The Antiviral Innate Immune Response in Fish: Evolution and Conservation of the IFN System

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Abstract

Innate immunity constitutes the first line of the host defense after pathogen invasion. Viruses trigger the expression of interferons (IFNs). These master antiviral cytokines induce in turn a large number of interferon-stimulated genes, which possess diverse effector and regulatory functions. The IFN system is conserved in all tetrapods as well as in fishes, but not in tunicates or in the lancelet, suggesting that it originated in early vertebrates. Viral diseases are an important concern of fish aquaculture, which is why fish viruses and antiviral responses have been studied mostly in species of commercial value, such as salmonids. More recently, there has been an interest in the use of more tractable model fish species, notably the zebrafish. Progress in genomics now makes it possible to get a relatively complete image of the genes involved in innate antiviral responses in fish. In this review, by comparing the IFN system between teleosts and mammals, we will focus on its evolution in vertebrates.

Introduction

Teleosts, the largest and best-known clade of ray-finned fish, constitute a highly successful and diverse group, including half of vertebrate species. Their line and ours diverged about 450 million years ago. Several species within this group, both commercial species and model organisms, have been studied to some depth by immunologists, and many details of their antiviral defenses are now known. Although fish genomes have a complex history of whole genome duplications (WGDs) and contractions, the remarkable conservation of the interferon (IFN) system underlines the critical importance of innate antiviral immunity in vertebrates.

Part 1. Architecture of Innate Immune Response in Fish: IFNφ, Receptors, General Structure of Pathways

Fish IFNs

Extensive studies performed in mammals in various contexts of viral infection demonstrated the importance of IFNs in antiviral responses. The name of this group of cytokines originates in their ability to "interfere" with the viral progression, as first described in 1957 by Isaacs and Lindenmann [1]. IFNs belong to class II helical cytokine family and, in mammals, can be divided into three different groups

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based on biological and structural features as well as receptor usage [2]: mammalian IFNs have been classified as type I (α, β, ω, ε, and κ), type II (γ), and type III (λ) IFNs. Actually, only type I and type III IFNs (often grouped under the label "virus-induced IFNs") are truly specialized as innate antiviral cytokines; IFNγ is rather a regulatory cytokine of innate and adaptive immunity, mostly active against intracellular bacteria.

IFN-like antiviral activity has been reported in fish 40 years ago [3,4]. However, teleost IFN genes could not be identified before the development of fish genomics [5–8]. These virus-induced fish IFNs were clearly responsible for a strong inducible activity against a range of viruses [5–7]. Although some fish species (e.g., fugu or medaka) appear to possess one single virus-induced IFN gene, the number of identified genes grew rapidly in other fish species. There are four virus-induced IFN genes in zebrafish (aka IFNϕ) [9,10], a number unlikely to change much considering the quality reached by the zebrafish genome assembly. Salmonids, however, have many more IFN genes; the current record is 11 genes in Atlantic salmon [11]. Two main subsets could be distinguished among fish virus-induced IFNs, corresponding to the number of cysteine (C) residues predicted to be engaged in disulfide bridges: two for IFNs of group I and four for IFNs of group II [9,11], as was later confirmed by three-dimensional crystallography [12]. The 4C configuration is found in all tetrapod type I IFNs, with the exception of mammalian IFNβ, which has only one disulfide bridge. However, the cysteine pair of IFNβ is different from the one of fish group I IFNs, and one should emphasize that the two groups of fish IFNs do not correspond to the alpha/beta subdivision of mammalian type I IFNs, which occurred after the divergence of avian and mammalian lineages.

Two different isoforms of some fish IFN transcripts, resulting from the usage of alternative promoters, show different levels of induction: upon viral infection, a short transcript encoding a protein with a signal peptide is induced in addition to a constitutively expressed isoform, which lacks signal peptide [13]. This particularity has been observed in a number of fish species, but not for all their IFN genes [14–16]. No function of the presumably non-secreted IFN isoform, unique to teleosts as far as we know, has been reported.

Importantly, the two groups of IFNs were found to signal via two different receptors in zebrafish (Fig. 1) [10]. IFNϕs of the first group (IFNϕ1 and ϕ4) bind to the cytokine receptor family B (CRFB)1–CRFB5

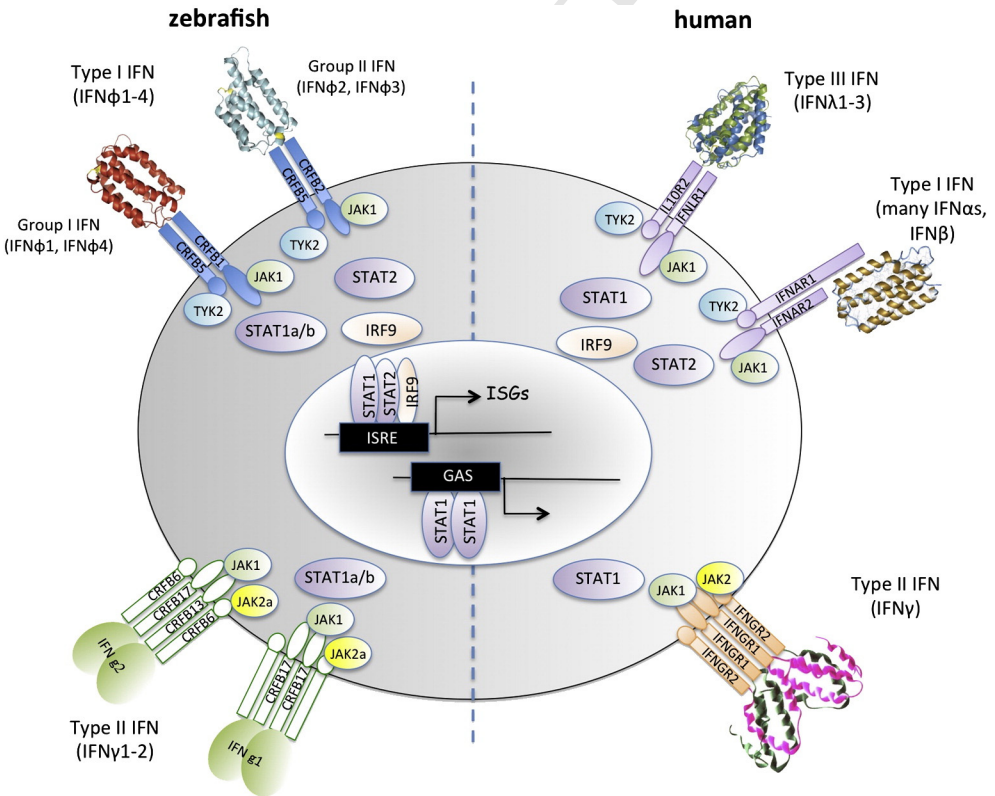

Fig. 1. Schematic representation of zebrafish IFNs and their receptors. Tridimensional representations of IFNs are from the Protein Data Bank (accession numbers: 3PIV, zebrafish IFNϕ1; 3PIW, zebrafish IFNϕ2; 3HHC, human IFNλ3; 1AU1, human IFNβ; 1HIG, human IFNγ).

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complex while the CRFB5 chain is associated to CRFB2 to form the receptor for group II (IFNϕ2 and ϕ3) [13,10]. Interestingly, both zebrafish IFNϕ4 and salmon IFNd—which are possible orthologues—seem to have lost antiviral activity and might be on their way becoming pseudogenes. Alternatively, they may even play a decoy role for other IFNs.

