Innate antiviral defense of zebrafish : from signalling to specialized cells

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Défenses innées antivirales du poisson zèbre: de la signalisation aux cellules spécialisées

Antiviral innate defense in zebrafish: from signalling to specialized cells

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Preamble

Microbes establish infections when they succeed to enter their host organism and cope with its defense mechanisms. Skin and mucosa play an important role of mechanical barrier to restrict pathogenic microbe invasion. If pathogens succeed to overcome this barrier, they must find its ecological niche and escape the immune reaction. The diversity of microbes infecting metazoans led to the evolution of a myriad of defense mechanisms that shape the host/pathogen interactions.

Among microbes, viruses are obligatory parasites that hijack cellular machinery, replicate and hence evolve very quickly. To cope with such pathogens, metazoans developed a number of different strategies, from downregulation of cellular machinery or induced cell death to synthesis of specific antibodies. In vertebrates, both innate and adaptive immune strategies are used in antiviral defense. As master cytokines of the innate antiviral response, type I interferons (IFN) elicit defense mechanisms at a molecular level via induction of specialized effector proteins that disrupt viral cycle at various points. They also induce chemokines that regulate leukocyte activation and migration.

IFNs are induced when a virus is detected by a number of specialized sensors constituting a surveillance network in membranes and cytosol. Viral RNAs can be detected in the infected cell by a number of specific sensors, including RNA helicases located in the cytoplasm and membrane toll-like receptors (TLR). Upon recognition of their viral ligands (nucleic acids, glycoproteins), these sensors relay signal through different cascades; this results in the activation of the transcription factors interferon-response factors (IRF3, IRF7) and NF-κB, leading to IFN induction and secretion.

Type I IFNs do not block viral infection directly, but act in an autocrine and paracrine manner to induce a large number of effector genes named "Interferon stimulated genes" (ISGs), via the Jak/STAT pathway. The signalling leading to the ISG upregulation is highly complex and tuned to achieve virus- and tissue-specific responses.

The type I IFN system is overall well-conserved across jawed vertebrates: in addition

to the cytokine itself that is found from sharks to mammals, the signalling factors (IRFs, NFκB, Jak/STATs, etc) are highly conserved. However, among fish, the teleosts which form the largest branch of bony fishes with more than 26 000 species, underwent whole-genome duplication in the beginning of its evolution and so many ISG can be found in duplicates, offering many opportunities for sub-functionalization. Teleosts are ray-finned fish as opposed to another branch of bony fish – the lobe-finned fish. As teleost group is very large there is a great diversity of shape, size, life span, and adaptations of fishes and they have colonized almost all aquatic environments, which provides a very interesting context for comparative studies of immunity. The immune system of fishes has been extensively studied in a few key fish species: mainly aquaculture fishes such as carp (cyrpinid), trout and salmon (salmonids), and among the model species, essentially the zebrafish (cyprinid, figure in Annex 1).

In my thesis I have compared teleost fish and mammalian type I IFN system. Just after hatching, fish larvae rely only on innate immunity to deter pathogens, including viruses, and hence constitute good models to investigate innate mechanisms, whereas adults also use adaptive immunity. Most of the classical components of IFN system are present in fish and a number of studies have revealed similar activation and response dynamics as in mammals, however there are also considerable differences. For example, fish have additional TLR that recognise long double stranded RNA and some fish species have several largely expanded families of ISGs. Exploration of fish immune system helps to highlight primordial antiviral mechanisms and reveals new ones that were triggered from ancestral building blocks and have diversified in evolution.

I have studied zebrafish antiviral immunity. Zebrafish are cyprinid fish belonging to the same group as carps, however zebrafish lineage did not have any additional whole genome duplications as carps (Annex 1). Zebrafish has recently become a valued model for the study of host/pathogen interactions on a whole organism level thanks to its utility for live-imaging and the availabilty of a wide array of genetic manipulation tools.

Firstly, I studied a transcription factor that was recently described as a novel participant in IFN signalling. Mammalian promyelocytic leukaemia zinc finger protein (PLZF) could be considered as one of the transcription factors that tune ISG profile specificity to a virus, as it participates in the upregulation of a particular subset of ISGs. In zebrafish there are two PLZF orthologues likely due to the whole-genome duplication event that occurred during the early evolution of teleosts. I found that zebrafish Plzf's are involved in IFN induction in IRF3 signalling axis. This finding highlights transcription factor versatility, and the complexity of IFN system as one protein can play a role at different levels of regulation.

I was also involved in the characterization of a tri-partite motif protein (TRIM) named Ftr83 that belongs to a fish-specific TRIM subset, the finTrims. TRIM form a large family of proteins that have E3 ubiquitin ligase activity; many of them have been shown to influence IFN system as signalling modulators and effector proteins. The finTrim protein that was studied in this thesis - Ftr83, was shown to participate in IRF3-dependent induction of Ifn and inhibit virus infection. At the organism level, Ftr83 is expressed at sites exposed to pathogens such as gills, where its expression level correlates with that of type I IFN. Thus, this study revealed a new strategy involving a TRIM-protein that should assure a local innate immune protection at virus-entry sites.

Local activation of IFN signalling molecules is one way of safeguarding tissues exposed to pathogens. Another way is the relocation/mobilization of specialized leukocytes at critical sites. Fish have a unique mechano-sensory organ for water movement perception - the neuromast, which is embedded in the skin. The sensory cells of neuromasts are constantly renewed, creating a possible epidermal breach and thereby an entry site for pathogens. We hypothesize that there might be specialized leukocytes to protect neuromasts. In my thesis, I characterized a subpopulation of myeloid cells that indeed patrol neuromasts. The model I established could be used to test myeloid cell behaviour in early events of viral entry into the organism.

Thus, in my thesis, I describe two different proteins - Plzf and Ftr83 - that constitute independent modulators of the Ifn pathway. Such studies in zebrafish demonstrate

how important is the enhancement of Ifn in the regulation of the response; it also illustrates how the regulation of a conserved system evolves and diversifies. In addition to studying antiviral immunity at the molecular level, I also studied immune response at the cellular level by characterizing a subset of myeloid cells that might be sentinel cells devoted to patrolling neuromasts.

INTRODUCTION

CHAPTER I

Type I IFN system of mammals and fish

Innate antiviral defense is primarily based on type I IFN– molecules that are secreted upon the recognition of viruses or intracellular bacteria, and elicit the expression of multiple genes with defensive functions (ISGs: IFN-stimulated genes). Type I IFNs were first described in mammals in the late fifties and through decades of work IFN signaling became one of the best known cytokine pathways (reviewed in (1)). Fish IFN was first described in the seventies (2). A lot of progress has been made in both fields. Here I outline and compare the main components of mammalian and fish antiviral IFN systems.

Virus sensing and conserved signalling pathways leading to type I IFN production

Mammals

Cellular sensors of viruses - PRRs (pattern-recognition receptors) - detect pathogen motifs such as viral genomes (RNAs or DNAs) and intermediates of replication and probably viral glycoproteins as well. Upon the detection of their ligand, PRR trigger signalling that leads to the activation of NF-κB, IRF3 and IRF7 transcription factors and type I IFN production. Three classes of PRR involved in virus sensing are Toll-like receptor (TLR) family members, retinoic acid inducible gene-I (RIG-I)-like receptors (RLR) and a heterogenic family of cytosolic DNA sensors (CDS). TLR detect nucleic acids in endocytic compartments from viruses that may have infected the cell or circulating viruses that were pinocytozed, whereas RLR and CDS recognize cytosolic nucleic acids of viruses that have infected the cell. Viral proteins, such as the VSV surface glycoprotein, may be detected in a TLR4-dependent fashion however direct evidence of ligand-binding is missing (3). Ligand specificities of PRR that sense nucleic acids are summarized in Table 1.

PRR name	Presence		Ligand	
	Mammals	Fish	Mammals	Fish
TLR family				
TLR3	Yes	Yes	dsRNA	dsRNA
TLR7	Yes	Yes	ssRNA	no direct evidence
TLR8	Yes	Yes	ssRNA	no direct evidence
TLR9	Yes	Yes	unmethylated CpG DNA	unmethylated CpG
TLR21	No	Yes/No	-	CpG DNA
TLR22	No	Yes	-	dsRNA
RLR family				
RIG-I	Yes	Yes	variety of RNA molecules	no direct evidence
MDA5	Yes	Yes/No	long dsRNA	no direct evidence
LGP2	Yes	Yes/No	RNA	no direct evidence
CDS				
DAI	Yes	No	DNA	-
DHX9	Yes	Yes	DNA	no direct evidence
DHX36	Yes	No	DNA	-
DDX41	Yes	Yes	DNA, cyclic diguanylate	no direct evidence
cGAS	Yes	Yes	DNA	no direct evidence

Table 1. Known PRR that mediate virus recognition in mammals and fish. Some PRR are present in some fish species and missing in others (indicated by Yes/No).

TLR

In 2001, the first virus-recognizing TLR - TLR3 - was identified, followed shortly by others (4), (reviewed in (5)). In mammals, TLR3 targets dsRNA, whereas TLR7 and -8 target ssRNA (6) and TLR9 unmethylated CpG DNA of bacteria and viruses (7,8). Upon ligand binding, TLR7, -8 and -9 interact with myeloid differentiation primary response protein 88 (MYD88) which recruits IL-1R-associated kinase 1, 2 and 4 complex (IRAK1, IRAK2, IRAK4). This leads to the phosphorylation of TNF receptor-associated factor 6 (TRAF6), that with TRAF family member-associated NF-κB activator kinase (TANK) and inhibitor of NF-κB activator kinase (IKKi), cause NF-κB or IRF7 translocation to the nucleus and type I IFN induction. TLR3 interacts with another adaptor - the TIR domain containing adaptor inducing IFNβ (TRIF aka TICAM-1 or Myd88-3) - that signals through TRAF3, TANK, TBK1 (TANK-binding kinase 1) and ultimately IRF3/IRF7.

RLR

There are three RLRs identified: the founding member RIG-I (aka DDX58),

melanoma differentiation-associated gene 5 (MDA5, aka IFIH1) and laboratory of genetics and physiology 2 (LGP2, aka DHX58). RIG-I binds a variety of RNA molecules: short dsRNA and ssRNA either with or without 5'-triphosphate (9). MDA5 recognizes high molecular weight dsRNA (10). LGP2 has RNA helicase activity, yet lacks a CARD domain, which is necessary for signal induction; hence LGP2 negatively regulates RLR signalling, however LGP2 can have positive roles as well (11). RIG-I and MDA5 have CARD domains, which mediate homotypic interactions with the adaptor molecule mitochondrial antiviral signalling protein (MAVS, also known as IPS-1, VISA or CARDIF). This interaction initiates downstream signalling via TRAF3 leading to TBK1 activation and subsequent phosphorylation of NFκB, IRF3 and IRF7 transcription factors (12).

CDS

Additionally there are viral DNA-recognizing CDS such as DNA-dependent activator of IFN-regulatory factors (DAI, aka ZBP1) (13), DExD/H-box helicases DHX9, DHX36 and DDX41 (15,17) and cyclic GMP-AMP synthetase (cGAS, aka MB21D1) (18). Downstream signalling adaptor of CDS is endoplasmic reticulum (ER)-associated stimulator of IFN genes (STING) protein (also called MITA, mediator of IRF3 activation; ERID, endoplasmic reticulum IFN stimulator; and MPYS, N-terminal methionine-proline-tyrosine-serine protein) that leads to IKK and TBK1-mediated activation of IRF3, then to IFN induction.

Fish

TLR

Most components of PRR signalling axis have also been found in fish (Figure 1). All virus-specific TLR have orthologues in fish and overexpression of TLR or treatments with ligands induce type I IFN, although there is not always evidence of direct ligand-binding/specificity. Fish Tlr3 recognizes dsRNA and induces antiviral response (19,20). Whether Tlr7 and Tlr8 function similarly to their mammalian counterparts is less clear, however TLR7 and TLR8 agonists induce a IFN response in salmonid leukocytes and in Japanese flounder (21,22). Additionally, Tlr7/8 leucine

rich repeats that contribute to ligand-binding are almost exactly conserved between fish and mammals, which indicate that they bind similar ligands (23). Two Tlr with no mammalian counterpart recognize nucleic acids: Tlr21 is found in birds and fish, Tlr22 exclusively in fish. Tlr21 is implicated in responses to CpG DNA (24), whereas Tlr22 is targeted to plasma membrane and recognizes long dsRNA (25). Downstream signalling adaptors and enzymes have been identified and for some there are functional studies as well. Signalling molecules are generally very well conserved although fish have often several copies, implying that the functional pattern is complex. Myd88 has been found in many fish; in zebrafish Myd88 knock-down impaired Tlr-mediated immune response thus confirming its central role in immunity and it was further confirmed with a true mutant (26,27). Tlr3 adaptor Trif was identified in zebrafish and was shown to elicit Ifn response in an Irf3/7-independent

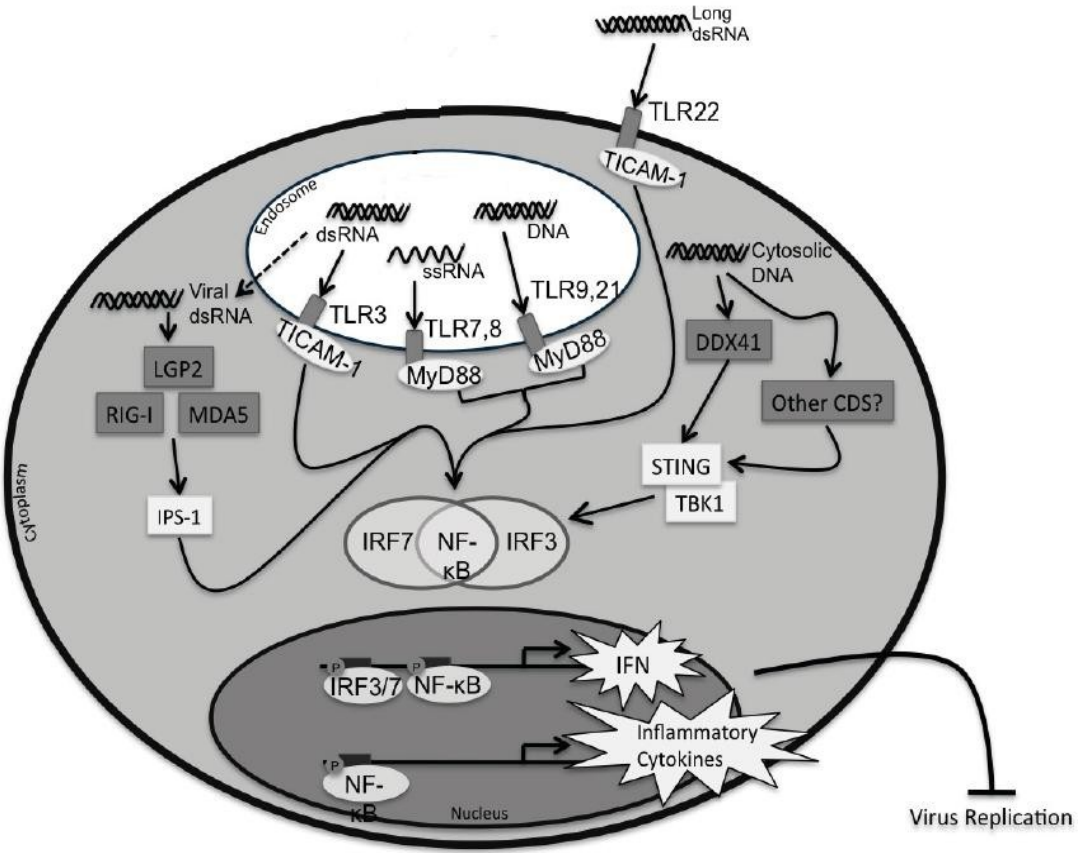

Figure 1. Virus sensing and Ifn induction in fish. As in mammals TLR initiate signalling either through adaptor Myd88 or Ticam-1 (aka Trif), RLR via Ips-1 (aka Mavs) and cytosolic DNA sensors via Sting. All pathways terminate in either transcription factor Irf7, Irf3 and/or Nf-κb activation. Intermediate steps involving other adaptors and kinases are described in the text. Scheme adapted from (33).

manner (28). Unlike mammalian TRIF it was seen to localize to Golgi complex (29). Fish-specific Tlr22 signals through Trif as well (25). Zebrafish Irak4 overexpression induced NF-κB reporter in zebrafish cells (20). On the contrary, grouper Irak4 had a negative effect on NF-κB activity in HEK293 cells – this could be due to the lack of fish interactor proteins in human cells or the lack of Irak2 interaction sites in grouper Irak4 (30). In addition, IRAK2 orthologues have not been found in many fish, which suggests that some pathways are different (31), whereas Irak1 is present in fish and is an ISG (32). Zebrafish Traf6 potentiated NF-κB activity (20).

RLR

All three RLR are conserved in zebrafish, grass carp and channel catfish whereas in other fish model species only one or two of RLR were found (12,34–36) Fish Lgp2 lacks a CARD domain just as its mammalian counterpart and seems to be nonetheless a positive regulator of RLR-signalling as well (35). Zebrafish express two isoforms for both Rig-I and Mda5. The two Rig-I isoforms were able to induce Ifn response, however with different efficiency (37). Expression of the two Mda5 isoforms created an antiviral state that was augmented by addition of Mavs (38). Mavs is found in many fish species (36,39–41). In zebrafish there are also two Mavs isoforms and both activate IFN reporter (42). Mavs-Traf3-Tbk1 axis leading to Irf3 and -7 activation is likely conserved in fish (12,41,43,44).

CDS

In fish only two CDS have been identified to date: Ddx41 and Dhx9 (45,46); surprisingly, cGAS, a key CDS in mammals, is conserved but seems dispensable in zebrafish (46). Their downstream adaptor STING/MITA is also conserved (47,48).

Thus virus sensing and IFN induction in mammals and fish relies on same basic components, however there are also species-specific differences between mammals and fish and also among fish - which is not surprising as fish are very diverse and their genomes have been subjected to one or several rounds of whole genome duplications resulting in multiple paralogs for some signalling components and their subfunctionalization.

Type I IFNs and their receptors

Mammals

Type I IFNs

Mammalian type I IFNs are encoded by a multigenic cluster. In humans it is situated on chromosome 9 and is comprised of 13 IFNα subtypes and single genes of IFNβ, -ε, -κ and -Ω. Two of the 13 IFNα proteins – encoded by *IFNA1* and *IFNA13*, are identical whereas overall the IFNα subtypes have 78-99% protein similarity and are less similar to other type I IFNs. Nonetheless they are structurally similar enough to bind to a common receptor (49,50).

Receptors

Type I IFN receptor consists of two chains: IFNAR1 and IFNAR2 (51–53). Receptor chains are ubiquitously expressed (reviewed in (54)) – although this dogma has been recently challenged (55). Different IFN proteins bind them with varying affinity, but generally bind more strongly to IFNAR2 than IFNAR1 (56). The effect of respective affinities of a given IFN towards each chain seems to result in biological outcome as antiviral signalling is stimulated by type I IFNs that have a higher affinity towards IFNAR2, whereas higher affinity towards IFNAR1 correlates with antiproliferative activity (56).

Expression of IFNs

The availability of multiple type I IFNs that have differential binding properties and induce downstream signalling likely allows the development of complex physiological responses *in vivo*. The prerequisite is differential regulation of the expression of type I IFNs. This notion is supported by the finding that type I IFN promoters have different IRF binding site combinations and functionality leading to differing expression dynamics (57). Furthermore, different TLR agonists stimulate responses that vary in the combination of expressed type I IFNs and the kinetics of induction that ultimately translates into differences in ISG repertoires (58). Additionally it has been observed during a systemic infection *in vivo* that a higher replication rate of a virus in a given tissue correlates to a more complex IFNα subtype

response showing that there is hierarchical upregulation of different type I IFNs (59). During a systemic infection there are two waves of IFN production – early (IFNα4 or IFNβ) and late (IFNα2,-5,-6,-8) (60). The early response type I IFN is produced by plasmacytoid dendritic cells, which are also the highest type I IFN producers (61).

Fish

Type I IFNs

The number of type I IFN genes in fish species varies from one (e.g. fugu) to at least eighteen (trout) (62,63). Unlike in mammals, teleost IFN genes have introns. Teleost type I IFNs are structurally further divided into two groups based on the presence of either one or two disulfide bridges (62). Zebrafish possesses two group I (Ifnφ1 and -4) and two group II (Ifnφ2 and -3) IFNs (64). The two groups of interferons have very low sequence homology (19%), but they have the same 3D structure, which is similar to alpha-helical topology of mammalian type I IFNs (65). An intriguing feature of some teleost IFNs is that they are transcribed in two forms: with and without a signal peptide (66,67) the secreted form being the one induced upon viral infection. However, overexpression of a non-secreted form of IFN from trout induced ISG and established protective state against viruses (68).

Receptors

Teleost type I IFN receptor consist of two different chains as in mammals, however the two groups of type I Ifn bind two different receptor-complexes that have only one chain in common. It was shown in zebrafish that group one Ifns signal via Crfb1 and Crfb5, whereas group two signals via Crfb2 and Crfb5 (64). Structurally, fish type I receptors differ from mammalian ones in that no chain exhibits the duplicated extracellular domain of IFNAR1, a feature (together with the multiple exons of fish IFN genes) that led to the now disproven hypothesis that fish IFNs may be more closely related to type III IFNs than to type I (67).

Expression of IFNs

As in mammals, the promoters of fish type I Ifns differ in the composition of transcription factor binding sites; hence fish type I Ifns show variability in expression

patterns (63). Furthermore, different agonists of PRR induce different set of Ifns (69,70). On a whole organism level, group one Ifns are usually inducible in most cells, whereas group two Ifns have a low basal expression and are upregulated in leukocytes (62). Plasmacytoid dendritic cells have not been described in fish, however it has been observed in trout that Ifns are produced by a heterogenous population of scattered cells (70). In zebrafish, it was more specifically discerned that during a systemic infection of larvae crucial producer of Ifnφ1 was a population of neutrophils (71). Salmonids who possess a large multigenic type I Ifn family also display sequential expression of early and late Ifn subsets (63).

It is intriguing that the number of IFN molecules across species is so variable and yet the antiviral defense is still efficient. Further studies of different fish Ifn system should bring new knowledge as to how the basic components can combine into functional systems.

Induction pathways of ISGs

Mammals

JAK-STAT-ISGF3 axis

The canonical signalling leading to ISG induction relies on janus kinase (JAK)-signal transducer and activator (STAT) proteins (Figure 2). A JAK-STAT pathway was first described in the context of the IFN system, but was later found to be involved in response to many other cytokines (reviewed in(1)). JAK family consists of four tyrosine kinases out of which JAK1 interacts with IFNAR2 chain and TYK2 with IFNAR1 chain (72,73). When type I IFN is bound to its receptor it causes activating conformational changes in JAKs that phosphorylate receptor chains and thereby create binding sites for STAT1 and -2 transcription factors which are then also phosphorylated by JAKs. Phosphorylated STATs heterodimerize and form a complex with IRF9 named ISGF3 that translocates to the nucleus where it triggers ISGs through the binding of Interferon-stimulated response elements (ISRE) (74–76).

Additional regulation

ISGF3-mediated ISG induction is the canonical view of signalling whereas there is

plenty of information regarding post-translational modifications that modulate the activities of its components and the involvement of other transcription factors that all serves to modulate the final ISG profile. For example, STAT1 serine residue phosphorylation by IKK-I is necessary for the induction of a subset of ISG such as OAS1g, IFI204, TLR3 and Mx1 (77). In addition to ISGF3 complex, IRF3 and IRF1 can also activate a subset of ISG without the presence of IFN, however these experiments were done through over-expression or constantly active protein forms implying that *in vivo* they still must be activated, for example downstream of a PRR (78,79). Apart from STAT it has been proven that other transcription factors can tune the expression of ISGs, for example the BTB/POZ transcription factor PLZF (80) (see chapter II).

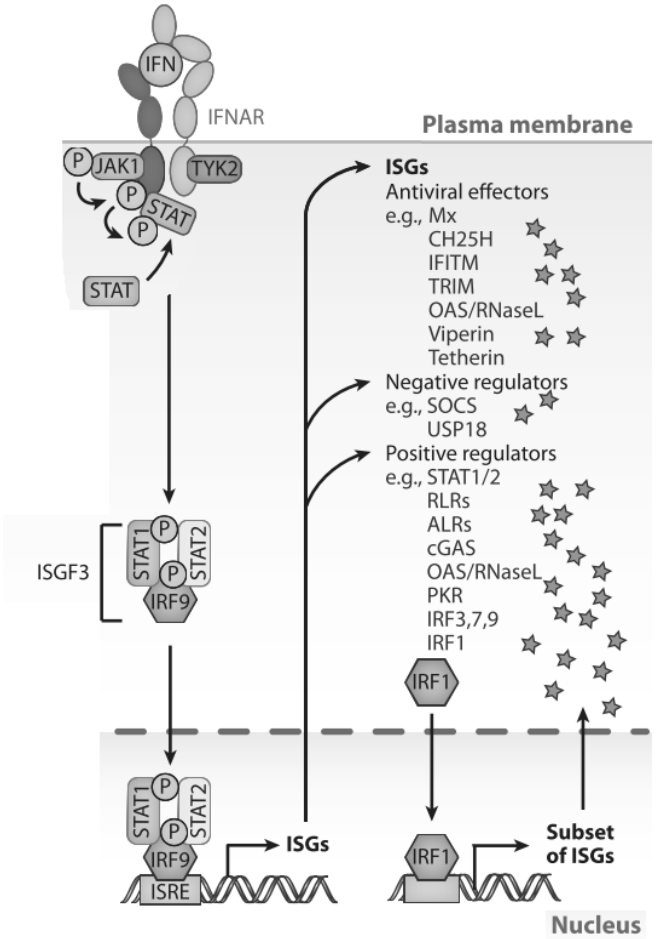

Figure 2. ISG induction in mammals. Type I IFN binding by its receptor elicits conformational changes in associated kinases JAK1 and TYK1 thereby causing several phosphorylation events that results in transcription factors STAT1 and STAT2 phosphorylation. STATs associate with IRF9 and activate transcription from ISRE elements. Some of the products reinforce or inhibit signalling, some have direct antiviral functions. Additionally, ISG can be transcribed by IRF1. Scheme adapted from (81).

Fish

All components of the JAK-STAT pathway have orthologues in fish, often in duplications. Fish JAK have the same domains and are upregulated upon PRR stimulus (31,82). Nonetheless, STAT1, STAT2 and IRF9 functional involvement in fish ISG induction is likely different from mammals. For example, all fish seem to have two STAT1 paralogues (Stat1a and Stat1b), and while zebrafish has a single Stat2, salmon possess two paralogues of STAT2 which are phorphorylated in response to type I Ifn although they can regulate type II IFN signalling as well (which in mammals involves STAT1 homodimers only) (31,83). Therefore, the JAK-STAT pathway that induces ISGs is present in fish, though functional data on ISG induction pathways is scarce.

ISG repertoire

The executive role of the type I IFN system is carried out by several hundreds of ISG that correspond to proteins with activities ranging from direct anti-viral properties to general impact on processes such as apoptosis and signalling pathways that either reinforce or inhibit immune defense. The great numbers of ISGs also reflect the need to have several mechanisms to overcome viral infections as viruses themselves have evolved ways to subvert IFN system by blocking ISG functions. Although the functions of many ISGs remain unknown, large-scale screens have started to assess systematically the implication of each ISG in defenses against different types of viruses (84).

Mammals

Diverse mechanisms of ISG

Viral cycle roughly consists of entry, uncoating, translation/replication, virion assembly and release – all these stages are subjected to intervention by many ISG that are not specific for any particular virus (but may be specialized in fighting virus classes). The entry of enveloped viruses can be blocked for example by cholesterol-25-hydroxylase that produces oxysterols, which modify plasma membrane (85). Mx (myxovirus resistance) proteins can influence several stages as MxB can disrupt

uncoating (86) and MxA captures viral nucleic acids in fibrillary structures thereby inhibiting their translation/replication (87). Many other indirect mechanisms are elicited to battle viral replication. dsRNA-activated protein kinase R (PKR) reduces host cell translation through the modulation of elongation initiation factor-2 subunit alpha (eIF2α) thereby inhibiting viral translation as well (88). Another type of ISGs that limit viral translation indirectly are oligoadenylate synthetase (OAS) and latent endoribonuclease (RNaseL) (89). OAS recognizes cytosolic RNAs and in response synthetises 2'-5'oligoadenylates that activate RNaseL which cleaves viral RNA. In addition to destroying viral genome, it also cleaves host RNA into fragments that stimulates PRR and thus augments immune response (90). One of the most highly induced ISG is RSAD2 (viperin), but its antiviral functions are not completely elucidated: it has been shown that RSAD2 affects several stages of viral cycle. For example, RSAD2 indirectly affects the properties of plasma membrane lipid content and thereby inhibits normal budding of virions and it could block the entry of viruses as well (91); also it inhibits transport of soluble proteins (92). Another broadly acting effector is ISG15 that can be covalently bound to more than hundred proteins to change their function and for instance inhibit viral budding (reviewed in (93)).

In addition to these generic antiviral proteins, there are ISG belonging to TRIM superfamily of proteins that are more specialized to certain viruses. For example, TRIM5α that causes retroviral capsid disassembly (94) or TRIM22 that targets viral nucleoprotein or TRIM56 that targets viral anti-host immunity protein for degradation (95,96).

Collectively, these examples illustrate the diversity of biochemical function and cellular processes through which ISGs fight the infection. Furthermore, it is known that combinatorial action of different ISGs is required for efficient virus inhibition and overexpression of only one effector-ISG does not generally protect against virus infection (84).

ISGs participate in IFN signalling retrocontrol loops

Many of the ISGs are PRR/IFN signalling components such as MYD88 and IRF1, which reinforces the activation of the IFN inducing pathways (97). Importantly, IRF1

can induce a subset of ISG that include broad anti-viral effectors such as ISG15 and viperin (79,98). IRF1 can act independently of JAK-STAT pathway – this branch of ISG induction has likely evolved since it assured that in case of subversion of JAK-STAT by viral proteins, key ISGs would still be induced (see above, Induction pathways of ISG). As such, IRF1 overexpression inhibited the replication of six different ssRNA viruses (99). The aforementioned PKR also stimulates NFκB-pathway by the binding of NFκB inhibitor (100). Among ISG are also molecules that repress IFN signalling as the ISGs clearly paralyze normal metabolism of the cell and it has to be tightly controlled to avoid detrimental hyperinflammation. For example, suppressor of cytokine signalling (SOCS) inactivates signalling through the binding of phosphorylated sites on IFN receptors or JAK proteins.

Context-specificity of ISG profile

ISG repertoire consists of hundreds of genes, but the full set is not expressed in a given cell during infection. ISG profile can vary depending on the virus, cell type as well as whether it is an infected or neighbouring cell. ISG profiles induced by different viruses are tuned and include shared and virus-specific ISGs. Among shared ISG are usually such molecules as RIG-I, IRFs, ISG15 and RSAD2 (99,101). Interestingly in a screen of TLR7 and-9 agonist the shared ISG were the ones with direct antiviral properties such as OAS1 and ISG20, whereas genes related to antigen presentation or cell mobilization were differentially expressed (58). ISG profile cell-specificity has been observed for example in subtypes of neurons: higher basal expression level of select ISG in granule cell neurons ensured greater resistance to virus than for cortical neurons (102). Since type I IFNs not only serve to eliminate viruses from infected cells and protect neighbouring cells but also have broader influence by activating leukocytes, the ISG profiles in these specialized cells are remarkably different as well. For example, mice natural killer (NK) cells required type I IFN receptor to express perforin, granzyme B and other crucial NK molecules in response to adenovirus infection (103) and type I IFN can upregulate antigen-presentation molecules in dendritic cells (104).

Fish

Most of the abovementioned ISGs are also found in fish, whereas some families are absent – for example OAS proteins. However, for the fish ISG orthologues there is usually no evidence that mechanisms are the same as in mammals.

Fish ISG orthologs

Several fish species have two paralogs of a number of ISGs, as a result of the whole genome duplication that occured in early evolution of fishes (105). For example, fish have two PKR homologues – Pkr and Pkz, both with PKR-like kinase domain; however they differ in nucleic acid-binding domains, and constitute a typical sub-functionalization example. PKR is functionally equivalent to zebrafish Pkr and Pkz as they were both induced by poly I:C treatments and were able to phosphorylate eIF2α (106). Amplification of ISG families can also result from tandem duplications, as for the Mx homologs in fish which have antiviral potency, however there are no mechanistic insights (107,108). Fish Rsad2 was described in late nineties just as mammalian viperin (109). It is a highly induced ISG in trout, carp, salmon, zebrafish and has antiviral properties (67,109–111). Interestingly, there is only one Rsad2 orthologue in a number of fish species, including zebrafish; and its degree of conservation between fish and mammals is the highest among all highly-inducible ISGs. For Isg15 there are several paralogues in many fish that can have different features. For example, cod has three homologues that were all poly I:C-inducible, but only one of them was shown to be conjugated to host proteins upon viral infection (112). In another study from our laboratory, overexpression of zebrafish Isg15 established strong antiviral state and many host and viral proteins were shown to be isgylated (113). Inhibitors of signalling such as SOCS are conserved in teleosts as well (114).

Large-scale ISG response studies

Microarray analyses of polyI:C-treated or virus infected cells revealed that many of the typical highly induced mammalian ISGs are also retrieved in fish (115,116). The comparison of genes induced by the strong IFN due to chikungunya virus infection of

zebrafish larvae with the human ISG repertoire revealed a core set of ISGs conserved across vertebrates counting about 100 genes (116). These data implies that although there are multiple gaps of knowledge about IFN and ISG induction pathways in fish, conserved ISG subsets with mammals can be retrieved in fish virus infection models.

In the beginning of my Phd I contributed to a review article about type I Ifn system in fish (117). During these years I participated in the study of fish-specific TRIM proteins in zebrafish. This study revealed a potent antiviral TRIM that seems to be a determining factor for higher basal level expression of IFN signalling components in certain tissues – this study is described in Chapter VI. I also studied a transcription factor named PLZF that has been recently described in mice as a novel ISG inducer. I confirmed that zebrafish Plzf can modulate type I Ifn system as well – this study is described in Chapter V.

CHAPTER II

PLZF transcription factor – an emerging regulator of innate responses to pathogens

The family of BTB/POZ transcription factors

BTB/POZ transcription factors have a N-terminal domain known as BTB (Broad-complex, Tramtrack, Bric-à-Brac) or POZ (Poxvirus and Zinc-finger) and one or more C-terminal Krüppel-like Zinc-finger regions. There are 49 BTB/POZ transcription factors in the human genome, yet the function of most of these proteins remains poorly known. The BTB/POZ domain was first identified in 1991 in genes involved in homeotic patterning during fruit fly development (118). Afterwards BTB/POZ proteins have been implicated in many other processes such as germ cell and leukocyte differentiation, tumorigenesis and cell cycle regulation (119). The biological functions of BTB/POZ proteins are most frequently associated with their roles as transcriptional repressors.

When dimerized, BTB domains can interact with different co-repressors and histone deacetylases (120–122), leading to modifications of chromatin conformation. Additionally, BTB/POZ proteins differ in their repression ability due to varying binding affinity to co-repressors (123). However, BTB/POZ proteins can also activate transcription, indicating that they can form chromatin-remodelling complexes with versatile functions. Zn-fingers bring these chromatin-remodelling complexes to target promoters (124). BTB/POZ proteins have a variable number of Zn-fingers in their C-terminus, and some of them have specialized Zn-fingers that bind methylated CpG – transcriptionally repressed regions of the genome (125).

Functional plasticity of BTB-POZ proteins is further enhanced by many post-translational modifications. They can be acetylated (126), ubiquitinated (127,128), SUMO-ylated (129,130) and phosphorylated (127,128,131) depending on cell type, cell cycle or physiological context.

While only a few BTB/POZ proteins have been studied in depth, 8 of them have been implicated in the differentiation of specific leukocyte lineages (reviewed in (132)). For example, ZBTB7b (ThPOK) is essential for the development of CD4+ T-cells, ZBTB19 (MAZR) for CD8+ T-cell development , and ZBTB27 (Bcl16) for B-cell development.

Promyelocytic leukemia zinc finger (PLZF) or ZBTB16

PLZF is one of the most studied members of the BTB/POZ family. PLZF was discovered in 1993 as a gene involved in a genomic translocation that causes a rare form of acute promyelocytic leukemia (133). Six years later the human *PLZF* locus on chromosome 11 was described; it is known to comprise 7 exons and 6 introns (134,135). The coding region starts in the second exon, which is also the longest exon and encodes the whole BTB/POZ domain, an unstructured region, and half of the nine Zn-fingers. The native configuration of the PLZF gene in shown in figure 3a.

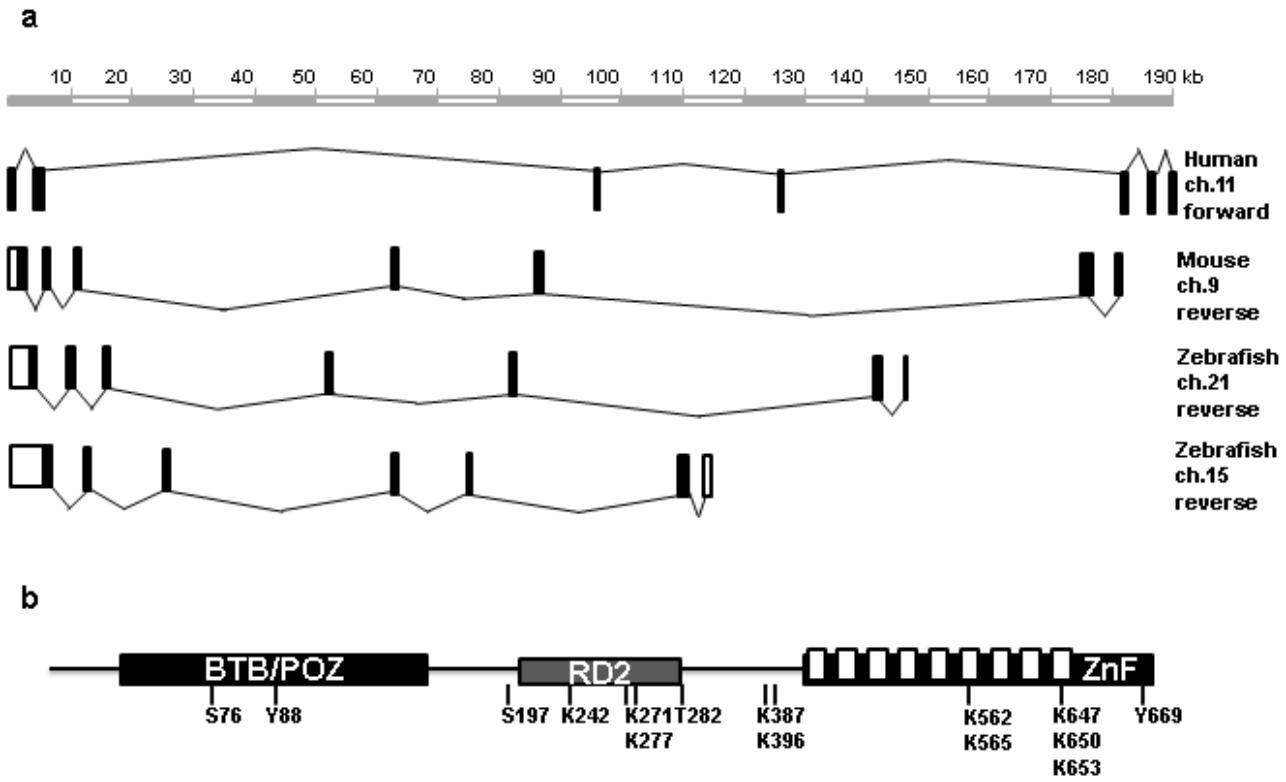

Figure 3. Schematic representation of PLZF genes and protein. a) gene structure of human, mouse and two zebrafish PLZF. Boxes correspond to exons and lines to introns. b) protein domains – post-translationally modified residues are highlighted, based on human protein sequence. RD2 is the ETO-binding region in the non-structured region of PLZF.

PLZF protein domain-function analysis

The BTB/POZ and Zn-finger domains as well as the non-structured hinge region must be dynamic as PLZF can both repress and activate promoters. All three domains are subjected to post-translational modifications (positions subjected to modifications are shown in figure 3b). It is likely that these modifications affect PLZF ability to interact with different proteins and promoters.

BTB/POZ

PLZF represses promoters when self-association of BTB/POZ forms a charged pocket that interacts with co-repressors such as SMRT (136), mSin3A (137) or N-cor (138) and thereby recruits different multiprotein chromatin-modifying complexes that involve histone deacetylases and in some cases DNA methylases (123,139). Lately it was also found that PLZF binds cullin 3, a E3 ubiquitin ligase which modifies several proteins in the repressor-complex by adding ubiquitin moieties (140). Loss of PLZF results in the increase of acetylation of histones around promoters that PLZF normally targets for repression (80,141,142). BTB/POZ domain was also implicated in transcriptional activation as induction of *RSAD2* promoter was reduced when S76 or Y88 were mutated (residues are highlighted in fig3b). It was shown that these residues were phosphorylated in response to IFNα highlighting a possible mechanism of PLZF switch to activator function. Interestingly, association with HDAC1 augmented PLZF-mediated transcriptional activation in this system (143).

Hinge region

The BTB/POZ and Zn-finger domains are linked by a hinge region that spans 268 amino acids. A strip of 100 amino acid residues in this region termed repression domain 2 (RD2) has been shown to interact with ETO – a protein which potentiates PLZF-mediated repression as it can recruit co-repressors SMRT, mSin3A and N-CoR (144). ETO-binding region is acetylated at lysine residues K271 and K277 upon TLR or TNF-α receptor activation. Acetylation of the latter residue is required for efficient repression of NF-κB-regulated genes and modulates inflammatory response (141). In addition to acetylation, lysine residues K387 and K396 right before Zn-fingers can be sumoylated, which is crucial for PLZF-mediated repression in HEK293T and COS1 cells (129,130).

