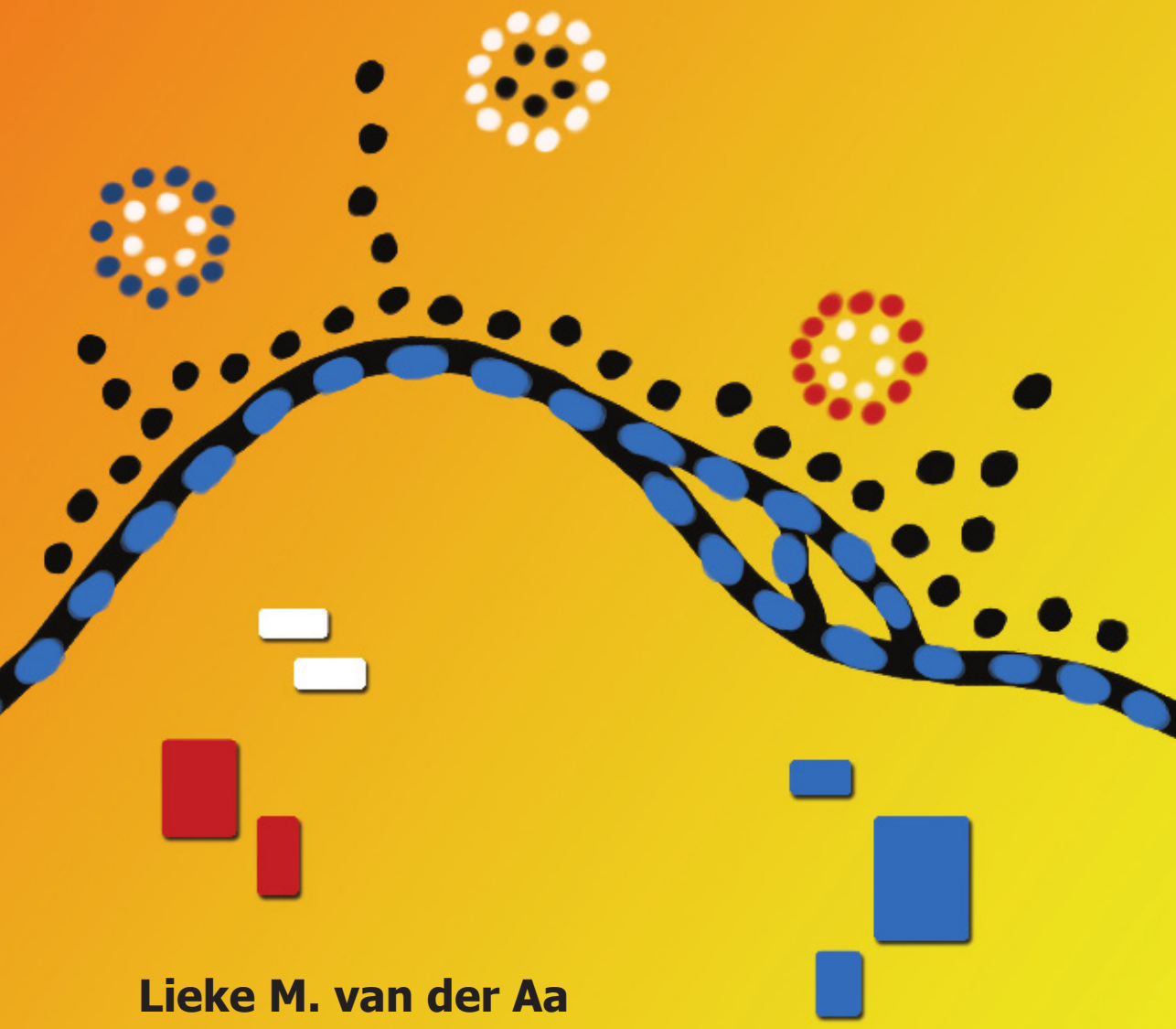
TRIM proteins and CXC chemokines

Evolutionary dynamics and functional characterization of two large protein families in teleost fish



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Thesis
submitted in fulfillment of the requirements for the degree of doctor
at Wageningen University
by the authority of the Rector Magnificus
Prof. Dr. M.J. Kropff,
in the presence of the
Thesis Committee appointed by the Academic Board
to be defended in public
on Friday 20 January 2012
at 4 p.m. in the Aula.

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TRIM proteins and CXC chemokines, Evolutionary dynamics and functional characterization of two large protein families in teleost fish

PhD thesis, Wageningen University, the Netherlands, 2012
With references, with summaries in English and Dutch

ISBN: 978-94-6173-120-3

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Un problème sans solution
est un problème mal posé.
- Albert Einstein -

Chapter 1

General Introduction

Pathogens are a major driving force for the immune system to evolve. Pathogens drive the immune system to constantly re-adapt and to create a well-balanced response to combat microbes that invade and inhabit them. An interesting class of the animal kingdom to study the evolution of the immune system are teleost fish, an extremely successful animal group that represents more than half of all living vertebrate species [1]. Teleosts originated 450 million years ago and because teleost fish have a well-developed innate immune system, and a functional adaptive immune system, they can provide interesting clues about the early evolved defense system against pathogens.

In this thesis, two large protein families were studied: the **TRIM protein family** and the **chemokine family**. TRIM proteins are intracellular proteins that control immunity from within the cell, while chemokines are secreted cytokines that orchestrate immunity at systemic levels. The evolution of both TRIM and chemokine genes is characterized by species-specific duplication events, followed by functional diversification. These processes generated two protein families that both play a crucial role in the immune system. The interest in TRIM proteins in mammals has grown enormously in the last few years when their importance in the antiviral immune response was discovered. At the start of this thesis project, research on TRIM proteins in fish was limited to the description of only two TRIM proteins in zebrafish (named bloodthirsty and moonshine), which both function in hematopoiesis [2, 3], a TRIM protein in Japanese eel that is expressed in branchial epithelial cells [4] and TRIM genes identified by a global screening of the genome of tetraodon and zebrafish [5]. Within the chemokine family, certain genes have clear orthologs in fish and mammals, but for many genes is still a matter of debate whether there is true orthology. Due to the low sequence similarity between chemokine genes from fish and mammals and the small size of these proteins, it is difficult to establish their evolutionary relatedness [6-9]. Although gene expression data indicate that certain fish chemokines play a role in immunity and inflammation, their biological function is not yet well known. In this thesis, the evolutionary dynamics of TRIM and CXC chemokine genes were investigated and a characterization of their functions was made in fish. Zebrafish (*Danio rerio*), common carp (*Cyprinius carpio*) and rainbow trout (*Onchorynchus mykiss*) were used hereby as model species.

1.1 Pathogen recognition and activation of the immune system

Pathogens present in air, water, food, or carried and transmitted by other organisms, form a continuous challenge to a host. The epithelial lining of the skin, respiratory airways and gastro-intestinal tract form the physical barrier between the outside and inside world of an organism. When a pathogen succeeds in trespassing one of these barriers, the immune system plays a crucial role in eliminating the intruder before it causes irreversible harm to the host. High numbers of innate immune cells are residing in the extravascular tissue, the space between the epithelium and blood vessels. The main task of these immune cells is to quickly detect and eliminate intruding pathogens. Pathogen recognition is a key step to initiate immune responses. Pathogens have characteristic well-conserved molecules that are important for their virulence and survival, and that are usually not expressed by the host. These so called pathogen-associated molecular

patterns (PAMPs) are recognized by pathogen recognition receptors (PRRs), which are expressed by cells of the host. Based on distinct protein subdomains of which PRRs consist, they are classified into four major classes: Toll-like receptors (TLRs), NOD-like receptors (NLRs), RIG-I-like receptors (RLRs) and the C-type lectins (CLRs) (reviewed by [10-13]). PRRs are mostly expressed by innate immune cells and are localized on the cell surface (TLRs), in phagosomal vacuoles (TLRs), or in the cytosol (NLRs, RLRs, CLRs). Recognition of PAMPs by PRRs facilitates the onset of an immune response tailored to a specific type of pathogen. Pathogen recognition by PRRs activates intracellular signaling pathways that lead to the activation of transcription factors. These include nuclear factor-κB (NF-κB), AP-1 and interferon-regulatory factors (IRFs). These transcription factors regulate cytokine gene expression, like the pro-inflammatory cytokines interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α), which together activate local immune cells to kill the pathogen. In case of viral infection, certain types of interferons are especially important for the induction of an anti-viral state in both immune and non-immune cells, by stimulating the production of anti-viral proteins. Proteins belonging to the **TRIM protein family** control the production of interferons and have also anti-viral activity (reviewed by [14-17]). In addition to evoking a local immune response, cytokines produced upon PRR activation mediate in the induction of a systemic immune responsive. Hereby, immune cells are recruited from the blood circulation and specialized lymphoid tissues and are activated to mediate in the process of pathogen killing and elimination, to prevent a systemic infection. The process of vascular dilation, increased permeability and edema, together with leukocyte accumulation at the site of infection, is called inflammation. **Chemokines** are specialized cytokines that play an important role in orchestrating cell recruitment during inflammation. A schematic overview of the first steps of the immune response is depicted in figure 1.

1.2 TRIM proteins: E3 ligases functioning in antiviral immunity

The immune response upon viral infection is becoming well characterized in mammals. Cytokines specifically induced upon detection of viral RNA and DNA by the PRRs TLR3, TLR7-9, RIG-I, MDA-5, PKR and DAI, are type I interferons (IFNs) [12, 18]. IFNs are classified into type I, II and III, based on their binding to distinct receptors. Type I IFNs include IFN-α and IFN-β, which are produced by both immune and non-immune cells upon virus detection. Type II IFN, IFN-γ, is induced upon viral and other pathogen detection and is expressed by specific cell types of the immune system. IFN-γ stimulates macrophages and plays a role in the onset of the adaptive immune response. Type III IFN, IFN-λ, also has anti-viral activity. Secreted IFN-α/β acts on nearby cells that express the receptor for IFNα/β (IFNAR). Triggering of the IFNAR, which signals through the Jak/Stat pathway, leads to the activation of transcription factors (e.g. IRFs, STATs) and subsequent expression of hundreds of genes that mediate in clearance of the viral infection (reviewed by [18, 19]). Among interferon-responsive genes are genes that encode for pro-inflammatory cytokines, which further elicit the immune response. Moreover, IFN-α/β induces the expression of intracellular anti-viral proteins that create an anti-viral state in cells. This anti-viral state allows cells to immediately fight incoming virus particles and reduces further viral replication and dissemination. Certain anti-

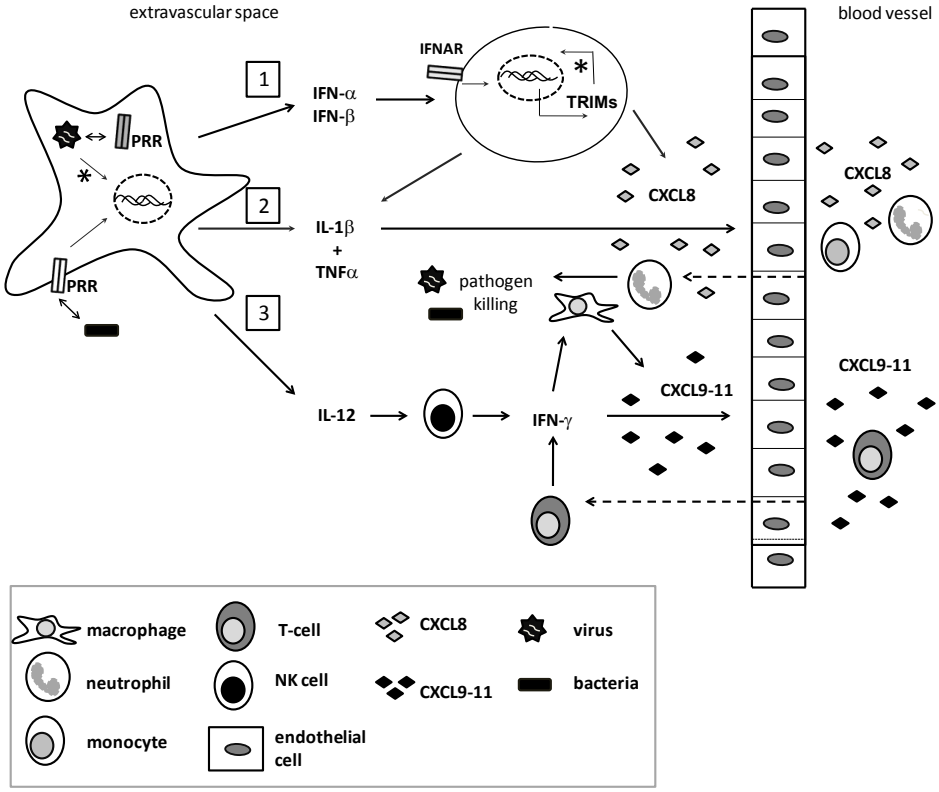

Fig. 1. Early steps of the immune response.
Macrophages express pattern-recognition receptors (PPRs) by which they can detect infiltrating viruses and bacteria. Upon pathogen detection, the macrophage produces three groups of important cytokines: type I interferons (IFNs) (**1**), the pro-inflammatory cytokines IL-1β and TNF-α (**2**), and IL-12 (**3**).
1) Type I IFNs are typically produced upon viral detection. IFNs are secreted and bind the IFN-α/β receptor (IFNAR) on neighboring cells. In these cells an antiviral state is induced by the expression of proteins with antiviral activities (e.g. **TRIM proteins**). IFNs also induce the production of IL-1β and TNF-α, to initiate the inflammatory response. Also the **CXCL8** chemokine is inducible by IFN. **TRIM proteins** not only have direct antiviral activity, but also regulate signaling pathways downstream the PRR that lead to IFN production, and the signaling pathway triggered upon IFNα/β binding to the IFNAR (indicated by *).
2) IL-1α and TNF-β bind receptors on endothelial cells of nearby blood vessels, which will then produce CXCL8. CXCL8 orchestrates recruitment of neutrophils and monocytes (that both express the CXCL8 receptors CXCR1 and CXCR2) from the blood circulation into the extravascular space. Infiltrated monocytes differentiate into macrophages and together with the neutrophils kill bacteria and viruses.
3) IL-12 produced by activated macrophages stimulates natural killer (NK) cells to produce IFN-γ. IFN-γ induces expression of **CXCL9**, **CXCL10** and **CXL11** by both immune cells (e.g. macrophages) and non-immune cells. CXCL9-11 recruit activated T-cells that express CXCR3. By the recruitment of T-cells, the adaptive immune response is initiated.

viral proteins and viruses co-evolved and these proteins restrict specific viruses.

In mammals, it is shown that a large number of IFN-induced genes encode for proteins that belong to the **TRIM protein family** [20, 21]. It is described for multiple TRIM proteins that they regulate the signaling pathways that lead to IFN production (recently reviewed by [14, 17]). Members of the TRIM protein family are characterized by a tri-partite motif (TRIM) that consists of a RING zinc finger, one or two B-boxes and a coiled coil domain (see Fig. 2a) [22-24]. The RING domain (Really Interesting New Gene), originally known as the A-box, is defined by a cysteine-histidine motif, $CX_2CX_{9-27}CXHX_2CX_2CX_{6-17}CX_2C$ (C_3-HC_4), which binds two zinc ions [25-27]. The B-box contains a cysteine-histidine motif: $CX_2HX_7CX_7CX_2CX_5HX_2H$, which binds one zinc ion [24, 28]. Both domains interact with other proteins, DNA and RNA. The coiled coil domain is an alpha helix that allows TRIM proteins to multimerize by self-association. Around seventy genes are identified on the human genome that encode for TRIM proteins. They are distinct not only in sequence, but also in the presence or absence of an additional domain at the C-terminal side of the protein [5, 22, 29]. Different types of additional domains are found together with the RBCC-motif and are an important determinant for ligand-binding specificity [29]. TRIM proteins are intracellular proteins with functions in conserved cellular processes and implicated in development, tumor suppression and immunity.

One biochemical mechanism of TRIM proteins is their E3 ubiquitin ligase activity [30]. Ubiquitination is a type of post-translational protein modification whereby ubiquitin molecules are covalently attached to a substrate protein. This reaction is catalyzed by three enzymes. Hereby, the E1 enzyme activates ubiquitin, an E2 enzyme conjugates E1 activated ubiquitin, and an E3 enzyme catalyzes the ligation of conjugated ubiquitin from E2 to a substrate (see Fig. 2b) [31]. The E3 ubiquitin ligase is an important determinant for substrate specificity. Initially, protein modification by ubiquitination was found to target proteins for degradation by the 26*S* proteasome [32]. It is now evident that protein modification by mono-ubiquitin, and structurally distinct polyubiquitin-chains, may result in functionally different outcomes. Ubiquitination plays a major role in regulating protein-protein interactions and E3 ligases are important regulators of intracellular pathways, including those of PRRs (reviewed by [33-36]). By their E3 ubiquitin ligase activity, TRIM proteins regulate viral PRRs and corresponding signaling pathways that lead to the production of type I IFNs (recently reviewed by [14, 16]). It was first demonstrated for TRIM25 that it activates RIG-I by ubiquitination, allowing RIG-I to interact with its adapter molecule MAVS (mitochondrial antiviral signaling protein) [37]. This was followed by the identification of other TRIM proteins that interact with proteins that make up the signaling pathways and transcription factors that lead to IFN production [38-42]. Hereby, TRIM proteins both mediate in the induction of type I IFNs, and act in a negative feedback loop to protect the host from a prolonged immune response. Next to regulating IFN responses, TRIM genes are inducible by type I and II IFNs and mediate in the direct restriction of a broad range of viruses [15, 20, 21], which includes retroviruses, pestiviruses, herpesviruses, hepadnaviruses, picornaviruses and flaviviruses ([43-48] and reviewed by [15]). One notable discovery was the identification of TRIM5α as a restriction factor for HIV-1 in

rhesus macaques [49]. This finding boosted research on intracellular restriction factors that may contribute to the development of a therapy against HIV-1.

1.3 CXC chemokines implicated in inflammation

It is described in mammals that signaling by PRRs upon pathogen recognition leads to expression of the pro-inflammatory cytokines IL-1β and TNF-α. Together, these two cytokines form the basis for the onset of inflammation. IL-1 is produced as a precursor protein (pro-IL-1) that is processed into IL-1β by the inflammasome. The inflammasome is a multiprotein complex that consists of either RIG-I, AIM2, or NLRP3, which upon ligand binding, interacts with the adapter protein ASC and activates caspase-1. Caspase-1 mediates processing of pro-IL-1 into IL-1β (reviewed by [50]). Both IL-1β and TNF-α act on cells that express the IL-1R and TNF-R. IL-1β and TNF-α not only activate local immune cells, but also act on nearby endothelial cells that line the

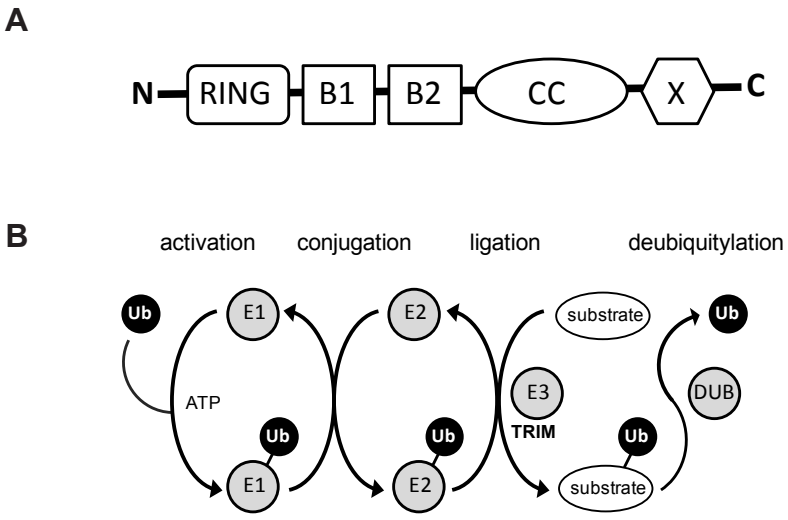

Fig. 2 TRIM protein structure and their activity in the ubiquitination cascade
A TRIM proteins contain a tri-partite motif that consists of a RING zinc finger, one or two B-boxes (B1 and B2) and a coiled coil (CC) domain. This motif is located in this order at the N-terminus of the proteins. Many TRIM proteins contain an additional domain at the C-terminus (noted as X). The B30.2 domain is the most frequently found C-terminal domain.
B In the ubiquitination cascade, ubiquitin (Ub) is first activated by the activating enzyme (E1). This is a high energy process mediated by ATP. In the second step, activated ubiquitin is conjugated by the conjugating enzyme (E2). The E3 ubiquitin ligase catalyzes the transfer of ubiquitin from E2 to a substrate. TRIM proteins are E3 ubiquitin ligases. Protein modification by ubiquitination is a reversible process: deubiquitinating enzymes (DUBs) remove ubiquitin from ubiquitinated proteins.

vessels of the vascular system. This results in the expression of cell-adhesion molecules and co-stimulatory molecules at the inner lining of blood vessels. The expression of these molecules allows 'capturing' of circulating immune cells and passage of these cells through the epithelial lining, into the extravascular space. In addition, upon stimulation by IL-1β and TNF-α, endothelial cells produce IL-1β and TNF-α themselves, which subsequently act on circulating immune cells, to express adhesion molecules and receptors. This further facilitates adhesion of leukocytes to the endothelium.

Chemokines are chemotactic cytokines that direct cell migration, chemotaxis, along a chemokine gradient. In mammals, four subgroups of chemokines are described, CXC, CX3C, CC and C chemokines, referring to a cysteine-motif at the N-terminus of the protein. Chemokines play a very important role in inflammation, as they determine the specificity of cells that are attracted towards the site of inflammation. Specific leukocytes display chemotaxis towards different types of chemokines. Differential expression of distinct chemokines over the course of the inflammatory response, allows recruitment of specialized leukocytes during the different phases of the inflammatory response. For example, CXC chemokines (e.g. **CXCL8**, also known as IL-8), which contain a specific ELR signature (Glu-Leu-Arg), are crucial for the recruitment of polymorphonuclear leukocytes (PMNs, e.g. neutrophils, basophils and eosinophils) towards the site of infection during the early phase of inflammation. CXC chemokines lacking the ELR signature (e.g. **CXCL9, CXCL10** and **CXCL11**) are generally expressed when inflammation has already initiated and specifically attract lymphocytes and monocytes [51]. CXCL8 is produced by locally activated immune cells, by IL-1β and TNF-α stimulated endothelial cells, and by newly recruited immune cells. A major cell type that responds to CXCL8 is the neutrophilic granulocyte, which expresses both CXCL8 receptor CXCR1 and CXCR2 [52]. Neutrophils make up a large proportion of circulating immune cells and are generally the first cells that are recruited to the site of inflammation. Monocytes are less abundant in circulation, and moreover enter the blood circulation when a systemic immune response is induced. Monocytes express CXCR1 and CXCR2 and are also responsive to CXCL8. Once entered into the extravascular space, neutrophils become activated, monocytes differentiate into macrophages, and both produce oxygen radicals, nitric oxide (NO, by iNOS) and lysozomal proteases to eradicate the microbes. In addition, the activated leukocytes will produce IL-1α, TNF-β and CXCL8, to recruit more leukocytes from the blood.

