



## Md1 and Rp105 regulate innate immunity and viral resistance in zebrafish



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### ABSTRACT

TLR4 was the first TLR family member identified in mammals and is responsible for the activation of the immune response by bacterial LPS. Later, MD1 and RP105 were shown to form complexes that directly interact with the MD2-TLR4 complex, acting as physiological negative regulators of LPS signaling. Despite the general conservation of various TLR families from fish to mammals, several differences can be appreciated, such as the high tolerance of fish to LPS, the absence of the crucial accessory molecules Md2 and Cd14 for Tlr4 signaling in fish, the absence of Tlr4 in some fish species, and the confirmation that LPS does not signal through Tlr4 in zebrafish. The present study has identified the Rp105 and Md1 homologs in zebrafish, confirming (i) Rp105 and Tlr4 evolved from a common ancestor before the divergence between fish and tetrapods and (ii) the presence of Md1 in teleost fish and the lack of Md2, suggesting that the divergence of these accessory molecules occurred in the tetrapod lineage. Biochemical and functional studies indicate that Md1 binds both Rp105 and Tlr4 in zebrafish. Genetic inhibition of zebrafish Md1 and Rp105 reveals that Md1 or Rp105 deficiency impairs the expression of genes encoding pro-inflammatory and antiviral molecules, leading to increased susceptibility to viral infection. These results shed light on the evolutionary history of Md1 and Rp105 and uncover a previously unappreciated function of these molecules in the regulation of innate immunity.

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### 1. Introduction

The innate immune system is an ancient type of host defense against infection which is present in invertebrate and vertebrate animals. This has evolved to recognize conserved molecular structures of pathogenic microorganisms, called pathogens-associated molecular patterns (PAMPs), through a limited number of germline-encoded receptors, named pattern recognition receptors (PRRs) (Akira

et al., 2006; Janeway and Medzhitov, 2002). Toll-like receptors (TLRs), the most relevant and studied group of PRRs, are type typically I transmembrane proteins. TLRs are composed of an extracellular domain containing leucine-rich repeats (LRRs), a conserved pattern of juxtamembrane cysteine residues, and an intracellular Toll/interleukin 1 (IL-1) receptor domain (TIR) that initiates signal transduction (Akira et al., 2006; Xu et al., 2000).

TLR4, the first mammalian homolog of *Drosophila* Toll to be discovered (Medzhitov et al., 1997; Rock et al., 1998), was identified in mammals as the PRR responsible for the signaling in response to LPS (Poltorak et al., 1998; Qureshi et al., 1999), the complex glycolipid that is the major component of the Gram-negative outer membrane. This role for TLR4 was confirmed by the observation that *Tlr4*<sup>-/-</sup> mice were hyporesponsive to LPS (Akira et al., 2006; Bryant et al., 2010). However, the transfection of cell lines with TLR4 was not sufficient to confer them the ability to respond to LPS even though NF-κB was constitutively activated (Kirschning et al., 1998), suggesting that another factor was essential for LPS signaling via TLR4 (Wright, 1999). MD2, also named lymphocyte antigen 96 (LY96), was found to bind LPS (Ohto et al., 2007) and, after its association with the extracellular domain of TLR4, confers LPS responsiveness on cells expressing TLR4 alone (Nagai et al., 2002a; Shimazu et al., 1999).

**Abbreviations:** Actb, β-actin; as, antisense; hpf, hours post-fertilization; hpi, hours post-injection; LBP, LPS-binding protein; LY96, lymphocyte antigen 96; MO, morpholino; Mxb, myxovirus (influenza) resistance b; PAMP, pathogens-associated molecular pattern; PRR, pattern recognition receptor; Ptgs2a, prostaglandin-endoperoxide synthase 2a; qPCR, quantitative PCR; RACE, rapid amplification of complementary DNA ends; RP105, radioprotective 105; SVCV, spring viremia of carp virus; VaDNA, *Vibrio anguillarum* genomic DNA; TCID<sub>50</sub>, 50% tissue culture infectious dose; TIR, Toll/IL-1 receptor domain.

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The TLR-like molecule radioprotective 105 (RP105), also named CD180, shares with TLRs a conserved extracellular leucine-rich repeat domain and a pattern of juxtamembrane cysteines. However, RP105 lacks the TIR domain, containing only 6–11 intracellular amino acids (Miyake et al., 1995). MD1, also named lymphocyte antigen 86 (LY86), was identified as a molecule associated with the extracellular domain of RP105 (Miyake et al., 1998), and like MD-2, directly interacts with LPS (Yoon et al., 2010). Similarly to TLR4 and MD2, RP105 expression and signaling depends on the coexpression of MD1 (Miura et al., 1998; Miyake et al., 1998; Nagai et al., 2002b; Shimazu et al., 1999). Further investigation demonstrated that RP105 is a physiological regulator of TLR4 signaling through the direct interaction of the RP105–MD1 and TLR4–MD-2 complexes and the subsequent inhibition of LPS binding (Divanovic et al., 2005a, 2005b; Kimoto et al., 2003). Besides MD1, MD2 and RP105, other accessory molecules are required for the regulation of the TLR4 function by mediating ligand delivery and/or recognition, e.g. LPS-binding protein (LBP), CD14 and CD36 (Akashi-Takamura and Miyake, 2008; Lee et al., 2012).

In zebrafish, 15 Tlrs have been identified, including Tlr 1, 2, 3, 4a/b, 5a/b, 7, 8a/b, 9, 14, 19, 20a, 21 and 22 (Meijer et al., 2004; Palti, 2011). Despite the fact that the zebrafish *tlr4* has been cloned and characterized (Jault et al., 2004; Meijer et al., 2004), the lack of the important accessory molecules Md2 and Cd14 in the zebrafish genome (Pietretti et al., 2013) and the inability of fish Tlr4 to recognize LPS (Sepulcre et al., 2009; Sullivan et al., 2009) indicate that the function of Tlr4, Md1 and Rp105 is unclear in teleost fish. In this study, we use bioinformatic analysis, gain and loss of function strategies, and different bacterial and viral infection models in zebrafish to throw light on a previously unappreciated role for Md1 and Rp105 in PAMP responses and antiviral defense.

## 2. Materials and methods

### 2.1. Animals

Zebrafish (*Danio rerio* H.) were obtained from the Zebrafish International Resource Center and mated, staged, raised and processed as previously described (Westerfield, 2000). The experiments performed comply with the Guidelines of the European Union Council (86/609/EU) the Spanish RD 53/2013, and the Bioethical Committee of the University of Murcia (approval number #537/2011).

### 2.2. Identification and sequence analysis of zebrafish Md1 and Rp105

Zebrafish Md1 and Rp105 sequences were analyzed for similarity with other known sequences, obtained from The Universal Protein Resource (UniProt) database (<http://www.uniprot.org/>) using the Similarity & Homology tool within The European Bioinformatics Institute (EMBL-EBI) website (<http://www.ebi.ac.uk/>). A direct comparison between two sequences was performed using the EMBOSS Needle program within the EMBL-EBI website, while multiple sequence alignment was carried out with the ClustalX version 2.1 program (Larkin et al., 2007). The molecular weights were estimated using the Protein Molecular Weight tool, from The Sequence Manipulation Suite (<http://www.bioinformatics.org/sms/index.html>). The domains of the proteins deduced from the nucleotide sequences were determined using the Simple Modular Architecture Research Tool (SMART), from the European Molecular Biology Laboratory (EMBL) website (<http://smart.embl-heidelberg.de/>) (Letunic et al., 2012; Schultz et al., 1998). Finally, unrooted phylogenetic trees were constructed based on those amino acid sequence alignments generated by the ClustalX version 2.1 program and then displayed using TreeViewX Version 0.5.0 program, developed by Professor Rod Page of the University of Glasgow.

