REVIEWS IN Aquaculture

Reviews in Aquaculture, 1–32 doi: 10.1111/raq.12349

Fish type I and type II interferons: composition, receptor usage, production and function

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Received 13 December 2018; accepted 27 March 2019.

Abstract

Interferons (IFNs) have been reported in vertebrates from fish to mammals. To date, two types of IFNs, i.e. type I and type II IFNs have been reported in a range of fish species in aquaculture. In fish, type I IFNs are clarified into group I and group II, with two cysteines and four cysteines, and further into subgroups a, d, e, h, and b, c, f, respectively. Group I IFNs appear to exist in all fish species, whereas group II IFNs are discovered only in certain species such as cyprinids, salmonids and perciform fish. It seems preferentially functional that group I IFNs signal through cytokine receptor family B (CRFB) 5 and CRFB1, while group II IFNs through CRFB5 and CRFB2. But they all initiate a same signalling pathway for the expression of interferon induced genes. The information about the production and the function of type I IFNs in fish are further summarized in the review. Interestingly, two members of type II IFNs, IFN-γ and a unique member, IFN-γ related gene (IFN-γrel) exist in fish, and IFN-γ and IFN-γrel can separately become homodimers for signalling through CRFB13, CRFB17, CRFB6 and CRFB17, respectively. In addition, the variation in the composition of type I and type II IFNs as well as in their receptors in some cyprinid and salmonid fish has also been reviewed. It is suggested that future perspectives for the research on type I and type II IFNs in fish are outlined from a comparative and evolutionarily point of view.

Key words: antiviral response, fish, IFN receptor, Interferon, type I IFN, type II IFN.

Introduction

Interferons (IFNs) are a subset of the class II cytokines playing crucial roles in host immune defense, especially in immune response against viruses (Pestka *et al.* 2004). It is perhaps not an exaggeration to say that the discovery and clinical use of IFNs have been a major advance in immunology and biomedicine over the past sixty years. Based on sequence similarity, genomic organisations, receptor usage and biological function, IFNs are classified into three types, i.e. type I, type II and type III IFNs (Pestka *et al.* 2004). Type I and type III IFNs are specialized as innate antiviral cytokines (Lazear *et al.* 2015; Teijaro 2016), and their main difference lies in their distribution and target cells: almost all nucleated cells respond to type I IFNs, whereas type III IFNs are restricted in tissues with high risk of viral exposure and infection, such as those with epithelial surfaces (Lazear *et al.* 2015; Teijaro 2016). By contrast, type II IFN is now known to represent a regulatory cytokine orchestrating both innate and adaptive immunity against viral and intracellular bacterial infections (Schoenborn & Wilson 2007).

From an evolutionary point of view, IFNs have been identified in vertebrates from fish to mammals (Zou & Secombes 2011; Gan *et al.* 2017, 2018; Langevin *et al.* 2013a; Santhakumar *et al.* 2017; Secombes & Zou 2017). Fish can be classified into three classes, i.e. Agnatha (jawless fish), Chondrichthyes (cartilaginous fish) and Osteichthyes

(bony fish), and the latter two are considered as the earliest vertebrates possessing the IFN system, representing an important model to expand the knowledge about the origin and evolution of the IFN system in vertebrates (Zou & Secombes 2011; Zhang & Gui 2012; Langevin *et al.* 2013a; Boudinot *et al.* 2016; Secombes & Zou 2017; Robertsen 2018). On the other hand, as a vital protein source for human consumption, farmed fish are now threatened with viral infectious diseases, and the understanding of IFN system in fish with aquaculture importance may provide clues for the development of strategies in relation with the prevention of diseases (Workenhe *et al.* 2010; Evensen & Leong 2013; Collet 2014; Collins *et al.* 2018; Oidtmann *et al.* 2018). Hence, the fish IFN system has been one of the research focuses for comparative immunologists, and significant progress has been achieved in understanding the fish IFN system in recent years. Although type III IFN genes are not to date identified in fish (Zou & Secombes 2011; Zhang & Gui 2012; Langevin *et al.* 2013a; Boudinot *et al.* 2016; Secombes & Zou 2017; Robertsen 2018), IFN ligands, receptors, signalling pathways, regulatory mechanism and functional properties of type I and II IFN systems have been extensively studied. This review attempts to summarize the recent discoveries on the IFN systems in fish.

Type I IFNs in fish

Discovery and phylogeny of type I IFNs

Although IFN was first discovered by Alick Isaacs and Jean Lindenmann as early as in 1957, the first fish IFN gene was identified in 2003 by three independent groups in zebrafish (*Danio rerio*) (Altmann *et al.* 2003), Atlantic salmon (*Salmo salar*) (Robertsen *et al.* 2003) and green spotted pufferfish (*Tetraodon nigroviridis*) (Lutfalla *et al.* 2003). Afterwards, the IFN genes have been identified in a multitude of fish species, including grass carp (*Ctenopharyngodon idella*), common carp (*Cyprinus carpio*), gibel carp (*Carassius auratus gibelio*), red crucian carp (*C. auratus* red var.), black carp (*Mylopharyngodon piceus*), rohu (*Labeo rohita*), rainbow trout (*Oncorhynchus mykiss*), mandarin fish (*Siniperca chuatsi*), large yellow croaker (*Larimichthys crocea*), orange-spotted grouper (*Epinephelus coioides*), seven band grouper (*E. septemfasciatus*), European sea bass (*Dicentrarchus labrax*), gilthead seabream (*Sparus aurata*), rock bream (*Oplegnathus fasciatus*), meagre (*Argyrosomus regius*), turbot (*Scophthalmus maximus*), Japanese flounder (*Paralichthys olivaceus*), channel catfish (*Ictalurus punctatus*), medaka (*Oryzias latipes*) and Japanese eel (*Anguilla japonica*) (Table 1) (Gonzalez *et al.* 2007; Aggad *et al.* 2009; Casani *et al.* 2009; Chang *et al.* 2009; Chen *et al.* 2014; Huang *et al.* 2015b; Ding *et al.* 2016, 2017; Hu *et al.* 2017; Huang *et al.* 2018a; Long *et al.* 2004, 2006; Zou *et al.* 2007, 2014a; Kitao *et al.* 2009; Lopez-Munoz *et al.* 2009; Purcell *et al.* 2009; Sun *et al.* 2009; Li *et al.* 2012; Wan *et al.* 2012; Svingerud *et al.* 2012; Parhi *et al.* 2014; Pereiro *et al.* 2014; Valero *et al.* 2015; Kuo *et al.* 2016; Liao *et al.* 2016; Maekawa *et al.* 2016; Wei *et al.* 2016; Yan *et al.* 2016; Yang *et al.* 2017; Laghari *et al.* 2018; Milne *et al.* 2018; Wu *et al.* 2018; Xia *et al.* 2018). In addition, the IFN genes are also found in cartilaginous fish, such as elephant shark (*Callorhinchus milii*) (Chang *et al.* 2009; Zou & Secombes 2011).

Nevertheless, the phylogenetic relationship of these fish IFNs with mammalian type I and III IFNs had been heavily debated among comparative immunologists in the last ten to twenty years (Lutfalla *et al.* 2003; Robertsen 2006; Levraud *et al.* 2007; Zou *et al.* 2007; Aggad *et al.* 2009; Chang *et al.* 2009). Several aspects of evidence based on sequence analysis suggested that fish IFNs are likely to be the homologues of mammalian type I IFNs. In particular, they possess two important sequence features which are conserved between fish and mammalian type I IFNs, including the cysteine pattern and the C-terminal CAWE motif (Robertsen 2006; Zou *et al.* 2007; Chang *et al.* 2009). In contrast, it was argued that fish IFNs were likely to be the homologues of mammalian type III IFNs, as these fish IFNs are encoded by intron-containing genes with five exons and four introns as seen in mammalian type III IFNs, being entirely different from mammalian type I IFN genes which are intronless (Lutfalla *et al.* 2003). Consistently, the receptors of these fish IFNs share features of mammalian type III IFN receptors rather than type I IFN receptors in terms of the domain organization (Lutfalla *et al.* 2003; Aggad *et al.* 2009; Levraud *et al.* 2007). The short cytoplasmic receptor chain of fish IFNs, named as cytokine receptor family B (CRFB) 5, only possesses two fibronectin type III (FNIII) domains, as observed in the corresponding receptor chain of mammalian type III IFNs (IL-10R2), being distinct from the one of mammalian type I IFNs (IFNAR1) which has a uniquely large extracellular region containing four FNIII domains (Lutfalla *et al.* 2003; Aggad *et al.* 2009; Levraud *et al.* 2007). It was once hard to claim with certainty that these fish IFNs were the orthologues of mammalian type I or type III IFNs before the possible emergence of new evidence, so some researchers proposed that fish IFNs should be named as IFNφ, representing a unique type of virus-induced IFNs in fish (Stein *et al.* 2007).

This puzzling controversy has been clarified in a crystallographic study to determinate the three-dimensional structure of zebrafish IFNs in 2011, in which both zebrafish IFN1 and IFN2 exhibit typical type I IFN structure with a straight F helix rather than a bend in type III IFN architecture (Hamming *et al.* 2011). Now, it is generally accepted that these fish IFNs belong to type I IFNs (Zou & Secombes 2011; Zhang & Gui 2012; Langevin *et al.* 2013a; Boudinot *et al.* 2016; Secombes & Zou 2017; Robertsen 2018), but two new intriguing questions need to be further resolved:

Table 1 Currently reported type I IFNs in fish

Order	Species	Gene	GenBank accession number	References
Cypriniformes	Zebrafish (*D. rerio*)	Dr-IFN1	NM_207640	(Aggad *et al.* 2009; Altmann *et al.* 2003; Lopez-Munoz *et al.* 2009)
		Dr-IFN2	NM_001111082	
		Dr-IFN3	NM_001111083	
		Dr-IFN4	NM_001161740	
	Grass carp (*C. idella*)	Ci-IFN1	DQ357216	(Li *et al.* 2012; Liao *et al.* 2016)
		Ci-IFN2-4	KU182641–KU182643	
	Common carp (*C. carpio*)	Cc-IFN1-2	AB376666 –AB376667	(Gonzalez *et al.* 2007; Kitao *et al.* 2009; Wei *et al.* 2016)
	Crucian carp (*C. auratus*)	Ca-IFNa	AY452069	(Xia *et al.* 2018)
		Ca-IFNc	MF574165	
	Red crucian carp (*C. auratus* red var.)	Car-IFNa	KU561831	(Yan *et al.* 2017; Yan *et al.* 2016)
		Car-IFNa2	KX839490	
	Black carp (*M. piceus*)	Mp-IFNa	KR265208	(Huang *et al.* 2015b; Wu *et al.* 2018)
		Mp-IFNb	MF497811	
	Rohu (*L. rohita*)	Lr-IFN	HQ667143	(Parhi *et al.* 2014)
Salmoniformes	Rainbow trout (*O. mykiss*)	Om-IFNa1 (IFN1)	CAM28541	(Chang *et al.* 2009; Purcell *et al.* 2009; Zou *et al.* 2007, 2014a)
		Om-IFNa2 (IFN2)	NP_001153977	
		Om-IFNa3–a4	HF931021–HF931022	
		Om-IFNb1 (IFN3)	NP_001153974	
		Om-IFNb2 (IFN4)	NP_001158515	
		Om-IFNb3–b5	HF931023–HF931025	
		Om-IFNc1–c4	HF931026–HF931029	
		Om-IFNd1 (IFN5)	NP_001152811	
		Om-IFNe1–e7	HF931030–HF931036	
		Om-IFNf1–f2	HF931037–HF931038	
	Atlantic salmon (*S. salar*)	Ss-IFNa	AY216594	(Robertsen *et al.* 2003; Sun *et al.* 2009; Svingerud *et al.* 2012)
		Ss-IFNb	JX524152	
		Ss-IFNc	JX524153	
		Ss-IFNd	JX524151	
Perciformes	Mandarin fish (*S. chuatsi*)	Sc-IFNc	KY768914	(Laghari *et al.* 2018)
		Sc-IFNd	KY768915	
		Sc-IFNh	KY768916	
	Large yellow croaker (*L. crocea*)	Lc-IFNd	KU144880	(Ding *et al.* 2016, 2017)
		Lc-IFNh	KU144879	
	Orange-spotted grouper (*E. coioides*)	Ec-IFN	JX443623	(Chen *et al.* 2014)
	Seven band grouper (*E. septemfasciatus*)	Es-IFN	AB585968	(Kuo *et al.* 2016)
	European sea bass (*D. labrax*)	Sb-IFN1–2	AM765846–AM765847	(Casani *et al.* 2009; Valero *et al.* 2015)
	Gilthead seabream (*S. aurata*)	Sa-IFN1–4	FM882241–FM882244	(Valero *et al.* 2015)
	Rock bream (*O. fasciatus*)	Rb-IFN	JX020703	(Wan *et al.* 2012)
	Meagre (*A. regius*)	Ar-IFNc	MG489872	(Milne *et al.* 2018)
		Ar-IFNd	MG489873	
		Ar-IFNh	MG489874	
Pleuronectiformes	Turbot (*S. maximus*)	Sm-IFN1–2	KJ150677–KJ150678	(Pereiro *et al.* 2014)
	Japanese flounder (*P. olivaceus*)	Jf-IFN1	BAH84776	(Hu *et al.* 2017)
		Jf-IFN2	AHB59752	
		Jf-IFN3–4	LC222627–LC222628	

Table 1 (continued)

Order	Species	Gene	GenBank accession number	References
Tetraodontiformes	Pufferfish (*T. nigroviridis*)	Tn-IFN	AJ544889	(Lutfalla *et al.* 2003)
Siluriformes	Channel catfish (*I. punctatus*)	Ip-IFN1	AY267538	(Long *et al.* 2004, 2006)
		Ip-IFN2	AY847295	
		IpIFN4	AY847296	
Beloniformes	Medaka (*O. latipes*)	Ol-IFNa	LC066594	(Maekawa *et al.* 2016)
		Ol-IFNd	LC066595	
Anguilliformes	Japanese eel (*A. japonica*)	Aj-IFN1–4	MH822129–MH822132	(Huang *et al.* 2018a)

how intron-containing type I IFN genes in fish might have evolved into intronless type I IFN genes in mammals, and CRFB5 with two FNIII domains in fish evolved into IFNAR1 with four FNIII domains in mammals.