Chemokines that play a later role in inflammation and that mediate in the onset of the adaptive immune response are the IFN-γ-inducible chemokines CXCL9, CXCL10 and CXCL11. Upon phagocytosis of microbes, macrophages produce IL-12, which stimulates natural killer cells (NKs) to produce IFN-γ. IFN-γ subsequently acts on macrophages to further activate them to kill microbes. Moreover, IFN-γ stimulates macrophages to produce CXCL9, -10 and -11. A well described cell type that is recruited by stimulated macrophages are activated T helper 1 (Th1) lymphocytes that express CXCR3, the receptor for CXCL9-11 [53]. In addition, activated Th1-cells produce IFN-γ themselves, and further activate macrophages. At the same time, IFN-γ stimulation leads to production of co-receptors on macrophages for T-cell stimulation, and IL-12 secretion, which also stimulates T cell differentiation. By this amplification loop, effector T cells further drive leukocyte recruitment and inflammation.

1.4 Teleost fish and its evolution of the immune system

TRIM proteins are ancient and first genes have been identified in cnidarians [54]. Genes of the chemokine family were first identified in lamprey and are restricted to vertebrates (see Fig. 3) [8, 55, 56]. Both the TRIM and chemokine family already highly expanded before the divergence of the fish and tetrapod lineages [5, 8, 9]. Zebrafish, common carp and rainbow trout are ray-finned fish. Zebrafish and carp are close relatives that both belong to the cyprinid lineage, whereas trout belongs to the salmonid lineage. Ray-finned fish represent the largest group of fish species, which together with the lobe-finned fish form the bony fish (teleosts) superclass. An important event in fish evolution is the occurrence of a third round of whole genome duplication. A first and second round of whole-genome duplication occurred during the early evolution of vertebrates, of which one occurred most probably after the divergence of the lamprey lineage. The third round of whole-genome duplication occurred in ray-finned fish early evolution [57, 58]. This extra round of whole-genome duplication has contributed to the diversification of teleosts and explains the high number of duplicated genes found in all fish genomes. For example, two gene copies have been identified for numerous fish cytokines, compared to single numbers in mammals. Moreover, additional rounds of whole-genome duplications have occurred in certain fish lineages, which include cyprinids and salmonids [59-61]. This has resulted in a tetraploid genome for multiple fish species (for example common carp and trout), while other species such as zebrafish have diploid genomes.

The innate immune system is of primary importance in fish, however, major features of the adaptive arm of the immune system already predated the divergence of the fish lineage and fish have a fully developed adaptive immune system [59, 60]. For example, an unique feature of the vertebrate adaptive immune system is the somatic development of clonally diverse lymphocytes. RAG genes and B and T cell receptor VDJ genes originated in jawed vertebrates [61]. The PRRs, the germline-encoded innate immune receptors, are partly conserved in invertebrates and vertebrates (see for recent reviews [62-65] and [66-69]). Some of the fish PRRs and actors of the downstream signaling pathways are structurally very similar to the mammalian PRRs and their signaling pathways. Other fish PRRs have distinct features. Moreover, as fish represent such a large animal class, with large phylogenetic distances, differences are observed among fish species. The inflammatory response in fish resembles the mammalian inflammatory response and is characterized by a first wave of expression of the pro-inflammatory cytokines IL-1β and TNFα, followed by the expression of chemokines and IL-12 [70]. The anti-inflammatory cytokine IL-10 is produced during the last phase of inflammation. IL-10 inhibits excessive activation of the immune response and possibly initiates processes of wound healing, tissue remodeling and recovery [71]. This correlates with an inhibited expression of IL-1β and CXC chemokines [70, 72].

The fish innate immune system involved in anti-viral defense resembles the mammalian anti-viral defense system. A functional IFN-system is present in fish and genomic surveys in fish indicate a global conservation of viral PRRs, downstream signaling pathways and ISGs (recently reviewed by [73-77]). Major TLRs and RLRs for viral recognition have been identified on fish genomes and functional studies indicated

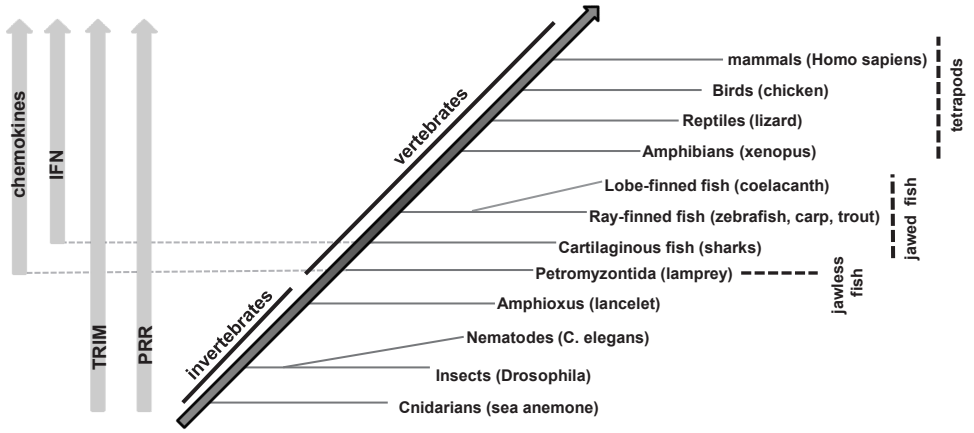

Fig. 3. TRIM proteins and chemokines in the evolution. Rainbow trout, common carp and zebrafish are ray-finned fish. Ray-finned fish and lobe-finned fish form together the bony fish (teleost) superclass. TRIM proteins are evolutionary old and first TRIM genes have been identified in cnidarians and insects. This is similar for Pattern Recognition Receptors (PRRs). Chemokines emerged at the divergence of the vertebrate lineage. First interferon genes have been identified in sharks.

that some fish PRRs indeed function as viral recognition receptors [62, 65, 68]. For example, rainbow trout MDA-5 and LGP2 recognize dsRNA (polyI:C) [78]. Moreover, downstream signaling appear conserved. In Atlantic salmon, the RIG-I adapter protein MAVS is localized to the mitochondria, activates the IFN-α1 and NF-κB promoter, and overexpression of MAVS induces ISG expression and a protective state against viral infection [79, 80]. Earliest species for which IFN-like genes have been found are sharks [81] and both type I IFN-like and typical type II IFNs are present in fish. Mammalian type I IFNs are encoded by genes with no introns, type II IFNs by genes with three introns and type III IFNs by four introns. In fish, first IFNs with antiviral activity and four-intron structures were described as type I IFNs, while their receptor is reminiscent of a type III IFN receptor [82-84]. Together with different patterns of antiviral activity for the multiple fish IFNs, this has complicated the nomenclature and fish IFNs are known under various names: type I IFN, virus-induced IFN, IFN, or IFNϕ [77, 83, 85, 86]. Elucidation of the crystal structure of members of the zebrafish IFN group I and IFN group II established that both groups resemble a typical type I IFN [87]. Multiple IFNϕ genes have been identified in many fish species, similar to the multiple IFN-α genes present in mammals (reviewed by [76]). For type II IFN, fish possess duplicated IFN-γ genes of which one copy seems to be involved in typical functions of type II IFNs [88-92]. Also downstream signaling of the IFN receptor, which signals via the Jak/STAT-pathway, appears at least partly conserved in fish. Mammalian type I IFNs bind the IFNAR, which typically induces the formation of the transcription factor complex STAT1/STAT2/IRF9 that binds IFN-stimulated response elements (ISRE). Type II IFNs bind the

IFNGR, leading to dimerization of two STAT1 molecules that bind gamma-activating-sequences (GAS) [18]. In atlantic salmon, it was shown that STAT1 dimerizes upon type I and II IFN signaling and localizes to the nucleus. In ginbuna crucian carp, upon IFN-γ signaling, STAT1 dimerizes and bind to GAS sequences, indicating a conserved downstream signaling pathway and gene activation [93, 94]. Typical mammalian ISGs have been reported in fish as well, including ISG15 and Mx proteins, which have antiviral restriction activity (reviewed by [75]).

1.5 Aims and outline of this thesis

It was discovered only recently that TRIM proteins play a key role in the antiviral immune response. Although some TRIM proteins had been reported in fish, the description was far from being complete and possible functions for TRIM proteins in the (anti-viral) immune system were not yet investigated at the start of this project. In **Part One** of this thesis, a detailed description of the TRIM gene family in fish was made by a computational approach. An experimental approach was used to determine a possible role for fish TRIM proteins in anti-viral immunity. In **Chapter 2**, the discovery of two multigene TRIM families in fish is described: fish novel TRIM (finTRIM) and TRIM39/bloodthirsty. In **Chapter 3**, a complete description is provided for the TRIM gene family in zebrafish and tetraodon. Further, the identification of a third multigene TRIM family in fish, TRIM35, is described. In **Chapters 2 and 4**, finTRIMs were further characterized and finTRIM gene expression upon interferon and virus stimulation is presented. Rainbow trout was used in these studies, as it is a well-established model for viral infection. In **Chapter 4**, E3 ubiquitin ligase activity is demonstrated for two finTRIMs from rainbow trout. This indicates that finTRIM proteins can modify other proteins by ubiquitination and possibly control intracellular signaling pathways of the anti-viral immune response.

Some of the mammalian CXC chemokines, such as CXCL12 and CXCL14 that are important in development, have unambiguous orthologs in fish. Other CXC genes form fish-specific lineages [95]. The alternative name CXCa was earlier proposed for CXC genes in fish that are most similar to mammalian CXCL8 in mammals, but no true orthologs. Analogously, the alternative name CXCb was proposed for fish CXC genes that are most similar to, but no true orthologs, to mammalian CXCL9, CXCL10 and CXCL11 [7]. Although previous gene expression studies indicate that fish CXCa and CXCb are implicated in immunity, their exact biological function was not yet clear. In **Part Two** of this thesis, the diversification of fish CXC chemokines was investigated and the biological function of CXCa and CXCb chemokines determined in cyprinid fish. In **Chapter 5**, the identification of two CXCL8-like lineages in cyprinid fish is described, which were named CXCa_L1 (lineage one) and CXCL8_L2 (lineage two). A role in inflammation for both CXCL8-like chemokines was demonstrated by measuring their gene expression during inflammation and their ability to induce phagocyte chemotaxis *in vitro*. In **Chapter 6**, the function of CXCa_L1, CXCL8_L2 and CXCb was further investigated. The effect of the different chemokines on phagocyte activation was studied and the ability of CXCa_L1

to induce calcium mobilization in granulocytes is shown. The induction of chemotaxis in different leukocyte subpopulations by the three chemokines was investigated in vitro and in vivo. In **Chapter 7**, the cloning of a second CXCb gene in carp, which was named CXCb2, and the identification of a CXCb gene cluster on the genome of zebrafish is described. The promoter of the individual zebrafish CXCb genes was analyzed and the induction of carp CXCb2 gene expression was studied. The results provided in **Part Two** of this thesis indicate a functional diversification of the chemokine lineages within the CXCa and CXCb subsets and a role during different phases of the inflammatory response for CXCL8-like and CXCb chemokines.

In **Chapter 8,** the evolutionary characteristics of multigene families is discussed, highlighted by the observations made for TRIM and CXC chemokines in fish. Further, the significance of ubiquitin(-like) modification in cell signaling is explained, with specific attention to the control of the viral pattern-recognition receptor signaling pathways by E3 ligases. With the functional data obtained for fish CXC chemokines, a model of the fish inflammatory response is proposed.

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L`ìmpossible n`est pas Français.

- Napoleon -

Chapter 2

A large new subset of TRIM genes highly diversified by duplication and positive selection in teleost fish

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BMC Biology 2009 Feb 5;7:7

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Abstract

In mammals, the members of the tripartite motif (TRIM) protein family are involved in various cellular processes, including innate immunity against virus infection. Viruses exert strong selective pressures on the defense system. Accordingly, antiviral TRIMs have highly diversified through gene expansion, positive selection and alternative splicing. Characterizing immune TRIMs in other vertebrates may enlighten their complex evolution. We describe here a large new subfamily of TRIMs in teleosts, called finTRIMs, identified in rainbow trout as virus-induced transcripts. FinTRIMs are formed of nearly identical RING/B-box regions and C-termini of variable length; the long variants include a B30.2 domain. The zebrafish genome harbors a striking diversity of finTRIM genes, with 84 genes distributed in clusters on different chromosomes. A phylogenetic analysis revealed different subsets suggesting lineage-specific diversification blasts. Accordingly, the number of finTRIM genes varies greatly among fish species. Conserved syntenies were observed only for the oldest *finTRIMs*. The closest mammalian relatives are *TRIM16* and *TRIM25*, but they are not true orthologs. The B30.2 domain of zebrafish finTRIMs evolved under strong positive selection. The positions under positive selection are remarkably congruent in finTRIMs and in mammalian antiviral TRIM5α, concentrated within a viral recognition motif in mammals. The B30.2 domains most closely related to finTRIMs are found among NOD-like-receptors (NLR), indicating that the evolution of TRIMs and NLRs was intertwined by exon shuffling. The diversity, evolution, and features of finTRIMs suggest an important role in fish innate immunity; this would make them the first TRIMs involved in immunity identified outside mammals.

Introduction

Newly discovered players in the antiviral immunity field are the proteins belonging to the TRIM family. The TRIM proteins are characterized by a tripartite motif that comprises from the N- to C-terminus a RING zinc finger domain, one or two B-boxes and a coiled-coil domain. They are therefore also known as RBCC proteins [1]. The RING finger and B-box are cysteine-rich domains and both domains bind zinc atoms, suggesting interaction with other proteins, RNA and DNA [2-5]. They are usually encoded as a single exon, and form together the "RBB" region. In addition, the RING finger has E3 ubiquitin ligase activity [6]. The coiled coil region seems to be predominantly necessary for multimerization, resulting in the formation of high molecular weight complexes. In many TRIM proteins, an additional domain is present at the C-terminus [7], with the B30.2-domain being the most frequent one [reviewed in [8]. The B30.2 domain is encoded by one exon [9, 10]. The domain is also found in butyrophilins and stonus toxins [11] and has evolved by a relatively recent juxtaposition of the PRY domain and the SPRY domain; it is therefore also known as the PRY/SPRY domain [12]. The B30.2 domain has been shown for several TRIM proteins to be essential for ligand binding [13-15]. Its tertiary structure has recently been elucidated for TRIM21, revealing two binding pockets formed by six variable loops [16]. Since the order and spacing of the domains are highly conserved, a TRIM protein presumably acts as an integrated structure [1]. TRIM proteins are evolutionarily old proteins that can be found in primitive metazoans [6]. Currently, 68

TRIM-encoding genes have been described in human [1, 7, 8, 17]. Most TRIM genes code for at least two isoforms that are generated by alternative splicing, resulting in full length and partial transcripts that lack the C-terminal encoding sequence.

The TRIM proteins play multiple roles in various cellular processes, which include cell growth, differentiation and apoptosis in mammals. Many TRIM genes are proto-oncogenes and severe diseases such as Opitz syndrome and acute promyelocytic leukaemia (APL) are caused by mutations in TRIM18 and TRIM19, respectively [18], reviewed in [19]. An antiviral activity has also been described for several TRIM proteins: TRIM1, -5α, -11, -15, -19, -22, -25, -28 -32 [8, 20-22]. These TRIM proteins can block viral infection by different mechanisms, as revealed by the functional characterization of TRIM5α, TRIM19 and TRIM25. A virus-specific interaction has been described for TRIM5α and TRIM19. TRIM5α was initially identified in rhesus macaques as the protein responsible for post-entry restriction of HIV-1 in this species, while its human ortholog could not block HIV-1 [23]. TRIM5α forms trimers that bind the nucleocapsid of incoming viral particles through a C-terminal B30.2 domain, which accelerates the uncoating of the viral core and hereby interferes with the reverse transcription [24, 25]. Among primates, this domain contains four hypervariable regions that have been subjected to a virus driven diversification and account for the species-dependent retrovirus restriction of TRIM5α [26-28]. The RING and B-box domains of TRIM5α are essential for localizing TRIM5α in specific cytoplasmic 'bodies' and may also be involved in inhibiting the assembly of progeny virions [6, 29-31]. The antiviral restriction activity of TRIM19, or promyelocytic leukaemia (PML) protein has been demonstrated for retroviruses (HFV, HIV, MLV), but also for an arenavirus (lymphocytic choriomenengitis virus), a rhabdovirus (VSV) and an orthomyxovirus (influenza A) [8, 32]. For example, TRIM19/PML binds the HFV Tas protein, a transactivator for HFV transcription, preventing binding of Tas protein to the viral genome and thereby transcription of viral ORFs [33]. As expected for proteins involved in antiviral defenses, several TRIM proteins are induced by interferon [34-37], but they can also participate in the induction of interferon synthesis. Thus, TRIM25 is involved in the production of IFN-β through the RIG-I pathway [20].

Here, we characterize a new subset of TRIM genes that were originally discovered in a screen for virus-induced genes expressed by fish [38], and named them finTRIM for fish novel TRIM. The finTRIM genes constitute a unique expansion of TRIM genes in different teleost species, with up to 84 genes identified in zebrafish (*Danio rerio*). In the zebrafish, these genes are located on several chromosomes, with three main clusters on the chromosome 2. The open-reading frames (ORFs) of this multigene family are highly similar in sequence, but variable in length. The most extended proteins contain a RING finger, two B-boxes, a coiled coil region and a B30.2 domain. Interestingly, the finTRIM B30.2 domains have evolved under diversifying selection, and are closely related to B30.2 domains present in a NOD-like-receptor (NLR) subfamily unique to teleost fish [39, 40]. The characterization of the finTRIM subset highlights the evolutionary dynamics of the TRIM family and strongly advocates a role in the innate antiviral response of fish.

Material and methods

Trout leukocyte preparation
Rainbow trout (*Oncorhynchus mykiss*) were raised in the Jouy-en-Josas experimental fish facility. The fish were sacrificed by overexposure to 1% 2-phenoxyethanol (2-PE). The entire pronephros were removed aseptically and dissected. Cells from the pronephros of a single fish were deposited on a Ficoll solution (Lymphocyte separation medium [d 1.077]; Eurobio, Les Ulis, France) and centrifuged 10 min at 900 g. The leukocyte fraction at the Ficoll-medium interface was collected and subsequently used in the finTRIM stimulation experiments and mRNA isolation. Homozygous rainbow trout clones were produced using a gynogenesis-based strategy [60]. A population of doubled haploids was first established, using a mitotic gynogenesis procedure described. At the next generation, homozygous clones (G-clones) were obtained using meio-gynogenetic reproduction of individual homozygous doubled haploid females. Within a G-clone, some progeny were sex-reversed by early hormonal treatment to obtain functional XX males, and these animals were crossed with females from the same G-clone to produce N-clones. Such animals (N-clones) were used in this study.

Cells and viruses
Trout RTG-2 and carp EPC cell lines were used for virus production and titration. They were cultured in BHK-21 medium (Invitrogen-Gibco), supplemented with 10% (V/V) fetal calf serum (FCS, Eurobio, Les Ulis, France), 10% (V/V) tryptose broth, streptomycin (50 µg/ml) and penicillin (50 units/ml). African green monkey COS-7 cells were used for rainbow trout recombinant interferon production. They were cultured in Dulbecco's Modified Eagle's Medium (Eurobio, Les Ulis, France), supplemented with 10 % (V/V) fetal calf serum. COS-7 cells were transfected with an expression plasmid encoding trout interferon (from AY788890) using FuGENE 6 Transfection Reagent (Roche, Neuilly, France) and supernatant was collected and titrated. One thousand units of interferon per ml were used to study induction kinetics. Cycloheximide (100 µg/ml) (Sigma Aldrich, Saint-Quentin, France) was used to block cell protein synthesis. Viral hemorrhagic septicemia virus (VHSV) strain 07-71 [61]) was inactivated by overnight treatment with diluted (1/4000) β-propiolactone (BPL). For the stimulation of finTRIM expression, 50 µg/ml Poly(I:C) (Sigma Aldrich, Saint-Quentin, France), 100 µg/ml of *Eschericha coli* lipopolysaccharide (LPS from *E. Coli* 0127B8; Sigma-Aldrich, Saint-Quentin, France) were used. Spring viraemia of carp virus (SVCV), and a heat-adapted variant of infectious hematopoietic necrosis virus (IHNV) were used for zebrafish infection experiments. SVCV was diluted to 10^7 pfu/ml and IHNV to $5x10^6$ pfu/ml in PBS containing 0.1% phenol red; viral suspensions were kept as much as possible on ice.

Zebrafish embryos experiments
Zebrafish (*Danio rerio*) of the AB strain, initially obtained from the Zebrafish International Resource Center (ZIRC, Eugene, OR) or F1 derived from this stock were raised in the Institut Pasteur facility and mated to obtain eggs. IFN-over-expressing embryos were obtained as described in [62]. Embryos were injected at the 1-cell stage with 12 pg of

plasmid DNA driving expression of zebrafish IFN1; as a control for successful injection, the DNA solution also included a plasmid driving expression of the fluorescent mCherry protein. As controls, some embryos were injected with the mCherry plasmid alone. Embryos were then allowed to develop at 28°C. At 24 hpf (hours post-fertilization) they were sorted under a fluorescence stereomicroscope to retain only mCherry-expressing animals; abnormally developing embryos were also discarded. At 72 hpf, the larvae were euthanized with 2-PE and homogenized in Trizol reagent (Invitrogen) for RNA extraction. For viral infections, non-manipulated larvae that had been left to develop for 3 days at 24°C were dechorionated, anesthesized with Tricaine (Sigma), and microinjected with 1 nl of viral suspension in the venous plexus located just posterior to the cloaca. They were then incubated at 24°C for 24h in the case of SVCV, or 48h in the case of IHNV (yielding larvae at a developmental stage corresponding roughly to 78 hpf and 96 hpf at 28.5°C, respectively); at this time point infection was well advanced, but all embryos were still alive. Embryos were then sacrificed and treated with Trizol as above. Uninjected control larvae, incubated at the same temperature and for the same durations, were processed in parallel from the same clutches. Ten to 15 larvae were included for each point.

RNA isolation , cDNA synthesis, 5' and 3' RACE PCR

Total RNA was extracted with the Trizol reagent according to the manufacturer's instructions, then treated with 5 units of RNAse free DNase (Invitrogen) to remove any remaining genomic DNA. cDNA was synthesized from total RNA using either an oligo(dT) primer or the SMART PCR cDNA Synthesis Kit (Clontech). The 5'-rapid amplification of cDNA ends (5' -RACE)-PCR and 3'-RACE-PCR were performed using the SMART RACE cDNA Amplification Kit (Clontech BD), according to the instructions of the manufacturer. 5'- and 3'-RACE PCRs were performed with relevant specific primers (see Table 1) and the universal primers from Clontech. In rainbow trout, the 3'-RACE-PCR was performed from VHSV-induced leukocyte cDNA using a universal primer specific for trout finTRIM localized in the highly conserved region around the start codon. This conserved region was confirmed through EST analysis and sequencing of ten clones generated by a 5'-RACE experiment using the same template. For zebrafish samples, 3'-RACE was performed using Invitrogen's GeneRacer kit, with two rounds of amplification (round one with primers zftruniv1S and GeneRacer 3' primer, round two with zftruniv2S and GeneRacer nested 3' primer); the consensus primers are located in the RING domain.