Do the two groups of fish virus-induced fish IFNs play distinct or redundant roles? By injecting recombinant IFNs in adult zebrafish and challenging them with different pathogens, Lopez-Munoz *et al.* found that both types would protect against a virus, but only the group I IFN would also protect against a bacteria [17]; they also observed an induction of distinct gene subsets. However, it is difficult to reach a firm conclusion from this study, because untitered culture supernatants were used as sources of recombinant IFNs, and because the slow kinetics of induction of most downstream genes (including the IFN themselves) suggests indirect effects. Most other studies found quantitative but not clearly qualitative differences between the responses induced by the different IFNs (e.g., Ref. [18]), although this remains to be analyzed in depth. Nevertheless, the distinct receptors for the two IFN groups raise the possibility of different target tissues; in addition, important differences in expression patterns of the different fish IFNs have been demonstrated. The spatial differences of IFN and interferon-stimulated gene (ISG) expression will be reviewed in later sections.

Classification of virus-induced fish IFN genes, relative to mammalian IFNs, has been controversial for some time. Molecular phylogenies were uncertain because the low overall similarity (<25%) between mammalian and fish proteins resulted in uncertain software-generated alignments. It was thus not possible to claim with certainty that fish virus-induced IFNs were closer to mammalian type I or type III IFNs (or co-orthologous to both groups as a set of paralogues), although some sequence features, such as the CAWE sequence at the beginning of the C-terminal helix, were noted by some as characteristic of type I IFNs [9,11,19]. By contrast, fish IFN genes are composed of five exons and four introns [11,19], as are mammalian type III IFN genes, while mammalian type I IFN genes contain a single exon; additionally, when receptors for IFNs were identified in zebrafish, their domain organization had features of the receptor of human IFN λ rather than type I IFN receptor, which has a uniquely large extracellular region in one chain (Fig. 1) [13]. However, the first argument was soon dismissed when frogs were found to have both type I and type III IFNs, all with five-exon structures, indicating that single-exon type I IFN genes were the result of a retrotransposition event in the amniote lineage, not an ancestral feature [20]. Finally, crystal structures revealed a characteristic type I IFN architecture for both groups of IFNϕs with a straight F helix, as opposed to the remaining class II cytokines, including IFN-λ, where helix F is bent [12].

Based on these considerations, different names have been proposed for fish IFNs: type I IFNs, virus-induced IFNs, IFNλ, or even simply IFNs. Following Stein *et al.* [21], zebrafish IFNs are now called IFNϕ (ϕ for fish). While it is now demonstrated that fish virus-induced IFNs are structurally type I IFNs, a consensus about a consistent nomenclature for these cytokines has still been reached. The current zebrafish nomenclature avoids orthology assumptions but does not clearly distinguishes group I and group II IFNs. The current nomenclature for salmonid IFNs, which groups the genes into four subgroups, IFNa, IFNb, IFNc, and IFNd [11,22], has the same issue (group 1 includes IFNas and IFNds; group 2 includes IFNbs and IFNcs) with the caveat that unaware readers could wrongly assume that IFNas are orthologous to mammalian IFNαs, and IFNbs to IFNβ. A self-explanatory nomenclature reflecting the phylogenetic relationships between IFN genes remains to be established.

Fish also possess clear orthologues of mammalian type II IFNs (γ), with many fish species having two type II *ifn* genes (*ifnγ1* and *ifnγ2*) [15,23–25]. In zebrafish, IFNγ1 and IFNγ2 bind to distinct receptors: the IFNγ2 receptor includes Crfb6 together with CRFB13 and CRFB17, while the IFNγ1 receptor does not comprise CRFB6 or CRFB13 but includes CRFB17 (Fig. 1) [26]. Genes encoding a trout receptor of IFNγ have also been identified [27]. Infection studies show that IFNγ signaling is involved in resistance against bacterial infections in the zebrafish embryo, with a proper level required for the fish to clear high doses of *Escherichia coli* or low doses of the fish pathogen *Yersinia ruckeri* [24]. However, a potent antiviral activity of IFNγ was also demonstrated in Atlantic salmon against infectious pancreatic necrosis virus (IPNV) and infectious salmon anemia virus (ISAV), which may partly depend on the coexpression of type I IFN [28]. However, fish IFNγ are not always induced by viral infections under conditions where type I IFNs are [26], indicating that in fish as well as in mammals, IFNγ are probably not specialized antiviral cytokines; they will therefore not be discussed further.

Virus sensors in fish and their signaling pathways

In mammals, viral infection is rapidly detected by specialized PRRs (pattern recognition receptors) such as RIG-I-like receptors (RLRs) and Toll-like receptors (TLRs). These cellular sensors of invading pathogens are directly involved in the activation of the IFN system.

Three RLRs, that is, RNA helicases containing canonical DExD/H motifs, have been identified to date in humans: retinoic acid-inducible gene I (RIG-I, also

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known as DDX58), melanoma differentiation-associated gene 5 (MDA5, or IFIH1), and laboratory of genetics and physiology 2 (LGP2, or DHX58). *In silico* analyses led to the identification of RLRs described in many teleost fish including zebrafish, Atlantic salmon, grass carp, Japanese flounder, rainbow trout, and fathead minnow [22,29–36]. These sequences are highly conserved between mammalian and fish orthologues [37]. LGP2 and MDA5 seem to be conserved in all fish species, while RIG-I has been retrieved only in some groups including salmonids and cyprinids [38]. Like their mammalian counterparts, expression of RLRs is modulated upon viral infection [29,31,32,36,39,40] and IFN stimulation through polyI:C treatment [33] or by ubiquitin-like ISG15 [41], which also modulates RIG-I activity [42]. Interestingly, LGP2 appears to be a positive activator of the IFN pathway in fish. Sequence analysis suggests a fair conservation of signaling pathways downstream of RLR (Fig. 2), with a critical role of for the mitochondrial antiviral signaling protein (MAVS, also known as CARDIF, VISA, or IPS-1) [22,29,34,43,44]. Association of MAVS with TRAF [tumor necrosis factor (TNF) receptor-associated factor] 3 and activation of the pathway by TBK1 (TANK binding kinase 1) via phosphorylation of IFN regulatory factor (IRF)3/7 transcriptional factors have also been shown in fish [44,45]. Nuclear translocation of these factors induced the transcription of different cytokines including IFN genes. The adaptor STING (aka "mediator of IRF3 activation" or MITA, ERIS, and MYPS), a transmembrane protein located in the endoplasmic reticulum, links signaling between MAVS and downstream cytosolic kinase TBK1 [46,47]. In mammals, STING is also involved in the induction of IFNβ by DNA viruses, connecting cytosolic DNA sensing to TBK1 and IRF3 activation [48]. STING has been identified in fish and plays an important role in the RLR/IRF3-dependent signaling [39,49]. The pathways induced by DNA viruses are still poorly known in fish, and the importance of STING is this signaling remains to be established. Interestingly, the DNA sensors AIM2 and IFI6-16 seem to be missing in fish.