Recently, studies on amphibian type I IFNs and their receptors provide reasonable evidence to explain the difference in genomic organisations between fish and mammalian type I IFNs as well as in the copy number of FNIII domains between the receptors (Gan *et al.* 2017, 2018). It is revealed that intronless and intron-containing type I IFN genes coexist in the amphibian species, the clawed frog *Xenopus* and the Tibetan frog *Nanorana parkeri*, providing key evidence to support the hypothesis that retroposition events may have resulted in the generation of intronless type I IFNs in vertebrates (Gan *et al.* 2017, 2018). More importantly, several lines of evidence based on homology, synteny, phylogeny and divergence time estimation indicate that the intronless type I IFN genes in *Xenopus, N. parkeri* and amniotes might have arisen from three independent retroposition events occurred in these three lineages (Gan *et al.* 2017, 2018). It can then be proposed that the intron-containing type I IFN genes in fish might have lost their introns and evolved into the intronless type I IFN genes in amniotes through the retroposition event (Gan *et al.* 2017, 2018). On the other hand, it is interesting to note that the extracellular region of IFNAR1 in *N. parkeri* contains only two FNIII domains, which is similar to CRFB5 in fish, suggesting that the two FNIII domain-containing CRFB5 gene in fish might have gained other two FNIII domains and evolved into the four FNIII domain-containing IFNAR1 gene in mammals via a tandem domain duplication event during a transit period from cold-blooded to warm-blooded tetrapods (Gan *et al.* 2018). As a result of these events during the evolution of tetrapods, fish type I IFNs harbour a combination of features observed in mammalian type I and type III IFNs.

Classification and nomenclature of type I IFNs

As described above, owing to the profound impacts of retroposition events in the evolution of type I IFNs, as well as to the lineage-specific expansion and divergence of type I IFNs in vertebrates, it is difficult to establish orthologous relationships between fish type I IFNs with their mammalian counterparts. It is clear that fish type I IFNs cannot be simply classified into IFNα or IFNβ or any other subtypes of type I IFNs according to the classification and nomenclature in mammals, and great efforts have been made already by fish immunologists to establish a new classification system for the type I IFNs in fish.

Like other vertebrates, fish also possess multiple copies of type I IFN genes which are linked in the genome, and the gene copy number varies in different species. For example, zebrafish appears to possess four type I IFN genes (Altmann *et al.* 2003; Aggad *et al.* 2009; Lopez-Munoz *et al.* 2009), whereas at least eleven type I IFN genes have been identified in Atlantic salmon (Robertsen *et al.* 2003; Sun *et al.* 2009; Svingerud *et al.* 2012). Based on the number of cysteine residues predicted to be involved in the formation of disulfide bond, fish type I IFNs were originally classified into two main groups, two cysteine-containing group I and four cysteine-containing group II (Zou *et al.* 2007; Chang *et al.* 2009). Group I IFNs appear to exist in all fish species, whereas group II IFNs are discovered only in certain species such as cyprinids, salmonids and perciform fish (Zou & Secombes 2011; Langevin *et al.* 2013a; Boudinot *et al.* 2016; Secombes & Zou 2017; Laghari *et al.* 2018; Robertsen 2018). Undoubtedly, it is rational to classify fish type I IFNs according to this criterium, because group I and group II IFNs have been proven to exhibit certain degrees of difference in their receptor usage, expression pattern, and antiviral activity, which will be discussed in the following sections. However, the copies of type I IFN genes in this classification system are distinguished by Arabic numerals according to their discovery order and chromosome location. In the case of zebrafish, four type I IFN genes are named as IFN1, IFN2, IFN3 and IFN4, with IFN1 and IFN4 being in group I, and IFN2 and IFN3 in group II (Altmann *et al.* 2003; Aggad *et al.* 2009; Lopez-Munoz *et al.* 2009). Obviously, this nomenclature does not distinguish group I nor group II IFNs, and may lead to the inconsistent classification among type I IFN genes from different species. For

instance, IFN1 or IFN2 from other species may fall in different groups as observed in zebrafish, and both IFN1 and IFN2 from the Japanese eel all belong to group I IFNs (Huang *et al.* 2018a).

To avoid the above-mentioned problem, a more complex classification system has been established, in which group I IFNs are further divided into subgroups a, d, e and h, and group II IFNs into subgroups b, c and f (Sun *et al.* 2009; Svingerud *et al.* 2012; Zou *et al.* 2014a; Ding *et al.* 2016; Laghari *et al.* 2018; Milne *et al.* 2018). Among the investigated fish species, salmonids appear to harbour the most complex type I IFN repertoire, containing subgroups a, b, c, d, e and f (Sun *et al.* 2009; Svingerud *et al.* 2012; Zou *et al.* 2014a). In addition, a recently identified IFNh, is only reported in perciformes (Ding *et al.* 2016; Laghari *et al.* 2018; Milne *et al.* 2018). According to the current sequence information, the type I IFN repertoire in most fish species appears to contain only three subgroups, including subgroups a, c and d (Zou & Secombes 2011; Langevin *et al.* 2013a; Boudinot *et al.* 2016; Secombes & Zou 2017; Robertsen 2018). Although it is convenient to recognize the subgroup of IFN genes in this classification system, this nomenclature may be easily confused with mammalian type I IFNs, as IFNα, IFNβ etc. are present in mammals. The names of fish type I IFNs should be clarified to the subgroup and any possible confusion should be avoided with mammalian denomination, and a better nomenclature still remains to be established.

Transcript variants and intracellular forms of type I IFNs

In mammals, the IFN proteins consist of a predicted signal peptide and a mature region, which are secreted into extracellular space to perform their functions via a classic protein secretion pathway (Pestka *et al.* 2004). Unexpectedly, type I IFN variants which lack a signal peptide have been reported in several fish species (Long *et al.* 2006; Levraud *et al.* 2007; Purcell *et al.* 2009; Chang *et al.* 2013). These variants are transcribed from a single gene that generates two kinds of variants, referred to as the shorter and longer transcripts, with the shorter transcript encoding the normal form of IFN with a signal peptide, and the longer transcript encoding the intracellular form of IFN without a signal peptide (Levraud *et al.* 2007; Purcell *et al.* 2009; Chang *et al.* 2013). In zebrafish, the shorter transcript of IFN1 was induced by viral infection, whereas the longer transcript was constitutively expressed at a relatively high level and was poorly inducible after viral infection (Levraud *et al.* 2007). By contrast, both shorter and longer transcripts of IFN1 in rainbow trout could be detected in most tissues and were induced following viral infection, and it is proven that these two forms were derived from alternative transcription under the control of two different promoters (Purcell *et al.* 2009).

Remarkably, the intracellular form of type I IFNs encoded by the longer transcript has been proven to be functional in rainbow trout, and its recombinant intracellular IFN can induce the expression of Mx and provide protection against viral infection *in vitro* and *in vivo* (Chang *et al.* 2013; Cao *et al.* 2016). Similar to the IFN genes, the IFN receptor genes in rainbow trout also have transcript variants translating into the intracellular form of IFN receptors without a signal peptide. The intracellular receptors seem to be mainly found in the perinuclear region and co-localized with the intracellular IFN. The overexpression of intracellular IFNs and receptors in HEK293 cells can lead to the phosphorylation of signal transducers and activators of transcription (STAT) 1 and STAT2 (Chang *et al.* 2013), confirming that intracellular IFN and receptors can indeed form a cellular ligand–receptor complex to induce the expression of interferon induced genes (ISGs) and a cellular antiviral state in a STAT-dependent manner. However, it remains to be determined whether intracellular IFNs are widely present in different fish species. Notably, all the fish intracellular IFNs stated above belong to the IFNa subgroup of group I IFNs. It is not clear whether fish type I IFN genes from other subgroups can also generate transcript variants encoding the intracellular IFN proteins.

Type I IFN receptors

In mammals, the initiation of type I IFN-mediated signalling pathway depends on the interaction between type I IFNs and their heterodimeric receptor complex comprised of IFNAR1 and IFNAR2 (Pestka *et al.* 2004). IFNAR1 is regarded as the low-affinity receptor chain with a uniquely large extracellular region containing four FNIII domains and a short cytoplasmic region, whereas the high-affinity receptor chain IFNAR2 has a two FNIII domain-containing extracellular region and a long cytoplasmic region (Piehler *et al.* 2012). Both IFNAR1 and IFNAR2 belong to the class II cytokine receptor family, which is called the cytokine receptor family B (CRFB) in fish by comparative immunologists (Lutfalla *et al.* 2003; Stein *et al.* 2007). Genome-wide sequence analyses revealed that at least 17 CRFB members are present in zebrafish and pufferfish (Lutfalla *et al.* 2003; Stein *et al.* 2007; Aggad *et al.* 2010). Phylogenetically, it is indicated that CRFB1 and CRFB2 can be the homologues of mammalian IFNAR2, and CRFB5 is of the mammalian IFNAR1 (Stein *et al.* 2007). The two group I IFNs in zebrafish, which are named as IFN1/4 in zebrafish and should be IFNa and IFNd, respectively, were found to signal through a receptor complex consisted of CRFB1 and CRFB5, while the two group II IFNs, i.e. IFN2/3 which should all be

classified as IFNc, signal through a distinct receptor composed of CRFB2 and CRFB5 (Levraud *et al.* 2007; Aggad *et al.* 2009).

The evidence obtained in other species of fish is almost consistent with the observation in zebrafish. As revealed by cross-linking reaction, recombinant grass carp IFNa (belonging to group I IFNs) can bind the recombinant extracellular region of CRFB1 and CRFB5 to form a ternary complex *in vitro* (Chen *et al.* 2015a,b). In mandarin fish, the co-transfection assays showed that IFNd and IFNh (group I IFNs) use preferentially the receptor complex comprising of CRFB1 and CRFB5, and IFNc (group II IFN) use preferentially the one consisting of CRFB2 and CRFB5, for their efficacious induction of ISGs (Laghari *et al.* 2018). Curiously, the co-transfection of mandarin fish IFNh, CRFB2 and CRFB5 expressing plasmids also had inductive effect on the expression of ISGs (Laghari *et al.* 2018). In consistency with the complex type I IFN repertoire, Atlantic salmon appears to have a more extended repertoire of type I IFN receptors than other fish species, with two CRFB clusters containing two CRFB1 members (CRFB1a and CRFB1b), one CRFB2 gene, and four CRFB5 members (CRFB5a, CRFB5b, CRFB5c and CRFB5x) (Sun *et al.* 2014). The Mx reporter assays suggested that salmon IFNa (group I IFN) signals through a receptor complex consisted of CRFB1a as the long cytoplasmic chain and either CRFB5a, or CRFB5b or CRFB5c as the short cytoplasmic chain. By contrast, IFNc (group II IFN) signals through the one composed of CRFB2 as the long chain and either CRFB5a or CRFB5c as the short chain, and that IFNb (group II IFN) through the one comprised of CRFB2 as the long chain and CRFB5x as the short chain (Sun *et al.* 2014).

It is therefore demonstrated that fish group I and group II IFNs may signal through distinct receptor complexes (Fig. 1), which is obviously different from the manner in mammals, since all mammalian type I IFN subtypes initiate signalling via the same receptor complex consisting of IFNAR1 and IFNAR2.

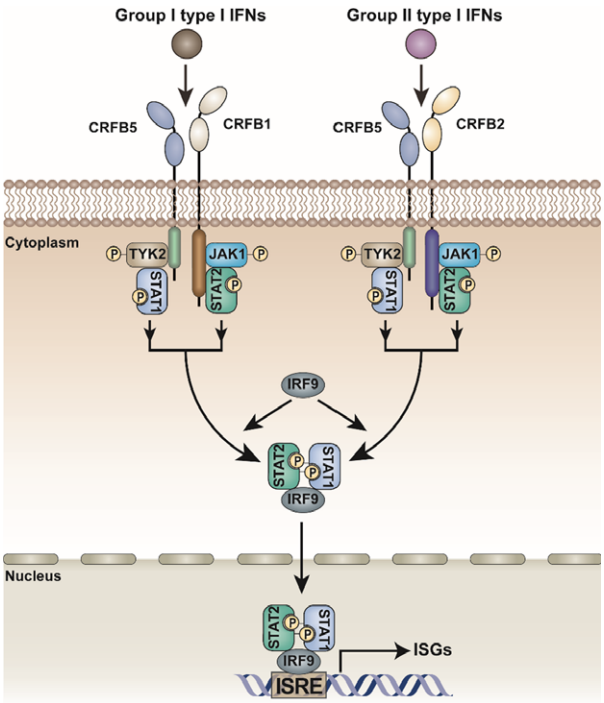

Figure 1 Signalling and ligand-receptor models for type I IFNs in fish. The two groups of type I IFNs, two cysteine-containing group I and four cysteines-containing group II IFNs, bind to receptor complexes with a common short cytoplasmic receptor chain CRFB5, but two distinct long cytoplasmic receptor chains, CRFB1 and CRFB2. The interaction between type I IFNs and their receptors triggers the recruitment and activation of TYK2 and JAK1. Subsequently, these activated kinases promote the phosphorylation of STAT1 and STAT2, which are dimerized and assembled with IRF9 to form a complex called ISGF3. This trimolecular complex translocates to the nucleus and binds to ISREs, thus activating the transcription of ISGs.

