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Contrasted Innate Responses to Two Viruses in Zebrafish: Insights into the Ancestral Repertoire of Vertebrate IFN-Stimulated Genes

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Ease of imaging and abundance of genetic tools make the zebrafish an attractive model host to understand host–pathogen interactions. However, basic knowledge regarding the identity of genes involved in antiviral immune responses is still lagging in this species. We conducted a microarray analysis of the larval zebrafish response to two models of RNA virus infections with very different outcomes. Chikungunya virus (CHIKV) induces a rapid and protective IFN response. Infection with infectious hematopoietic necrosis virus is lethal and is associated with a delayed and inefficient IFN response. A typical signature of IFN-stimulated genes (ISGs) was observed with both viruses, but was stronger for CHIKV. We further compared the zebrafish and human ISG repertoires and made a genomic and phylogenic characterization of the main gene families. We describe a core set of well-induced ISGs conserved across vertebrates, as well as multigenic families diversified independently in each taxon. The conservation of ISGs involved in antiviral signaling indicates conservation of the main feedback loops in these pathways. Whole-mount in situ hybridization of selected transcripts in infected larvae revealed a typical pattern of expression for ISGs in the liver, gut, and blood vessels with both viruses. We further show that some inflammatory genes were additionally induced through IFN-independent pathways by infectious hematopoietic necrosis virus and not by CHIKV. This study provides a useful reference set for the analysis of host–virus interactions in zebrafish and highlights the differences between protective and non-protective antiviral innate responses. The Journal of Immunology, 2014, 192: 4328–4341.

Viruses are ubiquitous pathogens, and all living organisms possess antiviral defenses. The evolution of these mechanisms, mirroring that of viral strategies, is fast and complex. Among metazoans, invertebrates appear to rely mainly on RNA interference–based mechanisms. In contrast, the innate antiviral defenses of vertebrates seem largely mediated by proteins, the expression of which is orchestrated by a group of cytokines known as IFNs. In mammals, type 1 IFNs directly induce the expression of a few hundred genes collectively known as IFN-stimulated genes (ISGs) (1, 2). ISGs encode a variety of proteins with diverse biochemical properties and functions (3, 4). Some have been shown to exert a direct antiviral role (e.g., MX proteins, PKR, TRIM5), which may act at any stage of the viral cycle. Others contribute to antiviral signaling, generating positive (e.g., STAT1, IFN regulatory factor [IRF] 7, TRIM25) or negative (e.g., SOCS proteins) feedback loops. A few ISGs also attract or activate cells with specialized roles in antiviral defense (e.g., CXCL9–11). The function of most, however, remains elusive. Recent approaches based on loss- and gain-of-function screens, performed on immortalized cell lines, allowed rapid progress of this field (2, 5). Nevertheless, the use of model organisms will be critical to unravel the role of these proteins, especially as many ISGs may be predicted to exert complex activity involving cell–cell cooperation or regulatory functions that cannot be detected by in vitro screening approaches. The quick evolution of most of these genes may complicate the transposition from model species to humans. However, the patterns of evolution of ISG families also convey functional information, as they bear witness to the evolutionary pressures in the arms race between host and pathogens. For example, host factors that directly interact with viral proteins

The microarray design and dataset presented in this article have been submitted to the Gene Expression Omnibus database (http://www.ncbi.nlm.nih.gov/geo) under accession numbers GPL7735 and GSE47057.

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Abbreviations used in this article: CHIKV, Chikungunya virus; dpf, days postfertilization; FC, fold change; hps, hour(s) postinfection; IFITM, IFN-inducible transmembrane protein; IHNV, infectious hematopoietic necrosis virus; IPA, Ingenuity Pathway Analysis; IRF, IFN regulatory factor; ISG, IFN-stimulated gene; RLR, RIG-I–like receptor; WISH, whole-mount in situ hybridization.

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may be predicted to evolve under positive selection, and much faster than others implicated in a cascade where they contact only other host proteins (6). Family expansions may also be expected to arise from such evolutionary pressures. Importantly, to draw functionally meaningful conclusions, it is necessary to determine whether ISG orthologs present in distant genomes are indeed IFN and/or virus-inducible in the corresponding species.

Several features of the zebrafish (Danio rerio) make this model attractive for functional characterization of ISGs. The relatively basal position of the tetrapod–teleost split in the vertebrate tree makes the phylogenetic comparisons highly relevant, and functional testing is facilitated by the optical and genetic tractability of the zebrafish larva. The zebrafish has therefore recently gained acceptance as an immunological model and has been used to study host–pathogen interactions in a variety of bacterial or viral infections (7–9). In recent studies, we have conducted detailed analyses of two experimental viral infections in zebrafish larvae. The fish nothvhabdovirus infectious hematopoietic necrosis virus (IHNV), a negative strand RNA virus that belongs to the Rhabdoviridae family, is systematically lethal in 3–4 d after inoculation, and primarily targets vascular endothelial cells; the host’s IFN response occurs too late to protect the larvae (10). In contrast, the human alphavirus Chikungunya virus (CHIKV), a positive strand RNA virus that belongs to the Togaviridae family, induces a powerful IFN response, which allows larvae to recover from the infection despite a strong early replication (11). A number of virus-induced genes have been identified in zebrafish in our and other’s work, but mostly based on homology approaches; no systematic study has been published so far in this species. Although IHNV and CHIKV are not natural viruses of zebrafish, none of which has been characterized so far, the comparison of the highly contrasted host responses to these two pathogens should provide useful insight as to the prototypical antiviral response of zebrafish to viruses. In particular, because CHIKV induces a very strong host IFN response, we expect a rather complete set of ISGs to be induced during this infection; the functional relevance of this response is demonstrated by the fact that knocking down IFN receptors results in death of CHIKV-infected larvae (11). In contrast, the inefficient host response to IHNV may arise from a generally lower IFN response, or from a selective blockade of a subset of ISGs; comparing the two responses will allow us to test this hypothesis. A global analysis of the host response to both viruses would also reveal possible additional response pathways to these viruses, which may interfere or synergize with the IFN response.

Large-scale analyses of transcriptome response to viral infection have been carried out in a number of other fish species, including Atlantic salmon head kidney cell response to SAV-1 (12), Atlantic salmon heart response to piscine myocarditis virus (13), orange-spotted grouper spleen response to Singapore grouper iridovirus (14), response of the grouper kidney cell line infected by betanodavirus (15), and Atlantic cod brain response to nervous necrosis virus (16). These studies generally addressed the transcriptional response of a particular tissue after infection with a given virus, and resulted in overlapping but largely distinct sets of modulated genes. To target the largest number of ISGs, these analyses were often performed with lymphoid tissues (i.e., spleen or kidney), thus revealing transcriptome modifications, which reflect mixed innate and adaptive responses in complex cellular contexts. Additionally, other studies have compared different states of transcriptome modulation between different conditions: Purcell et al. (17) studied the impact of infection with high and low virulence IHNV on kidney cells, whereas Martin et al. (18) compared the response of the salmon macrophage-like cell line SHK-1 to IFN type I (aka IFN-α) and type II in one of the first comprehensive microarray-based ISG screenings in fish (18).

To produce a comprehensive description of the genes modulated by the virus infection in the whole zebrafish larva, we chose to follow the modifications of the transcriptome of the whole larva, using the validated microarray platform available in this species (19). At the selected developmental stage (5 d postfertilization [dpf]), cells of the adaptive immunity are still absent and cannot skew the general description of the response. We carried out a comparative transcriptome analysis of the two highly contrasted infection models, IHNV and CHIKV. We then analyzed the kinetics and spatial expression of a series of genes, identifying a conserved pattern of expression for ISGs at the scale of the whole organism. Finally, we performed a genome-wide analysis of ISG-containing gene families.

