Supporting Information

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SI Materials and Methods

Reagents and Antibodies. CpG-oligodeoxynucleotides (CpG-ODNs) were purchased from Invitrogen or Genomics. DAPI, Alexa Fluor 594-labeled anti-mouse and anti-rabbit antibodies, and Alexa Fluor 488-conjugated anti-mouse and anti-rabbit antibodies were purchased from Invitrogen. Anti-Myc antibody and rabbit monoclonal antibodies for lysosome-associated membrane protein 1 (Lamp-1), apoptosis-inducing factor (AIF), endosome antigen 1 (EEA), RCAS1, and calnexin were purchased from Cell Signaling. Tricaine, chloroquine, and anti-FLAG M2 monoclonal antibody were purchased from Sigma-Aldrich.

Molecular Cloning of Zebrafish UNC93B1. A zebrafish UNC93B1 (zebUNC93B1) cDNA containing both 5′ and 3′ UTRs and a complete coding region was cloned by PCR amplification from a zebrafish kidney first-strand cDNA library prepared from total RNA using a SuperScript preamplification kit (Invitrogen). The forward and reverse primers were designed based on the 5′ and 3′ end sequences of a predicted zebUNC93B1 sequence (XM_002660536). The generated zebUNC93B1 cDNA was ligated into a PEF6 vector (Invitrogen) for sequencing. The nucleotide sequence of this cDNA was submitted to GenBank (accession no. KF697668).

Expression Constructs. Comparison of the cDNA sequences of zebrafish TLR9 (zebTLR9; NM_001130594) and TLR21 (zebTLR2; NM_001199335) with their genomic DNA sequences in the National Center for Biotechnology Information's zebrafish (*Danio rerio*) genomic BLAST database (http://blast.ncbi.nlm.nih.gov/Blast.cgi) indicated that both zebrafish TLRs are encoded by a single exon. Making use of this property, DNA fragments containing the complete coding region for zebTLR9 and zebTLR21 were PCR-amplified from their genomic DNA using the Expand High-Fidelity PCR System (Roche). The PCR products were then subcloned into a PEF6 expression vector (Invitrogen) encoding with a FLAG tag fusion to the C terminus of these TLRs. The genomic DNA was isolated from zebrafish using the QIAamp DNA Mini Kit (Qiagen) following the manufacturer's protocol.

To generate an expression vectors for human UNC93B1 and zebUNC93B1, cDNA fragments containing the complete coding regions of these proteins were PCR-amplified from a first-strand cDNA library prepared from poly(A^+)-mRNA isolated from a human spleen (purchased from Clontech) as described previously (1), and a zebrafish kidney first-strand cDNA library. The PCR product was subcloned into a PEF6 expression vector encoded with a Myc tag fusion to the C terminus.

Expression Constructs for zebTLR9 and zebTLR21 Point Mutants and Chimeras. DNA fragments encoding zebTLR9 and zebTLR21 point mutants and chimeras were generated by two-step PCR amplification as described previously (2). These amplified DNA fragments were restriction-digested and subcloned into a PEF6 vector to generate expression constructs.

Cell Culture, Transfection, and TLR Activation Assays. HEK293 cells were grown at 37 °C in DMEM supplemented with 10% FBS. Zebrafish ZF4 cells were grown at 28 °C in a 1:1 mixture of Ham's F-12 nutrient mixture and DMEM supplemented with 10% FBS. For TLR activation assays, these cells were seeded on 24-well plates, allowed to adhere overnight, and cotransfected using PolyJet (SignaGen) with zebTLR9 or zebTLR21 expression vector, β-galactosidase plasmid, and an NF-κB–driven luciferase reporter plasmid in the presence or absence of human UNC93B1 or zebUNC93B1 expression vector as indicated.

The next day, the transfected cells were treated with various CpG-ODN and different TLR ligands as indicated for 6 h and then lysed. Luciferase activity in each sample was measured using reagents in a Promega luciferase assay system. Relative luciferase activity was calculated as fold induction compared with an unstimulated control. Data are expressed as mean ± SD ($n = 3$).