Zn-fingers

The nine C2H2 Krüppel-type zinc-fingers in the carboxy-terminus of PLZF mediate DNA binding. The minimal PLZF binding site is 5'-TACTGTAC-3' (124). PLZF's DNA binding activity can be regulated by histone acetyltransferase p300 that

acetylates lysine residues K562 and K565 in the 6th and K647, K650 and K653 in the 9th zinc finger. The acetylation of the 9th zinc finger is critical for DNA binding and subsequent deacetylation of surrounding histones, hence transcriptional repression (145). Also, acetylation-deficient mutant did not localize to the nucleus. Although DNA binding is the most prominent feature of Zn-finger moiety, PLZF Zn-fingers can also mediate interactions with proteins (131,146). Additionally, phosphorylation of Y669 in the last zinc finger was necessary for PLZF translocation from cytosol to nucleus to activate transcription, implying that Zn-fingers were necessary for protein-protein interactions (147).

Thus, PLZF is subjected to a multitude of regulatory modifications resulting in great functional plasticity. Indeed, PLZF seems to have both activating and repressing ability on the same promoter. In human embryonic kidney 293T cell line and monocyte-like U937 cells PLZF was shown to directly bind and repress *c-myc* promoter (148), whereas overexpression of PLZF in human cord blood-derived myeloid progenitors caused augmentation of *c-myc* transcripts (149).

Thus the function of PLZF in a given context is largely determined by its post-translational modifications, and by the availability of co-interacting proteins in a particular cell type and/or cell cycle phase.

Intracellular shuffling of PLZF in the context of cell cycle regulation

PLZF can restrict cell growth and proliferation by inhibiting the expression of genes such as *cyclin A* (150) and *c-myc* (148). In keratinocytes, PLZF-dependent cell cycle arrest can be abolished by PLZF translocation to the cytosol. Translocation of PLZF was observed when heparin-binding EGF-receptor was activated and cleaved, its intracellular fragment then travelled to the nucleus where it bound PLZF and shuffled it out of the nucleus. PLZF translocation to cytoplasm resulted in cyclin A expression and cell cycle progression (131). Translocation-mediated inactivation of PLZF has been also observed in myeloid progenitors. In these cells, PLZF is involved in the maintenance of the progenitor pool as it represses the expression of transcription factors that control myeloid maturation. PLZF also increases the expression of negative regulators of differentiation, including *c-myc*. The PLZF mediated-control is

relieved upon stimulation by stress cytokines such as IL-3, that activate the ERK pathway and cause PLZF redistribution in the cytoplasm (149). Cell cycle progression can also be triggered by direct degradation of PLZF upon cyclin-dependent kinase 2 (CDK2) activation. CDK2 phosphorylates serine and threonine residues in PEST motifs contained in the hinge region of PLZF; phosphorylated PEST then serves as a signal for multi-ubiquitination, and subsequent PLZF degradation (127,128). In addition to repressing cell cycle propagation, PLZF can actively facilitate cell growth in cardiomyocytes, where it upregulates the expression of phosphatidylinositol-3 kinase subunit upon angiotensin II receptor activation that causes PLZF movement from cytosol to nucleus (147).

These data shows that changes in PLZF activity can be rapid and can trigger quick physiological responses as would also be expected in the context of antiviral signalling.

Biological functions

Progenitor maintenance and self-renewal

PLZF modulates cell growth and proliferation in the maintenance of many types of progenitor cells: cells in the developing limb bud (151), spermatogonial stem cells (152) and hematopoietic multipotential progenitors (149,153). PLZF knockout mice are viable, but as expected from PLZF functions in progenitor maintenance, these mice show obvious defects in limb and axial skeleton (151). They also have a shrinked spermatogonial progenitor pool rendering males infertile (152). In zebrafish, PLZF was also shown to maintain neuronal progenitors (154). PLZF likely is an essential factor in the nervous system development of other species as well, since in mouse and chicken embryos PLZF is expressed with distinct patterns at rhombomere boundaries in developing brain (155). PLZF also maintains CD34+ hematopoietic progenitors. PLZF-mediated cell cycle arrest is abrogated upon stress cytokine IL-3 – allowing their proliferation and differentiation into mature leukocytes during pathogen invasion (see above, *4. Intracellular shuffling of PLZF in the context of cell cycle regulation;*(149)).

Leukocyte differentiation

PLZF knockout mice have a defective hematopoietic system. Although T-cells and B-cells are present, their populations are much smaller than in wild-type mice. Alterations were also detected for a distinct subset of non-convenional T-cells (invariant natural killer T-cells, NKT) that were less abundant, did not home correctly, nor acquire proper effector-functions in the absence of PLZF ((156), reviewed in (157) and (158)). PLZF is expressed in the NKT lineages throughout the first two stages which correspond to NKT cells that produce type 2 T_{helper}-cell cytokines (such as IL-4), and gets post-trancriptionally down-regulated by let-7c microRNA during the final maturation into IFNγ-producing NKT cells (159).

PLZF is also crucial for the maturation of γδ T cells (160), and PLZF is necessary for the development of progenitor of innate lymphoid cells (ILC) in the fetal liver and adult bone marrow (161). ILCs are a set of mucosal and epithelial leukocytes that - as iNKT and γδ T cells - quickly respond during infection by secreting regulatory cytokines that modulate and set up the immune response. PLZF depletion also hampers the cytolytic ability of conventional natural killer cells (143), and is essential for the differentiation of megakaryocytes (162).

Promyelocytic leukemia

PLZF was discovered during the characterization of a rare form of acute promyelocytic leukemia in 1993 (133). It is caused by a genomic translocation between two loci : *PLZF* and *retinoic acid receptor-alpha* (*RARα*). Translocation results in the expression of two proteins: RAR-PLZF contains the BTB-domain and the first Zn-fingers of PLZF fused with the DNA-binding portion of RARα, and PLZF-RARα, that contains the transcriptional activation domain of RARα fused to the remaining Zn-fingers of PLZF. Both fusion proteins have oncogenic properties (reviewed in (163)). PLZF-RARα acts as a dominant-negative of the wild-type RARα by recruiting a co-repressor complex to RARα promoters and thereby blocking myeloid differentiation. However, promoters containing RAR-binding elements can also be upregulated, highlighting that PLZF-RARα may recruit co-activators. As wildtype PLZF is also expressed in hematopoietic CD34+ progenitors (153) the

disruption of its normal function might contribute to oncogenesis, as well as the RAR-PLZF fusion protein expressed instead of wild-type PLZF might deregulate PLZF target genes.

These data show that PLZF is essential for the maintenance of several types of progenitors, and is an important transcription factor for the maturation of many immune cells that participate in the early response to pathogens and also regulate adaptive immunity. This is consistent with the known functions of other ZBTB transcription factors: for example, ZBTB7A aka LRF (leukemia/lymphoma related factor) that is crucial for erythrocyte maturation or ZBTB27 aka BCL6 (B-Cell CLL/Lymphoma 6) that is important for the development of germinal centers (164).

PLZF involvement in anti-viral and-bacterial defence

PLZF controls inflammation induced by bacterial infections

PLZF depletion makes mice susceptible to overactive inflammatory response to bacteria, causing tissue damage and higher mortality. This was due to PLZF ability to restrict the NF-kB-mediated TLR and TNF-α response (80). In these studies, an increase of histone methylation and acetylation at NF-kB promoters was seen in PLZF knockout mice. Additionally, PLZF was shown to associate with HDAC3 and NF-kB p50 subunit.

PLZF is also involved in antiviral immunity

plzf knockout mice could not successfully fight infection by two neurotrophic single-stranded RNA viruses, semliki forest virus (SFV) and encephalomyocarditis virus (143). In fact, PLZF enhanced the upregulation of a subset of ISGs by IFN, while other ISGs were induced independently of PLZF. These PLZF-dependent ISGs comprise for example *OAS1* - a 2,5-oligoadenylate synthetase that in the presence of viral RNA triggers the activation of Rnase L and is crucial for the clearance of SFV, *rsad2/viperin* – a member of SAM superfamily of enzymes that suppresses progression of the viral cycle by several mechanisms such as alteration of plasma membrane fluidity, and *IFIT2* – a protein that contains several tetratricopeptide repeats mediating interactions with many cellular proteins and viral nucleic acids.

The promoter sequence of viperin and IFIT2 contained PLZF binding sites in close proximity to IRF1/2/4/7, and STAT3 suggesting that PLZF might interact with conventional IFN signalling factors to activate selected ISGs. In contrast, the promoters of ISG induced independently of PLZF did not contain PLZF binding sites.

Also, coexpression of HDAC1 and PLZF enhanced the induction of viperin-Luciferase reporter showing that histone deacetylation is important for transcriptional activation by PLZF. In addition, upon IFNα1 treatment, PLZF is localised in the nucleus and binds the TRIM family member PML (promyelocytic leukemia protein, aka TRIM19). PML itself is an ISG and integral part of major transcriptional regulation complexes termed PML nuclear bodies, which are clearly involved in anti-viral responses (165). Additionally, the indirect effects of the many abnormal leukocyte pools on *plzf*$^{/-}$ mice susceptibility to viruses remains to be clarified (see above, *PLZF biological functions*).

Thus, although much is still unknown about PLZF antimicrobial properties, the available data demonstrates that this transcription factor has a considerable impact on antiviral innate immune signalling at least via chromatin remodelling.

Fish PLZF

PLZF is a well-conserved protein across vertebrates that is present in mammals, birds and fish; a homolog is also found in insects (166,167). During the evolution of bony fishes, several duplications (whole genome duplications or local duplications followed by translocations) have occurred, so it is not a surprise that two or more paralogues of PLZF could be found in these species; for example two *plzf* genes were identified in zebrafish (154) and trout (168). However, their phylogenetic relationships to mammalian PLZF remain unclear, and whether the paralogues are functionally different has not been described.

Most studies in fish have focused on PLZF as a marker of male germ cells. In zebrafish, by using an antibody that was raised against one of the two Plzf paralogues, it was determined that this gene is a reliable marker for undifferentiated spermatogonia (169). Afterwards, a zebrafish spermatogonial cell line was made and

Plzf was used as a marker to monitor the cell stage (170,171). Plzf was used for the same purpose in studies of spermatogonial stem cells in rainbow trout (168). In carp, the *plzf* promoter region was mapped and cloned (172). A 3D model of BTB/POZ domain was created (166), indicating that the charged pocket serving as an interaction platform for co-repressors in mammalian PLZF was conserved in this fish species.

Only one study of fish *plzf* was performed in another field, in an attempt to understand regulatory loops of neuronal differentiation in zebrafish. It was reported that *plzfa* was maintaining neuronal progenitor state by suppressing the expression of a protein that would trigger differentiation. This repression was abolished by the ubiquitination of Plzfa and subsequent degradation. The two zebrafish paralogues *plzfa* and *plzfb* were both expressed in developing nervous system, although *plzfa* was more widely expressed than *plzfb* (154).

The data available on fish *plzf* genes shows that it is structurally well-conserved across vertebrates. Judged by its expression in spermatogonial cells and its function in neuronal progenitors, it also shares at least some of its biological functions with mammalian PLZF. As IFN signalling is well conserved between mammals and fishes, with many common components of the IFN pathway, one may expect that *plzf* genes have important functions in the regulation of IFN response in fish. My aim was to investigate whether zebrafish Plzf proteins could be functional counterparts of their human orthologue regarding IFN signalling.

In my thesis I showed that both zebrafish *plzfa* and *plzfb* are indeed involved in the control of anti-viral signalling; however, it augments type I IFN itself, while mammalian PLZF control the induction of ISGs, more downstream in the pathway. Thereby I confirmed that the implication of this transcription factor in the IFN system is ancient, but my results suggest that the PLZF transcription factor can participate at multiple steps in the pathway. These results have been submitted for publication and are described in detail in Chapter V.

CHAPTER III

TRIM antiviral immunity in mammals and fish

TRIM proteins – RING-B-box-CC superfamily

Humans and mice have about 70 *trim* genes. TRIM are defined by the presence of N-terminal RING (really interesting new gene) domain, B-box and Coiled-coil domain – the so-called tripartite motif, and grouped into eleven categories according to variable composition of C-terminus (173,174). TRIM-like proteins that lack one or two domains of the motif as well as differentially spliced variants proteins have been also detected (175). For some TRIM, different functions have been assigned to the isoforms, for example as negative regulators of the corresponding full-length TRIM (176) or they have specific functions different from the full-length TRIM (165).

Protein domains and their functions

RING

The RING domain, found in many proteins beyond TRIMs, is a ~50 AA long zinc-finger domain typically endowed with E3 ubiquitin ligase activity, however some can also ligate SUMO (small ubiquitin-like modifier) or ISG15. All TRIM possess a RING, hence likely have E3 ubiquitin ligase activity. The RING domain of some well-studied TRIM has been shown to have multiple activities. For example TRIM25 acts as ubiquitin and SUMO ligase (177,178). The ligation of ubiquitin (or ubiquitin-like proteins) requires the activity of three enzymes : E1 which activates the molecule that is then transferred to catalytic enzyme E2 which in cooperation with E3 ligates the target protein (reviewed in (179)). Ubiquitin itself can form polyubiquitin complexes through linkage on different ubiquitin lysine residues – the resulting macromolecules of various shapes affect targets in different ways, opening the possibility that the TRIM RING might have diverse roles through its E3 activity. For example, K63-linked ubiquitin activates signalling pathway proteins, whereas K48-polyubiquitin targets proteins for degradation.

In at least one case, a RING domain (with B-box 2) is required for the formation of high-order multimeric TRIM complexes (180).

B-box

TRIM can have two consecutive B-boxes, short zinc-finger domains of about 40 AA in length. B-box domains are classified in B-box 1 and 2 with different consensus sequences. Most TRIM have B-box 2 and some additionally B-box 1. B-boxes can mediate protein interactions. For example, the B-box 2 of TRIM5 was essential for its interaction with viral capsid (181). The ability of TRIM15 to interact and block virus release depended exclusively on its B-box, which interacted with viral protein (182).

Coiled-coil

Coiled-coil domains mediate dimerization of many proteins, and are accordingly required for homo-dimerization of TRIM; heterologous interactions of two TRIM have also been observed in some cases (175,183). Nonetheless, it can also be important for interactions with other proteins apart from TRIM (184).

C-terminal domains

TRIM superfamily members are grouped into eleven categories (I-XI) according to domain-composition of their C-terminus (173,174). All the known TRIM protein forms and designated members are shown in figure 5.

B30.2 domain

The ~200 AA long B30.2 domain (named from an exon in the CMH where it was first identified), also named PRY-SPRY (SpIA/Ryanodine receptor) domain, is present in approximately two thirds of TRIM, probably due to local duplications of these genes. It is generally accepted that this domain mediates protein-protein interactions. B30.2 is an immunoglobulin-like fold consisting of β-strands and connecting loops. Loops of some TRIM have hypervariable sites, suggesting that it binds diverse ligands – for example different pathogens. It has been shown that B30.2 sequences from antiviral TRIM22 and TRIM5 have evolved under diversifying selection at precise sites probably mediating interactions with viruses. In contrast, the B30.2 domain of TRIM6 and TRIM34 that are in the neighboring genomic region have been subjected to purifying selection and thus are likely involved in different functions (185).

Structural variety makes TRIM a functionally very diverse superfamily with members involved in various biological functions including neuroprotection, apoptosis and immune defenses.

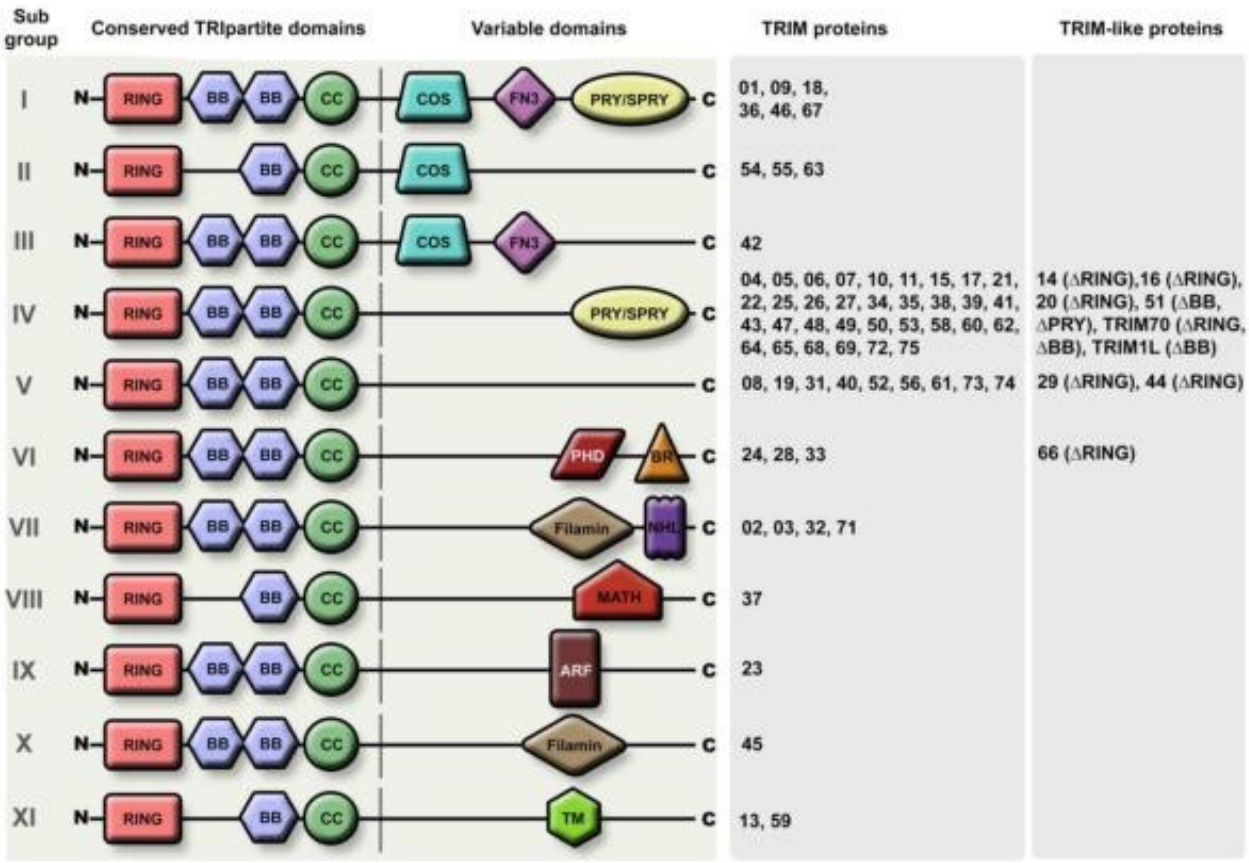

Figure 5. TRIM family sub-classification into groups. On the left schematic representation of different TRIM protein domain composition, on the right human group members are cited. Different domains are: COS (C-terminal subgroup one signature) which mediates binding to microtubules, FN3 (fibronectin type 3) that binds DNA and heparin, PRY/SPRY (B30.2) an Ig-like domain, PHD (plant homeodomain) and BR (bromodomain) can mediate chromatin remodeling, Filamin that can be involved in actin cross-linking, NHL (NCL-1, HT2A and LIN-41), MATH (meprin and TRAF homology), ARF (ADP ribosylation factor-like) that regulates intracellular trafficking and TM (transmembrane). Figure is from (186).

Intracellular localization

TRIM also display various intracellular patterns – diffused in cytosol, cytosolic filaments, nuclear or cytoplasmic speckles, reflective of involvement in different processes. Some of these patterns coincide with organelles: TRIM1 and TRIM18 interact with cytoskeletal structures via COS-domain, TRIM13 and TRIM59 have a transmembrane domain and localize to endoplasmatic reticulum, TRIM37 localizes to peroxisomes, whereas many other TRIM exhibit a localization not reminiscent to any known organelle (173,183,187,188). The speckles and aggregates likely reflect higher

order association of TRIM. Hence, it was proposed that TRIM define new cellular compartments (183). An important example of such compartment is the PML nuclear bodies (PML, for promyelocytic leukemia, is the most widely used name of TRIM19).

TRIM in antiviral innate immunty

TRIM as mediators of antiviral immunity

Driven by the initial discoveries of antiviral roles for several TRIM proteins (189–191), large-scale screens have been performed to get an overview of the extent that human TRIM family has in antiviral functions. These screens have clarified whether TRIM are themselves ISG, modulate IFN/NFκB transcription or inhibit/enhance (retro)virus different cycle phases (182,192–194). Overall, more than half of all TRIMs and members of each TRIM subgroup were implicated in IFN system at least in some way. Some TRIM had all aforementioned qualities: TRIM1, TRIM5, TRIM14, TRIM25 and TRIM26, all of which contain B30.2 domain. The mechanisms are well-known for some of these TRIM (see below). Out of the forty five B30.2-containing TRIM, twenty six were involved in antiviral activities. The ability of many TRIM to induce type I IFN has been confirmed by independent studies (192,194).

The type I IFN-induced modulation of TRIM expression has been tested at least in four different cell types and about twenty of human TRIMs were ISGs (192,193). TRIM5, -21 and -25 were upregulated upon IFN treatment in the different cell types, whereas others were more restricted and some TRIM were induced in one cell type and downregulated in other. Interestingly, TRIM had different expression dynamics during early phase of IFN treatment or infection – some were upregulated transiently, others continuously or sporadically suggesting that they are involved in different stages of antiviral actions (192).

Mechanisms

Antiviral TRIM may interact directly with viruses via different mechanisms: TRIM5 and TRIM22 recognize virus upon entry and cause early decapsidation or block the

assembly of virions (94,195). TRIM21 detects endocytosed virus-antibody complexes and leads to polyubiquitination that triggers anti-viral signalling and targets virions to proteasome (196,197). Additionally, half of the human TRIMs are able to enhance type I IFN response via the modulation of RIG-I pathway (192). Most TRIM affected signalling between RIG-I and TBK1-IKK-I, some between TBK1- IKK-I and IRF3, and TRIM1 and -49 likely interact with IRF3, but are not transcriptional regulators themselves as they did not relocate to nucleus during transfection. TRIMs can also inhibit IFN response. For example, TRIM11 that causes IRF3 hypo-phosphorylation probably by inhibiting TBK1 or TRIM27 that interacts with TBK1 and IKKβ and –α (184,198). TRIM21 can modulate IFN induction both negatively and positively. For example, it can promote IRF3 activity indirectly but also it can target IRF3 for degradation (199,200).

TRIM5 – a direct effector and a PRR

Because of its key role in species specificity of HIV (see below), TRIM5 is one of the best-studied retrovirus restriction factor, yet its restriction mechanism is still not entirely clear. The block occurs during early stage of infection and likely involves several mechanisms. First, upon virus capsid recognition TRIM5α (the longest protein isoform encoded by *trim5*) causes premature virus decapsidation (94). In addition, upon capsid lattice recognition, TRIM5α also catalyzes the Lys63-linkage of ubiquitin chains resulting in unanchored polyubiquitin that activates TAK1 kinase complex, which leads to anti-inflammatory cytokine production (201). Intact RING-domain (i.e ubiquitination capacity) is definitely necessary for efficient virus inhibition (191). Interestingly, TRIM5 of different species display different restriction capacity – reflective of diversifying evolution due to antagonistic relationships within species-specific viruses (185). For example rhesus monkey TRIM5α blocks HIV-1 replication whereas human TRIM5α cannot due to differences in B30.2 domain that interacts with the capsid protein (191,202).

TRIM25 – regulator of signalling

TRIM25 also is a B30.2 domain-containing TRIM that modulates RLR signalling by several ways. It can conjugate lysine-48-ubiquitin chains on MAVS, causing its degradation and the release of TBK1 that consequently phosphorylates IRF3 and induces type I IFN (203). TRIM25 can also synthesize polyubiquitin chains linked through lysine-63 that activate RIG-I; RIG-I ubiquitination is also necessary for downstream signalling (177,204). TRIM25 also participates in other pathways, for example in cell cycle regulation and is highly conserved across most vertebrates (205).

These data show that more than half of TRIM family can participate in antimicrobial defense. As many TRIM are ISGs and B30.2-domain containing TRIM have expanded in human and mouse and some of the members have been shown to evolve under diversifying pressure, their importance in immune defense was likely and now confirmed. Interestingly, members of other TRIM categories could modulate IFN system as well highlighting that TRIM superfamily important in innate immunity.

TRIMs in fish and mammals: different repertoires

Orthologues of TRIM from all categories except C-III are found in fish (206,207). As a consequence of a whole genome duplication of the teleost lineage (or additional ones), some of them have two or more remaining co-orthologues. There are several TRIM clusters in human genome likely due to local duplications, however even larger expansions occured in zebrafish making the fish TRIM family much bigger than in human and mouse – about 200 genes versus about 70. Examples of such expansions are two families orthologous to, respectively, TRIM39 (the first member of which was named *bloodthirsty,*(208)) and TRIM35, both of which contain at least 30 members (206). Interestingly, all expanded TRIMs have the B30.2 domain organization. Among B30.2-domain containing TRIM there are zebrafish orthologues for mammalian TRIM16, -25, -35, -39, -47 and -62 (206). There is no PML/TRIM19 orthologue in zebrafish.

Interestingly, *trim* genes with a B30.2 domain had been found in a screen for trout

viral-induced genes (209), and a search for related sequences in the zebrafish genome revealed an additional expanded subset of 84 B30.2-containing TRIM for which no tetrapod orthologues could be found; the family was named fish novel TRIM (FinTrim, abbreviated as Ftr)(210). The RING domain of some Ftr was capable of ubiquitination as common for TRIM proteins (211). The most similar mammalian counterparts to Ftr are TRIM16 and TRIM25, however both have their own orthologues in fish. The great trend of B30.2 domain to expand akin to finTRIMs is further illustrated by the fact that B30.2 domain is very similar to the B30.2 domain in nod-like receptors that are involved in immune function and also expanded in fish (31,212).

Flexibility and variety of TRIM evolution in fish species suggest that there might have been a strong pressure from diverse pathogens and fish have developed particular and complex anti-microbial strategies based on innate immunity. Additionally, some of the fish Trim could be involved in fish-specific biological processes.

The finTRIM subset: a large fish-specific group of B30.2 TRIM proteins

As mentioned above, the Ftr subset was found in our laboratories in an ISG screen in trout and then identified in other fish species. Interestingly, in leukocytes a broad range of Ftr isoforms was induced corresponding to C-terminally truncated forms as for human TRIM such as PML. The expansion of this subset likely involved local duplications of genes as half of zebrafish Ftr are located in three clusters on chromosome 2. Ftr within one cluster are more similar to each-other than to Ftr in the other clusters confirming that they resulted from local gene duplications (210).

Overall, zebrafish Ftr divide into three groups – A, B and C, based on the similarity of B30.2-domain, although phylogenies based on RING-B-boxes domains are largely congruent. Among different fish species only true orthologues of the group C (Ftr82, -83, -84) genes were found, and kept in a stable genomic context whereas no syntenic relationships were observed for group A and B genes. Group A contains most of the *ftr* genes and they are highly diverse (210). Furthermore, the B30.2 loops and some sites next to RING domain of group A Ftr evolved under diversifying selection

showing that these proteins might recognize pathogens and thus co-evolved with them and amplified thanks to a selective pressure that led to their fixation (206). On the other hand, group C Ftr occured early in the evolution of teleosts – they occupy a basal branch in trees of all ftr along with related TRIM genes, and were kept more alike.

Apart from being induced upon viral infection, when I started my PhD, there was no experimental evidence of a role for FinTrims in antiviral immunity. The study of many Ftr in zebrafish might be complicated by the fact that most Ftr likely detect specifically some viruses; however natural viruses of zebrafish have not been isolated yet and good model systems are not available. In this thesis zebrafish Ftr82 and -83 were chosen to address the question of a potential antiviral role of the finTRIM subset. Ftr82 and -83 seemed good candidates as they are ancient Ftr which were not subjected to many amplifications and thus might have retained a fundamental function of the group - for example in modulation of antiviral signaling as it appears to be a key function of the whole TRIM family in mammals.

We found that Ftr83 is a potent inducer of type I Ifn and as such protected against many (rhabdo)viruses, whereas Ftr82 did not. Ftr83 is predominantly expressed in mucosal tissues such as gills and skin and its expression in these organs correlated with a higher amount of constitutive level of Ifn, thus it might be the key factor in establishment of a low basal Ifn signal that would assure a quick response upon virus infection of these highly exposed organs. These results have been submitted for publication and are described in detail in Chapter VI.

CHAPTER IV

In vivo studies of leukocytes in zebrafish

Local activation of innate immune pathways in specific regions is an important component of the regionalization of responses to pathogens. Additionally, resident populations of specialized cells at critical sites, and selective recruitment of inflammatory leukocytes such as neutrophils and macrophages are also very important in this respect. The zebrafish model has been widely used to visualize the attraction of neutrophils and other leukocytes at sites of infection or inflammation; reporter transgenic lines in which a promoter active in a leukocyte lineage controls the expression of a fluorescent protein constitute exquisite tools for such approaches. In particular, the lines *mpx:gfp* and *lyzC:dsred* (for neutrophils) (213–215) and *mpeg1:gfp* or *mCherry* (for macrophages) (216,217) have been used to track leukocytes *in vivo* and *in toto* in infection models of viruses (71,218) and bacteria (217,219,220).

In my thesis, I undertook the characterization of a particular subset of leukocyte-like cells which express GFP driven by a promoter fragment of medaka's β-actin gene. In this fish trangenic line, named "medaktin", fluorescent cells with morphological features of leukocytes are located close to the neuromasts – mechano-sensory structures in fish skin, which evokes a possible function of sentinel cells. This section is an overview of the leukocytes in zebrafish, providing a frame for our studies of *medaktin:gfp*.

Zebrafish immune system : general anatomy

During the first three weeks of development zebrafish relies mainly on innate immunity, whereas afterwards adaptive immunity matures. Zebrafish adults have many of the basic leukocytes found in humans, however there are significant differences in the organization of immune system organs (Figure 6). Zebrafish do not have bone marrow - instead the main site of hematopoiesis in adult fish is kidney marrow, a place where granulocytes, myelomonocytes and B-cells mature (221). As in humans and in all jawed vertebrates, T-cells mature in the thymus or more exactly the thymi since they are bilateral in fish (222). Lymphatic vessels have been described, but zebrafish as all teleost fishes lacks lymph nodes (223). An analogous site for lymph node function - *i.e.* pathogen "filtration", and site for leukocyte

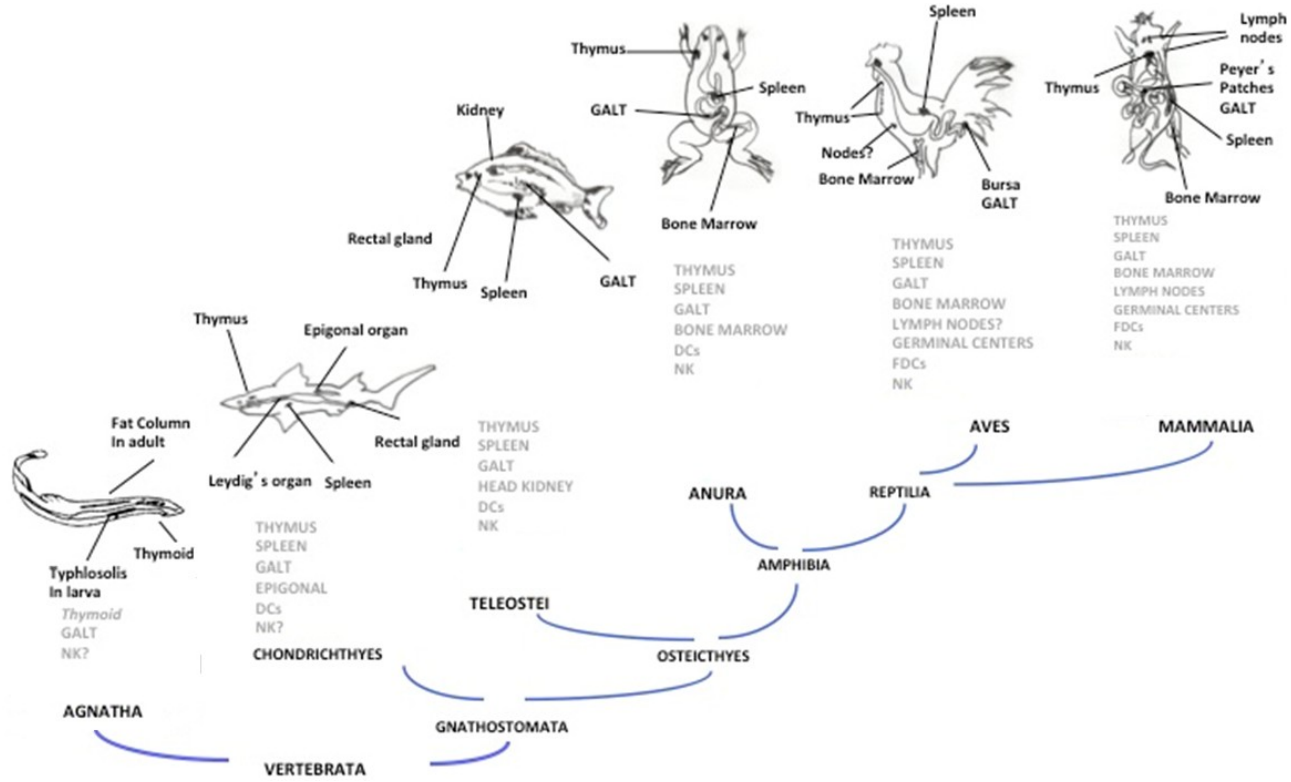

Figure 6. Organization of the immune system across vertebrates. Zebrafish belong to the infraclass of *Teleostei* that diverged from mammals approximately 450 millions years ago. Figure adapted from (225).

coordination for adaptive responses, might be the aggregations of macrophages and lymphocytes called melano-macrophage centers in kidney and spleen (224).

Zebrafish leukocytes

Zebrafish hematopoietic system is very similar to humans. The presence of all the basic leukocytes of innate and adaptive immunity has been confirmed in zebrafish (Figure 7), however the full repertoire of their functions and existence of various cellular subsets (*e.g.* innate lymphoid cells and natural killer T-cells) is not established.

Granulocytes – neutrophils and eosinophils

Neutrophils and eosinophils are prominent leukocytes in the destruction of pathogens via several mechanisms: exocytosis of toxic proteins (such as Rnases), synthesis of reactive oxygen species by cell-specific peroxidases and expulsion of DNA traps. Both cell types with corresponding functional characteristics have been identified in zebrafish (226,227). Zebrafish neutrophils also exhibit similar phagocytic traits as their mammalian counterparts. For example, zebrafish neutrophils were also shown to

avidly phagocyte bacteria - but only from surfaces, whereas macrophages could engulf pathogens in fluids (219). Although rarely remembered, this feature is also true of human neutrophils (228). Importantly, mammalian neutrophils are difficult to study as they rapidly die when cultured and *in vivo* studies are hampered due to inaccessibility and imaging difficulties. Zebrafish transgenic lines that mark neutrophils have proved very useful to study neutrophil behavior. For example, it was clarified in zebrafish model, that during the clearance of inflammation, most neutrophils do not undergo apoptosis, but instead leave the site via reverse migration and retain the capacity to attack pathogens (215,229). By using double-transgenic fish that label macrophages and neutrophils it was observed that macrophages can engulf parts of neutrophils leaving the neutrophil viable; however, the function of this interaction is not known (216). Importantly, neutrophils have been identified as key producers of type I IFNs in zebrafish larvae infected with Chikungunya virus (71) but it is not yet known if this happens in mammals.

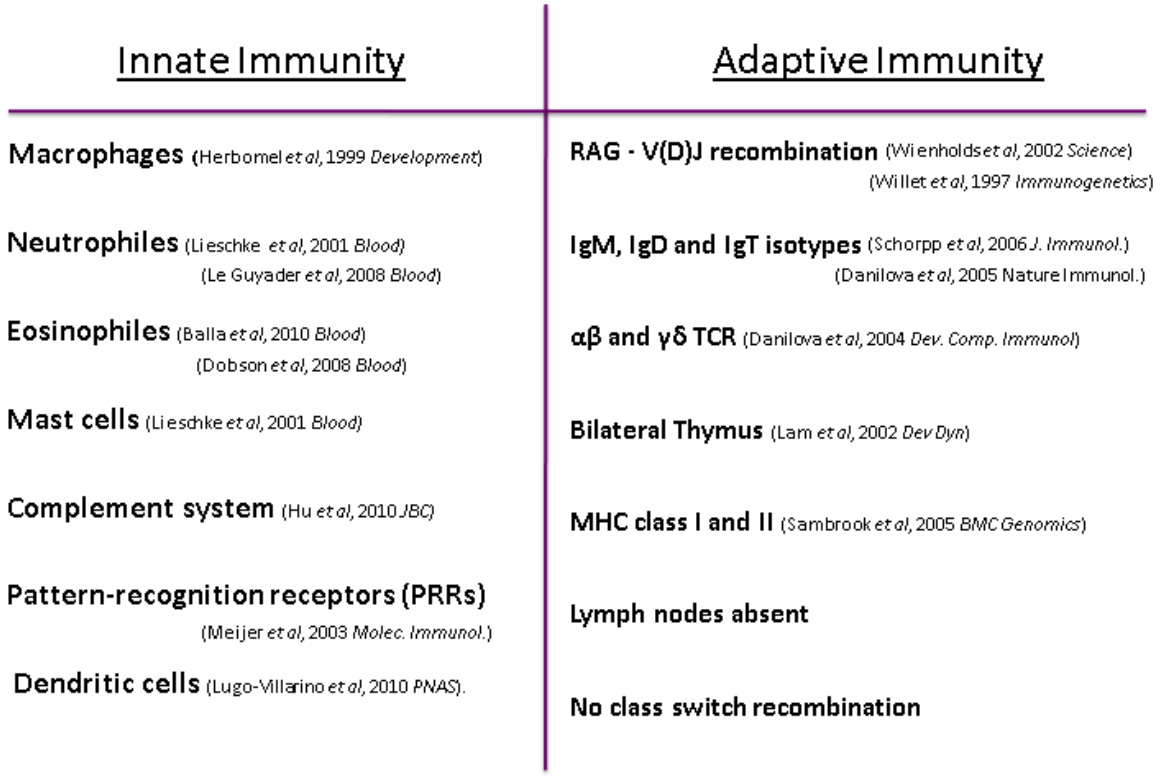

Figure 7. Zebrafish immune system. Summary of the basic components and features of zebrafish immune system.

Macrophages

Macrophages are highly phagocytic cells that were first observed by Ilya Mechnikov in the late 19th century. It was postulated that such phagocytotic cells could be involved in developmental sculpting, homeostasis and defense functions. This assumption has proven correct although the extent of physiological processes that can be influenced by macrophages is still not fully clear. In mammals, resident macrophages in many organs have been defined, as well as monocytes that home to infected sites and differentiate into dendritic cells or macrophages that either have pro-inflammatory (M1), wound healing (M2) or other regulatory functions. There is evidence that zebrafish have similar subsets of macrophages .

Just as in mammals and birds, macrophages are the first leukocytes that appear in zebrafish during development. In the 20 hpf embryo, cells with the morphology of macrophages and the capacity to scavenge apoptotic bodies and mount immune defense via phagocytosis appear (230). Zebrafish early macrophages populate retina and brain, where they transform into microglial star-shaped cells that have a high apolipoprotein E expression and become highly endocytic – likely reflective of the continuous uptake of neurotransmitters in synaptic cleft (231). The transcription factor Irf8 is crucial for the development of all embryonic and larval macrophages as proven with Irf8 knock-out zebrafish (232). Although, some Irf8-independent macrophage pools are established later in development, the microglial population does not appear if irf8 was not expressed during the development, showing the importance of the early macrophages for the establishment of microglial compartment. After the migration to anterior structures, macrophages invade other parts of the embryo such as epidermis where they have been observed to either patrol around epithelial cells or stay immobile and become ramified. At 4-5 dpf skin macrophage-like cells were seen mostly around neuromasts – mechanosensory structures, as assessed with differential interference contrast microscopy and L-plastin expression which is a marker of motile leukocytes (231). A broader network of macrophages in the skin morphologically reminiscent of Langerhans cells is detected starting from 8-9 dpf using fluorescent reporter lines of *mpeg1* (macrophage

expressed gene 1) and *mhc2dab* (Mhc Class II receptor β chain) genes (233,234). Kuppfer cells in the liver have not been identified in zebrafish, whereas functional osteoclasts that were necessary for proper bone remodeling exist in fish (235,236).

One of the highly used zebrafish transgenic lines labeling macrophage populations has been made with *mpeg1* promoter region (216). Mpeg1-reporter zebrafish have been used in the study of multiple aspects of macrophage biology *in vivo* in the whole animal: involvement in regeneration (237), interactions with other leukocytes (216) and with pathogens (71,220). It was demonstrated recently that zebrafish have three closely related *mpeg1* genes out of which one is a pseudogene and two others have been shown to be regulated during infection, however during normal homeostasis in larva the mpeg1-reporter recapitulates the expression of both functional *mpeg1* genes (238). It is not entirely clear if *mpeg1* is expressed in dendritic cells.

Dendritic cells - professional antigen-presenting cells

The main mammalian antigen-presenting cells are B-cells, macrophages and dendritic cells. Dendritic cells are considered to be the most potent naïve T-cell activators and as such the link between innate and adaptive immunity. Antigen-presentation and activation of T-cells is mediated via MHC Class I and Class II peptide-associated molecules together with co-stimulating molecules CD80, CD86 and CD209. A zebrafish transgenic line made with the regulatory region of *mhc2dab* gene faithfully marked all three types of antigen-presenting cells starting from 12 dpf (233). The smallness of dendritic cell population has made the isolation and functional characterization of these cells difficult. Nonetheless, the existence of dendritic cells has been proven through the enrichment of these cells with peanut agglutinin staining and subsequent biochemical and morphological characterization (239). The highest amount of dendritic cells was isolated from skin, yet they were also present in thymus, kidney, spleen and intestine, whereas none were detected in brain or liver (233). Functionally, zebrafish dendritic cells have been shown to cause antigen-dependent *in vitro* proliferation of T-cells. Importantly, this ability was significantly reduced by blocking the aforementioned receptors (240). Nonetheless, the many specific dendritic cell subsets defined in mammals such as plasmacytoid dendritic

cells have not been identified in fish.