Type I IFN-mediated signalling pathway

Mammalian type I IFNs exert their effects mainly through the JAK-STAT pathway (Stark & Darnell 2012). The interaction between type I IFNs and their receptors triggers recruitment and binding of tyrosine kinase 2 (TYK2) and Janus kinase 1 (JAK1) to IFNAR1 and IFNAR2, respectively. Subsequently, these activated kinases promote the phosphorylation of STAT1 and STAT2, which are dimerized and assembled with IFN regulatory factor (IRF) 9 to form a complex called IFN-stimulated gene factor 3 (ISGF3). This trimolecular complex is translocated to the nucleus and binds to IFN-stimulated response elements (ISREs), thus activating the transcription of several hundred ISGs (Platanias 2005). As revealed in several immunogenetics studies, the essential components involved in this pathway, including TYK2, JAK1, STAT1, STAT2 and IRF9, also exist in fish (Guo *et al.* 2009; Huang *et al.* 2010; Liongue *et al.* 2012; Jin *et al.* 2018; Xu *et al.* 2016). Mammalian IFNAR1 and IFNAR2 have no intrinsic enzymatic activity, and instead depend on the kinase activity of TYK2 and JAK1 for signal initialization (Piehler *et al.* 2012). Two recent studies suggested that this mechanism is likely conserved in fish. It is shown that the overexpression of Atlantic salmon TYK2 led to the autophosphorylation by a kinase domain-dependent way as seen in mammals, leading to the up-regulation of Mx expression (Sobhkhez *et al.* 2013). In grass carp, both JAK1 and TYK2 were up-regulated and phosphorylated following the stimulation by polyinosinic-polycytidylic acid [poly(I:C)], and the

overexpression of type I IFN receptor chains CRFB1 and CRFB5 also facilitated the phosphorylation of JAK1 and TYK2 (Hou *et al.* 2017).

Intriguingly, two STAT1 genes, named as STAT1a and STAT1b, were identified in zebrafish (Song *et al.* 2011). Zebrafish STAT1a contains all five domains of human STAT1a, whereas zebrafish STAT1b lacks the C-terminal transcriptional activation domain, resembling human STAT1b, a splice variant of human STAT1a (Song *et al.* 2011). Several reports from different fish species showed that fish STAT1a may play a vital role in type I IFN-mediated signalling (Oates *et al.* 1999; Skjesol *et al.* 2010; Zhang *et al.* 2017). Zebrafish STAT1a is able to rescue IFN-mediated growth suppression in a STAT1-deficient human cell line (Oates *et al.* 1999). Consistent with this finding, Atlantic salmon STAT1a was phosphorylated and transported rapidly into the nucleus following the treatment with recombinant IFNa1 (Skjesol *et al.* 2010), and the overexpression of orange-spotted grouper STAT1a significantly up-regulated the expression level of ISGs, thereby exerting antiviral activity against iridovirus and nodavirus (Zhang *et al.* 2017). However, gibel carp STAT1, which is similar to zebrafish STAT1b with the lack of the C-terminal transcriptional activation domain, has also been shown to be involved in the IFNa-triggered ISG induction as well as in the inhibition of viral infection (Yu *et al.* 2010). Similar to the highly extended repertoire of type I IFNs and their receptors, STAT1 and STAT2 genes exist as multiple copies in Atlantic salmon, including STAT1a, STAT1b and STAT1c, and STAT2a, STAT2b and STAT2c (Sobhkhez *et al.* 2014). Both Atlantic salmon STAT2a and STAT2b were phosphorylated and co-localized with STAT1a in the nucleus following the stimulation of recombinant IFNa1 (Sobhkhez *et al.* 2014). As revealed by Co-IP assay, both salmon STAT2a and STAT2b can bind to STAT1a and IRF9, suggesting that the ISGF3 complex composed of STAT1, STAT2 and IRF9 may conservatively exist in fish (Sobhkhez *et al.* 2014). This notion is further confirmed in a recent study in which mandarin fish STAT1, STAT2 and IRF9 can interact with each other *in vitro* (Laghari *et al.* 2018). Consistently, it has been demonstrated in studies from different fish species that fish IRF9 is essential for the type I IFN-mediated signalling (Shi *et al.* 2012; Huang *et al.* 2017a; Wu *et al.* 2017).

It is thus suggested that fish type I IFNs may mediate signalling downstream of the receptors in a similar way as in mammals (Fig. 1). Nevertheless, it remains to be determined whether multicopy STAT1 and STAT2 genes exist in a broad range of taxa in fish. Furthermore, it will be interesting to investigate the functional similarity and divergence of fish multicopy STAT1 and STAT2 genes in type I IFN signalling.

Regulation of type I IFN response

Extensive studies have shown that at least three families of pattern recognition receptors (PRRs), including the retinoic acid-inducible gene I (RIG-I)-like receptors (RLRs), Toll-like receptors (TLRs), and cytosolic DNA sensors, are involved in the activation of type I IFN response in mammals (Kawai & Akira 2011; Loo & Gale 2011; Wu & Chen 2014). When these PRRs are engaged with their corresponding ligands, they recruit downstream signalling molecules, resulting in the activation of key transcription factors, such as IRFs, for initiating eventually the transcription of type I IFN genes (Kawai & Akira 2011; Loo & Gale 2011; Wu & Chen 2014). The PRR-induced mechanism of type I IFN production seems to be conserved in fish (Aoki *et al.* 2013; Poynter *et al.* 2015) (Fig. 2), which will be discussed in this section.

RLR-mediated type I IFN response

Among all known PRRs, RLRs are a family of cytosolic receptors recognizing viral RNAs with three members, including RIG-I, melanoma differentiation-associated gene 5 (MDA5) and laboratory of genetics and physiology 2 (LGP2) (Loo & Gale 2011). In mammals, RIG-I senses primarily 5′ triphosphated double-stranded RNA (dsRNA), whereas MDA5 recognizes long dsRNA (Bruns & Horvath 2014). After the detection of appropriate viral RNAs, RIG-I and MDA5 can interact with the adaptor, mitochondrial antiviral signalling protein (MAVS), and recruit cytosolic protein kinase TANK binding kinase 1 (TBK1) through their caspase activation and recruitment domains (CARDs), leading to the activation of IRF3 and IRF7 and the transcription of type I IFN genes (Loo & Gale 2011). In contrast, LGP2 differs from RIG-I and MDA5 in lacking CARDs, but retains the ability to bind to dsRNA and the function to regulate signalling by RIG-I and MDA5 (Rothenfusser *et al.* 2005; Satoh *et al.* 2010). All three members in the RLR family, RIG-I, MDA5 and LGP2, as well as the key downstream molecules MAVS and TBK1, are found conservatively in fish (Zou *et al.* 2009; Chen *et al.* 2017b). Although MDA5 and LGP2 appear to exist in all fish species, RIG-I seems to be lost in fish of Acanthopterygii (Zou *et al.* 2009; Chen *et al.* 2017b). With regard to the ligand recognition by RLRs in fish, only a report from rainbow trout proved that MDA5 and LGP2 can bind synthetic dsRNA poly(I:C) by pull down assay *in vitro* (Chang *et al.* 2011). To date, there has been no experimental evidence for the ligand-binding activity of fish RIG-I.

Functionally, like its mammalian counterpart, fish RIG-I possesses the ability to induce the expression of type I IFNs, ISGs, and antiviral state, as supported by studies from

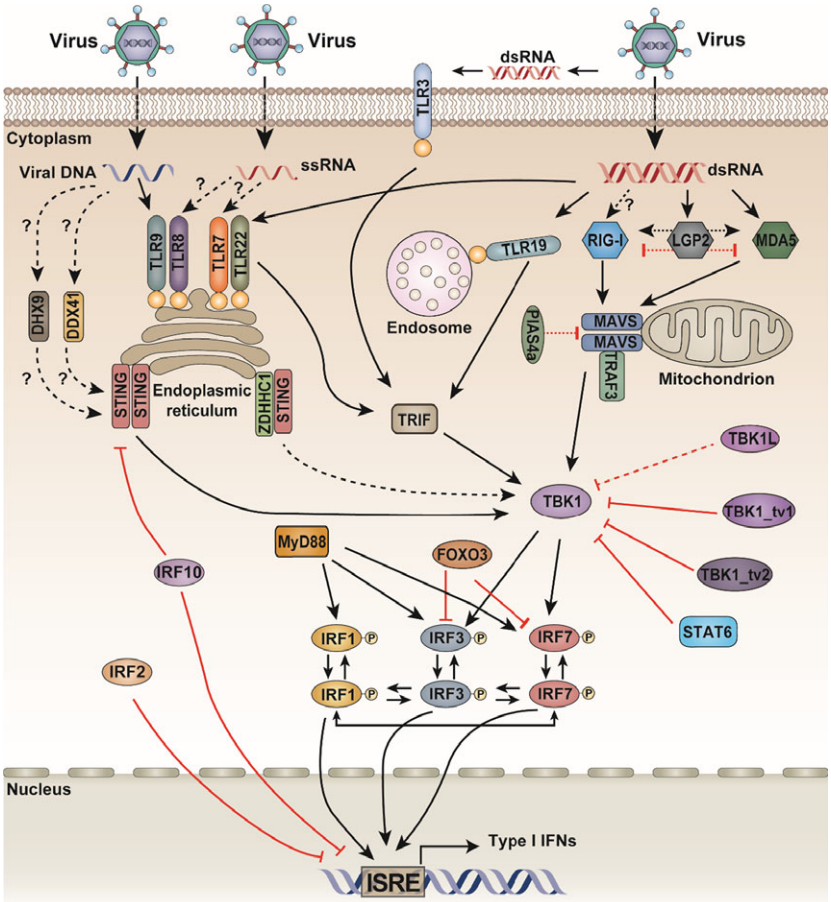

Figure 2 Regulation of type I IFN response in fish. Upon the recognition of dsRNA from viruses, RIG-I/MDA5 recruits MAVS, an adaptor protein located on mitochondria, which then become associated with TRAF3 and TBK1, leading to the phosphorylation and activation of IRF3/IRF7. LGP2 can be a positive or negative regulator of RIG-I/MDA5-mediated type I IFN response. TLR3, TLR19 and TLR22 can also recognize dsRNA from viruses and recruit TRIF and TBK1 to elicit type I IFN response. In addition, STING recruits TBK1 and IRF3 to trigger the expression of type I IFNs. IRF1, IRF3 and IRF7 can become either homo- or hetero-dimerized to recruit MyD88, for translocating into nucleus and then for binding ISRE motif, and for the production of type I IFNs and ISGs. Both IRF2 and IRF10 can inhibit type I IFN production by binding ISRE motif, and IRF10 can also inhibit type I IFN response through interacting with STING. FOXO3 can interact directly with IRF3 and IRF7, thereby suppressing IRF3/7-mediated type I IFN response. PIAS4a, TBK1L, TBK1_tv1, TBK1_tv2 and STAT6 negatively regulate the RLRs-MAVS-TBK1-mediated signalling pathway. In the signalling schematics, the negative regulation cascades are marked with red lines, and broken lines indicate the direct interaction or the molecules whose interaction need to be confirmed.

several fish species (Biacchesi *et al.* 2009, 2012; Sun *et al.* 2011a,b; Li *et al.* 2014a; Wang *et al.* 2014; Chen *et al.* 2015d; Zou *et al.* 2015; Wan *et al.* 2017). The transfection in EPC cells with N-terminal region of EPC RIG-I can activate strongly the EPC type I IFN promoter, and induce the expression of type I IFN and ISGs, thus resisting viral haemorrhagic septicaemia virus (VHSV) infection (Biacchesi *et al.* 2009). Similarly, the overexpression of gibel carp RIG-I enhanced significantly the gibel carp and zebrafish type I IFN promoters as well as the expression of viperin in CAB cells (Sun *et al.* 2011b; Wang *et al.* 2014), and the grass carp RIG-I when overexpressed in CIK cells increased significantly the expression of IFN2 and IFN4 (Wan *et al.* 2017). Research from zebrafish showed that the N-terminal region or wild-type of zebrafish RIG-I when overexpressed in EPC cells strongly activated zebrafish type I IFN promoter (Biacchesi *et al.* 2012; Li *et al.* 2014a; Zou *et al.* 2015). In addition, EPC cells transfected with wild-type RIG-I in zebrafish are resistant to spring viraemia of carp virus (SVCV) infection, with the increasing expression of Mx and IRF7, and these effects can be further improved by the co-transfection of zebrafish RIG-Ia, a longer spliced variant of wild-type RIG-I (Zou *et al.* 2015). Likewise, zebrafish IFN1, IFN4 and MxC genes were significantly induced when wild-type RIG-I was overexpressed in embryos (Chen *et al.* 2015d).

Like RIG-I, MDA5 in fish has also been proven to be capable of triggering type I IFN-mediated antiviral response, which was reported initially in rainbow trout and Japanese flounder, in which the overexpression of MDA5 in fish cells caused the increase in the expression of type I IFNs and ISGs as well as antiviral activity against viral infection (Chang *et al.* 2011; Ohtani *et al.* 2011). Similar results were reported in recent studies from other fish species. MDA5 in orange-spotted grouper when overexpressed in GS cells enhanced significantly type I IFN promoter activities as well as the expression of IRF3 and IRF7, thereby inhibiting cytopathic effect (CPE) progression and viral gene transcription during red-spotted grouper nervous necrosis virus (RGNNV) and Singapore grouper iridovirus (SGIV) infection (Huang *et al.* 2016a). Ectopic expression of black carp MDA5 in EPC cells resulted in the induction of promoter activity of zebrafish and fathead minnow type I IFNs, and improved antiviral ability against SVCV and grass carp reovirus (GCRV) infection (Liu *et al.* 2017). The overexpression of grass carp MDA5 in CIK cells significantly enhanced the expression of type I IFNs (Wan *et al.* 2017). Consistently, the knock down of MDA5 from sea perch (*Lateolabrax japonicas*) significantly increased the viral burden in RGNNV infected LJF cells (Jia *et al.* 2016a). More convincingly, several recent studies have shown that zebrafish MDA5 can enhance type I IFN-mediated antiviral response *in vitro* and *in vivo*. The activation of type I IFN promoters and the resistance against viral infection were found in fish cells transfected with zebrafish wild-type MDA5 (Li *et al.* 2014a; Zou *et al.* 2014b; Gabor *et al.* 2015), and such effect can be enhanced by the co-transfection of zebrafish MDA5b, a shorter spliced variant of wild-type MDA5 (Zou *et al.* 2014b). It is further confirmed that zebrafish MDA5 when overexpressed in embryos increased the expression of type I IFNs and ISGs (Chen *et al.* 2015d), and dominant negative MDA5 gene transgenic zebrafish displayed higher viral burden and mortality after snakehead vesiculovirus (SHVV) infection, which could be rescued by recombinant full-length MDA5 (Gabor *et al.* 2015).