RNA Extraction and RT-PCR Analysis. For analysis of zebTLR9, zebTLR21, and zebUNC93B1 expression levels at different developmental stages, samples were collected at different times after fertilization for total RNA isolation. For tissue distribution analysis, adult fish were euthanized with 4 mg/mL tricaine before tissue collection. Total RNA was isolated from samples with TRIzol reagent (Invitrogen) or the RNeasy Mini Kit (Qiagen) following the manufacturer's protocol.

RT-PCR analysis was performed as described previously (1). In brief, first-strand cDNA libraries were synthesized from the collected total RNA samples, after which PCR amplifications were performed. The sequences of the gene-specific primers are as follows: for zebGAPDH, 5′-gtcttcactactattgagaaggcttc-3′ and 5′-ctactccttggaggccatgtgtgc -3′; for zebTLR9, 5′-cactattgacactagcagcaacttgc-3′ and 5′-tcagaagaagctctccgtgacgttc-3′; for zebTLR21, 5′-ccataacctcacacagctgctcc-3′ and 5′-cagtgcatgggagttcttcagctt-3′; for zebUNC93B1, 5′-gggtttgttacagatgcagttgattc-3′ and 5′-ggcagactcttcaaaaccgtcttatt-3′; for zebIL-1, 5′-gattctgatgagatggactgttcag-3′ and 5′-ctagatgcgcactttatcctgcag-3′; for zebTNFb, 5′-ctgtacccgttaattcttcaagaagc-3′ and 5′-aatcacaacgcgaacaccccgaag-3′; for zebIFN-γ, 5′-gttttcgggatggatgacatacagtg-3′ and 5′-ggcactgatcaacctctatttagac-3′. PCR products were visualized by electrophoresis on a 1% agarose gel after staining with ethidium bromide, and then imaged with the UVP BioDoc-It Imaging System. The band intensities were quantified using ImageJ software.

Immunofluorescence Staining. To analyze cellular localization of zebTLR9, zebTLR21, and UNC93B1, HEK293 cells were transiently transfected overnight with an expression vector for FLAG-tagged zebTLR9 and zebTLR21 alone or in combination with an expression vector for Myc-tagged UNC93B1. These cells were treated with methanol or fixed in 2% paraformaldehyde, then permeabilized with 0.2% Triton X-100 in PBS. Fixed cells were blocked in phosphate buffered saline Tween 20 solution containing 1% of bovine serum albumin and then stained using the indicated primary antibodies, followed by Alexa Fluor-conjugated secondary antibodies. After mounting with Fluoroshield containing DAPI (GeneTex), the stained cells were observed under a fluorescence microscope (Leica TSC SP5II).

Immunoprecipitation and Immunoblotting. For immunoprecipitation, cell lysates were incubated with anti-Myc agarose beads at 4 °C overnight to form immunocomplexes. After extensive washing with lysis buffer, the immunocomplexes were analyzed by immunoblotting. For immunoblotting, immunocomplexes or cell lysates were resolved by polyacrylamide gels and transferred to PVDF membranes (Immobilon-P; Millpore). The membranes were immunoblotted with the indicated primary antibodies, followed by HRP-conjugated secondary antibodies. The immunoreactive bands were detected by adding a chemiluminescent HRP substrate (Immobilon Western; Millipore) and exposing the membrane in the UVP BioSpectrum Imaging System.

Histology Analysis. For histology analysis, fish from parallel groups of the bacterial infection studies were euthanized in 4 mg/mL tricaine and fixed in 10% formalin in phosphate buffered saline solution. The fish samples were embedded in paraffin wax, and sections were stained with H&E.

1. Fearns C, Pan Q, Mathison JC, Chuang TH (2006) Triad3A regulates ubiquitination and proteasomal degradation of RIP1 following disruption of Hsp90 binding. *J Biol Chem* 281(45):34592–34600.