### 2.3. Cloning of zebrafish Md1 and Rp105 and expression constructs

The full cDNA sequences of zebrafish Md1 and Rp105 were amplified by RT-PCR from samples obtained from adult zebrafish injected i.p. with 5 µg ultrapure *Escherichia coli* O111-B4 LPS (EclPS, Life Technologies). The primers used for that were designed from the previously annotated sequences for zebrafish Md1 and Rp105 (Supplementary Table S1). For zebrafish Rp105, the 5′ nucleotide sequence was confirmed by the rapid amplification of 5′ complementary DNA ends (5′-RACE) (Garcia-Castillo et al., 2002). The full zebrafish Md1 and Rp105 coding sequences were cloned into the expression vectors pFLAG-CMV<sup>TM</sup>-5a (Sigma-Aldrich) and pcDNA6/V5-His C (Life Technologies), respectively. Human TLR4-Flag and MD2-HA (Divanovic et al., 2005a), and zebrafish Tlr4ba-V5 and Tlr4bb-V5 (Sepulcre et al., 2009) have all been described previously.

### 2.4. MO and RNA injection

Specific MOs (Gene Tools) were resuspended in nuclease-free water to 1 mM (Supplementary Table S2). In vitro-transcribed RNA was obtained following the manufacturer's instructions (mMESSAGE mMACHINE Kit, Ambion). MOs and RNA (200 pg/egg) were mixed in microinjection buffer (0.5× Tango buffer and 0.05% phenol red solution) and microinjected into the yolk sac of one- to eight-cell-stage embryos using a microinjector (Narishige) (0.5–1 nl per embryo). The same amounts of MOs and/or RNA were used in all experimental groups. The efficiency of the MOs for Md1 and Rp105 was checked by RT-PCR.

### 2.5. PAMP-stimulation and infection assays in adult zebrafish

For the stimulation assays, adult fish were injected with PBS (control), 5 µg EclPS, 0.1 µg flagellin or 2.5 µg poly I:C (all from Invivogen) in the left epaxial muscle (Sepulcre et al., 2009). mRNA levels were measured by real-time RT-PCR in the injection site at 4, 24 and 36 hours post-injection (hpi) (see section 2.7). For the bacterial infection assays, adult fish were injected i.p. with PBS (control) or heat-killed or live *Streptococcus iniae* (10<sup>6</sup> cfu/fish) (Roca et al., 2008). For the viral infection assays, the spring viremia of carp virus (SVCV) isolate 56/70 virus stock was propagated in EPC cells and titrated into 96-well plates according to Reed and Muench (1938). Adult fish were injected i.p. with PBS (control) or 10<sup>5</sup> tissue culture infectious dose (TCID<sub>50</sub>) per fish SVCV. Infected fish were dissected and mRNA levels were measured by real-time RT-PCR in several heads at 24 and 48 hpi (see section 2.7). Basal levels of expression were also determined using real-time RT-PCR in different tissues from non-challenged fish. In all cases, fish were anesthetized by immersion in buffered tricaine (200 µg/ml, Sigma-Aldrich) before the injection of PAMPs, bacteria or virus.

### 2.6. PAMP-stimulation and infection assays in embryos/larvae

Morpholinos (Supplementary Table S2) and 6.5 ng/egg *Vibrio anguillarum* genomic DNA (VaDNA), 0.03 ng flagellin or 1.2 ng poly I:C were mixed in microinjection buffer and microinjected (0.5–1 nl) as described in section 2.6. mRNA levels were measured by real-time RT-PCR in dechorionated whole larvae 24 hours post-fertilization (hpf) (see section 2.7). For the viral infection assays, groups of 25–30 zebrafish larvae were challenged at 3 days post-fertilization in 5 ml egg water (60 µg/ml sea salts in distilled water) containing from 2.5 × 10<sup>7</sup> to 10<sup>8</sup> 50% tissue culture infectious dose (TCID<sub>50</sub>)/ml SVCV at 26 °C. Twenty four hours later, the virus was diluted by adding 35 ml of egg water, and the larvae were monitored every 24 hours over a 10-day period for clinical signs of disease and mortality (López-Muñoz et al., 2010). In addition, 25 larvae were

collected per experimental group at 48 hpi, pooled and processed for the analysis of gene expression by real-time RT-PCR.

### 2.7. Analysis of gene expression

Total RNA was extracted from pooled embryos/larvae or from adult tissues or organs with TRIzol reagent (Invitrogen) following the manufacturer's instructions and treated with DNase I, amplification grade (1 U/μg RNA; Invitrogen). SuperScript III RNase H<sup>-</sup> Reverse Transcriptase (Invitrogen) was used to synthesize first-strand cDNA with oligo(dT)18 primer from 1 μg of total RNA at 50 °C for 50 min. Real-time PCR (qPCR) was performed with an ABI PRISM 7500 instrument (Applied Biosystems) using SYBR Green PCR Core Reagents (Applied Biosystems). Reaction mixtures were incubated for 10 min at 95 °C, followed by 40 cycles of 15 s at 95 °C, 1 min at 60 °C, and finally 15 s at 95 °C, 1 min at 60 °C and 15 s at 95 °C. For each mRNA, gene expression was normalized to the ribosomal protein S11 (*rps11*) content in each sample using the Pfaffl method (Pfaffl, 2001). The primers used are shown in Supplementary Table S1. In all cases, each PCR was performed with triplicate samples and repeated with at least two independent samples.

### 2.8. Pull-down and western blot analysis

The physical interaction between zebrafish Md1 and Rp105 was analyzed by means of immunoprecipitation. Human embryonic kidney HEK293 cells were purchased from ECACC (UK) and grown at 37 °C in DMEM culture media (Gibco), supplemented with 10% fetal calf serum (FCS) and penicillin/streptomycin. Plasmid DNA was prepared using the Midi-Prep procedure (Qiagen) and transfected into HEK293 cells with LyoVec transfection reagent (InvivoGen), according to the manufacturer's instructions. Briefly, HEK293 cells were plated on 9 cm diameter Petri dishes (2,000,000 cells/dish) and transfected at the same time with 500 μl of transfection reagent containing 5 μg of human TLR4-Flag and MD2-HA or zebrafish Tlr4ba-V5, Tlr4bb-V5, Rp105-V5 or Md1-FLAG expression constructs. Forty-eight hours after transfection, the cells were washed twice with PBS and lysed in 200 μl lysis buffer (10 mM Tris-HCl pH 7.4, 150 mM NaCl, 1% Triton X-100, 0.5% NP-40, and a 1:20 dilution of the protease inhibitor cocktail P8340 from Sigma-Aldrich). Whole cell extracts were then mixed and incubated overnight at 4 °C with an ANTI-FLAG<sup>®</sup> M2 Affinity Gel (Sigma-Aldrich). After extensive washing with lysis buffer, the resin was boiled in SDS sample buffer and the bound proteins were resolved on 12% SDS-PAGE and transferred for 50 minutes at 200 mA to nitrocellulose membranes (BioRad). Blots were probed with specific antibodies to V5 (Invitrogen), FLAG (Sigma-Aldrich) or HA (GenScript), and then developed with enhanced chemiluminescence (ECL) reagents (GE Healthcare) according to the manufacturer's protocol.

### 2.9. Protein determination

The protein concentrations of cell lysates were estimated by the BCA protein assay reagent (Pierce) using BSA as a standard.