Materials and Methods

Ethics statement

All animal experiments described in the present study were conducted at the Institut Pasteur according to European Union guidelines for handling of laboratory animals (http://ec.europa.eu/environment/chemicals/lab_animals/home_en.htm) and were approved by the Direction Sanitaire et Vétérinaire de Paris under permit B-75-1061.

Zebrafish husbandry

Wild-type AB zebrafish, initially obtained from the Zebrafish International Resource Center (Eugene, OR), were raised according to standard procedures (20). Eggs obtained by natural spawning were bleached and raised at 28°C in Volvic source water. When eggs were used for imaging purposes, 1-phenyl-2-thiourea (Sigma-Aldrich, 0.003% final) was added at 24 h postfertilization to prevent melanin pigment formation. At 3 dpf, just before infections, larvae that had not hatched spontaneously were manually dechorionated.

Viruses and experimental infections

IHNV strain 25,70 and CHIKV strain 115 were produced as described previously (10, 11), and injections and handling of larvae were performed as described in Levraud et al. (20). Doses of ~100 PFU/50% tissue culture–infected dose were injected in a volume of 1 nl, either i.v. in the caudal vein or aorta, posterior to the urogenital opening, or in the center of the yolk syncytial cell. Control larvae were injected with PBS. Larvae were then incubated at 24°C (for IHNV) or 28°C (for CHIKV).

Microarrays design

The microarray slides were custom-designed by Agilent Technologies. They contained in total 43,371 probes (60-mer oligonucleotides). Of these probes, a total of 21,496 probes were identical to the probes present on the Agilent probe set that is commercially available (Agilent Technologies, catalog no. 013232_D). Most of the additional probes were designed using the eArray software from Agilent Technologies (http://earray.chem.agilent.com/earray). Settings used were based on the following settings: base composition methodology, best probe methodology, and design with 3 bias. The Agilent D. rerio transcriptome was used as a reference. A small number of probes were manually designed based on knowledge of particular polymorphisms for genes encoding protein families such as 14-3-3 proteins, chitinase-like proteins, and TLRs to obtain gene-specific probes. The microarray design has been submitted to the Gene Expression Omnibus database (http://www.ncbi.nlm.nih.gov/geo) under accession no. GPL7735. The following procedure was followed to get an updated defined annotation: 1) Ensembl biomart web interface (http://www.ensembl.org/biomart/) was used to extract the relationship between oligonucleotide identifiers and genes defined in Zv9 assembly (Ensembl gene identifier; gene symbol information). The extraction was restricted to oligonucleotides spotted on either the Agilent G2518A, “Spahn leiden 2.0,” or “Spahn leiden 3.0” microarray platform. 2) Oligonucleotide sequences were mapped on cDNA sequences corresponding to these genes using the alignment software bowtie (21), allowing fewer than three mismatches. All alignments were taken into account (using bowtie options “-a –best”). 3) We built a “zebrafish transcriptome” reference database with D. rerio RefSeq sequences. Oligonucleotide
sequences were again mapped on this sequence database using bowtie-
alignment software as above. Finally, all relationships between oligo-
nucleotide probes and Ensembl genes were gathered in a unique ano-
tation file, aggregating results from BioMart extraction, mapping of
probes on Ensembl transcripts, and mapping of probes against RefSeq
sequences.

RNA isolation, labeling, and hybridization
RNA was isolated from pooled larvae using TRizol (Invitrogen), following
the manufacturer’s protocol. The integrity of the RNA was confirmed by
laboratory-on-chip analysis using the 2100 Bioanalyzer (Agilent Technolo-
gies), using only samples with an RNA integrity number of at least 8. La-
beling and hybridization were performed as described in van Soest et al. (22).

Microarrays analysis
Statistical analysis was performed using R software (http://www.r-project.
org/). For probes linked to the same Ensembl gene, the average of signals
was considered. A lowess normalization was operated for each micro-
array using Limma R package. Limma functions were used to perform
differential expression analysis. Raw p values have been adjusted
using the Benjamini–Hochberg procedure. The entire dataset is available
at the National Center for Biotechnology Information Gene Expression
Omnius (http://www.ncbi.nlm.nih.gov/geo/info/linking.html; accession
no. GSE47057).

cDNA synthesis and quantitative real-time PCR
cDNA was synthesized using Moloney murine leukemia virus reverse
transcriptase (Promega) and dT17 primer. Real-time quantitative PCR was
then performed using the SYBR Green system (Applied Biosystems) in
triplicate wells on an ABI3700 system (Applied Biosystems). Amplifica-
tion yield was assessed in a titration assay (23) and used to deduce relative
transcript amounts. Absolute quantification was performed by comparing
levels in a reference cDNA mix with purified DNA fragments containing
transcript amounts. Absolute quantification was performed by comparing
the target sequence quantified by UV spectrophotometry; this reference
was considered. A lowess normalization was operated for each micro-
array using NucAway spin columns (Ambion). Sequences of morpholinos
are indicated close to the branches. The evolutionary history was inferred using the maximum likelihood
method with the JTT matrix–based model, pairwise deletion, and default
parameters of Mega5 (27). The bootstrap consensus trees computed from
1000 replicates are shown with branches to scale, and the percentage of
trees in which the associated taxa clustered together in the bootstrap test
are indicated close to the branches.

Results
Microarray analysis of the zebrafish larva response to IHNV and CHIKV
To compare responses to the highly IFN-inductive, nonlethal
CHIKV and to the lethal fish rhabdovirus IHNV, we performed