2. Liu J, et al. (2010) A five-amino acid motif in the undefined region of the TLR8 ectodomain is required for species-specific ligand recognition. *Mol Immunol* 47(5): 1083–1090.

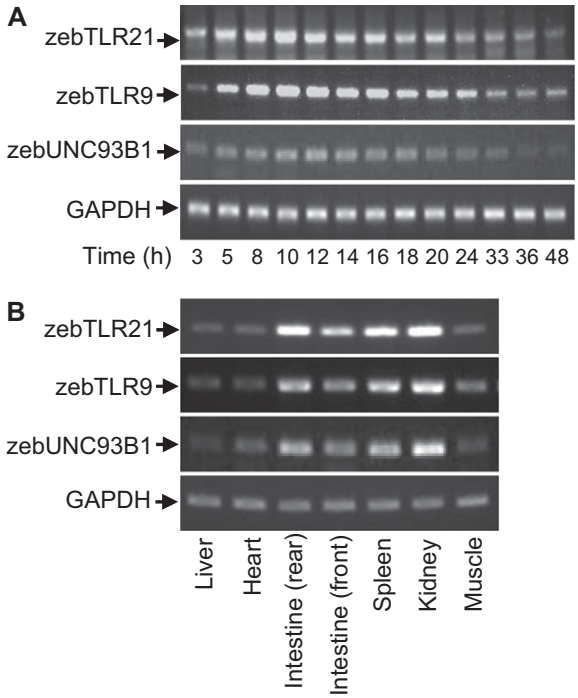


Fig. S1. Expression of zebTLR9, zebTLR21, and zebUNC93B1 at different times during embryonic development (*A*) and in different tissues of adult zebrafish (*B*). Expression of the indicated genes was analyzed by RT-PCR. Amplification of GAPDH was performed for control of amounts of cDNA used as a template. Blots are representative RT-PCR analyses of three independent experiments.

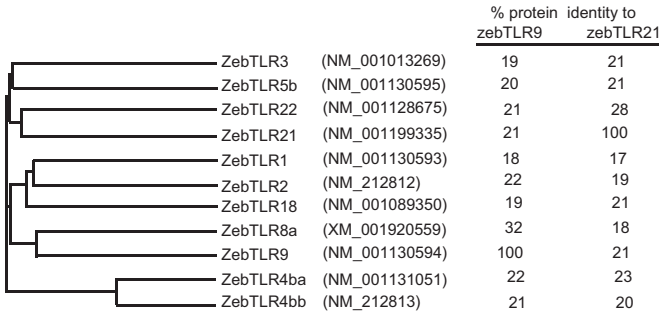


Fig. S2. Phylogenetic analysis of zebTLRs. The GenBank accession numbers of the zebTLR protein sequence used in this phylogenetic analysis are in parentheses. Numbers after the accession numbers are the percentages of protein identities of zebTLR to zebTLR9 and zebTLR21.