A diverse TLR repertoire has been found in fish [50,51]. Some TLRs have been described only in lower vertebrates including TLR14 and TLR23 [50]; TLR18, TLR19, and TLR20 [52]; TLR21 and TLR22 [53]; TLR24 [54]; and TLR25 and TLR26 [55]. TLRs, which are involved in the recognition of double-stranded RNA (dsRNA) (TLR3) or single-stranded

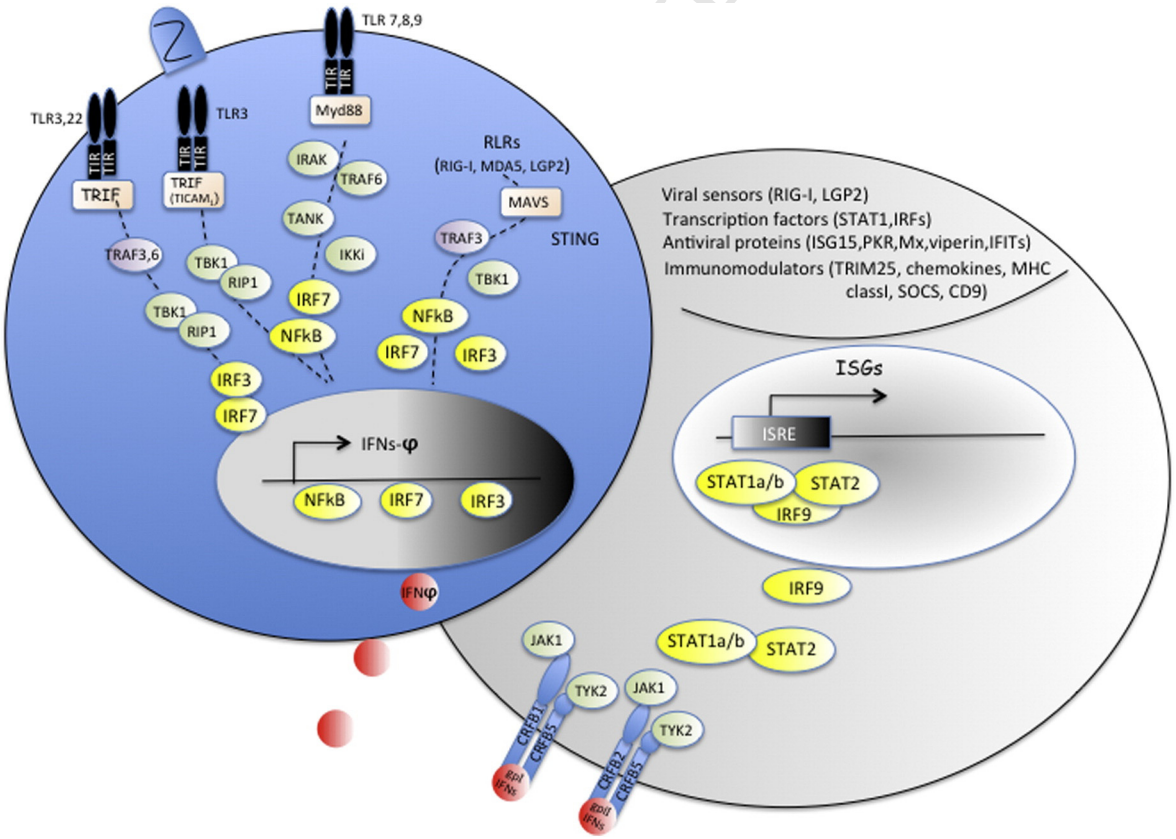

Fig. 2. Schematic representation of IFN signaling pathways in fish. Adaptor molecules are represented in orange, kinases are in green, TRAFs are in purple, transcription factors are in yellow, and IFNs are in red.

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RNA (TLR7 and 8) in mammals, have good orthologues in fish [52,53,56]. Both structural and functional evidence indicate that these TLR are also involved in virus sensing in fish: all critical residues for binding to dsRNA are conserved in fish TLR3 [55], and RTG-2 rainbow trout cells transfected with TLR3 showed increased IFN response after poly(I:C) stimulation [57]. Similarly, the leucin-rich repeats of TLR7 are remarkably conserved between mammals and fish [55,58], and a known ligand of TLR7 and TLR8 (R-848) induces a typical IFN response in salmonid leukocytes [18,59]. Additionally, among fish-specific TLRs, TLR22 is responsive to virus infections, poly(I:C), and dsRNA [57,60]. Fugu TLR22 recognizes long-sized dsRNA on the cell surface, while TLR3 binds short-sized dsRNA in the endoplasmic reticulum [57], which may represent a dual pathway for RNA virus sensing in fish.

Upon ligand binding, TLRs dimerize and their intracytoplasmic TIR (Toll-interleukin 1 receptor) domains recruit adaptor molecules through homotypic TIR/TIR interactions. In mammals, most TLRs signal through the Myd88 adaptor, which recruits interleukin-1R-associated kinase (IRAK) (Fig. 2). This protein then associates with TRAF6, subsequently involving TANK (TRAF family member-associated NfkB activator kinase) and IKKi (inhibitor of NFkB kinase) inducing NFκB nuclear translocation and type I IFN gene transcription. In contrast, TLR3 (specific for dsRNA) signaling occurs independently of Myd88 through the recruitment of TRIF (TIR domain containing adaptor inducing IFNβ, also known as TICAM-1 or Myd88-3), leading to TRAF3 signaling cascade, IRF3 phosphorylation preceding nuclear translocation, and recognition of IFN-stimulated responses elements on type I IFN promoters. Viral infection alternatively activates IRF7 via TLR7–9 in a TRAF6-dependent manner [61]. Although TLR families show distinct features among vertebrates, the components of signaling pathways are well conserved as suggested by the presence of kinase and adaptor molecule orthologues in zebrafish and pufferfish [21]. Myd88 and other TIR adaptors were identified in zebrafish [56], and morpholino approaches as well as infectious models demonstrated the functionality of Myd88 in the establishment of TLR-mediated immune response [62]. Further studies confirmed these observations using different stimulations [poly(I:C), flagelin, or chemical treatments] [63,64]. Since then, *myd88* has been identified in many fish species [64–68]. Zebrafish TRIF similarly triggered activation of type I IFN. The TRIF-dependent TLR pathway converges with the RLR pathway by activating the TBK1 kinase, which is conserved in fish as mentioned above. However, the TICAM1 signaling pathway observed in zebrafish is apparently independent of IRF3 and IRF7 and does not require interaction with TRAF6 [69]. Also, a gene coding for the IRAK2 kinase is missing from the genome of pufferfish, zebrafish, medaka, and stickleback [21], while an IRAK1 orthologue is present and can trigger innate immune response [70].

Thus, IFN-inducing signaling pathways are overall fairly well conserved between fish and mammals. Regarding the sensors, RLRs are also remarkably well conserved, while the fish TLR repertoire include a variety of receptors absent in mammals—some of which, at least, contribute to viral detection—in addition to well-conserved ones such as TLR3 and TLR7.