Table I. Primers used in this study

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Primer (5′ to 3′)</th>
<th>Reverse Primer (5′ to 3′)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCR primers</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ef1a</td>
<td>GCTGACTGTTGAGCTCAGCAA</td>
<td>ACGACTTGGACCTCTGCTGG</td>
<td>(10)</td>
</tr>
<tr>
<td>ifng1 (secreted)</td>
<td>GAGGATCCACATATTGACCTT</td>
<td>GGTCCACCACTTGGCCTG</td>
<td>(10)</td>
</tr>
<tr>
<td>ifng1 (intracellular)</td>
<td>ACGGACGCTGAAATTACCTT</td>
<td>GCTGCCACCTTGGGCTG</td>
<td>(10)</td>
</tr>
<tr>
<td>ifng3</td>
<td>GAGGACATAGCTCTTGGTGT</td>
<td>ACTGGCATGAGCCAACC</td>
<td>(10)</td>
</tr>
<tr>
<td>N-IHNV</td>
<td>CACTGAGCTGTAGACTCTACCTACCCAA</td>
<td>CTGGCAGAGTGCTGTTGGT</td>
<td>(11)</td>
</tr>
<tr>
<td>rsad2/ripen</td>
<td>TTGCGAAGAAGACGAGATGTC</td>
<td>AACACTGAGAGACACC</td>
<td>(11)</td>
</tr>
<tr>
<td>E1-CHIKV</td>
<td>AAATGCGACGCTTCCCTCTAGA</td>
<td>TCGATCGAGACAGGC</td>
<td>(24)</td>
</tr>
<tr>
<td>trin25</td>
<td>GAGCAGCCTCCCTCAAACC</td>
<td>ATCAATTTGCAAGCATGC</td>
<td></td>
</tr>
<tr>
<td>isg15</td>
<td>ACCTCGTGCAAGCTGCTGAC</td>
<td>TGGGACATGAAATGTC</td>
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<tr>
<td>isg12.2</td>
<td>CTCGAGATGCTCATAGCTT</td>
<td>TGACAGTGCCGCTGAGAA</td>
<td></td>
</tr>
<tr>
<td>isf1b</td>
<td>CGCGGAGATGCTCCCTCTA</td>
<td>TGGCGAGATGCTTAAAGA</td>
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<tr>
<td>c4b</td>
<td>CCAGACTGAGTGGTGT</td>
<td>ACGACTGAGTGGTGT</td>
<td></td>
</tr>
<tr>
<td>e6p</td>
<td>TGAAATGCTGCCTTTCCACAGGG</td>
<td>GATGCACCCCTCCCTGGAA</td>
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</tr>
<tr>
<td>mmp9</td>
<td>AGATCGATCGGTAGATGTCTG</td>
<td>GTCCCAAGCTTGTGAC</td>
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</tr>
<tr>
<td>ifb5p</td>
<td>ATTTCCGAGAGTGGCAC</td>
<td>TCCAGTTGCAAGCGACA</td>
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<td>Primers for antisense WISH probe production</td>
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<tr>
<td>isg15</td>
<td>ATTTCCGAGAGTGGCAC</td>
<td>TCCAGTTGCAAGCGACA</td>
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<tr>
<td>E1-CHIKV</td>
<td>AAATGCGACGCTTCCCTCTAGA</td>
<td>TCGATCGAGACAGGC</td>
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</tr>
<tr>
<td>e6p</td>
<td>TGACAGTGCCGCTGAGAA</td>
<td>GATGCACCCCTCCCTGGAA</td>
<td></td>
</tr>
<tr>
<td>rsad2</td>
<td>GTGACATCAGACATAGCTG</td>
<td>AACATTTGCAAGCATGC</td>
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</tr>
<tr>
<td>isg12.2</td>
<td>ATTTCCGAGAGTGGCAC</td>
<td>TCCAGTTGCAAGCGACA</td>
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</tr>
<tr>
<td>Morpholinos</td>
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<tr>
<td>e6f1b</td>
<td>CCGCAGAGTACATACCTCTTGGAAGTAGAA</td>
<td>(splice morpholino)</td>
<td></td>
</tr>
<tr>
<td>e6f2b</td>
<td>CCTAGAAATCCTTCACCTCAGTGTAAC</td>
<td>(splice morpholino)</td>
<td></td>
</tr>
<tr>
<td>control</td>
<td>GAAGACATCGACTGCGATCCTGACTGCA</td>
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<td></td>
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### Table II. Selection of up- and downregulated genes after CHIKV and/or INHV infection

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Description</th>
<th>Ensembl Gene ID</th>
<th>CHIKV FC and Adjusted p Value</th>
<th>INHV FC and Adjusted p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Upregulated genes</strong></td>
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<td></td>
<td></td>
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<td>IFN response</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>isg12-2</td>
<td>IFN-stimulated gene 12-2</td>
<td>ENSDARG00000039243</td>
<td>143.9****</td>
<td>6.7*</td>
</tr>
<tr>
<td>isg12-1</td>
<td>IFN-stimulated gene 12-1</td>
<td>ENSDARG00000017489</td>
<td>83.0****</td>
<td>4.8*</td>
</tr>
<tr>
<td>isg15</td>
<td>IFN-stimulated gene 15</td>
<td>ENSDARG00000086374</td>
<td>40.2****</td>
<td>5.4*</td>
</tr>
<tr>
<td>rsad2</td>
<td>Radical S-adenosyl methionine domain containing 2; viperin</td>
<td>ENSDARG00000004952</td>
<td>9.8**</td>
<td>3.8*</td>
</tr>
<tr>
<td>IFIT1</td>
<td>IFN-induced (tetratricopeptide)</td>
<td>ENSDARG00000007102</td>
<td>5.3**</td>
<td>2.7*</td>
</tr>
<tr>
<td>IFIT2</td>
<td>IFN-induced (tetratricopeptide)</td>
<td>ENSDARG00000057173</td>
<td>6.5*</td>
<td>1.6</td>
</tr>
<tr>
<td>drun1</td>
<td>Damage-regulated autophagy; viγ6</td>
<td>ENSDARG00000045581</td>
<td>5.0**</td>
<td>0.6</td>
</tr>
<tr>
<td>jf35</td>
<td>IFN-induced protein 35</td>
<td>ENSDARG00000075643</td>
<td>3.6**</td>
<td>1.1</td>
</tr>
<tr>
<td>isg12-4</td>
<td>IFN-stimulated gene 12-4</td>
<td>ENSDARG00000078389</td>
<td>7.3**</td>
<td>2.0</td>
</tr>
<tr>
<td>isg12-3</td>
<td>IFN-stimulated gene 12-3</td>
<td>ENSDARG00000074217</td>
<td>13.7****</td>
<td>1.6</td>
</tr>
<tr>
<td>3'UTR isg12-7</td>
<td>Similar to IFN-stimulated gene 12-7</td>
<td>A_15_P118496</td>
<td>4.1**</td>
<td>1.9*</td>
</tr>
<tr>
<td>Ifi441</td>
<td>IFN-induced protein 44-like</td>
<td>ENSDARG00000010729</td>
<td>9.1**</td>
<td>1.7*</td>
</tr>
<tr>
<td>mxa</td>
<td>IFN-induced GTP binding protein Mxa</td>
<td>ENSDARG00000021688</td>
<td>9.8**</td>
<td>2.1*</td>
</tr>
<tr>
<td>mxe</td>
<td>IFN-induced GTP binding protein Mxe</td>
<td>ENSDARG00000014427</td>
<td>8.2**</td>
<td>2.5*</td>
</tr>
<tr>
<td>Complement</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C4b</td>
<td>Complement component 4B</td>
<td>ENSDARG00000038424</td>
<td>5.1****</td>
<td>3.0*</td>
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<tr>
<td>MMP and signaling</td>
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<td></td>
<td></td>
<td></td>
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<tr>
<td>mmp9</td>
<td>Matrix metalloproteinase 9</td>
<td>ENSDARG00000042816</td>
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<td>7.6**</td>
</tr>
<tr>
<td>CD9c</td>
<td>CD9</td>
<td>ENSDARG00000044990</td>
<td>34.3****</td>
<td>4.0*</td>
</tr>
<tr>
<td>junBb</td>
<td>junB proto-oncogene</td>
<td>ENSDARG00000088371</td>
<td>1.7*</td>
<td>3.2**</td>
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<tr>
<td>Unc93b1</td>
<td>Unc-93 homolog B1</td>
<td>ENSDARG00000069114</td>
<td>0.56*</td>
<td>4.9*</td>
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<tr>
<td>Transcription factors</td>
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<td>ifi1b</td>
<td>IFN regulatory factor 1 (annotated ifrl1)</td>
<td>ENSDARG00000032768</td>
<td>7.1****</td>
<td>1.8*</td>
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<tr>
<td>if7</td>
<td>IFN regulatory factor 7</td>
<td>ENSDARG00000045661</td>
<td>11.0****</td>
<td>2.0*</td>
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<tr>
<td>Stat1b</td>
<td>STAT1b</td>
<td>ENSDARG00000076182</td>
<td>5.9**</td>
<td>1.7</td>
</tr>
<tr>
<td>Socs1</td>
<td>Suppressor of cytokine signaling 1</td>
<td>ENSDARG00000038095</td>
<td>3.6*</td>
<td>1.2</td>
</tr>
<tr>
<td>cebpb</td>
<td>CCAAT/enhancer binding protein</td>
<td>ENSDARG00000042725</td>
<td>1.3*</td>
<td>5.9**</td>
</tr>
<tr>
<td>Oxidative response and mitochondria</td>
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<tr>
<td>ndufs1</td>
<td>NADH dehydrogenase flavoprotein 1</td>
<td>ENSDARG00000036438</td>
<td>0.37***</td>
<td>4.4*</td>
</tr>
<tr>
<td>ndufs3</td>
<td>NADH dehydrogenase flavoprotein 3</td>
<td>ENSDARG000000113587</td>
<td>0.38**</td>
<td>2.6*</td>
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<tr>
<td>etfa</td>
<td>Electron transfer flavoprotein α</td>
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<td>mt-tRNA</td>
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<td>ENSDARG00000083519</td>
<td>1.5*</td>
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<td>Secreted factors and binding proteins</td>
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<tr>
<td>lgals3bpb-1</td>
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<td>lgals3bpb</td>
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<td>lgals91l</td>
<td>Galectin 9–like protein</td>
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<td>lecr2l</td>
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<td>CXCL-C1c</td>
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<td>MHC and proteasome</td>
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<td>b2m</td>
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<td>Trim</td>
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<td>3.1***</td>
<td>1.1</td>
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<td>ftr-like</td>
<td>fnfTRIM-related protein; ftr60</td>
<td>ENSDARG00000067661</td>
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</tr>
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<td>Translation</td>
<td></td>
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<td>rps5</td>
<td>Ribosomal protein S5</td>
<td>ENSDARG00000036875</td>
<td>1.0</td>
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</table>