Unlike RIG-I and MDA5, fish LGP2 may play dual roles in the type I IFN-mediated antiviral response, being similar to its mammalian counterpart (Rothenfusser *et al.* 2005; Satoh *et al.* 2010). Some reports showed that fish LGP2 can be a positive regulator of type I IFN response (Ohtani *et al.* 2010; Chang *et al.* 2011; Xiao *et al.* 2016). For example, the overexpression of Japanese flounder LGP2 in HINAE cells induced the expression of type I IFNs and ISGs, improving the antiviral ability against VHSV and hirame rhabdovirus (HIRRV) infection (Ohtani *et al.* 2010). Likewise, RTG2 cells transfected with rainbow trout LGP2 are resistant to infection by VHSV, with the enhancement of Mx expression (Chang *et al.* 2011), and ectopic expression of black carp LGP2 in EPC cells provides an immune protection in cells against SVCV and GCRV (Xiao *et al.* 2016). Conversely, there are some other reports which showed that fish LGP2 exerts inhibitory effect on antiviral response triggered by type I IFNs (Sun *et al.* 2011b; Yu *et al.* 2016a). For instance, the overexpression of gibel carp LGP2 in CAB cells decreased the activity of type I IFN promoter induced by poly(I:C), RIG-I or MDA5 (Sun *et al.* 2011b), and significant down-regulation of ISGs and increased severity of SGIV and RGNNV infections were observed in GS cells transfected with grouper LGP2 (Yu *et al.* 2016a).

The factor influencing the positive or negative role of fish LGP2 function in regulating type I IFN response is not yet fully understood, but may be related to the type and dose of pathogens as well as the stage of infection. In grass carp, the overexpression of LGP2 in CIK cells diminished the promoter activity and the expression of type I IFNs, as well as the synthesis and phosphorylation of IRF3 and IRF7 in the early phase of GCRV infection (Rao *et al.* 2017), but inhibited virus replication and protected cells from death in the late phase of GCRV infection (Chen *et al.* 2015e). Conversely, zebrafish LGP2 functions as a positive regulator of type I IFN signalling at the beginning of SVCV infection, but it switches to a negative regulator along with the replication of SVCV in cells up to a certain titre (Zhang *et al.* 2018). In addition, the functional switch of fish LGP2 has been revealed to be relevant to the dose of stimulator, which is supported by the fact that zebrafish LGP2 enhanced the production of type I IFNs in the absence or presence of poly(I:C) at limited concentrations, but inhibited type I IFN response when the concentrations of poly(I:C) is relatively high (Zhang *et al.* 2018). Thus, fish LGP2 may serve as a regulator to control the magnitude of type I IFN response, not only guaranteeing sufficient production of IFN molecules to elicit effective protection, and also preventing excessive production of IFN molecules to cause potential toxicities.

Unsurprisingly, as a key downstream molecule, MAVS seems to have conserved function on RLR-mediated type I IFN response in fish. It has been demonstrated that fish MAVS is associated with both RIG-I and MDA5 (Zou *et al.* 2014b, 2015), and can enhance both RIG-I- and MDA5-mediated type I IFNs response (Zhang *et al.* 2014; Zou *et al.* 2014b, 2015); Chen *et al.* 2015d. Mechanistically, fish MAVS is located at mitochondria in dimmers, and is likely to activate IRF3 and IRF7 to trigger the expression of type I IFNs through the recruitment of TBK1 (Lauksund *et al.* 2009; Biacchesi *et al.* 2009, 2012; Simora *et al.* 2010; Xiang *et al.* 2011; Kasthuri *et al.* 2014; Zhang *et al.* 2014; Zou *et al.* 2014b, 2015); Chen *et al.* 2015d; Feng *et al.* 2015b; Lu *et al.* 2015; Zhou *et al.* 2015; Jia *et al.* 2016b; Xiao *et al.* 2017; Huang *et al.* 2018b; Krishnan *et al.* 2018. Consistently, fish TBK1 can interact with IRF3 and IRF7 (Sun *et al.* 2011b; Hu *et al.* 2018b; Yu *et al.* 2018b), and the

overexpression of TBK1 alone can significantly up-regulate the expression of type I IFNs and ISGs *in vitro* and *in vivo*, thereby providing an efficient protection against virus infection (Sun *et al.* 2011b; Feng *et al.* 2014a; Zhang *et al.* 2016; Yan *et al.* 2017a; Hu *et al.* 2018a,b; Yu *et al.* 2018b). In addition, tumour necrosis factor receptor-associated factor (TRAF) 2 and TRAF3 may be also involved in the MAVS-mediated antiviral signalling (Xiang *et al.* 2011; Chen *et al.* 2017a).

However, two important and intriguing questions regarding fish RLR-mediated type I IFN response remain to be answered. Firstly, since RIG-I appears to be lost in certain groups of fish species (Zou *et al.* 2009; Chen *et al.* 2017b), it would be interesting to clarify the mechanism to compensate the deficiency of RIG-I in these species. Secondly, much more research is still required to fully elucidate the dual roles of fish LGP2 in type I IFN-mediated antiviral response, and to understand the factors influencing the functional switch of LGP2 in fish.

TLR-mediated type I IFN response

TLRs are type I integral membrane proteins comprising of an ectodomain, which contains leucine-rich repeats (LRRs) for the recognition of ligands, a transmembrane region, and cytosolic Toll-IL-1 receptor (TIR) domains which mediate downstream signalling pathways (Kawai & Akira 2010). Currently, 13 functional TLRs, named as TLR1–13, have been identified in human and mouse (Kawai & Akira 2010), in which TLR3, TLR7, TLR8 and TLR9 are the sensors of nucleic acids with the ability to initiate type I IFN response (Kawai & Akira 2011). TLR3 senses double-stranded RNA (dsRNA) and signals via TRIF, leading to the activation of IRF3 and the production of type I IFNs (Kawai & Akira 2011). By comparison, TLR7/8 and TLR9 recognize single-strand RNA (ssRNA) and cytosine-phosphate-guanosine (CpG)-containing DNA, respectively, and recruit MyD88 to activate IRF7-dependent type I IFN response (Kawai & Akira 2011).

Fish possess a larger TLR repertoire, with almost all orthologues of mammalian TLRs (TLR1–5, and 7–9) and non-mammalian TLRs (at least TLR5S, 18–23, and 25, 26) (Pietretti & Wiegertjes 2014). Among them, TLR3, TLR19 and TLR22 are verified to be involved in the activation of type I IFN response (Ji *et al.* 2018; Matsuo *et al.* 2008). The studies from fugu (*Takifugu rubripes*) and grass carp have shown that fish TLR3, TLR19 and TLR22 can all recognize dsRNA and recruit TRIF to elicit type I IFN response (Matsuo *et al.* 2008; Ji *et al.* 2018). TBK1 may be also involved in the TRIF-mediated type I IFN response, because it was proven that TRIF can bind TBK1 *in vitro* (Sullivan *et al.* 2007). The main difference among these three TLRs lies in their cellular localization and ligand preference. Firstly, fish TLR3 resides in endoplasmic reticulum, whereas TLR19 is present in endosome, and TLR22 is localized exclusively in plasma membrane (Matsuo *et al.* 2008; Ji *et al.* 2018). Secondly, fish TLR3 senses preferentially short dsRNA, while TLR22 recognizes relatively long dsRNA (Matsuo *et al.* 2008). Given that mammalian TLR3 is expressed in cytoplasmic compartments as well as on the plasma membrane of fibroblasts (Funami *et al.* 2004), TLR22 in fish might be a functional substitute for cell surface-expressed mammalian TLR3.

However, the ligand recognition and type I IFN-inducing activity of TLR3 and TLR22 were only defined in fugu, and TLR19 only in grass carp. The functional properties of these TLRs remain to be further characterized in other species of fish. Additionally, although fish TLR9 can bind CpG-containing DNA as in mammals (Iliev *et al.* 2013; Yeh *et al.* 2013), it remains to be demonstrated whether it can activate the production of type I IFNs. Future studies are also required to determine whether fish TLR7 and TLR8 can conservatively recognize ssRNA and trigger the type I IFN response.

Cytosolic DNA sensor-mediated type I IFN response

Apart from TLR9-mediated CpG-containing DNA sensing, a series of cytosolic DNA sensors are discovered and characterized functionally in mammals, including absent in melanoma 2 (AIM2), DNA-dependent activator of IFN-regulatory factors (DAI), interferon gamma-inducible protein 16 (IFI16), cyclic GMP-AMP synthase (cGAS), DEAD-box helicase 41 (DDX41) and DExH-box helicase 9 (DHX9) (Wu & Chen 2014). In downstream of these DNA sensors, the stimulator of IFN genes (STING, also known as MITA), which is a transmembrane protein located in the endoplasmic reticulum, acts as an adapter and triggers the production of type I IFNs through the activation of IRF3 (Ran *et al.* 2014).

In contrast with the significant advance in mammals, the understanding of cytosolic DNA sensors in fish is rather limited. Among the above-mentioned DNA sensors, only DDX41 and DHX9 have been shown to be involved in type I IFN-mediated antiviral response in fish (Ge *et al.* 2015; Quynh *et al.* 2015; Lazarte *et al.* 2017; Ma *et al.* 2018). In zebrafish, the induced expression of type I IFNs and ISGs following the stimulation of dsDNA virus infection or synthetic dsDNA analogue was significantly reduced by the separate knockdown of DDX41 and DHX9 (Ge *et al.* 2015; Ma *et al.* 2018), and was almost abolished in the DDX41/DHX9 double knockdown morphant (Ge *et al.* 2015). Consistently, the activity of the type I IFN promoter as well as the expression of type I IFNs were enhanced in Japanese flounder DDX41-overexpressing HINAE cells with the treatment of C-di-GMP (Quynh *et al.* 2015), and Japanese

flounder DDX41 is an effective adjuvant for the viral glycoprotein-based DNA vaccine against VHSV infection (Lazarte *et al.* 2017). Surprisingly, zebrafish cGAS seems to be dispensable for type I IFN production in response to dsDNA virus infection (Ge *et al.* 2015). In consideration of the importance of cGAS-STING pathway in the mammalian DNA sensing system (Chen *et al.* 2016), it appears possible that the foreign DNA sensing system may differ at least to some extent in fish.

Expectedly, fish STING is similar to the mammalian counterpart, functioning as a critical adapter molecule in type I IFN signalling. Mechanistically, fish STING recruits TBK1 and IRF3 to trigger the expression of type I IFNs and ISGs (Sun *et al.* 2011b; Biacchesi *et al.* 2012; Feng *et al.* 2014b; Ge *et al.* 2015; Huang *et al.* 2015a; Lu *et al.* 2017; Xu *et al.* 2017). Two serine residues (S_{379} and S_{387} in orange-spotted grouper, and S_{371} and S_{379} in black carp) in fish STING are essential for STING-induced type I IFN activation (Huang *et al.* 2015a; Lu *et al.* 2017). A new mechanism of fish STING-mediated type I IFN response was uncovered by a recent study from grass carp, in which STING was co-localized with zinc finger DHHC-type containing 1 (ZDHHC1) in endoplasmic reticulum, where it interacts with ZDHHC1 to produce the STING/ZDHHC1 heterodimer (Xu *et al.* 2017). This heterodimer complex elicits nuclear translocation of IRF3, thus initiating the transcription of type I IFNs (Xu *et al.* 2017). Interestingly, it has been reported that fish STING is also involved in RLR-mediated type I IFN response (Sun *et al.* 2011b; Biacchesi *et al.* 2012), suggesting that there exists a crosstalk between the viral RNA and DNA sensing system in fish.

Nevertheless, there is no direct experimental evidence to define the ligand recognition by cytosolic DNA sensors in fish. Moreover, the importance of fish STING in viral DNA sensor-mediated signalling remains to be established, and it is still unclear how fish cytosolic DNA sensors and RLRs cooperatively regulate type I IFN-mediated antiviral response.

Regulation of type I IFN response by IRFs

IRFs are a family of transcription factors playing diverse roles in the regulation of innate and adaptive immune responses (Ikushima *et al.* 2013). To date, 11 IRF members have been identified in vertebrates (Huang *et al.* 2010), among which, IRF3 and IRF7 are two best-characterized members in controlling the transcription of type I IFN genes (Ikushima *et al.* 2013). Based on the canonical model of IRF3/7-mediated type I IFN induction in mammals, IRF3 is constitutively expressed and is not up-regulated by type I IFNs, while IRF7 is considered as a typical type I IFN-induced gene. At the beginning of viral infection, constitutively expressed IRF3 is activated by virus-induced phosphorylation, resulting in the rapid induction of IFNβ and some IFNα genes, which are called early-phase IFN genes. These induced early-phase IFN genes then trigger a strong expression of IRF7, thus priming the expression of most IFNα genes, which exhibit delayed induction and are called late-phase IFN genes (Marie *et al.* 1998; Sato *et al.* 2000; Honda *et al.* 2005).