B-cells and T-cells

In zebrafish, T-cells appear during the first week of development and they start expressing T-cell receptor chains as well as RAG enzymes (222,241,242). Functional distinction between cytotoxic and helper T-cells is not well-established for lack of markers, however helper cells were recently characterized thanks to a anti-CD4-1 antibody (243). Functional B-cells seem to appear only around 3 weeks post-fertilization (244). Interestingly, zebrafish (and other teleost) B-cells have non-specific phagocytotic and microbicidal properties as well as great potency to activate T-cells which highlights them as important antigen-presenting cells in fish (245). Starting from 1-month zebrafish mount a mature adaptive response as witnessed by the production of immunoglobulins against T cell-dependent antigens (241,246). Hence, it is accepted that lymphocytes and adaptive immunity do not play a major role in immune defense of the larva.

This data show that despite some organizational differences in immune system, zebrafish have the same basic leukocytes subset than humans with similar features. In addition, zebrafish fluorescent reporter lines provide the means to study leukocyte interactions *in vivo* and *in toto*.

In this thesis, I undertook the characterization of a transgenic zebrafish line *medaktin:EGFP* in which high EGFP expression was seen in leukocyte-like cells surrounding neuromasts. I observed that these cells appear after mature neuromasts have formed, implying that they are not involved in neuromast formation. I have established that they are from myeloid lineage. Importanly, RNA-seq analysis of these cells showed that these cells have enriched expression of antigen-processing and -presentation pathway components as well as microbicidal proteins and several macrophage markers. Thus, our data implies that *medaktin:EGFP* labels a subpopulation of sentinel cells of neuromasts that belong to the macrophage lineage, and it is tempting to consider them as dendritic cells. We have started studies to discern if these cells are involved in neuromast protection and/or regeneration. These results have been summarized in Chapter VII.

RESULTS

Aims of the thesis and research projects

The basic components of type I IFN antiviral system have been identified in the last century. Nonetheless, IFN signaling must be much more complex than initially described to account for the differential ISG profiles that can be virus-specific and vary among tissues. The differential regulation of ISGs might be partly determined by additional modulators of PRR-IFN-ISG signaling axis, as well as feedback loops that change in various cell types. However the basis of such variations remains poorly known.

Prior to this thesis our laboratories discovered a highly expanded fish-specific TRIM ubiquitin ligase subset – the finTrims or Ftr. The expansion and diversification of this family suggests that Ftrs interact with pathogens, however the actual function is not known. To get insights, two members -Ftr82 and Ftr83- were cloned from zebrafish as they are well-conserved among fish species. A two-hybrid assay using Ftr82 as a bait recovered a transcription factor Plzf as an interactor. The mammalian orthologue of Plzf was recently described as an inducer of a subset of ISG. Thus, the first aim of this thesis was to initiate the study of immunomodulatory properties of fish-specific Ftr82-83 proteins and of the transcription factor Plzf as a protein potentially interacting with these Trims. This work will contribute to the understanding of the IFN pathway and its evolution.

In the first project, I showed that the two co-orthologues of PLZF found in zebrafish - *zbtb16a/plzfa* and *zbtb16b/plzfb*, induce a type I Ifn response when over-expressed *in vitro*. The effect was seen only early during infection which is a critical step during viral infection. The effect of Plzfb on Ifn induction was studied after infection by different non-enveloped RNA viruses, whereas responses to enveloped viruses were not affected. This work shows that *plzf* implication in the regulation of type I IFN responses is conserved across vertebrates and occurs at multiple levels of the pathway and through different mechanisms.

(**Manuscript presented in chapter V; accepted for publication)**

In the second project, we discovered that a zebrafish finTrim, Ftr83, participates to the regionalization of immunity. We showed that *ftr83* is constitutively expressed in the gills, skin and pharynx, and encodes a protein that strongly up-regulates the type I Ifn pathway. While *ftr83* was not Ifn-inducible, its *in vivo* expression in gills of healthy fish correlated with that of type I Ifn, suggesting it leads to local induction of basal Ifn expression. *In vitro*, overexpression of Ftr83, but not of its close relative Ftr82, induced Ifn and Ifn-stimulated gene expression, and afforded protection against different enveloped and non-enveloped RNA viruses. This antiviral activity was Ifn-dependent, and was abolished by a dominant negative Irf3 mutant. Our work indicates that TRIM proteins can contribute to the establishment of antiviral immunity by permanent type I IFN stimulation, creating a local anti-viral environment at sites exposed to pathogens. Our data also demonstrate that TRIMs were involved in antiviral immunity before the divergence between bony fish and tetrapods, early in vertebrate evolution.

(**Manuscript presented in chapter VI; submitted for publication)**

Hence, at a whole organism level, protection against viruses requires an augmented immune defense in regions more exposed to pathogens as proposed in the second project. Higher protection can also be achieved by the particular location of specialized sentinel leukocytes. The second aim of this thesis was to explore the potential sentinel role of leukocytes located close to the neuromasts of the fish. In the third project, I used a reporter zebrafish transgenic line, the "Medaktin-GFP" line in which leukocyte-like cells associated to these mechano-sensory organs in fish skin express GFP - facilitating *in vivo* imaging and sorting of these cells for further analysis. The data strongly suggest that these cells can be involved in antigen-presentation and could play a role of sentinels.

(**Manuscript draft presented in chapter VII; in preparation)**

These studies revealed to me the multiple levels of immune defense that organisms incorporate to fight infections. Additionally I learned about the evolution of antiviral

immunity from the same basic building blocks across vertebrates. My participation to a review paper helped me to better understand the complexity of the IFN pathway (**Annex; The antiviral innate immune response in fish: evolution and conservation of the IFN system C. Langevin, E. Aleksejeva, G. Passoni, N. Palha, J-P. Levraud, P. Boudinot Journal of Molecular Biology 425(24), 2013, 4904-4920**)

CHAPTER V

Zebrafish Plzf transcription factors augment type I IFN

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Zebrafish Plzf transcription factors enhance early type I IFN response induced by two non-enveloped RNA viruses

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ABSTRACT

The BTB-POZ transcription factor Promyelocytic Leukemia Zinc Finger (PLZF, or ZBTB16) has been recently identified as a major factor regulating the induction of a subset of Interferon stimulated genes in human and mouse. We show that the two co-orthologues of PLZF found in zebrafish show distinct expression patterns, especially in larvae. Although *zbtb16a/plzfa* and *zbtb16b/plzfb* are not modulated by IFN produced during viral infection, their over-expression increases the level of the early type I IFN response, at a critical phase in the race between the virus and the host response. The effect of Plzfb on IFN induction was also detectable after cell infection by different non-enveloped RNA viruses, but not after infection by the rhabdovirus SVCV. Our findings indicate that *plzf* implication in the regulation of type I IFN responses is conserved across vertebrates, but at multiple levels of the pathway and through different mechanisms.

1. Introduction

In vertebrates, antiviral innate immunity is primarily based on the stimulation of type I IFN pathway. After virus recognition, Pattern Recognition Receptors (PRR) trigger signalling pathways leading to the induction of type I IFNs. These cytokines are secreted, and, when bound on surface IFN receptors, promote via the Jak/STAT pathway the transcription of a large number of Interferon Stimulated Genes (ISGs), some of which have antiviral activity.

Teleost fish possess typical type I IFNs, which mediate potent antiviral activities as IFNα and IFNβ do in mammals (reviewed in (Langevin et al., 2013a)). Fish PRR specialized in virus recognition comprise RIG-I related receptors and Toll-like receptors, some of which are specific to fish - such as TLR22 that recognizes viral RNAs. Signalling pathways triggered by these sensors involve orthologues of key kinases and transcription factors of IRF or NFkB pathways, including TBK1, IRF3 and IRF7. Regarding the effectors of antiviral immunity, a core set of ISGs is conserved between fish and mammals, whereas multigenic families comprising ISGs have often diversified independently during fish and tetrapod evolution (Briolat et al., 2014). Many ISGs involved in the antiviral signalling are also highly conserved, denoting that the main feedback loops of the IFN pathway are similar in fish and mammals. Although canonical signalling pathways of the IFN antiviral axis are indeed well conserved across vertebrates, the implication of recently discovered mediators of these pathways often remains uncertain in fish.

The transcription factor Promyelocytic Leukemia Zinc Finger (PLZF) – aka ZBTB16 -is one of such recently discovered regulators of type I IFN system in men and mice. In a seminal study, Xu et al. found that in the presence of IFN, PLZF associates with Histone Deacetylase (HDAC)-1 and the TRIM protein PML to up-regulate the expression of an important subset of ISGs (Xu et al., 2008). PLZF is a member of the Broad-complex, Tramtrack, Bric-à-Brac – Poxvirus and Zinc finger (BTB-POZ) family of transcriptional regulators, which is characterized by a N-terminal BTB domain and C-terminal Zinc-fingers repeats connected by a hinge region. These transcription factors have been implicated in many processes such as development, germ cell and leukocyte differentiation, and cell cycle regulation (reviewed in (Siggs and Beutler, 2012)). The biological functions of BTB-POZ proteins are most frequently associated with their roles as transcriptional repressors: upon post translational modifications (Ball et al., 1999), (Costoya et al., 2008), (Kang et al., 2003), (Chao et al., 2007), (Nanba et al., 2003) BTB-POZ proteins can recruit co-repressors (Huynh and Bardwell, 1998; Melnick et al., 2000), histone deacetylases (Costoya et al., 2008) (Rui et al., 2012)

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and ubiquitin-ligases (Mathew et al., 2012), to build complexes that typically modify chromatin conformation and modulate gene expression. However, BTB-POZ proteins can also activate transcription as shown for PLZF with ISGs during the antiviral response. In mammals, PLZF directly regulated the promoters of targeted ISGs and overexpression of PLZF activated *rsad2*-Luciferase reporter. This activity is greatly enhanced by co-expression of HDAC1 and by the presence of type I IFN that triggers PLZF phosphorylation at positions that are critical for its role as a transcriptional inducer. As *plzf*-KO mice showed a severe defect in ISG induction and a higher susceptibility to different RNA viruses, this gene plays an important role in antiviral defenses *in vivo* and does not represent a secondary redundant level of regulation (Xu et al., 2008).

PLZF is conserved across vertebrates, but most fish species appear to possess two paralogues. To date, most studies of fish Plzf have focused on Plzfa as a marker of male germ cells (Ozaki et al., 2011) (Kawasaki et al., 2012) (Wong and Collodi, 2013), where it may play a similar role as in mammals where it is involved in spermatogonial stem cell self-renewal (Buaas et al., 2004) (Costoya et al., 2004). The degradation of Plzf also mediates a feedback loop that allows neuronal progenitors to undergo differentiation in zebrafish (Sobieszczuk et al., 2010). Altogether these reports indicate that the repressor activity of PLZF contributes to the maintenance of progenitors of diverse cell lineages in fish and mammals.

In this work, we show that besides these functions, fish Plzf proteins are also involved in the activation of type I IFN response, enhancing the expression of Ifnφ1 itself during the critical early stages of responses to viruses and poly I:C.

2. Materials and methods

2.1. Ethics statement

All animals were handled in strict accordance with good animal practice as defined by the European Union guidelines for the handling of laboratory animals (http://ec.europa.eu/environment/chemicals/lab_animals/home_en.htm) and by the Regional Paris South Ethics committee. Experimental protocols involving zebrafish were approved by the INRA institutional ethical committee "Comethea" (#12/114). All animal work was approved by the Direction of the Veterinary Services of Versailles (authorization number 78–28) as well as INRA (authorization number B78-720) or Pasteur institute (B75-15-22) fish facilities.

2.2. Fish

Wild-type AB, initially purchased from the ZIRC (Zebrafish International Resource Center, Eugene, OR), were raised in our fish facility. All staging in the text refers to the standard 28,5 C developmental time. Larvae were anesthetized with 200 μg/ml tricaine (Sigma–Aldrich). Adult fish were sacrificed by lethal anesthesia with eugenol (0.2% clove essential oil). Chikungunya infections of zebrafish larvae were performed as described in (Palha et al., 2013).

2.3. Whole mount in situ hybridization

RNA probes were designed to cover the hinge-coding region. Templates for RNA probe synthesis were PCR-amplified from cDNA (3dpf larvae) using following primers for *plzfa*: Plzfa ISH Fw and Plzfa ISH Rev (product size 717bp), and for *plzfb*: Plzfb ISH Fw and Plzfb ISH Rev (product size 805bp) (Table 1). Primers were removed with Illustra MicroSpin S-400 HR column (GE Healthcare). Antisense RNA probes were synthesized with T3 polymerase (Promega) in the presence of digoxigenin-11-UTP (Roche Applied Science) and purified with NucAway spin column (Ambion). Whole-mount *in situ* hybridization was done as in (Thisse and Thisse, 2008) with a hybridization temperature of 55 °C and using NBT/BCIP relevation (Sigma).

2.4. Immunocytochemistry

Cells overexpressing PLZF were fixed in 4% PFA/PBS for 20 min at 4 °C. Cells were then permeabilized in PBS/0.2% TritonX100 for 5 min at RT and before saturation in 2% BSA/PBS solution at RT for 1hr. Cells were then incubated with anti-HA (Roche) monoclonal antibody in 2%BSA/0.1% TritonX100/PBS for 1 h prior to incubation with an anti-mouse secondary antibody coupled to Alexa 594 (Molecular Probes) in 2% BSA/0.1% Triton X100/DAPI/PBS for 1 h at RT before mounting in Immuno Mount solution (Molecular Probes). Images were acquired on AxioObserver Z1 microscope (Zeiss) with a 63x Plan Neofluar objective using Photometrics CoolSNAP HQ2 Camera.

2.5. Plasmids

Zebrafish *plzfa* and *plzfb* open reading frames were amplified from cDNA prepared from 3 days post-fertilization whole zebrafish larvae respectively using Plzfa Fw and Rev primers and PlzfbFw and Rev primers (Table 1). Amplification product were cloned with a HA-tag into pcDNA3.3 expression vector using pcDNA™3.3-TOPO® TA Cloning® Kit (ThermoFisher). Similarly, Plzfb deletion mutant was synthesized by amplification of the C-terminal end comprising the last eight zinc-finger repeats, before cloning into the pcDNA3.3 expression vector.

2.6. Cells and viruses

Epithelioma papulosum cyprini (EPC) (ATCC CRL2872) cell line was maintained in Glasgow's modified Eagle's medium-HEPES 25 mM medium (Eurobio) supplemented with 10% fetal bovine serum (FBS, Eurobio), 1% tryptose phosphate broth (Eurobio), 2 mM L-glutamine (PAA) and antibiotics 100 μg/mL Penicilin, 100 μg/mL Streptomycin (Biovalley). The vesiculovirus spring viraemia of carp virus (SVCV) was produced at 20 °C on EPC cells in GMEM supplemented with 2% fetal bovine serum, 1% tryptose, 2 mM L-glutamine and antibiotics. The birnavirus Blotched snakehead virus (BSNV) was propagated as described in (Langevin et al., 2013b) at 20 °C on an *Ophiocephalus* cell line derived in the laboratory. BSNV does not replicate in EPC cells, while the reovirus Golden shiner virus (GSV) (Winton et al., 1987) can replicate, although with poor efficiency; the GSV was produced on another cell line from Fathead minnow, FHM cells (ATCC CCL42) at 24 °C.

2.7. Transfections

Twenty five millions of EPC cells were seeded on 6-well plates; the next day, cells were harvested from each well (about 4 millions cells per well), and were electroporated with 3–4 μg of plasmid per well using the nucleofector kit T (Lonza) following manufacturer's recommendations. Cells were kept for 3 days at 24 °C before proceeding with infections, poly I:C treatment or immunocytochemistry.

2.8. Virus infections

Virus absorption was performed on EPC cells in Glasgow's modified Eagle's medium-HEPES 25 mM medium (Eurobio) supplemented with 2% fetal bovine serum (FBS, Eurobio), 1% tryptose phosphate broth (Eurobio), 2 mM L-glutamine (PAA) and antibiotics 100 μg/mL Penicilin (Biovalley), 100 μg/mL Streptomycin (Biovalley) for 1 h at 14 °C. Four millions transfected EPC cells were seeded on a

Table 1
Primers used in this study.

Primer name	Sequence (5′→3′)	Reference or ID number
HA_Plzfa_Fw	AACATGTACCCATACGATGTTCCAGATTACGCTGGATCGGGATCGGATTTGACTAAAATGGGTGCG	ENSDARG00000007184
Plzfa_Rev	GTTTCACACGTAGCAGAGGTACAGG	ENSDARG00000007184
Plzfa_ISH_Fw	AATTAACCCTCACTAAAGGGAGATTGTCAGGCAGTTCAGCTC	ENSDARG00000007184
Plzfa_ISH_Rev	TAATACGACTCACTATAGGGAGAGGAAGGAACCTGGTCGGT	ENSDARG00000007184
Plzfa_QPCR_Fw	GAGCCTGAACGAGCGCTGCAA	ENSDARG00000007184
Plzfa_QPCR_Rev	CAGCAAGTGCATCCTTAGGCGC	ENSDARG00000007184
HA_Plzfb_Fw	ATGTACCCATACGATGTTCCAGATTACGCTGGATCGATGGATTTGACTAAAATGGGTGCG	ENSDARG00000074526
Plzfb_Rev	CACGGGCCTGTTTCACACGTAG	ENSDARG00000074526
Plzfb_ISH_Fw	AATTAACCCTCACTAAAGGGAGATTATCGGGCAGCTCCATAT	ENSDARG00000074526
Plzfb_ISH_Rev	TAATACGACTCACTATAGGGAGAGGATCAAGTACCTTAAGAGTG	ENSDARG00000074526
Plzfb_QPCR_Fw	ACCCAGAAAGAGCGGTGTGAGG	ENSDARG00000074526
Plzfb_QPCR_Rev	CAGCATGTGCATTCTCAGCCGT	ENSDARG00000074526
HA_ΔPlzfb_Fw	ATGTACCCATACGATGTTCCAGATTACGCTGGATCGGGATCGTGCGGAAAGCAGTTTCTGGACAGTT	ENSDARG00000074526
ΔPlzfb_Rev	CAGCATGCGCTATTTCGCACTTTTA	ENSDARG00000074526
Zfish-Isg12.1_QPCR_Fw	CTCAGATGGCATTCACAGCT	ENSDARG00000017489
Zfish-Isg12.1_QPCR_Rev	TGCACCTGCGCTTTGAAGAA	ENSDARG00000017489
EPC-Ifnφ1_QPCR_Fw	ATGAAAACTCAAATGTGGACGTA	(Biacchesi et al., 2009)
EPC-Ifnφ1_QPCR_Rev	GATAGTTTCCACCCATTTCCTTAA	(Biacchesi et al., 2009)
EPC-Vig1_QPCR_Fw	AGCGAGGCTTACGACTTCTG	(Biacchesi et al., 2009)
EPC-Vig1_QPCR_Rev	GCACCAACTCTCCCAGAAAA	(Biacchesi et al., 2009)
EPC-Isg15_QPCR_Fw	AACTCGGTGACGATGCAGC	(Biacchesi et al., 2009)
EPC-Isg15_QPCR_Rev	TGGGCACGTTGAAGTACTGA	(Biacchesi et al., 2009)
SVCV_Fw	GATTGGGATTCAGGGAGAGA	U18101, (Levraud et al., 2007)
SVCV_Rev	AGCAAAGTCCGGTATGTAGT	U18101, (Levraud et al., 2007)

P6 well, left for 3 days, then submitted to viral challenge. Cells were incubated with virus inoculum for 6 h at the optimal temperature for viral growth.

2.9. Poly I:C treatment

Twenty five millions EPC cells were seeded on 6-well plates and next day electroporated with 3–4 μg of Plzfa, Plzfb or delta Plfz plasmids as described above. Cells transfected with empty plasmid were used as control. Three days post transfection, these cells (about 4 millions per well) were transfected again with 15 ng of poly I:C mixed with 1.5 μg of empty vector using FugeneHD (Promega) according to the manufacturer's instructions. Five hours post transfection, cells were lysed and harvested for RNA preparation.

2.10. RNA extraction and real-time qPCR

Total RNA was extracted with TRIZOL (Invitrogen) from EPC cells or zebrafish tissues sampled from three months old zebrafish (strain AB). RNA was purified with RNeasy mini kit (QIAGEN) according to the manufacturer's instructions and digested with DNAse (QIAGEN). Reverse transcription was done on 1 μg of total RNA using 125 ng of random hexamer primers (Roche) in a Superscript II Reverse transcriptase kit (Invitrogen) following the manufacturer's instructions. Gene expression was measured by real time PCR with a Realplex^2 Mastercycler Instrument (Eppendorf) using Power SYBR® Green PCR Mastermix (Applied Biosystems). Each sample consisted of 5 μL of primers (300 nM each), 5 μL of cDNA (diluted 1/10 for cell samples and 1/5 for zebrafish samples) and 10 μL of PCR Mastermix. Program was following: 2 min at 50 °C, 10 min at 95 °C for enzyme activation, 40 amplification cycles (95 °C for 15 and 60 °C for 1 min), followed by conditions to obtain melting curve 15 s at 95 °C, 15 s at 60 °C, 20 min for 60 °C–95 °C and finally 15 s at 95 °C. Gene expression was presented after normalization to β-actin and converted to $2^{-\Delta Ct}$. Primers used for the RT QPCR experiments were presented in Table 1. Amplification products were validated by sequencing.

3. Results

3.1. Zebrafish possesses two homologs closely related to the human transcription factor PLZF

As previously reported, zebrafish possess two genes similar to human PLZF (Sobieszczuk et al., 2010). Tblastn analysis of the zebrafish genome using human PLZF as a bait identifies two homologous genes on chromosomes 15 and 21. These genes - named respectively *plzfb* and *plzfa* - encode typical BTB-POZ proteins with highly conserved BTB/POZ and Zinc finger domains linked by a more divergent connecting hinge region (Fig. 1a). Pair-wise comparisons show that Plzfa is more similar to human PLZF than Plzfb (Fig. 1a). Residues targeted by post-translational modifications in human PLZF are all well conserved in zebrafish sequences (Fig. 1a): phosphorylation sites (S66 and Y78, using human PLZF aminoacid numbering) of the BTB domain are important for PLZF-mediated ISG upregulation in human (Xu et al., 2008), while acetylation sites (K562/565 and K647/650/653) located in Zn-fingers 6 and 9 are important for promoter-binding (Guidez et al., 2005). Post translational modification-targeted residues located in the hinge region are also conserved in fish, including cyclin-dependent kinase 2 phosphorylation sites (S197 and T282) (Costoya et al., 2008) and sumoylated lysins (K242/387/396) (Kang et al., 2003), (Chao et al., 2007).

Teleost fish species typically possess two *plzf* genes on different chromosomes, located within regions corresponding to conserved syntenies as illustrated for zebrafish *plzfa* and *plzfb* in Fig. 1b. This observation supports that fish *plzf* paralogues originated through an ancient regional (or global) duplication, rather than via local recent duplication. Phylogenetic analysis of plzf sequences indicates that they constitute a well-supported cluster within *zbtb* genes; *plzfa* and *plzfb* sequences constitute two distinct clusters supported by high bootstrap values, but the relative position of these two clusters is not stable due to faster evolution of *plzfb* sequences (Fig. 1c). However, the evolution of neighbour genes such as *ncam1* supports a model in which this region was duplicated during fish evolution, likely at the whole genome duplication that occurred after the divergence of fishes and tetrapods (Meyer and

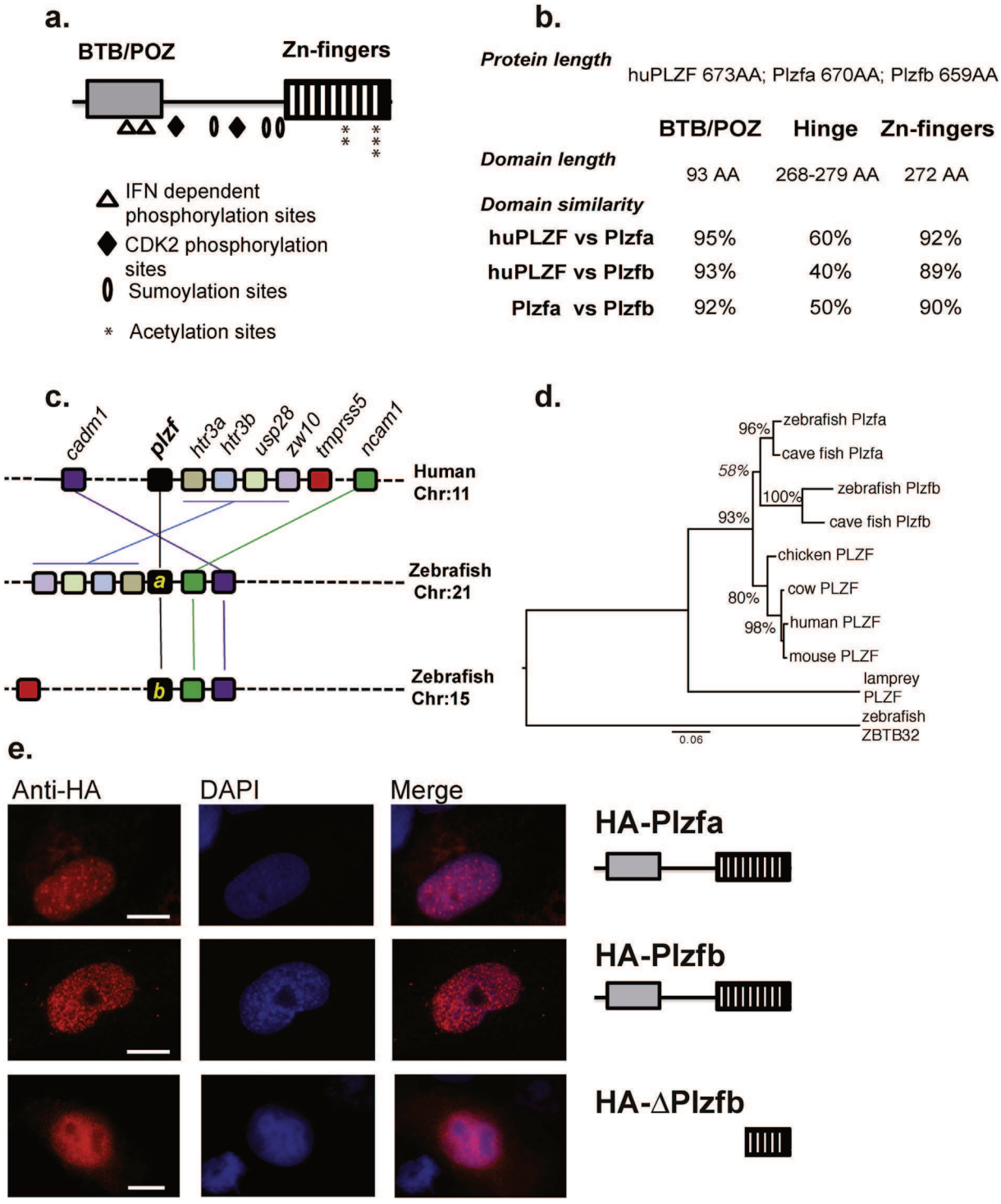

Fig. 1. Zebrafish Plzfa and Plzfb protein domains and gene organization. a) Protein domains of human and zebrafish PLZF proteins. Length of Plzf protein sequences and similarity between domains are indicated, as well as sites of human PLZF post-translational modifications conserved in zebrafish proteins b) Distance (N-J, pairwise deletion, bootstrap = 1000) tree based on zebrafish (A: gb|AAI65228.1|, and B: ref|XP_698274.3|), human (ref|NP_005997.2|), mouse (ref|NP_001028496.1|), cow (ref|NP_001032553.1|), chicken (ref|XP_417898.3|), cave fish (A: ref|XP_007250128.1|, and B ref|XP_007231497.1|) and lamprey (ENSPMAG00000002911) PLZF protein sequences and zebrafish ZBTB-32 (ref|XP_009290149.1|) as a more distant outgroup. Numbers are bootstrap values. c) Comparison of chromosome regions surrounding *plzf* locus in human and zebrafish d) HA-tagged Plzfa, Plzfb, and deletion mutant ΔPlzf localization in EPC cells. Cells were fixed 2 days post-transfection and proteins were visualized using HA-antibody and Alexa568-conjugated antibody. Nuclei were stained with DAPI.

Schartl, 1999). The phylogenetic tree of Plzf proposed by Ensembl, which is optimized to represent the evolutionary history of gene families (Vilella et al., 2009), also supports this hypothesis (see the tree at http://www.ensembl.org/Danio_rerio/Gene/Compara_Tree?db=core;g=ENSDARG00000007184;r=21:23124769-23271216;t=ENSDART00000007806;collapse=10290276). Thus, both (zebra) fish *plzfa* and *plzfb* likely represent co-orthologues of human *PLZF*, although *plzfb* has evolved faster than its paralogue.

When overexpressed in the cyprinid fish EPC cell line, both zebrafish Plzfa and Plzfb proteins showed a strong punctate staining in the nucleus. These patterns of expression were similarly observed in COS cells overexpressing zebrafish Plzfa or Plzfb (data not shown), and correspond to the well-described subcellular localization of mammalian PLZF (Mathew et al., 2012; Reid et al., 1995) (Fig. 1d). Interestingly, a Plzfb deletion mutant comprising only the C-terminal Zn-finger domains - a region highly similar in Plzfa and Plzfb – showed a distinct nuclear staining without granules, thus indicating that this protein was not associated to the same nuclear complexes as the wild type.

3.2. Zebrafish plzfa and plzfb expression patterns are distinct in larvae but more similar in adults

To determine the expression pattern of *plzfa* and *plzfb in vivo*, we first performed *in situ* hybridisations on 2, 3 and 4 dpf larvae (Fig. 2a). *plzfa* was transiently expressed in the pharynx, pectoral fins and intestine between 2 and 3 dpf and in thymus between 3 and 4 dpf. In contrast, *plzfb* was mainly expressed in the pronephric ducts and liver between 2 and 3 dpf. Both *plzf* genes were continuously expressed in the brain, but appear to partly segregate in different regions. Although, WISH was performed with similar concentrations of probes, the chromogenic reaction took 30 min longer to reveal a *plzfb* signal, suggesting that this transcript was

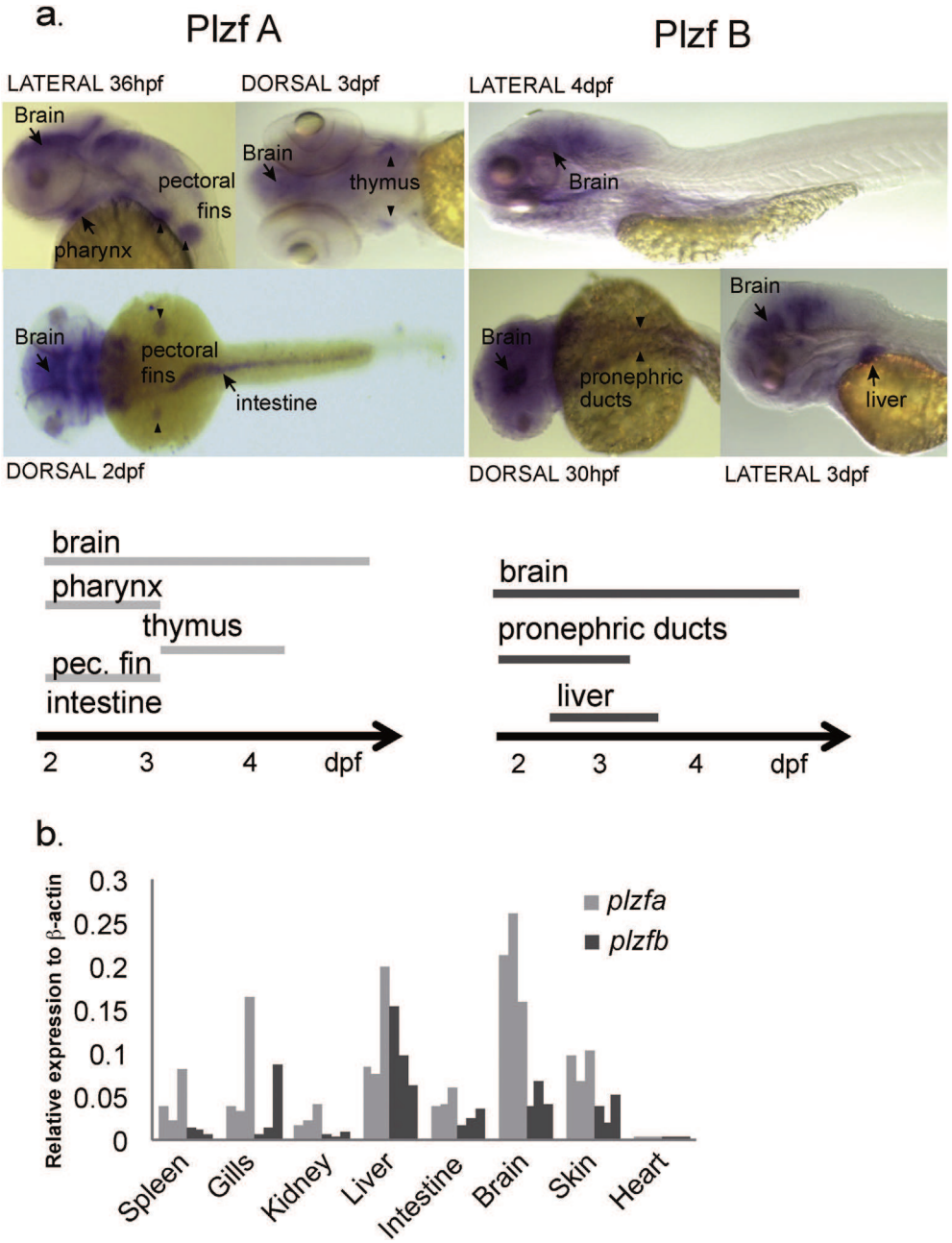

Fig. 2. *plzfa* and *plzfb* expression patterns in larvae and adult zebrafish. a) Whole mount *in situ* hybridisation on zebrafish larvae aged 2, 3 and 4 dpf. Two independent experiments were performed with at least 5 larvae per stage, and representative images are shown. b) *plzfa* and *plzfb* transcripts were quantified in different organs of 3 months-old zebrafish by RT-QPCR. Results were normalized on the basis of *ß-actin* expression. Three independent sampling groups of 15 fish were used. Bar represents one sampling group.

less abundant than *plzfa* at these stages. Thus, *plzfa* and *plzfb* expression patterns are distinct during zebrafish development (Fig. 2a, bottom panel).

The expression of *plzfa* and *plzfb* was also studied in a panel of adult tissues by RT-QPCR (Fig. 2b). At this stage, both genes displayed rather similar expression profiles: they were highly expressed in the liver, less expressed in skin, intestine, gills, and hematopoietic tissues (kidney and spleen). *plzfa* was significantly more expressed than *plzfb* in brain and skin. Thus, the expression pattern of the two *plzf* paralogues seems overall less contrasted in the adult than in the developing larvae.

The expression level of both *plzf* was also assessed from whole zebrafish larvae infected by the Chikungunya virus (ChikV), as this arbovirus induces a very high type I IFN response (Briolat et al., 2014; Palha et al., 2013). Fig. 3a–b showed that at this stage *plzfa* is expressed at a higher level than *plzfb*. Importantly, the strong IFN response induced by a ChikV infection, which is illustrated by the kinetics of *isg12.1* expression after infection (Fig. 3c), does not lead to a significant modulation of the expression of *plzfa* and *b*. Thus, in contrast to *isg12.1*, *plzf* genes are not interferon inducible, similar in this respect to their mammalian counterpart (Xu et al., 2008).

3.3. Zebrafish plzfa or plzfb do not induce ISG expression by themselves, but enhance early type I IFN response in a BTB-POZ domain independent manner

In *plzf* −/− mice, the induction of a subset of ISGs − comprising *oas1g*, *rsad2/viperin* and *ifit2/ifi54* - by type I IFN or viral infection was markedly reduced, while reciprocally, overexpression of PLZF by mammalian cells enhanced the IFN-induced expression of these genes (Xu et al., 2008). To investigate if fish *plzf* genes could play similar roles, we first over-expressed Plzfa or Plzfb in EPC cells (Fig. S1), and measured the expression of *rsad2*, one of the most conserved ISGs between fish and mammals. In absence of further stimulation of the IFN pathway, no change in *rsad2* expression was observed (Fig. 4a). Of note, Plzf overexpression did not lead to *ifnφ1* induction either (data not shown).

We then treated EPC cells over-expressing Plzfa or Plzfb with the viral PAMP mimic poly I:C. As intracellular poly I:C is a very powerful inducer of type I IFN, we set-up a treatment which did not induce a massive IFN induction to have a dynamic range of potential modulation by Plzf. EPC cells were transfected with growing amounts of poly I:C to determine a dose at which the IFN induction was only about ten fold after 6 h incubation. Using these conditions, we observed that Plzfa and Plzfb enhanced the early *ifnφ1* induction by about twofold and threefold, respectively (Fig. 4b). The up-regulation of two typical *isg* - *isg15* and *rsad2* - by poly I:C treatment was also significantly enhanced in EPC cells over-expressing Plzfb (Fig. 4c), indicating that the increased type I IFN response has an impact on the modulation of effector genes as early as 6 h post-stimulation. Thus, zebrafish PLZF proteins amplify the type I IFN response, as does their mammalian counterpart.

As the BTB-POZ domains recruit HDAC in transcriptional regulatory complexes, we also over-expressed a Plzfb deletion mutant comprising the C-terminal Zn-finger domains. As shown in Fig. 4b, this protein was also able to enhance significantly the Poly I:C mediated up-regulation of *ifnφ1*, indicating that the BTB-POZ domain is not required for this effect.

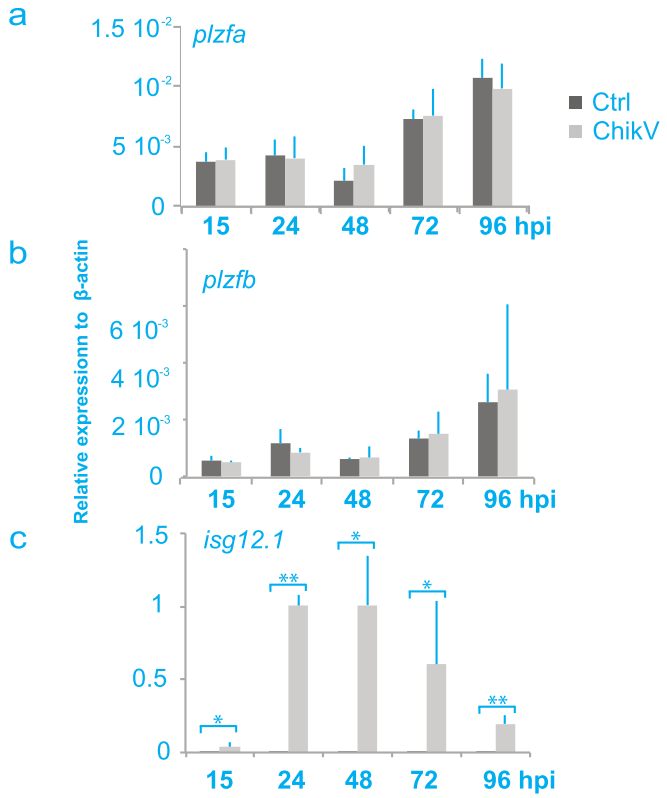

Fig. 3. Plzfa or Plzfb are not induced by type I IFN. Kinetics analysis after ChikV infection of zebrafish larvae: *plzfa (a), plzfb (b) or isg12.1 (c)* transcripts were quantified by QRT-PCR from three pools of three to five larvae infected by ChikV or from control pools. Results were normalized on the basis of *ß-actin* expression (mean ± SD). *: T-test $p<5\%$, **: T-test $p<1\%$.

3.4. Zebrafish PlzfB enhances early type I IFN response upon infection by RNA viruses

To further investigate if zebrafish Plzf enhances type I IFN response during viral infection, EPC cells over-expressing Plzfb were exposed to a warm water birnavirus, the Blotched Snakehead Virus (BSNV) (Da Costa et al., 2003). At 6 h post exposure, BSNV causes a strong induction of *ifnφ1* in non transfected EPC cells, which is significantly enhanced in cells overexpressing Plzfb (Fig. 5). Interestingly, Plzfb expression triggers IFN upregulation upon GSV exposure, another non-enveloped virus. To get insight into the effect of Plzfb on other viruses, similar experiments were performed with SVCV, an enveloped rhabdovirus typically produced at high titers by EPC cells. Cells exposure to SVCV inoculum leads to high levels of expression the nucleoprotein gene of SVCV 6 h post infection as determined by rtqpcr analyses (0.9 and 25 times the level of β-actin, respectively). As the *ifnφ1* transcript was hardly detectable in the mock infected cells, it was not possible to measure a precise fold change, but the induction rate of *ifnφ1* by SVCV infection was clearly more than 20 times at 6 h post infection. Overexpression of Plzfb did not significantly enhance this induction level (Table 2). Thus, the effect of PlzfB might be restricted to non enveloped viruses as infection with the enveloped rhabdovirus SVCV in similar conditions does not lead to any increase of the IFN response (Table 2).

The enhancement of the type I IFN induction by Plzfb was transient; at later time points (10 or 24hpi), the overall expression of *ifnφ1* was higher than at 6hpi, but not different between ctrl and Plzfb-expressing EPC cells (data not shown).