### 2.10. Statistical analysis

Data are shown as mean ± SEM of at least three separate assays for gene expression experiments. Data were analyzed by ANOVA and a Tukey multiple range test to determine differences between groups, while the differences between two samples were analyzed by Student's t test. Log-rank (Mantel-Cox) test was used for the survival curves.

## 3. Results

### 3.1. Identification and characterization of a homolog of mammalian MD1 in zebrafish

Since only a homolog of mammalian MD1 has been annotated in zebrafish and since its function is unknown, we analyzed its sequence in order to clarify the evolutionary history of vertebrate MD1 and MD2. The zebrafish *Md1* had a single open reading frame (ORF) encoding a putative polypeptide of 166 amino acids, with a predicted molecular mass of 18.97 kDa. The comparison of the zebrafish Md1 with other known MD1 and MD2 sequences from different species (Fig. 1A) showed, in all cases, a higher degree of amino acid homology to the MD1 sequences (30.5–39.7% identity and 45.1–54.3% similarity) than to those of MD2 (19.1–23.0% identity, 35.1–40.5% similarity) (Table 1). Analysis of the zebrafish Md1 domain organization (Fig. 1B) further confirmed that it shared a common domain distribution with other known MD1 and MD2, including a signal peptide (amino acids 1–23) and an ML domain (MD2-related lipid-recognition domain, amino acids 43–163), which has been described as a conserved domain involved in innate immunity and lipid metabolism (Inohara and Nuñez, 2002). Furthermore, the phylogenetic tree showed that all the MD1 sequences examined formed a cluster that included teleost Md1, while MD2 genes formed a separate branch (Fig. 1C).

### 3.2. Identification and characterization of a homolog of mammalian RP105 in zebrafish

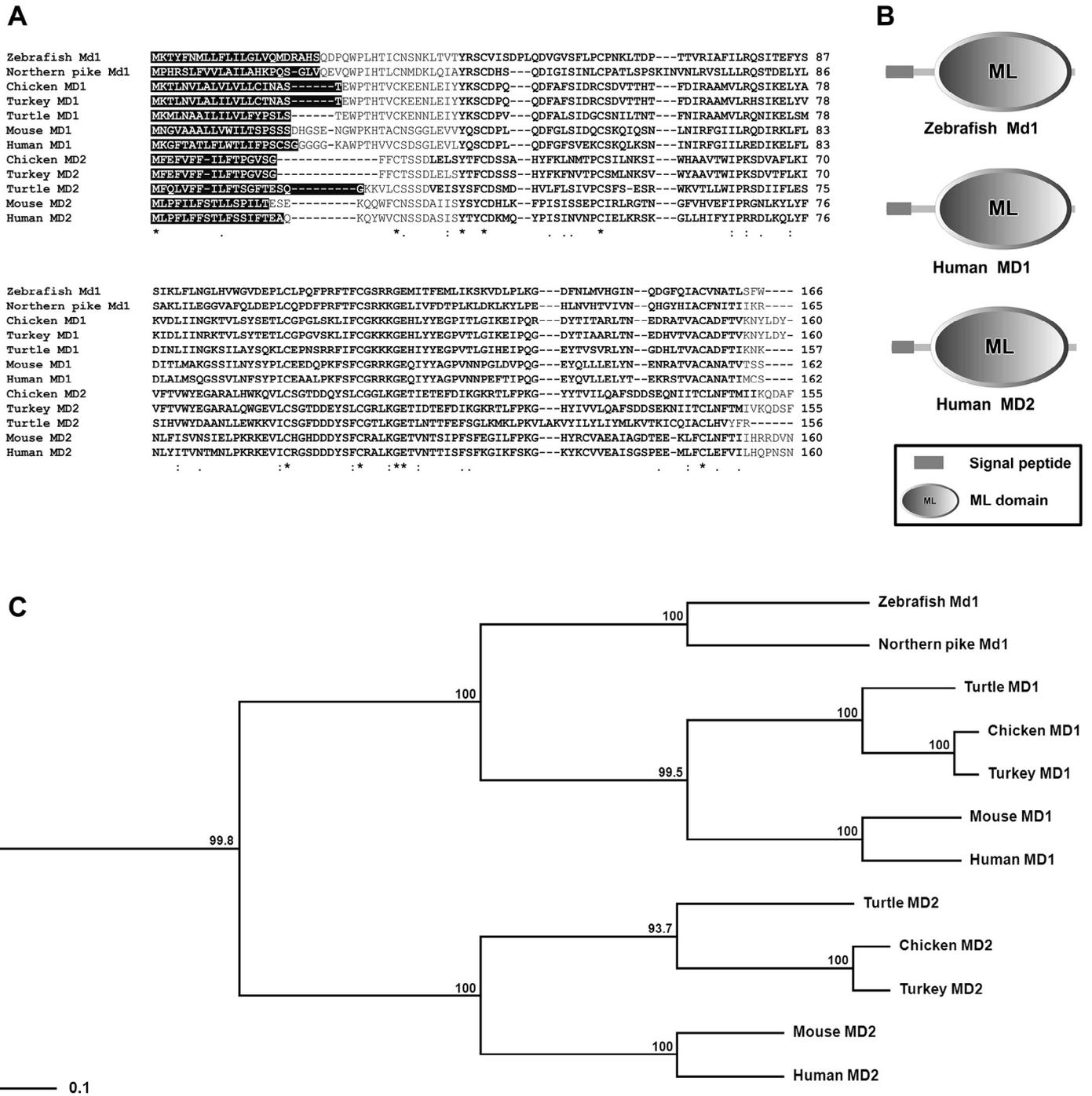
The zebrafish Rp105 had a single ORF encoding a 665 amino acid polypeptide, with a calculated molecular mass of 75.16 kDa. When this sequence was compared with other known RP105 sequences (Fig. 2A), 35.4–37.7% identity and 50.3–55.4% similarity were found (Table 2). Analysis of the zebrafish Rp105 domain organization (Fig. 2B) revealed a significant similarity with other known RP105 sequences, including a signal peptide (amino acids 1–24), seven leucine-rich repeats (LRR) and a transmembrane region (amino acids 631–653). Given the lack of any other sequences from fish to compare, the phylogenetic tree showed that the zebrafish Rp105 was settled in a branch separated from the other examined RP105 sequences (Fig. 2C).

**Table 1**

Amino acid identity and similarity between zebrafish Md1 and other vertebrate MD1 or MD2 sequences.

Species	Identity/Similarity (%)	
	MD1	MD2
<b>Teleosts</b>		
Northern pike	39.7/54.3	–
<b>Reptiles</b>		
Turtle	33.5/50.9	21.8/39.9
<b>Birds</b>		
Chicken	31.2/48.8	19.8/35.4
Turkey	31.2/45.1	23.0/35.1
<b>Mammals</b>		
Mouse	32.0/50.6	22.0/40.5
Human	30.5/47.3	19.1/39.9

Accession numbers are LN624113 for zebrafish (*Danio rerio*) Md1, C1BWW2 for northern pike (*Esox lucius*) Md1, XP\_005306030.1 and XP\_005306836.1 for western painted turtle (*Chrysemys picta bellii*, turtle) MD1 and MD2 respectively, Q90890 and E1C939 for chicken (*Gallus gallus*) MD1 and MD2 respectively, G1MXR9 and G1NG98 for turkey (*Meleagris gallopavo*) MD1 and MD2 respectively, O88188 and Q9JHF9 for mouse (*Mus musculus*) MD1 and MD2 respectively, and O95711 and Q9Y6Y9 for human (*Homo sapiens sapiens*) MD1 and MD2 respectively.