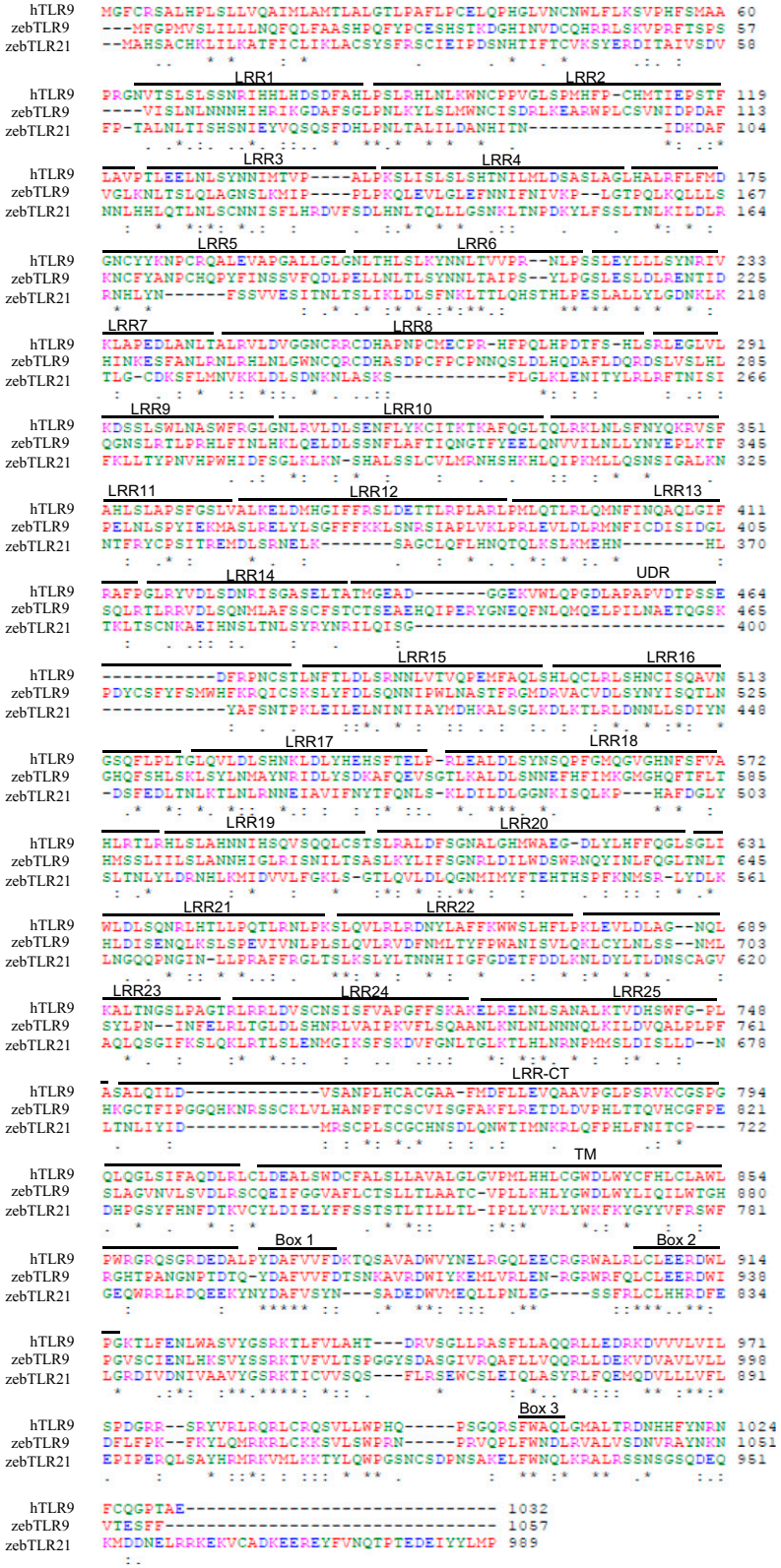
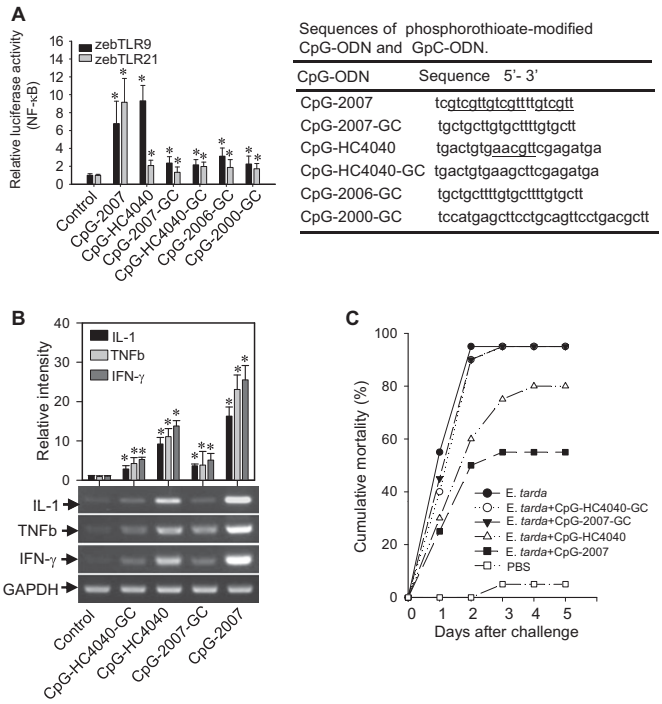