4. Discussion

In this work, we characterized the fish orthologues of the transcription factor PLZF, which has been recently identified as a key player in mouse type I IFN signalling (Xu et al., 2008) and TLR

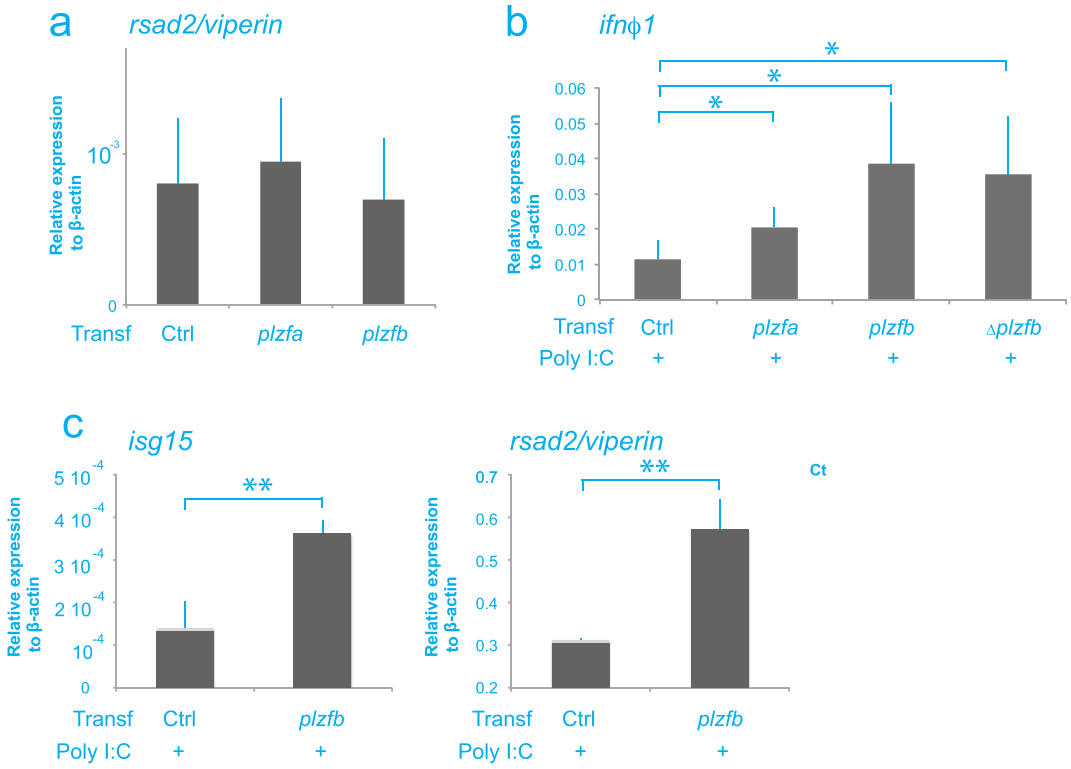

Fig. 4. *plzfa* and *plzfb* do not induce *rsad2*/*viperin* but enhance the early type I IFN response. EPC cells were transfected with expression plasmid for Plzfa, Plzfb, or a deletion mutant of Plzfb lacking the BTB-POZ domain (ΔPlzfb). Cells transfected with empty plasmid were used as control (Ctrl). Relevant transcripts were quantified by RT-QPCR, and results were normalized on the basis of *ß-actin* expression (mean ± SD). (a) Plzf over-expression does not induce the ISG *rsad2/viperin* (b) *ifnφ1* up-regulation upon poly I:C treatment is significantly enhanced in *plzfa-*, *plzfb- or* Δ*plzfb*-transfected cells. (c) *isg15* and *rsad2/viperin* up-regulation upon poly I:C treatment is significantly enhanced in cells overexpressing Plzfb. **: T-test $p<1\%$.

responses to bacteria (Sadler et al., 2015). Teleost fish have typically two paralogous *plzf* genes, likely due to the "3R" whole genome duplication that occurred in the early evolution of this lineage. We show here that both zebrafish Plzf paralogues increase the early antiviral response, as does mammalian PLZF although via mechanisms that apparently differ.

In PLZF-deficient mice, the induction of a subset of ISG was impaired during viral infections, leading to increased susceptibility to Semliki Forest Virus (Xu et al., 2008). No significant difference was observed regarding the level of IFN between wild type and PLZF-deficient mice. In fish epithelial cells, we did not observe a direct induction of ISGs by Plzf overexpression, but did observe an increase of ISG expression induced by polyI:C. A significant difference, however, was that type I IFN expression itself was also increased by zebrafish Plzfs, be it after polyI:C stimulation infection with two non-enveloped viruses.

This effect was observed during the early phase of IFN induction a few hours after stimulation, and had no impact on the final level of IFN expression later. However, this transient effect can have a significant impact on the outcome of a viral infection, as the success of the IFN mediated innate response often greatly depends on the early phase. This is well illustrated in fish by the contrasted susceptibility of rainbow trout lines to viral infection: while some resistant lines are able to mount strong early IFN responses and master the infection, susceptible lines develop a very high - but slower - IFN response that follows the spreading of the virus and is unable to stop it (Verrier et al., 2012).

In the mouse, PLZF associates with promyelocytic leukemia protein (PML) and histone deacetylase 1 (HDAC1) in the presence of IFN. This type of association of ZBTB with HDAC molecules is typically mediated by the transcription factor BTB-POZ domain (Melnick et al., 2002). PLZF also binds promoters of PLZF-regulated ISG at sites that are located close to ISRE, thus modulating the activation of ISG transcription (Xu et al., 2008). Interestingly, the

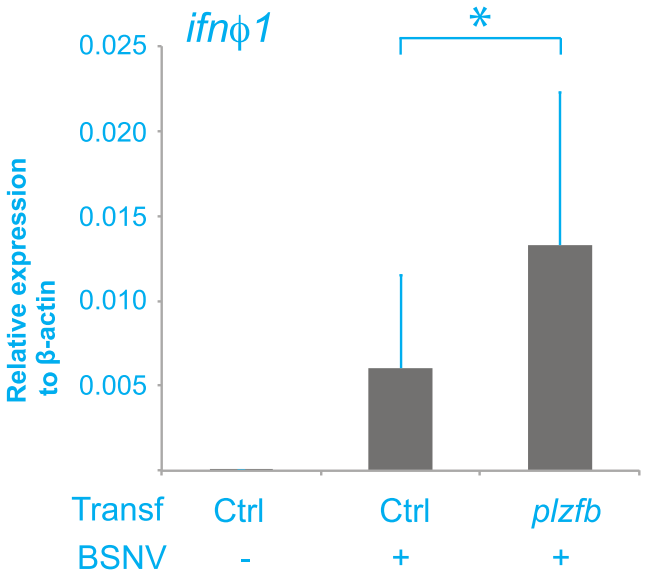

Fig. 5. Plzfb enhances ifnϕ1 response to BSNV infection. EPC cells were transfected with expression plasmid for Plzfb, and cells transfected with empty plasmid were used as control (Ctrl). *ifnφ1* up-regulation upon BSNV treatment is significantly enhanced in cells over-expressing Plzfb. *: T-test $p<5\%$.

Table 2
Enhancement of the early Ifnφ1 response by Plzfb.

Virus	Type	Relative increase of IFNφ1 induction in *plzf-b* transfected cells
BSNV (birnavirus)	Non Enveloped, bi segmented dsRNA	6.5 ± 3.4 (n = 4)
GSV (reovirus)	Non Enveloped, 11 segments dsRNA	6.3 ± 2.3 (n = 3)
SVCV (rhabdovirus)	Enveloped, ss RNA-	0.66 ± 0.11 (n = 2)

modulation of *ifnφ1* by zebrafish Plzf does not require the BTB-POZ domain, as the deletion mutant also increases IFN induction while it does not show the granular nuclear pattern observed for the complete molecule; these observations suggest that it may not involve the same type of mechanism as described in mammals. Alternatively, HDAC may be recruited at a sufficient level by a PLZF dimer consisting of the truncated Plzf and an endogenous, complete PLZF molecule (Ahmad et al., 1998). Of note, when a dominant negative mutant of IRF3 (IRF3DN) consisting of the C-terminal domain of the protein was co-expressed with Plzfb (data not shown), *ifnφ1* induction by poly I:C was significantly inhibited (more than 10 times, $p < 0.01$), suggesting that Plzf could modulate transcription at the *ifnφ1* promoter upstream from IRF3. Additional possible difference between mammalian and our findings could be that zebrafish Plzf may affect IFN pathway in response to non-enveloped double stranded RNA viruses, whereas mammalian PLZF affected responses to both enveloped and non-enveloped ssRNA viruses, suggesting that the transcription factor could be activated downstream from different PRR.

While mammals have a unique *plzf* gene, fish typically have two paralogues located in different regions corresponding to conserved synteny groups. As both Plzfa and Plzfb can increase IFN induction upon poly I:C treatment, and show divergent expression patterns in the zebrafish larvae, *plzfa* and *plzfb* may have acquired specialized, tissue-specific properties. These features and the general impact of Plzf proteins on the susceptibility to viral infections would have to be addressed by further loss-of-function experiments in zebrafish. Additionally, it would also be of interest to investigate if PLZF modulates the early induction of type I IFN in human and mice, as this effect might have been missed in previous studies focused on the PLZF impact on ISG induction.

In this study, we identified a new target for the Plzf transcription factor, showing that it can modulate the level of the early *ifnφ1* response at the transcript level This observation supports an early connection between *plzf* genes and type I interferon signaling, Our findings strengthen the role of PLZF in vertebrate antiviral innate immunity, and pave the way for further investigations on the role of this transcription factor in innate antiviral defense.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.dci.2015.12.016.

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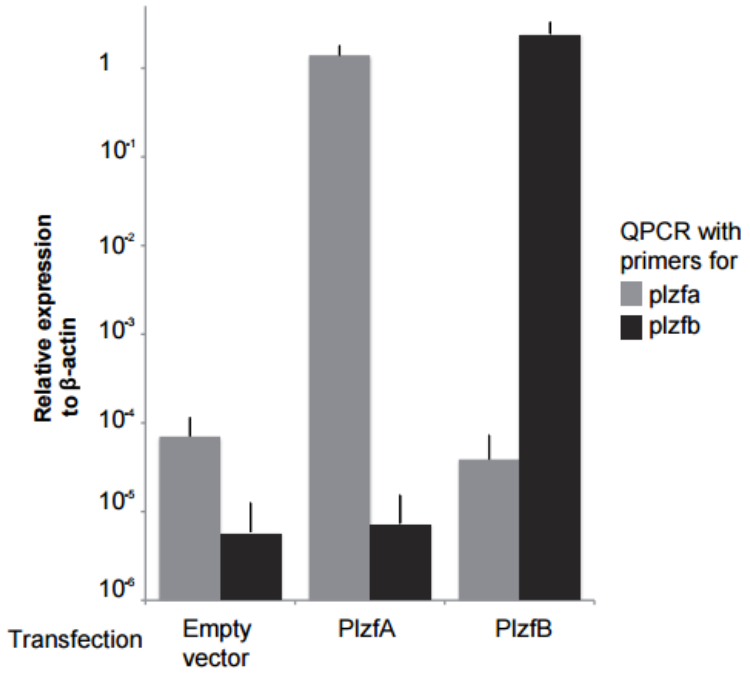

Figure S1. **Level of expression of plzfa and plzfb transcripts in EPC tranfected cells**

Zebrafish *plzfa and plzfb* transcripts were quantified by RTQPCR from EPC cells after transfection with expression plasmids, and results were normalized on the basis of *ß-actin* expression (mean +- SD).

CHAPTER VI

Steady state tissue-specific antiviral immunity: a finTRIM inducing constitutive IFN signaling is expressed at surfaces exposed to pathogens

Constitutive IFN induction by ftr83 in exposed surfaces of fish

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Abstract

The response to invading pathogens is controlled by the tissue environment. This regionalization determines the local capacity to face infection while avoiding deleterious side effects, and is a major aspect of immunity. The tripartite-motif-protein (TRIM) family includes many key components of our antiviral arsenal that are widely expressed in mammals; however, the contribution of TRIMs to local tissue-specific defense mechanisms remains poorly defined. In mammals, antiviral TRIMs mediate intrinsic viral restriction at diverse points of the viral cycle, or positively regulate innate immune signaling pathways. We show here that *ftr83*, a zebrafish *trim* gene constitutively expressed in the gills, skin and pharynx, encodes a protein that strongly up-regulates the type I interferon (IFN) pathway. While *ftr83* is not IFN-inducible, its *in vivo* expression in gills of healthy fish correlates with that of type I IFN, consistent with local induction of basal IFN expression by Ftr83. *In vitro*, overexpression of Ftr83, but not of its close relative Ftr82, induced IFN and IFN-stimulated gene expression, and affords protection against different enveloped and non-enveloped RNA viruses. This antiviral activity is IFN-dependent, and is abolished by a dominant negative IRF3 mutant. Our work indicates that TRIM proteins contribute to the establishment of antiviral immunity by permanent type I IFN stimulation. Hence TRIMs might create a local anti-viral environment at sites exposed to pathogens, a mechanism participating to the regionalization of immunity. Our data also demonstrate that TRIMs were involved in antiviral immunity before the divergence between bony fish and tetrapods, early in vertebrate evolution.

Introduction

Upon pathogen invasion, host immune response begins with pattern recognition receptor (PRR) activation by microbial products, leading to synthesis of inflammatory cytokines, which subsequently induce anti-microbial signaling and leukocyte activation. Immune response is strongly dependent of the tissue environment in term of specialized immune cells, local cytokine production and even microbiota for skin and mucosae. The integrity of tissues during the reaction is also critical, which influences the choice of effector mechanisms, as side effects of a

wrong defense response may be deleterious (1). Highly sensitive sites such as eye, brain and testis are protected from dangerous side-effects of inflammation and immune responses; the properties of such "immune privileged" regions, for example their capacity to tolerate heterologous grafts, were discovered a long time ago (2, 3) and showed that immune responses are compartmentalized and differ between tissues. As mucosae and skin are particularly exposed to invading pathogens, their local immunity should induce effective responses while maintaining tissue integrity and homeostasis.

Although most cell types are able to up-regulate type I interferon (IFN) upon viral infection, the amplitude and kinetics of the innate antiviral response and the repertoire of IFN stimulated genes (ISGs) are inconstant across different sites. The antiviral immunity within the nervous system illustrates the variability of the IFN response between tissues, and its importance. An elevated homeostatic type I IFN was found in neurons, higher than the basal expression in mouse fibroblasts. This was critical and sufficient for early control of viral infection, but many ISGs had a low basal expression in neurons suggesting unique IFN signaling (4). The frontiers between the organism and its environment also harbor particular responses to viruses, which are critical for the resistance to the infection. Thus, in lung epithelial cells, DDX58 (also known as RIG-I) activates IL-1β secretion upon influenza virus infection through type I IFN dependent or independent mechanisms that differ from those in macrophages (5). The situation can be further complexified by immunoregulatory functions of type I IFN itself, that can play anti-inflammatory role as for example in the gut, in addition to its antiviral effect (6). In addition, different cell types of the gut respond very differently to type I IFNs (7). Overall, the mechanisms of the regional variation of IFN responses remain poorly understood.

As a major protein superfamily involved in the regulation of type I IFN response and antiviral defence, TRIMs (tripartite motif) are likely to play important roles in this local regulation. These proteins are characterized by the presence of a RING/B BOX/Coiled coil tripartite motif, which can be followed by diverse C terminal extensions (8, 9). They are present across metazoans and highly diversified in

vertebrates; 75 TRIM genes are known in humans, and more than 200 in zebrafish (9-11). Recent studies revealed the great magnitude of the modulation of antiviral innate immunity by TRIMs (12, 13). Sixteen among 43 human TRIMs selected by Uchill et al. were able to modulate NFκB and/or AP1, and to restrict MLV infection (14). In another approach, Versteeg et al. evaluated the effect of 75 human TRIMs on IFN□, ISRE and NFκB luciferase reporter genes upon stimulation. Half of the TRIMs showed immunomodulary activities, and mainly appeared to be enhancers of innate immunity rather than direct sensors or effectors (13). In fact, TRIMs are involved in multiple antiviral mechanisms (for review (15, 16)). TRIM5α and TRIM22 directly interact with viral capsids, interfering with normal uncapsidation and activating immune signaling (17, 18) or blocking particle assembly (19). TRIM21 (Ro52), another TRIM with B30.2 domain, plays a role of immunoglobulin receptor and binds internalized antibody-coated viruses, which are then targeted to proteasome (20). In contrast, other TRIMs interact with innate immune factors and modulate antiviral pathways (15, 21). TRIM25 and TRIM4 activate DDX58-mediated IFN signaling pathway through polyubiquitination (22, 23). TRIM13 interacts with both IFIH1 and DDX58, down-regulating negatively the IFIH1 mediated response and increasing the DDX58 mediated IFN induction (24). Overall, the seminal work by Veersteg et al. reveals that TRIMs' action is often related to synthesis of antiviral cytokines and linked to the E3 ligase activity as shown by RING deletion mutants (13), identifying TRIMs as major PRR-triggered positive regulators of the IFN pathway.

These diverse functions strongly suggest that TRIMs could also be involved in natural barriers/resistance against viral infections. In fact, TRIM5α, which are potent restriction factors against specific retroviruses in different primate species, have been presented as interspecific intrinsic blocks rather than typical inducible antiviral factors of innate immunity. Study of mammalian TRIM expression patterns reveal a important heterogeneity of spatial distribution of individual TRIMs determined by RNA in situ hybridization on adult and embryonic mouse tissues ((25), and http://trim.tigem.it/). Also, TRIM9, a brain specific TRIM has recently been

described as a negative regulator of NFκB pro inflammatory signaling pathway activation in various cell lines and primary culture of rat neuronal cells (26). Altogether, these patterns are in favour of an implication of TRIMs in local tissue-specific regulation of innate immunity.

Fish constitute good models to study local mechanisms of the natural (antiviral) immunity in skin or gills, as an aquatic environment particularly rich in pathogens is in contact with the whole surface of the organism. As in other vertebrates, type I interferons (IFNs) are the master cytokines of fish antiviral defences (27). Upon infection, viral motifs are rapidly recognized by membrane or cytoplasmic sensors of the host cell, such as TLRs and RLRs, triggering activation of well-conserved signaling pathways. Subsequent translocation of IRF and NFκB transcription factors to the nucleus induce the expression of cytokines including type I interferons (IFNs) and a large number of interferon-stimulated genes (ISGs), some of which mediate specific antiviral mechanisms (28, 29). Fish also possess a large repertoire of TRIMs, with several large specific gene expansions including finTrims, bloodthirsty-related TRIM and TRIM35. FinTrims were initially discovered in salmonids as ISGs (30), and it was proposed they may have antiviral functions. In the zebrafish, *fintrim* (31) – *ftrs* - constitute a subset of about 80 functional genes over the 200 trim genes described in the whole genome. They are typically expressed at very low basal levels, and many are weakly induced by the viral infection. Interestingly, two members of the family (*ftr82* and *ftr83*) appeared to be expressed at a much higher constitutive level in zebrafish larvae, but were not induced by viral infection or IFN treatment, suggesting they might play a role in natural intrinsic immunity.

In this work, we show that *ftr83* is constitutively expressed in exposed sites such as gills and skin, and plays a role as an Ifn inducer. While it is not itself induced by Ifn, Ftr83 significantly increases basal Ifn expression and modulates expression of ISGs, mediating a potent antiviral activity against RNA viruses. In contrast, Ftr82 - another Ftr closely related to Ftr83 but with distinct expression pattern, does not possess such properties. Chimeras between Ftr82 and Ftr83 showed that both Ftr83 RBCC and B30.2 are required for antiviral functions. Our data show that positive regulation of

the IFN pathway and antiviral functions are a fundamental property of TRIMs across vertebrate immune systems, and support a new role of these proteins in intrinsic defence through local and permanent stimulation of the IFN pathway at steady state in sites highly exposed to pathogens.

Materials and methods

Ethics statement.

All animals were handled in strict accordance with good animal practice as defined by the European Union guidelines for the handling of laboratory animals (http://ec.europa.eu/environment/chemicals/lab_animals/home_en.htm) and by the Regional Paris South Ethics committee. All animal work was approved by the Direction of the Veterinary Services of Versailles (authorization number 78-28) as well as fish facilities (authorization number B78-720). Experimental protocols involving zebrafish were approved by the INRA institutional ethical committee "Comethea" (#12/114).

Primary antibodies

Anti-HA 3F10 monoclonal antibody was purchased from Roche and anti-V5 monoclonal antibody were from Molecular Probes.

Cloning of ftr82 and ftr83

Ftr82 and *ftr83* were also cloned in fusion with an HA tag respectively in pcDNA3.3 and pcDNA3.1 (Table S1). FTR chimeras were obtained by recombinant PCR using V5-ftr82 and ftr83-HA as templates. RBCC domains of ftr82 and ftr83 were respectively amplified with fwFTR82-Attb1/revFTR82-$_{B30.2ftr83}$ and fwFTR83-HA/revFTR83-$_{B30.2ftr82.}$ B30.2 domains of ftr82 and ftr83 were respectively amplified with fwFTR83-$_{B30.2ftr82}$/revHA-Ftr82, and fwFTR82-$_{B30.2ftr83}$/revFTR83B30.2-Attb2-nostop. These PCR products were then annealed and the full constructs were amplified with fwFTR82-Attb1 and revFTR83B30.2-Attb2-nostop for 82-83, and with fwFTR83-HA and revHA-Ftr82 for 83-82. 82-83 was cloned with the Gateway cloning system (Invitrogen) in pDSET 6.2V5 to be expressed with V5 tag fused to the C terminus. 83-82 was cloned with the TOPO TA cloning system (Invitrogen) in

pcDNA3.3 to be expressed with HA tag fused to the N terminus. *Ftr82* and *ftr83* coding region were also amplified using primers HA-ftr83 and ftr82-HAftr82-Attb1, ftr83-Attb1 and ftr82Attb2nostop, ftr83Attb2nostop (Table S1), cloned into the entry vector of the Gateway cloning system (Invitrogen) then transferred to the different destination vectors. Ftr82 was transferred in pDEST6.2-V5 or pDSET 47 to be expressed with V5 or GFP tag fused to the C terminus. Ftr83 was transferred to pDEST53 to be expressed with GFP tag fused to the N terminus. *Ftr83* deletion mutants were obtained using specific primers on ftr83-HA template. Ftr83B30.2 was constructed with Ftr83B30.2-Attb1 and Attb-2-nostop primers while FTR83ΔB30.2 was constructed with FTR83ΔB30.2-Attb1 and Attb2 primers (Table S1). PCR products were then cloned using the Gateway cloning system in pDSET 47 to be expressed with GFP tag fused to the C terminus.

Whole-mount in situ hybridization

Whole mount in situ hybridization were performed as described in (32), using NBT/BCIP revelation (Sigma). Antisense probes for ftr82 (product size 856bp) and ftr83 (product size 865bp) were generated with T3 polymerase (Promega). Templates for in vitro transcription were amplified using primers shown in Table S1, and PCR products were purified using Microspin™ S-400 HR colums (GE Healthcare).

Fish, cells and viruses

Zebrafish were raised in the fish facilities of Institut National de la Recherche Agronomique (Jouy en Josas, France). EPC (*Epithelioma papulosum cyprini*) cell line {ATCC® CRL-2872™} was maintained in Glasgow's modified Eagle's medium-HEPES 25mM medium (Eurobio) supplemented with 10% fetal bovine serum (FBS, Eurobio {produced and distributed in France under the veterinary authorization FR 91 692 200}), 1% tryptose phosphate broth (Eurobio), 2mM L-glutamine (PAA) and antibiotics 100µg/mL Penicilin (Biovalley), 100µg/mL Streptomycin (Biovalley). Transfection experiments, viral production and titration were performed in EPC cells. The novirhabdoviruses Infectious Hematopoietic Necrosis Virus 32-87 (IHNV) and Viral Hemorrhagic Septicemia Virus 07-71 (VHSV) and the vesiculovirus spring viraemia of carp virus (SVCV) were produced at 14°C on EPC in GMEM media

supplemented with 2% fetal bovine serum, 5% tryptose and 2mM L-glutamine. Cytopathic effect was evaluated 72 hours post-infection after cell fixation with 10% formol prior to coloration in 2% cristal violet.

Transfection

EPC cells were nucleotransfected with the nucleofector kit T (Lonza) following the manufacturer's recommendations. Briefly, 4 10^6 EPC cells were plated in P6 wells. The day after, cells were trypsinized, resuspended in 100µL of nucleofector solution with 3-5µg of DNA. After nucleotransfection, cells were resuspended in a P6 well plate for RTQPCR analyses or immunocytochemistry on PDL (10µg/ml) coated glass coverslips. Viral challenge was performed on P24 wells seeded with 1 million of transfected cells 24hrs before viral infections.

In vitro Infections

Ninety-six hours post transfection (hpt), EPC cell monolayers were infected with rhabdoviruses MOI1 by a 1 hour absorption step at 14°C in GMEM 2% FBS. After removal of the inoculum, cells were incubated in GMEM 2% FBS at 14°C for the rest of the experiment. Cell supernatant was taken post absorption and after 8, 24, 48 and 72hrs of infection for virus titration experiments. Infected cells were fixed at 72hpi to evaluate cytopathic effect by cristal violet coloration. Short SVCV infections were performed at 72hpt. Cells were exposed to virus inoculum (MOI1) 6hours at 14°C before analysis of gene expression. EPC cells were also infected with the birnaviruses Infectious Pancreatic Necrosis Virus (IPNV) strains VR299 or 31-75, in GMEM media supplemented with 2% fetal bovine serum, 5% tryptose and 2mM L-glutamine. Cytopathic effect was evaluated 72 hours post-infection after cell fixation with 10% formol prior to coloration in 2% cristal violet. The birnaviruses were propagated on BF cells at 14°C. Viruses were titrated on EPC by plaque assay as previously described in (33).

RNA isolation, cDNA synthesis

Total RNA extraction was performed by TRIZOL (Invitrogen) from 4 million EPC cells at 72hpt or from zebrafish tissues sampled from two-three months old Zebrafish

(strain AB). RNA was purified using the RNeasy mini kit (QIAGEN) according to the manufacturer's instructions and treated with DNAse. Reverse transcription experiment was performed on 1µg of total RNA using 125ng of random hexamer primers (Roche) in a Superscript II Reverse transcriptase kit (Invitrogen) according to the manufacturer's instructions.

Real time Q-PCR

Gene expression was measured by real time PCR with a Realplex² Mastercycler Instrument (Eppendorf) using Power SYBR® Green PCR Mastermix (Applied Biosystems). Each sample is componed by 5µL of primers (300nM each), 5µL of cDNA (diluted 1/10 for cell samples and 1/5 for zebrafish samples) and 10µL of PCR Mastermix. Samples were first incubated for 2 minutes at 50°C and for 10 minutes at 95°C, then subjected to 40 amplification cycles (95°C for 15 and 60°C for 1 minute), followed by 15 seconds at 95°C, 15 seconds at 60°C, 20 minutes from 60°C to 95°C and finally 15 seconds at 95°C, to establish the melting curve of PCR products. Gene expressions were computed according to the ABI Prism 7700 user bulletin (Applied biosystems) and normalized to the beta-actin expression level. All primers QPCR primers used in this study are shown in table I.

Immunocytochemistry

72 hours post transfection, cells were fixed in PBS pH7,4 PFA 4% for 20min at 4°C. After fixation, cells were permeabilized in 0,2% Triton X100 solution for 5 min at RT before saturation in PBS 2% BSA solution at RT for 1hr. Cells were then incubated with anti-HA (Roche) or anti-V5 (invitrogen) monoclonal antibodies in PBS, 2%BSA, 0,1% Triton X100 for 1hour. Primary antibodies binding sites were then revealed by incubation with Alexa coupled secondary antibodies (Molecular Probes) in PBS, 2%BSA, 0,1% Triton X100 and DAPI for 1 hour at RT before mounting in Immuno Mount solution (Molecular Probes). Images were acquired on AxioObserver Z1 microscope (Zeiss) with a 63x Plan Neofluar objective using Photometrics CoolSNAP HQ2 Camera.

Homology Modeling

Structural models of Ftr82 and Ftr83 B30.2 domains were built with Swis-model program (34) using the high resolution structure of mammalian TRIM25 as a template (PDB 4B8E). Chimera program was used for structure viewing and ftr B30.2 superposition (35).

Results

ftr82 and ftr83 are archetypal members of the large fish multigenic ftr family

The *fintrim* family extensively diversified in parallel in each fish lineage (11). Among the 80 genes found in the zebrafish, transcriptome studies ((36), and unpublished data) showed that *ftr82* and *ftr83* were constitutively well-expressed in the larva, but not induced by Ifn or viral infection. These two genes appeared to be those with true orthologues in other fish species. Phylogenetic analysis of Ftrs from different fish shows that both Ftr82 and Ftr83 sequences cluster in a well supported branch, as illustrated in Figure 1a for zebrafish and pufferfish. In contrast, other Ftr clusters comprise sequences from only one species and correspond to more recent, lineage-specific diversification. Within the conserved Ftr82/83 group, Ftr82 and Ftr83 each define a set of orthologs, indicating that *ftr82* and *ftr83* are representatives of ancestral genes, and were already present before the divergence of modern groups of fishes. *ftr82* and *ftr83* are part of a synteny group conserved between cypriniforms (zebrafish), percomorphs (medaka, stickleback, platyfish, pufferfish), and gadiforms (cod), comforting the idea they resulted from a local duplication of a common ancestor that occurred prior to teleost radiation (Figure 1b). *ftr82* and *ftr83* promoters have different potential binding sites of transcription factors (Figure 1c).

Ftr82 and Ftr83 have the typical domain structure of finTrims, comprising a RING/B-Box/Coiled coil tripartite motif and a typical B30.2 domain (Figure 1d). Zebrafish Ftr82 and Ftr83 protein sequences are 55% similar to each other (Figure 1e), but only 35 to 45 % similar to other zebrafish Ftrs. Hypervariable loops of the B30.2 domains are not highly divergent between Ftr82 and Ftr83 (Figure 1e and f), in contrast to what is observed for the whole Ftr group (31).

These observations indicate that *ftr82* and *ftr83* are "ancient" *ftrs* with conserved structure and genomic context across fishes, and may have a generic function different from the main set of *ftr* diversified during fish evolution. We therefore hypothesized that these widely conserved factors might be involved the natural antiviral immunity.

ftr83 expression pattern supports its implication in local intrinsic defence at pathogen-exposed sites

To determine the spatial pattern of expression of *ftr82* and *ftr83* in zebrafish larvae, we used whole-mount *in situ* hybridization. Figure 2a shows that *ftr82* and *ftr83* have distinct tissue distributions at 3.5 days post fertilization: while *ftr82* has a relatively wide range of expression with higher levels in the gut, *ftr83* is more restricted to the pronephric duct and pharyngeal area, notably gill arches. This pattern persists in the young adult, as shown by real time QPCR data from isolated organs of 3-month zebrafish (Figure 2b). At this stage, *ftr83* expression is mainly observed in gills, skin and pharynx; it can also be detected in hematopoietic tissues (spleen and kidney) although at a much lower level. In contrast, *ftr82* is well expressed in many tissues including gills, gut and liver and to a lesser extent in skin, pharynx, brain, spleen, kidney and heart. Altogether, these results indicate that *ftr83* is mainly expressed at exposed surfaces of the fish.

This pattern of expression suggests that a function of *ftr83 in vivo* would be to induce natural antiviral immunity in tissues directly exposed to the water, which could be achieved through constitutive type I Ifn expression. To test this hypothesis, and taking advantage of the inter-individual variation of expression previously observed for *ftr83* in gills (Figure 2b), we measured the expression of *ftr83* and of *ifnφ1* in gills of ten healthy adult individual zebrafish (Figure 2c). Interestingly, a fair correlation between the expression of the two genes was observed. As ftr83 is not induced by Ifn (Figure S1), this correlation supported the notion that Ftr83 might drive constitutive type I Ifn expression in the tissues most exposed to water-borne viruses in order to protect them from infection.

Ftr83 promotes the expression of key components of the Ifn pathway

To dissect the biological effect of Ftr83 (and Ftr82) overexpression on the Ifn pathway, we then used a cellular model previously developped in (33). We studied genes involved at different levels of the IFN/PRR signaling pathway including the molecular sensor *ddx58* (also known as *rig-I*); several kinases (*tbk1, ralbp1* also known as *rip1*, and *jak1*); key transcription factors as interferon regulatory factors (*irf3, 7* and *9*; Signal Transducers and Activators of Transcription (*stat*) *1a, 1b* and *2*); type I interferon *(ifnϕ1)* and its receptor *crfb5*, as well as two ISGs: *isg15* and *rsad2*. TLR signaling was also investigated through adaptor molecules (*Myd88* and *ticam1)*. We selected these genes based on the known structure of the IFN pathway, and on their induction rate in transcriptomic studies of virus- and IFN- induced responses in fish (and mammalian) models.

The expression of these genes was measured by real time QPCR from EPC fish cells over-expressing HA-tagged Ftr82 or 83 proteins seventy-two hours post transfection. Strikingly, *ddx58, irf7, ifnϕ1, stat1b,* and the ISGs *rsad2* and *isg15* were significantly upregulated upon expression of Ftr83 in absence of additional stimulation (Figure 3a; Figure S2a and b). While transfection of either Ftr82 or Ftr83-encoding plasmids led to similar expression levels (see Figure 4a), over expression of Ftr82 did not lead to any modulation of the selected markers. Interestingly, Ftr82 and Ftr83 presented distinct expression patterns in transfected cells as shown in Figure 3b. While Ftr82 proteins appeared as cytoplasmic inclusions heterogeneous in size and number, Ftr83 was much more diffuse in the cytoplasm. Overall, these observations were consistent with contrasted functions of *ftr82* and *ftr83*.

Altogether, these results indicate that Ftr83 is a potent inducer of DDX58-mediated Ifnϕ1 production. The impact of Ftr83 expression on the Ifn signaling pathway is summarized in Figure 3c.

Ftr83 affords protection against RNA viruses

To investigate the modulation of Ifn pathway by Ftr82 or Ftr83 in the context of an infection, EPC cells overexpressing finTrims were infected with the vesiculovirus

SVCV, and the expression of SVCV N transcript and components of the Ifn pathway was measured 6 hpi (hours post infection) (Figure 4a). Strikingly, the expression of the viral N was significantly reduced in cells expressing Ftr83 compared to cells expressing Ftr82 or to control cells, suggesting that Ftr83 may have an antiviral effect. Most importantly, while viral infection induces a strong Ifn response, the overexpression of Ftr83 did not lead to a significant increase of the expression level of *ifnφ1*, *rsad2* and *ddx58* mRNAs in infected cells at 6hpi, indicating that it promotes expression of Ifn and ISG independently of infection, and does not significantly enhance the rate of induction of interferon and antiviral proteins upon SVCV infection.

We then characterized the effect of the expression of Ftr82 and Ftr83 on the course of SVCV infection, and determined their impact on the cell sensitivity to several other RNA viruses including the novirhabdoviruses IHNV and VHSV.

Seventy-two hours post transfection, cells were subjected to distinct viral exposure (MOI1) and antiviral activity was evaluated by titration experiments from 0 to 96 hours post-infection. Figure 4b shows the effect of expression of the two Ftrs on growth kinetics of SVCV, IHNV and VSHV. Ftr83 over-expression strongly inhibited viral growth for both IHNV and VHSV as viral titers were reduced about 3000 fold upon expression of Ftr83 compared to Ftr82 or mock transfected cells at 72hpi. The inhibition of SVCV was less efficient, but still highly significant with a 15 fold difference of virus titers between Ftr83 and other conditions over the same period.

Accordingly, overexpression of Ftr83 prevented viral induced cytotoxicity and efficiently preserved the integrity of the cell monolayer after infection with IHNV, VHSV or SVCV, as demonstrated by crystal violet colorations (Figure 4c). In contrast, cytopathic effect of viral infections led to the complete destruction of the cell monolayer at 72hpi upon Ftr82 expression or in mock transfected conditions (Figure 4c). These observations were extended to non-enveloped viruses, as Ftr83 overexpression fully protected the cell monolayer against two strains of the birnavirus IPNV (Figure S3a).

To exclude that these phenotypes could be due to the HA tag, we constructed additional fusion proteins replacing this tag by V5 or GFP for Ftr82, and by GFP for Ftr83. While GFP-Ftr83 affords robust protection from IHNV and VHSV, none of the Ftr82 constructs showed a significant antiviral effect (Figure S3b and c).

Altogether, these results show that Ftr83 - but not Ftr82 - confers a potent resistance against several enveloped or non enveloped RNA viruses.

Antiviral effect of Ftr83 against RNA viruses relies on induction of type I interferon

To connect Ftr83 antiviral effect to its impact on the Ifn pathway, we then measured the up-regulation of type I Ifn expression in *ftr83*-transfected cells and monitored the kinetics of establishment of the antiviral state.

Ftr83 transfected cells were infected with IHNV (MOI1) at 24, 48 and 72 hpt (hours post transfection) (Figure 5a). As demonstrated by the viral titer reduction measured 72 hpi, IHNV restriction was gradually established after transfection. While no significant effect was detected at 24 hours post transfection, a 2-log decrease in virus titer was observed 48 hours post transfection, then a 3-log reduction 72 hours post transfection. Accordingly, evaluation of viral induced cytopathic effect over the same period showed a mild protection 48 hpt compared to control, while a full protection was observed 72 hpt (data not shown). In parallel, RTQPCR analyses showed increasing level of *ifnφ1* gene expression from 24 hpt to 72 hpt, while *ftr83* level remained stable over this period (Figure 5b). Altogether, our data reveal a very good correlation between type I Ifn expression and protection against IHNV infection.

To further investigate the importance of IFN pathway activation for Ftr83-dependent antiviral activity, a dominant negative mutant of Irf3 (Irf3DN) consisting of the C-terminal domain of the protein was co-expressed with Ftr83 in EPC cells. As Irf3 is a central mediator of type I IFN induction, it was a good candidate to test at which level Ftr83 activated the pathway. Of note, *irf3* itself is modestly but significantly upregulated in cells overexpressing Ftr83 (Figure 2b), thus enhancing its

effects on *ifn* induction. The induction of *ifnφ1*, *ddx58* and *rsad2* previously observed upon Ftr83 expression were abolished in cells overexpressing both Ftr83 and IRF3DN (Figure 6a), indicating that Ftr83 triggering of the IFN pathway occurs upstream of Irf3. Strikingly, the protection of the cell monolayer (Figure 6b) was abolished by the expression of Irf3DN, confirming that Ftr83 antiviral mechanisms mainly depend on Irf3 signaling.

RING and B30.2 domains are required for the FTR83 antiviral effect

To identify the domains required for the antiviral effect, we constructed chimeric proteins in which the B30.2 domain had been exchanged between Ftr83 and Ftr82 as represented in Figure 7a. Expression of both chimeras was investigated by RTQPCR (Figure S4a) and immunocytochemistry (Figure 7b). Similar expression was measured at the mRNA level for both chimeras, but distinct intracellular expression patterns were observed, as for wild-type Ftr82 and Ftr83: $Ftr83_{B30.2(82)}$ shows a diffuse pattern with discrete cytoplasmic inclusions as for Ftr83, while $Ftr82_{B30.2(83)}$ forms large cytoplasmic aggregates as previously visualized upon Ftr82 overexpression (Figure 7b).

EPC cells over-expressing chimeras did not show up-regulation of *ifnϕ1*, *rsad2 or ddx58* genes in comparison with mock transfected cells, indicating that both RBCC and B30.2 domain from Ftr83 are required for the modulation of the Ifn pathway (Figure 7c). Accordingly, no decrease of the IHNV and VHSV cytopathic effects could be observed in cells expressing these chimeras in contrast to Ftr83 (Figure 7d), indicating that both RBCC and B30.2 domains of Ftr83 are required for the antiviral function. This observation was extended to SVCV infected cells, in which the Ifn modulation triggered by the virus was similar to the one observed in control cells. No impact of chimeras was detected on the expression of the viral *N* transcript, which was consistent with a lack of antiviral activity (Figure S4a).

We further designed Ftr83 deletion mutants restricted to the B30.2 domain or lacking this domain. Overexpression of these Ftr83 mutants in EPC cells was not sufficient to reduce significantly IHNV or VHSV cytopathic effect (Figure S4b and

c), supporting the synergistic role of N and C-term part of the Ftr83 protein in the antiviral phenotype.

Altogether, these data indicated that both RBCC and B30.2 of Ftr83 are required for innate immunity modulatory effects and antiviral activity, and that neither RBCC nor B30.2 can be substituted by corresponding domains of Ftr82.

Discussion

Over the last decades, high-throughput screening has been driven to identify interferon-stimulated genes, which are the effectors of the innate antiviral immunity and might be exploited for the development of new anti-viral therapeutics. Many TRIM proteins have been identified as ISGs, and this family emerged as one of the key ISGs subset involved in anti(retro)viral defense. Antiviral TRIMs have been involved in different types of mechanisms, and recent large-scale studies revealed that TRIMs are frequently modulators rather than effectors of antiviral immunity (12, 13).

While TRIM genes are present across metazoans, whether their implication in antiviral mechanisms is a primordial feature of the family has remained an open and important question regarding their species-specific mechanisms of inhibition. In fishes, the large TRIM subset named "FinTrim" was suspected to play a role in antiviral immunity; these genes were discovered as virus- and IFN- induced genes and constituted a large and diverse group with strong signatures of positive selection (31). Within finTrims, a small set of genes has particular features suggesting they could be involved in regulatory functions rather than being direct effectors coevolving with viruses. These genes, that include *ftr83* and *ftr82* in zebrafish, are conserved across teleosts in contrast to the other *fintrim* genes, which apparently diversified independently in each fish lineage. In fact, they are at a "basal" position in the phylogenetic tree, and likely are representative of the primordial finTrims (31).

Our data show that Ftr83 mediates a strong antiviral activity against different RNA viruses, including enveloped and non-enveloped viruses, which indicates that the implication of TRIMs in antiviral defence is an ancestral function of this protein

family. Ftr83 triggers the Ifn signaling pathway, as transiently Ftr83-transfected cells showed up-regulation of type I *ifn* itself, as well as *ddx58, irf7, irf3,* and *stat1b*. It also leads to the up-regulation of two other ISGs, *rsad2* and *isg15*, which mediate antiviral mechanisms in fish and mammals (37, 38). Transfection of a truncated Irf3 composed of the C terminal domain only - acting as a dominant negative mutant -, was sufficient to inhibit both the Ftr83-mediated antiviral activity and induction of the Ifn pathway. Hence, Ftr83 antiviral activity mainly relies on the induction of the Ifn pathway upstream of Irf3. Altogether, these data show that immunomodulatory properties of TRIMs, a fundamental function of the family in mammals, are also found in fish and likely represent a primordial feature of vertebrate TRIMs.

The diversity of TRIM mechanisms and their specialization against different types of viruses suggested they might have site- or tissue-specific expression, and participate to the regionalization of immunity. Transcriptomic analyses and RTQPCR experiments showed that *ftr83* expression is not induced upon viral infection or Ifn treatment of zebrafish larvae ((36) and Figure S1a). *ftr83* has a restricted pattern of expression to gills, skin, pharynx, and to a much lesser extent hematopoietic tissues, as shown by ISH in the larva and by QPCR on dissected tissues in the adult. However, *ftr83* is expressed at a higher level than other finTrims that are generally almost undetectable in non-infected animals ((36) and unpublished data). Additionally, its expression level in gills is correlated to the Ifnφ1 expression in healthy individuals. Altogether, our data suggest that *ftr83* might be a constitutive finTrim and affords a selective, tissue-specific constant activation of the Ifn pathway in areas particularly exposed to pathogens. Such a system would lead to an increased basal level of expression of key genes of the pathway, hence allowing an overshooting response that reaches quickly an effective threshold of antiviral effectors at the critical sites of infection such as gills and skin. In keeping with this, Ftr83 expression does *not* lead to a higher final level of Ifn expression *after infection*, although it induces a significant decrease of the level of viral transcripts from 6 hours post infection extended to an inhibition of viral growth as determined by plaque assay up to 72hpi. Thus, we found a TRIM protein mediated effect on innate immunity via

the increase of the intrinsic expression level of a master cytokine in selected sites/tissues.