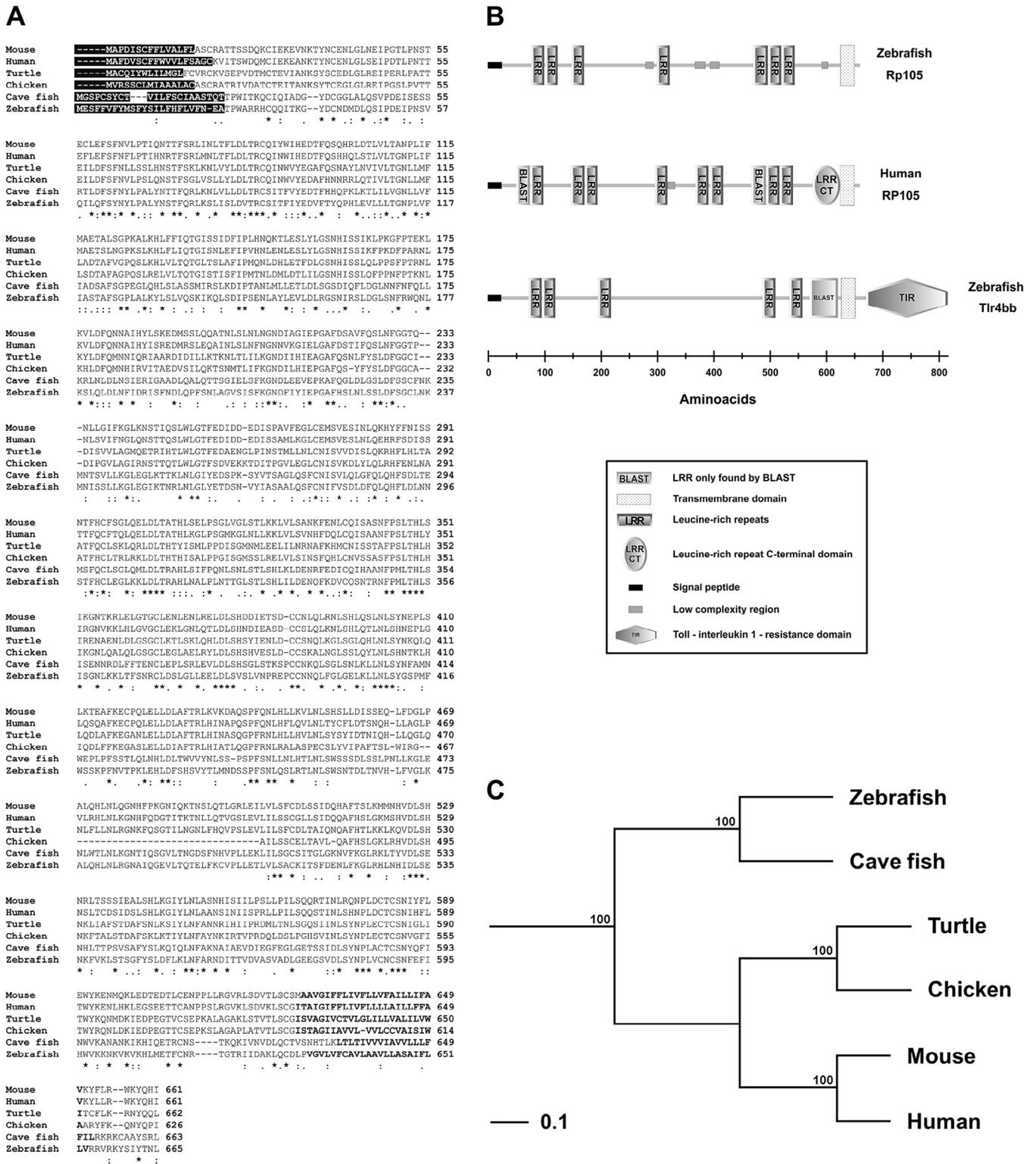


**Fig. 1.** Sequence alignment, molecular characteristics and phylogenetic relationships of zebrafish Md1 gene. (A) Multiple alignment of zebrafish Md1 sequence with other known MD1 and MD2 sequences. The predicted signal sequences are boxed and bold letters indicate the ML domain. (\*) Identity in one position; (.) conservative substitutions in one position; (:) semiconservative substitutions in one position. The accession numbers for MD1s and MD2s sequences are LN624113 for zebrafish (*Danio rerio*) Md1, C1BWY2 for northern pike (*Esox lucius*) Md1, XP\_005306030.1 and XP\_00530636.1 for western painted turtle (*Chrysemys picta bellii*, turtle) MD1 and MD2 respectively, Q90890 and E1C939 for chicken (*Gallus gallus*) MD1 and MD2 respectively, G1MXR9 and G1NG98 for turkey (*Meleagris gallopavo*) MD1 and MD2 respectively, O88188 and Q9JHF9 for mouse (*Mus musculus*) MD1 and MD2 respectively, and O95711 and Q9Y6Y9 for human (*Homo sapiens sapiens*) MD1 and MD2 respectively. (B) Diagrams showing the domain organization of zebrafish Md1, human MD1 and human MD2. The signal peptides are shown as black boxes and the MD-2-related lipid-recognition domains (ML) (SMART accession number SM00737) are shown as gray boxes. The position of each domain is indicated with respect to a ruler. (C) Phylogenetic tree of vertebrate MD1 and MD2 polypeptides. The tree was generated by the cluster algorithm using amino acid sequences. Numbers shown are percentages of 100 bootstrap replicates in which the same internal branch was observed. The horizontal lines are drawn proportional to the inferred phylogenetic distances, while the vertical lines have no significance.

3.3. Md1 interacts directly with Rp105 as well as with the two orthologs of Tlr4 present in zebrafish, Tlr4ba and Tlr4bb

Despite the absence in zebrafish of the TLR4 accessory molecule CD14, and the functional evidence showing that fish Tlr4 does

not recognize LPS (Sepulcre et al., 2009), the high homology found for zebrafish Md1 and Rp105 compared to their mammalian counterparts prompted us to study whether both proteins were able to physically interact as they do in mammals. HEK293 transfectants expressing zebrafish Md1 and Rp105, tagged with the Flag and V5



**Fig. 2.** Sequence alignment, molecular characteristics and phylogenetic relationships of zebrafish Rp105 gene. (A) Multiple alignment of zebrafish Rp105 sequence with other known Rp105 sequences. The predicted signal sequences are boxed and bold letters indicate the transmembrane domains. (\*) Identity in one position; (:) conservative substitutions in one position; and (.) semiconservative substitutions in one position. The accession numbers for Rp105 sequences are LN624114 for zebrafish (*Danio rerio*), ENSAMXG00000016677 for cave fish (*Astyanax mexicanus*), XP\_005301078.1 for western painted turtle (*Chrysemys picta bellii*, turtle), ENSGALP00000037878 for chicken (*Gallus gallus*), Q3U0U7 for mouse (*Mus musculus*), and Q99467 for human (*Homo sapiens sapiens*). (B) Diagrams showing the domain organization of zebrafish Rp105, human RP105 and zebrafish Tir4b. The signal peptides are shown as black boxes, the transmembrane domains as dotted boxes, the low complexity regions as grey boxes, the leucine-rich repeats domains (LRR) (SMART accession number SM00370, consensus sequence LGNL-TFSLQWN---RVLP---AGLFAH) as grey boxes labeled with the name of the domain, the LRR domains only found by BLAST as grey boxes labeled with the word “BLAST”, the leucine-rich repeat C-terminal domains (LRRCT) (SMART accession number SM00082) as circles, and the Toll-interleukin 1-resistance domains (TIR) (SMART accession number SM00255) as hexagons. The position of each domain is indicated with respect to a ruler. (C) Phylogenetic tree of vertebrate RP105 polypeptides. The tree was generated by the cluster algorithm using amino acid sequences. Numbers shown are percentages of 100 bootstrap replicates in which the same internal branch was observed. The horizontal lines are drawn proportional to the inferred phylogenetic distances, while the vertical lines have no significance.