Cloning and sequencing of PCR products

The trout PCR products were purified with Sephacryl S-400 columns (Pharmacia) and then cloned using the TOPO T/A Cloning Kit (Invitrogen). Upon transformation of E. coli, plasmid was isolated by the Nucleospin Plasmid Quickpure kit (Nucleospin; Macherey-Nagel, Duren, France). Purified plasmids were subjected to automated sequencing with M13 direct and reverse primers and with internal primers for long transcripts. Zebrafish RACE products were treated in a comparable manner but with different reagents: PCR products were purified on Qiaquick spin columns (Qiagen) and cloned in the pGEMT-

Table 1. Primers

	Primer sequences
Rainbow trout - Q PCR	
omOLIB144F	AGGACATGAGGGCTTTCTGCTT
omOLIB144R	GGACCAGGACCAGTTCTGTTGT
IFNGF	GCTGTTCAACGGAAAACCTGTTT
IFNGR	TCACTGTCCTCAAACGTG
Rainbow trout - 3'RACE-PCR	
omOLIB32ALL	GTGAACAACCGTCCAAATGGCTCA
Zebrafish - 3'RACE-PCR	
zftrall1S	TGTGGACACAGTTACTGTATGAGCTG
zftrall2S	TGCAGACAGACCTTCACTCCAAGACC

easy vector (Promega); plasmids were purified with QiaPrep Miniprep kits (Qiagen) and sequencing was performed initially with SP6 and T7 primers.

Real-time RT-PCR assay
Real-time RT-PCR reactions were performed using the SYBR green reagent from Applied Biosystems (Applied Biosystems, Les Ulis, France) and Eppendorf Mastercycler realplex2 S (Eppendorf, Le Pecq, France), according to manufacturer's instructions. All reactions were performed in duplicate. Data analysis was performed as described in the ABI PRISM 7700 sequence detection bulletin #2 from Applied Biosystems, following the ΔCt method. Oligonucleotides used for real-time RT-PCR are indicated in table 1.

Strategy for identification and alignment of finTRIM sequences
The finTRIM sequences were assembled using the tools of the Genetic Computer Group (GCG; Madison University, Wisconsin). The sequences were subjected to multiple sequence alignments using GCG (pileup) or ClustalW, and multiple alignments were edited using Boxshade software. Systematic searches for finTRIM were done using the tblastn program with rainbow trout or zebrafish sequences as a query, on available EST and genome databases. Searches in EST databases were mainly performed at the NCBI and Dana Farber Institute websites. Blast queries on complete genomes were sent to Ensembl and NCBI. The last assembly of the zebrafish genome (Zv7) was searched at the Ensembl site. When relevant genomic regions were identified, potential exons were manually identified by comparison with known sequences, notably RACE clones, with the help of predictions made by Genscan (http://genes.mit.edu/GENSCAN.html).

Detection of positive selection
The dataset for positive selection analysis was prepared from the zebrafish group A finTRIM (ftr) sequences that were found on the Ensembl zebrafish Zv7 assembly.

Three datasets were prepared, corresponding to sequences coding for the RING and B-box 1 and 2 (RBB) domains (55 sequences) and the B30.2 domain (38 sequences). Domains were identified by the web-based tool Simple Modular Architecture Research Tool (SMART) at http://smart.embl-heidelberg.de/. A multiple sequence alignment was made for each domain with ClustalW within the MEGA4 software and gaps were removed from the alignment. The final datasets consisted of 155 codons for the RING and B-box domains and 145 codons for the B30.2 domain. The phylogenetic trees were constructed by the Neighbour-Joining method using MEGA4.

The Codeml program of the Phylogeny Analysis by Maximum Likelihood (PAML) package [63], retrieved from http://abacus.gene.ucl.ac.uk/software/paml.html, was used for the detection of positive selection. The models M0, M1a, M2a, M7 and M8 were employed. The ratio of synonymous (dS) to non-synonymous (dN) substitution rates, ω=dS/dN, is determined by the program. A value of $\omega<1$ indicates negative, purifying selection, $\omega=1$ indicates neutral evolution and $\omega>1$ indicates the occurrence of diversifying, positive selection. We used the site-specific model that allows ω to vary among sites. The null models M0, M1a and M7 do not allow the existence of positively selected sites ($\omega>1$), while the alternate models M2a and M8 allow $\omega>1$. M8 follows a beta(p,q)-distribution and is less stringent than M2a. Within the models, a Maximum Likelihood algorithm is used, whereby the sites are allocated under classes of different ω probabilities. Sites allocated under the class with $\omega>1$ are considered as being under positive selection and were identified by a Bayes Empirical Bayes (BEB) analysis. Significance of outcome was confirmed by a likelihood ratio test (LRT). In the LTR we took twice the difference in log likelihood ($2\triangle$lnL) between the nested models and used the chi-square test with the degrees of freedom (df) being the difference in free parameters between the two models (M1a vs. M2a and M7 vs. M8). Tests were considered positive when $p<0.001$. Sites identified by BEB with a posterior probability higher than 95 percent were considered significant.

Analysis for recombination

To test for interference of recombination on the PAML results, we implemented a test by the algorithm PARRIS [64]. Under PARRIS, the PAML models M1a-M2a are employed with incorporation of site-to-site variation in synonymous substitutions rates and partitioning of data. We used the codon model for evolution GY94 x HKY85 and a discrete distribution of three bins for synonymous and for non-synonymous rates. Significance of results was tested by a LRT. We detected recombination breakpoints by the algorithm GARD [65]. We used the HKY85 model with general discrete distribution of rates across sites. We performed two screenings, for 2 or 20 breakpoints. The detection was validated by corrected Akaike`s information criterium (c-AIC) for best-fitted model selection. Both PARRIS and GARD are integrated in the HyPhy software package that was retrieved from http://www.hyphy.org.

Nucleotide sequence accession numbers

Sequences of rainbow trout and zebrafish finTRIM experimentally produced have been deposited in the EMBL database under accession no AM887792-AM887863 (rainbow

trout) and AM941305-AM941371 (zebrafish).

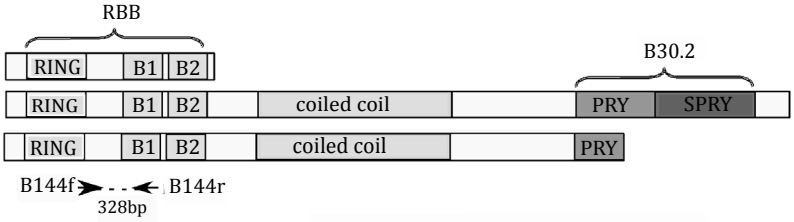
Results

FinTRIM, a new group of TRIM proteins induced by viruses in rainbow trout
In order to identify virus-induced transcripts in fish leukocytes, we previously used the method of subtractive suppressive hybridization on rainbow trout leukocytes that were either incubated with viral hemorrhagic septicemia virus (VHSV) or mock-infected. This approach identified 24 virus-induced sequences [38]. One of them (clone [Genbank:AF483536]) contained a 200 aa-ORF with a RING finger and two B-Box motifs, resulting in a RBB domain typical of TRIM proteins. No coiled coil region could be found in this sequence. A naive trout spleen cDNA library was then screened to confirm the structure of this TRIM cDNA and we identified two other full-length sequences (clones [Genbank:AM887799 and AM887838]). These sequences contained a RBB region almost identical to that of the AF483536 clone (96% identity over 200 residues), associated with a coiled-coil region (CC). In addition, the clone AM887799 contained a C-terminal B30.2 domain (Fig. 1A). A multiple alignment of these sequences suggested that they did not result from alternative splicing of the same gene. Taken together, these results already suggested that these trims belong to a multigenic family with a modular structure. We could not find any obvious counterpart of these trims in sequence databases from mammals or other tetrapods. We thus assumed that they may belong to a new subfamily, and named them fintrims for Fish Novel Trims.

The induction of finTRIM transcripts by the virus was further confirmed using real-time quantitative PCR (Fig. 1B), using primers B144f/r located in the RBB region and matching the three clones (and in fact all trout finTRIMs; see Fig. 1A for primer location). The induction ratio measured by this real time PCR therefore corresponded to an average value, and may conceal disparities of the induction level for different genes. An induction ratio higher than 10 was measured after the viral infection in leukocytes or poly(I:C) treatment in fibroblasts, while no induction was noted in leukocytes after incubation with LPS from *E. Coli.* In LPS treated leukocytes, IFN-γ transcript was induced 7-fold (range 5.8-8.4), demonstrating an effective stimulation. These experiments established that at least some finTRIMs are induced by the virus infection.

Fig. 1. Variants of rainbow trout finTRIM ORFs. **A** Schematic representation of the structure of the rainbow trout finTRIMs from SSH and cDNA library. ORFs were predicted from the sequence of full-length transcripts. The RING, B1 and B2 boxes, coiled coil and PRY/SPRY domains are represented by boxes. **B** Quantitative RT-PCR analysis of finTRIM induction in trout leukocytes (Leuk.) incubated with the virus (VHSV) or LPS for 40 hours or in RTG-2 fibroblasts (Fibr.) incubated with poly(I:C) for 24 hours, respectively. Normalized values for finTRIM expression were determined by dividing the average finTRIM value by the average β-actin value. Then, normalized finTRIM values were subjected to a calibration relative to the basic expression in control (Ctrl) leukocytes or RTG2 cells. The error bars represent standart deviation. **C** Complex profiles produced by 3'RACE amplification of rainbow trout finTRIMs from leukocytes incubated with (V) or without VHSV (Ctrl). **D** 3'RACE profiling of finTRIM from

A FinTRIMs ORFs from SSH (AF483536) or from trout spleen phage library (AA887838 and AA8877999



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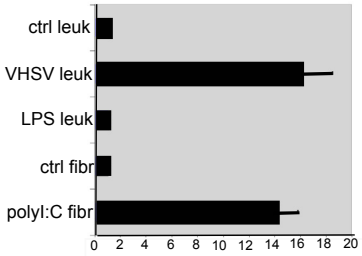

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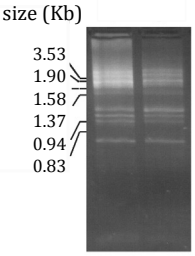

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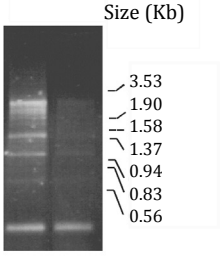
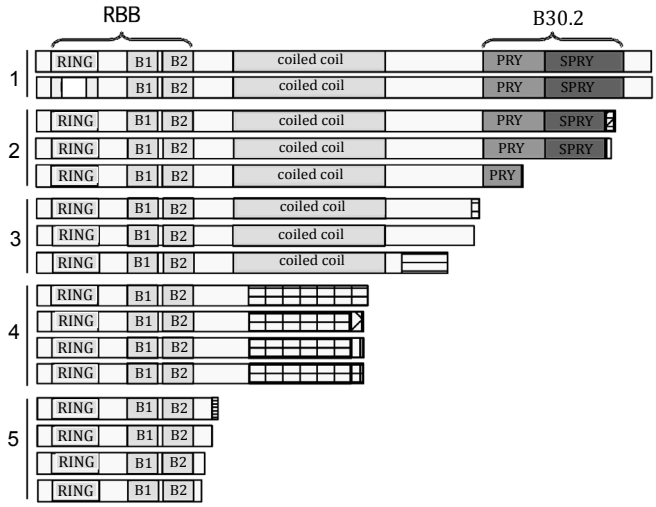
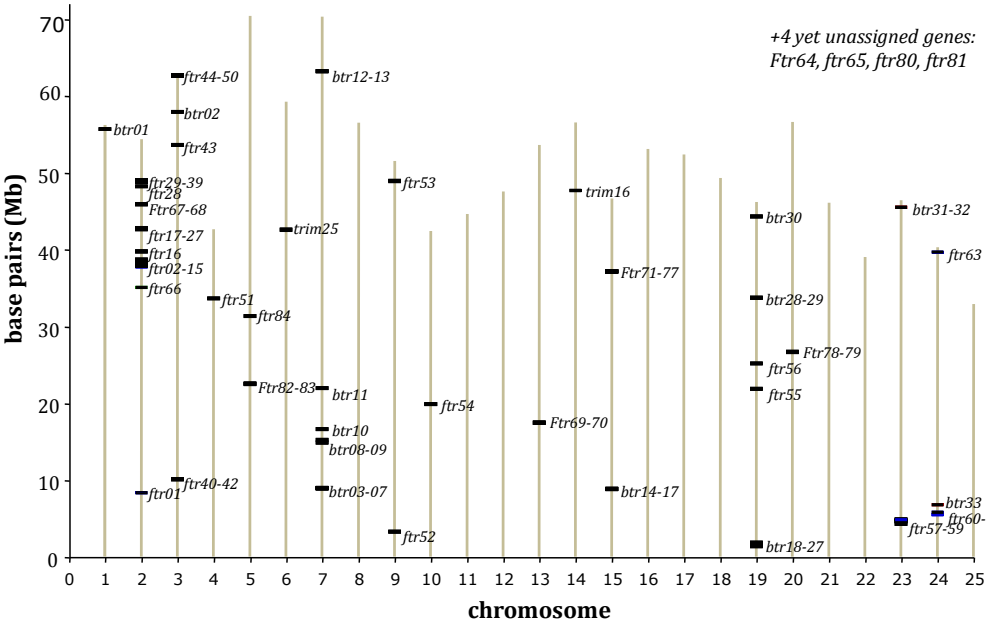


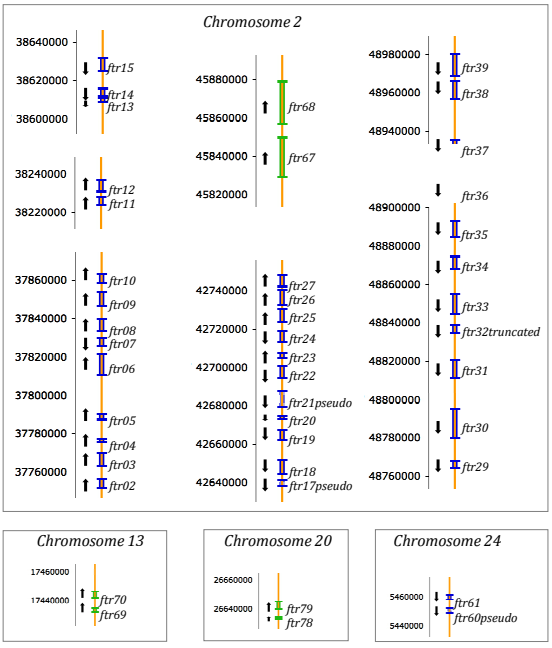
E FinTRIM ORFs from polyI:C-induced RTG2, VHSV-induced or control leukocytes



RTG2	Leukocytes	
polyI:C	VHSV	ctrl
11 +1	14	2
2	6	3
0	4	1
1	5	2
2	11	6

fibroblasts incubated with poly(I:C) (I:C) or without poly(I:C) (Ctrl). **E** Structure of the rainbow trout finTRIM ORFs from the 3'RACE products. Five classes could be distinguished on the basis of motifs and ORF lengths. Striped boxes indicate regions without similarity to the finTRIM consensus, perhaps corresponding to alternative splicing to additional exons. The number of clones corresponding to the different classes from the fibroblasts incubated with poly(I:C), or from leukocytes incubated with or without VHSV are indicated on the right.


+4 yet unassigned genes:
Ftr64, ftr65, ftr80, ftr81
base pairs (Mb)
chromosome
btr01
ftr44-50
btr02
ftr43
ftr29-39
ftr28
Ftr67-68
ftr17-27
ftr16
ftr02-15
ftr66
ftr51
ftr84
Ftr82-83
ftr01
ftr40-42
trim25
btr12-13
btr11
btr10
btr08-09
btr03-07
ftr53
ftr54
ftr52
trim16
Ftr69-70
Ftr71-77
btr14-17
btr30
btr28-29
Ftr78-79
ftr56
ftr55
btr18-27
btr31-32
ftr63
btr33
ftr60-
ftr57-59


Chromosome 2
ftr15
ftr14
ftr13
ftr12
ftr11
ftr10
ftr09
ftr08
ftr07
ftr06
ftr05
ftr04
ftr03
ftr02
ftr68
ftr67
ftr27
ftr26
ftr25
ftr24
ftr23
ftr22
ftr21pseudo
ftr20
ftr19
ftr18
ftr17pseudo
ftr39
ftr38
ftr37
ftr36
ftr35
ftr34
ftr33
ftr32truncated
ftr31
ftr30
ftr29
Chromosome 13
ftr70
ftr69
Chromosome 20
ftr79
ftr78
Chromosome 24
ftr61
ftr60pseudo

To characterize further the diversity of the finTRIMs, we performed a 3'RACE PCR on VHSV-induced leukocyte cDNA using a universal primer specific for trout finTRIM localized in the highly conserved region in the vicinity of the start codon. These experiments on infected leukocytes revealed a rich profile of amplified bands, which suggested that finTRIM sequences are highly diverse (Fig. 1C). A diverse profile was also observed in the absence of infection, but the signal appeared weaker and the profile less complex. To investigate finTRIM expression in a non-lymphoid cell type, similar 3'RACE PCR experiments were performed on the fibroblast cell line RTG2. Poly(I:C) was used to mimic viral infection, since VHSV replicates in these cells and kills them quickly, in contrast to leukocytes. Almost no expression of finTRIMs could be detected in untreated fibroblasts, while a number of bands appeared after poly(I:C) stimulation (Fig. 1D). This profile was less diverse than in leukocytes, with a bias towards long transcripts. This observation indicated that different arrays of finTRIMs are expressed in different cells, and corroborated the notion that some forms are inducible by viral infection.

The rainbow trout finTRIM transcripts are highly diverse

To further characterize the finTRIM diversity and to assess the specificity of the 3'RACE PCR amplification, we cloned the PCR products and sequenced clones picked at random. Fifty-four clones from leukocytes and 16 clones from RTG2 cells were completely sequenced. All clones contained a finTRIM sequence, confirming the high specificity of

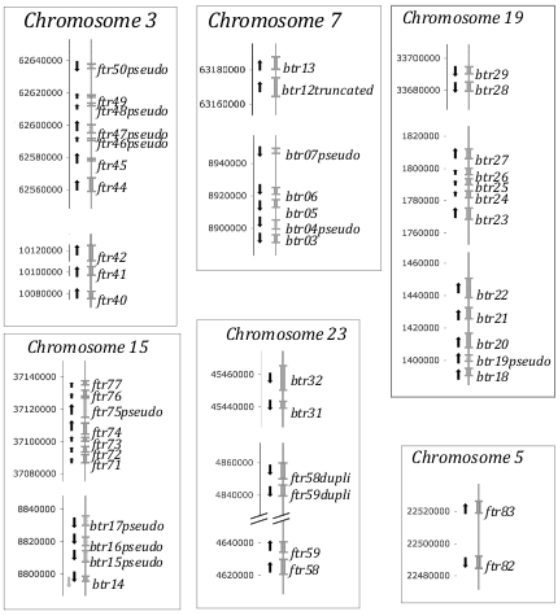


Fig. 2. Genomic location of zebrafish finTRIMs and related genes, based on the Zv7 assembly. On the left page, top: global distribution on the whole zebrafish genome; bottom (left and right page): detailed views of multigenic loci, with gene orientations. Gene limits depicted here correspond to the predicted start and stop codons (see details on table S1).

the amplification. The size of finTRIM transcripts was highly diverse, as expected from the 3'RACE profile. Different C-terminal regions of variable length were associated with the N-terminal RBB region shared by all sequences. For some clones, the C-terminus was encoded by short unique sequences unrelated to the longer ones. Based on their length or on the motifs present in the C-terminus, we classified the deduced finTRIM proteins into 5 different groups (Fig. 1E). In fact, although the N-terminal RING/B-box regions of the different finTRIM sequences were highly similar to each other, they were not identical due to single nucleotide substitutions. These substitutions were more frequent than the error frequency due to PCR and sequencing (2 to 5 %), and most of them were restricted to a number of conserved sites, which indicates that they did not correspond to artifacts. Therefore, the finTRIM diversity could not be due to alternative splicing of an unique RBB exon to multiple and diverse C-terminal exons. Moreover, the different sequences from leukocyte cDNAs were derived from a homozygous rainbow trout and therefore represent as many different genes and not allelic diversity. Taken together, these observations suggest that rainbow trout finTRIMs are encoded by a large number of different genes.

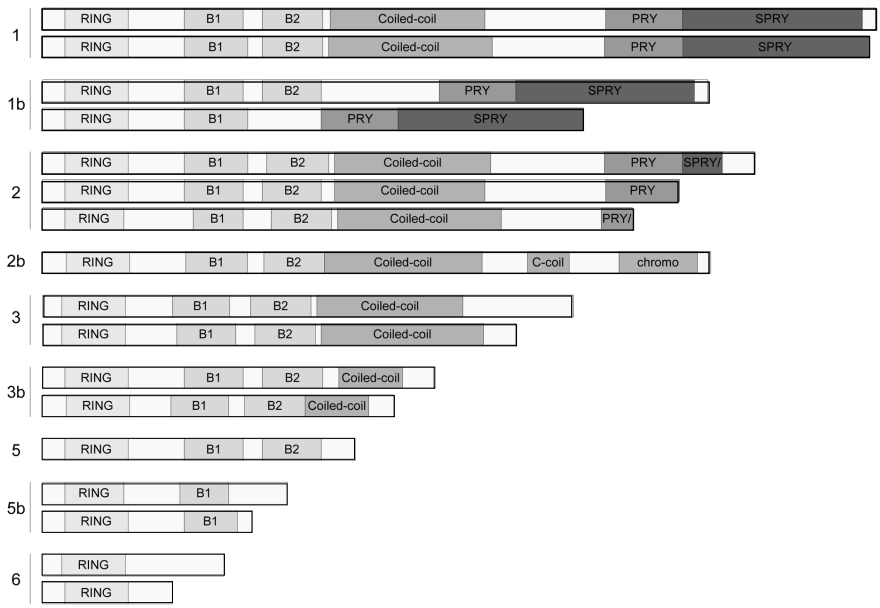


Fig. 3. Variants of zebrafish finTRIM ORFs. Schematic representation of the zebrafish expressed ftr sequences from group A. Protein sequences were predicted from 3'RACE clones that included a polyA tail. Since the consensus RACE primer was designed in the coding region (in the RING region), the N-termini of the proteins was extrapolated from the corresponding genomic sequences in zv7. Note that class 2b contains a chromo-domain at the C-terminus.