Conserved signaling pathways downstream of IFN receptors

In mammals, IFN binding to their membrane receptors leads to the activation of the JAK-STAT signaling pathway (Fig. 1). Type I IFN association to its receptor triggers recruitment and binding of the kinases TYK2 and JAK1 to IFNAR1 and IFNAR2, respectively. Subsequently, these kinases promote the phosphorylation of STAT1 and STAT2 proteins preceding their oligomerization. Conjugation of cytoplasmic IRF9 to the STAT1/2 oligomers generates the complex ISGF3 (IFN-stimulated gene factor), which induces the transcription of ISGs after binding nuclear IFN-stimulated responses elements on their promoter. In fish, the *stat1* gene has been described in many species [67,71–73]; the zebrafish genome encodes two different paralogues, *stat1a* and *stat1b* [21]. Functional studies highlighted their role in the regulation of the type I IFN pathway in different species [67,71,73]. However, the respective roles of the different STAT1s in IFN pathway regulation remain unclear in zebrafish. Kinases JAK1 and TyK2 as well as STAT2 and IRF9 are also present in fish genomes [21]. Aggad *et al.* proposed that TYK2 would be associated to CRFB5, while JAK1 would be associated to CRFB1 and 2, thus leading to the activation of the IFN signaling pathway and to *viperin* transcription (Fig. 2) [10].

In contrast, type II IFNs signal after binding to IFNGR1–2 by recruiting JAK1 and JAK2; these kinases promote phosphorylation of STAT1 homodimer, which directly translocates to the nucleus and bind a GAS element (IFN gamma-activated site), thus mediating up-regulation of a broad repertoire of genes, partly overlapping with the type I IFN-mediated response. In zebrafish, IFN-γ1 and IFN-γ2 bind distinct receptors (CRFB6–CRFB13 and CRFB17 for IFN-γ2 and CRFB17, plus unidentified chains, for IFN-γ1) with conserved binding regions of JAK1 and 2 kinases [26]. Two JAK2 kinases are expressed in this species (JAK2a and b), and only JAK2a has been involved in IFNγ signaling using constitutively active mutants (Figs. 1 and 2) [26]. Future studies will be required to determine which of the two STAT1 paralogues constitutes the active protein involved in the signaling pathway of type I and type II IFNs.

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Part 2. ISGs and Their Diverse Evolutionary Patterns

Type I IFNs do not possess antiviral activity *per se* but interfere with viral infection through induction of a vast repertoire of ISGs via the JAK/STAT pathway. A few hundred ISGs have been identified in human [74,75], with a rich diversity of molecular functions. Some ISGs exert a direct antiviral activity such as MX, VIPERIN/VIG1, ISG15, PKR, and TRIM5. However, the connection of most ISGs to antiviral mechanisms, and even their role in the biology of the cell, remain unknown.

While ISGs are intrinsically located downstream of IFN in the antiviral pathways induced by viral infections, a number of them are able to up-regulate type I IFNs and are therefore involved in positive feedback regulatory loops (e.g., *trim25*, *rigI*, *stat1*, *irf7*, and *viperin*/*vig1* [76–79], while some also feedback negatively on IFN signaling (e.g., *socs1* and *2*). Furthermore, the recognition of viral compounds by cellular sensors can up-regulate some ISGs directly, that is, independently of IFN induction; such bypass has been shown for example for Mx [80,81] and for viperin in human and fish [82,83]. Hence, while IFN definitely plays a central role in the innate antiviral response, a complex and redundant network of regulatory loops and bypass mechanisms is also involved, which makes the whole system more resistant to subversion by viruses.

Orthologues of human ISGs involved in IFN amplification have often been retrieved as ISGs in fish, which may indicate that they belong to the primordial IFN pathway: for example, *trim25*, *rigI*, *stat1*, *irf7*, and *viperin*/*vig1* are conserved in teleost fish and are induced by type I IFN in these organisms [84]. In fish, this list includes also *irf3* [45,85], which is not an ISG in mammals. Although their induction pathways are partly unknown, IFN-independent induction has been observed for some of them. Whether regulatory loops of signaling pathways for type I IFN and ISGs induction are ancestral, or have been shaped independently during fish *versus* tetrapod evolution, remains to be clarified.

The evolution of teleost fish was marked by an early WGD event, followed by a gene loss phase, and as a consequence, the fish genomes sequenced to date do not contain more genes than humans, but paralogous pairs that arose from this WGD are frequent [86]. To further complicate things, additional WGD episodes occurred in some branches among teleosts—for example, in salmonids—while other fish underwent strong genome contraction, such as the tetraodon/fugu family. Of note, zebrafish has a relatively large genome with many highly expanded gene families, compared to other fish model species [87]. Since genes involved in effector mechanisms of immunity tend to diversify to escape subversion by pathogens, one might expect that fish would have retained many ISG duplicates and would possess larger repertoires of ISGs.

In fact, this hypothesis is still difficult to validate, since the diversity of fish ISGs is not fully defined. A few typical ISGs were first identified using primers or probes targeting conserved sequences such as *Mx* [88–90] and genes of the MHC class I presentation pathway [91]. Then, PCR-based approaches for differential display of transcripts (differential display PCR, subtractive suppressive hybridization, etc.) led to the discovery of genes with high induction level; for example, *viperin*/*vig1* and 20 other viral hemorrhagic septicemia virus (VHSV)-induced genes (*vig*) including *isg15* and two chemokines were identified in rainbow trout leukocytes by DDPCR and SSH [83,84,92]. *cd9* and *isg15* were found induced by the rhabdovirus infectious hematopoietic necrosis virus (IHNV) in Atlantic salmon with the same methods [93,94], which were applied to many fish species. In grass carp (*Carassius carassius*), subtractive approaches showed that an *irf-like* [95], *jak1* and *stat1*, two *Mx* [96], two *isg15* [96,97], and a number of genes encoding tetratricopeptide-containing proteins [96] are up-regulated by the grass carp hemorrhage virus. In Atlantic cod (*Gadus morhua*), SSH screening after poly(I:C) stimulation identified a number of genes including those encoding ISG15; IRF-1, IRF-7, and IRF-10; MHC class I; VIPERIN/VIG1; and the ATP-dependent helicase LGP2 [98]. In the sea bass (*Dicentrarchus labrax*), brain nodavirus-infected tissue was analyzed and C-type lectins, pentraxin, and an anti-inflammatory galectin were found [99,100]. A more comprehensive representation of the fish transcriptional response to viral infection came only with genome and EST high-throughput sequencing, opening the way to the microarray technology. Microarray analyses were applied to characterize the response induced by different viruses [64,101–105], IFN inducers [106,107], or recombinant IFN itself [108]. These transcriptome analyses from multiple cell types and tissues suggested that a “core” set of 50–100 genes is typically induced [109]. To get a more comprehensive repertoire of ISG in a whole fish, we recently characterized the response of the zebrafish larva to the Chikungunya virus (CHIKV), a virus that induces a powerful type I IFN response [110]. A set of highly induced ISGs was found, which is also typically retrieved in human [75,111]: *rsad2*, *CD9*, *isg12*, *isg15*, *ifit* and *ifi44* family members, *stat1*, *trim25*, *socs1*, *irf1*, and *irf7*. This gene set was concordant with the major list of fish ISGs predicted from different tissues of other species (see above, reviewed in Ref. [109]). A list of zebrafish orthologues of human ISGs was similar to the repertoire of genes up-regulated by CHIKV infection, which also further confirmed the size of this core set [110].

The above-mentioned analysis of the zebrafish orthologues of all human ISGs also revealed some

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important mammalian ISGs that are almost certainly lacking an orthologue in the zebrafish genome [110]. Zebrafish (and apparently all teleosts) lacks the APOBEC3, OAS, IFI16, and CLEC4 families altogether. Among other notable absent genes, one may cite *bst2*/*tetherin*; several *trim* such as *trim5*, *trim22*, or *pml*/*trim19*; and *isg20*.