* (Table continues)
a transcriptome analysis of infected zebrafish larvae. Based on our previous data (10, 11), we chose 48 h postinfection (hpi) as the time point that would allow us to detect a well-developed response to both viruses at a comparable stage of development. At this time point that would allow us to detect a well-developed response to both viruses at a comparable stage of development.

These experiments required infecting a large number of larvae, which was performed by i.v. microinjections in our previous studies (10, 11). Because this is challenging and time-consuming, we tested alternative infection methods that would still lead to the expected outcome in a reproductive fashion. Immersion of 3 dpf larvae in water containing either virus did not yield infection. However, in the case of CHIKV, we knew that i.v. inoculation was performed by i.v. microinjections in our previous studies leading to death.

transcriptome analysis should not mainly reflect the general distress leading to death. 

Table II. (Continued)

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Description</th>
<th>Ensembl Gene ID</th>
<th>CHIKV FC and Adjusted p Value</th>
<th>IHNV FC and Adjusted p Value</th>
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<td>rps15</td>
<td>3’ UTR of ribosomal protein gene</td>
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<td>rps24</td>
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<td>rpsa</td>
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<td>ENSDARG00000019181</td>
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<td>elf1g</td>
<td>Elongation factor 1-g</td>
<td>A_15_P110855</td>
<td>0.48**</td>
<td>2.7**</td>
</tr>
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<td>sars</td>
<td>Seryl-tRNA synthetase</td>
<td>ENSDARG00000008237</td>
<td>0.7**</td>
<td>5.1**</td>
</tr>
</tbody>
</table>

**Enzymes**

| mov10b.1 | Putative helicase mov-10 | ENSDARG00000061177 | 6.6*** | 1.5 |
| zgc:154040 | Putative phospholipase A2 | ENSDARG00000071326 | 11.4*** | 1.4 |
| ctsb | Cathepsin S.b.1 | ENSDARG00000075176 | 4.3*** | 0.5* |
| gpxs1b | Glutathione peroxidase 1b | ENSDARG0000006207 | 3.6*** | 0.8 |
| cnpk2 | Thymidylate kinase LPS-inducible (tyki) | ENSDARG00000031359 | 3.5** | 1.7 |
| rnaes3 | RNase-like 3 | ENSDARG00000036171 | 3.3*** | 1.1 |
| adam8a | Metalloproteinase domain 8a | ENSDARG00000014452 | 2.6** | 0.5** |
| atp2a1 | ATPase, Ca2+-transporting, fast twitch 1 | ENSDARG00000020574 | 0.8* | 5.4** |
| tgm1 | Transglutaminase 1 | ENSDARG00000019024 | 1.0 | 3.0** |
| wath3 | N-acetyltransferase 8-like (EC 2.3.1.-) | ENSDARG00000077256 | 0.5*** | 2.9* |
| pfββ3 | 6-Phosphofructo-2-kinase | ENSDARG0000001953 | 1.2 | 2.5* |

**Apolipoproteins**

| apoeC1 | Apolipoprotein C1-like | A_15_P115491 | 0.2*** | 8.0* |
| zgc:193613 | Apolipoprotein like | ENSDARG00000015866 | 0.8 | 3.6** |
| apoaI | Apolipoprotein A-1 precursor | ENSDARG00000012076 | 0.4** | 2.6** |

**Downregulated genes**

**IFN response**

| prkrir | Protein kinase, IFN-inducible dsRNA-dependent inhibitor, repressor | ENSDARG00000042489 | 0.8** | 0.3** |
| bril | Bone-restricted IFITM-like protein (aka IFITM5) | ENSDARG00000091273 | 0.3*** | 0.4* |

**Oxidative and inflammatory response**

| fkp5 | FK506 binding protein 5 | ENSDARG00000028396 | 1.0 | 0.06** |
| dhrs1 | Dehydrogenase/reductase 1 (SDR family) member 1 | ENSDARG00000054300 | 0.3*** | 1.8 |
| pld2 | Phospholipase A2, group XIIB | ENSDARG0000015662 | 0.3*** | 2.4* |
| prss35 | Protease, serine, 35 | ENSDARG0000017213 | 0.3*** | 1.1 |
| dhrs1 | Dehydrogenase/reductase 1 | ENSDARG00000054300 | 0.3*** | 1.8 |
| zgc:64031 | Abhydrolase domain-containing 14B | ENSDARG00000023880 | 0.3* | 1.6 |
| ucmab | Unique cartilage matrix-associated protein | ENSDARG0000005485 | 0.2*** | 2.4 |
| cyp26b1 | Cytochrome P450 26B1 (Cyp26B1) | ENSDARG00000077121 | 1.0 | 0.3** |
| hamp2 | Hemicid-2 precursor | ENSDARG00000053227 | 1.9 | 0.3* |