The closest relative of *ftr83* in the zebrafish genome is *ftr82*, but its function – which remains to be understood – is clearly very different. In contrast to the strong effect of *ftr83*, *ftr82* overexpression was not sufficient to afford significant protection of transfected cells against any of the RNA viruses we tested. Our data show that Ftr82 does not affect RLR or TLR signaling pathways. It failed to induce any detectable Ifn up-regulation either upon basal conditions (Ftr82 overexpression) or after short exposure of transfected cells to the RNA vesiculovirus SVCV. Additionally, the modulation of the Ifn pathway by Ftr83 was not affected by the co-expression with Ftr82 (data not shown), indicating that Ftr82 does not function as a direct regulator of Ftr83 as sometimes observed for close paralogues (39), at least not in EPC cells in the context of our experiments. This is remarkable given the high similarity of Ftr82 and Ftr83 sequences. *ftr82* is not mainly expressed in the tissues where we found high levels of f*tr83* but showed a more widely distribution in the embryo as well as in the adult. Even at the subcellular levels, Ftr82 and Ftr83 do not seem to share a common expression pattern. In transfected EPC cells, Ftr82 proteins accumulated in cytoplasmic inclusions while overexpression of Ftr83 in similar conditions gave a diffuse pattern in the cytosol. These phenotypes likely reflect interactions with different cellular partners, and are consistent with parallel sub-functionalization of those genes and with the differences observed in the non-structured loops of their B30.2, constituting the interface of interactions with cellular or viral partners.

As *ftr82* and *ftr83* are closely related paralogues, their contrasted functional properties constituted a perfect system to investigate which domain(s) was responsible for the antiviral activity of Ftr83 by exchanging the domains and making chimeric proteins. This approach has been previously used to demonstrate the important role of TRIM5**α** B30.2 domain to mediate antiretroviral activity (40). None of the Ftr chimeras in which B30.2 domains had been swapped between Ftr82 and Ftr83 did afford protection against VHSV or IHNV. This observation is directly

correlated with the absence of modulation of Ifn signaling pathway triggered by the $Ftr83_{B30.2(82)}$ and $Ftr82_{B30.2(83)}$ chimeras evaluated by RTQPCR. Altogether, our data indicate that Ftr83 antiviral mechanism required both RING and B30.2 domains as previously reported for several mammalian TRIMs (12, 13, 17, 41, 42). While RING domain supports E3 ubiquitin ligase, ISGylation or SUMOylation activity and determines the specificity of the E2 conjugase (43), the selection of target proteins generally occurs through the C-terminal domain (10). This scheme is also consistent with the E3 ubiquitin ligase activity of finTrim we showed previously (44).

As they constitute a paramount system that regulates the antiviral response, TRIM represent a potential resource for therapeutical developments based on the manipulation of the innate immune responses (45). Interestingly, several examples illustrate the efficiency of cross species TRIM-mediated immunity. Gene therapies using human/rhesus TRIM5α chimera and TRIMCyp are actually tested in preclinical applications to block HIV infections. Importantly, the absence of viral anti-TRIM5 mechanisms may confer to such approaches a greater robustness compared to other anti-HIV treatments (45). In the same line, a transgenic cat expressing macaque *trimcyp* - a HIV restriction factor - in lymphocytes was resistant to the feline immunodeficiency virus (46). While the pathways of antiviral immunity are highly conserved across vertebrates (27), such results also advocate the potential importance of TRIM modulating IFN pathways for future therapeutics.

In this work, we demonstrate that a fish TRIM expressed in gills and skin constitutes a potent amplifier of the type I IFN expression, and yields an antiviral activity against several viruses, showing that modulation of antiviral innate immunity is an ancestral property of TRIM proteins. We propose a model in which this affords an intrinsic protection of sites particularly exposed to pathogens, where Ftr83 is expressed constitutively. This finding also provides a theoretical framework to understand the repeated TRIM gene expansions during vertebrate evolution.

Acknowledgements
We thank Dr. Stephane Biacchesi for IRF3 DN vector, and Aurélie Lunazzi for excellent technical help. We are grateful for assistance with microscopy received from the Plateforme Microscopie et Imagerie des Micro-organismes, Animaux et

Aliments in Jouy en Josas.

Figures

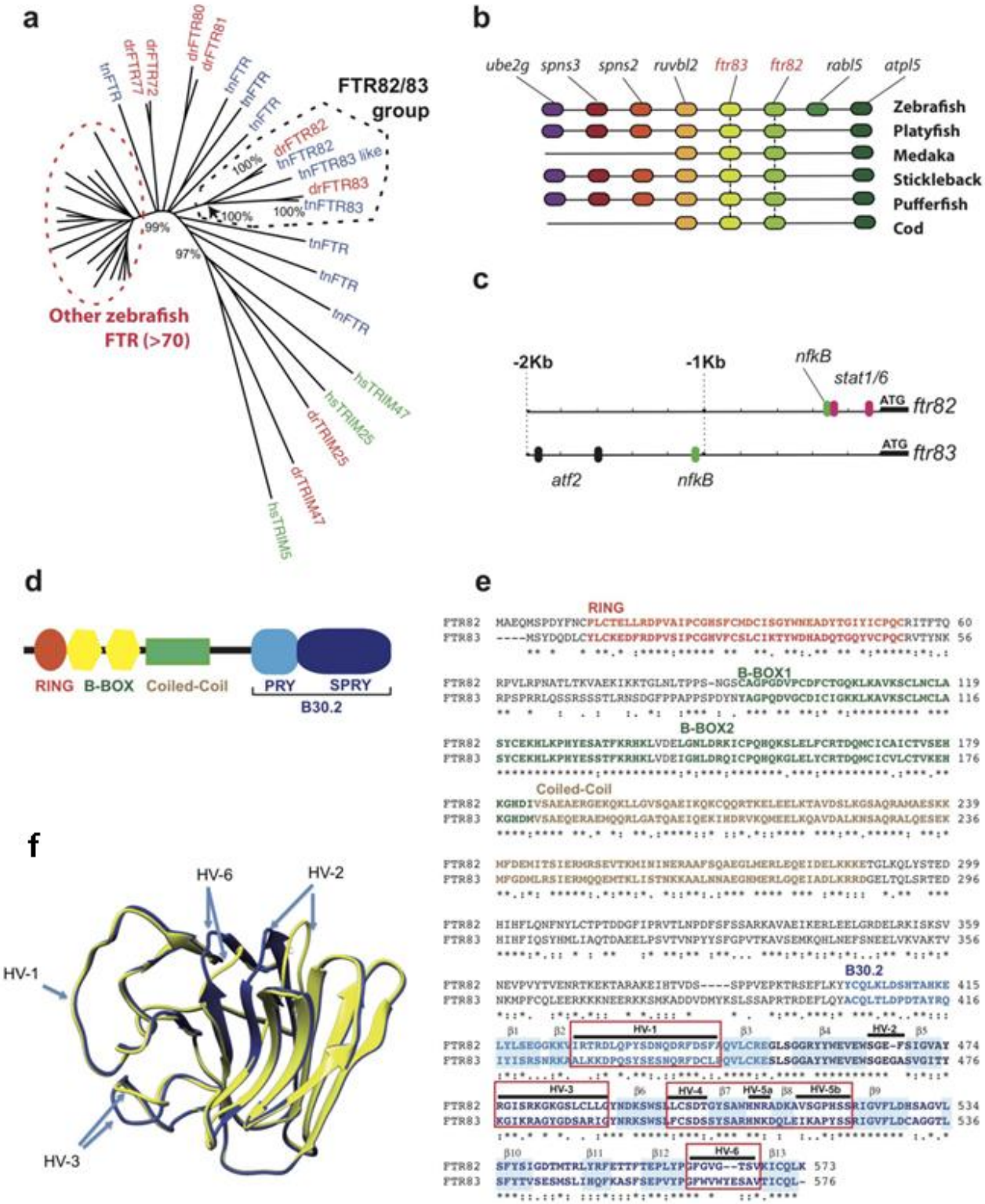

Figure 1. ***ftr82* and *ftr83*, two members of the fintrim family conserved across teleost fish.** .(a) Neighbour joining tree including zebrafish and pufferfish FTR sequences and their most similar human TRIM homologs. (b) Schematic representation of zebrafish *ftr82* and *ftr83* promoters with predicted binding sites of selected transcription factors. (c) Comparison of the conserved genomic context of

ftr82 and *ftr83* genes in different fish species, as shown using the Genomicus software. (d) Typical domain structure of finTrims. (e) Alignment of Ftr82 and Ftr83 sequences with hyper variable loops of the B30.2 domain highlighted. (f) Molecular modelling of B30.2 domains from Ftr82 (yellow) and Ftr83 (blue) derived from homology modelling based on crystal structure of huTRIM25 B30.2 domain. Visualization of models superposition was performed with the program Chimera.

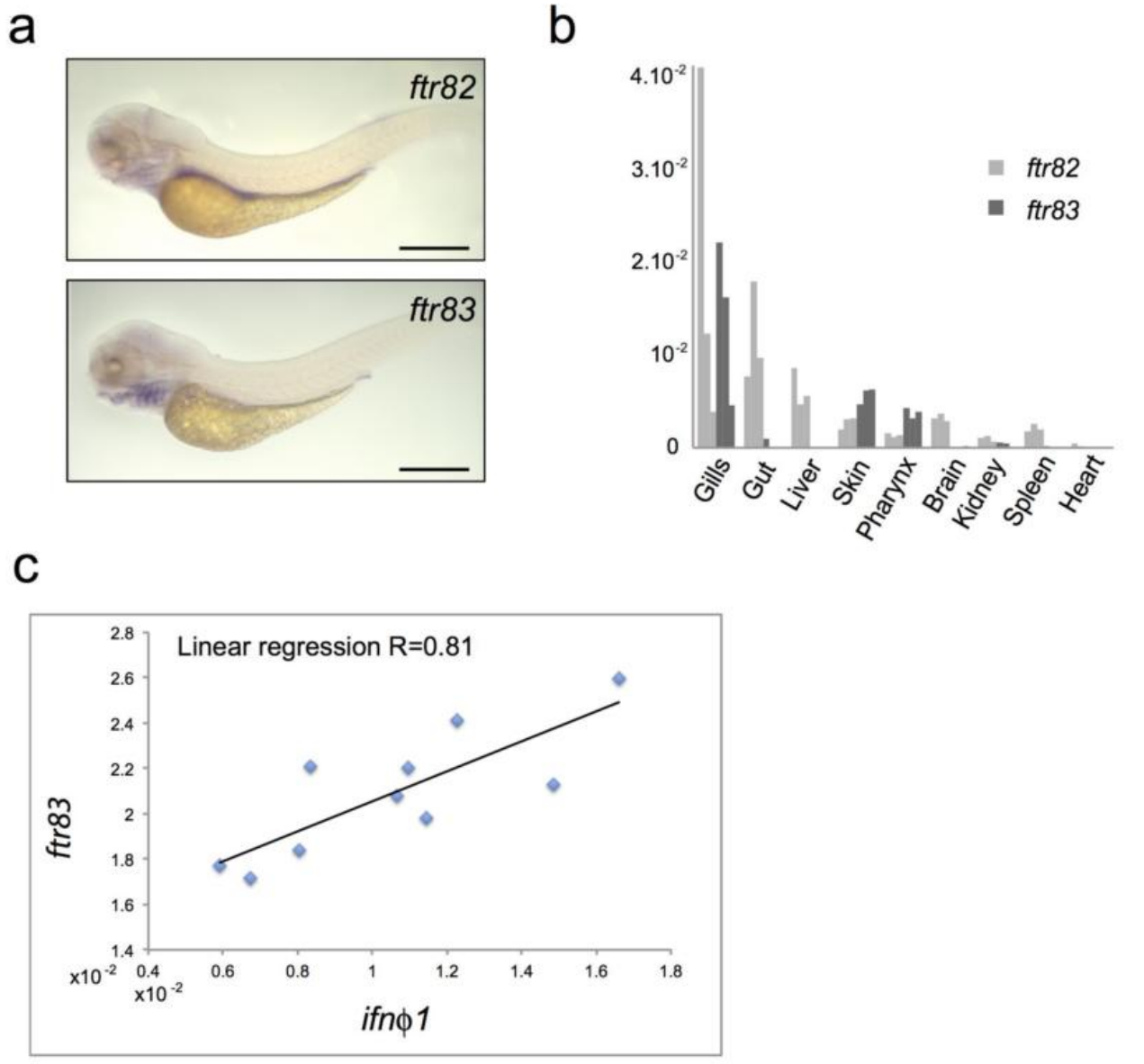

Figure 2. ***ftr83* expression pattern suggests its implication in natural antiviral immunity in gills.** (a) Spatial expression of *ftr82* and *ftr83* in 3.5 dpf zebrafish larvae. WISH using antisense probes indicated on each panel. Scale bar: 0.5 mm. (b) Genes expression in 2-3 months old juvenile zebrafish, measured by RTQPCR in various dissected tissues. Transcript copy numbers were normalized to *ß-actin* expression (measured ratio of mRNA of interest/*ß-actin* mRNA). There are three biological replicates, each being a pool of organs from 15 fish. (c) Positive correlation between *ftr83* and *ifnφ1* expression (normalised on *ß-actin* mRNA) in gills of 10 healthy individuals.

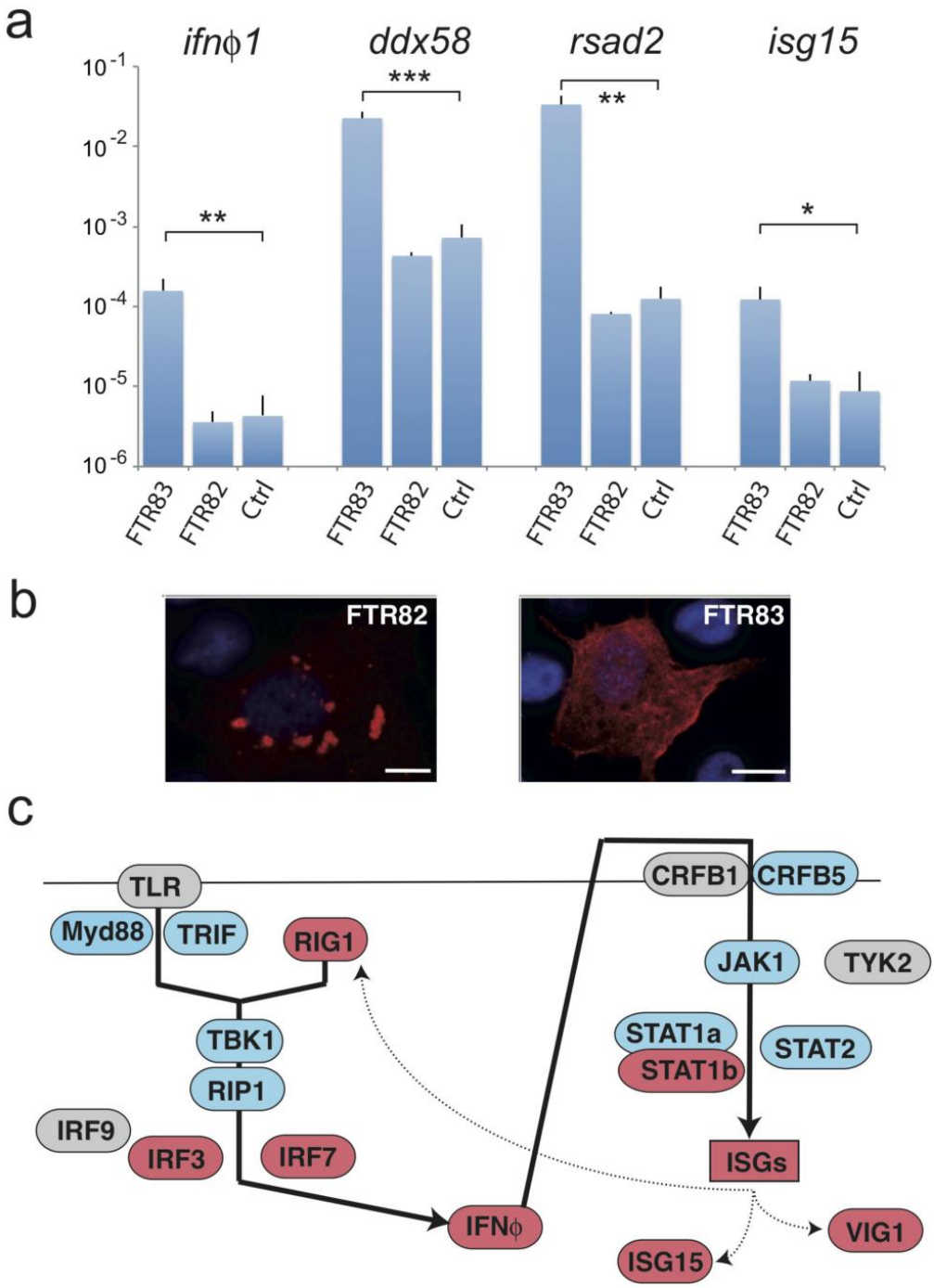

Figure 3. **Ftr83 is an inducer of IFN signaling pathway.** (a) EPC cells were transfected with expression vectors for *ftr82*, *ftr83* or with empty plasmid (Ctrl), and analyzed 72 hours post transfection for modulation of genes of the IFN pathway: *ifnϕ1*, *ddx58*, *rsad2*, *isg15*. RTQPRC results were normalized on the *ß-actin* expression. Mean and SD are shown, for three independent experiments; stars indicate significant differences using student T test (***: $p<0.001$, **: $p<0.01$, *: $p<0.05$). (b) Subcellular expression pattern of Ftrs. HA immunostaining in tagged Ftr82 (left) and Ftr83 (right) transiently transfected EPC cells, 72 hours post transfection. Ftr proteins appeared in red and nuclei in blue after DAPI staining. Scale bars: 10µm. (c) Schematic view of the type I IFN signaling pathway, with proteins whose genes were significantly up-regulated by Ftr83 highlighted in red. Proteins in blue were tested and did not appear modulated; proteins in grey were not tested.

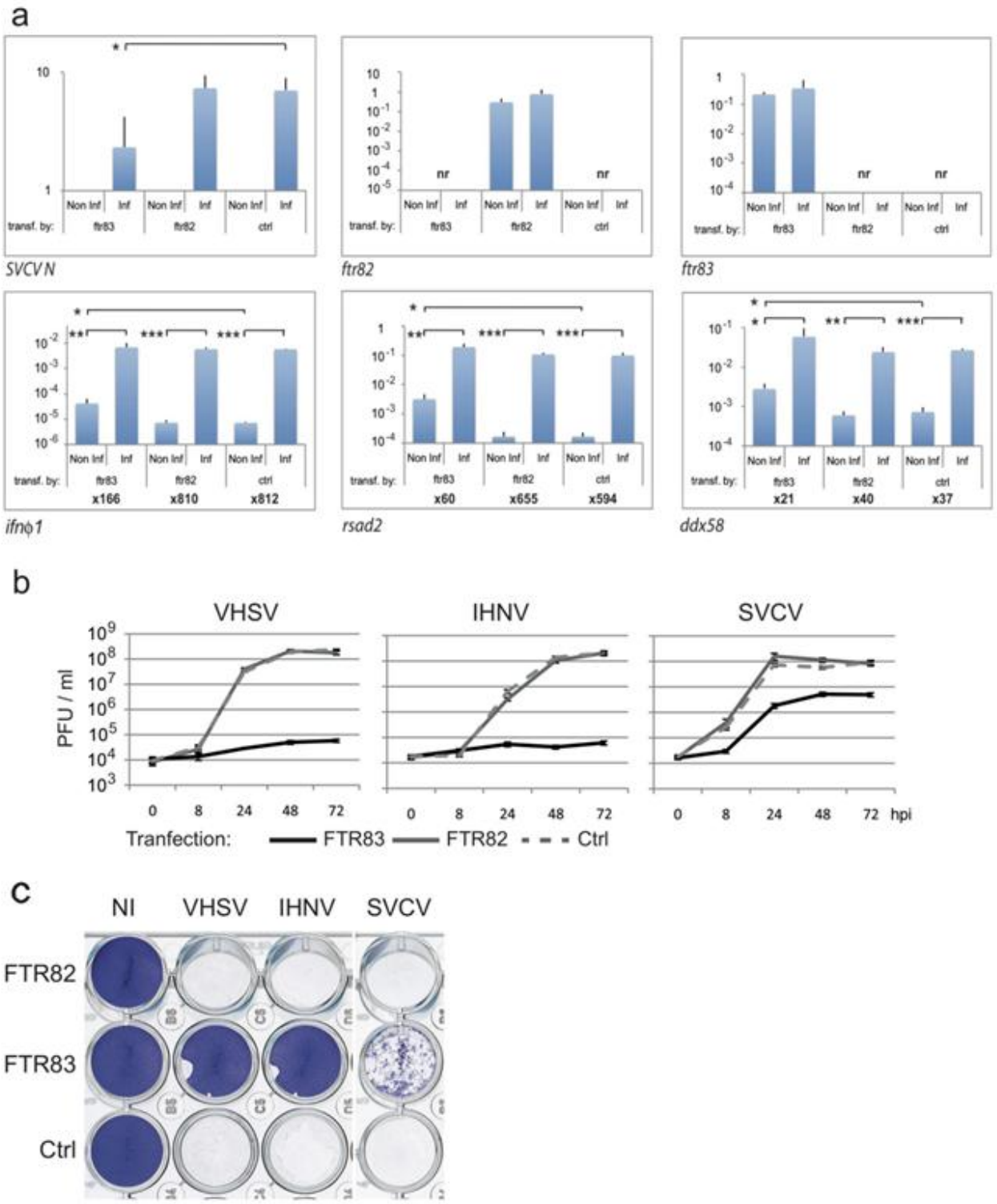

Figure 4. **Ftr83 induces key genes of IFN pathway independently of infection, and inhibits rhabdovirus infections.** (a) EPC cells were transfected with expression vectors for ftr82, ftr83 or empty plasmid (Ctrl) and at 72 hours post transfection cells were infected with SVCV (MOI 1). Transcripts of interest were quantified by RTQPCR 6 hours post infection. Results were normalized on the ß-actin expression. Mean and SD are shown, for three independent experiments, and the average of induction or repression fold between infected and non infected cells is shown when relevant. Stars indicate significant differences using student T test (***: $p<0.001$, **:

$p<0.01$, *: $p<0.05$). (b) Kinetic of viral growth measured by viral titration from 0 to 72hpi in the supernatants of cells transfected with expression plasmids for ftr83 (black line) or ftr82 (grey line) or with empty vector as control (dotted line). Cells were infected at 72 hpt with IHNV, VSHV or SVCV (MOI 1). The mean and SD of three independent experiments are presented. (c) Cytopathic effect of viral infections. Cells were infected 72 hours post transfection at MOI1 and viral induced cytopathic effect was assessed by crystal violet staining at 72hpi. Non-infected cells are presented as a control (NI).

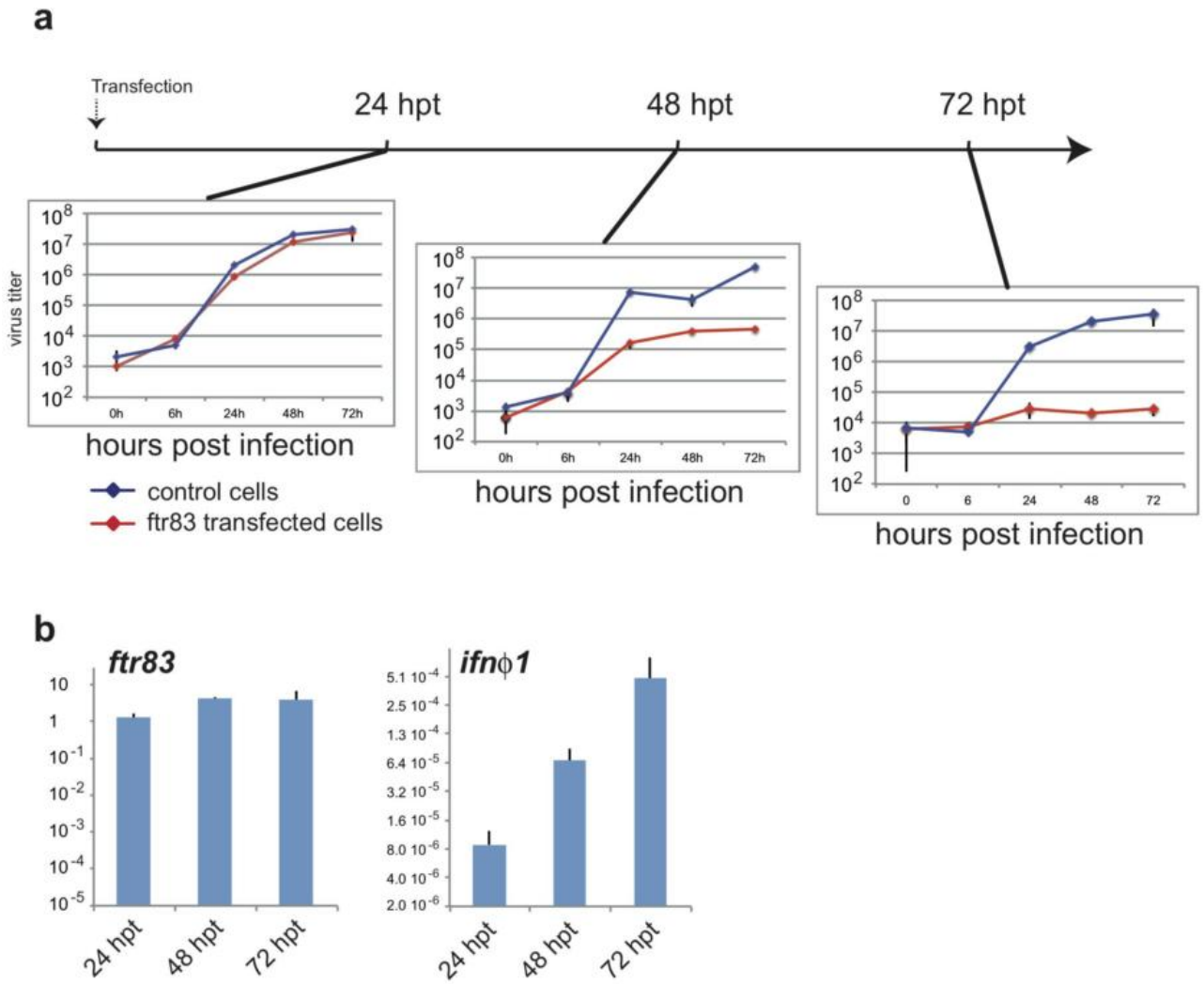

Figure 5. **Antiviral effect of Ftr83 is paralleled by stimulation of the type I Ifn pathway.** (a) EPC cells were transfected with ftr83 encoding or empty plasmid, and were infected with IHNV (MOI1) 24, 48 or 72 hours post transfection. Kinetic of IHNV growth was measured by viral titration from 0 to 72hpi. The mean and SD of three replicates are presented. (b) *ftr83* and *ifnϕ1* transcripts quantified by RTQPCR at the onset of infection, *ie* 24, 48 or 72 hours post transfection. Results were normalized on the *ß-actin* expression; mean and SD of three independent experiments.

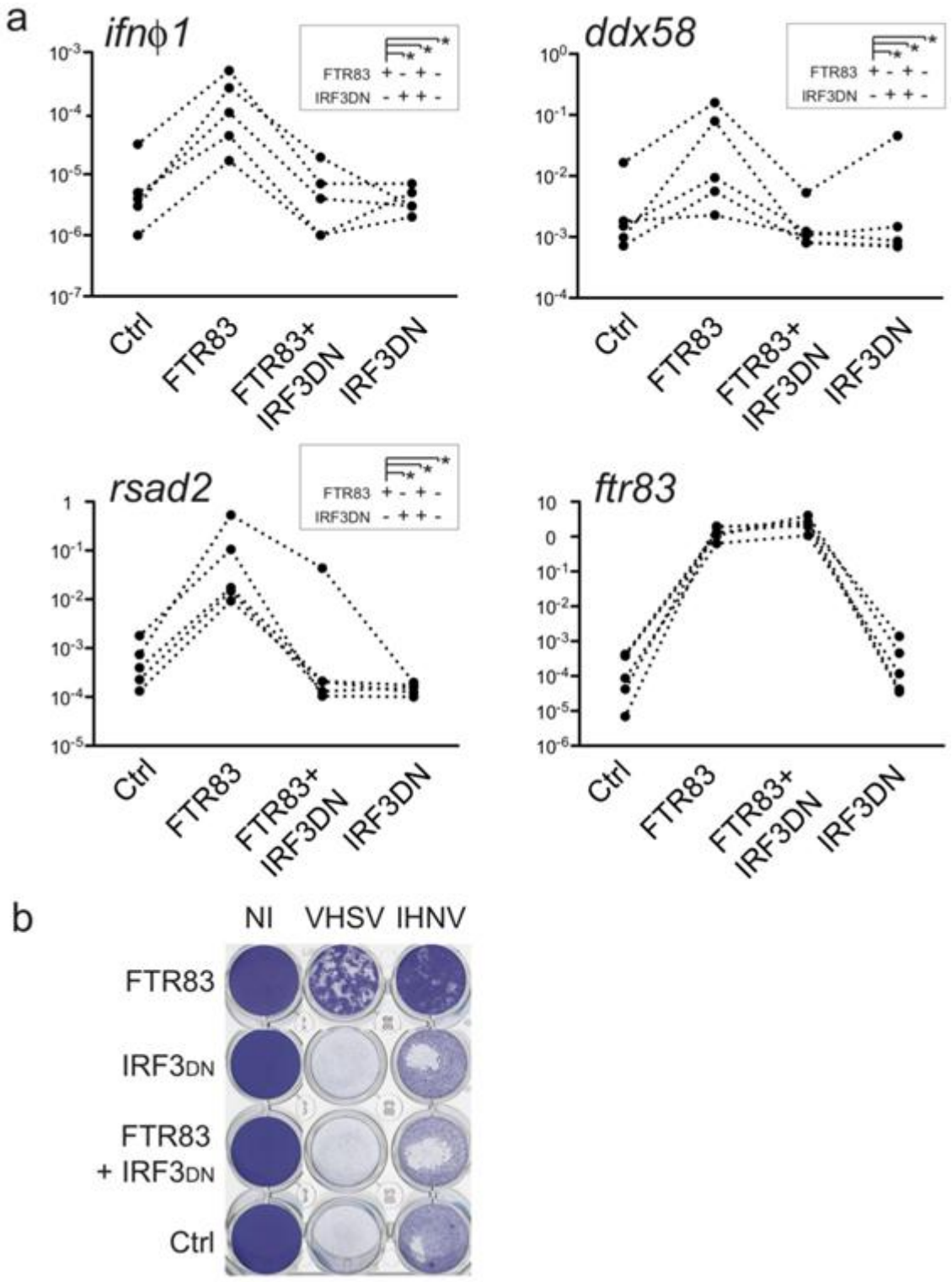

Figure 6. **Ftr83 acts through Ifn signaling pathway stimulation upstream of Irf3.** EPC cells were transfected with expression plasmid for *ftr83*, *irf3DN* or co-transfected (*ftr83+irf3DN*). Cells transfected with empty plasmid were used as control (Ctrl). (a) Transcripts of interest were quantified by RTQPCR, and the results normalized on the basis of *ß-actin* expression. Five representative experiments are represented, and correspond to dotted lines. (*: p<5%, Wilcoxon Signed-Rank test). (b) Transfected cells were infected by IHNV or VHSV (MOI 1) at 72hpt, and viral-induced cytopathic effect was assessed by crystal violet staining 72hpi. Mock transfected cells (Ctrl) and non infected cells (NI) were used as controls.

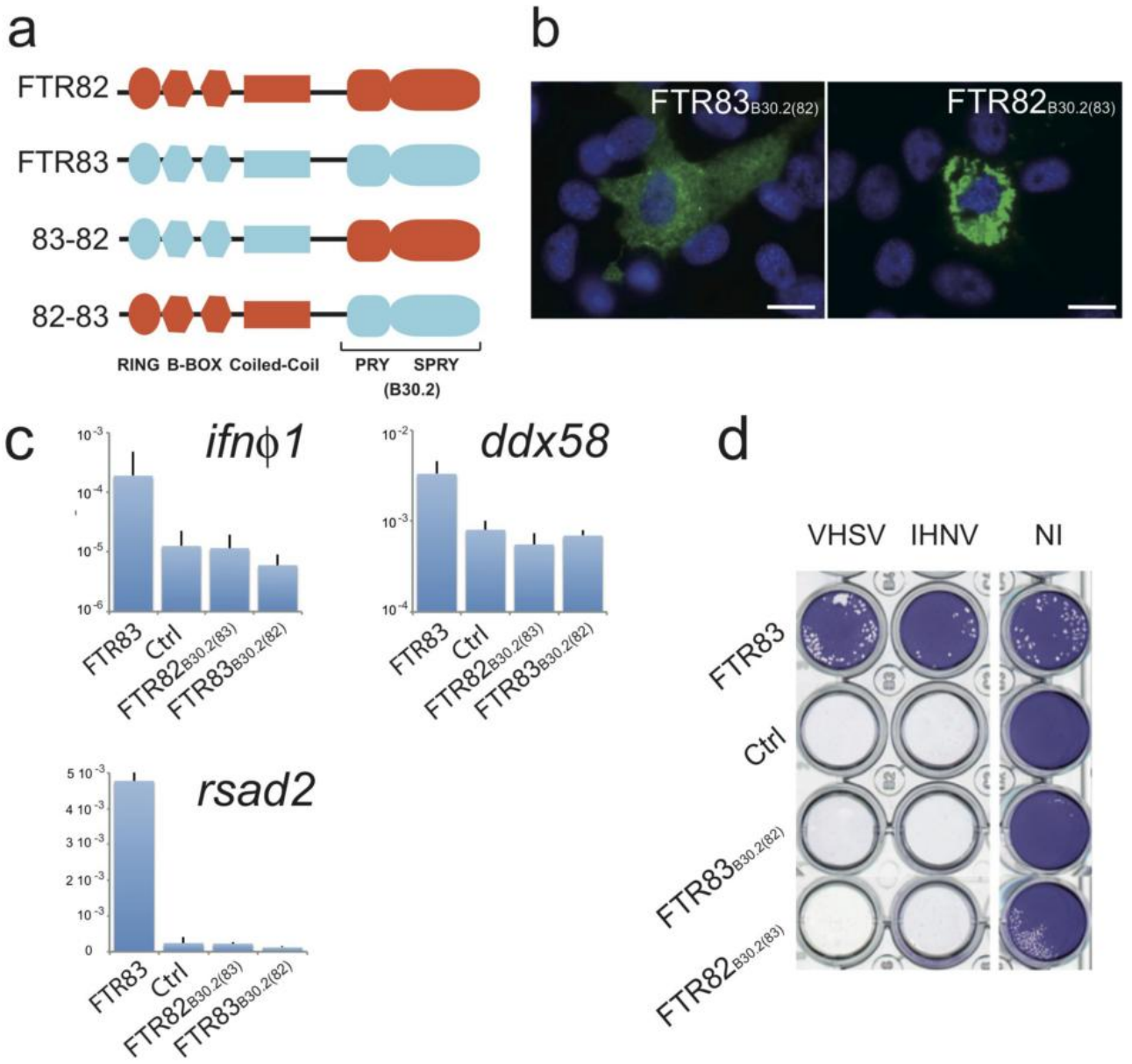

Figure 7. **Ftr83 domains involved in viral restriction.** (a) Schematic representations of finTRIM chimeras produced by combination of Ftr82 and Ftr83 domains. (b) Subcellular expression pattern of finTrim chimeras. HA tagged $Ftr83_{B30.2(82)}$ and V5 tagged $Ftr82_{B30.2(83)}$ were immunostained with relevant antibodies on transiently transfected EPC cells at 72 hpt. Ftr proteins appeared in green and nuclei in blue after DAPI staining. Scale bars: 10µm. (c) *Ifnφ1*, *ddx58* and *rsad2* transcripts are not induced by $Ftr82_{B30.2(83)}$ and $Ftr83_{B30.2(82)}$ chimeras. (d) Cytopathic effect of novirhabdoviruses (IHNV and VHSV) on transiently transfected EPC expressing full length Ftr83 (Ftr83), Ftr chimeras $Ftr83_{B30.2(82)}$ or $Ftr82_{B30.2(83)}$, or mock-transfected cells (Ctrl). Transfected cells were infected at MOI 1 72hpt, and viral induced cytopathic effect was assessed by crystal violet staining at 72hpi. Mock transfected cells (Ctrl) and non infected cells (NI) were used as controls.

Supplementary materials.

Supplementary table. Primers used in this study.

	Forward primers	Reverse primers
WISH		
Ftr83	AATTAACCCTCACTAAAAGGGAGATCTC AAGCTGGTCTTTGTTG	TAATACGACTCACTATAGGGAGACCATT GAAAGAATGCAGCAGG
Ftr82	AATTAACCCTCACTAAAGGGAGAACTAA CAGCCTTGTCTGCCC	TAATACGACTCACTATAGGGAGACTATC GAGAGGATGAGGTCAG
RT QPCR		
Ftr83	CGGAAATGCAGCAACGGCT	CTGAGTTCTTAAGAGCATCCA
Ftr82	GAGGGGAAAAACAGAAACTTC	TGCAGAGCCTTTGAGAGAGT
SVCV-N	GATTGGGATTCAGGGAGAGA	AGCAAAGTCCGGTATGTAGT
rsad2	AGCGAGGCTTACGACTTCTG	GCACCAACTCTCCCAGAAAA
ddx58	TGCTGGACCGGATGTGTTATCT	TGGTGATCGATGGTTCGATTCT
ifnphi1	ATGAAAACTCAAATGTGGACGTA	GATAGTTTCCACCCATTTCCTTAA
myd88	CTGAAGCTTTGTGTGTTTGAC	ACCACCATCCTCTTACACCT
trif	TGGCGTACAGGAGAGCCCTT	GCCACCTGCGGCCCGCAGAC
tbk1	TGCTGGTGATGGAGTACTGC	ATGTTCCCTGGCTTGATGTCTC
ripl	TGGCTCGTAAACTGGGTCTG	CAGTTTACCCACGGTGATGCC
irf7	GAGCAAATACGCTTCCCGA	CTTGTCCTGACGAAAGCCATA
crfb5	CTAGGTCCTCCGTCCAGTGTGA	CATGTCCTGCACCAGCGTCTTC
jak1	GCCAGGATCTCTGGTATGCTCC	CCATGCCAGTTTGTGAAATAAA
irf9	AATCCCTGCTACCCTTCATG	AAGACCCGTCCCTGGCAGAA
statla	AGGCTGAGCTTTGCTGGTCT	TCAGCATGTTGTACCACAT
statlb	GTGGAAGAAGAGACAGCAGAT	GTGAACCAGTTCTGCAGTTG
stat2	TTCAGGTAAGGATTCTCCTCTC	CGGATCATGGTAGCAGAAAA
irf3	AGTTATCCTGGAGTGTGTTGGACC	AAGTACCATCTTGAAGCCTTTG
isg15	AACTCGGTGACGATGCAGC	TGGGCACGTTGAAGTACTGA
CLONING		
FTR83		
FTR83-HA	ATGTCGTATGACCAGGACCTCTGTTACC	CTAAGCGTAATCTGGAACATCGTATGGG TACGATCCCGATCCCAGCTGACAAATAG TAACAGCAGACTCG
FTR83-Attb1	GGGGACAAGTTTGTACAAAAAAGCAGGC TTCGAAGGAGATAGAACCATGAACTATG CTGGACCCCAAGATGTGG	cloning in gateway pDONR
FTR83-stop-Attb2	GGGGACCACTTTGTACAAGAAAGCTGGG TCTCAGAGCTGGTTGGACATTTGGTGGT G	cloning in gateway pDONR
FTR83-nostop-Attb2	GGGGACCACTTTGTACAAGAAAGCTGGG TCGAGCTGGTTGGACATTTGGTGGTG	cloning in gateway pDONR
FTR83 mutants		
Ftr83ΔB30.2-Attb1	GGGGACAAGTTTGTACAAAAAAGCAGGC TTCGAAGGAGATAGAACCATGGCCTGCC AGCT	cloning in gateway pDONR
Ftr83ΔB30.2-Attb2	GGGGACCACTTTGTACAAGAAAGCTGGG TCACCATCTACAGCTGACAAATAG	cloning in gateway pDONR
FTR83B30.2-Attb1	GGGGACAAGTTTGTACAAAAAAGCAGGC TTCGAAGGAGATAGAACCATGGCCTGCC AGCTTACTCTGGACC	cloning in gateway pDONR
FTR83B30.2-	GGGGACCACTTTGTACAAGAAAGCTG	cloning in gateway pDONR

Attb2-nostop	GGTCCAGCTGACAAATAGTAACAGC	
FTR83-Attb1	GGGGACAAGTTTGTACAAAAAAGCAGG CTTCGAAGGAGATAGAACCATGAACTA TGCTGGACCCCAAGATGTGG	cloning in gateway pDONR
FTR82		
Ftr82-Attb1	GGGGACAAGTTTGTACAAAAAAGCAGG CTTCGAAGGAGATAGAACCATGGCTGA GCAAATGTCTCCAG	cloning in gateway pDONR
Ftr82-stop-Attb2	GGGGACCACTTTGTACAAGAAAGCTGG GTCTCATTTCAATTGGCAGATCTTGA	cloning in gateway pDONR
Ftr82-nostop-Attb2	GGGGACCACTTTGTACAAGAAAGCTGG GTCTTTCAATTGGCAGATCTTGAC	cloning in gateway pDONR
HA-Ftr82	ACCATGTACCCATACGATGTTCCAGAT TACGCTGGATCGGGATCGGCTGAGCAA ATGTCTCCAG	TCATTTCAATTGGCAGATCTTGAC
Chimeras		
Ftr83-B30.2 ftr82	GAGTTCCTTCAGTATTACTGTCAGCTG AAA	TTTCAGCTGACAGTAATACTGAAGGAAC TC
Ftr82-B30.2 ftr83	GAGTTTCTGAAATACGCCTGCCAGCTT ACT	AGTAAGCTGGCAGGCGTATTTCAGAAAC TC

Supplementary figures

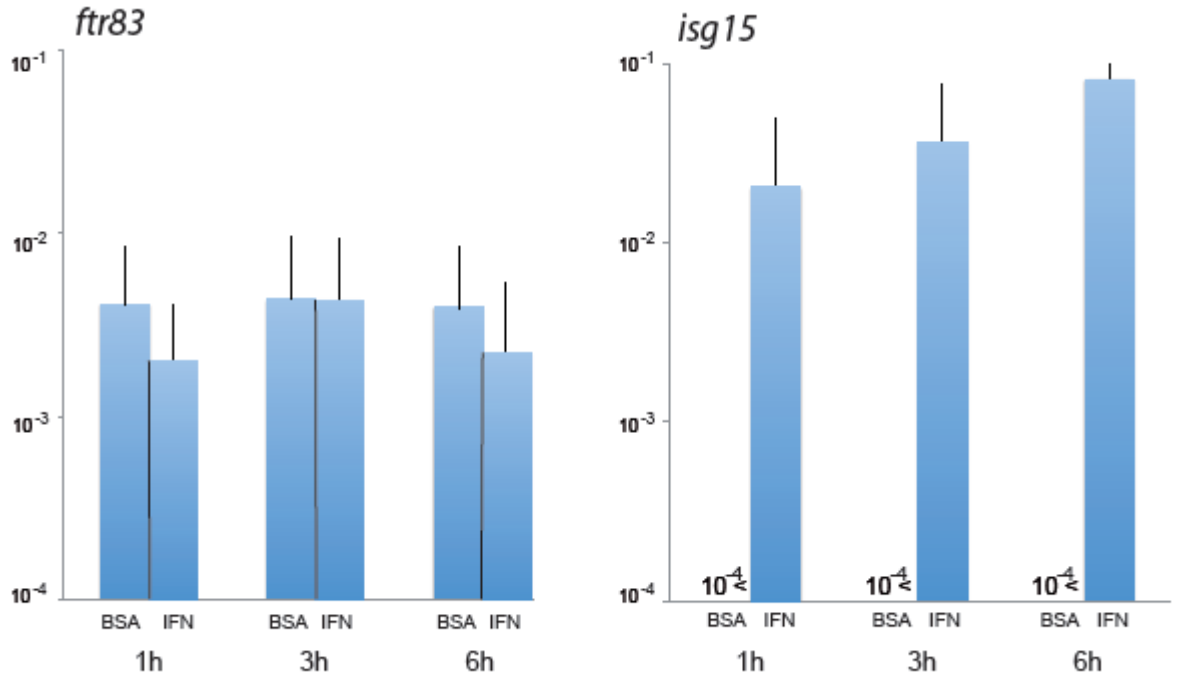

Figure 1S. **Ftr83 expression is not induced by type I IFN.** Zebrafish larvae were micro-injected intravenously with BSA or IFNϕ1 before measurement of ftr83 and isg15 expression 1,3 and 6 hours post-injection. While isg15 mRNA was induced as soon as 1 hour post injection (and not detectable in BSA injected larvae), IFNϕ1 did not induce ftr83 expression. Consistent results were observed at later timepoints. Each point represents the average of 2 individual larvae.