Analogously, IRF3 and IRF7 are also primary transcriptional factors priming the type I IFN response in fish. It has been shown that fish IRF3 or IRF7 can bind and activate type I IFN promoter, thereby increasing the expression of type I IFNs and ISGs as well as antiviral activity against viral infection (Holland *et al.* 2008; Kileng *et al.* 2009; Bergan *et al.* 2010; Sun *et al.* 2010, 2011b; Cui *et al.* 2011; Hu *et al.* 2011; Iliev *et al.* 2011; Feng *et al.* 2016; Zhao *et al.* 2017; Wang *et al.* 2018). A striking divergence between fish and mammals is that both IRF3 and IRF7 are type I IFN-induced genes in fish (Holland *et al.* 2008; Sun *et al.* 2010; Ding *et al.* 2016; Laghari *et al.* 2018). Importantly, different fish type I IFN members appear to be regulated by distinct IRFs. In the case of zebrafish, IFN1 (group I IFN) promoter is primarily primed by IRF3, being similar to the fashion of mammalian IFNβ, and IFN3 (group II IFN) promoter is predominantly primed by IRF7, resembling those of mammalian IFNα (Sun *et al.* 2011b; Feng *et al.* 2016). Additionally, fish IRF1 has been shown to be a key transcriptional factor involved in the activation of type I IFNs (Caipang *et al.* 2005; Bergan *et al.* 2010; Lai *et al.* 2013; Kung *et al.* 2014; Feng *et al.* 2015a, 2016). Akin to IRF3 and IRF7, fish IRF1 can bind directly to type I IFN promoter and activate type I IFN-mediated antiviral response (Caipang *et al.* 2005; Bergan *et al.* 2010; Lai *et al.* 2013; Kung *et al.* 2014; Feng *et al.* 2015a, 2016). Interestingly, these three IRFs can become either homo- or hetero-dimerized to recruit the adaptor myeloid differentiation primary response 88 (MyD88), cooperatively tuning the type I IFN response (Iliev *et al.* 2011; Feng *et al.* 2015a, 2016). Recent studies have shown that fish IRF5 and IRF6 are likely to be the positive regulators of type I IFN response (Wickramaarachchi *et al.* 2014; Li *et al.* 2016; Ai *et al.* 2018), but more experimental data from different fish species may be needed to further support this notion.

Negative regulation of type I IFN response

Type I IFNs have the capacity to activate the expression of ISGs (Schoggins *et al.* 2011), but uncontrolled type I IFN response can be harmful to host and may cause autoinflammation and tissue damage (Kretschmer & Lee-Kirsch 2017). To minimize potential detrimental outcome, the strength and duration of type I IFN response must be tightly controlled by a range of negative regulatory

molecules (Hertzog & Williams 2013). Fish appear to possess a finely balanced regulatory network to tune the immune response (Rebl & Goldammer 2018), and several negative regulation mechanisms of type I IFN response have been elucidated recently in fish.

Among several hundred ISGs induced by mammalian type I IFNs, suppressor of cytokine signalling (SOCS) 1 and ubiquitin carboxy-terminal hydrolase (USP) 18 are typical negative regulators of type I IFN signalling (Hertzog & Williams 2013; Yim *et al.* 2016; Yu *et al.* 2018a). The inhibitory role of SOCS1 and USP18 on type I IFN response seems to be conserved in fish as observed in mammals. Unsurprisingly in fish, both SOCS1 and USP18 can be significantly induced by type I IFNs (Nie *et al.* 2014; Skjesol *et al.* 2014; Chen *et al.* 2015a; Sobhkhez *et al.* 2017a). A preliminary study from zebrafish has shown that the overexpression of SOCS1 in embryos suppressed markedly the IFN1-induced ISG activation (Nie *et al.* 2014). Simultaneously, the suppressive mechanism of SOCS1 in fish was uncovered in studies from Atlantic salmon, in which SOCS1 can directly interact with TYK2 and STAT1, and inhibit type I IFN-induced nuclear localization of STAT1, thus strongly impairing type I IFN signalling and antiviral defense (Skjesol *et al.* 2014; Sobhkhez *et al.* 2017a). It has recently been revealed that gibel carp USP18 can dramatically down-regulate the induced expression of ISGs triggered by type I IFNs (Chen *et al.* 2015a), but the molecular mechanism of this inhibitory process is yet to be determined.

Similarly, not all the members of IRF family are transcriptional factors to prime the expression of type I IFNs, and some of them can even inhibit the activation of type I IFN response. For example, zebrafish IRF10 can interact with STING to block the activation of IFN1 and IFN3, and can also inhibit the induction of IFN1 and IFN3 by binding to the ISRE motif in their promoters (Li *et al.* 2014a). Analogously, grass carp IRF2 binds to the promoter of IFN1, thus down-regulating the expression of IFN1 and ISGs (Gu *et al.* 2015). On the other hand, some transcriptional factors from other gene families may play antagonistic roles in the IRF3/7-induced type I IFN activation. For instance, zebrafish forkhead box O (FOXO) 3 can interact directly with IRF3 and IRF7, thereby suppressing IRF3/7-mediated type I IFN response (Liu *et al.* 2016). Consistently, the higher expression of IFN1 and ISGs were observed in FOXO3-null zebrafish larvae, which display stronger antiviral capability when compared with wild-type larvae (Liu *et al.* 2016).

As stated above, fish RLR signalling plays vital roles in the induction of type I IFN expression, which is strictly controlled by several negative regulatory molecules. As revealed in zebrafish model, the spliced variants of some RLR members and key downstream molecules were identified as the negative regulators of type I IFN response. For instance, the wild-type LGP2 in zebrafish has dual functions in the regulation of type I IFN signalling, but its two spliced isoforms LGP2v1 and LGP2v2 only retain the inhibitory role (Zhang *et al.* 2018). Similarly, the overexpression of zebrafish MAVS_tv2, a spliced variant of wild-type MAVS, suppressed the activation of IFN1 promoter and the enhancement of IFN1 and ISG production triggered by IRF7 (Chen *et al.* 2015d). By contrast, the negative regulation of fish TBK1 may be much more complicated, which is related with the two spliced variants of zebrafish TBK1, namely TBK1_tv1 and TBK1_tv2, and a paralogous gene, termed TBK1L (Zhang *et al.* 2016; Hu *et al.* 2018b). Mechanistically, TBK1_tv1 and TBK1_tv2 can competitively interact with TBK1 and IRF3 to disturb TBK1-mediated IRF3 phosphorylation, thereby suppressing the activation of type I IFN signalling, and TBK1L has a similar inhibitory effect on TBK1-mediated type I IFN response (Zhang *et al.* 2016; Hu *et al.* 2018b). Similarly, zebrafish STAT6 can become associated with TBK1, thereby impeding the phosphorylation of IRF3 and the expression of IFN1 (Li *et al.* 2017). In addition, zebrafish protein inhibitor of activated STAT4a (PIAS4a) and von Hippel–Lindau (VHL) may be involved also in the repression of RLR signalling, which is supported by the fact that the co-overexpression of PIAS4a and MAVS in embryos reduced dramatically the MAVS-induced IFN1 expression (Xiong *et al.* 2012), and that VHL induced the degradation of MAVS *in vitro* and inhibited type I IFN-mediated antiviral response *in vivo* (Du *et al.* 2015). Apart from the zebrafish model, studies from orange-spotted grouper have shown that several members of tripartite motif (TRIM) protein family, including TRIM13, TRIM16L, TRIM35 and TRIM62, may negatively regulate type I IFN response by suppressing the RLR signalling (Huang *et al.* 2016a, 2017b; Yang *et al.* 2016b; Yu *et al.* 2016b).

Indeed, fish type I IFN signalling is tightly controlled at virtually every step of the signal process, including ligand production, signalling adaptors and enzymes, and transcriptional factors. However, the inhibitory mechanisms of type I IFN signalling at the level of receptors are still poorly understood in fish and require further investigation. Interestingly, a more recent study from gibel carp has shown that the type I IFN promoter activity could be suppressed by diverse immunoglobulin domain-containing proteins (DICPs) (Gao *et al.* 2018), which belong to a fish-specific gene family (Rodriguez-Nunez *et al.* 2016). It seems likely that fish may have some lineage-specific mechanisms in type I IFN signalling.

Expression characters of type I IFNs

Based on the current knowledge in mammals and birds, most type I IFNs are ubiquitously expressed at a relatively low level in unstimulated organs/tissues compared with housekeeping gene, and are highly induced by poly(I:C) stimulation or viral infection (Pestka *et al.* 2004; Teijaro 2016; Secombes & Zou 2017). This basic rule may be also true for most type I IFNs in fish, but extensive studies have revealed a diversity in the pattern of type I IFN expression in fish (Altmann *et al.* 2003; Lutfalla *et al.* 2003; Robertsen *et al.* 2003; Long *et al.* 2006, 2004; Gonzalez *et al.* 2007; Zou *et al.* 2007, 2014a; Casani *et al.* 2009; Chang *et al.* 2009; Kitao *et al.* 2009; Purcell *et al.* 2009; Li *et al.* 2012; Wan *et al.* 2012; Chen *et al.* 2014; Parhi *et al.* 2014; Pereiro *et al.* 2014; Valero *et al.* 2015; Huang *et al.* 2015b, 2018; Ding *et al.* 2016, 2017; Kuo *et al.* 2016; Liao *et al.* 2016; Maekawa *et al.* 2016; Wei *et al.* 2016; Yan *et al.* 2016, 2017b; Hu *et al.* 2017; Laghari *et al.* 2018; Milne *et al.* 2018; Wu *et al.* 2018; Xia *et al.* 2018). Firstly, considerable divergence may exist in the expression of group I and II type I IFNs, and expression difference may even exist among the members in each groups. For instance, an initial study in rainbow trout indicated that IFN1 (also known as IFNa1, belonging to group I IFNs) was not detected in all analysed tissues from unstimulated fish. By comparison, IFN2 (IFNa2, group I IFNs) was detected in all examined tissues, and IFN3 (IFNb1, group II IFNs) was mainly expressed in ovary and testis (Zou *et al.* 2007). Secondly, distinct expression is observed among fish type I IFNs in response to different immunological stimuli. Subtypes A of type I IFNs in Atlantic salmon were expressed differently upon the *in vivo* treatment of poly(I:C) (a ligand for TLR3/19/22 in fish) or S-27609 (a putative ligand for TLR7) (Sun *et al.* 2009). Following the stimulation of poly(I:C), IFNa was strongly induced during the first 24 h followed by decline, whereas IFNb was only slightly induced at 14 h, and IFNc level persistently increased during the experimental period. In contrast, the treatment with S-27609 led to a persistent up-regulation of IFNa, a transient but very strong induction of IFNb, and a short induction of IFNc (Sun *et al.* 2009). Thirdly, different patterns in the expression may be observed in some type I IFN members at different life stages of fish (Aggad *et al.* 2009). IFN2 in zebrafish is not expressed and not induced by viral infection in larvae, but it is expressed at relatively high level and well induced by viral infection in adults (Aggad *et al.* 2009).

In mammals, nearly all nucleated cells can secrete type I IFNs, but the capacity in producing the cytokines may vary in different cell types (Liu 2005). Plasmacytoid dendritic cells (pDCs) are considered as a predominant source of type I IFNs, which can rapidly produce a large amount of IFNα and IFNβ by TLR7/TLR8/TLR9–MyD88 signalling pathway upon viral infection (Liu 2005). Intriguingly, cell type-dependent expression of type I IFNs may also exist in fish (Zou *et al.* 2007; Svingerud *et al.* 2012; Palha *et al.* 2013). The expression of type I IFNs in different cell types were initially compared in a study from rainbow trout, and all examined type I IFNs, including IFN1 and IFN2 (group I IFNs) and IFN3 (group II IFNs), were expressed in primary head kidney leukocytes following poly(I:C) stimulation, but only IFN1 and IFN2 were expressed in RTG-2 cells, a kind of fibroblast-like cell line (Zou *et al.* 2007). More clearly, as revealed in a transgenic reporter zebrafish, hepatocytes and neutrophils were identified as the two main cell types expressing IFN1 in larvae upon chikungunya virus (CHIKV) infection (Palha *et al.* 2013). In addition, a fluorescence *in situ* hybridization (FISH) study in Atlantic salmon has shown that IFNa and IFNc were expressed in a minority of cells in head kidney, spleen, gills, liver and heart from poly(I:C)-stimulated fish, with some cells co-expressing these two IFNs (Svingerud *et al.* 2012). Importantly, IFNb and IFNc were co-expressed in a few cells of head kidney and spleen from R848-stimulated fish (Svingerud *et al.* 2012), and these high IFNb/IFNc-producing cells in the lymphoid tissues may be similar to mammalian pDCs, functioning as specialized high type I IFN producers in fish since R848 is a putative ligand for TLR7. Despite the scarcity in understanding type I IFN-producing cells in fish, it seems true, as observed in mammals, that different members of type I IFNs may be expressed in certain types of cells, which may need to be further clarified.

Functional properties of type I IFNs

The well-demonstrated function of type I IFNs is their antiviral activity. Type I IFNs can establish an antiviral state in cells through the induction of antiviral ISGs. Similar to mammals, fish possess a large repertoire of ISGs, which is supported by a recent study in which over 2000 genes were up-regulated by recombinant salmon IFNa in TO cells (Xu *et al.* 2015). A small subset of ISGs have been shown to possess direct antiviral effect in fish, such as Mx, viperin, ISG15, PKR and PKZ (Larsen *et al.* 2004; Liu *et al.* 2011, 2018; Schoggins & Rice 2011; Langevin *et al.* 2013b; Wang *et al.* 2014). Obviously, the antiviral activity of fish type I IFNs is closely related to the antiviral activity of their induced ISGs.

Currently, the antiviral activity of type I IFNs has been clearly defined in a wide range of species, including cyprinids, salmonids and perciform fish. The understanding of antiviral activity of type I IFNs in cyprinid fish is mainly derived from the studies in zebrafish (Altmann *et al.* 2003; Wang *et al.* 2006; Levraud *et al.* 2007; Aggad *et al.* 2009; Lopez-Munoz *et al.* 2009, 2010; Li *et al.* 2010; Palha *et al.* 2013). Among the four type I IFN members in zebrafish,

IFN2 and IFN3 (group II IFN, subgroup c) can elicit a rapid and transient expression of antiviral ISGs to protect fish against SVCV and infectious haematopoietic necrosis virus (IHNV) (Aggad *et al.* 2009; Lopez-Munoz *et al.* 2009, 2010). By contrast, IFN1 (group I IFN, subgroup a) induces a delayed but higher level of antiviral ISGs, thus providing an efficient protection against different viruses including SVCV, IHNV, snakehead rhabdovirus (SHRV), infectious spleen and kidney necrosis virus (ISKNV), and CHIKV *in vitro* and *in vivo* (Altmann *et al.* 2003; Wang *et al.* 2006; Levraud *et al.* 2007; Aggad *et al.* 2009; Li *et al.* 2010; Lopez-Munoz *et al.* 2009, 2010; Palha *et al.* 2013). It is indicated that the two type I IFN groups may have complementary antiviral roles at different stages of virus infection. Nevertheless, zebrafish IFN4 (group I IFN, subgroup d) could not protect embryos against IHNV infection, which may be resulted from a much weaker up-regulation of antiviral ISGs (Aggad *et al.* 2009). In addition, the ISG-induced activity and antiviral activity of type I IFNs have been characterized also in other cyprinids, including common carp, grass carp, black carp, gibel carp, and red crucian carp (Kitao *et al.* 2009; Li *et al.* 2013; Huang *et al.* 2015b; Yan *et al.* 2016, 2017b; Wu *et al.* 2018; Xia *et al.* 2018). Consistently, all these type I IFNs belong to subgroups a or c (Kitao *et al.* 2009; Li *et al.* 2013; Huang *et al.* 2015b; Yan *et al.* 2016, 2017b; Wu *et al.* 2018; Xia *et al.* 2018), and to date no IFNd in cyprinids has been proven to have antiviral activity.