**Others**

| epd | Ependymin precursor | ENSDARG00000014745 | 0.7 | 0.26** |
| mcam | Melanoma cell adhesion molecule | ENSDARG0000005368 | 0.9 | 0.27** |
| fnb5 | Fibulin 5 | ENSDARG00000039273 | 0.7** | 0.29** |
| vsg1 | Vessel-specific 1 | ENSDARG00000045003 | 1.7** | 0.17** |
| tppp3 | Tubulin polymerization-promoting | ENSDARG00000030463 | 0.7* | 0.31** |
| acta2 | Actin, α2 | ENSDARG00000045180 | 0.9 | 0.32** |
| ppdc2a | PPPDE peptide domain-containing 2a | ENSDARG00000033140 | 1.0 | 0.32** |
| meox1 | Mesenchyme homeobox 1 | ENSDARG0000007891 | 0.8* | 0.32** |
| rnc3 | Retinol-binding 3, EF-hand calcium binding | ENSDARG00000037961 | 0.6** | 0.34 |
| mad211 | MAD2 mitotic arrest deficient-like 1 | ENSDARG0000004713 | 0.9* | 0.33** |
| zgc:113183 | SWI/SNF-related containing DEAD/H-box 1 | ENSDARG00000014041 | 0.8 | 0.33** |
| kctd12.2 | Potassium channel tetramerization | ENSDARG00000053542 | 1.1 | 0.34** |
| taf6 | TAF6 RNA polymerase II, TATA box BP | ENSDARG00000029135 | 0.7* | 0.34** |
| il13ra2 | IL-13R, α2 | ENSDARG00000039436 | 0.8 | 0.35* |
| col8a2 | Collagen, type VIII, α2 | ENSDARG00000060893 | 0.7* | 0.35* |
| stk25a7 | Solute carrier family 25, member 27 | ENSDARG00000042873 | 0.55*** | 0.35** |
| gla2a | GLI-Kruppel family member GLA2a | ENSDARG00000025641 | 0.7** | 0.36** |
| mbhfd2 | Methylene-tetrahydrofolate dehydrogenase 2 | ENSDARG00000026666 | 0.7** | 0.36** |
| cx23 | Connexin 23 | ENSDARG00000054150 | 0.75 | 0.38* |

**FC < 0.5 and FC > 2 are in bold.**

**Adjusted p values:** *p < 0.01–0.05, **p < 0.005–0.01; ***p < 0.001–0.005.
Supplemental Fig. 1A. We also found that yolk injection of CHIKV resulted in only slightly lower levels of expression of the CHIKV E1 transcript compared with i.v. injection, and to a slightly lower, but still very strong, upregulation of *ifn* transcripts, with similar kinetics (Supplemental Fig. 1B). Thus, we chose to perform yolk injections for the microarray experiments. In contrast, injection of IHNV in the yolk did not result in infection, and we had to perform i.v. injections.

For each virus, three independent experiments were performed, and RNAs were extracted from pools of 30–70 larvae. RNAs from infected and control uninfected larvae from the same batch were labeled and hybridized to custom Agilent 44K microarray chips enriched in probes for predicted immune genes (19), which covers approximately half the zebrafish genome. After setting an arbitrary cutoff “fold” value at 2.5 (induction) or 0.4 (repression), we identified 583 features whose target expression was modulated by one or both viruses. A selection of these genes (based on strength of modulation and on predicted functions) is shown on Table II. Genes upregulated by CHIKV were induced by IHNV at a lower level (Fig. 1A, black ellipse). This pattern is well consistent with an induction mediated by type I IFNs, because CHIKV induces a much stronger IFN response compared with IHNV. In contrast, most genes well induced by IHNV infection were not significantly modulated by CHIKV (Fig. 1A, gray ellipse), suggesting induction of distinct pathways. The correlation between the two responses was relatively poor, with only 15 genes significantly up regulated >2.5-fold (and none downregulated) by both viruses (Fig. 1B).

**Kinetics of gene induction**

By performing quantitative RT-PCR on a subset of genes, we confirmed these findings and extended them to obtain a kinetic picture of the response (Fig. 2 and data not shown). Modulation of expression observed from microarray data was confirmed for this selection of genes, with the notable exception of two genes presumably downmodulated by CHIKV, *apoCIL* and *ucmbh*. Both were represented by a single probe on the array in their predicted 3′ untranslated region, whereas our PCR primers targeted open reading frames, suggesting that for these genes, CHIKV infection may be associated with use of alternative polyA sites, but this matter was not investigated further. Kinetics of infection, measured by expression of viral genes (Fig. 2, *top panels*), were consistent with previously published studies (10, 11), with CHIKV reaching a peak at 24 hpi then slowly declining, whereas IHNV continuously increased until the death of the larvae. Interestingly, genes orthologous to well-known ISGs (*rsad2/viperin*, *isg12.2*, *isg15*, *trim25*, *irf7*, *irf1b*) all followed the same kinetics, even though some may be induced 100-fold more than others; these kinetics matched very well the kinetics of the induction of the secreted isoform of *ifnb1*. The only exception was a decrease in *isg12.2* expression at the most advanced stage of IHNV infection. However, this was not observed in all experiments and is likely to be an artifact linked to the very low level of expression of this gene (see Fig. 2, *left center panel*). Lower fold changes (FCs) and different kinetics of induction upon CHIKV infection were observed for other inducible genes not expected to be ISGs such as *c4b* or *mmp9*, consistent with their induction by separate pathways (Fig. 2, *middle bottom panel*). The kinetics of gene induction by IHNV infection are more complex, but suggest that *mmp9* was upregulated early and did not show the delayed observed for the IFN response (see Fig. 2, *right panel center and bottom*).

**Spatial patterns of expression**

We used WISH to determine the site of expression of some selected genes. This analysis was performed at the peak of the response for CHIKV (24 hpi), and at 48 hpi for IHNV, as responses were quite weak at 24 hpi, whereas infection-induced damage at 72 hpi caused larvae to fall apart during the hybridization process. Fig. 3 illustrates spatial expression pattern of different ISGs regarding virus tropism represented by localization patterns of CHIKV- or IHNV-infected cells (Fig. 3A). Consistently with quantitative RT-PCR results, which showed that the basal expression of strongly inducible ISGs such as *isg15* was quite low, very little signal was detected in control, uninfected larvae; however, a strong signal was detected after infection with CHIKV, and a weaker but visible signal was detected in IHNV-infected larvae (Fig. 3B). The strongest *isg15* signal was detected in the liver after both virus infections: specific signal was also detected in the gut and, for CHIKV-infected larvae only, in the vasculature. Remarkably, the other ISGs assessed, that is, *irf7*, *rsad2/viperin*, and *isg12.2*, had a globally similar pattern of expression (Fig. 3C). However, closer examination revealed some preferences, with *irf7* being expressed more strongly in vessels (and also visible in dispersed cells, probably leukocytes), whereas *rsad2* and *isg12.2* are only modestly expressed in the gut and liver, respectively.

We also tested *mmp9*, for which a different pattern might be expected to arise from a different stimulus, because it is not
a typical ISG. Indeed, the pattern of mmp9 expression induced by IHNV was strikingly different from that of ISGs, with a staining of superficial cells, probably in the epidermis (Fig. 3D). Finally, we also tested a few downmodulated genes. Although no clear pattern of expression for fkbp5 could be observed (not shown), a striking pattern was observed in uninfected larvae for the epd gene encoding ependymin (Fig. 3E), in agreement with the pattern available on the Zebrafish International Resource Center Web site (http://zfin.org/cgi-bin/webdriver?MIval=aa-fxfigureview.apg&OID=ZDB-FIG-070503-464) (28); a subset of cells in the head (and possibly in the endocrine pancreas) expresses epd. Despite the gene name, these cells do not line brain ventricles and are thus not ependymal; instead, they are located on the brain external surface, apparently ensheathing some blood vessels. IHNV infection induced a dramatic reduction in the number of epd-expressing cells, especially in the dorsal cranium. This, consistent with the infection patterns (see figure 2D of Ref. 10), suggests that epd-expressing cells are targeted and killed by the virus. Similarly, vsg-1, an endothelium-specific gene (29), is downmodulated by IHNV, and this expression was lost in many vessels in IHNV-infected larvae (not shown), in accordance with our previous finding that vascular endothelium is a primary target of IHNV (10).