The finTRIM genes are highly diverse in zebrafish, where they constitute a multigene family

In order to extend the generality of our findings, we searched for finTRIM genes in another teleost, the zebrafish (*Danio rerio*). Tblastn searches were made on the most recent zebrafish genome assembly (Zv7), using as query the trout protein sequence AF483536 containing a RING and 2 B-Box motifs. A set of more than one hundred significant hits was obtained. More than half of them, associated with the most significant E-values, were more similar to the trout fintrims than to any other described trim gene. Unexpectedly, among low-ranking hits, we also found many genes with sequences most similar to *bloodthirsty* (*bty*), a known zebrafish TRIM gene with a B30.2 domain, involved in erythrocyte differentiation and closely related to human trim39 [41]. This observation was in accordance with a recent survey of vertebrate TRIM sequences reporting a high diversity of TRIM sequences in the fish genome [17].

As a consequence of our search criteria, all these hits corresponded to N-terminal RBB exons (truncated in three of them). We extended our search by looking for B30.2-domain encoding exons in the downstream genomic sequence and found one in most cases (>80%). Several genes appeared to be likely pseudogenes, either because of early frameshifts (allelic in some cases, see below), or absence of an identifiable start or stop codon. However, in several instances this may be due to a genome assembly defect or to unusual gene structure with extra undetected exon(s) upstream the RBB or downstream the B30.2. The deduced protein sequences were aligned and similarity trees were established (Fig. S1). The trees obtained with the RBB domains and with the B30.2 domains were highly congruent and allowed us to define two families: one that contained 84 finTRIM genes (hereafter named *ftr01* to *ftr84*) and one that contained 33 bloodthirsty-related genes (named *btr01* to *btr33*). Three subgroups, based on apparent phylogenetic age, were defined among the finTRIM family. The major subgroup (Group A), which includes 65 genes (*ftr01* to *ftr65*), represents the "crown group", which appears to have evolved most recently. Group B, including 17 genes (*ftr66* to *ftr81*) is not monophyletic, and contains genes that appear to have diverged at about the same time when the clade that now includes the zebrafish separated from the main teleost lineage. Finally, group C consists of only three genes (*ftr82*, *ftr83*, and *ftr84*) which seem to be the most ancient ones. Within each subgroup, genes were named according to their genomic position. Because the zebrafish genome assembly Zv7 is still imperfect, a few difficulties appeared with the annotation. For instance, the *bty* gene itself was not found in Zv7 (*bty* has been mapped to a relatively telomeric position on chromosome 19 [41], and therefore may reside within the same cluster as *btr18,* its closest relative among our dataset). An inverted duplication on chromosome 23 results in the presence of twins (in coding as well as in intronic sequences) for the closely linked *ftr58* and *ftr59* genes, which we named *ftr58dupli* and *ftr59dupli*. An assembly gap just downstream of the *ftr20* gene is probably responsible for its lack of a B30.2-containing exon. Finally, the contigs containing four genes (*ftr64*, *ftr65*, *ftr80*, and *ftr81*) are not yet assigned to a given chromosome.

The genomic distribution of all *ftr* and *btr* genes is shown on figure 2: detailed positions are given in table S1. As can be readily observed, most of these genes are

Table 2. FinTRIM gene expression in zebrafish embryos.

Class	sequences from control embryos (n=32)	sequences from virus or IFN-stimulated embryos (n=35)
1	11 (34%)	9 (26%)
	ftr14 (2x), ftr15 (3x), ftr39 (3x), ftr56, ftr65 (2x)	ftr02 (2x), ftr15, ftr23, ftr39, ftr56 (2x), ftr64, ftr65
1b	-	2 (6%)
		ftr15, ftr39
2	2 (6%)	3 (9%)
	ftr15*, ftr65*	ftr15* (3x), ftr39
2b	1 (3%)	1 (3%)
	ftr06	ftr06
3	5 (16%)	6 (17%)
	ftr14*, ftr15*, ftr19l, ftr51, ftr56	ftr07, ftr14, ftr19l (2x), ftr39, ftr56
3b	4 (13%)	8 (23%)
	ftr03 (3x), ftr65	ftr02(3x), ftr03 (3x), ftr23, ftr34
5	2 (6%)	-
	ftr15, ftr65	
5b	7 (22%)	3 (9%)
	ftr15, ftr23, ftr39* (3x), ftr65 (2x)	ftr39*, ftr64*, ftr65
6	-	2 (6%)
		ftr56*, ftr64

Group A finTRIM genes expressed in zebrafish embryos, as deduced from 3'RACE. Control embryos were either 72 hpf nonmanipulated embryos (4 sequences); 72 hpf, mcherry-transduced embryos (6 sequences); 78 hpf nonmanipulated embryos (10 sequences) or 96hpf nonmanipulated embryos (12 sequences). Stimulated embryos were either 72 hpf, zIFN1-transduced embryos (10 sequences); 78 hpf, SVCV-infected embryos (5 sequences); or 96 hpf, IHNV-infected embryos (20 sequences). Multiple occurences of ftr in different classes correspond either to allelic (*) or to splicing variants. Protein isoforms were classified according to Fig. 3. Accession numbers [GenBank:AM941305-AM941371].

arranged in clusters of genes in the same orientation. Half of the *ftr* genes are localized on chromosome 2, with 3 major clusters. Phylogenetic analysis indicates that genes within a cluster are more related to each other than to genes in other clusters. In addition to the long and readily detectable exons encoding the N-terminal RBB and C-terminal B30.2 domains, middle exons could be predicted for the majority of genes, with the help of our subsequent RACE analysis (see below) and with GNOMON-predicted sequences deposited in Genbank. For *ftr* genes, organization was very well conserved, with a first RBB-encoding exon of 555 to 594 (coding) bp, two 96 and 234-bp long exons encoding the coiled-coils, two 154 and 60-bp long exons with no clear domain associated, and a final ~545 coding bp exon encoding the B30.2 domain.

To get an insight into the expression pattern of zebrafish *finTRIM* genes, we designed consensus primers to perform 3'RACE-PCR simultaneously on all members of group A, the largest but also most homogeneous one. RACE-PCR products from zebrafish larvae were cloned and more than seventy clones were sequenced to get an approximate image of the relative fintrim expression in different settings of virus-induced activation. We tested three different stimuli: IFN1-overexpression, and experimental infection with either Spring Viraemia of Carp Virus (SVCV) or a heat-adapted variant of Infectious Hematopoietic Necrosis Virus (IHNV). Results are summarized in figure 3 and table 2. Given the very high number of zebrafish *ftr* genes in group A, it would be difficult to get a statistically significant picture of the expression of any single gene, but the aggregated data reveal interesting trends. At the developmental stage examined (72 to 96 hours post fertilization), expression was restricted, or at least highly skewed, towards a subset of genes, most of them on chromosome 2. Genes frequently found in control samples were also expressed in stimulated samples. Some genes, such as *ftr02*, *ftr23* and *ftr64* were detected only in stimulated samples. Short and long finTRIM proteins were expressed with or without B30.2 domain, in a striking parallel to what we have found in the trout 9 (compare Fig. 1D and Fig. 3). Viral or IFN-stimulation did not affect the overall pattern of length distribution, perhaps because RNA was extracted from whole embryos, which may dilute the specific response. Remarkably, alternative splicing did not account for all the diversity in protein size; allelic variation also played a prominent role (note that standard laboratory zebrafish are not inbred, and that pools of larvae were used). For instance, two different groups of ftr39 transcripts were found, some coding for canonical, full-length finTRIM proteins, while others, similar in this respect to the allele of the zv7 genome assembly, contain a 2-bp-deletion in the first exon, resulting in a transcript coding for a truncated protein with only a RING domain, irrespective of subsequent splicing.

The finTRIM family is present in many teleost fish but shows various levels of diversity
Systematic searches were performed in other available fish genome databases: medaka (*Oryzias latipes*), stickleback (*Gasterosteus aculeatus*) and pufferfish (*Tetraodon nigroviridis*), with tblastn and blastp using the trout and zebrafish conserved N-terminus of finTRIM as bait (Table S2). When highly similar hits were found, other finTRIM exons were searched close by, and the predicted sequences were subjected to multiple alignment with trout and zebrafish finTRIMs (Fig. 4A). We checked that these sequences retrieved zebrafish finTRIMs or allies when used as a query in the reciprocal blast searches. In medaka, this approach revealed a large number of close *finTRIM* homologs, as in zebrafish. The significant hits are mainly clustered on chromosomes 17 (29 hits, including 19 in the same region) and 18 (15 hits). A few genes are also dispersed on other chromosomes (Chr 1, 2, 5, 6, 14, 15, 21 and 24). In addition, 24 hits were found which were unanchored to the assembly. Interestingly, the whole ORF is encoded by one exon for most of these hits (47 genes among 55), in contrast to the zebrafish. These intronless genes correspond most probably to retrotransposed genes. In the pufferfish, the best hit is localized on chromosome 14, and additional homologs are found on chromosomes 7, 9, 17, 18 and 3 (9 hits in total), or among the unanchored

sequences. In the stickleback genome, at least four genes located in the same region of linkage group (LG) III and three genes in LG VII, showed high similarity with trout and zebrafish fintrims. A finTRIM encoding EST ([GenBank:BM424798]) was also identified in the channel catfish. Taken together, these observations suggested that fintrim genes constitute a multigenic family in all teleost genomes, with a highly variable number of genes (less than 10 to more than 80 genes).

The finTRIMs do not possess obvious orthologs in higher vertebrates

We used fish sequences of finTRIM RBB or B30.2 domains with tblastn program to search for their mammalian homologues in Genbank or genome databases. Both RBB and B30.2 regions from finTRIM sequences and the related sequences found in tetrapods were then subjected to phylogenetic analysis using NJ or parsimony methods, which produced congruent phylogenetic trees. This analysis revealed that the *finTRIMs* do not possess direct orthologs in mammals or in other tetrapods (Figure 4B and C). The mammalian proteins most similar to finTRIMs were TRIM16 and TRIM25. However, reciprocal blast queries using mammalian TRIM25 sequences identified in the zebrafish genome one single gene (gb#AY648763) more similar to *TRIM25* than to *finTRIMs*; a relative of this gene was also identified in trout and salmon. Similar queries with mammalian TRIM16 retrieved yet another gene (gb#BC155346). In contrast, no mammalian sequence appeared as a finTRIM ortholog. Orthologs of finTRIMs could not be found in chicken or *Xenopus* either, and therefore seem to be absent from tetrapods.

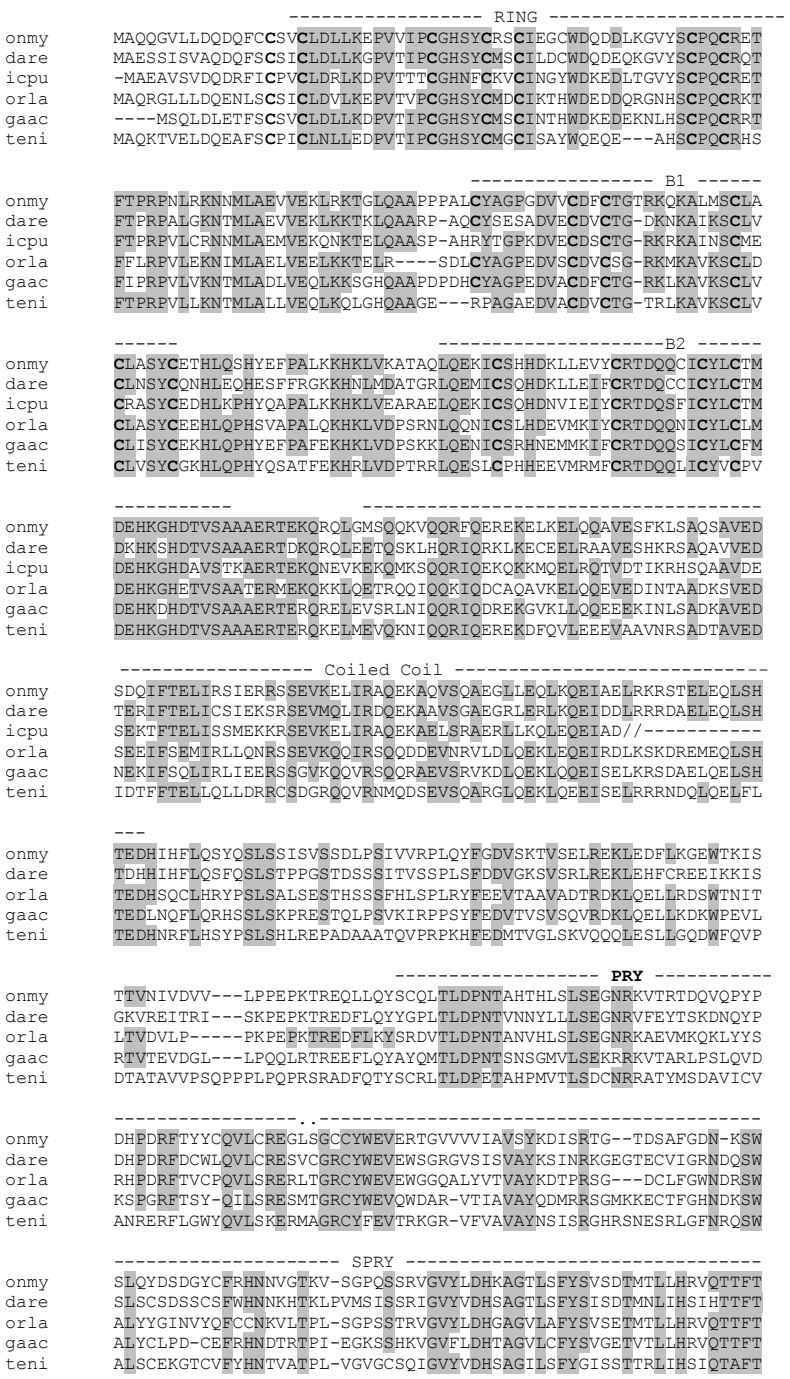
The phylogenetic analysis showed that finTRIMs sequences from different teleosts grouped in a separate branch supported by high bootstrap values in phylogenetic trees generated by either NJ or parsimony methods for both RBB and B30.2 regions (Fig. 4B and C). FinTRIMs therefore appear as a teleost-specific subset. In contrast, TRIM16-like and TRIM25-like sequences from fish or clawed frog grouped with their mammalian counterparts, identifying these trim genes as fish orthologs of mammalian trim16 and trim25. Interestingly, in the elephant shark genome, no finTRIM counterpart could be found, while sequences highly similar to TRIM16 and 25 were present (TRIM16: AAVX01048456; TRIM25: AAVX01256120; intermediate TRIM16/TRIM25: AAVX01115558, available at http://esharkgenome.imcb.a-star.edu.sg/). Although this database is still incomplete and shark *TRIM6/25* related sequences were partial, these observations reinforces the notion that *TRIM16* and *TRIM25* are ancient genes, while *finTRIMs* appeared and expanded in the teleost fish.

In addition, since *bty* has been described as the counterpart of *TRIM39*, we investigated phylogenetic relationships of the *btr* subgroup and tetrapod *TRIM39* with *TRIM16*, *TRIM25* and *finTRIMs*. Members of the btr subfamily (including *bty*) were indeed most similar to tetrapod *TRIM39*, and reciprocally; they also clustered together in the B30.2-based tree (Fig. 4C).

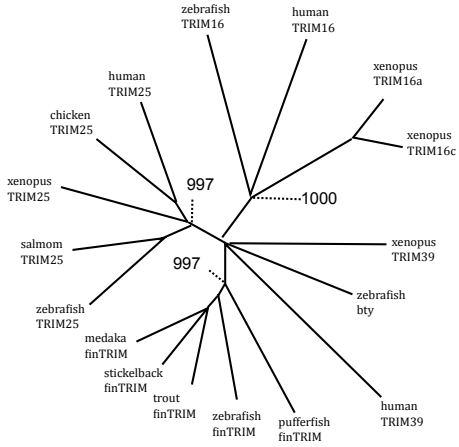
Conserved syntenies between teleost fish and tetrapods for fintrim-related genes

To further investigate the origin of finTRIMs, conserved markers in the vicinity of tetraodon, stickleback or zebrafish *ftr* clusters were identified and used to search for conserved syntenies in other genomes. No clear conserved synteny could be identified

A



B RBB domains



C B30.2 domains

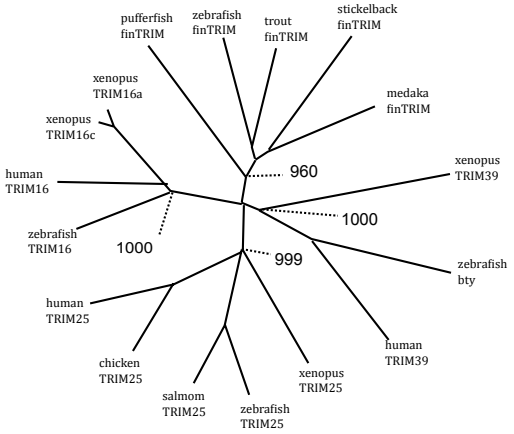


Fig. 4. FinTRIMs represent a teleost-specific multigenic family. **A** (previous page) Multiple alignment of representative teleost finTRIM sequences. Rainbow trout (onmy) [GenBank:AM887799], zebrafish (dare) (ftr14, [GenBank:XM_692536], channel catfish (icpu) ([GenBank:BM424798], medaka (orla) (Ensembl: ENSORLP00000003320), Stickleback (gaac) (Ensembl, Linkage groupIII:14324861-14326501; GENSCAN00000022585), Pufferfish (teni) (Ensembl GSTENT00020235001). **B** and **C** (above) Phylogenetic trees (NJ, boostrap=1000) of the finTRIM and their relatives, based on the RBB (**B**) and B30.2 (**C**) regions. finTRIM accession numbers are as in (A); other sequences from zebrafish: Bty (bloodthirsty) [GenBank: NP_001018311]; TRIM25, [GenBank: NP_956469]; TRIM16, [GenBank: XR_029737]; from human : TRIM16, [SwissProt: Q99PP9]; TRIM25, [SwissProt: Q14258]; TRIM39, [GenBank: NP_742013]; from chicken: TRIM25, [GenBank: XP_415653]; from salmon : TRIM25 [gene index TC35355 accessible at http://compbio.dfci.harvard.edu/]; from xenopus: TRIM16a, [GenBank: AAH74300]; TRIM16b, [GenBank: NP_001086184]; TRIM25, [Ensembl Xenopus genome scaffold255: 821309_819660]; TRIM39, [Ensembl Xenopus genome scaffold709: 241758_272825].

between the regions encoding group A or group B finTRIMs in different teleosts, suggesting that finTRIM genes were subjected to genus or species-specific duplication and expansion episodes during the evolution of teleosts.

In contrast, we could identify a conserved synteny between regions comprising the "ancient" (group C) *ftr82* and *ftr83* genes associated with the markers INTS2, RSPS6, ATP5L, RUVBL2 and SPSN2 in zebrafish, medaka, and stickleback (Fig. 5). Conserved syntenies were also observed for markers flanking *TRIM16* and *TRIM25* in these three species, supporting the hypothesis that these genes are older and kept in a more stable genomic configuration than recent fintrims. Interestingly, the orthologs of all the markers involved in these conserved synteny groups (*ftr82/83*, *TRIM25*, *TRIM16*) in teleosts were located on the same human chromosome 17, distributed over 70 megabases (Fig. 5). In addition, they were also retrieved on the mouse chromosome 11 and on the chicken mini chromosomes 18 and 19 that correspond to large regions of the human chromosome 17.

Taken together, these observations in teleosts and tetrapods suggest that the genomic configuration of *TRIM16* or *TRIM25* (as opposed to finTRIM genes) cannot be explained by recent sporadic events that occurred in particular species. They rather suggest that the regions containing the ancestral *TRIM16* and *TRIM25* moved apart in the early fish evolution and were kept as synteny groups on two different chromosomes while most fintrims appeared and differentiated by multiple duplications in the fish lineages. The situation is more complex for group C *ftrs 82/83* (see discussion).

The zebrafish finTRIM proteins have evolved under positive selection

To get further insight into the meaning of finTRIM sequence variability, we analyzed the pattern of variable positions, in the context of the tertiary structure of the B30.2 domain that has been determined for human TRIM21 [16, 42]. The B30.2 domain forms a distorted β-sandwich of two antiparallel β-sheets, made up by the PRY and SPRY subdomains [16, 42, 43]. The β-strands are connected by six variable loops that define regions of hypervariability and form the ligand-binding surface in TRIM5α and TRIM21. We determined variability in finTRIM B30.2 by performing two multiple alignments of both trout finTRIM B30.2 and zebrafish finTRIM group A B30.2 sequences (see Fig. S2) and determined site-by-site variation. We then aligned the B30.2 domains of trout and zebrafish finTRIM with human TRIM5α and TRIM21 (Fig. 6). The trout finTRIM B30.2 sequences are rather conserved and the majority of variable sites, 23 of 29, are located in the regions that correspond with the variable loops of TRIM21. Fifteen variable sites of trout correspond with regions that are hypervariable in primate TRIM5α. The zebrafish finTRIM B30.2 sequences are even less conserved with 78 variable sites that are also associated with the predicted variable loops of TRIM21 or to the hypervariable regions of TRIM5α, albeit more loosely.