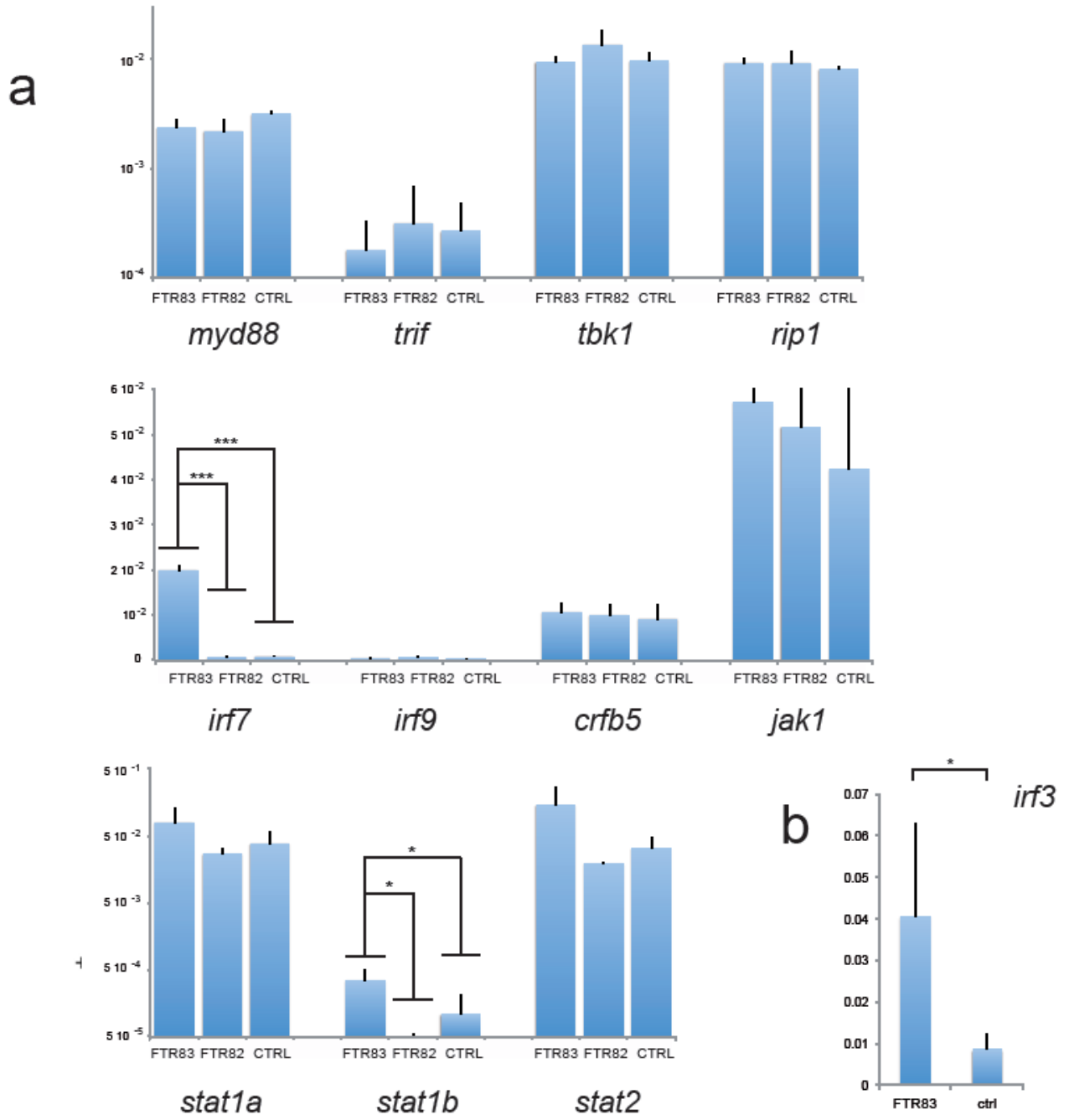

Figure 2S. **Impact of Ftr83 overexpression on key genes of the type I IFN pathway.** (a) EPC cells were transfected with expression vectors for ftr82, ftr83 or empty plasmid (Ctrl) and analyzed 72 hours post-transfection for modulation of IFN signaling genes: myd88, trif, tbk1, rip1, irf7, irf9, crfb5, jak1, stat1a, stat1b and stat2. RT-QPCR results were normalized on the β-actin expression. Mean and SD are shown for three independent experiments; stars indicate significant differences using T-test (***:p <0.001, **:p <0.01, *:p <0.05). (b) Over-expression of FTR83 in EPC cells up-regulates irf3 expression. Cells were transfected with expression plasmids for ftr83 or empty vector as control and irf3 transcripts were quantified by RT-QPCR 72 hpt. Results were normalized on the β-actin expression. The mean and SC of three independent experiments are presented (Student T-test,* :p<0.05).

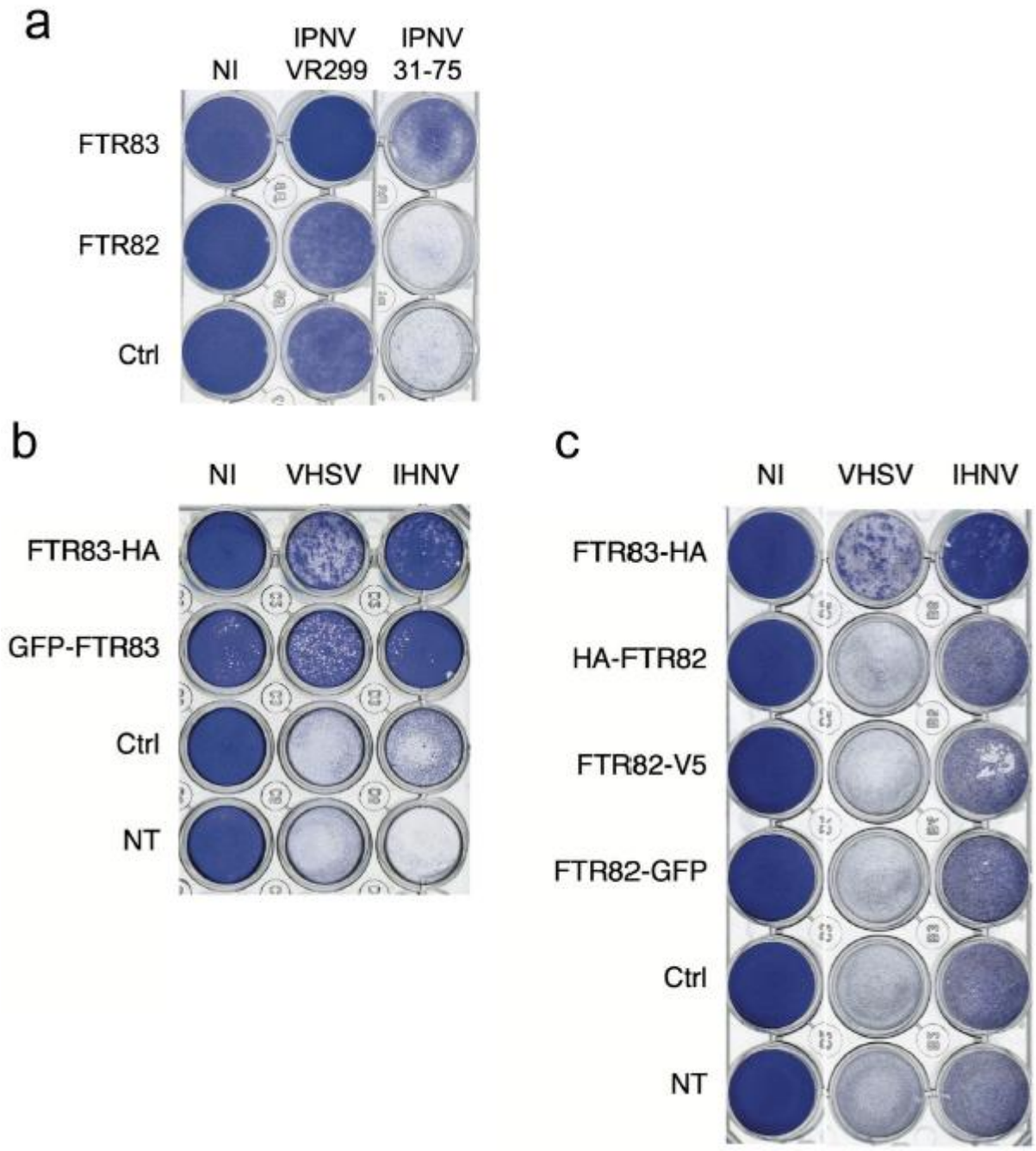

Figure 3S. **Ftr83 effect targets non-enveloped viruses and is not affected by different tags or their position.** (a) Cytopathic effect of birnavirus infection on transiently transfected EPC expressing FTR82, FTR83 or mock-transfected cells (Ctrl). Cells were infected at MOI1 with IPNV strains (VR299 or 31-75) 72 hours post-transfection and virus-induced cytopathic effect was assessed by crystal violet staining 72 hpi. Non-infected cells are presented as control (NI). (b) Cytopathic effect of IHNV and VHSV on transiently transfected EPC expressing FTR83-HA, GFP-FTR83 or mock-transfected cells (Ctrl). (c) HA-FTR82, FTR82-V5, FTR82-GFP or mock-transfected cells (Ctrl). Cells were infected 72 hours post-transfection at MOI1 and viral induced cytopathic effect was assessed by crystal violet staining 72 hpi. Non-infected cells (NI) and non-transfected cells (NT) are presented as controls.

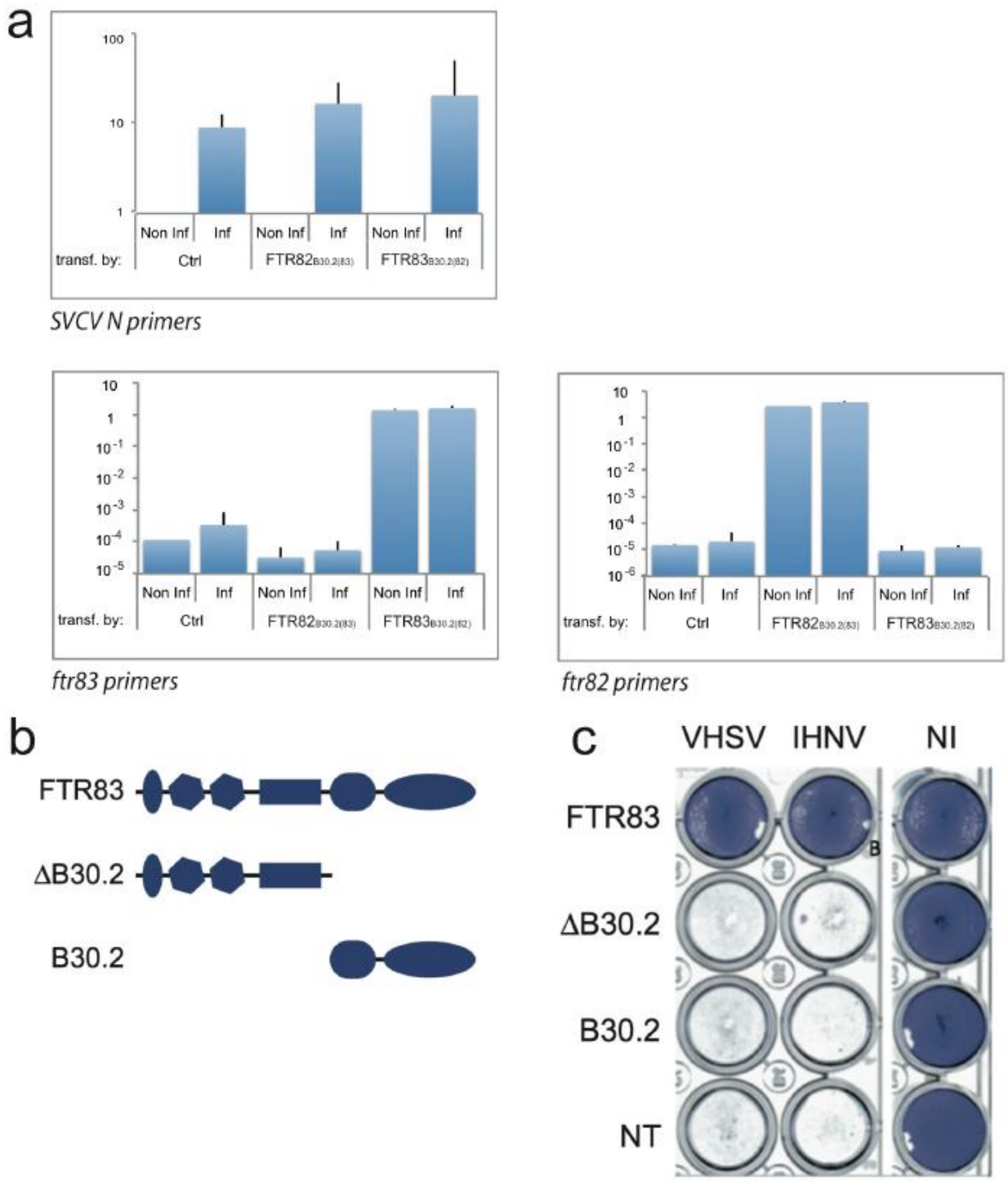

Figure 4S. **RING and B30.2 domains of FTR83 are required to induce type I IFN pathway and establish anti-viral mechanisms.** (a) FTR chimeras have no effect on SVCV transcript N expression levels. EPC cells were transfected with expression vectors for FTR chimeras $FTR83_{B30.2(82)}$ and $FTR82_{B30.2(83)}$, or with empty plasmid (Ctrl). At 72 hours post-transfection, cells were infected with SVCV (MOI1) for 6 hours. SVCV N, $FTR83_{B30.2(82)}$ and $FTR82_{B30.2(83}$ transcripts were quantified by RT-QPCR (for finTRIMs using primers located in the RBCC region) and results normalized to β-actin expression. Mean and SD are shown for four independent experiments. (b) Schematic representations of FTR83 deletion mutants. (c) Ftr83 deletion mutants do not confer cell protection against viral infection. Cytopathic effect of IHNV and VHSV on transiently transfected EPC expressing full-length

FTR83 (FTR83) and deletion mutants FTR83ΔB30.2 (ΔB30.2), FTR83B30.2 (B30.2) or non-transfected cells (NT).

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CHAPTER VII

Sentinel macrophages of zebrafish neuromasts

Sentinel macrophages of zebrafish neuromasts

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Manuscript in preparation

Introduction

Neuromasts are mechano-sensory organs embed in fish skin that detect water movements and as such allow fish navigate and react to surroundings. Neuromast is a volcano-shaped organ consisting of mechano-sensitive cells (hair cells), stem cell population, supporting cells and over-lying epithelial mantle cells (Figure 1a). The hair cells are functional equivalents of fish and mammalian inner ear hair cells, however neuromast hair cells continuously die and are replaced by new ones from the neuromast stem cell pool (247–249). The ability to induce hair cell death with antibiotics or copper sulphate has been extensively used to study hair cell regeneration (247,249–251).

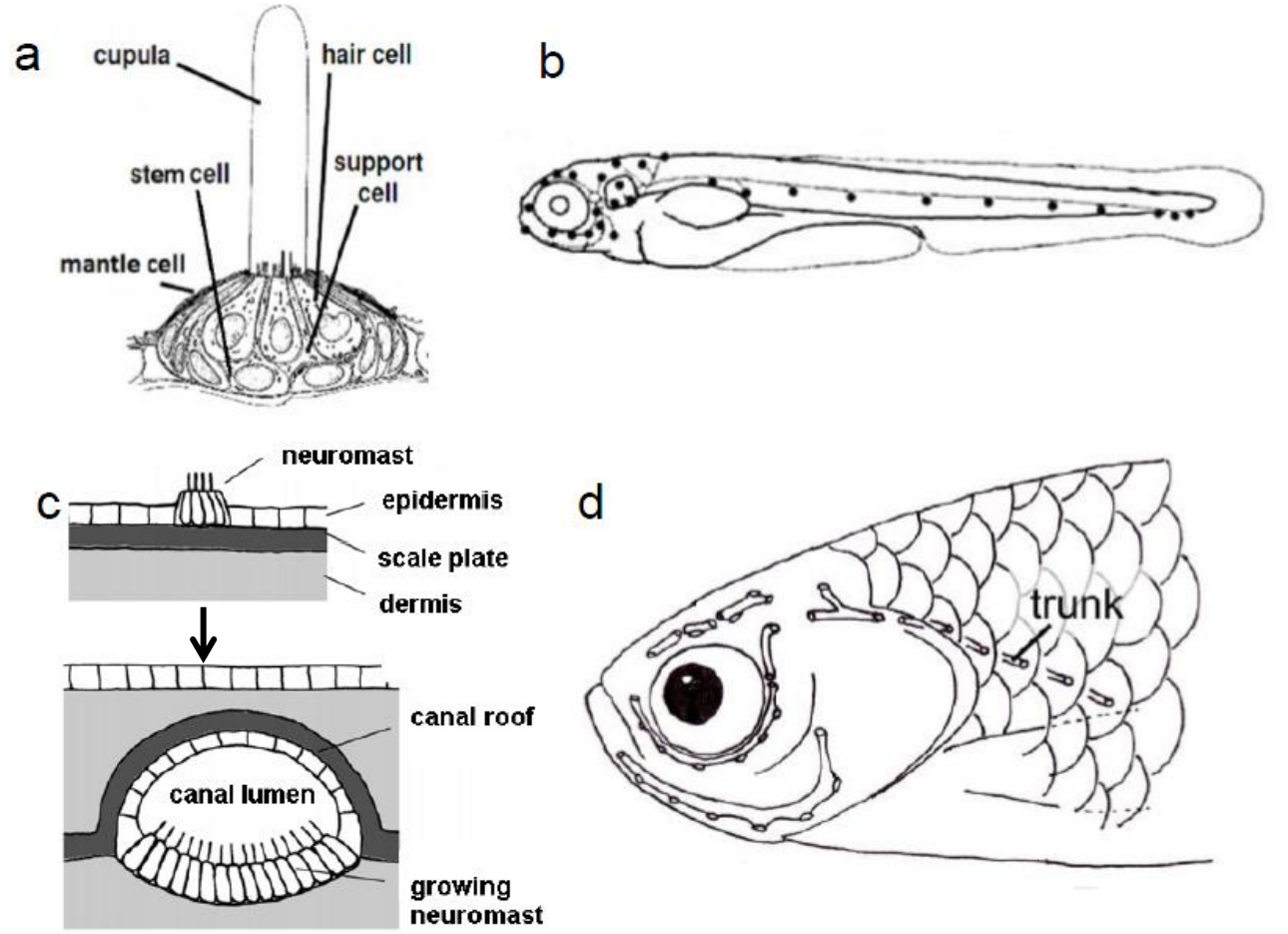

Figure 1. Neuromasts and lateral line organization. Schematic representations of a) cell types in neuromast, cupula is a mucous secretion in which the hairs of hair cells are embed, b) positions of neuromasts in a young larvae along the anterior and posterior lateral line, c) in adults some neuromasts are buried in canals that form in scales. Canals have pores through which water enters, d) anterior canal system. Partly adapted from (252).

Neuromast formation starts in the head and proceeds posteriorly through the migration of a primordium that deposits stem cells which differentiate into neuromasts – thus forming a primary network that covers the whole body by 76 hours post-fertilization (Figure 1b) (253). The differentiation of neuromast cells induces changes in overlying epidermal cells which form a pore that becomes neuromast opening through which hair cells protrude their hairs (253). The basal epidermal layer seems to be absent below neuromasts in larva, suggesting that neuromasts cause epidermal breach and might be a portal for pathogen entry (254). Furthermore, the sensory axis of neuromast might make it a vulnerable site since hair cells have a highly endocytotic apical plasma membrane as witnessed by spontaneous uptake of dyes and there are neurons synapsing with hair cells basolaterally which makes another possible route for pathogen entry (255–258). In adults the epidermis is multilayered and scales cover the body, yet regions where neuromasts are embed remain structurally different (Figure 1c,d) (252,259).

Leukocyte involvement in neuromast maintenance, protection or hair cell regeneration is scarce. Regarding hair cell regeneration, it has been documented that upon the destruction of hair cells with ototoxic agent circulating neutrophils and macrophages flock to neuromasts (238,260), however the use of anti-inflammatory drugs that can inhibit macrophages actually potentiated hair cell regeneration (261), thus the role of leukocytes in hair cell regeneration is uncertain. Interestingly, during early development neuromasts seem to be one of the first sites that get populated with macrophages which is consistent with the notion that neuromasts are vulnerable sites in epidermis (230).

Zebrafish fluorescent transgenic lines are popular tools to study leukocyte behavior *in vivo* and on the whole organism level. In an attempt to make a EGFP-reporter zebrafish with ubiquitous expression, Hsiao *et al* generated a transgene with medaka's β-actin promoter fragment flanked by adeno-associated virus inverted terminal repeats (262). However, although GFP expression is even in embryos, a tissue-specific pattern was consistantly observed in adults: a higher EGFP expression in the olfactory epithelium, in epithelial cells lining the scales, in gills, in the

urogenital duct, and in female gonads (262,263). Additionally, in the line that we received (bearing allele zf477Tg, derived from the β9 founder; which we named here medaktin::EGFP), we observed a very high EGFP signal in rare cells with leukocyte appearance in the skin. We have begun to characterize these scattered cells and found that they are myeloid cells associated with neuromasts. By sequencing their transcriptome we found that they express a high level of transcripts involved in antigen processing and presentation, as well as many anti-microbicidal genes. We have started functional studies to test the effect of different inflammatory stimuli (wound, bacteria) and are currently setting up protocols and doing pilot experiments to test their behavior during a local neuromast inflammation caused by the destruction of hair cells with neomycin/copper sulphate.

Materials and methods

Ethics statement

Animal experiments described were conducted either at the Institut Pasteur according to European Union guidelines for handling of laboratory animals (http://ec.europa.eu/environment/chemicals/lab_animals/home_en.htm) and were approved by the Direction Sanitaire et Vétérinaire de Paris under permit #B-75-1061 or at INRA Jouy-en-Josas, approved by the INRA institutional ethical committee "Comethea" (#12/114), the Direction of the Veterinary Services of Versailles (authorization number 78-28) as well as INRA (authorization number B78-720).

Fish

Medaktin::GFP (zf477Tg) were kindly provided by H.J Tsai (National Taiwan University, Taipei). Wild-type AB fish were purchased from ZIRC (Zebrafish International Resource Center, Eugene, OR). Transgenic lines used in this study are following: *mpx*:EGFP (i114Tg) (213), *lyzC*:DsRed (nz50Tg)(214), *mpeg1*:mCherry-F* (ump2Tg)(264), *mpeg1*:EGFP (gl22Tg) (216), *rag2*:loxP-dsRED2-loxP-EGFP-mMyc, here named *rag2*:dsRED (zdf7Tg)(265), *cd45*:DsRed (sd3Tg) (266).

Imaging

For time-lapse *in* vivo imaging, larva were anaesthized using eugenol (clove essential oil at 0,00725 %) and mounted in 1% agarose gel (Invitrogen 15510-027) in a 8 Well µ-Slide (Ibidi) and covered with ~ 300 µL anaesthetic solution. Time-lapse acquisition was made with Axio Observer Z1 microscope equipped with a CoolSnap HQ2 camera. Larva treated with whole-mount immunohistochemistry were imaged using Leica TCS SP8 MP microscope. Adult fish were imaged with Leica DFC450 camera or Leica SPE confocal microscope with 16x (NA 0,5) oil immersion objective.

Whole-mount immunohistochemistry

Larva were sacrificied by lethal anaesthesia using eugenol oil at 1500 µL/L. Immunohistochemistry protocol was adapted from (267). Antibodies used were following: anti-acetylated tubulin (t7457, Sigma-Aldrich), anti-parvalbumin (mab1572, Millipore), secondary antibody with conjugated Alexa594 (Molecular Probes).

Cell isolation for cell cytometry

All dissected organs were kept in ice-cold 0,9xPBS (phosphate buffer saline)/ 2% FBS (fetal bovine serum) until further processing. Blood was collected by cardiac puncture. Organs were distrupted with a polypropylene pestle (Sigma-Aldrich) directly on cell strainer. Alternatively, opercula and skin were dissociated by incubation in 0.2% trypsin/1mM versene solution under weak stirring for 4 min at room temperature. Trypsin was inhibited with 10% FBS and cells were pelleted by centrifugation at 250G for 5 min. Cell suspension were passed through 40µm strainer twice (BD Falcon).

Cell cytometry and sorting

Cytometric analysis of different organs was done with FACSCalibur (Becton Dickinson). For May-Grünwald-Giemsa stainings or deep-sequencing, opercular cells were sorted using Astrios 5L High Speed Sorter (Beckman Coulter). Sorting conditions were set up using whole kidney marrow suspension. Cellular debris was

excluded, duplets/triplets were excluded based on FSC-H/FSC-A plot and dead cells using cell dye (ethidium bromide, 7-Amino Actinomycin D or Draq7). For deep sequencing, two independent sets of cells were sorted. As the cells of interest were very scarce medaktinGFP^{high} cells were first collected together with medaktinGFP^{intermediate}, thus obtaining 30 000 cells enriched in medaktinGFP^{high} cells and equal amount of medaktinGFP$^{low/neg}$ cells were collected as a reference sample. In the second experiment, 7 500 medaktinGFP^{high} cells were sorted and equal amount of medaktinGFP$^{int./low}$ was used as a reference. Cells for RNA-seq were collected directly in lysis buffer, whereas cells for MGG were collected in 100% FBS.

Transcriptome sequencing and analysis

RNA was extracted from sorted cells using RNAqueous® Micro Total RNA Isolation Kit (Ambion). Quality of RNA was controlled with 2100 Agilent Bioanalyzer and Total RNA Pico kit. RNA fragmentation, first strand cDNA synthesis with oligo dT, adaptor ligation, and PCR amplification were performed using TotalScript™ RNA-Seq (Epicentre) in the first experiment and SMARTER V4 kit (Clontech) followed by NexteraXT kit (Illumina) in the second experiment. Libraries were sequenced either with Illumina MiSeq or Illumina NextSeq 500. At least 6 millions reads were obtained. Reads from each RNA-seq sample were filtered to remove adaptors and mapped using Tophat2 (268) on *Danio rerio* reference genome (Zv9), in which we manually added GFP sequences. Then, we used featureCounts (269) to assign read counts to genes using gene model available on Ensembl web site (release 78). For each sample, counts were normalized using upper quartile method (270) and relative enrichment was computed as the ratio between medaktinGFP^{high}-containing sample to the control sample. Functional annotation clustering of top 1000 genes with at least two-fold enrichment was done with Database for Annotation, Visualization and Integrated Discovery (DAVID) 6.7 online resource (http://david.abcc.ncifcrf.gov/) using general zebrafish background and Gene Ontology terms all together (BP/CC/MF).

Cytospin and May-Grünwald-Giemsa staining

FACS-sorted 15 000 medaktinGFP^{+} cells and 100 000 medaktinGFP^{-} cells were kept

in 100% FBS and spun on a microscopy slide at 900 rpm for 5 min, then left to dry for 30 min. MGG staining procedure was following: 2 min incubation in May-Grünwald stain, rinse in 1x Sorensen, 2 min wash in 1x Sorensen, 7 min incubation with Giemsa dye diluted in 1/20 Sorensen, rinse with ddH_2O, four washes with ddH_2O for 1min30s, drying over-night at room-temperature, mounting with Histolacque. Slides were imaged with Leica DMR microscope and a 100x objective and images taken with Olympus DP71 camera.

Results

Medaktin:EGFP transgenic labels leukocyte-like cell population in fish skin near neuromasts

Inspection of medaktin::EGFP transgenic adult fish had revealed that there is a high EGFP expression in rare cells reminiscent of leukocytes that are positioned all along the anteroposterior lateral line (Figure 1A). These cells start appearing around 7-9 dpf long after first mature neuromasts have formed. To clarify if the circular arrangement often seen for these cells reflects their association to neuromasts, we labelled larvae with antibodies against acetylated tubulin which marks neuronal axons as well as the kinocilium of neuromast hair cells and/or with parvalbumin-antibody which specifically labels hair cells (271)(Figure 1B). MedaktinEGFP^{hi} cells were indeed in close vicinity to neuromasts (Figure1B'-B''') and they were occasionally seen to sprout and form contacts with the inner core of neuromasts (Figure1B''). To make sure that medaktinEGFP^{hi} cells do not correspond to any known neuromast cell type such as epithelial mantle cells, we imaged the neuromasts on adult tailfins in bright-field to see the epidermal cell layers (Figure 1C). MedaktinEGFP^{hi} cells did not co-locate with mantle cells and were generally found in deeper layers even below the level of stem cell population. As these cells are seen near neuromast in larvae and adults under different conditions and intact hair cells could be visualized (Figure1B'), medaktinEGFP^{hi} cells could be cells that are associated to neuromast under normal conditions and not just leukocytes that responded to neuromast damage caused by handling.

In addition to the posterior lateral line, GFP-bright leukocytes were also observed

surrounding the neuromasts of the head, including those that are inside channels. They were especially numerous around the giant neuromast that is located in the channel just posterior to the eye. The number of GFP-bright cells around lateral line neuromasts in adult fish was typically 1, and some neuromasts were devoid of such cells. However, the giant neuromast that is located in the channel just posterior to the eye was associated with a larger group of ~10 medaktinEGFP^{hi} cells.

MedaktinEGFP^{hi} cells are only detected in the skin

We wondered if GFP-bright cells are also present in hematopoietic organs or peripheral blood as we were interested when the higher GFP expression is initiated. To set up FACS gating of hematopoietic cells whole kidney marrow was used as a reference (Figure2b). No medaktinEGFP^{hi} cells were detected by cytometric or microscopic analysis of whole kidney marrow, spleen and peripheral blood of adult fish(data not shown) suggesting that these higher GFP expression in these leukocyte-like cells is triggered in skin. Analysis of cell suspensions made from opercula and skin showed that the cell population with the strongest GFP expression had myelogranulocytic cell scatter characteristics (Figure2c). As expected from the distribution of neuromasts on the body (Figure 2a), the frequency of EGFP^{hi} cells was higher in cell suspension from opercular than trunk skin. We performed May-Grünwald-Giemsa staining on the whole EGFP-positive cell population on FACS-sorted cells from medaktin::EGFP operculums and could see cells reminiscent of monocytes and myeloid cells with many vesicules among the heterogenous population that included epithelial-like cells (the frequency of cells with vesicules among GFP+ cells was 5/38 and in GFP- population 0/24) (Figure 2d).

Most medaktinGFP^{hi} cells are mpeg1-positive motile cells

To get insights about the lineage of these cells we performed crosses with established RFP Tg-lines labelling various types of hematopoietic cells. MedaktinEGFP^{hi} cells around neuromasts did not co-express DsRed driven by *rag2*:*DsRed*, or *lyzC*:*DsRed* transgenes (not shown), indicating they are neither immature lymphocytes nor neutrophils. They also did not co-express the *cd45*:DsRed transgene (Figure 3a and data not shown), which is more difficult to interpret because expression of this

transgene in the skin is only partially characterized, however it suggests they are neither eosinophils nor T cells. At any rate, it did not rule out a hematopoietic lineage since this transgene is not active in B cells (233). By contrast most of medaktinEGFP^{hi} cells expressed mCherry driven by the *mpeg1*:mCherryF transgene (Figure 3b), suggesting they belong to the macrophage lineage. *In vivo* time-lapse imaging of 10 dpf medaktin:EGFP and mpeg1:mCherry transgenic revealed that double-positive cells are motile – showing circular patrolling behaviour (Figure 3c). Some rare medaktinEGFP^{hi} cells that appear to be single-positive were seen that did not seem to move but instead sampled surroundings by sprouting and thereby resembled dendritic cells, thus medaktinEGFP^{hi} cell subset might contain different types of myelomonocytic cells or this observation just confirms the notion that dendritic cell-macrophage lineage commitment is dynamical and interlinked.

MedaktinGFP^{hi} express antigen-presentation as well as microbicidial molecules

To further characterize these cells we performed whole-transcriptome sequencing.We first set up a FACS protocol to sort cells from opercula as it has a higher density of medaktinEGFP^{hi} cells than skin covering the body (Figure 2a, 2c).

As the medaktinEGFP^{hi} cells are very scarce (approximately 150 cells per operculum) we first sorted them together with medaktinEGFP^{intermediate} cells and obtained 30 000 cells and took an equal amount of medaktinEGFP$^{low/neg}$ cells as controls (Figure 4a). We got 6 million reads covering 21 000 genes (there are 26 459 coding genes in zebrafish). Judged by the expression of housekeeping genes the two samples contained the same amount of nucleic acid. As the medaktinEGFP$^{low/neg}$ cell population contained 5 reads of GFP transcript we considered 5 reads as a cutoff value – after the exclusion of genes with less than 5 reads in medaktinEGFP^{hi} cell population, 17 247 genes remained in the list. We did functional annotation clustering with DAVID resource using the 1000 top genes enriched in medaktinEGFP^{hi} cells at least two-fold. The most enriched cluster corresponded to ‘antigen processing and presentation’, and the second to ‘cell-cell junctions’ likely reflecting that the sorted cell population was heterogenous, containing leukocytes and keratinocytes, although keratinocytes are known to have the ability to present antigens via MHCII class as

well (Table I). Next, we checked the expression of some cell type markers. We confirmed previous findings obtained from transgenic crosses, namely that medaktinEGFP^{hi} cells express *mpeg1* and do not express neutrophil marker *mpx* nor lymphocyte marker *rag2* or hematopoietic cell marker *cd45* the expression of which was generally low in the tissue (Table II). Several transcripts expected from GFP-expressing epithelial cells were enriched: several keratins (*krt4*, *krt8, krt17, krt18*) as well as *mesothelin* and *cldne*. Many components of MHCI/II pathway were enriched in medaktinEGFP^{hi} cells-population as well. Additionally, several genes which are involved in immune defense were enriched: for example, autophagy (*dram1, sqstm1*, *vamp8*) and microbicidial genes (*nos2a, noxa1*, *irg1l, mpeg1.2*) as well as some IFN system components (*nfkbiab*, *nfkbiaa, tbk1*). Importantly some macrophage markers were enriched: *cd68, rgs, il13ra2* and *mpeg1.2*. Interestingly, zebrafish have three *mpeg1* paralogues: *mpeg1* (also known as *mpeg1.1*), *mpeg1.2* and *mpeg1.3*. The mpeg1:mCherry transgenic was made with the promoter fragment of *mpeg1*, however our data implies that medaktinEGFP^{hi} have a low expression of *mpeg1* and higher expression of *mpeg1.2* transcripts instead, suggesting that the transgenic does not recapitulate expression of *mpeg1* only. We analyzed with RT-QPCR the expression levels of *mpeg1* and *mpeg1.2, cd68, mhc2dab, mesothelin, cd74b, krt4* and also *ftr83* as it has been implicated in the pathogen-exposed tissue upregulation of type I IFN signalling (Figure 4C). All of the results obtained with RNA-seq were confirmed, expect for *krt4*. To distinguish between GFP-intermediate epithelial cells and GFP-high antigen presenting cells, we attempted to sort a pure population of medaktinEGFP^{hi} cells and sorted medaktinEGFP^{intermediate} cells as controls (Figure 4B). 7 500 cells total were obtained and as Illumina NextSeq 500 sequencer was used instead of MiSeq, allowing a greater sequencing depth. We then compared the relative enrichment of selected genes in the two experiments (Table II). Genes expected from keratinocytes were generally several folds less enriched in medaktinEGFP^{hi} pure population, however they were still highly expressed and thus the sorting could still be improved. Most of the aforementioned myeloid and microbicidial genes, as well as MHCI/II pathway components were still enriched in medaktinEGFP^{hi} cells, although the fold-difference was lower in reference to

medaktinEGFP^{intermediate} than to the whole opercular cell population sorted in the previous experiment. Thus, it is possible that GFP-positive keratinocytes also express these molecules at a lower level. Nonetheless, medaktinEGFP^{hi} cells are enriched for antigen presenting molecules as well as IFN signaling components and microbicidial molecules and thus could represent a neuromast-specialized sentinel cell.

MedaktinEGFP^{hi} cells stay near neuromast after skin injury

To start assesing the functional involvement of medaktinEGFP^{hi} cells in different inflammatory processes, we first asked if these cells would react to tailfin-amputation on adult medaktin:EGFP fish and did not see any medaktinEGFP^{hi} that would react to this inflammatory cue as none of them approached the cut site (data not shown), thus further suggesting that medaktinEGFP^{hi} cells is a myelomonocyte subset strictly associated to neuromasts. Importantly, newly regenerated neuromasts in tailfin got repopulated with GFP-bright cells suggesting that these leukocytes are not necessary for the regeneration of whole neuromast structure, but are still part of the final neuromast structure.

MedaktinEGFP^{hi} cells involvement in hair cell regeneration

As these leukocytes did not respond to distant inflammatory cues we next evaluated they role in hair cells regenerative models. To do so, 10dpf medaktin larve were exposed to the amninoglycoside neomycin, which induce hair cell death (Owen 2008). LyzC and medaktin fish were treated with 250 µM of neomycin for 1 hour. Hair cells state was determined in distinct neuromasts in operculum and along the lateral line after immunostaining with parvalbumin antibody. While neutrophils were rapidly detected in the neuromast of the tailfin after Hair cells degeneration (Figure 5a and c, right panel), medaktin cells observed in the operculum do not relocate when compared to non-treated fish (Figure 5b and c, left panel). In addition, neomycin treatment does not lead to an increase in GFPhi cells number around neuromasts as compared to non-treated fish.

We further determined medaktin cells phenotype upon copper treatment, which damage hair cells populations inducing stronger leukocytes recruitment in neuromasts

that after neomycin treatment. Data are under analyses but we did observed hair cell degeneration in absence of significant relocalization of induced GFP^{hi} cells. Initial observation is that copper treatment leads to modification of GFP^{hi} cells morphology after treatment with 1-50 μM copper sulphate treatment for 10-15 minutes.