Pathway analysis of the responses

**IFN-related pathways.** Analysis of the list of modulated genes by GenMAPP and Ingenuity Pathway Analysis (IPA) identified several biological pathways induced by the viral infections. IFN signaling was found enriched by IPA for both viruses, although with more genes involved and a lower p value for CHIKV; type I IFNs were predicted as main upstream regulators in both cases. Many genes previously characterized as ISGs or homologous to well-known human ISGs were found to be highly upregulated by CHIKV, and to a lesser extent also by IHNV (Fig. 4, Table II). Analysis of genes presumably involved in signaling pathways upstream of IFN induction was not as straightforward, probably reflecting that activation of these pathways involves posttranslational modifications more often than transcriptional modulation. The detection of viruses may happen either in the cytosolic compartment of cells via the RIG-I–like receptor (RLR) pathway, or in endosomal compartments via the TLR pathway. We have previously implicated the RLR pathway in the zebrafish response to CHIKV, because knockdown of MAVS/IPS-1, a central adaptor of this pathway, was found to strongly reduce IFN induction (11). No modulation of the mavs transcript was seen with either virus, which was consistent with previous observations (30, 31); this was also the case for most genes involved in the RLR pathway (Supplemental Fig. 2), with the notable exceptions of trim25, isg15, and irf7, all orthologs of known ISGs, involved in signaling feedback loops. A similar picture emerged from the analysis of the TLR pathway (Supplemental Fig. 2); neither virus modulated most genes in this pathway. A few genes induced by IHNV included proteins typically involved in responses to bacteria rather than to viruses (e.g., mmp9). Again, this suggests that in addition to the typical virus-induced IFN response, IHNV triggers other inflammatory pathways.

Conspicuously absent from the list of IHNV- or CHIKV-induced genes were the type I IFNs themselves, ifnα1–4. The microarray did not include probes for ifnα3 and ifnα4; ifnα4 does not appear to be involved in the antiviral response (32), and we
The liver X receptor/retinoid X receptor pathway was also high-
lighted after IHNV infection, comprising genes upregulated by both viruses such as complement genes or mmp9, but also the apolipoproteins, which were modulated by IHNV only, and phospholipase A2, which was induced by IHNV and repressed by CHIKV. Finally, “liver necrosis, cell death and inflammation” was among the top “tox lists” of IPA for both viruses, which could be related to the strong cytopathic effect of these viruses, as well as to the spatial expression pattern of modulated genes (see above).

IFN signaling is involved in the upregulation of key virus-induced genes

Genes strongly induced by CHIKV, but less so by IHNV, are presumably induced by the strong and protective IFN response in CHIKV-infected fish; indeed, most of these genes are orthologous to well-known mammalian ISGs. To check the IFN dependency of the upregulation observed during the infection for a selection of genes, we knocked down the two zebrafish type I IFN receptors (26) using antisense morpholinos, infected larvae with CHIKV, and measured gene expression by quantitative RT-PCR at the peak of the response. When compared with control morphants (Fig. 5), induction of genes such as rsad2/viperin, isg12.2, isg15, trim25, and irf7 was severely reduced, indicating that their induction was IFN-dependent, as expected. In contrast, mmp9 and irf1b induction appeared to be IFN-independent. This was expected for mmp9, but is surprising for irf1b, the human ortholog of which is an ISG. Therefore, we should be aware that induction by CHIKV does not necessarily equate induction by type I IFN, even for ISG orthologs. Nevertheless, it does seem to be the case for most of the genes we tested, which prompted us to perform a genome-wide analysis of the orthologs of human ISGs.
Genomic and expression analysis of ISG orthologs

Are zebrafish genes orthologous to human ISGs generally IFN inducible? Although our initial GenMAPP and IPA analysis identified a number of genes as being induced downstream of type I IFNs (displayed in Fig. 4), there are many other, less well-known ISGs. We took advantage of the exhaustive human ISG list compiled by Schoggins et al. (2) based on a compilation of a dozen human microarray studies. We searched the zebrafish genome for the orthologs of these genes, with the help of the phylogeny tools at Ensembl.org (release 72); the results of this analysis are provided as a searchable Excel file with links to gene pages (Supplemental Table I). This table includes 445 human genes for which a total of 593 zebrafish orthologs were found, of which 280 were represented on the microarray.

As expected, we found within this dataset the genes highly induced by the viral infection. However, a significant enrichment was detected even among genes with modest levels of induction (Fig. 6). Also, the proportion of genes reproducibly modulated between replicates (corrected $p < 0.05$, regardless of the actual FC) was higher within ISG orthologs (146 of 280 genes) compared with the whole dataset (5,188 of 12,524) ($\chi^2$ test, $p < 0.001$). Thus, in addition to the well-known, strongly induced ISGs, a large number of modestly inducible genes should also be included in the core vertebrate group of IFN (or rather, virus)-inducible genes. These observations led us to propose a tentative estimation of the size of the core set of ISGs conserved between zebrafish and humans; subtracting the global distribution of induction of CHIKV-induced genes to the distribution of induction of zebrafish orthologs of human ISGs, one obtains a rough assessment of 53 ISGs represented on the microarray (Fig. 6, highlighted area). Given that approximately half of genes are represented on the array, both for the entire dataset and for the ISG orthologs, the order of magnitude of the size of the core ISG subset shared by fishes and mammals would be 100 genes.

Analysis of ISG-containing multigene families

A large number of mammalian ISGs constitute multigene families; Table III shows that it is also the case in the zebrafish. Phylogenetic relationships between human ISGs and their zebrafish orthologs indicate that these genes are prone to extensive duplication in each evolutionary branch: 35 examples of “many-to-many” orthology relationships can be observed in Supplemental Table I, which are ~7-fold more frequent than for the overall genome comparison (233 such relationships in total) (35). Fami-
Studies of the virus-induced transcriptional response in fish were performed in a variety of infectious systems, identifying genes induced in specific tissues. In our study, we measured the FC in the infected larvae. Quantitative RT-PCR; means ± SEM for three pools of five larvae; one representative experiment out of two. At the one cell stage, embryos had been injected with a control morpholino oligonucleotide (blue bars) or with a mixture of CRFB1- and CRFB2-specific antisense morpholinos (red bars) to knock down IFN receptor expression. Levels of FC compared by unpaired t test: *p < 0.05, **p < 0.01, ***p < 0.001.

FIGURE 5. IFN dependence of response. Gene induction by CHIKV at the peak or response (24 hpi) after i.v. infection; FC compared with uninfected larvae. Quantitative RT-PCR; means ± SEM for three pools of five larvae; one representative experiment out of two. At the one cell stage, embryos had been injected with a control morpholino oligonucleotide (blue bars) or with a mixture of CRFB1- and CRFB2-specific antisense morpholinos (red bars) to knock down IFN receptor expression. Levels of FC compared by unpaired t test: *p < 0.05, **p < 0.01, ***p < 0.001.

FIGURE 6. Distribution of CHIKV-inductions of ISG orthologs, compared with the overall dataset. Distribution of genes with an Ensembl ID on the microarray dataset, according to FC by CHIKV (when corrected p value of < 0.05). For clarity, only the upper limit of the considered interval is indicated under the horizontal axis. Comparisons of the entire set and of the subset of ISG orthologs are shown. The shaded area depicts our estimate of the number of specifically ISGs. Differences in frequencies of ISG orthologs versus other genes within a given interval were tested by a χ² test (*p < 0.05, **p < 0.01, ***p < 0.001).