For primate TRIM5α it has been demonstrated that the B30.2 domain has evolved under diversifying selection pressure, with the positively selected sites predominantly located in the four regions that are hypervariable among primate TRIM5α [27, 44]. We investigated whether the zebrafish *finTRIM* genes have also evolved under diversifying selection. We used a test that is based on the estimation of synonymous (dS, silent

mutations) and non-synonymous (dN, amino acid altering) substitution rates of all codons among a set of sequences. The ratio ω= dN/dS is an indication for negative selection (ω < 1), neutral evolution (ω =1), or positive selection (ω >1). If an amino acid change is neutral, it will be fixed at the same rate as a synonymous mutation, and ω=1. If the amino acid change is deleterious, purifying (negative) selection will reduce its fixation rate, thus ω < 1. An amino acid change is fixed at a higher rate than

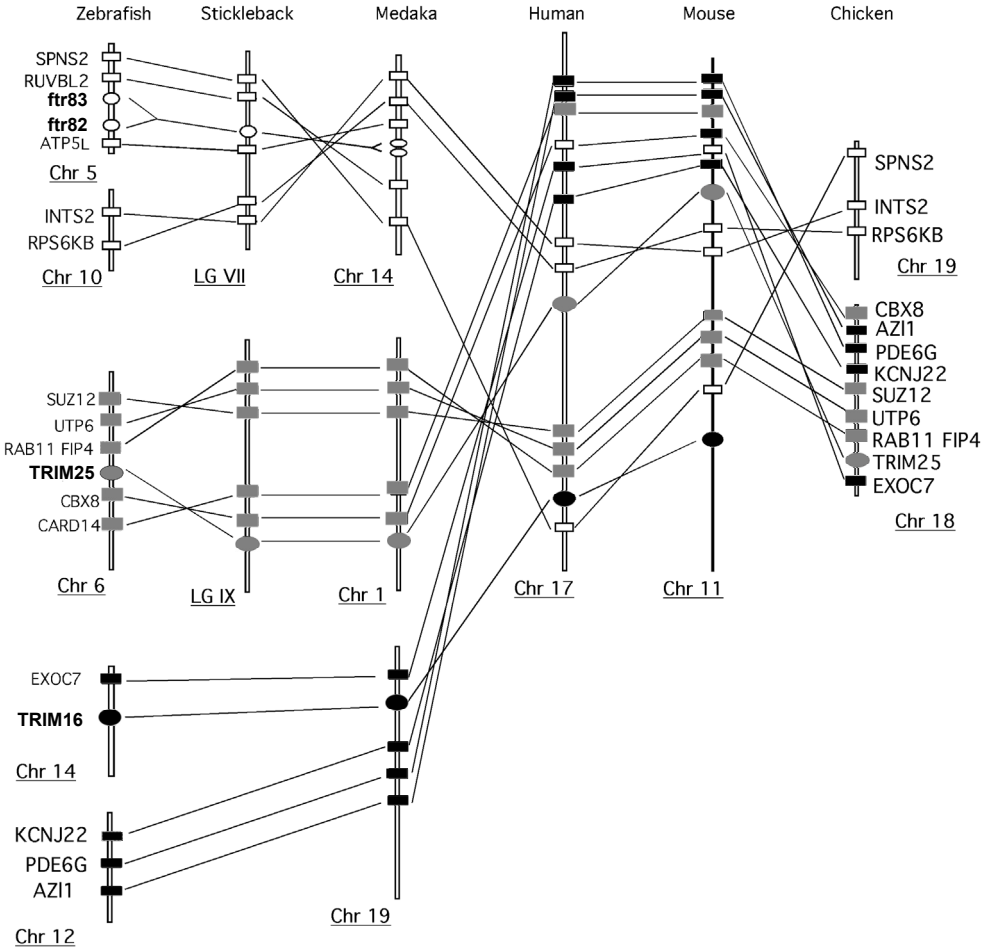


Fig. 5. Conserved synteny of finTRIM-like genes in teleosts. The locations of ftr82/83, TRIM25 and TRIM16 is schematically represented in the context of gene markers in zebrafish, stickelback, medaka, human, mouse and chicken to show conserved synteny groups. The gene locations are indicated (in Kb) in the table, according to the last assembly available for each species at the ensembl website (medaka: HdrR; stickleback: BROAD S1; zebrafish: Zv7; human: NCBI 36; mouse: NCBI m37).

a synonymous mutation (ω > 1) only when it offers a selective advantage. The site-specific model within the PAML software package allows heterogeneity in evolutionary pressure along a protein encoding sequence and can identify the specific sites that are under positive selection. Two models of substitution distribution, M2a and M8, can be used to test the positive selection hypothesis against the nested null models: M2a against M1a and M8 against M7.

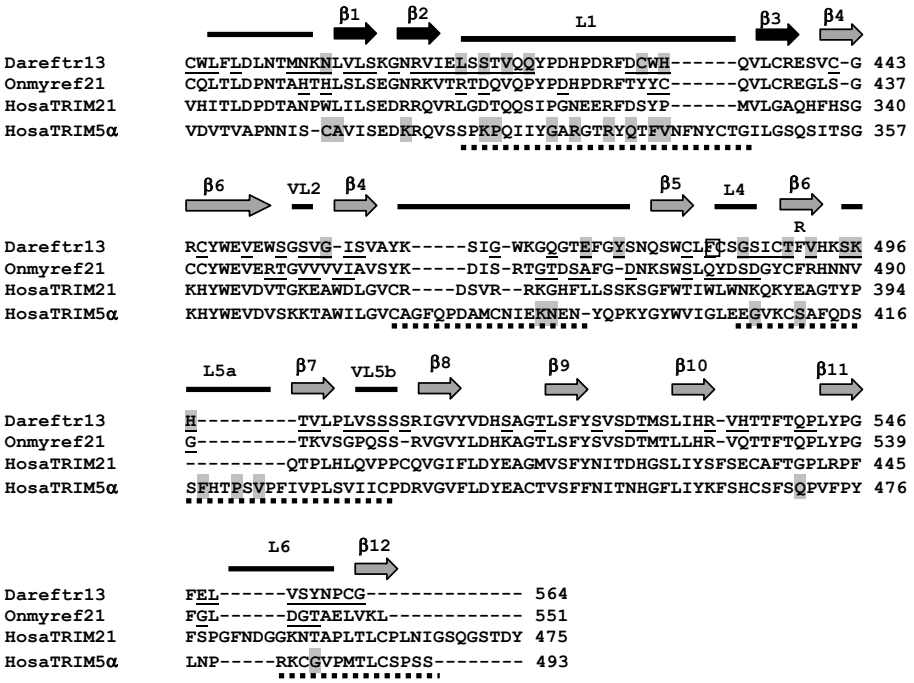


Fig. 6. Positive selection in the B30.2 domain. Distribution of hypervariable and positively selected residues in a multiple alignment of B30.2 domains from representative zebrafish and rainbow trout finTRIMs (Dareftr13: [GenBank: XM_695031], Onmyref21: [GenBank: XM_695031], human TRIM21 (HosaTRIM21) and TRIM5α (HosaTRIM5α). Sites that are less than 80 percent conserved among zebrafish or among trout sequences are underlined Positively selected sites (among zebrafish finTRIMs: this study; among primate TRIM5α: previous work of Sawyer, Emerman et al. [44]) are boxed in grey when detected under models 2a and 8. One site was positive under M8 but not under M2 and is boxed. β-strands identified from the TRIM21 structure B30.2 sequence are indicated by black (PRY) or grey (SPRY) arrows [16]. The variable loop-connecting strands are named VL1-VL6. The four hypervariable regions of the TRIM5α B30.2 domain are underlined by a dotted line [27]. The detailed PMAL results for each position under positive selection are available in figure S3. "R" indicates the recombination site identified by GARD.

We took the complete sequences of zebrafish finTRIM group A B30.2 domains and analyzed them with the PAML models M1a, M2a, M7 and M8. A value of ω >1 was detected for 15.6 % of sites under M2a and 17.6% of sites under M8 (see Table 3). The likely-hood-ratio test (LRT) was significant with p<0.001 for both models (see Table 4). The estimation of substitution rates by PAML is based on branch lengths of sequences in the phylogenetic tree. As a result of recombination, sites within one sequence are no longer similar in branch length and this can interfere with the results of PAML since it is assumed that all sites within a sequence are similar in branch length in the model. To investigate whether the detection of positive selection was not perturbed by recombination, we implemented the algorithm PARRIS on our dataset. With the PARRIS program, a partitioning approach is used and site-to-site variation in both synonymous and non-synonymous rates is integrated in the M1a and M2a models. We analyzed the zebrafish finTRIM group A B30.2 sequences with PARRIS and could still detect positive selection by the LTR with p<0.001, indicating that whether or not recombination did occur, the B30.2 has evolved under positive selection. To search for recombination sites, we employed the program GARD. The algorithm subdivides the sequence alignment in putative non-recombinant fragments, phylogenies are inferred for each fragment and the goodness of fit is assessed by the c-AIC, predicting whether or not the fragments are derived from two different ancestor sequences due to recombination. We searched for either two or up to twenty breakage points and could identify one breakage point by both searches, with ∆c-AIC= 42,96 under the two-breakage-point model and ∆c-AIC= 28,67 under the twenty-breakage-point model (see Table S3).

We re-analyzed our zebrafish finTRIM group A dataset of B30.2 by subdividing the alignment in two parts, containing the sequence regions before, or after the detected recombination point. For both regions, we detected positive selection, with p<0,001 in the LRT under M1a-M2a and M7-M8 models (see Table 3 and Table 4). The specific sites under positive selection according to the models 2a and 8 were identified by a Bayesian approach. For the B30.2 domain we could identify 16 sites under both model 2a and 17 under model 8. Fourteen sites were located in regions corresponding to the predicted variable loops of TRIM21. In addition, the majority of the sites were located within the regions corresponding with the four hypervariable regions described for TRIM5α, with six sites falling in the hypervariable region 1, one in region 2 and six in region 3. (See Figure 6 for positions of sites and Table S4 for posterior probabilities).

We used a similar approach to detect positive selective sites in the RING and two B-box domains. First we analysed with PAML the complete data-set from all RING-Bbox sequences from zebrafish finTRIM group A. Positive selection was detected for 6.3 % of sites under M2a and 7.1 % of sites under M8 with p<0.001 in the LTR of both M1a-M2a and M7-M8. With PARRIS we confirmed that the RBB has evolved under positive selection with p<0.001 in the LTR of M1a-M2a. We used GARD to search for recombination and we could identify as well a breakage point, with a significant value of ∆c-AIC= 254.63 under the two-breakage point model and ∆c-AIC= 263.22 under the twenty-breakage point model. We therefore divided the RBB multiple alignment in two segments and re-analyzed the sequences located before and after the breakage site using PAML. For the sequences located before the predicted breakage point we could find three sites under

Table 3. PAML results.

Region[1]	n[2]	c[3]	Parameters in ω distribution under M2a[4]		Parameters in ω distribution under M8[5]	
B30.2	38	145	$\omega_{>1}$ = 2.98787	$p_{>1}$ = 0.15644	$\omega_{>1}$ =2.64795	p_1 = 0.17627
complete 1-435			ω_1 = 1.000	p_1 = 0.25983		p_0 = 0.82373
			$\omega_{<1}$ = 0.18514	p<1 = 0.58373	p = 0.64985	q = 1.07859
B30.2	38	95	$\omega_{>1}$ = 2.79395	$p_{>1}$ = 0.15969	$\omega_{>1}$ = 2.38329	p_1 = 0.20431
fragment 1-285			ω_1 = 1.000	p_1 = 0.28631		p_0 = 0.79569
			$\omega_{<1}$ = 0.17036	p<1 = 0.554	p = 0.62590	q = 1.00411
B30.2	38	50	$\omega_{>1}$ = 3.28071	$p_{>1}$ = 0.09143	$\omega_{>1}$ = 2.84563	p_1 = 0.09925
fragment 286-435			ω1 = 1.000	p_1 = 0.28492		p_0 = 0.90075
			ω <1 = 0.17611	p<1 = 0.62365	p = 0.70827	q = 1.26680
RBB	55	155	$\omega_{>1}$ = 2.1211	$p_{>1}$= 0.06286	$\omega_{>1}$ = 1.58831	p_1 = 0.07109
complete 1-465			ω_1 = 1.000	p_1 = 0.44597		p_0 = 0.92891
			ω <1 = 0.11812	p<1= 0.49117	p = 0.47665	q = 0.86554
RBB	55	78	$\omega_{>1}$ = 2.53603	$p_{>1}$= 0.06002	$\omega_{>1}$= 1.98626	p_1 = 0.06121
fragment 1-234			ω_1 = 1.000	p_1 = 0.43598		p_0 = 0.93879
			ω <1 = 011843	p<1= 0.50400	p = 0.93879	q = 1.04448
RBB	55	76	$\omega_{>1}$ = 2.05674	$p_{>1}$= 0.02961	$\omega_{>1}$ = 1.49995	p_1 = 0.02779
fragment 238-465			ω_1 = 1.000	p_1 = 0.46960		p_0 = 0.97221
			ω <1 = 0.12722	p<1= 0.50079	p = 0.47756	q = 0.81657

[1] for sequence fragments, the numbers correspond with the position of first and last nucleotides in the alignment with excluded gaps

[2] n, the number of sequences in the alignment and tree

[3] c, the number of codons

[4] parameters determined under M2a with ω the ratio of non-synonymous rates (*dN*) and synonymous rates (*dS*) and p the corresponding proportion of sites for each ω-class

[5] parameters determined under M8 with ω the ratio *dN/dS*, the corresponding proportion (p_1=1-p_0) of sites and *p*- and *q*-estimates in the β(*p*,*q*)-distribution

positive selection under M2a and under M8, with $p<0.001$ in the LTR under both nested models. These sites are located just upstream the RING motif. For the region after the breakage point, the test for positive selection was no longer significant, with $p=0.155$ in the LTR of M1a-M2a and $p= 0.455$ in the LRT of M7-M8, (see Table 2).

Taken together, these results firmly establish that the loops of the zebrafish GroupA finTRIM B302 domains have been diversified under positive selection, as previously described for the sites determining virus specificity in TRIM5α suggesting a selective pressure on this domain for a binding to diverse ligands. A few positions located close to the RING motif are also subjected to diversification.

Table 4. Results of LRT for positive selection

Region[1]	**Model**[2]	**2 Δ lnL**	***p*-value**	**No sites**
B30.2 complete	PAML M1a-M2a	208.21	p<0.001	16
	PAML M7-M8	231.62	p<0.001	21
B30.2 complete	PARRIS M1a-M2a	29.38	p<0.001	ND
B30.2 1-285	PAML M1a-M2a	124.15	p<0.001	13
	PAML M7-M8	129.55	p<0.001	14
B30.2 286-485	PAML M1a-M2a	41.33	p<0.001	3
	PAML M7-M8	47.95	p<0.001	3
RBB complete	PAML M1a-M2a	25.25	p<0.001	4
	PAML M7-M8	18.20	p<0.001	2
RBB complete	PARRIS M1a-M2a	20.95	p<0.001	ND
RBB 1-234	PAML M1a-M2a	21.04	p<0.001	3
	PAML M7-M8	17.94	p<0.001	3
RBB 238-465	PAML M1a-M2a	3.73	p= 0.155	0
	PAML M7-M8	1.57	p= 0.455	0

[1] for fragmented regions, numbers correspond with positions of first and last nucleotides in the alignment

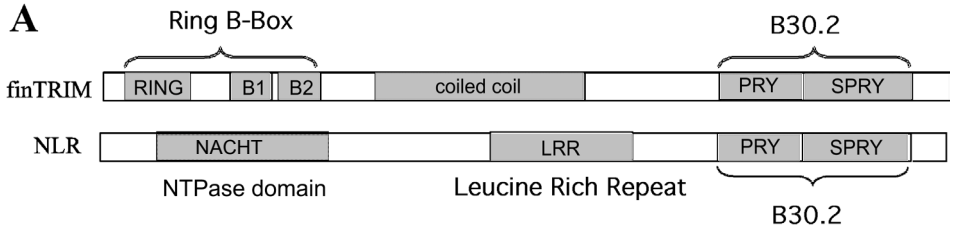
[2] The models M1a, M2a, M7 and M8 were employed, using either the program PAML or program PARRIS.

Exon shuffling of B30.2 domain occurred between finTRIMs and NLR
Since finTRIM B30.2 features suggest that they may be involved in interactions with diverse ligands, similar B30.2 domains may be used in other TRIM subfamilies for comparable purposes. We therefore searched the zebrafish genome for sequences closely related to typical finTRIM B30.2 domains. Unexpectedly, the closest counterparts of finTRIM B30.2 sequences were not found in other TRIM, but associated with NACHT and Leucine-rich repeats (LRRs)- ribonuclease inhibitor (RI)-like motifs in NLR (NOD-like receptors) proteins (Fig. 7A). These proteins that possess a B30.2 and a NACHT domain, and are very likely involved in innate immunity, have been identified very recently; such a combination is not found in mammals [40]. While the protein sequences of B30.2 from Group A ftrs were about 40% similar to B30.2 domains from close TRIM relatives such as bloodthirsty or TRIM25, they were 55 to 65% similar to B30.2 from these NLR proteins, and about 50-55% similar to those of the Group B or C ftr sequences (Fig. 7B). A phylogenetic analysis showed that B30.2 sequences of group A finTRIM and a subgroup of NLR (named here group 1) are joined as a cluster supported by a high bootstrap value (Fig. 7C). This cluster is quite distinct from that of other TRIMs including group B or group C *ftr* and *btr,* or even from the rest of B30.2-bearing NLRs (group 2), suggesting an exon shuffling event between group A *finTRIMs* and group 1 *NLRs*.

Discussion

We have described here a large set of closely related genes and transcripts that contain the three motifs typical of TRIM proteins, namely the RING zinc finger, two B-boxes and a predicted coiled coil region. Their close relatedness allowed us to group these sequences in one multigene family, the finTRIMs. The finTRIM genes were identified in all teleost fish species for which a genome database is available. In addition, we characterized a number of transcripts in rainbow trout and zebrafish, confirming that these genes are actively transcribed. Although the gene numbers vary among the different teleost species, the wide distribution of the fintrim genes suggests an important role, one in which diversity offers a selective advantage to the host. Since finTRIMs are specifically induced by viruses and poly(I:C), they probably play a role in antiviral immunity, as several other TRIMs do in mammals.

FinTRIM diversity suggests that they recognize multiple ligands
The highly diverse finTRIMs are encoded by a large number of genes. This TRIM subset is a teleost-specific group, well distinct from other TRIMs shared by fish and mammals as TRIM16, TRIM25, TRIM39 and others. Next to the variety generated by the large number of genes, we demonstrate here that the B30.2 domain of finTRIM genes has evolved under diversifying selective forces. This indicates that these proteins bind a diverse range of ligands, making an immune function very plausible. Consistent with a role in immune recognition, our current data suggest that there is a high allelic polymorphism among zebrafish fintrim genes. There are numerous examples of large polymorphic multigene families that are involved in innate immunity. The proteins they encode either recognize a variety of microbial patterns, or bind diverse receptors of



B

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FTR35           SQQFTLDPNTAHKYLVLSEGNTAVEDKDSVQPYPDHPDRFDGWRQVLCRESVCGRSYWEL
NLR group1      TCFLTLDPNTANTLLILSEENKKVKRVKEKQPYPDHPDRFDVHPQVLCRESVCGRCYWEI
                :  :********:. *:*** *. *:  .. ***********    ***********.***:

FTR35           EWSGDGVYLSVSYKSISRKGSGNECGFGHNDQSWSLCCSDYSFSFRHNNIKTKLPKPSVS
NLR group1      DWSGDVVEISVLYKSIRRKGDSVECVFGCNDQSWSLRCSSSSFSFRHNNEDTDVRVEPLS
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FTR35           SSRIGVYVDHSAGTLSFYSVSDTTMSLMHTLHTTFTHTLYPGFCVYYSSRLKL
NLR group1      R-RIGVFVDHSAGTLIFYNIYRDTMSLIHSVQTTFTEPLCPGFTVYSGSSVKL
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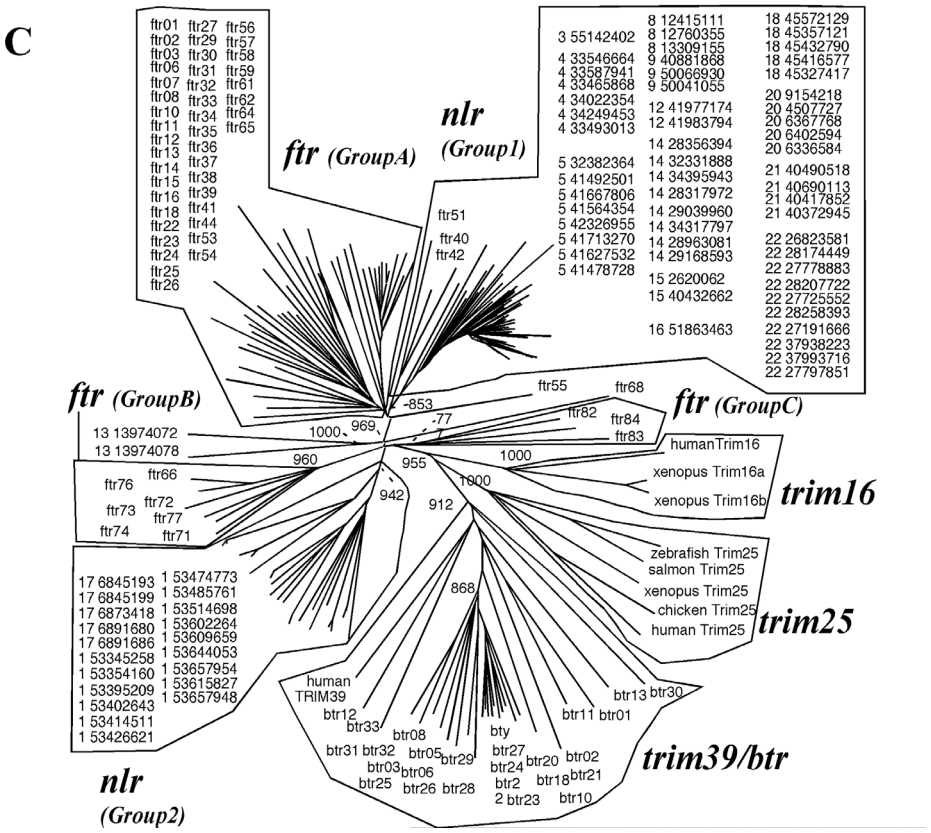

the host immune system and participate to the tuning of complex activation pathways. Such receptors include some mammalian TRIMs [45-47], but also the mammalian killer cell immunoglobulin-like receptors (KIR) [48] and Ly-49 related proteins [49], chicken Ig-like receptor (CHIR) [50, 51], fish novel immune-type receptors (NITR) [52, 53], fish leukocyte immune-type receptors (LITR) [54], and sea urchin Toll-like receptors [55]. If finTRIMs are specifically involved in virus recognition or act as virus restricting molecules, the high number of fintrim genes opens the possibility for parallel and simultaneous selection by different viruses [46].

Signatures of positively selected residues in finTRIM B30.2 equate with canonical motifs of the virus-binding sites in TRIM5α

The finTRIM B30.2 domain contributes dominantly to finTRIM diversity, and seems to be generally similar in structure to the B30.2 domain of TRIM21. Among the sequences of the multiple finTRIMs described in both trout and zebrafish, the variable sites are predominantly located in the variable loops of the domain, in a way that strongly suggests that the B30.2 domains interacts with their ligands as TRIM5α does for viral proteins. In particular, the sites identified in zebrafish as subjected to significant diversifying ("positive") selection were concentrated in the variable loop 1, between the β-strands 2 and 3. This loop was earlier designated as a 'hotspot' region, since sites within this region determine the lentivirus restriction-specificity of TRIM5α and mutations in this region are correlated with the disease susceptibility associated with TRIM20 (familial Mediterranean fever) and TRIM21 (an autoantigen in multiple diseases) [16]. Such a distribution of sites diversified under positive selection in the loop 1 (loop β2-3) of the B30.2 domain strongly supports both the reality of a diversifying selection and the diversity of ftr B30.2 ligands.

In addition, we could also find several sites under diversifying selection within the RBB domain, suggesting that additional sites may also be involved in binding of an(other) ligand. A few sites were earlier shown to have evolved under diversifying selection in the coiled coil region of proteins TRIM5α and TRIM22 [44], but positive selection of RING and B-box domains was not reported. Besides, the roles of the RING and B-box domains in TRIM function are still not fully understood, although deletion or mutations can abrogate the activity of TRIM5α, and the significance of the positively selected RBB residues in ftrs remains therefore elusive. In this context, one may also question the function of the finTRIM genes that do not contain a B30.2 domain (1 out of 5 in the zebrafish, but probably more in trout). It is tempting to consider them as

Fig. 7. Distance tree of finTRIM B30.2 domains and related sequences. The domain organization of finTRIM and NLR proteins is represented in (**A**). A typical alignment of the B30.2 sequences from group A ftr (ftr35) and NLR (chr5_32382364) is shown in (**B**). B30.2 domains of the zebrafish finTRIMs and btrs as well as those extracted from related trims of other vertebrates (human and xenopus TRIM16, human TRIM39, Atlantic salmon, zebrafish, xenopus, chicken and human TRIM25; see figure 4 for accession numbers) were aligned with related B30.2 sequences from nlrs, and a distance tree was produced using mega4 (NJ, boostrap=1000); high boostrap values of key nodes are indicated (**C**).

potential inhibitors/regulators of an antiviral response triggered by B30.2-containing genes.