Fig. S3. Alignment of zebTLR9 and zebTLR21. Protein sequences of zebTLR9 and zebTLR21 were aligned together with hTLR9 for comparison. Amino acids are color-coded to indicate their chemical properties: blue indicates acidic; green indicates hydroxyl/amine/basic/Q; pink indicates basic; and red indicates hydrophobic (including aliphatic Y). Identical residues are denoted by an asterisk; conservative substitutions, by a single dot; highly conservative substitutions, by two dots. LRR, leucine-rich repeat; UDR, undefined region; LRR-CT, C-terminal LRR; TM, transmembrane domain; Box1, Box2, and Box3, box 1, box 2, and box 3 in the cytosolic Toll/interleukin-1 receptor domain.



Sequences of phosphorothioate-modified CpG-ODN and GpC-ODN.

CpG-ODN	Sequence 5'- 3'
CpG-2007	tcgtcgttgtcgtttgtcgtt
CpG-2007-GC	tgctgcttgtgcttttgtgctt
CpG-HC4040	tgactgtgaacgttcgagatga
CpG-HC4040-GC	tgactgtgaagcttcgagatga
CpG-2006-GC	tgctgcttttgtgcttttgtgctt
CpG-2000-GC	tccatgagcttcctgcagttcctgacgctt

Fig. S4. Requirement of the CpG motif for immunostimulatory activities of CpG-ODNs to zebTLR9 and zebTLR21. The activities of CpG-2007 and CpG-HC4040 were compared with those of their GpC-variant forms, CpG-2007-GC and CpG-HC4040-GC, as well as CpG-2006-GC and CpG-2000-GC, to determine the requirements of the CpG motif for CpG-ODNs to activate zebTLR9 and zebTLR21 in cell-based assays (*A*), cytokine production in zebrafish (*B*), and antimicrobial response in zebrafish (*C*). The experimental methods are the same as those described in Figs. 1, 4, and 5. The sequences of CpG-ODN and GpC-ODN used in these experiments are listed in *A*, *Right*.

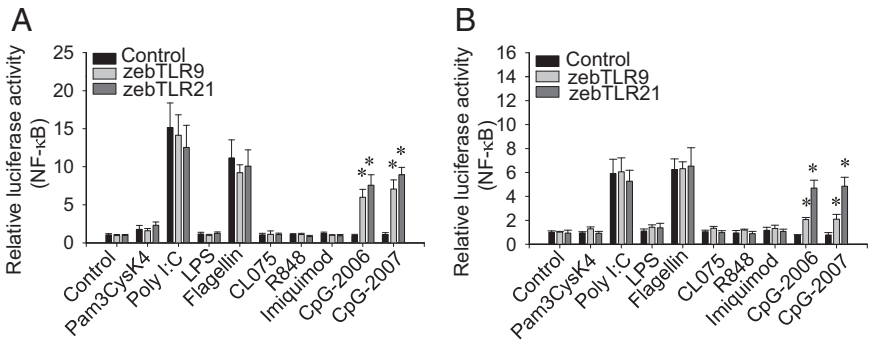


Fig. S5. Activation of zebTLR9 and zebTLR21 by different TLR ligands. HEK293 cells (*A*) and ZF4 cells (*B*) were transfected with expression vector for zebTLR9 and zebTLR21 as indicated, plus an NF-κB luciferase reporter gene, and then treated with 0.2 μg/mL Pam3CysK4, LPS, and flagellin; 5 μg/mL polyI:C; and 3 μM CL075, R848, imiquimod, CpG-2006, and CpG-2007. Relative luciferase activity was measured. Data are mean ± SD ($n = 3$). *$P < 0.05$ vs. control cells.