The CHIKV-induced response is mainly IFN-dependent and defines a core set of highly induced ISGs conserved across vertebrates

Studies of the virus-induced transcriptional response in fish were performed in a variety of infectious systems, identifying genes induced in specific tissues. In our study, we measured the FC in the...
whole larva, thus integrating the contribution of all tissues, either infected or put in contact with the secreted IFNs. The arbovirus CHIKV leads to a strong systemic IFN response in the zebrafish larva, and the genes with the strongest inductions could be immediately identified as a core set of canonical ISGs conserved in other fish, including in adult tissues, and in mammals. This set of IFN responsive genes was also induced, although more modestly, by the rhabdovirus IHNV in the zebrafish larva. In fact, rhabdoviruses can inhibit IFN responses through different mechanisms likely involving the P and NV proteins (38–43), and IHNV induces a relatively mild IFN response (44). This inhibition appears to play mostly upstream of IFN induction because recombiant IFN injection protects zebrafish from IHNV (26). Although ISGs are typically less strongly induced by IHNV compared with CHIKV, if features on the arrays were significantly induced by CHIKV but in fact repressed by IHNV, suggesting that antagonist pathways were implicated. Although the mechanisms of inhibition of IFN response by IHNV are beyond the scope of this study, our dataset might therefore provide clues about the genes involved.

In addition to highly induced orthologs of human ISGs, our analysis suggests that many ISGs common to fish and mammals can be induced at a more moderate level by CHIKV. For example, genes with an FC of 1.56–1.95 (with a p value of <0.05) were five times more frequent among zebrafish orthologs of human ISGs than they were for the whole gene set (Fig. 6). In fact, this observation suggests that 80% of the zebrafish orthologs of human ISGs within this FC interval are indeed truly upregulated by IFN. This provided us with a basis to estimate that the zebrafish possesses 100 IFN-induced genes among its orthologs of human ISGs.

Apart from this core set of ISGs common to fish and humans, do we have evidence of lineage-specific gene families modulated by the viral infection? A striking feature of the CHIKV-induced responses of zebrafish larva was the lack of additional families of highly induced genes, compared with the human ISG set. Several large multigene families such as finTRIM and NLR are likely playing important roles in the fish (inmate) immune responses because of their diversification patterns (45–47); strikingly, none of their members was found in our list of induced genes in the immature state of the zebrafish larva. There are, however, a few genes that are specifically induced in fish; for instance, the autophagy regulator dram1 was strongly induced by CHIKV and found to be induced in an IFN-specific manner in trout (48), although it is not a typical ISG in humans. Alternatively, we identified four tetrapod specific gene families containing ISGs: aim2/IFI16, Apobec3, Clec4, and OAS.

Additionally, noncoding RNAs, which are poorly conserved between species, constitute potentially important genes induced by viruses in a species-specific fashion. The trout gene vig2 (restricted to salmonids) and the human LINCO0969 represent good examples of such ISGs (2, 49). In our microarray data, a few strongly induced probes apparently hybridized in regions encoding long coding transcripts, but we did not investigate this further.

Altogether, these observations suggest the presence of a shared core set of ISGs in fish and mammals, which we predict to contain at least 100 genes. Importantly, however, our study has been performed on larvae, and it is quite possible that a larger set of ISGs may be found in adult zebrafish, in which the adaptive immune system is functional. This should be investigated in future studies.

How old is the core ISGs set?

ISGs follow several types of evolutionary patterns, and the analysis of families is particularly interesting in that respect. We observed that some families had diversified in the common ancestor of fish and tetrapods: as most human family members have a single orthologue, most tetrapod specific genes families containing ISGs: aim2/IFI16, Apobec3, Clec4, and OAS.

Table III. ISG-containing families

<table>
<thead>
<tr>
<th>Selected Families*</th>
<th>Human Membersb</th>
<th>Zebrafish Membersb</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tetrapod only</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AIM2/IFI16</td>
<td>aim2; ifi16; mnda; pyhnil1</td>
<td>—</td>
</tr>
<tr>
<td>AP0BEC3</td>
<td>apobec3a, b, c, d, f, g, h</td>
<td>—</td>
</tr>
<tr>
<td>CLEC4</td>
<td>clec4a, c, d, e, 6a</td>
<td>—</td>
</tr>
<tr>
<td>OAS</td>
<td>oas1, 2, 3, 1</td>
<td>—</td>
</tr>
<tr>
<td>Teleosts and tetrapods independently diversified in teleosts and/or tetrapods (young)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>APOL</td>
<td>apol1–3; apol6; apol4–5</td>
<td>—</td>
</tr>
<tr>
<td>CLEC2</td>
<td>cd69; clec2b; clec2-a, -d, -l; klrg12</td>
<td>—</td>
</tr>
<tr>
<td>GBP</td>
<td>gbp1-5; gbp6-7</td>
<td>—</td>
</tr>
<tr>
<td>IFI27/ISG12</td>
<td>ifi6; ifi27; b271; b2712</td>
<td>—</td>
</tr>
<tr>
<td>IFI44</td>
<td>ifi44; ifi44l</td>
<td>—</td>
</tr>
<tr>
<td>IFIT</td>
<td>ifit-1, -2, -3, -5, ifitb</td>
<td>—</td>
</tr>
<tr>
<td>LGAL</td>
<td>lgals-9, -s9b, -s9c</td>
<td>—</td>
</tr>
<tr>
<td>MS4A</td>
<td>ms4a4a; ms4a-2, -3, -5, -8, -12, -14, -15, -18</td>
<td>—</td>
</tr>
<tr>
<td>Ms</td>
<td>mx1; mx2</td>
<td>—</td>
</tr>
<tr>
<td>Conserved orthologous members in teleosts and tetrapods (old)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD9</td>
<td>cd9, cd81, tspn2</td>
<td>cd9-a, -b, cd9-e, cd81-a, -b, tspn2-a, -b</td>
</tr>
<tr>
<td>RLR</td>
<td>ddx58/riq1; ifi1/mda5; ddx58/lg2p</td>
<td>ddx58/riq1; ifi1/mda5; ddx58/lg2p</td>
</tr>
<tr>
<td>IRF</td>
<td>irf-1, -2, -7, -9 irf3-6, -8</td>
<td>irf-1a, -b, irf2a, -2b, -37, -4a, -b, -5, -6, -7, -8, -9, -10</td>
</tr>
<tr>
<td>SOCS</td>
<td>socs-1; socs-2; socs-4 to -7; cish</td>
<td>socs-1a; socs1b; socs3a; socs3b; socs4; socs5a; socs5b; socs6b; socs7b; socs8; cish</td>
</tr>
<tr>
<td>STAT</td>
<td>stat1; stat2; stat3; stat4; stat5a; stat5b; stat6</td>
<td>stat1a; stat1b; stat2a; stat3; stat4; stat5; stat5a; stat5b</td>
</tr>
</tbody>
</table>

*Only families with at least two ISGs were considered.

bISGs are in bold (same criteria as for Fig. 7); fish genes not represented on the array are on gray background. Overall, the nomenclature was chosen to fit best the phylogenetic relationships; similar names were not used for nonorthologs. Identity of zebrafish genes can be searched in Supplemental Table I.
conserved (Fig. 7, Table III). This pattern typically corresponds to regulatory components such as transcription factors or signal transduction proteins (e.g., IRFs, STATs, SOCS). A sar e result, the virus-responsive signaling machinery appears to be very well conserved between fish and mammals, with very similar feedback loops at play (Supplemental Table I). In contrast, we called "young" other families differentiated in parallel in each lineage from a single common ancestor gene. Those would be expected to evolve under high diversifying pressures, such as the ones dictated by the modalities of direct interactions with pathogens. This is indeed the case for such proteins belonging to the young MX or IFIT families (50). We can therefore predict that functionally uncharacterized ISGs from such young families, such as members of the ISG12/IFI27 or IFI44 families, should directly interact with some viral components.