A significant antiviral activity was demonstrated in fish for several of the ISGs. For example, overexpression of a Japanese flounder PKR homologue increased eIF-2 phosphorylation and inhibited the replication of the *Scophthalmus maximus* rhabdovirus [112]; MX proteins blocked the birnavirus IPNV [113], but not the rhabdovirus IHNV [89]; fish ubiquitin-like ISG15 shares with its mammalian homologues the anchor LRGG motifs and interacts with cellular and viral proteins [114], and an ISGylation-dependent activity of the zebrafish ISG15 was recently demonstrated against different RNA and DNA viruses [41]. A cytokine-like activity was also reported for the ISG15 secreted form in the tongue sole [115], as previously for mammals [116].

Altogether, these observations indicate that a number of essential ISGs were already important players of the IFN-mediated antiviral response rather early in the vertebrate history, at least in the common ancestor of tetrapods and fish. It starts to be possible to assess the extent of functional conservation of this core gene set, not only by direct comparison of the functions of individual genes but also using global comparative analyses. For example, some ISGs are typically induced more than others. Do human ISGs and their zebrafish homologues show similar response patterns? Figure 3A shows a tentative correlation of the response of zebrafish larva to CHIKV with the response of human liver to IFNα [117] and illustrates that orthologues of strongly induced human ISGs tend to be strongly induced by CHIKV infection in zebrafish as well.

Genes involved in immune responses typically show high rates of evolution due to selection pressures exerted by pathogen subversion. Under this rule, ISGs should show a similar trend, and we should observe a negative correlation between ISG sequence similarity in fish and human and their induction level. The relationship between induction rate and sequence similarity/conservation is obviously complex, and these two parameters are not merely correlated (Fig. 3B). However, the global pattern may suggest a loose negative correlation, and outliers such as *rsad2*/*viperin*, which are highly conserved and well induced by IFN, constitute interesting exceptions.

Many ISGs are members of gene families, with different evolutionary dynamics of expansion/diversification during the evolution of tetrapods *versus* that of fish. Among families containing ISGs, two different patterns were observed: families that differentiated in parallel in tetrapods and fishes from a single common ancestor gene ("young" families) and families that had already diversified in the common ancestor to fishes and mammals ("old" families) [110]. Young families (such as MX or IFIT) would likely bind viral components and quickly diversify under strong selection pressure. On the contrary, old, stabilized families typically contain regulatory factors or signal transduction components (i.e., IRFs, STATs, and SOCS) and constitute key molecules in the conserved antiviral machinery.

To illustrate how comparative analysis of human and fish transcriptional responses might suggest important new genes to be targeted in future studies, we will focus on the subset of human ISGs that have a one-to-one orthologue in zebrafish, because they are the easiest to test experimentally, for example, by morpholino knockdown assays. This list includes 178 human genes [110]. Strikingly, among these ISGs, 140 (80%) are not annotated as having a potential role in antiviral defense in the current Ensembl GO classification. Some of those genes surely play important, but for the moment overlooked, roles in antiviral responses. Good candidates for further research would be ancestral ISGs, identifiable within this list by having a zebrafish orthologue induced by IFN. At least four genes fulfill this criterion based on the microarray analysis of the response to CHIKV: *cmpk2*, *phf11*, *upp2*, and *ftsjd2*. The kinase CMPK2 participates in dUTP and dCTP synthesis in mitochondria and may play a role in monocyte differentiation, PHF11 is a positive regulator of Th1-type cytokine gene expression, UPP2 is involved in nucleoside synthesis, and FTSJD2 mediates mRNA cap1 2′-*O*-ribose methylation to the 5′-cap structure of mRNAs—a feature that, remarkably, distinguishes host mRNAs from some viral mRNAs [118]. More genes shall be added to this list in the future as RNA-seq analysis and improved stimulation protocols will yield a more exhaustive list of zebrafish ISGs.

Part 3. IFN-Producing Cells

The current paradigm for type I IFN production in mammals is that all cell types are able to produce IFNβ upon sensing a virus, and in addition, some specialized sentinel cells such as plasmacytoid dendritic cells can produce very high levels of IFNα. The specialized cells have a different array of sensing molecules (e.g., TLR7) and are poised for rapid IFN expression by constitutive expression of some signal-transducing molecules that need to be induced in other cell types (e.g., IRF7). Is the situation similar in fish?

A few studies have addressed the tissue-specific differences in expression of fish type I IFNs and sometimes identified the cell types involved. Zou *et al.* [9] found important differences between leukocytes and fibroblasts upon poly(I:C) stimulation *in vitro*: thus, head kidney cells would express all IFNs tested, while RTG-2 fibroblasts would express the group I IFNs

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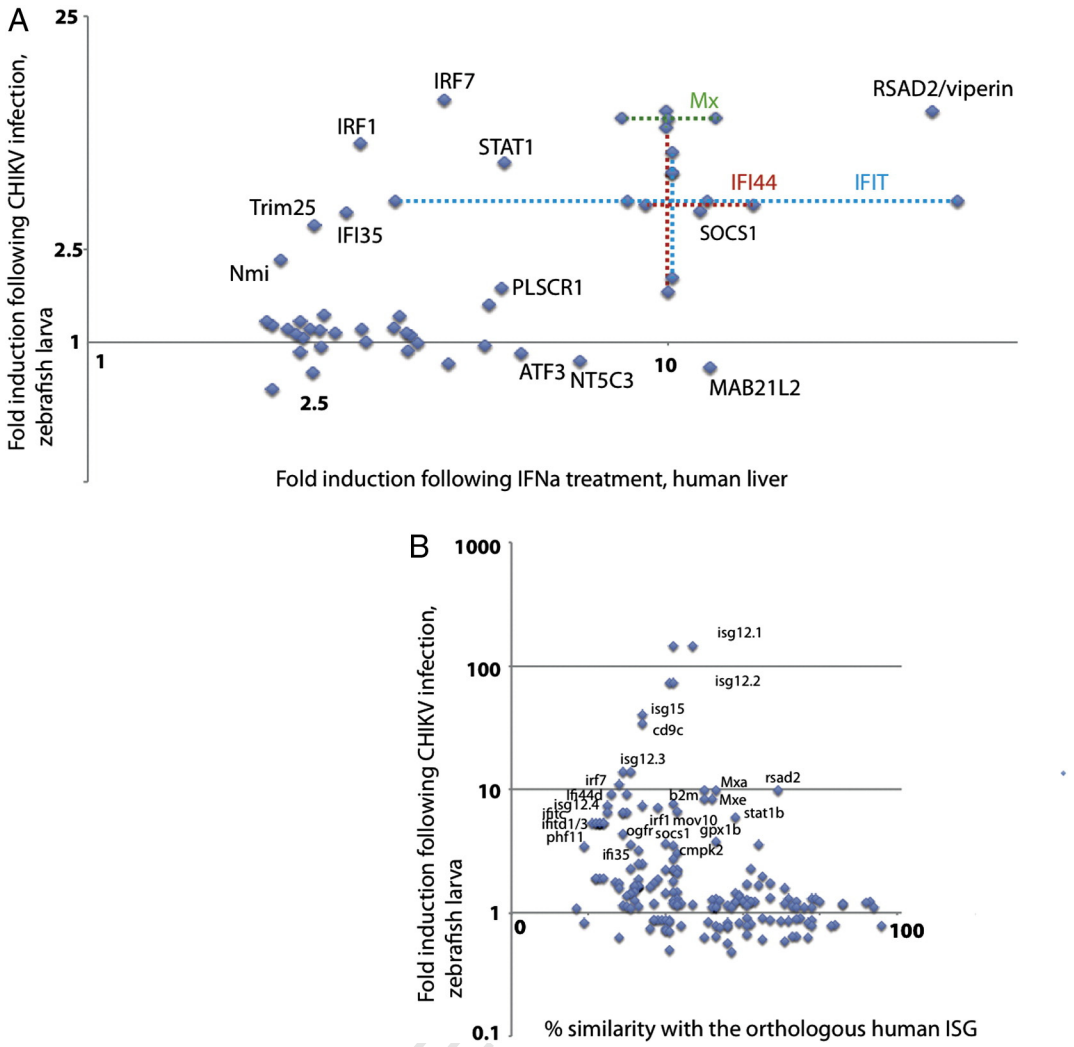