As described above, salmonids appear to have the most complexed type I IFN repertoire containing subgroups a, b, c, d, e and f (Sun *et al.* 2009; Svingerud *et al.* 2012; Zou *et al.* 2014a), which is a remarkable model to study functionalization of type I IFNs in fish. Most research regarding antiviral activity of type I IFNs in salmonids has been conducted in Atlantic salmon (Ooi *et al.* 2008; Xu *et al.* 2010a; Svingerud *et al.* 2012, 2013; Chang *et al.* 2014, 2016), with discovery of considerable divergence in antiviral effects among different type I IFN subgroups. *In vitro* experiments have shown that salmon IFNa and IFNc display potent antiviral activity against infectious pancreatic necrosis virus (IPNV) and salmonid alphavirus (SAV) 3 (Ooi *et al.* 2008; Xu *et al.* 2010a; Svingerud *et al.* 2012; Chang *et al.* 2016), but only IFNa can protect fish cells against infectious salmon anaemia virus (ISAV) infection (Svingerud *et al.* 2013). In contrast, IFNb induces lower antiviral effect against IPNV than IFNa and IFNc *in vitro* (Svingerud *et al.* 2012). Nevertheless, distinct results have been obtained when testing antiviral activity of IFNs *in vivo* by injecting fish intramuscularly with expression plasmids expressing IFNa, IFNb and IFNc, respectively (Chang *et al.* 2014). All three IFNs induced similarly the expression of antiviral ISGs at the muscle injection site, but only IFNb and IFNc up-regulated the expression of antiviral ISGs in head kidney, liver, heart and pancreas of injected fish (Chang *et al.* 2014), suggesting that IFNa is active mainly at the injection site, whereas IFNb and IFNc can be systemically active. In addition, IFNc plasmid induced stronger Mx protein expression in heart and liver than IFNb plasmid (Chang *et al.* 2014). Being consistent to the antiviral ISG-induced activity of these IFNs, the treatment of fish with IFNc plasmids before ISAV or SAV3 infection gave a strong protection, but IFNb a weak protection, and IFNa no protection (Chang *et al.* 2014, 2016). Interestingly, salmon IFNd showed no ISG-inducing ability and antiviral activity against IPNV in fish cells (Svingerud *et al.* 2012), and the antiviral activity of IFNe and IFNf in salmonids remains to be assessed.

Apart from cyprinids and salmonids, type I IFNs have been characterized in a few species of perciform fish, which appear to harbour subgroups c, d and h (Casani *et al.* 2009; Wan *et al.* 2012; Chen *et al.* 2014; Valero *et al.* 2015; Ding *et al.* 2016, 2017; Kuo *et al.* 2016; Laghari *et al.* 2018; Milne *et al.* 2018). Recently, it has been shown that IFNc and IFNh in perciform fish can trigger a strong expression of antiviral ISGs to have an efficient protection against viral infection (Ding *et al.* 2017; Laghari *et al.* 2018). By contrast, conflicting results have been reported on the function of IFNd in this lineage of fish. IFNd has been proven to have ISG-inducing activity and antiviral activity in several perciform fish including rock bream, orange-spotted grouper, seven band grouper and large yellow croaker (Wan *et al.* 2012; Chen *et al.* 2014; Ding *et al.* 2016; Kuo *et al.* 2016), and in some species from other orders, such as channel catfish and medaka (Long *et al.* 2006; Maekawa *et al.* 2016). However, a most recent study showed that mandarin fish IFNd has no effect on the phosphorylation of STAT1 and STAT2, nor on the expression of antiviral ISGs, thus providing no protection against viral infection (Laghari *et al.* 2018), which is similar to IFNd in zebrafish and Atlantic salmon as mentioned above (Aggad *et al.* 2009; Svingerud *et al.* 2012). It is interesting to investigate the mechanism underlying the functional divergence of IFNd in these species of fish.

Extensive studies have shown that recombinant type I IFNs or type I IFN expression plasmids when injected into fish can provide *in vivo* efficient protection against viral infection (Levraud *et al.* 2007; Aggad *et al.* 2009; Lopez-Munoz *et al.* 2009, 2010; Li *et al.* 2010, 2013; Palha *et al.* 2013; Chang *et al.* 2014, 2016; Pereiro *et al.* 2014; Kuo *et al.* 2016; Xia *et al.* 2018), indicating that fish type I IFNs are promising candidates for potential antiviral agents in the prevention of infectious viral diseases in aquaculture. More intriguingly, fish type I IFNs seem to be able to trigger adaptive immune response, and hence have the potential as novel adjuvants in antiviral vaccines. This notion is supported by recent studies from Atlantic salmon, in which

the fish were immunized with the injection of a plasmid encoding the haemagglutinin-esterase (HE) gene of ISAV alone or together with a plasmid expressing either IFNa, IFNb or IFNc (Chang *et al.* 2015; Robertsen *et al.* 2016; Sobhkhez *et al.* 2017b). Remarkably, HE plasmid alone only provided a weak protection against ISAV infection, with the induction of low IgM antibody titre, whereas all three IFN plasmids together with HE plasmid enhanced markedly the protection with a strong and prolonged antibody response against ISAV (Chang *et al.* 2015; Robertsen *et al.* 2016). The mechanism behind the adjuvant effect of fish type I IFNs is not yet fully understandable, but may be related to the increasing expression of chemokines induced by type I IFNs, which may lead to the attraction of lymphocytes at the muscle injection site (Chang *et al.* 2015; Sobhkhez *et al.* 2017b).

Type II IFNs in fish

Discovery of type II IFNs

IFN-γ, belonging to the type II IFN family, is encoded by a single copy gene with the genomic structure of four exons and three introns in mammals (Savan *et al.* 2009). Human *IFNG* is located on chromosome 12 containing another two members of class II cytokine family, IL-22 and IL-26. Mammalian IFN-γ is a multi-functional cytokine. For example, IFN-γ can modulate $CD4^+$ T helper 1 (Th1) cell response and is pivotal for the clearance of intracellular pathogens (Boehm *et al.* 1997). On the other hand, IFN-γ is a key regulator for macrophage activation (Green *et al.* 2017) and for promoting antigen presentation process to inhibit viral replication and tumour growth by the induction of immune-related ISGs (Schoenborn & Wilson 2007).

The first identified type II IFN in fish was the IFN-γ gene in fugu by gene synteny analysis (Zou *et al.* 2004). In fact, type II IFN loci have conserved synteny in vertebrates from fish to mammals (Savan *et al.* 2009; Chen *et al.* 2013). However, a unique gene as the new member, named as IFN-γ1 (also known as IFN-γ related gene, IFN-γrel, see below), of type II IFN family was identified at the up-stream of IFN-γ2 gene (the orthologous gene of fugu IFN-γ) in zebrafish and pufferfish (Igawa *et al.* 2006). Indeed, the teleost IFN-γrel (or the so-called IFN-γ1) contains IFN-γ signature motif and has moderate sequence similarity with IFN-γ (IFN-γ2) which possesses more conserved vertebrate IFN-γ sequence features, such as nuclear localization signal (NLS) motif at C-terminal region (Igawa *et al.* 2006; Zou & Secombes 2011; Chen *et al.* 2013). The NLS sequence of IFN-γ is conserved from fish to human, with usually several continuously encoded lysines or arginines (for example, RRKR and KRKR in trout and human respectively), which have been proven to be essential for the function of fish and mammal IFN-γ (Subramaniam *et al.* 1999; Zou *et al.* 2005), although ginbuna crucian carp (*C. auratus langsdorfii*) IFN-γrel1 (one of IFN-γrel isoforms) also has functional NLS sequence (KHHHR) at C-terminal region (Shibasaki *et al.* 2014). On the other hand, more than two copies of fish type II IFN gene are present in several species of fish (Zou & Secombes 2011). To avoid any confusion, it is suggested that fish IFN-γ1 and IFN-γ2 gene were renamed as IFN-γ related molecule (IFN-γrel) and IFN-γ, respectively (Zou & Secombes 2011), and this nomenclature has been generally accepted among fish immunologists.

To date, IFN-γ genes have been identified in many different species of fish, such as goldfish (*C. auratus*), ginbuna crucian carp, grass carp, Atlantic cod (*Gadus morhua*), Atlantic salmon, Atlantic halibut (*Hippoglossus hippoglossus*), black seabream (*Acanthopagrus schlegelii*), channel catfish, common carp, medaka, large yellow croaker, rainbow trout, orange-spotted grouper, Japanese flounder, turbot, Nile tilapia (*Oreochromis niloticus*), European seabass and barramundi perch (*Lates calcarifer*) (Table 2) (Zou *et al.* 2005; Milev-Milovanovic *et al.* 2006; Robertsen 2006; Stolte *et al.* 2008; Furnes *et al.* 2009; Grayfer & Belosevic 2009a; Matsuyama *et al.* 2009; Yabu *et al.* 2011; Overgard *et al.* 2012a; Yang *et al.* 2013; Kuznetsova *et al.* 2014; Nuñez Ortiz *et al.* 2014; Chen *et al.* 2015c; Mohapatra *et al.* 2015; Pereiro *et al.* 2016; Velazquez *et al.* 2017; Xiang *et al.* 2017; Zhong *et al.* 2017; Peng *et al.* 2018). Similar to other vertebrate counterparts, teleost IFN-γ has a conserved signal peptide, IFN-γ signature sequence and NLS motif (continuous lysines or arginines). Among the species with known IFN-γ gene structure, four exons and three introns are observed, except in medaka which has two splicing forms of IFN-γ gene (designated as IFN-γ2a and 2b in the original publication) derived from eight exons and six exons, respectively, by comparing to the genomic sequences (Mohapatra *et al.* 2015). It seems likely that the two isoforms are chimeric sequences based on the *IFNG* locus data from medaka RNA-seq exon coverage (NCBI *Oryzias latipes* Annotation Release 103) and genomic assembly (ASM223467v1). Although it is unclear whether all teleost IFN-γ genes possess conserved four-exon gene organization, the splicing of IFN-γ gene transcripts is likely to have effect on biological activities of IFN-γ.

Except for the presence in zebrafish, fugu and spotted green pufferfish, IFN-γrel genes were also discovered in channel catfish, common carp, goldfish, ginbuna crucian carp, grass carp, rohu and orange-spotted grouper (Table 2) (Chen *et al.* 2010; Grayfer *et al.* 2010; Milev-Milovanovic *et al.* 2006; Peng *et al.* 2018; Shibasaki *et al.* 2014; Stolte *et al.* 2008; Swain *et al.* 2015; Yabu *et al.* 2011). These teleost IFN-γrel molecules contain conserved signal peptide and IFN-γ signature sequences, but without NLS motif of continuous lysines or arginines. In addition, it was failed to identify IFN-γrel gene in other different

Table 2 Currently reported type II IFNs in fish

Order	Species	Gene	GenBank accession number	References
Cypriniformes	Zebrafish (*D. rerio*)	IFN-γ1 (IFN-γrel)	NM_001020793	Igawa *et al.* (2006)
		IFN-γ2 (IFN-γ)	NM_212864	Igawa *et al.* (2006)
	Grass carp (*C. idella*)	IFN-γrel	FJ695519	Chen *et al.* (2010)
		IFN-γ2 (IFN-γ)	JX196701.1	Yang *et al.* (2013)
	Common carp (*C. carpio*)	IFN-γ1 (IFN-γrel)	AM261214	Stolte *et al.* (2008)
		IFN-γ2 (IFN-γ)	AM168523	Stolte *et al.* (2008)
	Crucian carp (*C. auratus*)	IFN-γ1 (IFN-γrel)	GQ149696	Grayfer and Belosevic (2009a)
		IFN-γ2 (IFN-γ)	EU909368	Grayfer and Belosevic (2009b)
	Ginbuna crucian carp (*C. auratus langsdorfii*.)	IFN-γrel1	AB570433	Yabu *et al.* (2011)
		IFN-γrel2	AB614642	Shibasaki *et al.* (2014)
		IFN-γ1 (IFN-γ)	AB570431	Yabu *et al.* (2011)
		IFN-γ2 (IFN-γ)	AB570432	Yabu *et al.* (2011)
	Rohu (*L. rohita*)	IFN-γrel	KJ874352	Swain *et al.* (2015)
Siluriformes	Channel catfish (*I. punctatus*)	IFN-γ1 (IFN-γrel)	DQ124249	Milev-Milovanovic *et al.* (2006)
		IFN-γ2a (IFN-γ)	DQ124250	Milev-Milovanovic *et al.* (2006)
		IFN-γ2b (IFN-γ)	DQ124251	Milev-Milovanovic *et al.* (2006)
Salmoniformes	Rainbow trout (*O. mykiss*)	IFN-γ	AJ616215	Zou *et al.* (2005)
	Atlantic salmon (*S. salar*)	IFN-γ	AY795563	Robertsen (2006)
Gadiformes	Atlantic cod (*G. morhua*)	IFN-γ	FJ356235	Furnes *et al.* (2009)
Cichliformes	Nile tilapia (*O. niloticus*)	IFN-γ	NM_001287402.1	(Velazquez *et al.* 2017; Zhong *et al.* 2017)
Perciformes	Large yellow croaker (*L. crocea*)	IFN-γ	KM501500.2	Chen *et al.* (2015a,b,c,d,e)
	Orange-spotted grouper (*E. coioides*)	IFN-γ1 (IFN-γrel)	-	Peng *et al.* (2018)
		IFN-γ	JX013936	–
	European sea bass (*D. labrax*)	IFN-γ	KJ818329	Nuñez Ortiz *et al.* (2014)
	Barramundi perch (*L. calcarifer*)	IFN-γ	NM_001360734.1	Kuznetsova *et al.* (2014)
Spariformes	Black seabream (*A. schlegelii*)	IFN-γ	KY921614	Xiang *et al.* (2017)
Pleuronectiformes	Turbot (*S. maximus*)	IFN-γ	KX360748	Pereiro *et al.* (2016)
	Japanese flounder (*P. olivaceus*)	IFN-γ	AB435093	Matsuyama *et al.* (2009)
	Atlantic halibut (*H. hippoglossus*)	IFN-γ	GU985450	Overgard *et al.* (2012)
Tetraodontiformes	Spotted green pufferfish (*T. nigroviridis*)	IFN-γ1 (IFN-γrel)	KJ524454	Yi *et al.* (2014)
		IFN-γ2 (IFN-γ)	KJ524455	Yi *et al.* (2014)
	Fugu (*T. rubripes*)	IFN-γ	AJ616216.2	Zou *et al.* (2004)

vertebrates, including elephant shark, tropical clawed frog, green anole (*Anolis carolinensis*), chicken (*Gallus gallus*) and human, (Chen *et al.* 2013; Qi & Nie 2008; Savan *et al.* 2009; Venkatesh *et al.* 2014). However, all these vertebrates have IFN-γ gene, and a scenario is accepted to explain the occurrence of IFN-γrel in teleost fish that the teleost-specific IFN-γrel gene is likely derived from the duplication of IFN-γ gene (Igawa *et al.* 2006; Savan *et al.* 2009). However, much more effort is needed to understand how and when the duplication event might have occurred.