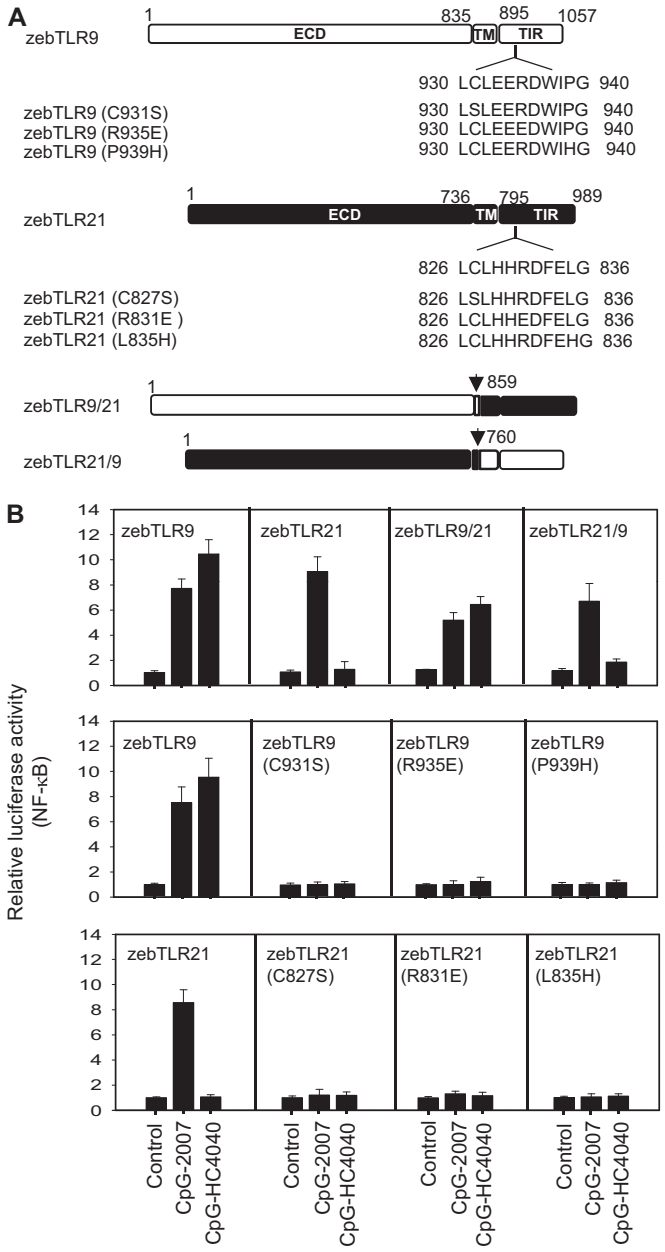


Fig. S6. Role of the ectodomain and amino acid residues in box 2 in mediating zebTLR9 and zebTLR21activation. (*A*) Schematic of the zebTLR9 and zebTLR21 chimera and point mutant constructs. ECD, extracellular domain/ectodomain; TM, transmembrane domain. (*B*) Responses of chimera and point mutants to ligand stimulation. HEK293 cells were transfected with different expression vectors, as indicated, along with an NF-κB luciferase reporter gene. The cells were stimulated with 3 μM CpG-ODN for 6 h, after which relative luciferase activity was measured. Data are mean ± SD (n = 3).