**Table 2**

Amino acid identity and similarity between zebrafish Rp105 and other vertebrate RP105 sequences.

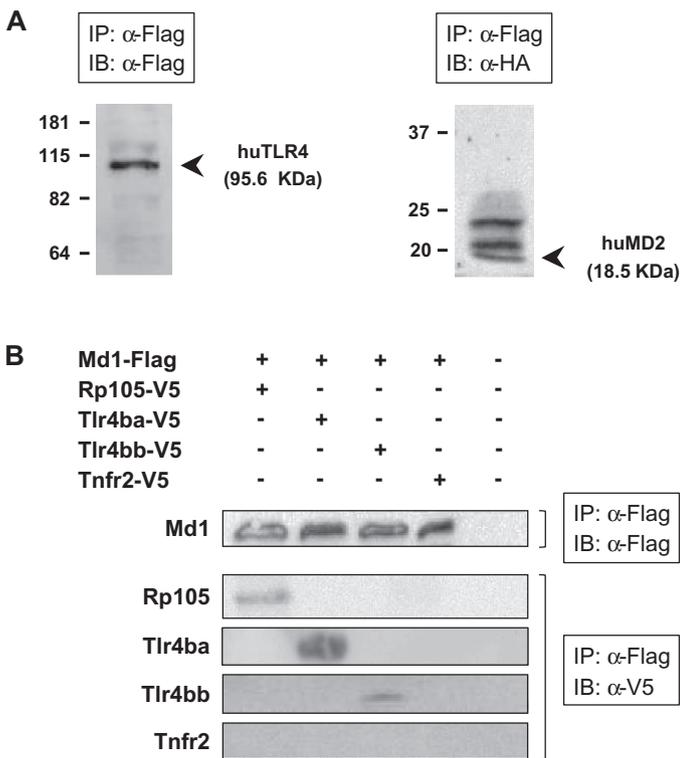
Species	Identity/Similarity (%)
<b>Reptiles</b>	
Turtle	37.7/54.9
<b>Birds</b>	
Chicken	35.4/50.3
<b>Mammals</b>	
Mouse	37.1/55.0
Human	37.6/55.4

The accession numbers are LN624114 for zebrafish (*Danio rerio*), XP\_005301078.1 for western painted turtle (*Chrysemys picta bellii*, turtle), ENSGALP00000037878 for chicken (*Gallus gallus*), Q3U0U7 for mouse (*Mus musculus*), and Q99467 for human (*Homo sapiens sapiens*).

epitopes, respectively, were used to probe the physical association between these proteins using coimmunoprecipitation assays. As expected, human TLR4 and MD2 were able to physically interact (Fig. 3a). Similarly, it was found that zebrafish Rp105 coimmunoprecipitated with Md1 (Fig. 3b). Surprisingly, both zebrafish Tlr4 orthologs also coimmunoprecipitated with Md1 (Fig. 3b), even though MD1 is not able to interact with TLR4 in the absence of MD2 in mammals (Divanovic et al., 2005b).

#### 3.4. Expression patterns of md1 and rp105 genes in zebrafish

The fact that Md1 and Rp105 interact physically led us to measure *md1* and *rp105* mRNA levels in embryos during the first stages of



**Fig. 3.** Physical interactions between zebrafish Rp105 and Md1. HEK293 cells were transfected with TLR4 tagged with the Flag epitope and the MD2 tagged with the HA epitope human expression constructs (A) or Tlr4ba-V5, Tlr4bb-V5, Rp105-V5 or Tnfr2-V5 (negative control) alone or in combination with pFLAG (empty plasmid, control for FLAG) or Md1-Flag zebrafish expression constructs. Forty-eight hours after transfection, cells were washed twice with PBS, lysed, pulled down using the ANTI-FLAG® M2 Affinity Gel and then probed with anti-HA (A) or with anti-FLAG and anti-V5 (B) mAbs. The results are representative of several independent experiments.

development (Fig. 4A) and in different adult tissues and organs (Fig. 4B). The analysis revealed that the *md1* transcript is maternally transferred since it was detected from the fertilization time, peaked at 9 hpf and was not detected again until 3 dpf. However, the *rp105* transcript was not detected until 9 hpf, showing gradually increasing levels until it peaked at 5 dpf (Fig. 4A).

The analysis of *md1* and *rp105* mRNA levels in different zebrafish adult tissues and organs showed that both genes are constitutively expressed in all cases, their expression levels being very similar in all the tissues and organs studied, including spleen, head kidney, gut and gill (Fig. 4B), which play a key role in the immune response of fish.

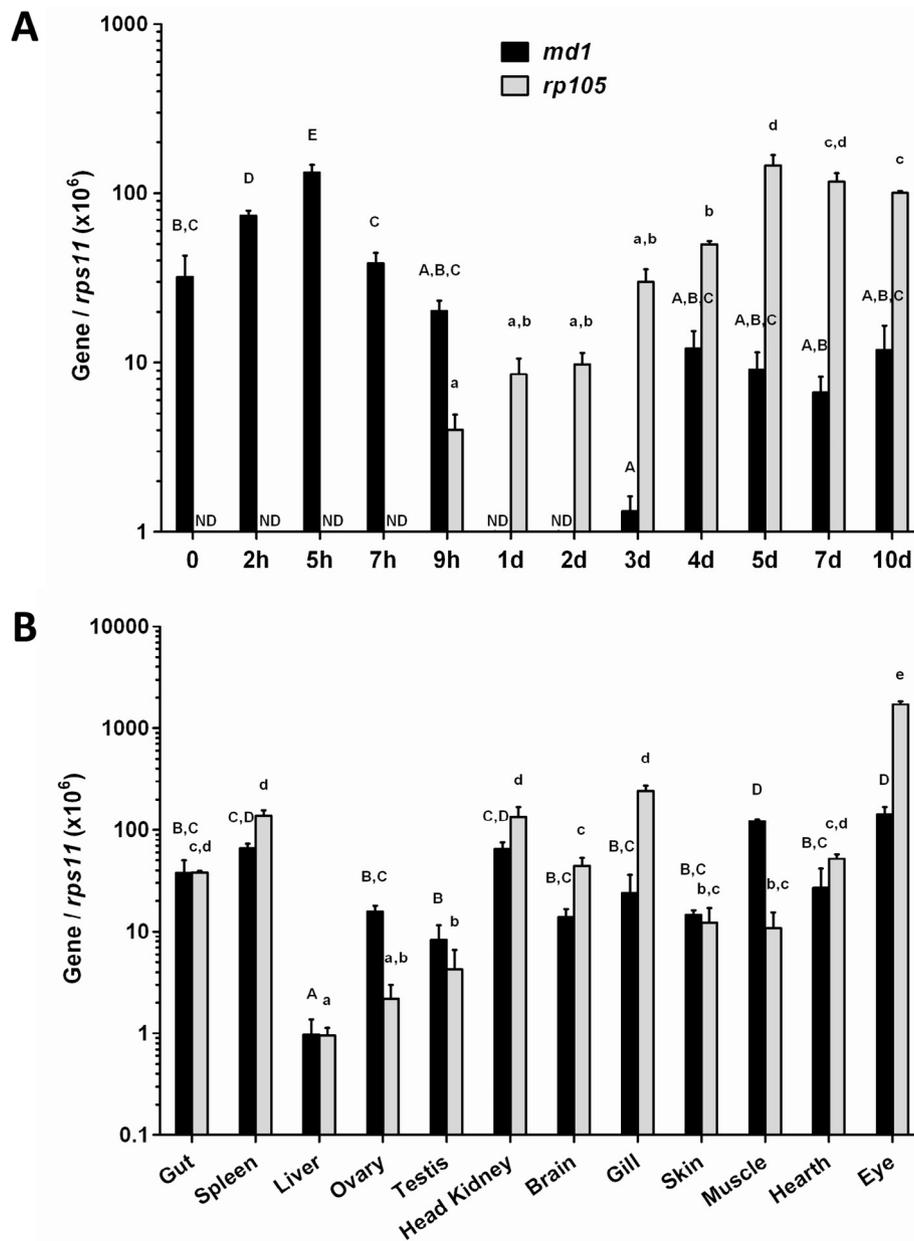
Expression studies with adult zebrafish injected i.m. with different PAMPs showed that while the *md1* mRNA levels increased at a later time point in the injection site only in fish stimulated with *E. coli* LPS (Fig. 5A), the *rp105* transcript levels rapidly increased with all PAMPs (Fig. 5B). Similarly, the *md1* and *rp105* transcripts were weakly, but significantly, modulated in the case of adult fish injected with live or heat-inactivated *S. iniae* (Fig. 5C). However, *rp105* mRNA levels decreased upon viral infection with SVCV (Fig. 5D).