The evolutionary affinities of finTRIM B30.2 domains suggest a domain shuffling between NLR and TRIM molecules

An interesting finding was the close relationship of finTRIM B30.2 domains with the B30.2 domains present in a subset of NLR proteins recently described in the zebrafish. NLR proteins, also known as CATERPILLER, NACHT or NOD-LRR proteins are large cytoplasmic proteins involved in inflammation and apoptosis. They are characterized by a NACHT domain and a Leucine rich repeat (LRR) region at the C-terminus and vary in their N-terminal effector domain, which is either a CARD, pyrin, or TIR domain. As for TRIM proteins, the physiological functions of NLRs are diverse, and several NLRs are inhibitors/activators of the inflammatory and immune responses. For example, NALP3/cryopyrin is a key component of the inflammasome and inhibits TNF-α and TRAF6- induced NF-κB activation [56], while NOD2 plays a role in NF-κB activation [57]. Large groups of novel, fish-specific NLR proteins have been recently described in fish and appear to be highly related within each species, indicating recent species-specific expansions [39]. Some of these teleost NLR proteins contain the NACHT and LRR domains at the C-terminus in combination with a B30.2 domains, as the NLR-C described in [40]. The close relatedness of these B30.2 domains to the group A finTRIM B30.2 domain suggests that the corresponding exon(s) have been subjected to shuffling between NLR and group A finTRIM, ie during the *ftr* evolution (Fig. 8). The exchange of a ligand recognition module evolving under positive selection with another protein family mainly involved in inflammation, immune regulation and possibly pathogen sensing, constitutes another argument supporting an immune function for group A finTRIMs.

Phylogenetic analyses suggest that ftr appeared and diversified during the teleost evolution while TRIM16/25/39 are more ancient genes common to all vertebrates

The *finTRIMs* and their relatives (*TRIM25*, *TRIM16*, *TRIM39*) followed different evolutionary pathways (Figure 8). Both teleosts and mammals possess single orthologous *TRIM25* and *TRIM16*, as evidenced by phylogenetic analysis and conserved synteny. Sequences coding for partial *TRIM*16 and *TRIM*25 were also identified in the elephant shark genome, confirming that these genes were already present in the early vertebrates. In contrast, *finTRIMs* seem to be unique to teleost fish and could not be found in any other group of animals. Since they were present in all fish for which a significant amount of genomic data was available, finTRIMs most probably appeared during the early evolution of teleosts. While they are represented by large gene sets in zebrafish, salmonids, or medaka, several fish species such as fugu or stickleback only possess a few copies of finTRIM genes. Thus, finTRIM genes have been probably subjected to parallel - and independent - duplication blasts in the different branches of teleosts. This hypothesis is strengthened by the fact that zebrafish ftr genes were generally most similar to their immediate neighbors, suggesting tandem duplication within clusters rather than *en bloc* duplication as the main gene amplification mechanism.

This view is supported by the absence of obvious synteny between the regions

encoding almost all finTRIMs in different teleosts. Also, diverse mechanisms of gene spreading appear to have been used in different species, since many medaka *finTRIMs* are intronless in contrast to zebrafish *ftr*, suggesting that one or more retro-transposition events have been involved in the multiplication of these sequences. This observation is a good argument for a fast expansion in this species, and reinforces the idea of strong selection pressures towards finTRIM diversification. Such selection pressures exerted by species- or family- specific viruses are expected to be highly variable between different fish taxa. Whether another TRIM set constrains or balances the evolution of finTRIMs, as described for anti-retroviral TRIM5α and TRIM22 [44], remains to be established. Also, the B30.2 exon shuffling has complicated the finTRIM evolutionary pathway.

Group C finTRIMs is closest to other TRIMs in the phylogenetic trees, suggesting that they appeared during the early evolution of teleosts. They are present in the different fish investigated so far, but no counterpart was found in other vertebrates. Interestingly, a conserved synteny of *ftr82/83* and a few neighboring markers was established in zebrafish, stickleback and medaka, indicating that they were kept in a more stable genomic context than other *finTRIMs*; it is also worth noting that in the

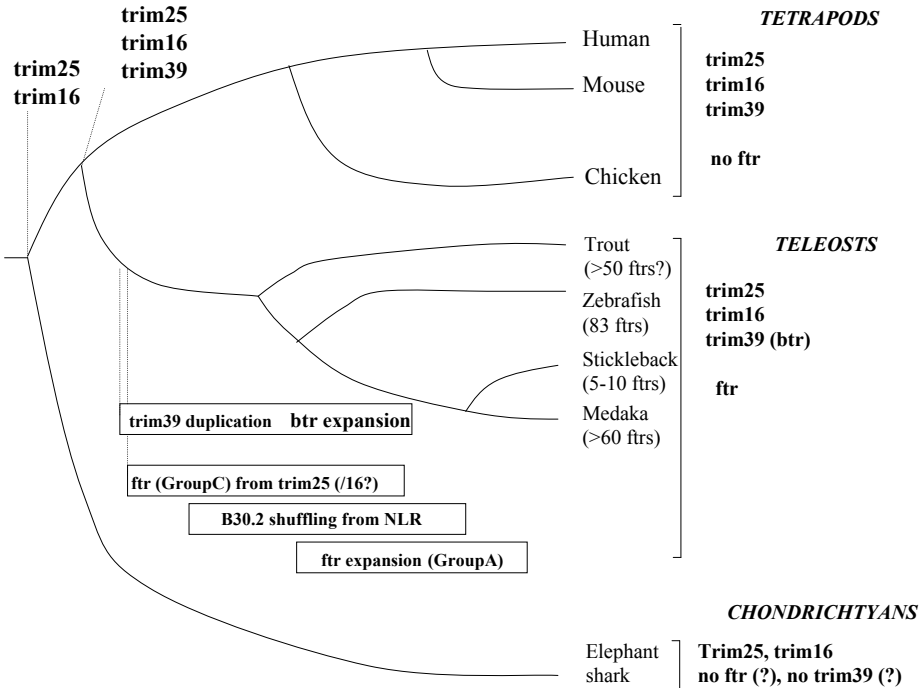

Fig. 8. Tentative evolutionary pathway of fintrims and their relatives in vertebrates.The genes identified in the genomes of different vertebrate groups are indicated on the right. Since the current draft of the elephant shark genome is still partial, the absence of fintrim and btr is unsure, which is indicated by "?".

medaka, these genes have retained the six-exon structure. Also, several markers located close to *ftr82/83* in fish possess counterparts on the human chromosome 17, where both *TRIM16* and *TRIM25* are located together with the markers defining their own conserved syntenies among vertebrates. This loose association is unlikely to have occurred by chance, and may suggest that certain group C *finTRIMs* could constitute an intermediate between the main finTRIM family and the older TRIM genes (presumably *TRIM25*; *TRIM16* is an unlikely candidate since its RBB domain is truncated) from which they appeared by duplication. This hypothesis fits well with the whole-genome duplication that occurred in the beginning of teleost evolution [58]. Along the same line, *ftr82* and *ftr83*, together with *ftr84*, are the most similar to *TRIM25* among the *finTRIM* relatives (see *ftr/btr* tree in Fig S1, and Fig 7). TRIM25 is involved in IFN signaling, but interacts with endogenous RIG-I and not with viral proteins [20]. It is therefore tempting to speculate that *ftr82* and *ftr83* have been restrained from duplications and diversifying selection by such a functional specialization. Such a contrasted evolutionary history has been observed for example for the CytP450 superfamily: enzymes with endogenous substrates are phylogenetically stable, while xenobiotic detoxifiers are encoded in unstable gene islands that appeared by tandem duplication [59]. Actually, one may propose that the quick radiation of group A finTRIMs was triggered when a TRIM25-like *ftr* acquired a B30.2 exon derived from a NLR gene, allowing the newly created finTRIM to directly detect a pathogen motif. Such an event would have occurred after the teleost-tetrapod split, and perhaps even after the divergence of main teleost lineages, as illustrated in figure 8. It has to be underlined here that the phylogenetic analysis does not support a monophyletic origin of group B *finTRIMs*, which therefore constitute probably the tracks of several duplication/differentiation events in the finTRIM group. Such duplication events have been suggested to explain the expansion of TRIM genes in fish and other species [17]. A recent extensive survey of TRIM proteins divided these proteins into two large groups: an evolutionary conserved group I comprising TRIM with various C-terminal domains, and a more recent group 2 that groups sequences containing a B30.2 domain and showing species-specific diversification. The presence of two B-Box motifs, although the B-Box-1 is rather degenerated, and the sequence similarity to TRIM16 and TRIM25 suggests that finTRIMs may be closer to the group I. However, the finTRIM evolutionary pathway described here fits better the properties of the group II. Our observations seem to reflect a fish-specific evolutionary pathway of a TRIM subset derived from ancestral group I members by an ancient duplicatio

The evolutionary pathways of *TRIM39* is rather different since it was retrieved as a single gene in mammals but as a multigene set in several teleosts. TRIM39 is a member of the Group II as described in [17] and the diversification observed in the zebrafish is well in accordance with the evolutionary properties of this group. Thus, there are more than 30 orthologs of *TRIM39* in zebrafish. We named these genes *btrs* for "bloodthirsty-like TRIMs", as one of them is known as *bloodthirsty,* a gene involved in erythropoiesis [41]. These observations indicate that *TRIM39* was already present in the common ancestor to fish and mammals, but was subjected to a successful expansion by duplication in at least some lineages of teleosts.

In conclusion, our results indicate that the finTRIM family has been subjected to a quick, extensive diversification by duplication and specialization under positive selection exerted on positions concentrated in the B30.2 domain. The sharing of B30.2 domains with NLR emphasizes the shuffling of a putative target-binding module between two major protein families involved in immune recognition of pathogen specific motifs [39, 40]. While the targets of finTRIMs have yet to be identified, this first survey suggests that finTRIMs are involved the antiviral innate immune system. Our future work will be aimed at a further understanding of the biological functions of the finTRIMs.

Acknowledgements

We acknowledge Anthony Levasseur for his help with the PAML software and positive selection analysis. We thank Louis du Pasquier and Pierre Pontarroti for many helpful discussions, Lidy Van Kemenade and Huub Savelkoul for critical reading of the manuscript, and Bertrand Collet for the IFN-γ primers. This work was supported by the Institut National Recherche Agronomique, Wageningen University, and by the projects PTR_INRA/Institut-Pasteur#231 and ANR_MIME2007 Zebravirus.

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Historien: monsieur qui predit le passé.
- Maurice Michaux -

Chapter 3

Origin and evolution of TRIM proteins: new insights from the complete TRIM repertoire of zebrafish and pufferfish

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PLoS One, 2011; 6(7):e22022

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Abstract

Tripartite motif proteins (TRIM) constitute a large family of proteins containing a RING-Bbox-Coiled Coil motif, followed by different C-terminal domains. Involved in ubiquitination, TRIM proteins participate in many cellular processes including antiviral immunity. The TRIM family is ancient and has been greatly diversified in vertebrates and especially in fish. We analyzed the complete sets of TRIM genes of the large zebrafish genome and of the compact pufferfish genome. Both contain three large multigene subsets, adding the hsl5/TRIM35-like genes (*hltr*) to the finTRIM (*ftr)* and the bloodthirsty (*btr)* that we previously described, all containing a B30.2 domain that evolved under positive selection. These subsets are conserved among teleosts. By contrast, most human TRIM genes of the other classes have only one or two orthologues in fish. Loss or gain of C-terminal exons generated proteins with different domain organizations; either by the deletion of the ancestral domain or, remarkably, by the acquisition of a new C-terminal domain. Our survey of fish TRIM genes in fish identifies subsets with different evolutionary dynamics. TRIMs encoding RBCC-B30.2 proteins show the same evolutionary trends in fish and tetrapods: they evolve fast, often under positive selection, and they duplicate to create multigenic families. We could identify new combinations of domains, which epitomize how new TRIM classes appear by domain insertion or exon shuffling. Notably, we found that a cyclophilin-A domain replaces the B30.2 domain of a zebrafish finTRIM gene, as reported in the macaque and owl monkey antiretroviral TRIM5α. Finally, TRIM genes encoding RBCC-B30.2 proteins are preferentially located in the vicinity of MHC or MHC gene paralogues, which suggests that such TRIM genes may have been part of the ancestral MHC.

Introduction

The tripartite motif (TRIM) family, also known as the RING finger/B-box/coiled coil (RBCC) family, plays major roles in development, tumor suppression, disease pathology and viral restriction/sensing [1,2]. This tripartite motif is associated with diverse C-terminal domains, which often determine the specificity of the interactions of TRIMs with other proteins. Hence, TRIM proteins associate a RING-dependent E3 ubiquitin ligase activity with the capacity to build multiprotein complexes though interactions with their CC and C-terminal domains. Human TRIM proteins have been classified into nine architectural subsets on the basis of their C-terminal domains, subcellular compartmentalization and functionality ([3], Fig. 1). The B30.2 domain [4] found in Class-I and Class-IV TRIM proteins is constituted by the juxtaposition of a PRY and a SPRY domain, and is also known as PRY/SPRY domain [5].

In a survey of the TRIM family in various species, Sardiello *et al*. distinguished two groups: the trim genes from group 1 contain a variety of C-terminal domains and are generally well conserved among distant species, while members of group 2 correspond to the structural Class IV subgroup which evolve much faster, display lower levels of amino acid conservation in distant species and are subjected to different selection pressures [6]. Importantly, the Class IV TRIM proteins include multiple members involved in antiviral immunity at various levels of the interferon (IFN) signalling cascade.

For instance TRIM25 is required for viral RNA sensing performed by the cytoplasmic helicase RIG-I, leading to IFN production [7]. Other class IV TRIM proteins also control signalling pathways that lead to IFN production: TRIM27 represses NF-κB and IRF3/IRF7 [8] while TRIM21 ubiquitylates IRF3/IRF7 and IRF8 [9,10,11]. On the other hand, TRIM5α, which was described as a strong restriction factor for HIV-1 in rhesus macaque, directly targets retroviruses [12]. The TRIM5α B30.2 domain binds the nucleocapsid of incoming viral particles and accelerates the uncoating of the viral core, while the RING/B-box domains are essential for the localization in specific cytoplasmic 'bodies' [13,14,15] and mediate a TRIM5α higher-order self association that increases avidity for retroviral capsids [16, 17]. The structure of the B30.2 domain is a β-sandwich core with ligand-binding loops at the top that are variable and determine the specificity of the interaction. Ligand-binding loops of the TRIM5α B30.2 domain diversified during the evolution of primates, ensuring efficient restriction of specific retroviruses in the different species [18,19]. Thus, while TRIMs constitute an ancient family with members involved in basic cellular processes in practically all bilateria and pre-bilateria phyla [20], it seems that several subsets have been recruited and diversified for antiviral immunity during the evolution of vertebrates. However, the specific modalities of these apparently independent multiplication and diversification events are still poorly understood.

Teleost fishes offer an intriguing model for a comparative study of the TRIM family because of their ancient separation from the tetrapods, their great diversity and the considerable variation in the number of trim genes in their genome. In addition, the zebrafish (*Danio rerio,* Hamilton) is an important vertebrate model for developmental biology, and more recently, for host-pathogen interactions. Therefore the identification and classification of its many TRIM genes is important for these fields of biological study, and the further development of zebrafish as a model for vertebrates. Sardiello *et al*. listed 240 zebrafish trim genes, but without providing classification [6]. During our investigation of IFN-inducible class IV TRIM homologues in trout, we identified 84 finTRIM (*ftr*) and 33 TRIM39/bloodthirsty-like (*btr*) genes [21], implying that the zebrafish genome contains at least 117 class IV genes, and probably many more. Ftrs do not have true orthologues in mammals, thus should have a specific function in fish defense. Apart from *ftrs* and *btrs,* the zebrafish gene database at zfin.org currently lists only 21 additional TRIM genes. We therefore performed an exhaustive description of trim genes in two fish species that followed different genomic evolutionary histories, zebrafish (*Danio rerio*) and spotted green pufferfish (*Tetraodon nigroviridis*), updating and completing the lists provided in [6]. In contrast to other TRIM genes that are generally conserved through vertebrates with conserved expression patterns, the vast majority of fish class IV TRIM genes belongs to three multigenic families of which the B30.2 domain has evolved under positive selection. Our systematic analysis of TRIM genes also led to the identification of genes that have lost, gained or replaced their C terminus domain, providing a good illustration of the mechanisms giving birth to new TRIM classes.

Materials and Methods

Identification of a complete array of genes from the trim family in zebrafish
Zebrafish TRIM genes, defined as encoding proteins with a RING-B-Box-Coiled Coil (RBCC) motif, were searched in the Zv8 genome assembly available at http://www.ensembl.org/. The survey was later updated from the current assembly (Zv9, made available at the end of 2010). The new assembly Zv9 did no show any major change in number, structure or location of trim genes. Both lists are given in Supplemental Figure S1 for an easier comparison with previous reports. Several strategies were followed in parallel to try to get a complete list of zebrafish TRIM. First, all zebrafish ensembl proteins with a motif RING (ipr IPR001841) or B box (ipr IPR000315) were extracted using the biomart tool, and the intersection of the two lists kept as a first set of trim sequences (Set#1). The ensembl Ids, annotation, locations and status were also extracted. Second, the protein sequences belonging to the TRIM Ensembl families detected in zebrafish (ENSFM00300000079125, ENSFM00400000131833, ENSFM00250000004079, ENSFM00250000005797, ENSFM00390000126422, ENSFM00500000272256, ENSFM00500000271543, ENSFM00500000272036, ENSFM00390000126385, ENSFM00250000006428, ENSFM00250000001082, ENSFM00500000270185, ENSFM00250000001642, ENSFM00400000131788, ENSFM00250000004429, ENSFM00250000008223 , ENSFM00500000287404): were collected and combined with the set #1 (set#2). Third, the zebrafish ensembl orthologs of all human trim gene were collected; the human orthologue of each gene was then checked, and this information was used to annotate the genes previously identified.

To compare the sequences to our previous work on two multigenic *TRIM* subsets performed on the zebrafish Zv7 assembly, the *finTRIMs* and the bloodthirsty-related *(btr*) *TRIMS*, we used the TBLASTN program at http://www.ensembl.org/ to align the FTR and BTR protein sequences with the current genome assembly. We also compared the sequences of *ftr* and *btr* genes extracted from Zv7 to the current assembly. Using both alignment scores and hit location, the *ftr* and *btr* sequences were identified in the set#2. For the new *ftr* and *btr* present in Zv8, as well as for the *TRIM35* multigenic family, sequences were manually edited from gene models available in both Ensembl and Genbank. When the *ftr* or *btr* genes were fully retrieved in Zv8, we kept our previous manual annotation rather than the Ensembl automatic assignment. For the other TRIM, the protein models from Zv8 and Zv9 were considered, and the most recent annotation available. Finally, the protein sequences corresponding to this trim list was subjected to a domain analysis using Interproscan. The sequences unassigned yet were then manually annotated one by one. Starting from each zebrafish *TRIM* we searched for the possible orthologues and paralogues in pufferfish. The orthologues of each human and zebrafish trim were searched in the Ensembl database. All proteins including a RING and a B30.2 domains were also extracted, which confirmed that the previous list was comprehensive.

Cloning of ftr52 sequences
Transcript sequences were amplified from cDNA of pooled 5dpf AB larvae with AccuStar

DNA polymerase (Eurogentec) using primers ATGAATTCGTGTAAATACAGCGAAATGGCA and ATGCGGCCGCACCTAGGCTCACAGCTG. A band of ~2 kb was gel-purified, digested with EcoRI and NotI, and cloned in the pBK-CMV plasmid. The genomic region encompassing the RING-encoding domain was PCR-amplified with primers TACAGTGGCTCGTCAAGTGA and TGCACTCTTCATCCGTGTGA.

Detection of positive selection in B30.2 domain

The dataset for positive selection analysis was prepared from *btr* and *trim35/hltr* sequences that were found on the Ensembl zebrafish assembly. Domains were identified by the web-based tool Simple Modular Architecture Research Tool (SMART) at http://smart.embl-heidelberg.de/. A multiple sequence alignment was made for each domain with ClustalW within the MEGA4 software and gaps were removed from the alignment. The Codeml program of the Phylogeny Analysis by Maximum Likelihood (PAML) package [80], retrieved from http://abacus.gene.ucl.ac.uk/software/paml.html , was used for the detection of positive selection. The models M0, M1a, M2a, M7 and M8 were employed. The ratio of synonymous (dS) to non-synonymous (dN) substitution rates, $\omega=dS/dN$, is determined by the program. We used the site-specific model that allows ω to vary among sites. The null models M0, M1a and M7 do not allow the existence of positively selected sites ($\omega>1$), while the alternate models M2a and M8 allow $\omega>1$. M8 follows a beta(p,q)-distribution and is less stringent than M2a. Within the models, a Maximum Likelihood algorithm is used, whereby the sites are allocated under classes of different ω probabilities. Sites allocated under the class with $\omega>1$ are considered as being under positive selection and were identified by a Bayes Empirical Bayes (BEB) analysis. Significance of outcome was confirmed by a likelihood ratio test (LRT). In the LRT we took twice the difference in log likelihood ($2\Delta lnL$) between the nested models and used the chi-square test with the degrees of freedom (df) being the difference in free parameters between the two models (M1a vs. M2a and M7 vs. M8). Tests were considered positive when $p<0.001$. Sites identified by BEB with a posterior probability higher than 95 percent were considered significant.

Analysis for recombination

To test for interference of recombination on the PAML results, we implemented a test by the algorithm PARRIS [81]. Under PARRIS, the PAML models M1a-M2a are employed with incorporation of site-to-site variation in synonymous substitutions rates and partitioning of data. We used the codon model for evolution GY94 x HKY85 and a discrete distribution of three bins for synonymous and for non-synonymous rates. Significance of results was tested by a LRT. We detected recombination breakpoints by the algorithm GARD [82]. We used the HKY85 model with general discrete distribution of rates across sites. We performed two screenings, for 2 or 20 breakpoints. The detection was validated by corrected Akaike's information criterium (c-AIC) for best-fitted model selection. Both PARRIS and GARD are integrated in the HyPhy software package that was retrieved from http://www.hyphy.org

Fish, RNA isolation and real time quantitative PCR
RNA was extracted from either single fish or pooled organs from five to ten two-year old zebrafish of AB background. All the animal experiments described in the present study were conducted at the Institut Pasteur according to the European Union guidelines for the handling of laboratory animals (http://ec.europa.eu/environment/chemicals/lab_animals/home_en.htm) and were approved by the Institut Pasteur animal care and use committee and by Direction Sanitaire et Vétérinaire de Paris under permit #A-75-12-22. Dissected organs, or entire fish cut in 3 mm pieces, were stored for a few days in RNALater (Ambion) before RNA extraction using TriZol (Invitrogen). DNA contaminations were removed by DNAse I treatment followed by phenol-chloroform extraction; integrity of the resulting RNA was checked on an 2100 bioanalysis station with a RNA nano chip (Agilent). cDNA was generated using M-MLV H- reverse-transcriptase (Promega) with a dT17 primer. Quantitative PCR was then performed on an ABI7300 thermocycler (Applied Biosystems) using SYBR green reaction power mix (Applied Biosystems). Primers are indicated in table 1. Quantifications were performed on triplicate wells, and taking into account the previously measured yield of the reaction as described in [83]. To normalize cDNA amounts, we have used the housekeeping gene EF1α, chosen for its high and stable level of expression over development and among tissues [84]. After calculations of trim/Ef1α transcript expression ratios, data have been normalized to the average expression in entire fish (using the geometric mean of the results obtained on the whole male and the whole female), to highlight which organs express higher or lower levels of a given gene compared to the rest of the body. Results are reported as mean ± standard deviation of the measured ratios.