Structure of type II IFNs

The recombinant human IFN-γ contains six helices (A-F) and is crystallized in the form of homodimer (Ealick *et al.* 1991). Similar to human, homologous dimerization of recombinant or native IFN-γ was observed in zebrafish, goldfish, ginbuna crucian carp and Japanese flounder (Grayfer & Belosevic 2009a,b; Yabu *et al.* 2011; Yoon *et al.* 2016; Zahradnik *et al.* 2018). The crystal structure of the flounder IFN-γ is also in general similar to that of other known IFN-γ, despite of some significant differences, including additional helix G at C-terminal region and a different angle between helices C and D, in the fold of flounder IFN-γ when compared with mammals (Zahradnik *et al.* 2018). However, there are converse opinions on the dimerization of IFN-γrel. Recombinant goldfish IFN-γrel is capable to form homodimer (Grayfer & Belosevic 2009a,b), while monomers of IFN-γrels possess biological activity in ginbuna crucian carp (Shibasaki *et al.* 2014). To date, little is known in relation with the crystal structure of IFN-γrel and type II IFN receptors in teleost fish.

Receptors and signalling of type II IFNs

In mammals, the homodimer of IFN-γ activates the canonical JAK-STAT signalling pathway through the tetrameric IFN-γ receptor (IFN-γR) complex, which includes two

subunits of IFN-γR1 and IFN-γR2, respectively (Boehm *et al.* 1997; Green *et al.* 2017; Ivashkiv 2018; Stark *et al.* 1998). Human IFN-γR1 is encoded by a single copy gene and is also called ligand binding chain due to its high affinity binding to IFN-γ, and its intracellular region has two key anchor sites ($L_{266}PKS_{269}$ and $Y_{440}DKPH_{444}$) for docking and activation of JAK1 and STAT1, respectively (Bach *et al.* 1997; Stark *et al.* 1998). In fact, the phosphorylation of JAK1 and STAT1 needs first the activation of JAK2, which binds to intracellular region of IFN-γR2 (Stark *et al.* 1998). Activated STAT1 molecules become dimerized and can bind to the DNA sequences called gamma-interferon-activated site (GAS) to initiate the transcription of ISGs in the nucleus (Green *et al.* 2017). Furthermore, IFN-γ is also able to activate other transcriptional factors, such as STAT3 and the complex of STAT1 and IRF9 (Green *et al.* 2017).

Unlike in mammals, IFN-γR1 gene in teleost was duplicated as two copies in consideration of phylogenetic and syntenic analyses, which were designated as CRFB13/IFNGR1-2/IFNGR1-like and CRFB17/IFNGR1-1/IFNGR1 respectively (Grayfer & Belosevic 2009a,b; Chen *et al.* 2013; Lu *et al.* 2014; Liao *et al.* 2016; Zahradnik *et al.* 2018), although four copies of IFN-γR1 gene are present in some salmonid fish, such as Atlantic salmon, due to the separate duplication of IFNGR1-1 and IFNGR1-2 (Zahradnik *et al.* 2018). Actually, the two teleost IFN-γR1 genes are separated on different loci, which all show conserved synteny when compared with other vertebrates: CRFB13/IFNGR1-2 gene is located adjacent to oligodendrocyte transcription factor 3 (OLIG3), and CRFB17/IFNGR1-1 gene is linked with LTV1 ribosome biogenesis factor (LTV1), respectively (Grayfer & Belosevic 2009a,b; Chen *et al.* 2013; Lu *et al.* 2014). Expectedly, many studies have shown that both CRFB13/IFNGR1-2/IFNGR1-like and CRFB17/IFNGR1-1/IFNGR1 are main subunits of type II IFN receptor complex based on experimental evidence revealed by various approaches, such as ligand-receptor cross-linking, receptor-specific knockdown/knockout, expression of receptors in xenogenic cells, ligand-receptor immunoprecipitation and affinity analysis (Gao *et al.* 2009; Grayfer & Belosevic 2009a,b; Aggad *et al.* 2010; Yabu *et al.* 2011; Lu *et al.* 2014; Sawamiphak *et al.* 2014; Zahradnik *et al.* 2018). However, the IFN-γR1 model of teleost type II IFN receptor complex seems to be not very clear, or there is no universal model but species-specific ligand-receptor binding. For example, goldfish IFN-γ2 (IFN-γ) and IFN-γ1 (IFN-γrel) can be cross-linked with IFNGR1-2 (CRFB13) and IFNGR1-1 (CRFB17) respectively *in vitro*, while no binding activity has been detected after the exchange of receptors (Grayfer & Belosevic 2009a,b). But in zebrafish, the limitation of CRFB17 translation has significant effect on signalling and function of both IFN-γ2 (IFN-γ) and IFN-γ1 (IFN-γrel), and simultaneously IFN-γ2 (IFN-γ) signalling also needs CRFB13 (Aggad *et al.* 2010). Similarly, both IFNGR1-1 (CRFB17) and IFNGR1-2 (CRFB13) are likely to be associated with IFN-γ in pufferfish, and IFNGR1-1 is the main component of IFN-γrel receptor complex (Lu *et al.* 2014). In addition, IFN-γ1 (but this is not IFN-γrel) and IFN-γ2, the two isoforms of IFN-γ, use different IFN-γR1s, IFNGR1-2 (IFNGR1-like/CRFB13) and IFNGR1-1 (IFNGR1/CRFB17), respectively, in ginbuna crucian carp (Yabu *et al.* 2011). Anyway, the two teleost IFN-γR1 genes may be originated from one ancestral gene, with similar protein features in having a signal peptide, an extracellular region with FNIII domains, a transmembrane region and an intracellular region. It is implied that cross-talk may occur possibly in the IFNGR1-1 or IFNGR1-2 mediated signal pathway for teleost type II IFNs.

In fact, the potential STAT1 binding sites (consensus sequences: YDxxP[K/H]) are found in intracellular region of both CRFB13/IFNGR1-2 and CRFB17/IFNGR1-1 (Grayfer & Belosevic 2009a,b; Aggad *et al.* 2010; Zou & Secombes 2011). Both IFN-γ and IFN-γrel are able to induce STAT1 expression and its phosphorylation in goldfish, zebrafish etc. (Grayfer *et al.* 2010; Skjesol *et al.* 2010; Lopez-Munoz *et al.* 2011; Collins *et al.* 2014; Ruan *et al.* 2017). Simultaneously, mutants of GAS sequences on promoters or intron of teleost ISGs led to dramatically depressed activation of ISG luciferase reporter plasmids mediated by IFN-γ or STAT1 (Shi *et al.* 2013; Cheng *et al.* 2014; Ruan *et al.* 2017; Liu *et al.* 2018). Moreover, IFN-γ can also activate STAT2 and STAT3 (Sawamiphak *et al.* 2014; Sobhkhez *et al.* 2014). The ISREs on promoters of some ISGs are also essential for response to recombinant IFN-γ (Castro *et al.* 2008). It is thus revealed that STAT1/3-involved signal transduction of type II IFN is likely to be conserved from teleost to human. However, the role of JAK1 in signalling of teleost type II IFNs is still unclear, although the intracellular region of IFN-γR1s contains a conserved binding motif (consensus sequences: PxxL) for JAK1 (Aggad *et al.* 2010; Zou & Secombes 2011).

The fish IFN-γR2 (also known as CRFB6) has been identified in zebrafish, grass carp, rainbow trout and Dabry's sturgeon, and it possesses a signal peptide, an extracellular region, a transmembrane region and an intracellular region (Gao *et al.* 2009; Aggad *et al.* 2010; Liao *et al.* 2016; Luo *et al.* 2018). The IFN-γR2, as in human, is a single copy gene and links with transmembrane protein 50B (TMEM50B) gene on a conserved locus of vertebrate class II cytokine receptor, except in zebrafish (Stein *et al.* 2007; Chen *et al.* 2013; Lutfalla *et al.* 2003; Zahradnik *et al.* 2018). Unsurprisingly, IFN-γR2 is a component of IFN-γ receptor complex (Gao *et al.* 2009; Aggad *et al.* 2010). However, the knockdown of all known class II cytokine receptor (CRFB1-2, 4-8 and 12-17), including CRFB6 (IFN-γR2), could not block the signalling mediated by IFN-γ1 (IFN-γrel), except that the knockdown of CRFB17

(IFNGR1-1) in zebrafish led to the failure of IFN-γ1 (IFN-γrel) signalling (Aggad *et al.* 2010). It is bewildering that there might be another unknown IFN-γR2-like receptor in teleost genome, or that CRFB17 (IFNGR1-1) alone is sufficient for the signal transduction of IFN-γrel (Aggad *et al.* 2010), although IFN-γR2 is essential for immune-related function of IFN-γ in mammals (Lu *et al.* 1998). In addition, zebrafish JAK2a, rather than JAK2b, plays a role in signalling transmission of IFN-γ (Aggad *et al.* 2010).

Functional properties of type II IFNs

Antiviral activity of type II IFNs

To date, it has been observed that the expression of teleost IFN-γ or IFN-γrel genes was significantly induced by many kinds of viruses, including SVCV, GCRV, VHSV, red RGNNV, IHNV, crucian carp haematopoietic necrosis virus (CHNV) or synthetic mimics of virus, such as poly(I:C) in different species of fish (Zou *et al.* 2005; Igawa *et al.* 2006; Furnes *et al.* 2009; Grayfer & Belosevic 2009a,b; Purcell *et al.* 2009; Aggad *et al.* 2010; Chen *et al.* 2010; Xu *et al.* 2010b; Overgard *et al.* 2012b; Du *et al.* 2013; Wang *et al.* 2013; Shibasaki *et al.* 2014; Somamoto *et al.* 2015; Swain *et al.* 2015; Pereiro *et al.* 2016; Velazquez *et al.* 2017; Xiang *et al.* 2017; Yang *et al.* 2017), although the induction of IFN-γ and IFN-γrel is tissue-specific or developmental stage-specific in some species of fish (Aggad *et al.* 2010; Lopez-Munoz *et al.* 2010; Velazquez *et al.* 2017). Meanwhile, type II IFNs are able to promote antiviral genes, including Mx, ISG15, RSAD2 etc., and can inhibit viral replication *in vitro* (Lopez-Munoz *et al.* 2009; Sun *et al.* 2011a; Shibasaki *et al.* 2014; Kwak *et al.* 2017; Xiang *et al.* 2017), despite that a minor inhibitory effect was observed for Atlantic salmon IFN-γ on the replication of virus in TO cell (Xu *et al.* 2010a). Furthermore, the antiviral activity of IFN-γ is partially dependent on the induction of type I IFN in TO cells (Sun *et al.* 2011a). These data suggest that teleost type II IFNs are involved in antiviral immune responses. However, the *in vivo* overexpression of IFN-γ or IFN-γrel could not protect zebrafish larvae against SVCV infection but surprisingly led to a higher mortality rate than that infected with SVCV only, although the overexpression of these two genes significantly increased the expression of antiviral genes (Lopez-Munoz *et al.* 2011). A possible explanation is that type II IFNs activate phagocytes and induce excessive inflammation due to strongly up-regulated expression of pro-inflammatory genes (Lopez-Munoz *et al.* 2011). In fact, it is still unclear whether viral proliferation is repressed *in vivo* by type II IFNs. However, some results seem to indicate viral inhibition led by IFN-γ *in vivo*: cumulative mortality of Japanese flounder was decreased when the fish were infected with recombinant VHSV which expressed the flounder IFN-γ comparing to the control groups by other recombinant or wild-type VHSVs (Kwak *et al.* 2017).