Fig. 3. Assessment of the conservation of ISGs: comparison of induction levels and sequence similarity between human ISGs and their zebrafish orthologues. (A) Induction levels of human ISGs (liver biopsy cells treated for 4 h with IFNα, from Sarasin-Filipowicz *et al.* [117], GEO accession GSE11190) compared with induction levels of their zebrafish orthologues (larvae infected for 48 h with the strong IFN-inducing CHIKV, GEO accession GSE47057). When homologous genes from human and zebrafish were not linked by a one-to-one orthology relationship, they were linked by a colored dotted line and set at the geometric average of the fold changes values of the other species. In these cases, the name of the gene family is indicated in the corresponding color. (B) Level of induction by CHIKV of zebrafish genes orthologous to human ISGs [same data set as for (a)], compared with their degree of similarity with their human orthologues (retrieved from the Ensembl database).

(IFN1 and IFN2) but not the group II IFN (IFN3). *Ex vivo* analysis of tissues from infected trout suggested a similar picture, with IFN3 being expressed in lymphoid tissue (kidney and spleen) but much less in liver [9]. In Atlantic salmon, Sun *et al.* [11] also found a much more restricted expression of IFN subtype by fibroblast-like TO cells, where only IFNa (a group I IFN) was induced more than twofold, while head kidney leukocytes would also express the group II IFNb and IFNc [11]. In these cells, polyI:C would induce IFNa and IFNc, while S-27609 (a TLR7 agonist) would preferentially induce IFNb. Similar outcomes were found *in vivo* at early time points after poly(I:C) or S-27609, but the pattern changed strongly after a few days, likely as a result of complex feedback loops [11]. More recently, Svingerud *et al.* published a study that largely confirmed these findings (using R848, a TLR7/8 agonist, instead of S-27609) and added much spatial information, notably by performing *in situ* hybridization on tissue slices [18]. Quite remarkably, in all tissues, expression of all tested IFNs was restricted to a minority of cells. IFNa and IFNc were sometimes coexpressed by the same cell in poly(I:C)-injected animals, while IFNb and IFNc could be coexpressed after R848 injection. Cell types that could be identified as expressing IFNs were

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endothelial cells and gill pillar cells for IFNa and gill pillar cells for IFNc. No IFNb-expressing cell could be positively identified, but the data suggest that they were distinct from IFNa-expressing cells. IgM-positive B cells did not express any IFN; neither did melanomacrophages [18].

More recently, this question has been addressed in zebrafish using IFN-reporter transgenes. In larvae, among the four zebrafish *ifn* genes, only *ifnφ*1 (a group I *ifn*) and *ifnφ*3 (a group II *ifn*) are considered to play a role, because *ifnφ*2 is expressed only at the adult stage and *ifnφ*4 does not seem to exert a significant antiviral effect [10]. An *ifnφ*1 reporter transgene has been recently reported [119] and analyzed in the context of CHIKV infection, which induces a strong IFN response. The transgene was mainly expressed in two cell populations: neutrophils and hepatocytes—a pattern entirely consistent with expression of the endogenous *ifnφ1* gene as seen by *in situ* hybridization, although the transgene expression was somewhat delayed [119]. The pathways inducing *ifnφ1* in these two populations are not yet unraveled but are likely to be different since hepatocytes were a target of CHIKV while neutrophils were not infected. A small macrophage-like population also expressed the transgene. Depletion studies demonstrated that neutrophils, but neither hepatocytes nor macrophages, were critical to control the infection. Interestingly, in control, uninfected fish, a small population of neutrophils (10–30 cells/larva) express the transgene at a weak level [119]. An *IFNφ3* reporter line has also been generated (V. Briolat, N.P., G. Lutfalla, and J.-P.L., unpublished results). The pattern of expression of this transgene during CHIKV infection is very different from that of the *ifnφ1* reporter and includes fibroblasts, endothelial cells, hepatocytes, and muscle fibers, all cell types that may be infected by CHIKV; however, expression of the transgene was only observed in virus capsid-negative cells (N.P., unpublished results).

As a general conclusion, fish IFNs generally appear to be expressed by discrete, scattered cell populations with little overlap between IFN subtypes. Some IFNs are expressed in an "IFNβ" pattern, by fibroblasts and other tissue cells that may be direct targets of the viruses, while others are expressed in an "IFNα" fashion by more specialized immune cells. Surprisingly, however, while group II IFNs are those that are preferentially expressed by hematopoietic cells in salmonids, the reverse seems true in zebrafish: group I is preferentially expressed by neutrophils.

There is so far no evidence for a cell type similar to plasmacytoid dendritic cells in fish, but these studies are still in their infancy. Neutrophils seem to play such a role in zebrafish larvae, which came as a surprise. It remains to be tested whether neutrophils are also major IFN-producing cells in adult zebrafish, in other fish species, and possibly during some viral infections in tetrapods.

Part 4. Kinetics of the Different IFN Responses in Fish

Early studies in fish cell lines described a quick and early production of IFN-like activity after viral infection or incubation with UV inactivated viruses [4,120]. IFN production following a virus infection was also demonstrated *in vivo* in rainbow trout, with higher amount on day 1 post-VHSV infection and declines to background level by day 14 post-infection [3]. In keeping with this, in carp injected with 10^7 pfu of virulent spring viremia of carp virus, the IFN-like activity peaked as early as days 1 and 2, started to decline at day 3, and had disappeared by day 14 [121].

In the 1990s, the kinetics of the antiviral response was studied in further detail using (semi)Q RT-PCR to assess expression of ISG transcripts. After the first fish type I IFN genes were cloned in the 2000s, the kinetics of the IFN mRNA itself could be measured in various infection contexts. Different types of kinetics were obtained, a few of which will be illustrated. McBeath *et al.* compared the kinetics of type I IFN in Atlantic salmon after infection by ISAV and IPNV [122]. Type I IFN and Mx expression peaked twice on days 3 and 6 after IPNV infection and declined progressively. This biphasic response might rely on a positive feedback loop depending on IRF induction by the first burst of IFN production as described in mammals [123]; however, the mechanisms underlying the biphasic salmon IFN response to IPNV remain unknown. In contrast to this kinetics, a later, monophasic type I IFN response occurred after ISAV infection; IFN shortly peaked on day 5 or 6, while Mx peaked on day 6, declined to day 9, and remained expressed until day 30 post-infection. These differences likely reflected that these viruses use different mechanisms for dealing with the host response. Early up-regulation of IFN and ISG like Mx by the IPNV probably contributed to the good survival recorded after this infection. In contrast, high mortality and late response were observed after ISAV infection, which could be due to viral anti-IFN mechanisms [124]. Transcriptome profiling of the response induced by recombinant IFN in macrophage-like SHK1 cells showed that Mx and other ISGs were induced after 6 h of incubation and peaked at 24 h [108], supporting other observations reported for different tissues (e.g., trout kidney leukocytes in Ref. [84]).