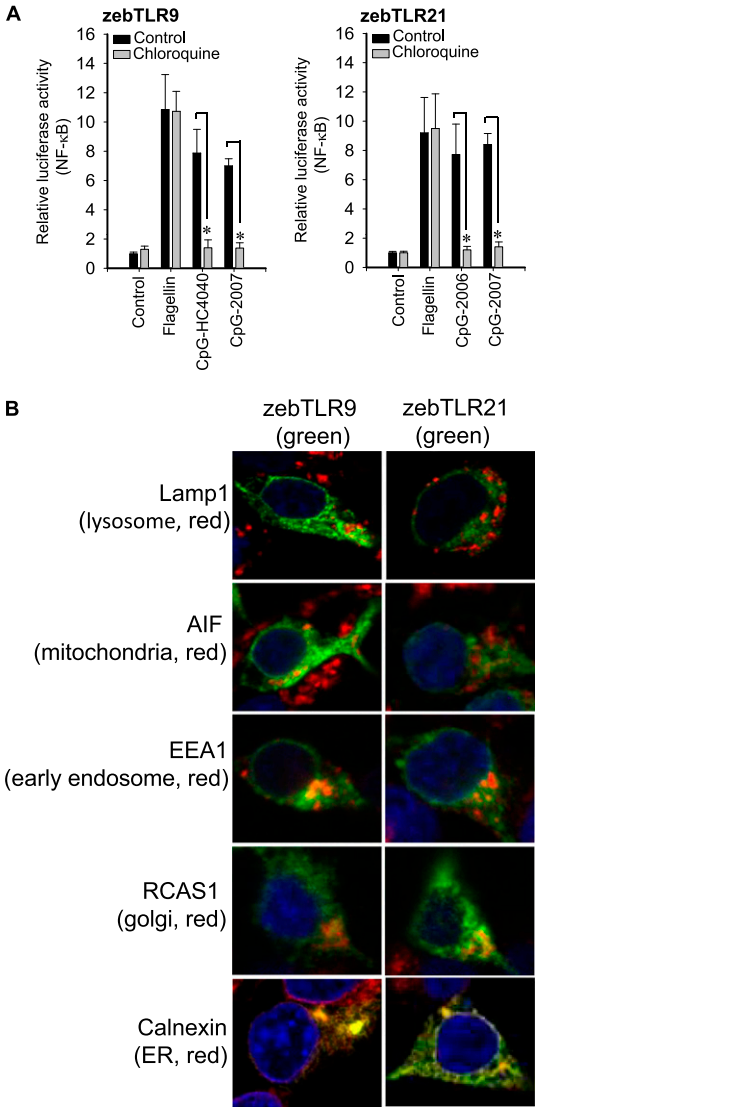


Fig. S7. Intracelullar activation and localization of zebTLR9 and zebTLR21. (*A*) HEK293 cells were cotransfected with expression vector for zebTLR9 (*Left*) and zebTLR21 (*Right*) and NF-κB luciferase reporter gene. These cells were pretreated with 2 μM chloroquine, and then treated with 0.2 μg/mL flagellin and 3 μM CpG-ODN. Relative luciferase activities were determined. Data are mean ± SD ($n = 3$). *$P < 0.05$ vs. cells without chloroquine treatment. (*B*) HEK293 cells were transfected with expression vector for zebTLR9 and zebTLR21. Expression of zebTLR9 and zebTLR21 was visualized by immunofluorescence staining using anti-FLAG M2 mAb, followed by an Alexa Fluor 488-labeled anti-mouse antibody. Cellular organelles were stained by anti-Lamp1, anti-AIF, anti-EEA1, anti-RCAS1, and anti-calnexin antibodies, followed by an Alexa Fluor 594-labeled secondary antibody. Nuclei were stained by DAPI. Representative merged confocal images are shown for the cellular localization of zebTLR9 and zebTLR21. Green indicates zebTLR9 or zebTLR21; red, organelle markers; blue, nuclei stained with DAPI.