The role of type II IFNs in antibacterial immunity

Teleost type II IFNs may have antibacterial activity. First, IFN-γ and IFN-γrel are capable of inducing the expression of nitric oxide synthase (iNOS) gene, enhancing nitric oxide (NO) production in phagocytes and increasing phagocytic activity of immune-related cells (Grayfer & Belosevic 2009a,b; Arts *et al.* 2010; Grayfer *et al.* 2010, 2018; Yang *et al.* 2013, 2017; Biswas *et al.* 2016; Peng *et al.* 2016, 2018; Wiegertjes *et al.* 2016). NO is essential for antimicrobial activity of macrophages in mammals (MacMicking *et al.* 1997). Second, IFN-γ and IFN-γrel are able to induce cytochrome b-245 beta chain (CYBB, also known as $gp91^{phox}$ and NOX2, a subunit of the NADPH oxidase), neutrophil cytosolic factor 1 (NCF1, also known as $p47^{phox}$ and NOXO2) and respiratory burst, including the production of reactive oxygen species (ROS), in phagocytes (Zou *et al.* 2005; Grayfer & Belosevic 2009a,b; Grayfer *et al.* 2010, 2018; Wiegertjes *et al.* 2016; Yang *et al.* 2017; Peng *et al.* 2018), which are involved in killing bacteria in mammals (Schroder *et al.* 2004). Third, the knockdown of type II IFNs (IFN-γ1/IFN-γrel and IFN-γ2/IFN-γ) and their receptors, including CRFB13, CRFB17 and CRFB6, resulted in significantly increased susceptibility to the infection of *Yersinia ruckeri* in zebrafish larvae (Sieger *et al.* 2009; Aggad *et al.* 2010). On the other hand, excessive IFN-γ and IFN-γrel in zebrafish may lead to higher mortality rate than in wild-type fish, and it seems possible that the expression level of IFN-γ and IFN-γrel is strictly controlled in response to bacteria (Sieger *et al.* 2009), although both IFN-γ and IFN-γrel are up-regulated by a variety of bacteria (Raida & Buchmann 2008; Furnes *et al.* 2009; Metzger *et al.* 2010; Gjessing *et al.* 2011; Evenhuis & Cleveland 2012; Hodgkinson *et al.* 2012; Kondo *et al.* 2014; Chen *et al.* 2015c; Swain *et al.* 2015; Pereiro *et al.* 2016; Takizawa *et al.* 2016; Velazquez *et al.* 2017). Moreover, intraperitoneal injection of recombinant IFN-γ2 (IFN-γ) and IFN-γ1 (IFN-γrel) in pufferfish could not provide protection against the infection of *Vibrio parahaemolyticus*; simultaneously, recombinant IFN-γ1 (IFN-γrel) reduced survival rate and induced stronger inflammatory response than IFN-γ2 (IFN-γ) (Peng *et al.* 2016). Nevertheless, the survival rate of Japanese flounder with recombinant IFN-γ injected intraperitoneally was increased when the fish was infected with *Edwardsiella tarda*, with the expression of inflammatory factors up-regulated remarkably by recombinant IFN-γ (Jung *et al.* 2012). Similarly, recombinant ginbuna crucian carp IFN-γ also protected fish against *Nocardia seriolae* infection (Nayak *et al.* 2014). The contradictory results may be impossible to explain at present, but may be associated with IFN-γ dose or different pathogens, as *E. tarda*

and *N. seriolae* are intracellular bacteria and are able to replicate in fish cells (Tan *et al.* 2005; Nayak *et al.* 2014; Xie *et al.* 2014; Matsumoto *et al.* 2017). However, *in vitro* pre-treatment of monocytes/macrophages with recombinant IFN-γ enhanced the invasion of another intracellular bacterium (*Francisella noatunensis* subsp. *noatunensis*) in Atlantic cod, which may have been resulted from macrophage activation as an early step of host defense, because the internalization of *F. noatunensis* subsp. *noatunensis* is likely mediated by phagocytosis (Bakkemo *et al.* 2016). Furthermore, Atlantic salmon IFN-γ plays a role in the TLR9-mediated innate immune response *in vitro* (Skjaeveland *et al.* 2008; Iliev *et al.* 2013). It is thus indicated that teleost type II IFNs may exhibit some functional complexity in antibacterial immunity.

The role of type II IFNs in cell proliferation and development

Teleost type II IFN signalling may have an effect on cell proliferation and development. In zebrafish embryos, type II IFN signalling is able to enhance the formation of haematopoietic stem and progenitor cells (HSPCs) in the aorta-gonad-mesonephros (AGM) region (Li *et al.* 2014b). Indeed, IFN-γ signalling is involved in the development of haematopoietic stem cells (HSCs), and endothelial-to-HSC conversion is positively regulated by IFN-γ and CRFB17 (IFNGR1-1), whose expression is controlled by Notch signalling and blood flow in the AGM region (Sawamiphak *et al.* 2014). In fact, the transcription of type II IFN receptor genes, including CRFB13 (IFNGR1-2) and CRFB17, is also maintained by RNA polymerase II (Pol II) pausing, whose deficiency may result in the defective HSPC formation and decreased chromatin accessibility on promoters of type II IFN receptor genes, thus suggesting that Pol II pausing is required for HSC emergence mediated by type II IFN signalling in zebrafish embryos (Yang *et al.* 2016a). Furthermore, IFN-γ signalling participated in developmental biliary defects in zebrafish (Matthews *et al.* 2011; Cui *et al.* 2013). Additionally, the expression of medaka IFN-γ2b (one of IFN-γ2 splicing forms) was modulated by steroid and was detected only and predominantly in female germ cells whose proliferation is possibly associated with IFN-γ2b (Mohapatra *et al.* 2015).

Other functions of type II IFNs

As in mammals, type II IFNs in teleost are multi-functional molecules that play roles in many other biological processes, including allograft rejection, antigen presentation etc. For instance, the treatment of recombinant IFN-γrel1 (an isoform of IFN-γrel) enhances the scale allograft rejection and increases the number of $CD4^+$, $CD8^+$ and $sIgM^+$ cells in grafted scales of ginbuna crucian carp (Shibasaki *et al.* 2016). Teleost type II IFNs can strongly induce antigen-presentation-associated genes, including proteasome subunit beta 9 (PSMB9, also known as LMP2), transporter 2 (ATP binding cassette subfamily B member, TAP2), beta-2-microglobulin (B2M), major histocompatibility complex (MHC) molecules etc. (Zou *et al.* 2005; Martin *et al.* 2007a,b,c; Castro *et al.* 2008; Lopez-Munoz *et al.* 2011). However, type II IFN mediated antigen presentation is not well understood in fish. Moreover, the expression of IFN-γ was reduced in those zebrafish less responsive to social stimuli (Kirsten *et al.* 2018), and zebrafish IFN-γ promotes anti-pathogen response in brain, which may be associated with social/aggregation behaviour (Filiano *et al.* 2016).

MicroRNAs (miRNAs) are small non-coding RNAs that target messenger RNA (mRNA) to regulate post-transcription of genes to impact many biological processes (Ambros 2004; Bushati & Cohen 2007; Krol *et al.* 2010). It has been reported that teleost miRNAs are regulated by type II IFNs and are involved in biological functions mediated by type II IFNs. In green spotted pufferfish, the expression of over 1500 miRNAs has changed in spleen cells after the treatment of recombinant type II IFNs (Yi *et al.* 2014). Orange-spotted grouper IFN-γ2 (IFN-γ) is likely to be involved in anti-inflammatory response through the induction of microRNA-146a (miR-146a) which possibly inhibits TRAF6 (Peng *et al.* 2018). In addition, the deletion of miR-142-3p in zebrafish causes impaired neutrophil development mediated by the abnormally activated IFN-γ signalling (Fan *et al.* 2014). Zebrafish IFN-γ is likely to inhibit the over-inflammation induced by miR-223 deficiency, although this requires more experimental evidence (Zhou *et al.* 2018).

Conclusions and future perspectives

From an evolutionary point of view, fish possess a unique type I IFN repertoire, which contains only intron-containing type I IFNs (Zou & Secombes 2011; Langevin *et al.* 2013a; Boudinot *et al.* 2016), and fish may represent an important model to study the origin and evolution of type I IFNs in vertebrates. Different from other lineages of vertebrates, teleost fish have two members of type II IFNs, i.e. IFN-γ or IFN-γ2 and IFN-γrel or IFN-γ1, which use different receptor systems to activate JAK-STAT signalling. Although the understanding on the fish type I IFN and type II systems has been significantly increased in recent years, some significant issues remain to be resolved.

(1) RNA virus-induced type I IFN response in RIG-I-absent fish species. As RIG-I seems to be lost in certain groups of fish species, such as in Acanthopterygii (Zou *et al.* 2009; Chen *et al.* 2017b), and also RIG-I is absent in chicken, which is probably a key factor for the high susceptibility to RNA viruses, such as zoonotic influenza viruses (Barber *et al.* 2010; Santhakumar *et al.* 2017), whether RIG-I-absent fish species are more susceptible to RNA viruses, and whether these species of

fish may have some undiscovered mechanisms to compensate the absence of RIG-I remains to be investigated. Hence, one important future direction is to gain a deeper understanding of type I IFN-inducing mechanism in RIG-I-absent fish species upon RNA virus infection.

(2) The cell type-specific expression of type I IFNs in fish. In mammals, type I IFNs are mainly produced by pDCs through TLR7/TLR8/TLR9–MyD88 signalling after viral infection (Liu 2005). Whether fish possess a cell type specific expression pattern similar to mammalian pDCs remains as a mystery. It is necessary to analyse cell surface markers of type I IFN-producing cell populations in fish, and to elucidate the molecular mechanism in which these cell populations can produce large amount of type I IFNs.

(3) The functional property of IFNd in fish. Unlike other IFN subtypes, IFNd has been proven to have antiviral activity in some fish species, but not in some other fish species, such as zebrafish, Atlantic salmon and mandarin fish (Aggad *et al.* 2009; Svingerud *et al.* 2012; Laghari *et al.* 2018). The factors or mechanisms related with the functional or disfunctional IFNd in different species of fish is of value for further research.

(4) The ligand-receptor models for type II IFNs in fish. There are three possible models for IFN-γ (IFN-γ2), homodimer of IFN-γ binds to the receptor complex with 2 × IFN-γR2 (CRFB6) subunits + 2 × IFN-γR1-2 (CRFB13) subunits, 2 × IFN-γR2 + 2 × IFN-γR1-1 (CRFB17) and 2 × IFN-γR2 + heterodimer of IFN-γR1-1 and IFN-γR1-2 (Fig. 3). It would be necessary to investigate if these ligand-receptor relationships are species-specific or coexist in some cell-types of a species. On the other hand, for IFN-γrel (IFN-γ1), four cases of ligand-receptor complex may be possibly included as the followings: homodimer of IFN-γrel binds to 2 × unknown molecules + 2 × IFN-γR1-1 molecules or binds to homodimer of IFN-γR1-1 only (Aggad *et al.* 2010); monomer of IFN-γrel binds to homodimer or monomer of IFN-γR1-1 (Fig. 3). All these receptor models need to be experimentally tested or verified in future (Aggad *et al.* 2010).

(5) The possible signalling and functional difference mediated by fish IFN-γ and IFN-γrel. It has been reported that the signalling pathway or function of IFN-γ and IFN-γrel are quite different (Grayfer *et al.* 2010; Lopez-Munoz *et al.* 2011; Peng *et al.* 2016; Shibasaki *et al.* 2016), and that IFN-γ and IFN-γrel may target possibly different cell-types. It would be a novel breakthrough to understand the functional similarity or divergence, if any, of the two members of type II IFNs in teleost.

The most challenging question would be "Does type III IFN exist in fish"? To date, type I and II IFNs have been identified in elephant shark, a cartilaginous fish, but not type III IFN (Venkatesh *et al.* 2014). In fact, it is reported that the elephant shark contains IFNLR1 gene, which

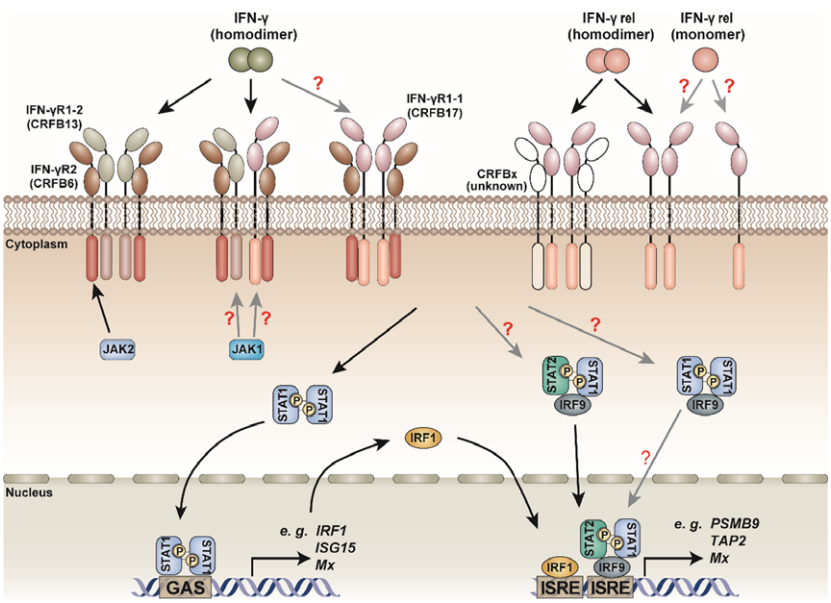

Figure 3 Signalling and ligand-receptor models for type II IFNs in fish. The homodimer of IFN-γ likely binds to the receptor complex with 2 × IFN-γR2 (CRFB6) subunits plus 2 × IFN-γR1-2 (CRFB13) subunits, 2 × IFN-γR2 plus 2 × IFN-γR1-1 (CRFB17) or 2 × IFN-γR2 plus heterodimer of IFN-γR1-1 and IFN-γR1-2, while the receptors of IFN-γrel contain at least IFN-γR1-1 (CRFB17). IFN-γ and IFN-γrel regulate the expression of ISGs (IRF1, PSMB9 and etc.) via JAK-STAT signalling. In addition, IRF1, as the secondary transcription factor, is likely involved in signal transduction mediated by type II IFNs.

encodes the specific receptor chain of type III IFNs (Hemann *et al.* 2017), with conserved synteny in comparison with tetrapods (Venkatesh *et al.* 2014; Secombes & Zou 2017). It is worthwhile to search for type III IFN in cartilaginous fish on the basis of ligand-receptor coevolution hypothesis (Moyle *et al.* 1994).

Acknowledgements

This work was financially supported by grants (Nos. 31320103913 and 31702369) from the National Natural Science Foundation of China (NSFC), and also by China Agriculture Research System (CARS-46), and by a special talent programme "One Thing One Decision (Yishi Yiyi)" and "First class fishery discipline" programme in Shandong Province, China.

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