Figures

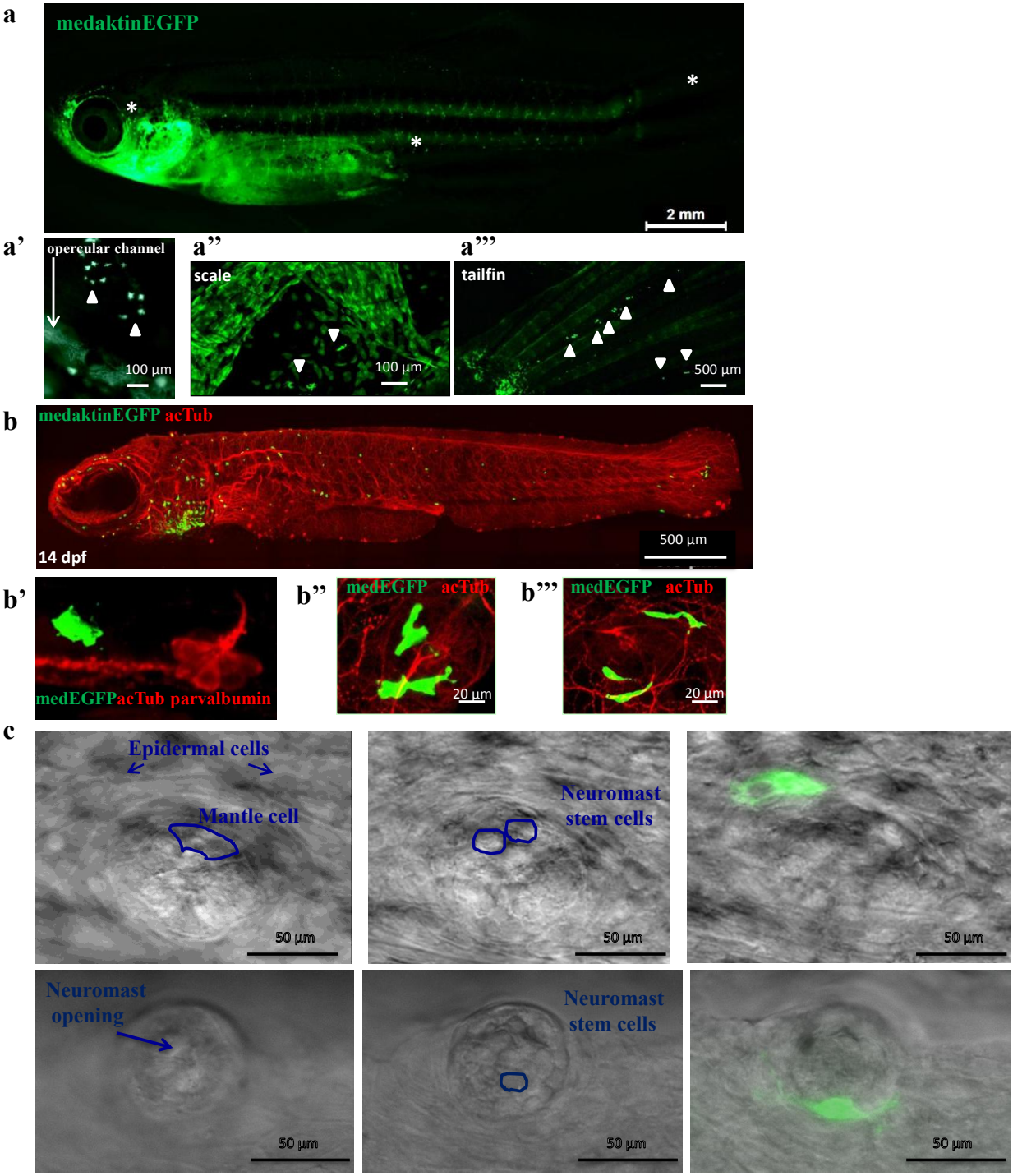

Figure 1. Medaktin:EGFP transgenic line marks leukocyte-like cells near neuromasts. (a) Lateral view of a 1-month medaktin::EGFP transgenic fish showing transgene expression imaged with stereo-microscope. Asterisks indicate regions that were imaged with higher magnification along lateral line in operculum (a'), trunk skin (a'') and tailfin (a'''). (b) GFP-high cells distribution in medaktin::EGFP transgenic larvae along the lateral line determined by two photon microscopy after immunohistochemistry with acetytlated tubulin antibodies. Higher magnification showed GFP cells distribution in the viscinity of the neuromast detected after anti-acetytlated tubulin and anti-parvalbumin immunohistochemistry (b') or anti-acetylated tubulin treatment only (b''- b'''). (c) GFP-bright cells distribution in neuromast in adult tailfin. Z-stack acquisitions of neuromast structure is presented showing surface epidermal cells/mantle cells, neuromast stem cells.from adults were clipped, fixed with 4% PFA and mounted on a microscope slide. GFP signal of medaktinEGFP^{hi} cells does not after fixation.

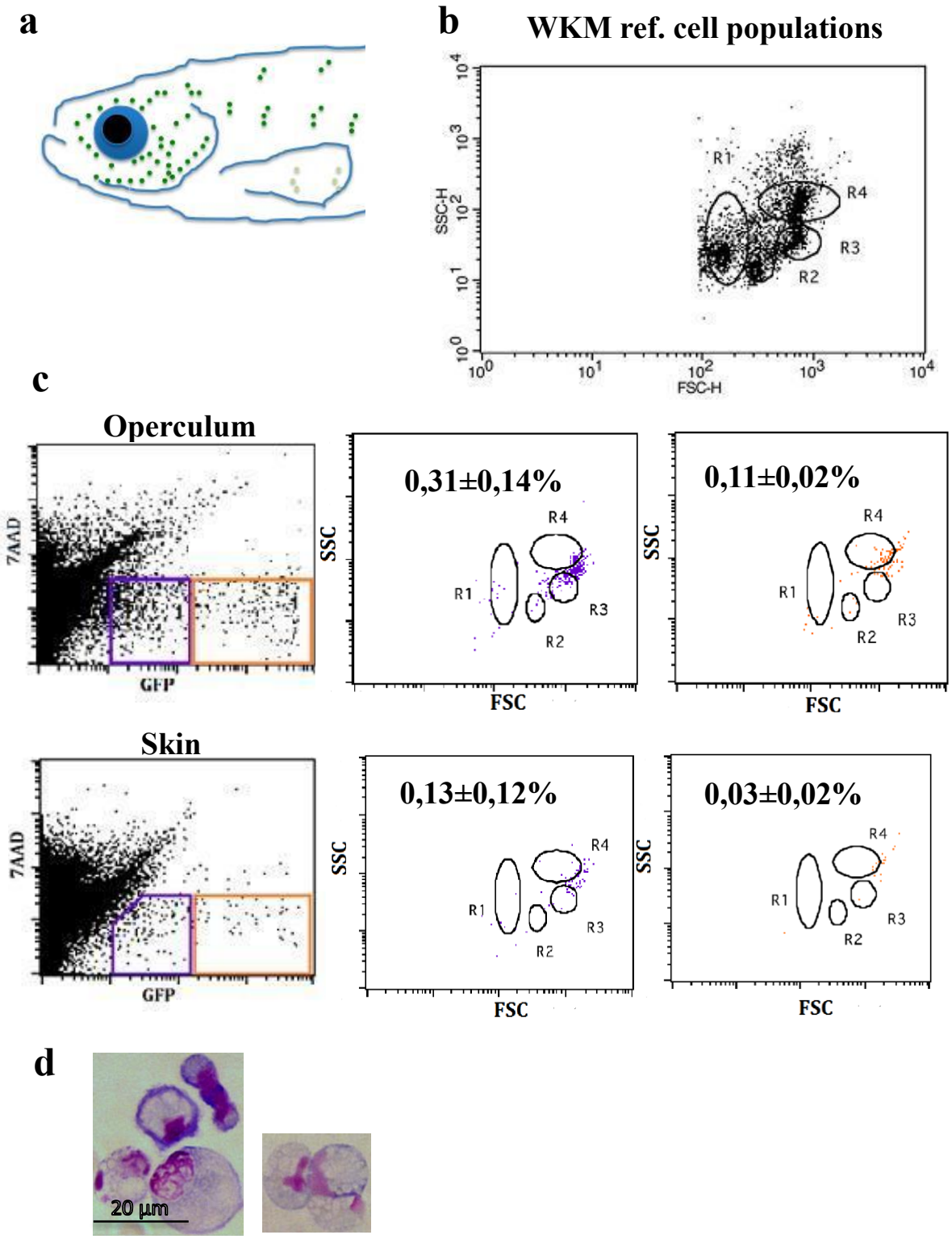

Figure 2. MedaktinEGFP^{high} cells are only detected in skin and have myeloid characteristics. (a) Schematic representation of approximate neuromast positions on operculum and body of an adult fish. Neuromasts are illustrated with green dots. (b) representative plot of whole kidney marrow (WKM) cell populations which were used as a reference. R1:erythrocytes, R2:lymphocytes, R3: myeloid progenitors, R4: myeloid cells and granulocytes. (c) flow cytometrie analyses of cell suspensions obtained from medaktin:EGFP fish operculum (upper panel) or skin (bottom panel). Gates were drawn with the assumption that GFP^{hi} (purple square) and GFP^{low} (orange square) cells represent different cell populations. Percentage of total 100 000 events. (d) MGG staining of cytospinned whole medaktinGFPpositive population.

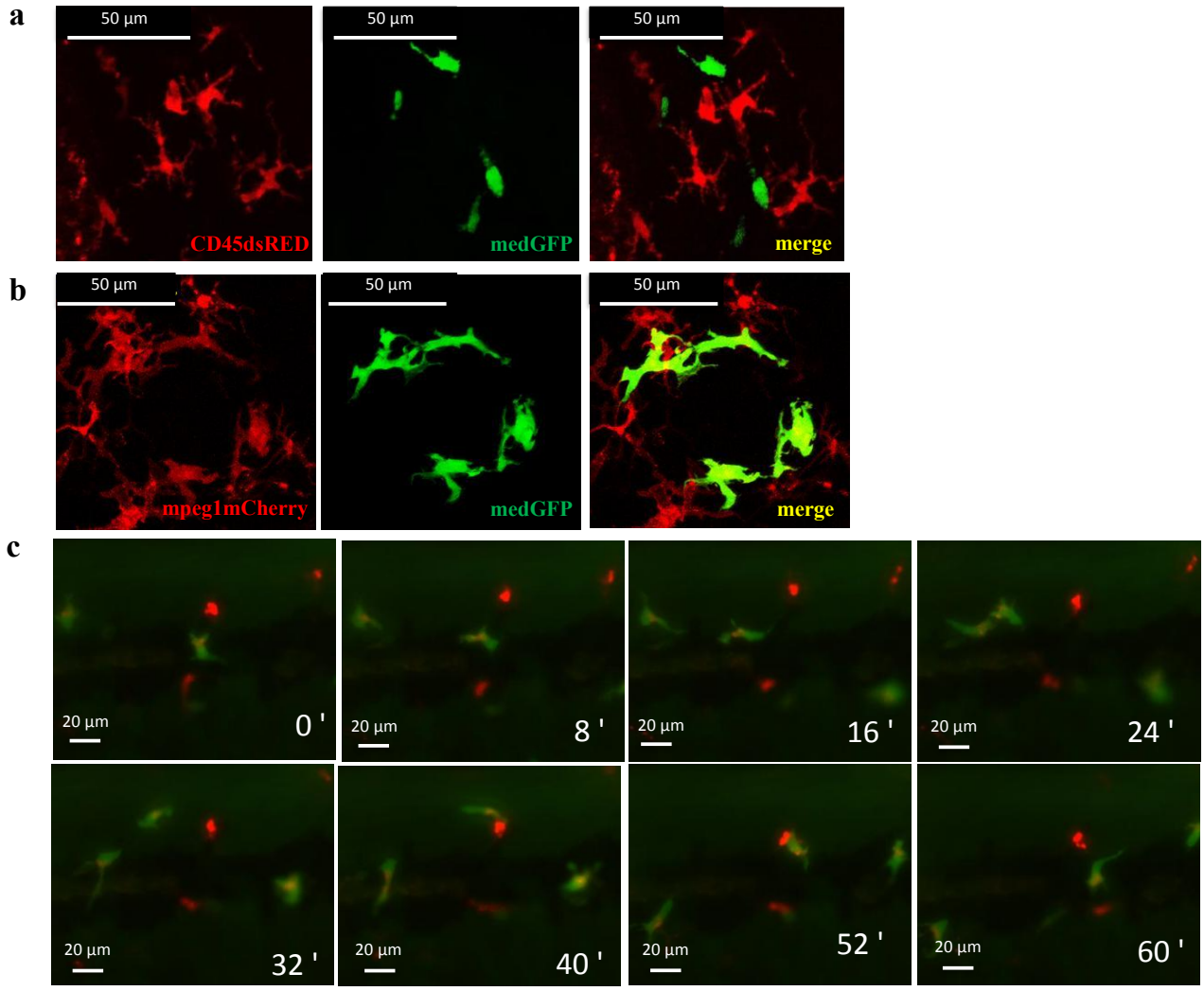

Figure 3. MedaktinEGFP^{high} cells are mpeg1+ motile cells .
(a) confocal microscopy imaging of skin of cd45::DsRed, medaktin::EGFP double transgenic larvae at 8dpf. (b) skin from a mpeg1mCherry, medaktin::EGFP double transgenic adult fish imaged with a confocal microscope. (c) frames from time-lapse imaging of mpeg1::mCherry, medaktin::EGFP double transgenic fish at 10 dpf.

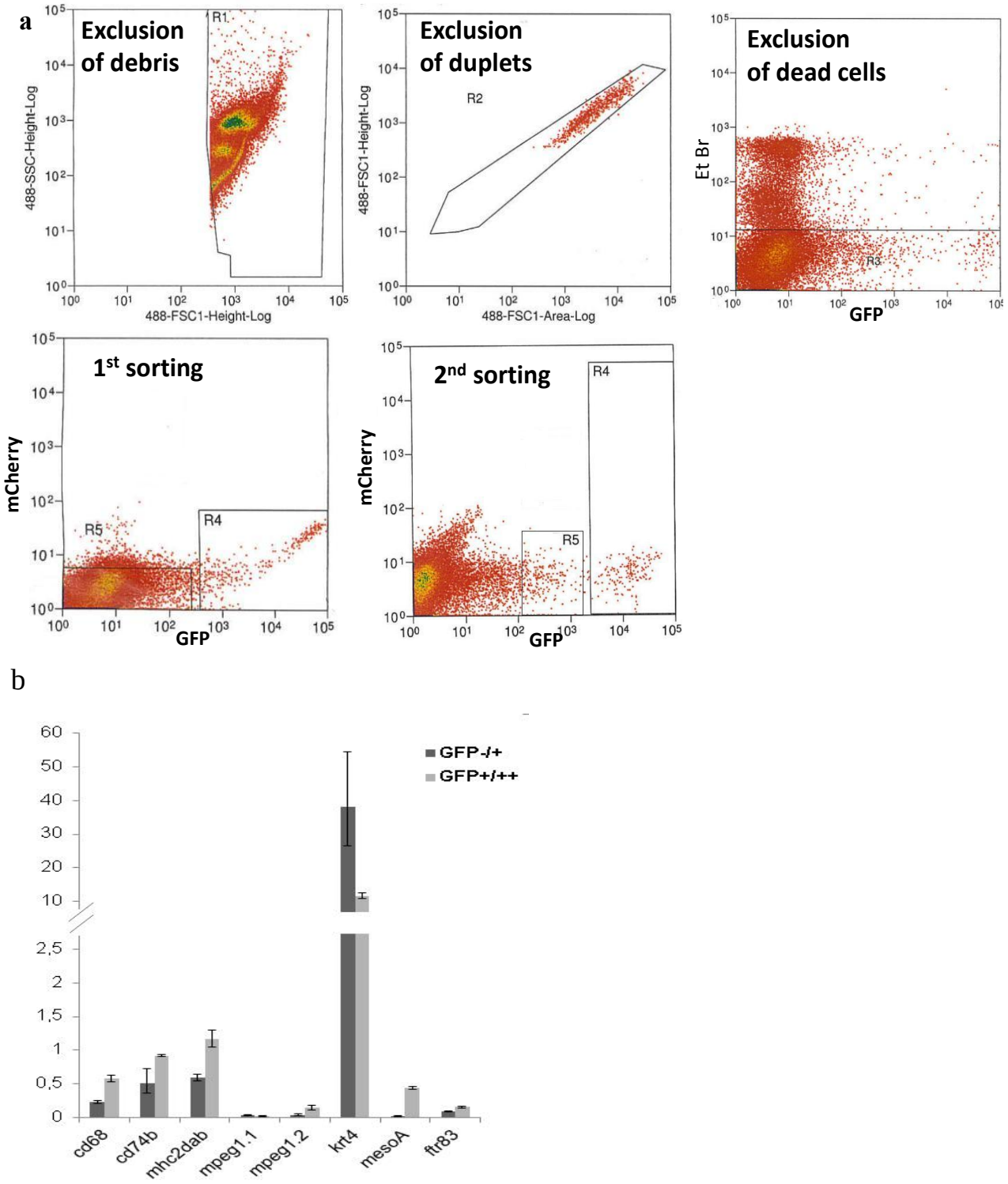

Figure 4. Cell isolation from opercula for RNA-seq.

(a) FACS-plots illustrating general GFP cell sorting procedure (debris-,duplets- and dead cells exclusion) and selection of distinct cell populations in two independent sorting experiments. In the first experiment, medaktinGFP cells were sorted without distincting between GFP ^{high} and GFP ^{intermediate} cells (R4) (30 000 cells). Equal amount of the remaining cells were sorted as control. In the second experiment, medaktinGFP^{high} cells (R4) were selectively isolated (7 500 cells), while medaktinGFP^{intermediate} cells (R5) were used in control. (b) RT-QPCR of selected genes performed on cDNA synthesized from GFP hi and GFP intermediate cells (dark grey) or GFP high cells only (light grey). RTQPCR results were normalized on the β-actin expression. SD are shown for three technical replicates.

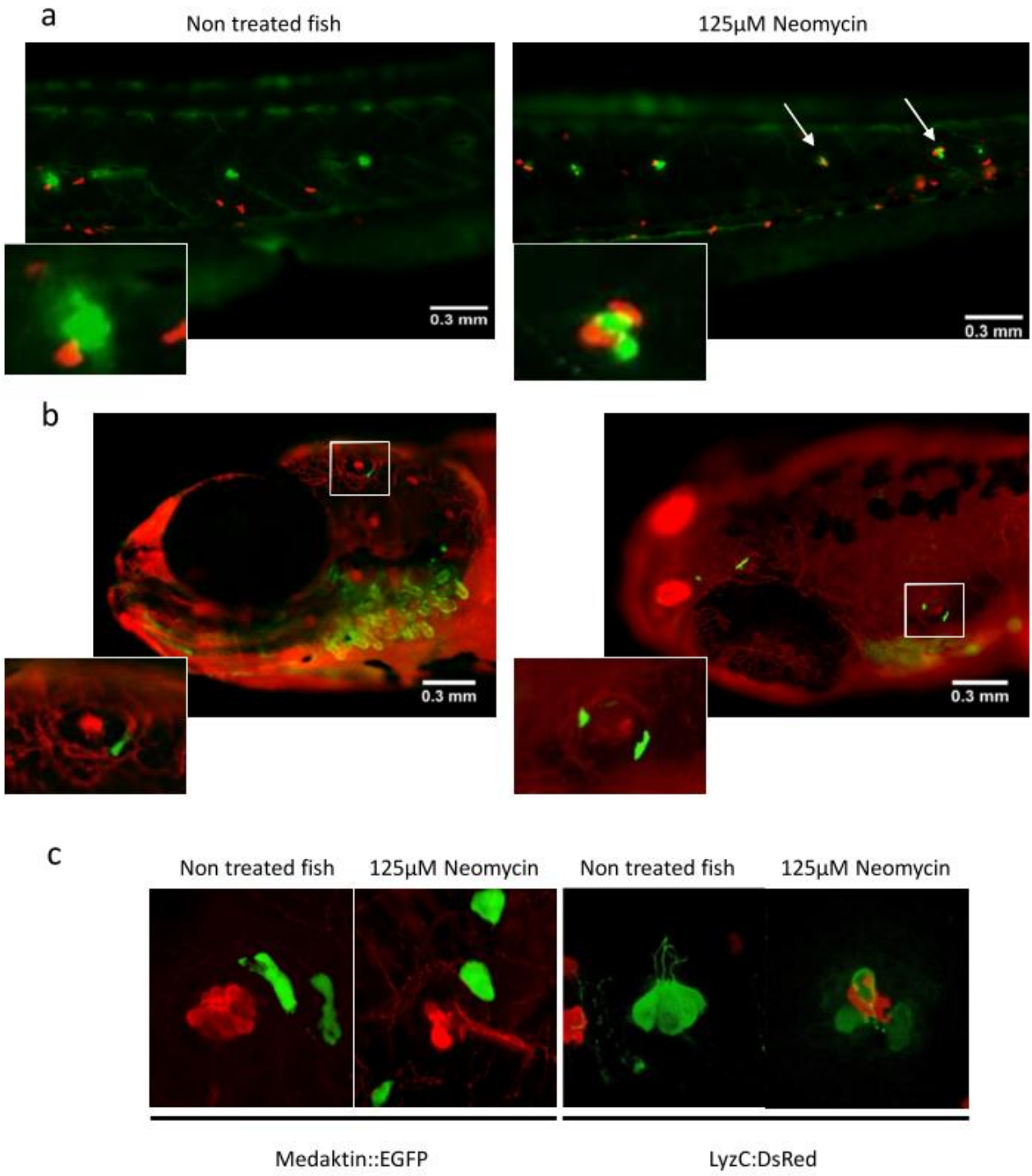

Figure 5. Leukocyte recruitment to neuromast upon neomycin.
LyzC::Dsred (a) or medaktin::EGFP (b) larvae were used to study leukocytes behavior after hair cells degeneration induced by neomycin treatment. LyzC:DsRed larvae were observed in neuromast of the tail region while medaktin:EGFP were observed in operculum. Hair cells were detected with anti-parvalubumin and anti-acetylated tubulin (green in the upper panel and red in lower panel). (c) Confocal images of leukocytes surrounding hair cells in control fish or under neomycin treatment.

Table I. DAVID functional annotation clustering of top 1000 genes for which there was at least 2-fold change.

Annotation Cluster 1	Enrichment Score: 5.22		
Category	Term	Gene count	P-Value
GOTERM_BP_FAT	**GO:0019882~antigen processing and presentation**	17	2,22E+06
GOTERM_CC_FAT	**GO:0042611~MHC protein complex**	14	1,81E+07
GOTERM_CC_FAT	**GO:0042613~MHC class II protein complex**	10	6,20E+08
GOTERM_BP_FAT	**GO:0006955~immune response**	20	8,68E+09
GOTERM_CC_FAT	**GO:0044459~plasma membrane part**	32	2,67E+11
GOTERM_CC_FAT	**GO:0005886~plasma membrane**	39	0.0020
GOTERM_BP_FAT	**GO:0002504~antigen processing and presentation of peptide or polysaccharide antigen via MHC class II**	5	0.0023

Annotation Cluster 2	Enrichment Score: 3.46		
Category	Term	Gene count	P-Value
GOTERM_CC_FAT	**GO:0070160~occluding junction**	11	1,38E+11
GOTERM_CC_FAT	**GO:0005923~tight junction**	11	1,38E+11
GOTERM_CC_FAT	**GO:0043296~apical junction complex**	11	2,86E+11
GOTERM_CC_FAT	**GO:0016327~apicolateral plasma membrane**	11	2,86E+11
GOTERM_CC_FAT	**GO:0044459~plasma membrane part**	32	2,67E+11
GOTERM_CC_FAT	**GO:0005911~cell-cell junction**	11	0.0026
GOTERM_CC_FAT	**GO:0030054~cell junction**	12	0.0385
GOTERM_MF_FAT	**GO:0005198~structural molecule activity**	20	0.0533

Table II. Comparison of the expression of selected genes in medaktinEGFP^{high} cells and control cells in the two sorting experiments.

	1st experiment			2nd experiment		
Gene	**Expression in GFP+/− cells**	**Expression in GFP++ cells**	**Relative enrich. (GFP++ / GFP+/−)**	**Expression in GFP+ cells**	**Expression in GFP++ cells**	**Relative enrich. (GFP++ / GFP+)**
Housekeeping						
tbp	85	113	**1,28**	1274	1054	**0,93**
gapdhs	2025	2632	**1,25**	89248	72364	**0,91**
actb2	27435	31190	**1,09**	871094	575903	**0,74**
actb1	15878	14664	**0,89**	385310	247392	**0,72**
eef1a1a	14	8	**0,55**	140	39	**0,31**
hprt1	75	23	**0,30**	5070	2649	**0,59**
gapdh	2	1	**0,51**	79	36	**0,51**
GFP	5	2261	**423,59**	1627	286960	**198,03**
Keratinocytic						
krt4	12196	34241	**2,69**	1628979	2422265	**1,67**
krt18	508	2522	**4,76**	122868	237212	**2,17**
krt17	1182	7755	**6,30**	93090	125429	**1,51**
krt8	799	5745	**6,90**	39364	106719	**3,04**
epcam	5859	7681	**1,26**	476390	800981	**1,89**
cldn23	69	2063	**28,64**	70058	408791	**6,55**
cldne	1488	8604	**5,55**	80370	286872	**4,01**
mesothelin	123	5644	**43,99**	9682	57109	**6,62**
Leukocyte						
ptpn6	18	62	**3,29**	3588	1943	**0,61**
lcp1	0	0	**1,00**	1769	1697	**1,08**
coro1a	98	66	**0,65**	9932	9273	**1,05**
rag2	3	2	**0,65**	126	15	**0,13**
cxcr3.2	26	10	**0,37**	1982	749	**0,42**
ptprc (CD45)	65	6	**0,09**	4637	246	**0,06**
Neutrophil						
mpx	17	1	**0,063**	226	0	**0,0006**
T cell						
plcg1	77	125	**1,56**	1279	1045	**0,92**
cd3eap	43	36	**0,80**	1225	389	**0,36**
camk4	8	4	**0,49**	128	672	**5,89**
cd8a	20	8	**0,39**	276	20	**0,08**
lck	11	4	**0,36**	338	144	**0,48**
DC-Mph						
mfap4.9	0	5	**40,19**	207	431	**2,34**
cd68	1182	5080	**4,12**	62391	104682	**1,88**
itgax	3	11	**3,42**	820	960	**1,31**
il13ra2	109	567	**4,99**	46259	82227	**2,00**
rgs2	596	2220	**3,57**	2419	7447	**3,46**

	1st experiment			2nd experiment		
Gene	**Expression in GFP+/− cells**	**Expression in GFP++ cells**	**Relative enrich. (GFP++ / GFP+/−)**	**Expression in GFP+ cells**	**Expression in GFP++ cells**	**Relative enrich. (GFP++ / GFP+)**
MHC I						
mhc1uba	166	1100	**6,35**	205818	225157	**1,23**
mhc1uca	52	283	**5,21**	41241	84121	**2,29**
mhc1zaa	1	5	**4,38**	3027	6314	**2,34**
mhc1zba	2515	9951	**3,80**	385419	675302	**1,97**
tapasin	5	41	**7,70**	1452	1363	**1,05**
TAP1	52	63	**1,16**	1102	1280	**1,30**
b2m	4187	10576	**2,42**	131292	201706	**1,73**
b2ml	4232	9024	**2,05**	108108	184206	**1,91**
TAP binding protein	63	133	**2,02**	3770	2599	**0,77**
tapbpl	8	72	**8,52**	3350	3782	**1,27**
MHC II						
mhc2dab	1718	4943	**2,76**	27908	47899	**1,93**
mhc2dbb	25	270	**10,32**	2752	8609	**3,51**
cd74a	8064	23882	**2,84**	148324	255383	**1,93**
cd74b	3078	8561	**2,67**	92590	167139	**2,03**
lgmn	649	3127	**4,62**	50748	37893	**0,84**
ifi30	1030	2242	**2,09**	37260	89485	**2,70**
ly75	335	683	**1,96**	4998	8676	**1,95**
ctso	0	11	**87,22**	660	1740	**2,96**
ctssb.2	708	2977	**4,03**	9719	13246	**1,53**
Autophagy						
dram1	8	59	**6,99**	4112	1889	**0,52**
sqstm1	787	2960	**3,61**	49194	131677	**3,01**
vamp8	299	1429	**4,58**	26803	77112	**3,23**
Microbicidial						
nos2a	2277	5498	**2,32**	913	2001	**2,46**
noxo1a	217	1265	**5,59**	1110	8175	**8,27**
noxa1	26	37	**1,36**	577	571	**1,11**
irg1l	3914	16095	**3,95**	612	1436	**2,63**
mpeg1.3	1	9	**7,80**	200	57	**0,32**
mpeg1.2	281	3039	**10,37**	20270	59211	**3,28**
mpeg1	201	68	**0,33**	25498	6781	**0,30**
IFN signalling						
tlr3	27	25	**0,89**	1334	1509	**1,27**
tlr4a1	4	7	**1,66**	240	1032	**4,83**
tlr8b	0	0	**1,00**	389	882	**2,55**
tlr2	2	8	**3,67**	253	513	**2,28**
irf1b	535	1133	**2,03**	6636	6492	**1,10**
irf7	200	333	**1,60**	10137	13913	**1,54**
irf3	22	18	**0,79**	327	565	**1,94**
nfkbiab	894	2882	**3,09**	49321	72991	**1,66**
nfkbiaa	1564	4913	**3,01**	32574	45439	**1,57**
myd88	54	168	**2,98**	5728	9189	**1,80**
irak3	34	117	**3,29**	3125	5178	**1,86**
ifi35	285	445	**1,50**	10148	12329	**1,36**
tbk1	254	621	**2,35**	9035	11828	**1,47**
cd9	837	2798	**3,21**	74447	159147	**2,40**

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General discussion

This thesis focuses on (antiviral) immunity using zebrafish as a model to understand the evolution and the essential conserved features of the innate defences of vertebrates. It focuses on mechanisms regulating IFN induction either upon infection – as demonstrated for transcription factor Plzf, or locally in sites particularly exposed to pathogens – with the impact of Ftr83 on steady state type I IFN level in gills and skin. On the whole organism level, the regionalization of immune response was investigated through the study of potential "sentinel" role of a subset of leukocyte-like cells located close to neuromasts in fish skin.

Zebrafish as a model to study the immune system: advantages and pitfalls

The zebrafish is a small tropical freshwater fish, easy to grow at relatively low cost. It became a very popular model for developmental studies as the 1mm-diameter egg are transparent, allowing observation of the embryo. While pigment cells progressively appear, addition of PTU in the water blocks the process and allows imaging for much longer period, and at the whole body scale. Among the many mutants there are also available mutant fish that lack pigment cells.

When larvae hatch, they only have innate immunity, as lymphocyte populations will progressively appear during the first weeks of the life of the fish. This natural "knock out" system has been used a lot to investigate the mechanisms of innate immunity, in absence of interference with adaptive immunity and lymphocyte responses (241,272). As this innate system is well-conserved across vertebrates, at molecular and cellular levels, it constitutes a very useful model to explore immune response to infections, including viral infections.

A number of techniques and resources available in the zebrafish model have proved very useful for the study of immunity. In addition to live imaging allowed by the transparency of the embryo and to some extent larva, whole-mount *in situ* hybridization (WISH) and immunohistochemistry (WIHC), are also very useful as these methods provide whole body assessment of relevant gene expression, and also an easy localization of pathogens in infected animals. This characteristic is very important to the study of host-pathogen interactions as well as the regionalization of the antiviral immunity, and makes the zebrafish a good model for it.

Regarding genetic resources, a genome sequence is available for zebrafish as well as a high-quality assembly (quality akin to mouse and human) making it the best available reference genome among fish (273). In addition to that, transgenesis and mutagenesis protocols are well established, and vast amount of mutants and transgenic lines of zebrafish are available. Of key importance for immunologists, a number of reporter transgenic lines in which promoters of key immune genes, or markers specific for particular immune cell types are available, and allow to follow leukocyte activation, migration and recruitment at inflammatory sites (213,214,216,266). The good genome assembly is also a very favorable context for using CRISPR editing methods, and will likely lead to easy and systematic directed mutagenesis of immune genes. Another approach for loss of function experiments is transient gene knock-down, that is possible *in vivo* by injection in zebrafish eggs of morpholinos (antisense oligonucleotides blocking translation or splicing of mRNAs). For example, such an approach was used to identify zebrafish Ifn receptors.

Regarding antiviral innate immunity, zebrafish is certainly one of the non-mammalian vertebrates that is the best studied. Type I Ifn's have been found as well as many of the key signaling factors involved in their induction after virus sensing. Virus PRR such as TLR and RLR have been identified, and transcriptome studies after infection already provided a good overview of the ISG repertoire, including fish specific genes as well as a core list conserved through vertebrates.

Concerning, host-pathogen interactions, zebrafish has been proven to be a good model for recapitulating key events of important diseases. The most famous disease model in zebrafish is tuberculosis, as it allowed the discovery of the key events leading to granuloma formation.

With respect to viruses and viral diseases, zebrafish models have been set up for a number of viruses (Table I), including some human pathogens. In particular, a chikungunya virus model - a re-emerging alphavirus that caused recently an epidemic in the Caribbean islands was developed in zebrafish larva by Jean-Pierre Levraud and colleagues; this virus induced a very strong type I IFN response that usually restrains infection (71,274). There are many unresolved questions about chikungunya

infection, such as how the persistence of virus is established and does it cause chronic symptoms. Unlike chikungunya and other virus models in mice, following virus infection in zebrafish allows to discern viral invasion mechanisms *in toto* and *in vivo* and study how, where it replicates, spreads, persists and individual differences in infection course can be studied. The model already proved to be very good to follow the propagation of the virus in the whole organism, and to identify the tissues producing IFN using a type I IFN reporter transgenic line (71).

Fish viruses (especially rhabdoviruses SVCV and IHNV) were also shown to cause

	Human viruses	**Fish viruses**
DNA viruses		
Adenoviridae	Adenovirus 5	
Herpesviridae	Herpes Simplex Virus-1(HSV-1)	
Iridoviridae		Lymphocystis disease virus (LCDV) Infectious spleen and kidney necrosis virus (ISKNV) Epizootic hematopoietic necrosis virus (EHNV) Tiger frog virus (TFV) Rana grylio virus (RGV)
	Hepatitis B Virus (HBV) Hepatitis C Virus (HCV)	
RNA viruses		
Rhabdoviridae		Spring Viremia of Carp Virus (SVCV) Infectious hematopoietic necrosis virus (IHNV) Snakehead rhabdovirus (SHRV) Viral hemorrhagic septicemia virus (VHSV)
Birnaviridae		IPNV(like)
Nodaviridae		Nervous necrosis virus (NNV)
Alphaviridae	Chikungunya virus (CHIKV)	
Orthomyxoviridae	Infuenza virus A	
Retroviridae		Zebrafish endogenous retrovirus (ZFERV)

Table 1. Viral infection models in zebrafish. Taken from (272).

infections in the zebrafish larva, and are interesting tools to characterize the zebrafish antiviral response. The capacity of other fish viruses such as birnaviruses to induce IFN response in zebrafish and other fish species, has also been studied in the lab. Such models are promising to develop antiviral drug screens in the future - as zebrafish larvae may be tested in 96 well plates, and also provide a favorable context to characterise the IFN system and its evolution.

With respect to other disease models, the zebrafish has also become a popular model for recapitulating different genetic diseases or to characterize tissue regeneration thanks to all the advantages mentioned above. The last part of this thesis is a characterization of leukocyte-like cells located close to neuromasts – mechano-sensory organs which have hair cells at the core. Importantly, neuromast is a highly dynamic structure as hair cells constantly degenerate and are replaced by new ones from the local stem cells – a feature not shared by the hair cells of the inner ear (275). Loss or defects of sensory hair cells in the inner ear is a major cause of deafness (276). The zebrafish has become an excellent model to study the regeneration of hair cells, as their superficial localization in the skin - in the neuromasts, the sensory organs of the fish lateral lines - enables time-lapse imaging. As the lateral line develops within the first week of development, it makes it accessible to the advanced imaging and manipulation of gene expression using morpholinos. The development of fluorescent reporter transgenic lines - such as the medaktin:EGFP in this thesis, allowing *in vivo* visualization of potentially relevant cell types is an important point to develop a zebrafish model to study the hair cell biology and the importance of inflammation for their loss or damage.

The zebrafish also have pitfalls. Growing size and pigmentation make imaging more and more complicated when fish develops and genetic loss-of-function approaches using morpholinos become hardly possible after one week. Another important disadvantage is the lack of zebrafish cell lines; only a few are available and two have been developed in the lab, but they are not easy to transfect. This raised difficulties for the characterization of Plzf during my project, and we had to use fathead minnow cells (EPC cell line, from another cyprinid fish), instead. Lack of cell markers,

specific antibodies against membrane receptors of leukocytes and also lack of knowledge about leukocytes subsets greatly complicate the study of immune responses and antiviral defenses. Also, there is no natural zebrafish virus available (277), and the route of infection in most infection models is therefore artificial (generally microinjection). These few negative features will likely be overcome in the future through development of CRISPR genome editing, tissue transparization for microscopy (although this excludes *in vivo* imaging), or via identification and characterization of new infection models.

Type I IFN signalling is multilayered – insights from the zebrafish model about non-canonical modulators

Canonical pathway leading to ISG induction, that consists of the activation of transcription factors like STAT1, STAT2 and IRF9, has been outlined decades ago; nonetheless large-scale studies have shown that there must be additional mechanisms in place as the ISG profiles differ depending on tissue and virus (102). Furthermore, a large group of ISG consists of regulators and of signaling factors, and participate in feedback loops that affect the type I IFN system (97). This indicates that IFN signaling is very complex and there is still much to learn about it. In fact, most of the factors influencing the IFN response and the mechanisms of most ISG remain unknown, and a number of teams are working to reveal them (82, 184).

How type I IFN signalling is controlled? Crosstalk between different pathways

To counteract the various subversion mechanisms used by viruses, IFN and ISG induction does not rely solely on one pathway. For example, IRF1 can upregulate a subset of ISG in an IFN-independent manner (278). Also, IRF3, which is a chief IRF in IFNβ induction, can induce a subset of ISG – thus, depending on the infection dynamics, IRF3 can modulate IFN system at multiple levels via the upregulation of IFNs and/or ISG (279).

The cellular context is another variable that might affect IFN response as different signalling pathways converge or intermingle with the IFN pathway. In fact, type I IFNs can affect broad biological functions in immune system. It has been proposed

that the effect of IFNR stimulation depends on the cellular availability of different type of transcription factors as well as the activation of other pathways (Figure A), such as B-cell differentiation, apoptosis etc. One can envisage that the differential induction of ISG profiles could be affected by the specific set of transcription factors that are present in a particular cell type as well. The convergence and redundancy of pathways is illustrated by the fact that IRF1-activation depends on enzymes other than JAKs: indeed, a mutation in IFN receptor hindered the binding of JAK and therefore abrogated canonical type I IFN signalling, but did not block the activation of IRF1 (280).

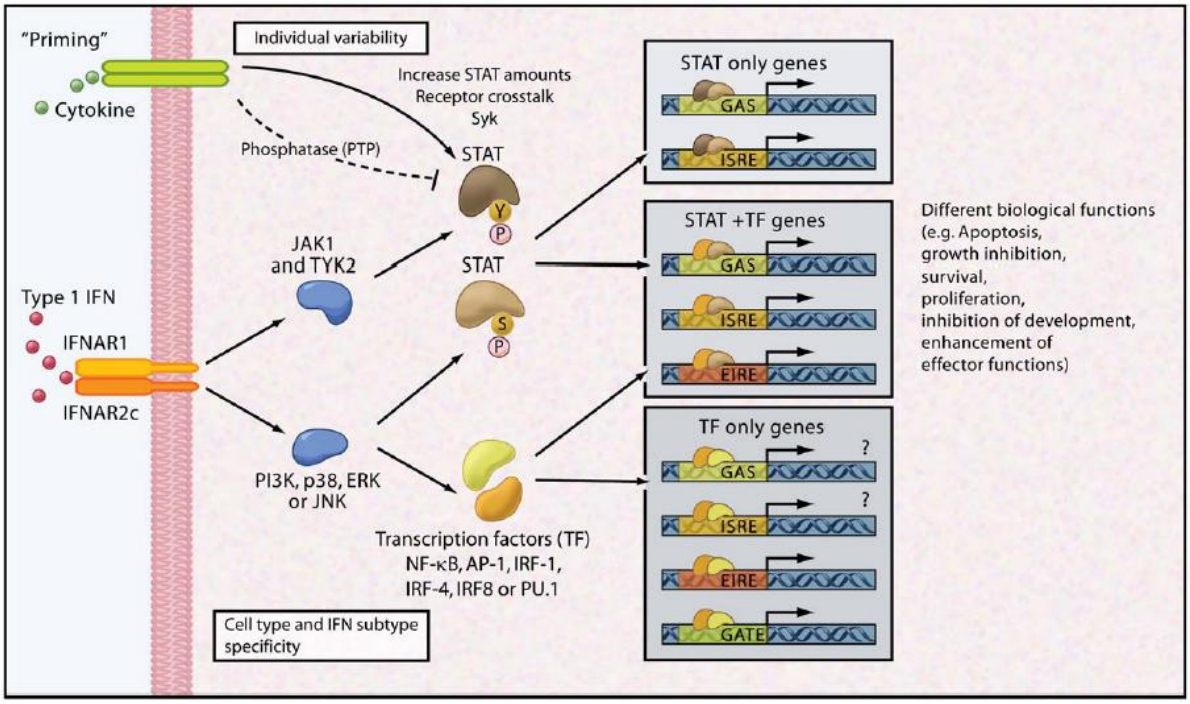

Figure 1. Model to explain the different biological outcomes of IFN signalling. The activation of other cytokine pathways and availability of non-canonical transcription factors results in differential expression of genes. Schematic from (281).

PLZF – a non-canonical modulator of type I IFN system

PLZF is a BTB/POZ transcription factor that has been implicated in a variety of biological processes: spermatogonial stem cell self-renewal (152), tumorigenesis (133), leukocyte differentiation/maturation (150,159,282) and just lately in anti-microbial responses (141,282). Regarding IFN signaling, PLZF was shown to upregulate a subset of ISG *in vitro* in response to type I IFN. Furthermore, these ISGs were not inducible any more in PLZF knock-out mice. PLZF directly induced the

promoter of selected ISG, and this effect was augmented by HDAC1 and a TRIM ubiquitin ligase PML (282). Interestingly, JNK kinase activity was crucial for PLZF's capacity to induce ISG. This seminal work positioned a BTB/POZ protein in the regulation system of type IFN I response, supporting the notion that there remains likely unknown factors that can modulate IFN signaling.