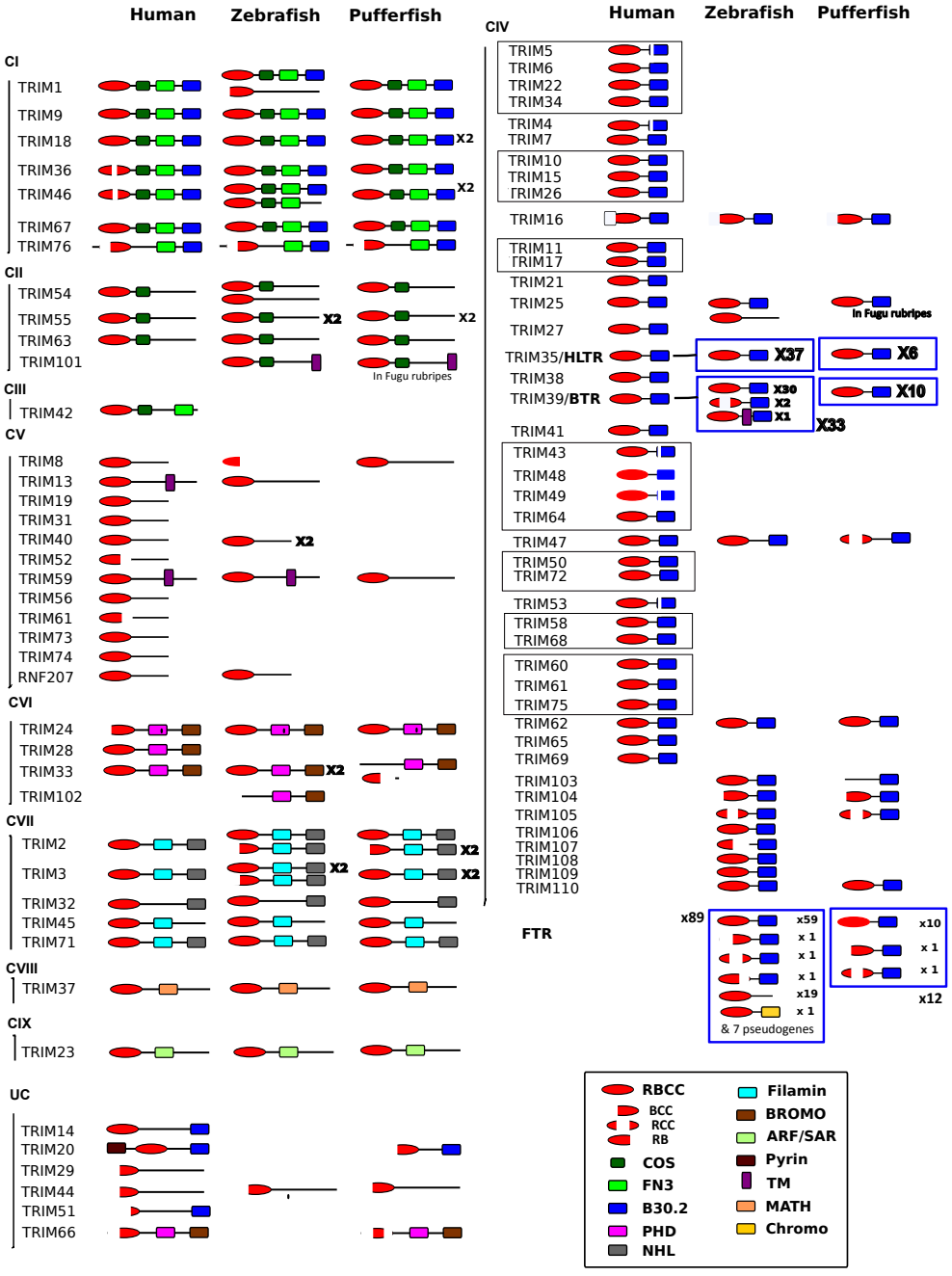
Results

TRIM classes reflect two distinct evolutionary pathways in fish
The complete repertoire of trim genes was determined in two fish species using combined approaches of genome scanning for protein domains and sequence comparison (see Material and Methods for details). Among fish species with available genomes, we chose the zebrafish and the spotted green pufferfish because they are phylogenetically distinct with an estimated 300 My time of divergence [22] and followed distinct

Table 1. Primers

Gene	forward primer	reverse primer
EF1α	GCTGATCGTTGGAGTCAACA	ACAGACTTGACCTCAGTGGT
TRIM1	CAAAACCAACAGTCAGCCTTT	AAGAGCGTACCATGTAGAGG
TRIM13	CAGGTAGACAAACTTTGCGC	CAGTCCGACGGAAGAAAGTT
TRIM25	GAGCGGCGCTTCAAACAAAA	ATCAATTGCCAGCATGGCCT
TRIM33	GTTCCTACCTCGGTTCCTAA	GAATCGGCCTGGACATTACT
TRIM54	GGAGCATCAAGGACAATGGT	CTTCGTGCTCTGCAGGAATA
TRIM59	CTGGTGCAGAAAGATCGAGA	CTCGTAGGCCTGATTGAGAA

genomic evolutions. They have different gene contents (15315 genes in the pufferfish compared to 23569 in the zebrafish, in Ensembl release 57) and have been subjected to different events of genome expansion/contraction. Thus, we expected that zebrafish genome would contain an expanded repertoire of TRIM genes while the compact pufferfish genome may have a "minimal" TRIM repertoire. The zebrafish and pufferfish TRIM repertoires are presented in Figure 1, where they are compared to the human repertoire. Detailed information about the genes is provided in Supplemental Figures S1, S2 and S3. Fish TRIM genes were named after the human orthologues, following the Ensembl annotations confirmed by the analysis of the domain organization of the protein. When a close paralogue of such a fish *TRIM* was found with a highly similar organization but lacking the terminal domain, it was considered as another orthologue (i.e. a co-orthologue) and was therefore given the same name with a "like" suffixe. When a fish *TRIM* had no orthologue in human or in the mouse, we named it from available publications, or we attributed a temporary "*TRIM*101-110" annotation, waiting for a definitive nomenclature. We found 208 trim genes in the zebrafish (Zv9 assembly) and 66 in pufferfish (Fig. 1), compared to 75 and 67 genes reported in human and in the mouse respectively [23,24]. Sardiello *et al*. had reported 240 trim genes in zebrafish and 58 in pufferfish [6]; the large discrepancy observed in zebrafish was due to the fact the list established by Sardiello *et al*. was derived from a search in ESTs that were not matched to the genome sequence. This procedure resulted in frequent inclusion of the same sequence under two accession numbers, or inclusion of allelic variants. Orthologues of human *TRIM* genes for all classes excluding C-III (RBCC-COS-FN3) were present in both zebrafish and pufferfish. The main TRIM categories, with the possible exception of C-III, were therefore already defined in the common ancestor of fishes and tetrapods. As shown below, TRIM genes could be separated into two main subsets reflecting their evolutionary dynamics, in complete agreement with Sardiello et al. [6].One or two counterparts were found in fish for the majority of the human TRIM genes belonging to the classes I, II and VI-IX (Fig. 1, left column). The presence of two co-orthologues of a given human gene in both fish species likely reflects the ancestral duplication of teleost genomes [25,26]. The term "co-orthologue" is employed here to describe the evolutionary relationship of two or more paralogous genes with their counterpart in another species. Co-orthologue is synonymous of "inparalogue" [27]. For these *TRIM* classes, pufferfish and zebrafish *TRIM* repertoires were overall very similar, with a few exceptions: *TRIM40* (ClassV), *RNF207* (ClassV), *TRIM102* (ClassVI), as well as a *TRIM1*-like, a *TRIM54*-like and a *TRIM3*-like were found in zebrafish only while *TRIM20*, *TRIM66*, *TRIM18-2* and *TRIM2*-like were found only in pufferfish. These exceptions are likely due to local events of gene duplication or loss. TRIM lacking a unique C terminal domain (Class C-V) could also be included into this subset as they never have more than two fish orthologues, even though many class V human genes lack a fish counterpart: only 6 zebrafish and 2 pufferfish counterparts were found for 12 human genes. In fact, genes closely related to members of other classes that have lost their C terminal domain, for example zebrafish *TRIM54-like* could fall into the class V as defined above. Thus, at least in fish, TRIM genes with no specific C-terminal domain do not constitute a homogeneous group.


Human
Zebrafish
Pufferfish
CI
TRIM1
TRIM9
TRIM18
TRIM36
TRIM46
TRIM67
TRIM76
CII
TRIM54
TRIM55
TRIM63
TRIM101
In Fugu rubripes
CIII
TRIM42
CV
TRIM8
TRIM13
TRIM19
TRIM31
TRIM40
TRIM52
TRIM59
TRIM56
TRIM61
TRIM73
TRIM74
RNF207
CVI
TRIM24
TRIM28
TRIM33
TRIM102
CVII
TRIM2
TRIM3
TRIM32
TRIM45
TRIM71
CVIII
TRIM37
CIX
TRIM23
UC
TRIM14
TRIM20
TRIM29
TRIM44
TRIM51
TRIM66
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TRIM104
TRIM105
TRIM106
TRIM107
TRIM108
TRIM109
TRIM110
FTR
& 7 pseudogenes
RBCC
BCC
RCC
RB
COS
FN3
B30.2
PHD
NHL
Filamin
BROMO
ARF/SAR
Pyrin
TM
MATH
Chromo

Fig. 1. TRIM proteins from zebrafish and pufferfish. Classification of fish TRIM proteins based on their C-terminal domain(s) and the categories proposed by Short & Cox in Ref. 3. Previously unreported TRIM proteins found in fish were tentatively numbered TRIM101-111. Conserved TRIM proteins are represented on the left panel (Classes I-III and V-IX). Other TRIM proteins are shown on the right panel (Class IV). Dotted lines delimit groups of closely related human TRIM (modified from Ref. 6) corresponding to a diversification that occurred during tetrapod evolution. Blue frames indicate multigenic families observed in teleost fish. RBCC: Ring-BBox-Coiled Coil; COS: C-terminal subgroup one signature; FN3: Fibronectin, type III; B30.2: PRY/SPRY domain; PHD: Plant Homeo Domain; NHL: NCL-1, HT2A and Lin-41 repeat; Filamin: named from the protein Filamin; Bromo: acetylated lysine binding domain; ARF/SAR: from ARF and SAR GTP binding proteins; Pyrin: member of the six-helix bundle death domain-fold superfamily; TM: transmembrane; Math: meprin and TRAF homology domain; Chromo: CHRromatin Organization Modifier domain.

The conservation of these TRIM proteins between teleosts and mammals strongly suggests that they carry out conserved functions. Such a hypothesis would be reinforced if these proteins had similar tissue-specific expression patterns. To test this hypothesis, we selected a subset of *TRIM* genes with a clear orthology relationship between human and zebrafish (Fig. S4) and tissue-specific expression described in mammals, and measured their expression in various organs of adult zebrafish by qRT-PCR (Fig. 2). *TRIM1* (FXY2 or MID2) has been reported to be expressed in low abundance in brain and lung, with even lower levels in heart, liver and kidney by northern blot analysis of mouse tissues [28]. Indeed, in zebrafish, *TRIM1* was expressed at a higher level in brain than in heart, liver or kidney (Fig. 2). In the case of *TRIM13* (aka RFP2) strongest expression was found in the testis (ovary was not tested) for both mouse and human [29]; the situation was different in the zebrafish, where only moderate levels of *TRIM13* were measured in the testis, although levels were high in the ovary (Fig. 2). Apart from gonads, the zebrafish tissue with the highest level of expression was the brain, in agreement with mouse, but not human northern blot data [29]. Note however that strong staining with anti-RFP2 antibody is detected in human brain samples (www.proteinatlas.org). Expression of *TRIM25* (efp), the function of which may suggest a rather uniform expression [7], has been tested by RNAse protection assay in mouse tissues; abundant levels were observed in uterus, ovary and placenta, medium levels in the mammary gland, and lower levels in liver, spleen, kidney, heart, lung and thymus, and only a faint band in brain [30]. A rather ubiquitous pattern was found in zebrafish (Fig. 2); a discrepancy with mouse data was the relatively high expression in brain. By Northern blot, *TRIM33* (TIF1γ) expression was found to be highest in mouse testis, then in liver, heart, brain and kidney, weak in spleen and lung and very low in skeletal muscle [31]. Among the corresponding zebrafish organs, *TRIM33* expression was highest in brain, then in testis; however, it was fairly low in liver, while intermediate in muscle (Fig. 2). *TRIM54* (MURF) constitutes the most clear-cut example or tissue-specific expression, with an almost exclusive expression in heart and skeletal muscle [32]. A similar pattern was observed in zebrafish: expression was strong in skeletal muscle, and extremely low in visceral organs – the low heart expression was, however, a remarkable difference (Fig. 2). Finally, *TRIM59* (Mrf1) expression data in mammals are rather conflicting; in

mouse, expression was found to be strong in testis, moderate in spleen, weak in brain and heart, and very low in lung, liver, kidney or skeletal muscle [33]; while in humans, highest levels were detected in skeletal muscle, heart, liver and lung [34]. In zebrafish, strong expression was found in ovary, and low levels in gut, level or muscle (Fig. 2). In conclusion, although the variety of techniques and organs sampled in the various published studies makes comparisons quite difficult, similar patterns of expression can often be observed between mammalian and fish tissues (if one excludes gonads, where extreme expression levels are frequent), likely reflecting conservation of function for these genes.

Class IV trims (RBCC-B30.2) followed a different evolutionary pathway. No obvious counterpart could be found in fish of the majority of human and mouse trim genes belonging to this class (Figure 1, right column). Our analysis identified fish orthologues only for *TRIM16*, *TRIM25*, *TRIM35*, *TRIM39* (*btr*), *TRIM47* and *TRIM62*. Reciprocally, several fish class IV genes had no counterparts in mammals, such as the *finTRIM* and the *TRIM103-110*. Strikingly, several Class-IV members constitute multigenic subsets in fish. Two of these multigene subsets possess a unique human counterpart *TRIM39* and *TRIM35*, respectively. The third multigene set is composed of sequences that lack counterparts in tetrapods, *finTRIMs* (*ftr*) [21]. These different gene expansion events explain the high *TRIM* numbers observed in fish compared to human. Interestingly, the repertoire of class IV genes was also more divergent between zebrafish and pufferfish than for the other classes. Indeed, the multigenic class IV *TRIM* subsets contains much

	brain	gills	gut	heart	kidney	liver	muscle	ovary	skin	spleen	testis	whole female	whole male
trim1	6.17 ± 0.68	0.42 ± 0.07	0.05 ± 0.009	0.60 ± 0.18	0.18 ± 0.02	0.05 ± 0.003	0.59 ± 0.016	25.21 ± 2.28	0.16 ± 0.04	0.54 ± 0.06	1.86 ± 0.17	1.36 ± 0.04	0.74 ± 0.08
trim13	34.13 ± 4.28	0.90 ± 0.11	0.15 ± 0.004	0.65 ± 0.02	0.25 ± 0.03	0.15 ± 0.010	1.50 ± 0.18	8.40 ± 0.89	0.41 ± 0.03	0.69 ± 0.02	1.18 ± 0.11	0.75 ± 0.06	1.33 ± 0.07
trim33	10.29 ± 1.35	1.55 ± 0.18	0.19 ± 0.02	0.48 ± 0.04	0.69 ± 0.05	0.10 ± 0.004	1.27 ± 0.21	24.45 ± 2.56	0.47 ± 0.05	0.80 ± 0.03	4.67 ± 0.55	1.25 ± 0.06	0.80 ± 0.10
trim54	0.010 ± 0.006	1.17 ± 0.14	0.0005 ± 0.0002	0.028 ± 0.006	0.025 ± 0.0014	0.00011 ± 0.0002	5.15 ± 0.70	0.10 ± 0.02	0.45 ± 0.05	0.05 ± 0.010	0.07 ± 0.012	0.44 ± 0.04	2.29 ± 0.15
trim59	1.23 ± 0.27	0.69 ± 0.22	0.09 ± 0.005	0.28 ± 0.015	0.31 ± 0.11	0.010 ± 0.009	0.24 ± 0.22	13.63 ± 1.11	0.38 ± 0.06	1.21 ± 0.15	1.54 ± 0.54	1.70 ± 0.09	0.59 ± 0.03
trim25	4.30 ± 0.46	1.62 ± 0.20	0.78 ± 0.03	0.87 ± 0.07	0.60 ± 0.04	0.26 ± 0.02	1.17 ± 0.13	2.48 ± 0.33	1.13 ± 0.09	1.21 ± 0.04	2.35 ± 0.25	0.55 ± 0.06	1.82 ± 0.10

Figure 2. Expression profile of selected trim genes. The expression of 6 zebrafish trim genes was measured by quantitative RT-PCR in pools of tissues from 10-12 animals. E1f-α was used as a housekeeping gene, and the relative expression levels of trim genes were normalized on the geometric mean of the values measured for "whole males" and "whole females", to take both sexes into account in the normalization. Expression level and standard deviation is indicated for each condition.

less genes in pufferfish compared to zebrafish: 12 *ftr* for 89, 6 *TRIM35* instead of 37 and 10 *btr* instead of 33. Additionally, several genes including *TRIM106-109* and *TRIM25* were absent from pufferfish while found in zebrafish.

Most remarkably, three instances of gain of domain were also detected (Fig. 3). The *ftr06* gene acquired a C-terminal chromodomain via the insertion of a single exon between the original 5th and 6th exons (Fig. 3A). Thus, although the sequence encoding for a B30.2 domain is still present in the genome downstream of the chromodomain, it is not included in the transcribed gene, as established previously by RACE analysis ([21] see sequences #AM941366 and AM941342). Along the same line, just downstream of

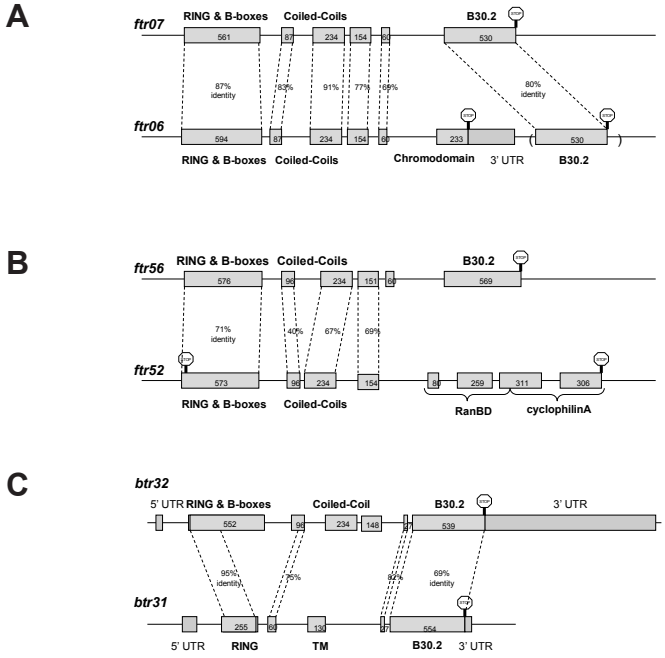

Figure 3. Three different ways to generate TRIM proteins with new domains. For these three cases observed in the zebrafish genome, the new gene is shown on the bottom of panel, and is compared with a closely related, typical member of the ftr or btr family illustrated on top. Percentages of identity refer to DNA sequences. Rectangles represent exons, numbers refer to nucleotides of coding sequence, stop included. Introns not to scale. **A** The ftr06 gene, contained within a large cluster of tandem ftr genes on chromosome 2, encodes a protein with a C-terminal chromodomain instead of a B30.2. This is due to the insertion of a single chromodomain-encoding exon just upstream of the usual exon 6. The previous B30.2 exon, shown in parenthesis, is still present downstream, nonmutated, but is not included in the chromodomain-encoding transcript. **B** The ftr52 gene, isolated on chromosome 9, encodes for a TRIM protein with a C-terminal RanBD/cyclophilin A domain instead of a B30.2. In this case, the new C-terminal domain is encoded by multiple exons; no B30.2-encoding sequence can be detected in this genomic area. **C** The btr31 gene, located on chromosome 19 tandemly to its close relative btr32, encodes for a protein with the typical N- and C- ends of bloodthirsty-like proteins, but the B-boxes and the coiled-coil regions in the middle have been replaced by a transmembrane domain.

the *ftr52* gene, one can find a Ran binding-domain (RanBD) and a cyclophilin A (CypA) domain, encoded by four exons, while no B30.2 domain can be detected in this genomic region (Fig. 3B). Such a configuration could happen in one single step by the insertion of a piece of DNA containing exons 1 to 4 of a ftr gene within a pre-existing RanBP2-like gene. *Ftr52* was believed to be a pseudogene since it contains a predicted stop codon in the N-terminal RING-encoding region (found on the previous and current genomic assemblies). To test whether *ftr52* was effectively transcribed into mRNA, we PCR-amplified cDNA from AB fish with primers upstream of the RING and downstream of the CypA regions, and cloned and sequenced the product (accession number JF295002). The retrieved sequence does correspond to a properly spliced transcript that would encode a RBCC-RanBD-CypA protein, if not for a premature stop codon in the N-terminal exon. This stop codon is identical to the one found in the current genomic sequence, derived from a Tü strain fish. We PCR-amplified genomic DNA of several independent AB and Tü zebrafish (the most used "wild-type" laboratory strains) and found this stop codon in all products. A slightly more complicated sequence of events took place to generate the *btr31* gene that encodes a protein with a predicted TM domain between a N terminal RING domain and a C terminal B30.2 domain, while B-Boxes and the Coiled Coil have disappeared. This gene is clearly a product of recent duplication of a *btr* gene, and is most similar to its neighbour *btr32* that possesses the bona fide domains. The genesis of *btr31* would require at least two genomic rearrangement events: the replacement of exons 3 and 4 by a DNA stretch containing a TM-encoding exon, and the deletion of the end of exon 1; however the gene structure is confirmed by several ESTs (i.e., EH489524 and EH515884), excluding an assembly artefact. Similar to the first subset of *TRIM* genes, the loss of the specific C-terminal domains was also frequently observed in pairs of co-orthologues. Such events of gain of domain were not found in the pufferfish.