However, these studies do not reflect the whole complexity of the type I IFN response since (1) most of the first QPCR and array systems did not take into account the IFN alternative transcripts discovered in zebrafish and in other species; hence, measures of IFN up-regulation integrate both secreted and non-secreted isoforms, which provides a partial view of the kinetics of the effective response; (2) fish genome and EST sequences revealed many

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type I IFN genes, especially in salmonids; (3) IFNγs may also contribute to the induction of some ISGs [28].

It is difficult to compare kinetics of IFN gene induction by two different viruses; not only is there a large range of antiviral mechanisms potentially at play (as discussed later), but viral burden (and thus signal) is likely to be different in both cases; comparing induction of different genes in the same context is more informative. For instance, in the zebrafish CHIKV infection model, expression of *ifnφ1* was sustained, while *ifnφ3* expression was more transient [119]. This likely reflects the different pathways (and cell types, as discussed above) involved in their induction, consistently with results of luciferase assays suggesting the variable contribution of IRF3 and/or IRF7 to activate the promoters of the various zebrafish IFNs [49].

Part 5. Tissue-Specific Responses

Expression of IFNs is induced upon detection of viruses and is thus expected to be fairly organ specific, depending on the tropism of the particular virus considered. By contrast, since type I IFN receptors are ubiquitously expressed in mammals and IFNs diffuse via the blood, ISGs would be expressed in a more uniform fashion. However, recent findings have shown this idea to be simplistic. For instance, type III IFNs induce the same set of ISGs than type I IFNs, but their receptor is expressed in a tissue-restricted fashion, allowing for targeted induction of ISGs, notably in epithelia exposed to outer environment such as the gut [125]. In addition, even upon systemic type I IFN administration, ISG expression has been found to be highly variable from tissue to tissue [126]. Do we find a similar situation in fish?

As mentioned above, fish also possess two groups of virus-induced IFNs that signal via two distinct receptors [10]. Although both groups are phylogenetically related to mammalian type I (rather than type III) IFNs [12], it has been proposed that the group I/group II and type I/type III dichotomies may have evolved in a convergent manner in teleosts and tetrapods, respectively [10]. A potential selective advantage of the dichotomy would be that a response restricted to external tissues may deal with most viruses with few of the side effects associated with a full-blown IFN response, which would be triggered only upon the most severe viral infections. Unfortunately, there are as yet no data published regarding the tissue-specific expression of the receptors for the two groups of IFNs. Both receptors share the CRFB5 chain, which is expressed ubiquitously at a relatively high level, but the weak expression of the specific CRFB1 and CRFB2 chains precluded their detection by whole-mount *in situ* hybridization in zebrafish embryos [13].

We also recently used whole-mount *in situ* hybridization to establish the expression pattern of four ISGs (*isg15*, *rsad2*/*viperin*, *isg12.1*, and *irf7*) in zebrafish larvae, notably in the CHIKV infection model, which results in a very strong endogenous IFN expression [110]. Basal levels of expression were below detection level, but upon infection, strongly tissue-dependent induction was observed, with an overall pattern of expression in liver, gut, and blood vessels, with some gene-specific differences (e.g., *viperin* was comparatively less induced in the gut while *isg12.1* was less induced in the liver). A rather similar, if weaker, pattern was observed after IHNV infection [110] or after intravenous injection of recombinant zebrafish IFNs (J.-P.L., unpublished results), suggesting that it mostly reflects the differential susceptibility of organs to circulating IFNs.

It is still unclear whether this pattern seen in zebrafish larvae can be generalized, as tissue variability in ISG expression has been addressed in relatively few studies. Lymphoid organs constitute the site for the activation of a proper immune response and, therefore, the majority of the studies present in literature focus their attention on the specific responses activated in those tissues. Responses have also sometimes been analyzed in some tissues for which viruses were known to have a preferred tropism. The following paragraphs focus on such studies.

One of the gateways of viral entry and replication in fish is fin bases, for example, for novirhabdoviruses [127]. In response to lethal VHSV infection of Pacific herring (*Clupea pallasii*), *Mx*, *psmb9*, and an *MHC class I* gene were found to be induced both in the spleen and in the fin bases, with a moderately stronger induction in the spleen attributed to the higher viral burden in this organ [128]. Transcriptomic and proteomic studies performed in adult zebrafish during VHSV infection have shown that a number of infection-related genes/proteins are overexpressed in the fins but not in other organs. Among these are complement components, interleukin genes, *hmgb1* protein, *mst1*, and *cd36* [129]. This does not seem to reflect a typical ISG response, and indeed *ifnφ1* transcripts were not identified in this study, possibly because the low temperature required for VHSV replication was suboptimal for induction of a response in zebrafish. Infection of rainbow trout fin bases with VHSV, on the other hand, determines the up-regulation of the chemokines CK10 and CK12, as opposed to those overexpressed in the gills (CK1, CK3, CK9, and CK11). These expression variations may be due to a different permissivity of the tissues (fins or gills) to viral replication [130].

Several fish viruses are also known to have a tropism for the heart. Fish alphaviruses and, more recently, members of the *Totiviridae* family (e.g., piscine myocarditis virus) are associated with cardiac and/or skeletal myopathies. In particular,

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alphaviruses, such as salmonid alphavirus subtype-1, are capable of causing acute heart lesions with necrotic foci and hypertrophy of the cardiac muscle. Unlike adult fish, smolts can replace damaged cardiomyocytes by cell division and may, therefore, be subjected to a decreased pathogenesis and impact [131]. Recently, the determinants of resistance of two strains of Atlantic salmon to salmonid alphavirus have been investigated, comparing responses in heart, kidney, and gills (a possible port of virus entry). The two strains displayed significantly different basal expressions of *ifna1* and ISGs (*Mx*, *viperin*, and *cxcl10*); however, the induction by viral infection was comparable in the three organs [132]. Similar results were obtained from Atlantic salmon infected with piscine myocarditis virus [133].

Several fish viruses also have a preferred tropism for the central nervous system. One of the most serious viral diseases affecting marine fish is represented by nodavirus encephalopathy. The central nervous system and the eye constitute the specific targets for nodavirus replication, leading to mass mortality in larvae and juvenile fish. Numerous studies have, therefore, been conducted to determine the immune responses activated in the brain tissue upon infection, but comparison with other tissues remain scarce. Infection of zebrafish larvae with nervous necrosis virus (NNV), for example, leads to mortality rates higher than 95%. This has been linked to the lack of IFN and Mx expression, not detectable in the larval stage but expressed by infected adults [104]. A thorough transcriptomic analysis conducted in Atlantic cod (*G. morhua*) has revealed that NNV infection affects mainly neural processes and their regulation and cellular differentiation (down-regulated genes). Many ISGs were found to be induced in the brain, but expression in other tissues was not reported [104]. NNV infection in turbot (*S. maximus*) is followed by overexpression of *Mx*, *irf-1*, and *tnf-α* [134]. Finally, in European sea bass (*D. labrax*), two different *x* genes (*MxA* and *MxB*) were differentially expressed during NNV infection. While *MxA* is highly up-regulated in the brain, *MxB* expression does not differ substantially from controls, thereby suggesting that the former is the predominant isoform and that *MxB* may play a different and independent functional role [135].