**Temporal and spatial regulation of ISGs**

To complement the microarray study, performed at a single time point on extracts from entire larvae, we analyzed the kinetics (Fig. 2) and the spatial expression (Fig. 3) of a gene subset. All ISGs in this subset showed a very comparable kinetic that faithfully mirrored that of secreted ifn-1 induction. Remarkably, and despite it being widely accepted that all nucleated cells express type I IFN receptors, ISGs were expressed in a tissue-restricted pattern, with a prominent expression in the liver, the vasculature, and the gut, with minor gene-specific tissue preferences superimposed on this general pattern. Interestingly, this pattern matches partially the one of IFN-1 itself, with a strong expression in the liver (11). Such contrasted patterns were not expected, yet they are reminiscent of studies performed in mice with an MX/luciferase

**FIGURE 7.** Different types of phylogenies of ISG-containing families. (A and B) Two examples of old families: IRF and CD9. (C and D) Two examples of new families: ISG12/IFI27 and IFIT. Background colors: orange for ISGs (see below), green for non-ISGs, gray if unknown. Human genes are shown with black letters; zebrafish genes are shown in white. Maximum likelihood trees with bootstrap values out of 1000 replicates are indicated. Human genes classified as ISGs according to their presence or absence on the list established by Schoggins et al. (2). Zebrafish genes were classified as ISGs when found induced >2.5-fold by CHIKV on our microarray dataset, with the exception of ifr3, not present on the microarray but induced by IFN in fish as shown by Sun et al. (37) and verified by N.P. (unpublished data). ISG12.7 is on green background (non-ISG) but was slightly induced by CHIKV (FC = 1.88; p < 1%). Accession numbers of sequences used: IRF: HsIRF1, NP_002189; HsIRF2, NP_002190; HsIRF3, NP_001562; HsIRF4, NP_002451; HsIRF5, NP_116032; HsIRF6, NP_006138; HsIRF7, NP_001563; HsIRF8, NP_002154; HsIRF9, NP_006075; DrIRF1, NP_001035442; DrIRF2, ENSDARP0000005229; DrIRF2A, B8JIT4; DrIRF3, NP_001137376; DrIRF4A, NP_001116182; DrIRF4B, XP_697730; DrIRF5, NP_998040; DrIRF6, NP_956892; DrIRF7, NP_956971; DrIRF8, NP_001002622; DrIRF9, NP_991273; DrIRF10, NP_998044; DrIRF11, NP_991310. CD9: HsCD9, NP_001760; HsCD81, NP_004347; HsTSPAN2, NP_005716; CD9a, NP_997784; CD9b, NP_998593; DrCD9A, NP_998180; DrCD9A, NP_571953; DrTSPAN2A, NP_001035442; DrTSPAN2B, ENSDARP000000710. ISG12/IFI27: HsIFI27, NP_001123552; HsIFI27L1, NP_660292; HsIFI27L2, NP_114425; HsIFI6, NP_075011; DrISG12.3, XP_704861; DrISG12.1, NP_00107133; DrISG12.2, NP_001007134; DrISG12.3, ENSDARG0000007217; DrISG12.4, ENSDARG0000007389; DrISG12.6, ENSDARG00000074754; DrISG12.7, ENSDARG00000075115; DrISG12.8, ENSDARG00000092895. IFIT: DrIFIT1, EAW50136; DrIFIT1B, NP_00101967; DrIFIT5, NP_036552; DrIFIT2, NP_001538; DrIFIT3, NP_001540; DrIFIT8, XP_695838; DrIFIT4, XP_695770; DrIFITb2, NP_001177393; DrIFITb1, NP_001177394; DrIFITa, NP_701096; DrIFITc, NP_001032564; DrIFITd, XP_001334809; DrIFITd1, XP_001334809; DrIFITd2, XP_001334890. DrISG12.5 appeared to be partial and was not included.
reporter gene (51), where liver was observed to respond much more strongly than other organs to injected IFN or to Thogoto virus infection. Thus, strongly organ-biased expression of ISGs may well be the norm among vertebrates. It remains to be established whether this reflects local availability of IFNs or differential responsiveness of other organs.

**Alternative pathways at play during IHNV infection**

Whereas CHIKV infection induced a strong and protective IFN response, IHNV infection invariably leads to the death of the zebrafish larva. Many genes are induced by the INHV infection but poorly or not induced by CHIKV. Their upregulation is likely linked to the activation of IFN-independent pathways. This may be largely caused by extensive cell death due to viral cytopathic activity and subsequent inflammation.

Many mitochondrial genes were induced by IHNV, suggesting that this virus induces a general mitochondrial response potentially linked to apoptosis and oxidative reaction. Interestingly, a cellular oxidative response can suppress the IFN-α-α-induced assembly of STAT factors at the ISG promoters, thus potentially contributing to the knockdown of the IFN-induced response (52). Such an oxidative response may also be responsible for the induction of mmp9 in epidermal cells that we observed by ISH, similar to what has been observed during bacterial infections (53).

A variety of ribosomal proteins are also modulated by IHNV, possibly in response to translational shut-off of host proteins achieved by rhadoviruses. A downregulation of protein synthesis by IHNV infection have been reported before in rainbow trout (44).

Fkbp5 was strongly downregulated by the IHNV infection, although it is a mammalian ISG, and it is induced in chicken by the Newcastle disease virus (54). In fact, fkbp5 is probably not an ISG in zebrafish, because it is not induced by CHIKV (nor, for that matter, is fkbp4, another possible ortholog of human FKBPs). FKBP5 links glucocorticoids to NF-kB signaling during viral infection (55), but its role in antiviral responses is still elusive. This immunophilin with peptide-prolyl-isomerase activity reminds one of cyclophilins, which are other targets of cyclosporine A and may be either deleterious or beneficial for virus infections (56). Whether FKBP5 downregulation has a positive or a negative impact on the response to IHNV remains to be determined.

Our study identified ependymin, a marker specific of brain perivascular cells, as a gene repressed by the INHV, but not by CHIKV. The progressive reduction of expression during infection suggested that peripheral brain cells in which it was expressed might be targeted and killed by the virus. The WISH supported this notion, because the infected larvae lost completely ependymin expression in the dorsal cranial. Thus, transcriptome survey may lead not only to the identification of responding cells through localization of gene upregulation, but may also reveal virus cellular targets by extinction of specific markers.

In conclusion, our study characterizes the contrasted transcriptional responses of zebrafish to a fish rhabdovirus, IHNV, and a human arbovirus, CHIKV. The strong type I IFN response induced by CHIKV allowed us to greatly extend the list of zebrafish ISGs, many of which belong to multigene families. Their different evolutionary stories reflect their functional implication in antiviral pathways. Our transcriptome survey also led to the characterization of the spatial expression pattern of key up- or downregulated markers, providing an important reference set for the study of host-virus interactions in zebrafish, a powerful model where one can perform gene silencing, mutagenesis, or drug screening.

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**Disclosures**

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**References**