#### 3.5. Md1 or Rp105 deficiency impairs the expression of genes encoding pro-inflammatory and antiviral molecules

We next examined the impact of Md1 and Rp105 on the regulation of innate immunity. Therefore, we assayed the expression of the genes encoding interleukin 1β (Il1b), prostaglandin-endoperoxide synthase 2a (Ptgs2a, also known as Cox2a), interferon phi 1 (Ifnphi1) and myxovirus (influenza) resistance b (Mxb), by using a gain (RNA) and loss of function (MOs, Supplementary Figs. S1 and S2) strategy in embryos/larvae that had been injected upon fertilization with bacterial DNA (Sepulcre et al., 2009). The results showed that the stimulation with bacterial DNA triggered the expression of all the genes studied, with the exception of *mxb*, in control larvae (Std-MO) (Fig. 6A and 6B). Interestingly, the mRNA levels of all the genes studied were strongly reduced in Md1 and Rp105 morphants in both basal and bacterial DNA stimulated conditions (Fig. 6A and 6B). In addition, overexpression of *md1* or *rp105* mRNAs rescued the impaired induction of *ifnphi1* in Md1 and Rp105 morphants, respectively (Fig. 6C). Notably, overexpression of both mRNAs simultaneously resulted in increased transcript levels of *il1b* upon poly I:C and flagellin stimulation, and *ifnphi1* upon poly (I:C) stimulation (Fig. 6D). These results indicate that Md1 and Rp105 play a key role in the activation of the immune system in response to viral and bacterial stimuli.

#### 3.6. Md1 and Rp105 deficiency results in increased susceptibility to viral infection

The impaired antiviral response of Md1 and Rp105 morphants prompted us to examine their viral susceptibility. The survival analysis in SVCV infection assays revealed a significantly increased susceptibility in single and double Md1 and Rp105 morphants compared to control fish (20% vs. 40% survival at 7 dpi) (Fig. 7A). Notably, the overexpression of Md1 or Rp105 mRNAs separately was unable to reverse the increased susceptibility of Md1 and Rp105 morphants (Fig. 7B), further indicating their non-redundant role. In addition, the overexpression of Md1, Rp105 or Md1+Rp105 mRNAs failed to increase the resistance to the virus (data not shown). However, the simultaneous overexpression of Md1 and Rp105 was able to fully rescue the increase susceptibility of the double morphants (Fig. 7B), confirming the specificity of the MOs used and that both molecules act together to regulate the antiviral response and viral clearance.



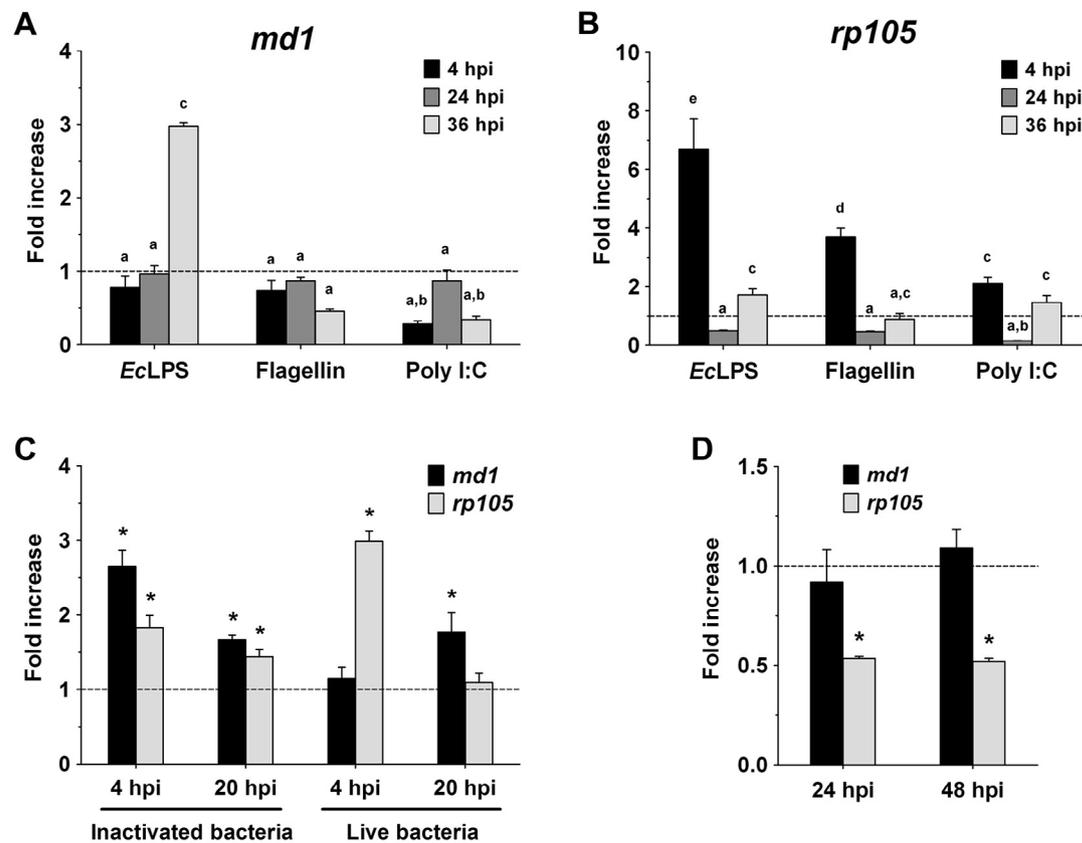
**Fig. 4.** mRNA levels of zebrafish *md1* and *rp105* during the first stages of development and in different adult organs. (A) Zebrafish embryos/larvae were collected at different times post-fertilization, pooled and the expression of *md1* and *rp105* genes was measured by RT-qPCR. (B) Different tissues and organs were obtained from adult zebrafish and the expression of *md1* and *rp105* genes was measured by RT-qPCR in those samples. In both cases, the gene expression is normalized against *rps11*, and the different letters denote statistically significant differences among the groups ( $p < 0.05$ ).