Part 6. Subversion Mechanisms by Viruses in Fish

The complexity of antiviral signaling pathways reflects the dynamic interactions between viruses and their hosts and has been shaped by the highly diverse strategies developed by these pathogens to evade antiviral immunity. In mammals, a vast number of strategies have been discovered, targeting immunity (pattern recognition receptors, IFN signaling, MHC class I presentation, cytokine or chemokine networks, etc.) as well as basic mechanisms of virus–host interactions (autophagy, cell cycle, protein synthesis, etc.).

Such mechanisms are certainly used by fish viruses as well, but remain poorly described. Subversion of host immune response has been mainly studied for novirhabdoviruses, birnaviruses, and orthomyxoviruses.

Novirhabdoviruses are negative-sense single-stranded RNA viruses infecting fishes. They have a small genome encoding four structural proteins (N, P, M, and G) plus a polymerase (L), like other rhabdoviruses, and one specific nonstructural protein (NV), which is a good candidate for subversion of immune pathways. Recombinant IHN and VHS viruses lacking NV were able to replicate in cell culture, although the growth of the IHNV-ΔNV was severely impaired [136–138]. The importance of NV protein for pathogenicity was also strongly suggested by *in vivo* challenges with mutant viruses that caused only 20% mortality, whereas the wild-type control virus causes 100% mortality [136–138]. Although the sequence of the NV protein is not highly similar between novirhabdoviruses, the attenuated phenotype of VHSV-ΔNV can be rescued by re-introduction of NV from IHNV and vice versa [137,139], suggesting that the function of NV during infection is conserved. In fact, cells infected by NV-deletion mutants express higher levels of type I IFN transcripts, suggesting that NV is used to evade the innate antiviral immune response [140]. Moreover, growth of IHNV-ΔNV was inhibited by poly(I:C) treatment at 24 h post-infection, while the wild-type virus was not blocked. The overexpression of VHSV NV protein also reduced the TNFα-mediated activation of NFκB, which likely contributes to its impact on the innate response [141].

"Multitask" properties are known for M and P proteins of prototypical rhabdoviruses infecting higher vertebrates, rabies virus (RV), and vesicular stomatitis virus (VSV) [142]. RV was shown to diminish IFNβ induction through the viral protein P, which blocked IRF3 phosphorylation [143]. The P protein of RV also inhibited IFN downstream signaling by blocking the nuclear import of STAT1 [144] and has an impact on viral transcription and nucleocapsid formation. In fish, such mechanisms have not been reported yet, but the P protein of IHNV (as well as NV) is targeted by ISG15, which may represent a cell countermeasure [41]. Indeed, overexpression of ISG15 in EPC cells is sufficient to trigger antiviral activity against novirhabdoviruses (IHNV, VHSV), birnavirus (IPNV), or iridovirus (EHNV). ISGylation, which targets cellular proteins such as TRIM25 and viral proteins such as the P and NV of IHNV, is required for viral inhibition: the $ISG15_{LRAA}$ mutant (incapable of functional ISGylation)

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does not afford any protection. Subversion of IFN induction has also been demonstrated for fish birnaviruses and orthomyxoviruses. The proteins VP4 and VP5 of the birnavirus IPNV had antagonistic properties towards an IFN reporter [145]; however, *in vivo* comparison of IPNV field isolates with different levels of pathogenicity did not clearly confirm the importance of an intact VP5 protein for virulence [146]. Similarly, two ISAV proteins encoded by the genomic segments 7 and 8—respectively named s7ORF1 and s8ORF2—are involved in the modulation of the IFN signaling [124,147]. While s7ORF1 expression is restricted to the cytoplasm [147], s8ORF2 possesses two NLS signals responsible for nuclear expression and binds both dsRNA and polyA RNA [124]. The IFN antagonist activity of s7ORF1 was shown by Mx-Luc reporter assay or RT QPCR on Mx and IFN upon poly(I:C) treatment [147]. Another study determined that s7ORF1 and s8ORF2 expression down-regulates the activity of a type I IFN promoter upon poly(I:C) exposure [124].

Large DNA viruses often possess genes blocking IFN pathways or inhibiting ISG function. For example, the ranavirus RCV-Z (*Rana catesbeiana* virus Z), a pathogen of fish and frogs, circumvents host-induced transcriptional shutoff and apoptosis by expressing a pseudosubstrate for PKR [148]. Other fish iridoviruses and herpesviruses can also possess such "mimickry" genes: for example, the koi herpesvirus encodes an IL-10 homologue [149], the Singapore grouper iridovirus encodes IgSF members, and another fish iridovirus encodes a B7-like sequence [150].

Viruses also dysregulate a number of basic cellular functions, which they use for their own replication and to block intrinsic antiviral mechanisms. For instance, IHNV has an acute life cycle during which it causes global blockage of cellular transcription, very similarly to the well-studied VSV [151,152]. The M protein of VSV, in addition to repressing cellular transcription, was shown to inhibit nuclear trafficking of RNA and proteins, thereby also inhibiting antiviral responses [153]. Both VSV and IHNV elicit cell rounding, probably by interfering with cytoskeletal dynamics [151,154]. Shutoff of basic cellular machinery eventually leads to apoptosis. Programmed cell death being also one of the host's antiviral strategies, many viruses developed strategies to delay apoptosis and complete their infection cycles. In fish, VHSV was able to block experimentally induced apoptosis in EPC cells in an NV-dependent manner [139].

Conclusion

Antiviral immunity has been studied only in a few fish species, either aquaculture fishes or model species. Fish are vertebrates and share with humans and mice most of the key antiviral pathways. However, fishes had a long and complex genome history and developed a specific adaptation to the aquatic environment (and to its pathogens). Hence, the fish antiviral immunity represents an alternative version of what could evolve upon highly selective pressures of host–virus interactions, from the ancestral system present in the early vertebrates. Comparison of mammalian and fish innate antiviral mechanisms will be certainly beneficial to distinguish the core system, which is resilient to the subversive selective pressures exerted by the viral world, from the specialized systems that emerged during the evolution of each branch in response to particular viral strategies. In addition, the imaging possibilities offered by model fish species such as the zebrafish will be instrumental, in the future, to unravel the spatiotemporal dynamics of these core antiviral responses shared by all vertebrates.

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Abbreviations used:
IFN interferon; WGD whole genome duplication; CRFB cytokine receptor family B; IPNV infectious pancreatic necrosis virus; ISAV infectious salmon anemia virus; RLR RIG-I-like receptor; TLR Toll-like receptor; RIG-I retinoic acid-inducible gene I; LGP2 laboratory of genetics and physiology 2; TNF tumor necrosis factor; TRAF TNF receptor-associated factor; TBK1 TANK binding kinase 1;

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IRF IFN regulatory factor; dsRNA double-stranded RNA; TIR Toll-interleukin 1 receptor; IRAK interleukin-1R-associated kinase; VHSV viral hemorrhagic septicemia virus; IHNV infectious hematopoietic necrosis virus; CHIKV Chikungunya virus; NNV nervous necrosis virus; RV rabies virus; VSV vesicular stomatitis virus

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