

Gui, J., C. Greene, C. Sullivan, W. Taylor, J. Moore, **C.H. Kim**. 2014. Testing multiple hypotheses through IMP weighted FDR based on a genetic functional network with application to a new zebrafish transcriptome study. *Submitted*.

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