#### 4. Discussion

RP105 was identified as a murine B cell surface molecule that transmits an activation signal to B cells following ligation with anti-RP105 mAbs, leading to protection of B cells from irradiation- or dexamethasone-induced apoptosis, and to B cell proliferation (Miyake et al., 1995). MD1 was later found to be associated with RP105 in B cells and to regulate its surface expression in mouse (Miyake et al., 1998) and human (Miura et al., 1998). However, it was then reported that the RP105/MD1 complex was not specific to B cells but mirrors the expression of TLR4 in macrophages and dendritic cells (Divanovic et al., 2005b). In addition, the RP105/MD1 complex interacted directly with the TLR4 signaling complex, inhibiting its ability to bind LPS (Divanovic et al., 2005b). Although additional functions of this complex have not been extensively examined, a recent

study added to the mystery since it showed that the RP105/MD1 complex cooperates with TLR4 to promote proliferation and IgM-secreting plasma cell differentiation of B cells in response to lipid A and LPS (Nagai et al., 2012).

Despite the conservation of various TLR families from fish to mammals (Jault et al., 2004; Meijer et al., 2004; Palti, 2011; Roach et al., 2005), several differences have been noted, such as the existence of fish-specific TLRs (Roach et al., 2005), the absence in fish of the crucial accessory molecules Md2 and Cd14 for TLR4 signaling (Iliev et al., 2005; Pietretti et al., 2013), the absence of Tlr4 in some fish species, such as the fugu (Roach et al., 2005), and the confirmation that LPS does not signal through Tlr4 in zebrafish (Sepulcre et al., 2009; Sullivan et al., 2009). In the present study, we have identified the Rp105 and Md1 homologs in zebrafish and demonstrated their regulation by different immune challenges. These results



**Fig. 5.** mRNA levels of *md1* and *rp105* in adult zebrafish injected with PAMPs, bacteria or virus. (A, B) Adult zebrafish were injected with 5  $\mu$ g *E. coli* LPS, 0.1  $\mu$ g flagellin or 2.5  $\mu$ g poly I:C in the left epaxial muscle. The expression of *md1* (A) and *rp105* (B) was measured by RT-qPCR in the injection site at 4, 24 and 36 hours post-injection. Different letters denote statistically significant differences between the groups ( $p < 0.05$ ). (C) Adult zebrafish were injected i.p. with heat-killed or alive *S. iniae*. Fish were dissected, and the expression of *md1* and *rp105* was measured by RT-qPCR in head kidney at 4 and 20 hpi. (D) Adult zebrafish were infected i.p. with the SVCV and at 24 and 48 hpi the head kidney were collected and the expression of *md1* and *rp105* measured by RT-qPCR. In all cases, the gene expression is normalized against *rps11* and is shown as relative to the mean of PBS-injected larvae. The results are representative of two independent experiments. \* $p < 0.05$ .

confirm those of a previous *in silico* study that suggested that Rp105 and Tlr4 evolved from a common ancestor before the divergence between fishes and tetrapods about 450 million years ago (Iliev et al., 2005). They further reveal the presence of Md1 in teleost fish but the lack of Md2, suggesting that the divergence of these accessory molecules occurred in the tetrapod lineage. Notably, the pull-down experiments indicate that zebrafish Md1 is able to physically interact with both Rp105 and Tlr4, in contrast to mammalian MD1 which fails to associate with TLR4 in the absence of MD2 (Divanovic et al., 2005b). Collectively, these results suggest that zebrafish Md1 might regulate the activity of both Rp105 and Tlr4. However, the absence of Md2 in fish and the failure of Tlr4 to recognize LPS, together with the tolerance of fish to relatively high concentrations of LPS, suggest that Md1 is not involved in the recognition of LPS in this group of animals. Curiously, however, the highest induction of *rp105* and *md1* was observed in fish stimulated with LPS.

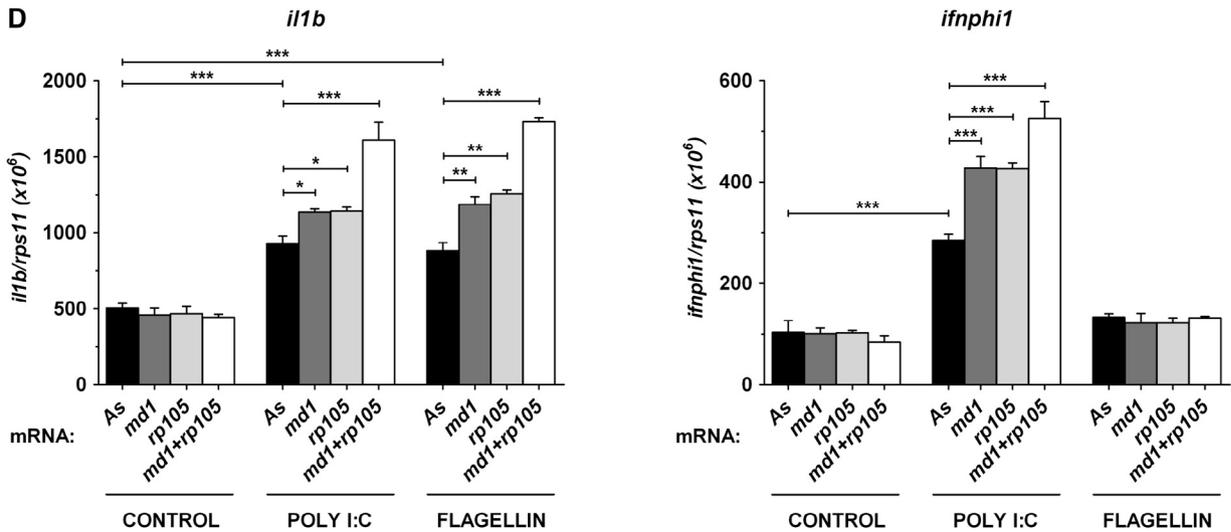
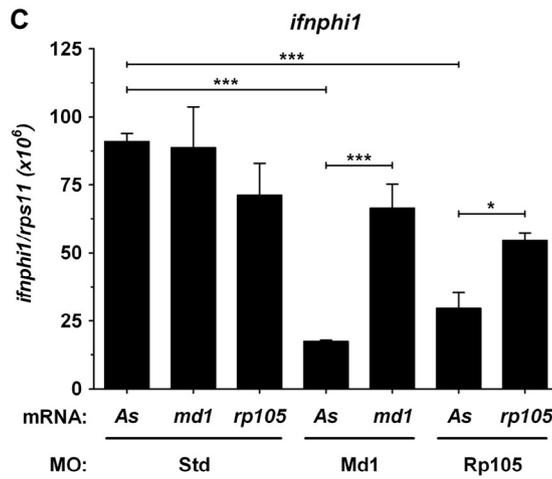
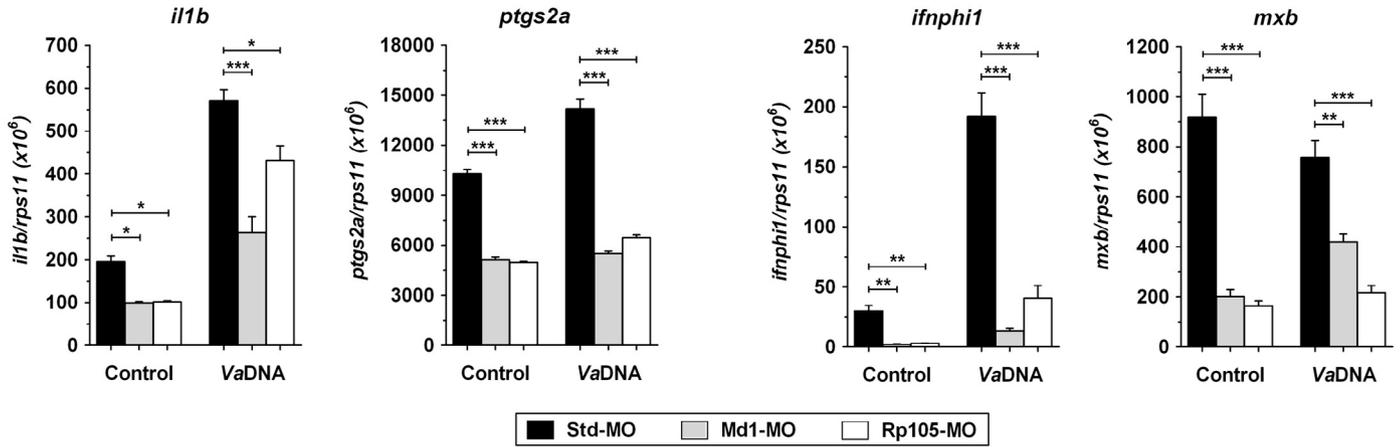
This puzzling scenario led us to investigate the function of Rp105 and Md1 using gain- and loss-of-function strategies in zebrafish. Surprisingly, genetic inactivation of either Rp105 or Md1 resulted in reduced transcript levels of the genes encoding key pro-inflammatory and antiviral molecules in basal conditions and upon stimulation of the fish with different PAMPs, known to signals via different TLRs and accessory molecules, i.e. TRIF- (poly I:C) vs. Myd88-dependent (bacterial DNA and flagellin) signaling pathways (Fan et al., 2008; Sepulcre et al., 2009). In addition, while overexpression of either molecule alone resulted in a weak induction of pro-inflammatory *il1b* and antiviral *ifnphi1* in response to