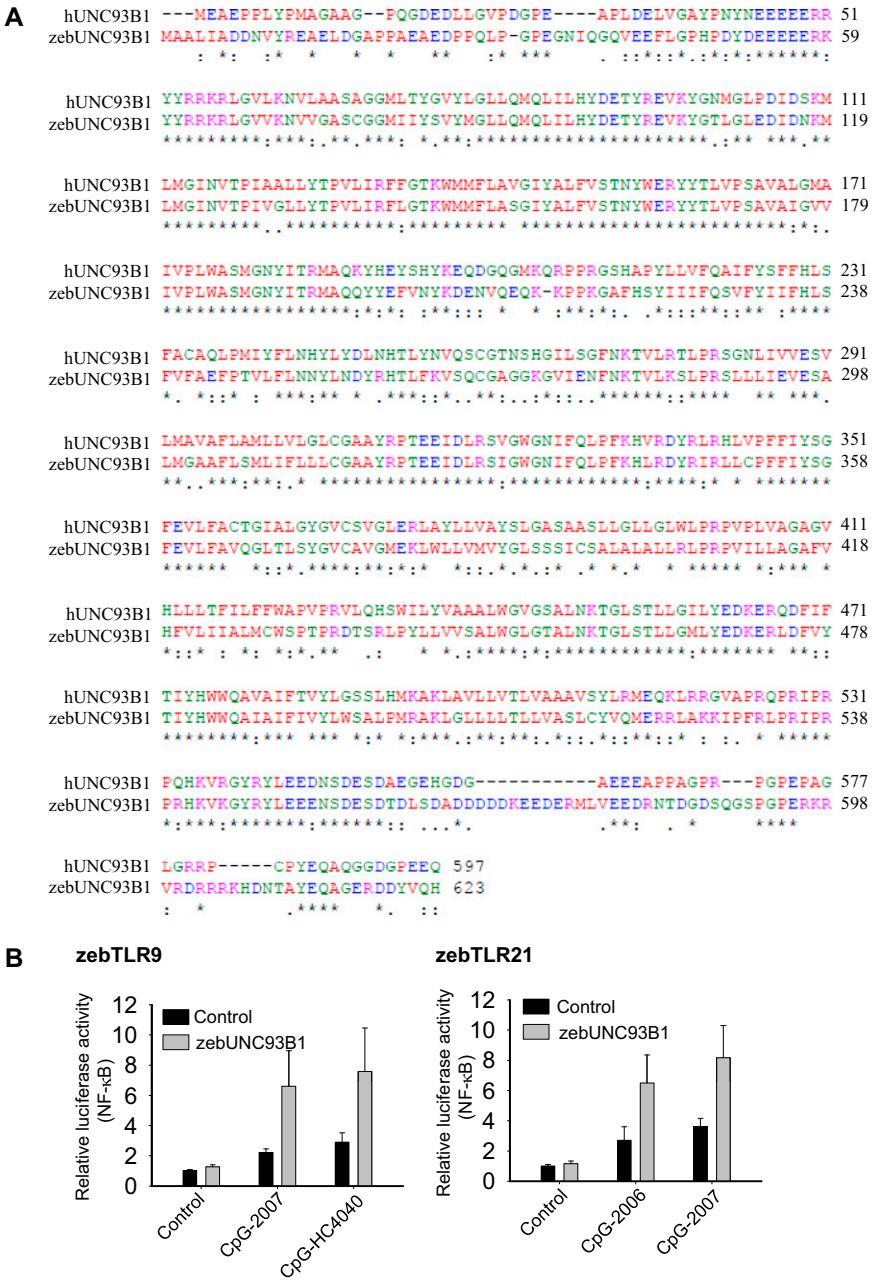


Fig. S8. Regulation of zebTLR9 and zebTLR21 activation by zebUNC93B1. (*A*) Alignment of human UNC93B1 and zebUNC93B1. Zebrafish UNC93B1 cDNA was cloned. The deduced protein sequence contains 623 amino acid residues. This sequence was aligned with the protein sequence of human UNC93B1 for comparison. Amino acids are color-coded to indicate their chemical properties. Blue indicates acidic; green, hydroxyl/amine/basic/Q; pink, basic; and red, hydrophobic (including aliphatic Y). Identical residues are represented by an asterisk; conservative substitutions, by a single dot; and highly conservative substitutions, by two dots. (*B*) ZF4 cells were cotransfected with NF-κB luciferase reporter gene and expression vectors for zebTLR9, zebTLR21, and zebUNC93B1 as indicated. The cells were treated with 3 μM CpG-ODN, after which relative luciferase activity was measured. Data are mean ± SD ($n = 3$).