In my thesis, I explored whether zebrafish Plzf is involved in type I IFN signaling. Zebrafish have two Plzf paralogues – Plzfa and Plzfb. Although both are orthologues of mammalian PLZF, Plzfb has diverged more than Plzfa. In contrast to mammalian PLZF which upregulated ISGs, both zebrafish paralogues acted by augmenting type I IFN transcription, at an early step of the response to non-enveloped dsRNA viruses *in vitro*. In the presence of dominant-negative Irf3, Plzfb transcription factor did not increase IFN induction, suggesting that Plzf modulate *ifnφ1* promoter upstream from IRF3. Interestingly, BTB-domain was dispensable for PLZF activity on IFN regulation.

Although the mechanism remains unknown, the fact that BTB-domain was not necessary suggests that IFN augmentation is achieved via different mechanism than ISG-induction observed in mammals as HDACs interact with PLZF transcription factors via adaptors that bind BTB-domain (120,123). Nonetheless, it can also be assumed that HDAC might interact with Zn-finger moiety instead. It has been shown that PLZF can be acetylated on selected Zn-fingers by histone acetyl-transferase (HAT)p300 (145). Thus, it is possible that HDAC can interact with the same sites as HATp300, and complex with PLZF via Zn-finger moiety. If this is the case then zebrafish Plzf might upregulate Ifn when complexed with HDAC as well. This interaction might affect the promoter binding specificity of PLZF as well as the activity of other proteins in the complex and chromatin.

The lack of PML (TRIM19) in zebrafish also suggests a different mechanism, from what is known in the mouse. Interestingly, we have data showing that zebrafish Plzf proteins can interact with another TRIM protein – Ftr82. The interactions of mammalian PLZF with PML and zebrafish Plzf with Ftr82 are both mediated by Zn-finger moiety. In our manuscript "Constitutive IFN induction by Ftr83 in exposed

surfaces of fish", we show that Ftr82 does not modulate Ifn responses, in contrast to Ftr83. However, we have not yet fully analysed the possible interaction between Ftr83 and Plzf, or the impact of co-expressing Plzf's and Ftr82 (and/or Ftr83). Hence, the possibility remains that together they have an impact on IFN system.

The most direct mechanistic insight into zebrafish Plzf function is that Plzf-mediated augmentation of Ifn was "upstream" of Irf3. It is possible that Plzf complexes with Irf3 and modulates its activity. However, as we overexpress Plzf prior to the stimulation of IFN system for several days, it is also possible that Plzf regulates the expression of other genes, which upon the triggering of PRR pathway are activated and amplify Ifn transcription in an Irf3-dependent manner. Importantly, it has to be noted that in our approach, zebrafish transcription factor modulates IFN signalling in a cell line derived from another, closely related fish species - the fathead minnow. This is legitimated by the high conservation of most signalling factors within cyprinid fishes, but would deserve further investigation in zebrafish cells.

In conclusion, the finding that zebrafish Plzf can modulate type I IFN suggests that PLZF-like protein in the common ancestor of fish and mammals was involved in anti-viral immunity. This is interesting as it is a rare example of non-canonical transcription factor that participates in type I IFN system. However, the initial descriptions of mammalian and fish PLZF suggest that it has evolved to play different roles. Furthermore, it is also possible that in zebrafish, which has two Plzf paralogues, subfunctionalization has occurred as these genes have slightly different expression patterns, and Plzfb seemed to be a more potent type I Ifn amplifyer.

Perspectives

The involvement of HDAC in the regulation of IFN responses has not been extensively studied. In one study, HDAC inhibition suppressed the transcriptional activation from ISRE showing that HDACs are likely part of the transcriptional complex (283). The fact that ISG-induction by mammalian PLZF was further enhanced in the presence of HDAC1 exemplifies that HDACs could also be essential components in IFN system. Thus, it would be interesting to clarify if zebrafish Plzf also co-operates with HDAC in the augmentation of Ifn and if so via which

mechanism.

The PLZF knock-out mice were less resistant to infections with neurotropic viruses which suggests that PLZF could also influence tissue-specific responses. Interestingly, the zebrafish Plzf genes are both highly expressed in nervous system. It would be interesting to know if Plzf knock-out in zebrafish would also make them more susceptible to infections with (neurotropic) viruses.

Additionally, the involvement in type I IFN system of other BTB/POZ family members could be addressed. Many of them are good candidates as they have been shown to be necessary in the differentiation or maturation of leukocytes (*e.g.* ZBTB46 in dendritic cells, BCL6 in macrophages).

Regionalization of immune response

One reason for differential IFN response on an anatomical level could lie in the biological function of a given organ. For instance, respiratory system is continuously exposed to pathogens and thus requires additional means of protection (mucus) and means to trigger quick responses (low basal level of signaling), whereas brain or eye have to be protected without eliciting massive inflammation that would cause irreversible damage. Indeed, the existence of regional immunological differences has been known for a long time as some organs such as eye are dubbed immune-privileged since they do not mount destructive immune response towards foreign material. As for type I IFN system, transgenic IFN reporter mouse and zebrafish have constitutive expression in some organs: thymus for mouse and liver and leukocytes for zebrafish(71,284). However, such expression might be related to other IFN biological functions and transgene might not recapitulate the endogenous expression of IFN fully. More importantly, it has been observed that even ISG expression can be detected in some tissues during non-infected state and during infection these tissues respond more quickly and resolve the infection whereas tissues that lack basal expression of ISG succumb to infection (285,286). The question remains what could be the intrinsic regulators that maintain the constitutive activation of IFN signaling.

Is the constitutive activation of type I IFN signaling in mucosa a mechanism by which TRIM protect surfaces exposed to microbes?

TRIM is a large family of ubiquitin ligases of which many are implicated in type I IFN signaling (182,192). Some TRIM are effectors that restrict viruses directly via different mechanisms (94,195,196), whereas others modulate type I IFN signaling (198,199), however for most the exact role is not known. Indirect suggestion for the involvement in anti-viral interactions is the fact that TRIM genes have specifically expanded in different species and have undergone positive selection – diversification, which implies that they are interacting with highly evolving pathogens (185,206,210).

During my thesis I participated in the study of two fish-specific TRIM proteins – Ftr82 and Ftr83. These protein share 55% of sequence similarity, can heterodimerize and yet have completely different activity. Ftr82 did not potentiate type I IFN signaling, whereas Ftr83 induced type I IFN very strongly and thus protected cells against rhabdoviruses which are highly virulent. This effect was Irf3-dependant. Intriguingly, Ftr83 expression in the gills correlated with higher basal level of type I IFN at steady state. Overall, Ftr83 expression was mainly restricted to mucous pathogen-exposed organs (pharynx, gills and skin) in larvae as well as adult zebrafish. Furthermore, its expression was not induced by type I IFN or virus infection making it an intrinsic factor of the tissue. Therefore, this TRIM protein might be a factor determining the tissue-specific difference in IFN response in gills which along other factors such as the presence of mucus and specific adaptive responses as described for fish mucosal IgT (287), secures a greater protection in an organ highly exposed to pathogens.

Perspectives

Although Ftr83 expression correlated to that of type I Ifn in the gills, we do not know which particular cell types expressed it. Is it more expressed in specialized leukocytes or epithelial cells or is it homogenously expressed in the whole organ? Furthermore, it would be interesting to test if same correlation persists in germ-free animals as the continuous presence of pathogens might keep the tissue in slightly inflamed state. Nonetheless, *in vitro* experiments demonstrated the potency of Ftr83 as a viral

restriction factor. Although Ftr83-mediated induction of Ifn was dependent on Irf3 we still do not know how it regulates this axis. The importance of RING-domain (ubiquitination capacity) and its potential targets could be investigated. Potential candidates could be transcription factors Plzf's which also have the ability to augment type I IFN, albeit much less. Plzf can interact with Ftr82, interaction with Ftr83 is not known, but Plzf's are quite expressed in the skin and gills of adult animals, which suggests that they might interact *in vivo*.

Ftr82 and Ftr83 belong to a fish-specific TRIM subfamily for which mammalian orthologs do not exist. It would be interesting to know if a number of mammalian TRIM play a similar role to Ftr83 in the pathogen-exposed organs, as already described for TRIM9 (288).

Leukocytes specialized in sentinel/homeostatic functions of particular sites

Additional aspect of immune defense is the strategic localization of specialized leukocytes at different sites. For instance, many macrophage subtypes exist with distinct properties (Figure 2). Zebrafish neuromasts are special structures in the skin with several features that could suggest that the presence of specialized leukocytes is likely. Firstly, the epidermal structure in the region of neuromast as well as hair cell properties (fast cell turn-over and apical endocytosis rate) make it susceptible for pathogen entry and suggests that additional means to protect this area from pathogens is required (248,253–256). Secondly, it represents a dynamical environment where stem cells differentiate into new hair cells as mature hair cells degenerate – although there is currently no evidence that leukocytes are crucial for hair cell regeneration, this possibility remains (245,289–291). Additionally, neuromast hair cells are innervated by sensory neurons – as water movement can be rapid these synapses are likely very active and might require similar pruning as the cerebral neurons by microglia (257).

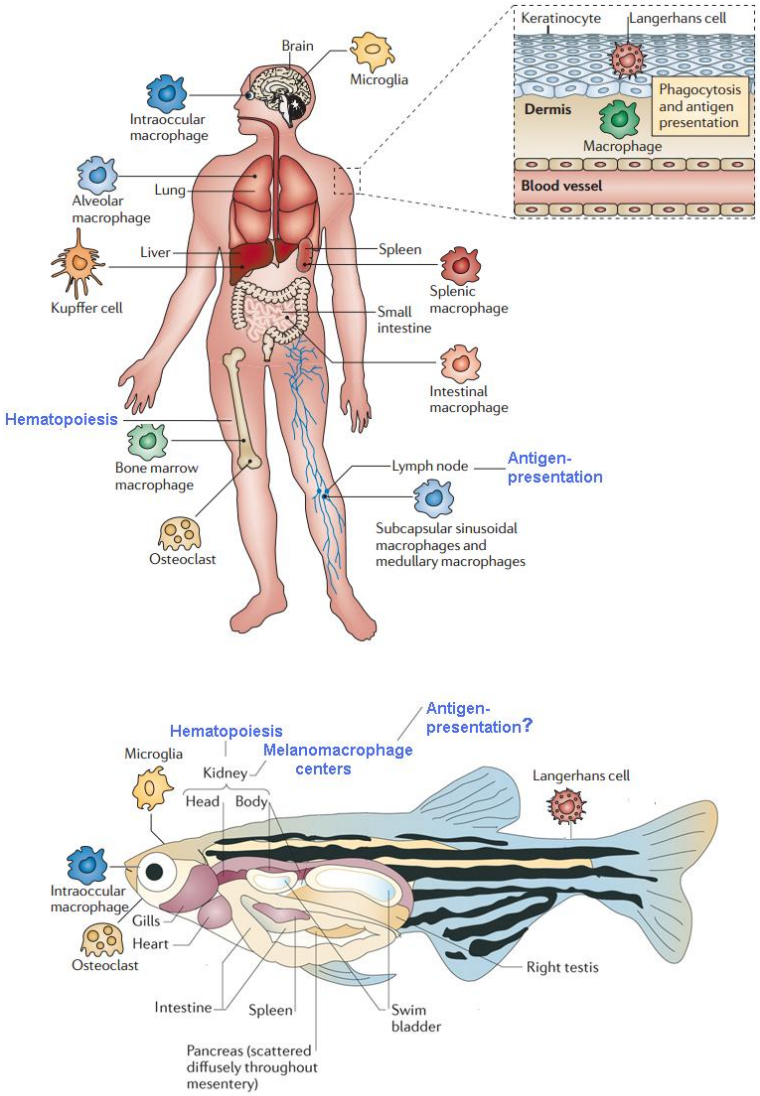

Figure 2. Macrophage subsets in human and fish. In humans many resident macrophages have been described: for example, alveolar macrophages that eliminate dust and allergens, Kupffer cells in liver that eliminate pathogens and toxins, microglia that emit trophic signals to neurons and clear neurotransmitter debris, osteoclasts that are involved in bone remodeling. In zebrafish the existence of several resident macrophages is suggested: microglia, osteoclasts, macrophages in retina, as well as Langerhans cell-like macrophages. Schematics adapted from (292,293).

Medaktin:EGFP – a transgenic reporter line marking leukocytes associated to neuromasts

In this thesis I have characterized with the aid of a transgenic fluorescent reporter line a subset of leukocytes close to neuromasts. These cells express GFP very highly and appear at 7-8 dpf – at a time when the first mature lateral line has already formed thus implying that these leukocytes are not necessary for the development of neuromasts, however the order of appearance recapitulates the development of lateral line, meaning first cells appear in the head (292). Time-lapse imaging of larvae revealed that these cells have to phenotypes: most of them are motile cells that patrol in

circular movements whereas some rare cells were less motile and reached out dendrites reminiscent of sampling the environment – showing that this might be a slightly heterogenic population in which cells might acquire specific phenotypes in some surroundings. The transcriptome of these cells implies that they have antigen presentation capacity as well as microbicidial properties. Additionally, they express several macrophage/dendritic cell markers. The first functional studies imply that they are indeed sentinel cells of neuromast as they do not leave their positions to different inflammatory cues (tailfin amputation, peritoneal inflammation) which is true for other macrophages (235,294). However, the local destruction of hair cells with neomycin or copper did not cause any significant behavioral change either – although it was observed in some cases that these cells become round and thus could be entering apoptosis or mitosis, this observation needs to be confirmed. As this transgenic has GFP expression in other cell types as well, it is interesting to note that high expression of the transgene is also observed in the gills – an organ where additional means of immune defense are likely required. Albeit, there the GFP expression is not in leukocyte-like cells.

Perspectives

One of the most immediate questions about this subset of cells is what is the gene which expression is recapitulated. This transgenic was made with the promoter fragment of medaka's β-actin gene, however the mosaic expression pattern implies that there is an insertional effect, thus the sequencing of the transgenes' insertion area is one perspective. The fact that no medaktinGFP^{hi} cells were observed in organs other than skin suggests that the transgene recapitulates the expression of some homing receptor or adhesion molecule for "neuromast-associated" cells. As two behaviors were observed, and when medaktin:EGFP were crossed with mpeg1:mCherry fish a major double-positive but also a rare GFP-single positive cellular population was seen, it would be of interest to define these subsets better. Furthermore, the mpeg1 reporter transgenic line has not been extensively studied at adult stage, so it is not entirely clear which cell types it labels – our data strongly suggested that at least a subset of GFP positive cells from the medaktin:EGFP line

belongs to myeloid lineage, possibly being particular dendritic cells. As we have not observed striking differences in medaktinGFP^{hi} cells localization in neuromast during hair cell destruction, it would be of interest to isolate the cells during this process and sequence their transcriptome. Thus, activation state of Medaktin cells could be determined and cytokine/chemokines expression investigated. Current analyses will be extended to model of nerve degeneration to evaluate the potential role/interaction of medaktinGFP^{hi} cells with injured nerves as previously demonstrated for macrophages (295).

Furthermore, medaktin:GFP transgenic fish will be exposed to invading pathogens (viruses and bacteria) by immersion to determine the possible role of these cells as sentinels in the skin during infection processes.

Conclusions

During the preparation of this thesis I enjoyed working on two research lines: the particularities of antiviral signalling and the study of specialized leukocytes associated to neuromasts. This and the insights from evolutionary comparisons provided me with a synthetic view on innate immune defenses.

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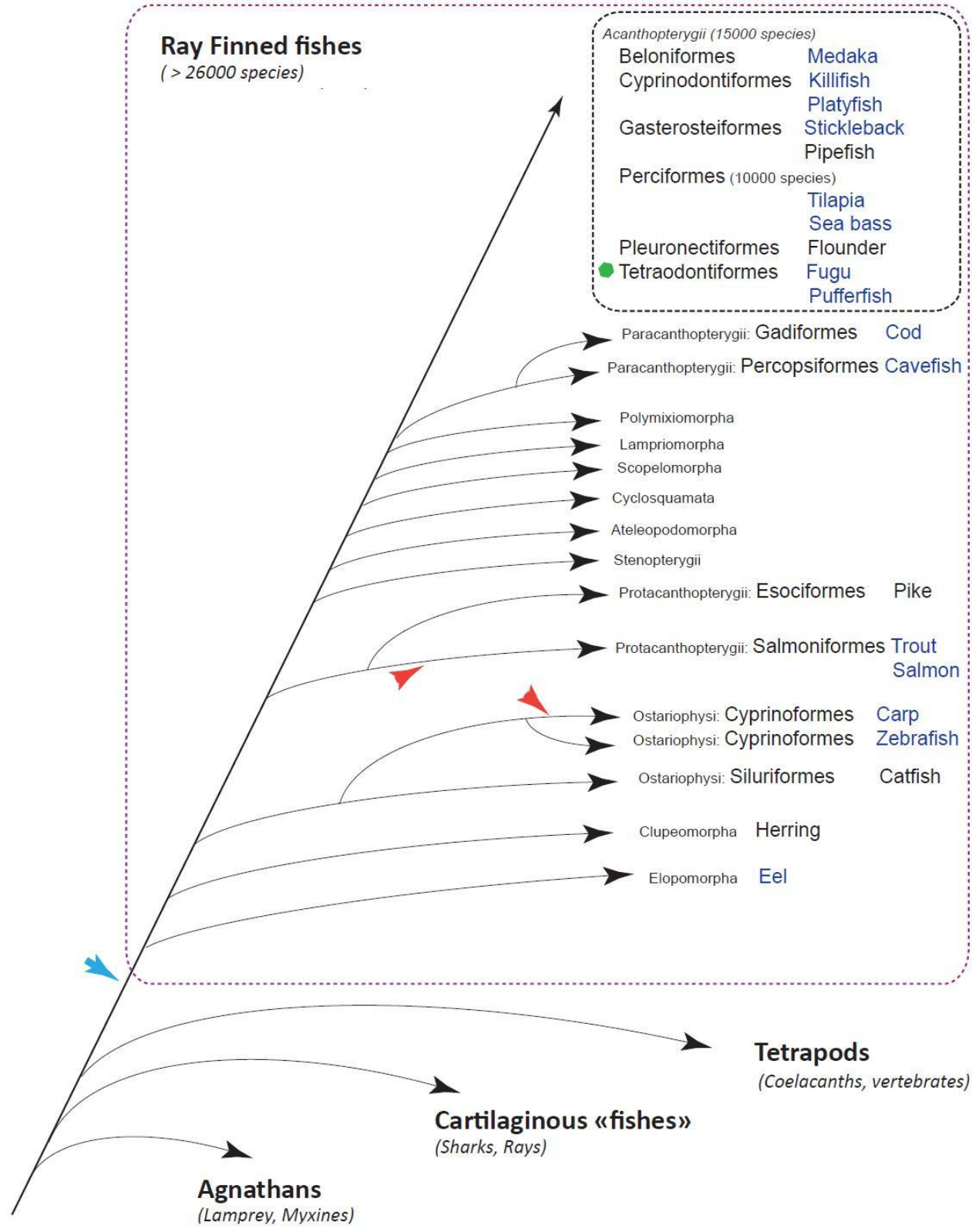
Annexes

Phylogenetic tree of fishes

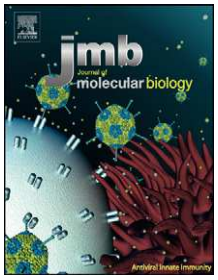


Annex 1. **Schematic phylogenetic tree of Fishes.**
Red arrows indicate examples of posterior WGD in specific branches. The green dot denotes an example of genome contraction in tetraodontiformes. A genome sequence is available for all species in blue. Adapted from Magadan, 2015.

Review paper

The antiviral innate immune response in fish: evolution and conservation of the IFN system

Review



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

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Abstract

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

Introduction

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

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

Fish IFNs

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

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

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

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

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

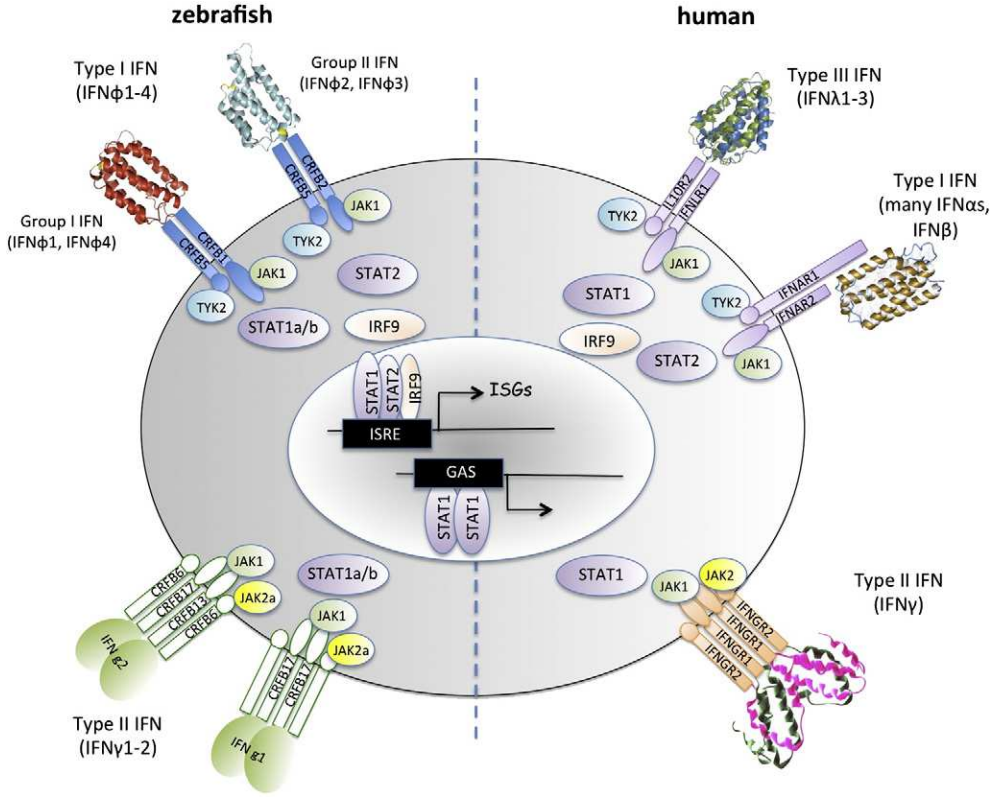

Fig. 1. Schematic representation of zebrafish IFNs and their receptors. Tridimensional representations of IFNs are from the Protein Data Bank (accession numbers: 3PIV, zebrafish IFNϕ1; 3PIW, zebrafish IFNϕ2; 3HHC, human IFNλ3; 1AU1, human IFNβ; 1HIG, human IFNγ).

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

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

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

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

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

Virus sensors in fish and their signaling pathways

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

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

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

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

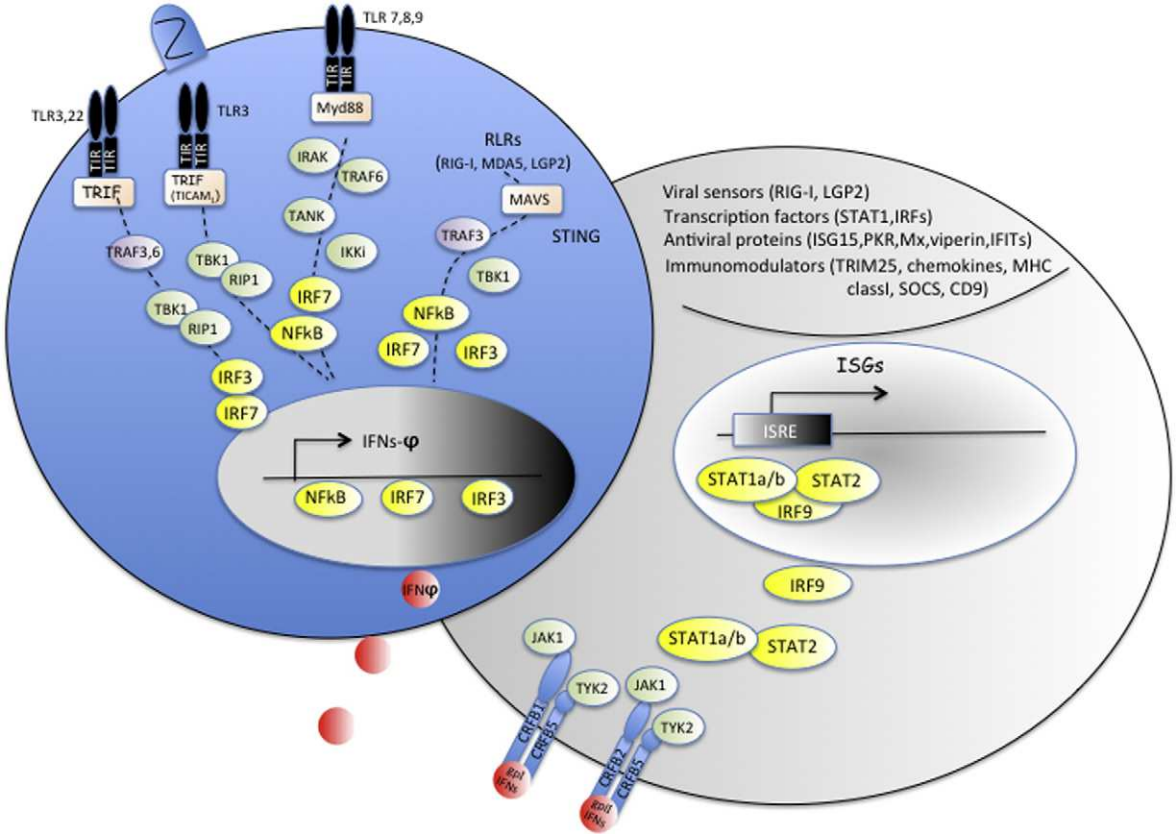

Fig. 2. Schematic representation of IFN signaling pathways in fish. Adaptor molecules are represented in orange, kinases are in green, TRAFs are in purple, transcription factors are in yellow, and IFNs are in red.

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

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

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

Conserved signaling pathways downstream of IFN receptors

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

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

Part 2. ISGs and Their Diverse Evolutionary Patterns

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

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

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

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

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

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

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

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

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

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

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

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

Part 3. IFN-Producing Cells

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

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

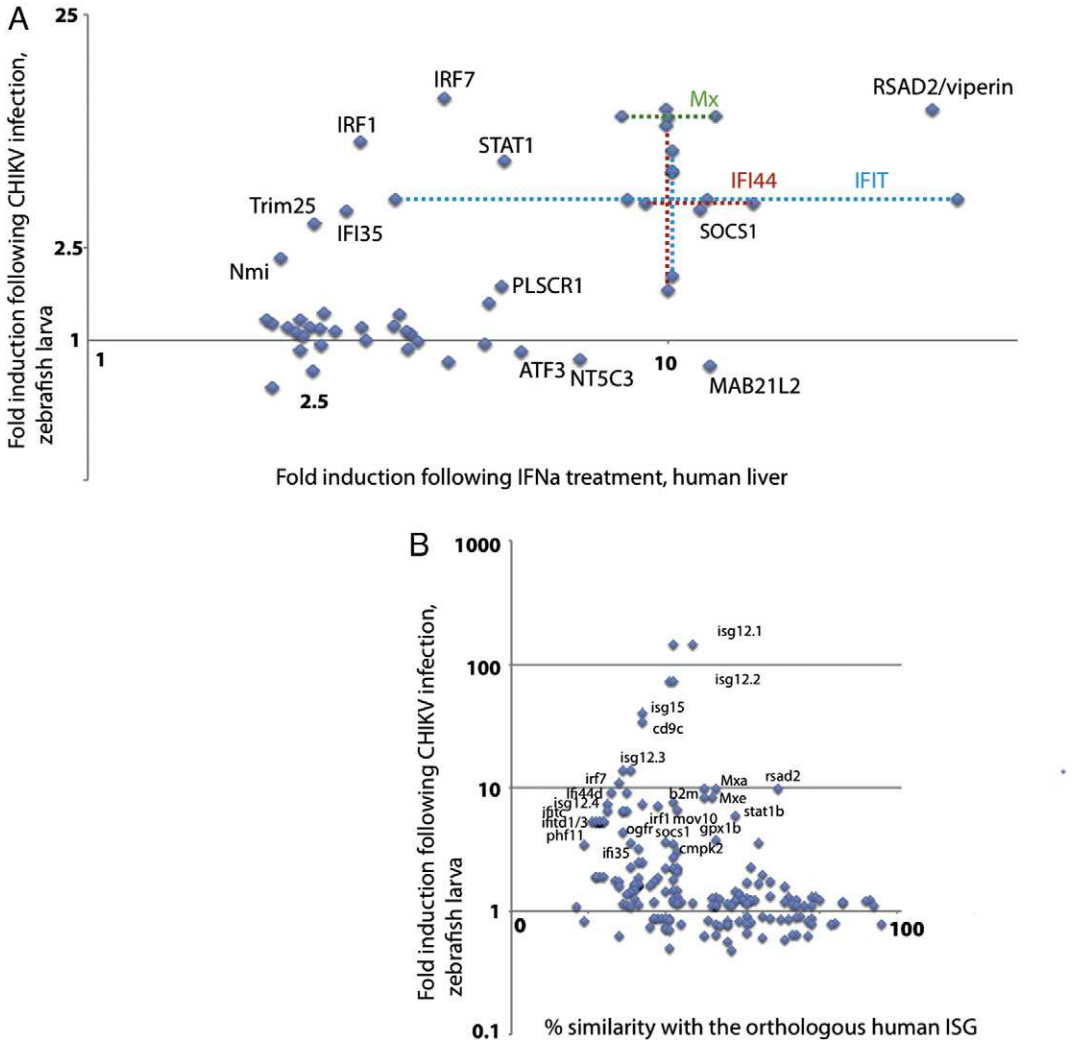

Fig. 3. Assessment of the conservation of ISGs: comparison of induction levels and sequence similarity between human ISGs and their zebrafish orthologues. (A) Induction levels of human ISGs (liver biopsy cells treated for 4 h with IFNα, from Sarasin-Filipowicz *et al.* [117], GEO accession GSE11190)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

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

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

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

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

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

Part 4. Kinetics of the Different IFN Responses in Fish

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

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

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

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

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

Part 5. Tissue-Specific Responses

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

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

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

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

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

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

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

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

Part 6. Subversion Mechanisms by Viruses in Fish

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

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

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

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

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

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

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

Conclusion

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

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

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

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Résumé en français (version longue)

Le système immunitaire des poissons possède les types cellulaires et les facteurs moléculaires fondamentaux du système immunitaire des Vertébrés, alors que leur organisation anatomique et leurs adaptations physiologiques au milieu aquatique en font un groupe très particulier. Cependant, les poissons et les tétrapodes ayant divergé il y a plus de 400 millions d'années, les gènes des facteurs du système de défense ont évolué en parallèle sur une très longue période. Ce groupe est donc à la fois pertinent pour étudier les caractéristiques fondamentales et conservées de l'immunité des Vertébrés, et pour analyser les patrons évolutifs associés à des adaptations et à des histoires génomiques différentes, en comparant par exemple les poissons aux mammifères.

Dans cette thèse, nous avons étudié différents aspects de l'immunité d'un poisson modèle, le poisson zèbre ou danio rayé (Danio rerio): nous nous sommes attachés à mieux comprendre certains mécanismes de l'immunité innée antivirale, à travers l'étude d'un facteur de transcription, PLZF, et d'une famille de facteurs de restriction viraux , les TRIMs. Nous avons également mis à profit les avantages étonnants du poisson zèbre pour l'imagerie en caractérisant une lignée "rapporteur" où des cellules de morphologie leucocytaire localisées à proximité des neuromastes expriment fortement la GFP. Ces cellules présentent de nombreuses caractéristiques suggérant qu'elles appartiennent au lignage myéloïde, et pourraient constituer des cellules sentinelles associées à ces structures sensorielles.

Les poissons possèdent des interférons (IFN) de type I qui possèdent une forte activité antivirale et qui, comme leurs homologues chez les mammifères, orchestrent la réponse antiviral innée. Ces cytokines sont codées par des gènes possédant des introns et se lient à des récepteurs qui rappellent les récepteurs des IFN de type III, mais leur structure en fait des IFN de type I parfaitement typiques. Ces IFNs sont induits après détection de l'infection virale par la cellule via un répertoire de récepteurs appartenant aux familles de senseurs de molécules d'origine virale (récepteurs intracytoplasmiques de la famille RIG-I, récepteurs de type Toll ciblant les acides nucléiques viraux, ...) et des pathways de signalisation bien conservés entre

les poissons et les mammifères.

Les IFN de type I du poisson zèbre induisent de nombreux gènes (ISGs) dont un certain nombre codent des protéines effectrices possédant une activité antivirale, ou des protéines régulant la réponse. Bien qu'un groupe central d'ISG soit bien conservé à travers les vertébrés, et joue un rôle central dans la réponse, les fonctions et le mode d'action de la plupart des ISGs demeurent mal compris.

Le facteur de transcription PLZF pour " Promyelocytic Leukemia Zinc Finger ", aussi nommé ZBTB16, appartient à la famille des BTB/POZ et possède un domaine BTB et un domaine à doigts de Zinc. Il a été récemment identifié comme un régulateur majeur de l'induction d'un sous ensemble d'ISGs chez la souris et l'homme. Les souris chez qui ce gène a été muté sont beaucoup plus sensibles à différentes infections virales. Nous nous sommes intéressés à l'implication des homologues de ce gène dans la réponse antivirale des poissons. Le poisson zèbre possède deux (co)orthologues de PLZF (Plzfa et Plzfb), qui ont des patrons d'expression différents chez la larve de poisson zèbre en développement, et chez l'animal adulte. Bien que Plzfa et Plzfb ne soient pas modulés par l'IFN de type I induit lors de la réponse antivirale, leur sur expression dans la cellule conduit à une augmentation du niveau d'induction de l'IFN par le poly I:C. Cet effet est observé durant les phases précoces de la réponse, à une période critique de la course entre le virus et la mise en place des défenses immédiates cellulaires. L'effet de Plzfb a été aussi observé après infection par deux virus non enveloppés, un birnavirus, le BSNV et un réovirus le GSV, mais pas après infection par un virus enveloppé, le rhabdovirus SVCV. L'effet semble indépendant de la présence du domaine BTB/POZ, un mutant de délétion étant aussi actif de la protéine complète. Ce travail suggère que le facteur de transcription PLZF est impliqué dans la réponse antivirale chez différents groupes de Vertébrés, mais via des mécanismes variés intervenant à différents niveaux du pathway. Ce travail suggère qu'une étude détaillée de l'implication de Plzf à différentes étapes de la réponse IFN des mammifères serait intéressante.

La famille TRIM comprend des protéines caractérisées par l'association d'un domaine RING, de domaines B-BOX, d'une région Coiled Coil et de domaines variés en région C terminale. Cette famille compte 70 membres chez l'Homme, dont près de la

moitié interviennent dans la réponse antivirale à différents niveaux du pathway. Le facteur de restriction de HIV TRIM5 en est un exemple bien connu. Chez le poisson zèbre, cette famille comporte plus de 200 gènes, dont plusieurs sous groupes de gènes récemment amplifiés: TRIM39, TRIM35 et un groupe ne possédant pas d'équivalent en dehors des poissons téléostéens, appelé finTRIM. Les gènes de ce groupe ont été identifiés au laboratoire il y a une dizaine d'années comme des gènes induits par l'infection virale, et leur domaine C-terminal a évolué sous sélection positive (diversifiante), suggérant qu'il reconnaît un ensemble de ligands diversifiés. Ces observations suggéraient que les finTRIM jouent un rôle dans lés réponses antivirales.

Nos résultats montrent que le gène ftr83 est exprimé constitutivement dans les branchies, le pharynx et la peau du poisson zèbre, et que son niveau d'expression dans la branchie est corrélé au niveau d'expression de l'IFN de type I dans ce tissu. FTR83 n'est pas induit par les IFN de type I, mais lorsqu'il est surexprimé dans la cellule, induit lui même une forte réponse IFN, avec une augmentation considérable de l'expression de différents ISGs. Cette réponse est capable de bloquer différents virus à ARN enveloppés ou non. L'effet de FTR83 est aboli par la co-expression d'un mutant dominant négatif de IRF3. Différentes chimères de FTR83 et d'un autre FTR apparenté qui n'a pas d'activité antivirale montre que la présence du domaine RBCC et du domaine C terminal SPRY de FTR83 sont requis pour l'induction de l'IFN de type I. Ces données indiquent que l'expression de FTR83 dans les tissus exposés aux pathogènes de l'environnement probablement assure une expression basale accrue de l'IFN et de ses effecteurs et assure une meilleure résistance locale à l'infection. Ce mécanisme participant à la régionalisation de l'immunité serait décrit ainsi pour la première fois pour une protéine TRIM. Ces données montrent aussi que le rôle antiviral des TRIM est conservé à travers les Vertébrés.

La spécialisation locale des défenses immunitaires antivirales peut aussi relever de la présence de cellules sentinelles aux sites critiques. La lignée transgénique de poisson zèbre "medaktin", dont le transgène correspond à la GFP placée sous le contrôle du promoteur de l'actine du Médaka (Orysias latipes) montre un profil de fluorescence inattendu : des cellules très fluorescentes, dont la morphologie suggère qu'il s'agit de

leucocytes, sont localisées à proximité immédiate des neuromastes, et sont concentrées sur la tête, les opercules et le long de la ligne latérale. La position de ces cellules GFP++ , et leur mobilité dans le voisinage de l'organe mécano-sensible qu'est le neuromaste, ont été caractérisées par microscopie. En croisant la ignée medaktin avec la lignée rapporteur mpeg1:mcherry, où les macrophages sont fluorescents (rouges), il a été possible de montrer que la plupart des cellules GFP++ de la lignée medaktin sont double positives et donc représentent très probablement des cellules apparentées aux macrophages. Des expériences de cytométrie de flux ont montré que les cellules GFP++ sont uniquement localisées dans la peau, et pas dans les tissus lymphoïdes par exemple. Pour aller plus loin dans la caractérisation de ces cellules, les opercules, où elles sont nombreuses, ont été isolés, et les cellules préparées puis triées au FACS, et leur ARN séquencé. Une analyse différentielle entre le transcriptome de cette population et celui de la population complémentaire de l'opercule a permis de mettre en évidence un fort enrichissement en transcrits de différents gènes du pathway de présentation de l'antigène par le MHC de classe II (MHCl II, CD74, ...). Le marqueur de macrophage mpeg1 était également très bien exprimé. Ces données confirment que les cellules GFP++ appartiennent à la lignée myéloïde, et indiquent qu'elles sont probablement des cellules présentatrices d'Ag. Enfin, la reconstitution de la population de cellules GFP++ à proximité des neuromastes qui réapparaissent dans la queue du poisson après régénération post ablation a été étudiée et suivie. Ce projet continue afin de mieux comprendre les fonctions de ces cellules qui apparaissent comme des cellules sentinelles potentielles.

université PARIS-SACLAY | ÉCOLE DOCTORALE Structure et dynamique des systèmes vivants (SDSV)

ED SDSV

Titre : Défenses innées antivirales du poisson zèbre: de la signalisation aux cellules specialisées

Mots clés : interferon, poisson zebre, leukocyte, neuromaste

Résumé : Cette thèse est basée sur deux projets principaux: (1) l'étude de la réponse innée antivirale du poisson zèbre, en particulier des voies de signalisation des interférons de type I et (2) l'étude de leucocytes particuliers localisés au voisinage des neuromastes, structures permettant au poisson de percevoir le flux d'eau qu'il traverse et constituant potentiellement des brèches dans la peau de l'animal.
La voie des IFN de type I est le principal composant de l'immunité antivirale innée. Dans cette thèse, deux types de protéines de poisson-zèbre capables d'augmenter l'induction des IFN de type I ont été étudiés. Nous avons montré que les deux orthologues chez le poisson zèbre du facteur de transcription à domaine BTB/POZ nommé PLZF (Promyelocytic leukemia zinc finger) augmentent l'induction de l'Ifn par différents stimuli. Ce travail montre que l'implication de PLZF dans la régulation de la voie IFN est ancienne et peut intervenir à différents niveaux de la voie Ifn. Le second modèle étudié est le gène Ftr83 (finTRIM83), qui appartient à un groupe de TRIM très diversifié et spécifique des poissons. L'expression de cette protéine TRIM induit une très forte induction des Ifn de type I et une protection contre différents virus, via la surexpression de différents ISGs. Ftr83 est exprimé dans la peau et dans les branchies, régions très exposées aux pathogènes, et son niveau d'expression est fortement corrélé au niveau d'expression de l'Ifn.
Dans cette thèse, une lignée transgénique où les cellules spécifiquement fluorescentes évoquent des leucocytes localisés à proximité des neuromastes a été étudiée. Ces cellules ont été observées, leurs mouvements suivis et leur transcriptome analysé par séquençage profond après tri au FACS. Cette analyse a identifié des marqueurs typiques de cellules myéloides (macrophages, dendritiques); ces observations sont cohérentes avec l'idée de cellules sentinelles autour des neuromastes.

Title : Innate antiviral defense of zebrafish: from signalling to specialized cells

Keywords : interferon signaling, macrophages, zebrafish, neuromast

Abstract : This thesis is based on the studies of two aspects of innate immunity in zebrafish: 1) proteins involved in the regulation of type I interferon (Ifn) and 2) specialized myeloid cells that patrol neuromasts – mechano-sensory organs embed in the skin that could be pathogen entry sites. In this thesis two different proteins are described for the capability to enhance Ifn production. In one part, two zebrafish orthologues of mammalian transcription factor PLZF (Promyelocytic leukemia zinc finger) are shown to augment type I Ifn and ISG in response to double-stranded RNA viruses. PLZF is a BTB/POZ transcription factor that was recently shown to induce a subset of ISG, in human and mouse. Thus, zebrafish Plzf proteins can operate at multiple steps in the Ifn system. Furthermore, their activity was not dependent on the presence of BTB-domain implying that the underlying mechanism is different from the usual mode of action of BTB/POZ transcription factors. In the second part, fish-specific TRIM ubiquitin ligase - Ftr83 (Fish novel tripartite motif protein 83), mounted a strong anti-viral protection through the upregulation of Ifn. Interestingly a strong correlation between the expression of Ftr83 and Ifn was seen in the gills suggesting that Ftr83 might maintain a low basal level of Ifn signalling in organs constantly exposed to pathogens. In the second part, a GFP reporter transgenic line called medaktin:EGFP has been characterized. It marks leukocytes in the skin surrounding neuromasts. Deep sequencing revealed that these cells express several macrophage and dendritic cell markers, including genes involved in autophagy, microbicidial functions and antigen presentation, thus highlighting them as possible sentinel cells.