PAMP stimulation, the co-expression of both molecules resulted in a stronger induction of these genes, further suggesting that zebrafish Rp105 and Md1 act as a signaling complex, but which is not directly involved in the recognition of PAMPs. In addition, Rp105 and/or Md1 deficiencies increased the susceptibility of zebrafish larvae to viral infection. More importantly, this higher susceptibility of Rp105/Md1 morphants to viral infection was reversed by the simultaneous expression of *rp105* and *md1* RNAs but not by the expression of either of these molecules alone. These results are not completely unexpected, since it has been shown that RP105-deficient mice suffer the accelerated onset and increased severity of arthritis, concomitant with increased IFN $\gamma$  and TNF $\alpha$  production by spleen cells (Tada et al., 2008). Notably, RP105-deficient mice also showed more severe arthritis induced by collagen when injected with IFA, suggesting that endogenous TLR ligands play a role in this phenomenon (Tada et al., 2008). Furthermore, another study has recently reported that murine RP105 is involved in macrophage activation by Pam3CSK4 through TLR2 signaling and that this signaling is even able to overcome the RP105-mediated regulation of TLR4 signaling (Liu et al., 2013). These data, therefore, suggest that RP105 is an essential accessory molecule for immune responses through TLR2 signaling. Unfortunately, none of these studies investigated whether Md1 is required for Rp105 functions.

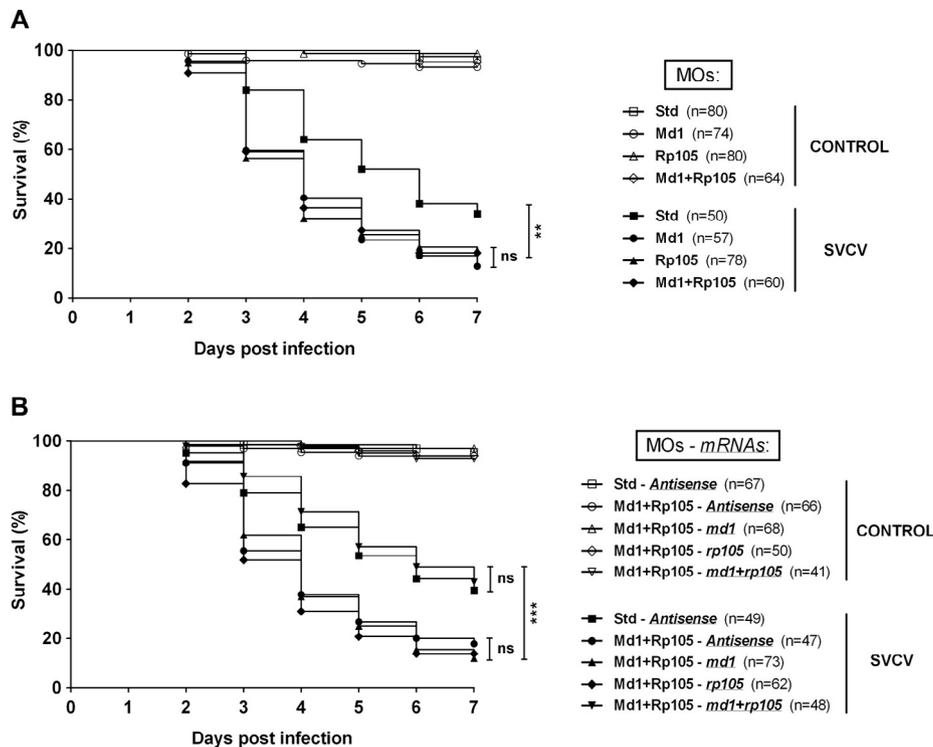
In conclusion, we have identified and characterized the zebrafish homologs of the mammalian Rp105 and Md1. Their physical interaction and their non-redundant role in regulating the expression of genes encoding pro-inflammatory and antiviral mediators,

**A: Pro-inflammatory molecules**

**B: Antiviral molecules**



**Fig. 6.** Md1 or Rp105 deficiency impairs the expression of genes encoding pro-inflammatory and antiviral molecules. Zebrafish one-cell embryos were injected with standard control (Std), Md1 or Rp105 MOs alone or in combination with antisense (As), *md1*, *rp105* or *md1+rp105* mRNAs in the presence of PBS (control), 6.5 ng VaDNA, 0.03 ng flagellin or 1.2 ng poly I:C. The expression of the genes encoding the pro-inflammatory *il1b* (A, D) and *ptgs2a* (A), and antiviral *ifnphi1* (B, C, D) and *mxb* (B), was measured at 48 hpi by RT-qPCR in pooled larvae. The results are representative of three independent experiments. \*p < 0.05; \*\*p < 0.01; \*\*\*p < 0.001.



**Fig. 7.** Md1 or Rp105 deficiency results in increased susceptibility to viral infection. Survival of zebrafish larvae injected at the one-cell stage with standard control (Std), Md1, Rp105 or Md1+Rp105 MOs, alone (A) or in combination with antisense (As), *md1*, *rp105* or *md1+rp105* mRNAs (B). In both cases, larvae were challenged by immersion with  $2.5 \times 10^7$  to  $10^8$  TCID<sub>50</sub>/ml SVCV at 3 days post-fertilization. mRNA names are shown in *italics* and underlined in the legend to facilitate interpretation of the graph. ns, not significant; \* $p < 0.05$ ; \*\* $p < 0.01$ ; \*\*\* $p < 0.001$ .

together with their involvement in viral immunity, uncover a previously unappreciated function of these molecules in the regulation of innate immunity. This paves the way for future studies aimed at clarifying their complex role in the immune defense of vertebrates.

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## Appendix: Supplementary material

Supplementary data to this article can be found online at doi:10.1016/j.dci.2015.01.005.

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