TRIM39/btrs and TRIM35-related multigene families derive from ancient duplications
Fish genomes contain three large multigene subsets of class IV trims: finTRIMs *(ftrs*), bloodthirsty-like *TRIMs* (*btrs*) and Hematopoietic lineage switch-5/trim35-like trims (*hltrs*). In an attempt to understand the selective constraints that give rise to such large families, we analyzed the diversity of the *ftr* family from a prior study [21] and extend this analysis to include *btr* and TRIM35/hltrs genes. The *btr* family, orthologous to *TRIM39*, has been previously reported in zebrafish and other teleosts [21]. These genes were named bloodthirsty-related genes, or *btr,* from the first described member of the family that is involved in erythropoiesis, *bloodthirsty* [35]. The *btr* genes are relatively dispersed in the zebrafish genome but do not colocalize with the *ftr* clusters. *btr* clusters are found on chromosomes 7, 15 and 19 (Fig. 4). As previously seen for *ftr*, a minority of *btr* genes are part of synteny groups conserved in zebrafish, pufferfish and in other fish. *Btr1* (chr1), *btr2* (chr3) and *btr33* (chr24) belong to gene sets found in conserved synteny (Fig. 5). Additionally, *btr* genes located on zebrafish chr5 and 15 are linked to the paralogous markers encoding the alpha-crystallins *cryabb* and *cryaa* respectively, suggesting a common origin for the corresponding regions. Thus, at least the *btr* that are involved in conserved syntenies were produced by regional and global duplications which occurred relatively early during fish evolution. The other *btr* constituting clusters

are probably more recent.

Thirty-seven TRIM35/hltr genes were identified in the zebrafish genome (Zv9 assemby), all containing a B30.2 domain. In contrast, only six *TRIM35*/*hltr* were found in the pufferfish genome. When all zebrafish *TRIM35*/*hltr* sequences were included in a distance tree with representative genes from class IV, they grouped in a specific cluster, confirming that they constitute a distinct subset in the TRIM family (Fig. 6A). Separate phylogenetic analyses were performed for RBB and B30.2 regions using NJ (Fig. 6B and 6C) and PHYML. These analyses indicate that the fish TRIM35/HLTR sequences group with the reptile and mammalian TRIM35, while its closest relatives TRIM21, 11 and 60 as well as FTR and TRIM25 determine distinct clusters each supported by high

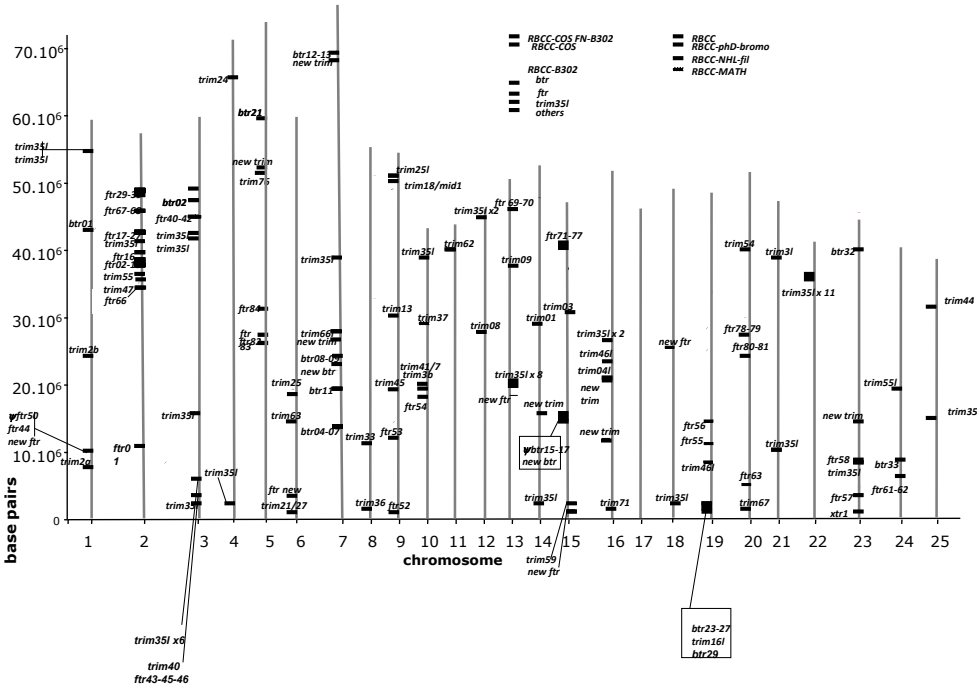

Figure 4. Genomic location of zebrafish trim genes. This representation is based on the Zv8 assembly. RBCC: Ring-Bbox-Coiled Coil; COS: C-terminal subgroup one signature; FN3: Fibronectin, type III; B30.2: PRY/SPRY domain; PHD: Plant Homeo Domain; NHL: NCL-1, HT2A and Lin-41 repeat; Filamin: named from the protein Filamin; Bromo: acetylated lysine binding domain; ARF/SAR: from ARF and SAR GTP binding proteins; Pyrin: a member of the six-helix bundle death domain-fold superfamily; TM: transmembrane; Math: meprin and TRAF homology domain; Chromo: CHRromatin Organization Modifier domain.

boostrap values in phylogenetic trees. Both RBB and B30.2 trees are congruent and strongly suggest that fish trim35/hltr genes are good co-orthologs of their mammalian unique (i.e. non-duplicated) counterpart. This hypothesis could not be further validated by examining conserved synteny because the markers defining a conserved 2 Mbp-neighbourhood of TRIM35 in tetrapods (Fig. 7A) are not found in the same synteny group in teleosts. In contrast, more than 25 of the TRIM35/hltr genes found in the teleost stickleback (*Gasterosteus aculeatus*) are part of synteny clusters conserved in medaka (*Oryzias latipes*) and even in pufferfish (Fig. 7B), indicating that some duplications predated the split between these lineages. Only the regions involving *TRIM35*-12 and *TRIM25*-28 have counterparts in zebrafish. In zebrafish, the multiple copies of *TRIM35/hltr* are scattered on 15 different chromosomes (Fig. 4) and they are often grouped in clusters as previously observed for *ftr* and *btr* ([21], see above). In striking similarity

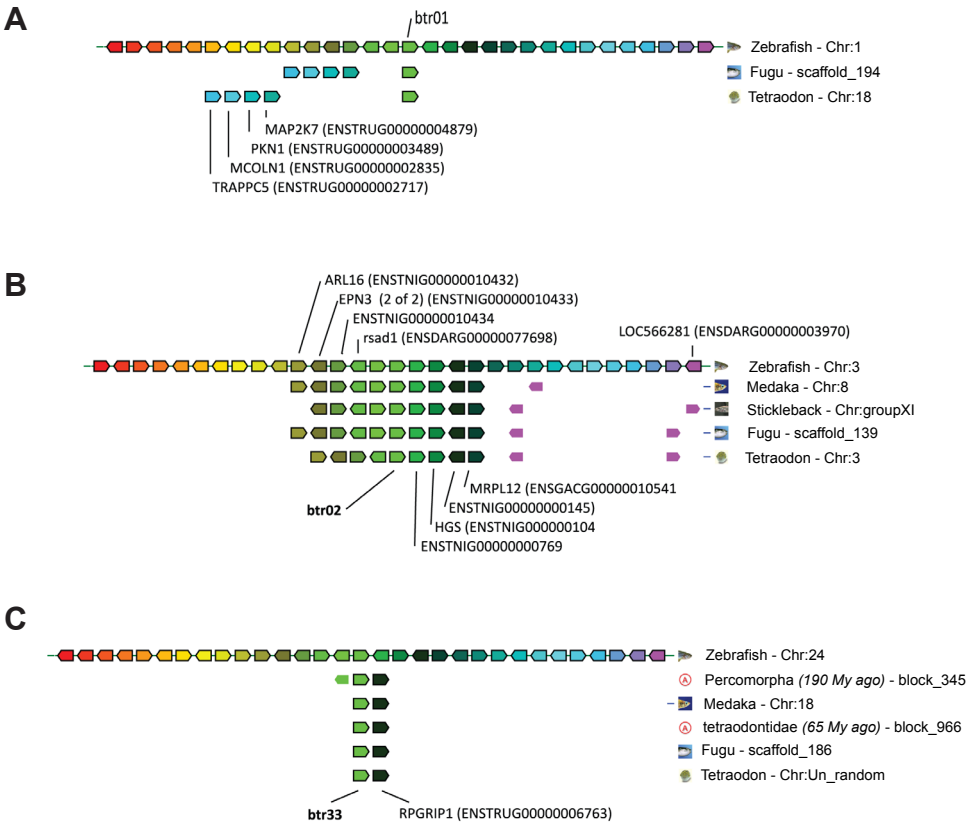

Figure 5. Group of conserved synteny around btr 01, 02 and 33. Synteny groups were determined from Ensembl assemblies using the genomicus database and browser (http://www.dyogen.ens.fr/genomicus-59.01/cgi-bin/search.pl) [85]. The figure is edited from the PhyloView taking btr 01 (**A**), 02 (**B**) and 33 (**C**) genes as references. The reference *btr* gene and its orthologues are shown in light green over a thin vertical line, and are indicated in bold.

with the *ftr* genes, the TRIM35/hltr genes involved in conserved syntenies are not found among the large sets of recently duplicated sequences represented by the distal branches in the phylogenetic tree.

Positive selection of B30.2 domains of btr and trim35 families

The B30.2 domain consists of two subdomains, PRY and SPRY and forms a distorted b-sandwich of two layers of antiparallel β-sheets [36,37,38]. The β-strands are connected by six variable loops (VL) that define hypervariable regions and form the ligand-binding surface in TRIM5α. The loops also contain the Ig-binding regions in TRIM21 [39]. We observed earlier in FTRs hypervariable regions similar to those of TRIM5α [21], and we showed that they evolved under positive selection. To determine whether zebrafish *TRIM35/hltr* and *btr* share the same evolutionary pattern and show diversified regions in their B30.2 domains, these genes were subjected to a similar analysis calculating the Shannon entropy site by site (Fig. S4).

The distribution of variable sites in TRIM35/HLTR and BTR is remarkably consistent with the patterns observed for FTRs and for TRIM5α: 39 among 59 and 26 among 37 hypervariable sites of TRIM35/HLTR and BTR, respectively, are shared with FTR (Fig. 8). Variable regions corresponding to the loops joining the B30.2 domain β-strands were retrieved, including those involved in the binding of the virus by TRIM5α. Interestingly, conserved variable sites were concentrated in the β2-β3 loop (VL1), which is responsible of retroviral binding specificity of TRIM5α and was identified as an evolutionary hotspot in TRIM5α and TRIM22 [40]. To test whether TRIM35/hltr and btr B30.2 domains evolved under diversifying selection in zebrafish, we used a test based on the estimation of synonymous (dS, silent mutations) and non-synonymous (dN, amino acid altering) substitution rates of all codons among a set of sequences: the ratio $\omega = dN/dS$ is an indication for negative (purifying) selection of deleterious changes ($\omega < 1$), neutral evolution ($\omega = 1$), or positive (diversifying) selection when changes offer a selective advantage ($\omega > 1$). This approach is often used on paralogues to detect the accumulation of non-synonymous changes that suggests a positive selection driving the evolution of new functions following gene duplications [41,42]. This method requires that the paralogue sequences are not too divergent i.e. that good quality multiple alignments can be easily produced. We verified that this condition was met for each dataset subjected to PAML analysis (Fig. S5). B30.2 domain sequences of zebrafish *TRIM35/hltr* and *btr* were analyzed under different evolutionary models M1a, M2a, M7 and M8 by PAML. Positive selection was detected for ~10% of sites of TRIM35/hltr under both M2a and M8, while for btr 4.5 % of sites were positively selected under M2a and 8.5 % of sites under M8 (Table 2). These results were validated by significant likelihood ratio test (LRT) with $p < 0.001$ for both models for TRIM35/hltr and btr (Fig. S6).

To investigate whether the estimation of positive selection under PAML was not perturbed by recombination between similar trim sequences during the evolution of the zebrafish genome, we re-analyzed our dataset with the algorithm PARRIS, which uses a partitioning approach to test whether sequences have been subjected to positive selection even if recombination occurred. Positive selection was still indicated by the

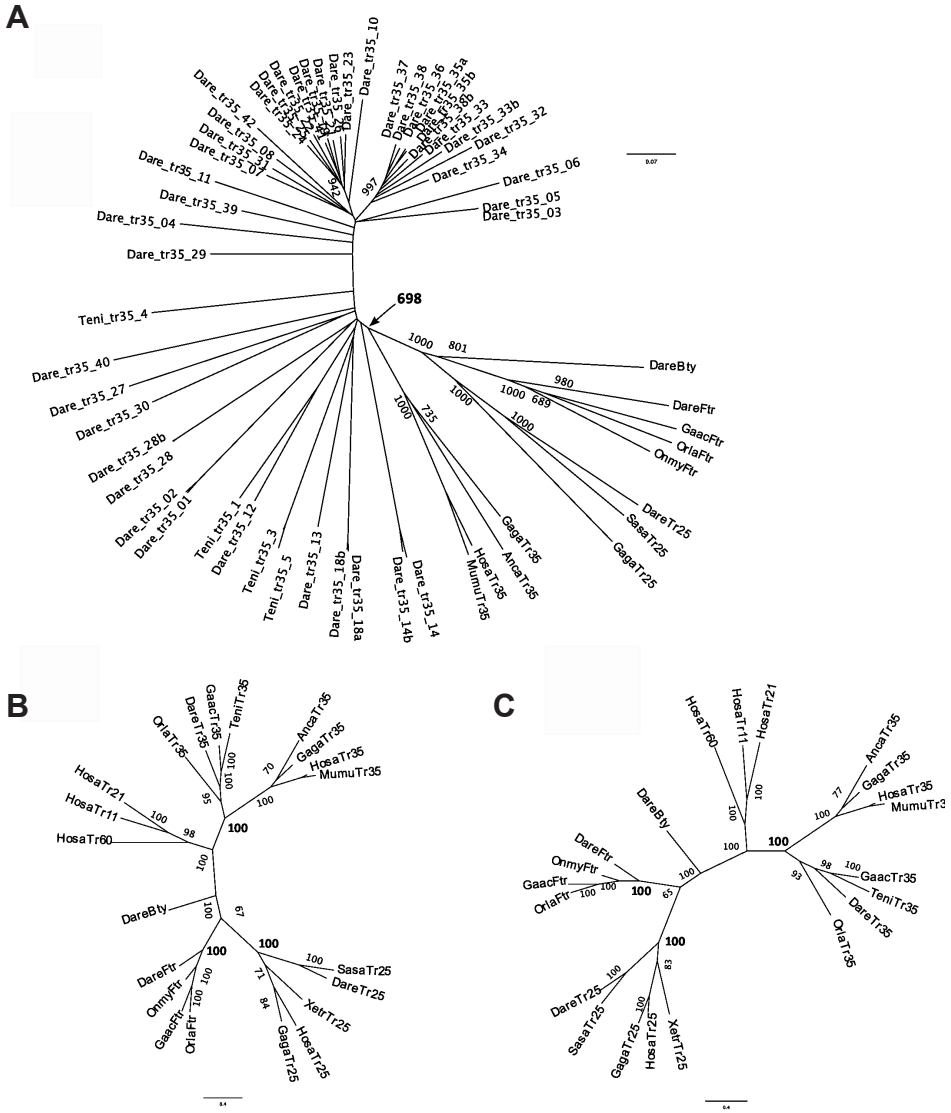

Figure 6. Fish counterparts of TRIM35 constitute multigenic subsets. **A** Distance tree produced by ClustalW (Neighbor joining; boostrap=1000) for the zebrafish TRIM35/HLTR sequences and representative TRIM sequences from other species. Relevant boostrap values are indicated. Separate phylogenetic analyses of the RBB (**B**) and B30.2 (**C**) regions of TRIM35 and other representative TRIM using Clustalw (Neighbor joining; boostrap=1000). The same analyses were performed with PHYML and led to consistent trees. Sequences integrated into the trees: DareBty: zebrafish bloodthirsty (NP_001018311); DareFtr: zebrafish fintrim (XM_692536); GaacFtr: stickleback fintrim; OrlaFtr: medaka fintrim (ENSORLP00000003320); OnmyFtr: rainbow trout fintrim (AM887799); DareTr25: zebrafish trim25 (NP_956469); SasaTr25:

LRT with p <0.001 for both TRIM35/HLTR and BTR. This indicated that whether or not recombination did occur, the B30.2 domains of TRIM35/HLTR and BTR have most probably evolved under positive selection. To search for recombination sites, we used the program GARD, which subdivides a sequence alignment in putative non-recombinant fragments, infers phylogenies for each fragment and assesses the quality of the fit for these phylogenies. This comparison therefore determines if the fragments are derived from two different ancestor sequences due to recombination. No evidence for recombination was detected for TRIM35/hltr. In contrast, seven breakpoints were identified between btr sequences at the positions 262, 269, 271, 288, 399, 405 and 427 of the btr B30.2 multiple alignment (Fig. S5 and Δc-AIC values in Fig. S7). These breakpoints suggested the existence of three segments of btr B30.2 domain where no recombination has occurred (see segments 1, 2 and 3 in Fig. 8). We detected positive selection in fragment one and three, with p < 0.001 in the LRT under M1a-M2a and M7-M8 models. No positive selection was detected in fragment two (Fig. S8). Finally, the specific sites where non synonymous changes accumulated were identified by a Bayesian approach using the complete gene set for TRIM35/hltr and the fragmented gene set for btr. For TRIM35/hltr, we found 12 sites under M2a and 11 sites under the more restrictive model M8. The majority of the sites fall in the predicted variable loops corresponding to those reported for TRIM21 and TRIM5α. For the btrs, we found 7 sites under both M2a and M8. Hence, a significant number of sites showing hints of positive selection in TRIM35/hltr and btr B30.2 domains were located in the β2-β3 loop, at positions matching well those previously noted for ftr and for the same region in TRIM5α (Fig. 8).

Do fish TRIM genes colocalize with the MHC paralogs?

The major histocompatibility complex (MHC) is a genetic region that plays a key role in self/non-self recognition and T-cell responsiveness. The presence of Class IV trim genes in the MHC is a conserved feature in mammals and birds [43,44,45]. Based upon this feature we addressed whether the co-localization predates the split (>450 Mya) between fish and tetrapods. This gene dense region has an ancient history, as the mouse and human genome contain four well established MHC paralogous regions of the MHC, that are believed to be the result of two whole genome duplications in the early evolution of vertebrates [46]. In teleosts, a variable number of global genome duplications followed by genome contraction and rearrangement events have “broken”

salmon trim25 (gene index TC35355 accessible at http://compbio.dfci.harvard.edu/); GagaTr25: chicken trim25 (XP_415653); XetrTr25: Xenopus tropicalis Trim25 (Ensembl Xenopus genome scaffold255: 821309_819660); HosaTr25: human Trim25 (Q14258); GagaTr35 : chicken trim35 (ENSGALP00000026735); AncaTr35 : lizard trim35 (ENSACAP00000002320); HosaTr35 : human trim35 (NP_741983.2); MumuTr35 : mouse trim35 (ENSMUSP00000022623); GaacTr35 : stickleback TRIM35 (ENSGACP00000004694); OrlaTr35: medaka Trim35; TeniTr35 : pufferfish Trim35; dareTr35: zebrafish Trim35-8 (ENSDARP00000064945); HosaTr21: human Trim21 (NP_003132); HosaTr11: human Trim 11 (NP_660215); HosaTr60: human Trim 60 (AAI00986). The IDs of the other TRIM35 sequences from zebrafish used in (A) are available in Figure S1.

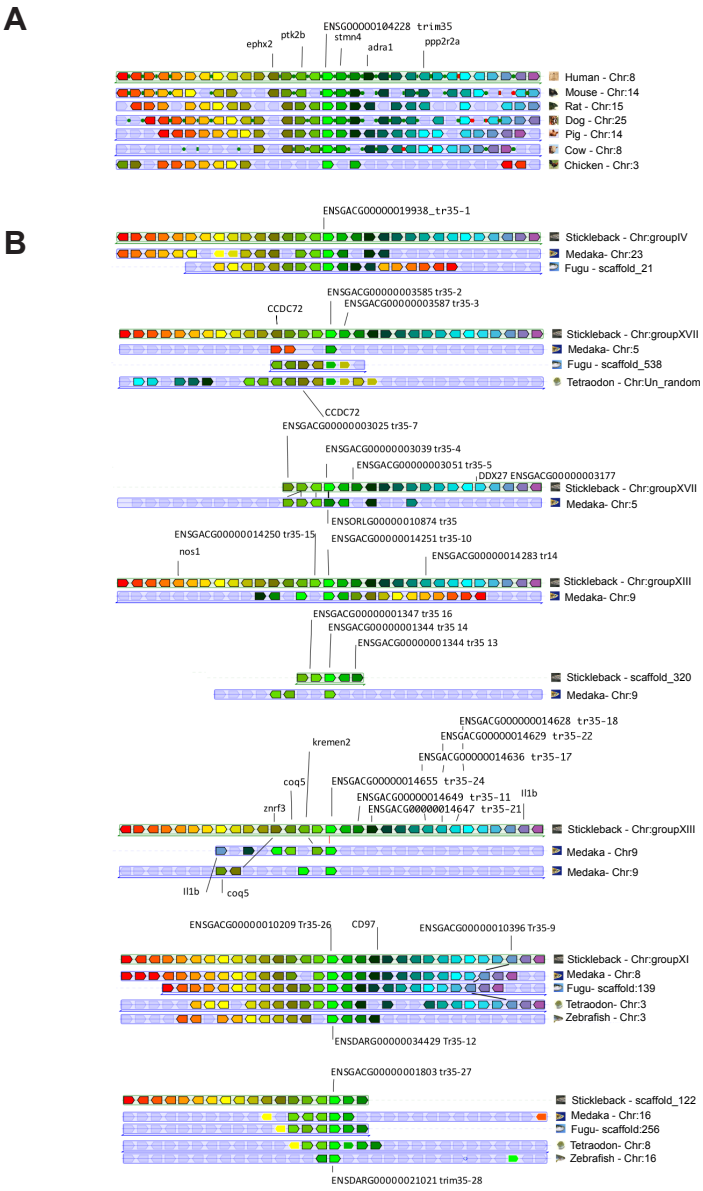

Figure 7. Group of conserved synteny around TRIM35 genes and gene clusters. Synteny groups were determined from Ensembl assemblies using genomicus database and browser (http://www.dyogen.ens.fr/genomicus-59.01/cgi-bin/search.pl) [85]. The figure is edited from the PhyloView taking the human TRIM35 gene (ENSG000000104228) as a reference (**A**), or taking the stickleback TRIM35-01, TRIM35-02, TRIM35-04, TRIM35-10, TRIM35-14, TRIM35-24,TRIM35-26 and TRIM35-27 genes as references (**B**). The reference gene and its orthologues is shown in light green over a thin vertical line and is indicated with its Ensembl ID.

the MHC into multiple regions in the genome of fish such as the MHC class I and II regions are on different chromosomes [47]. To trace the existence of an early association of ClassIV TRIM genes with the "primordial" MHC, it was therefore relevant to examine the different MHC regions and all of their associated paralogues in fish genomes. A loose linkage of ftr, btr and the MHC or its paralogues has been previously reported in zebrafish [20]. The MHC regions and their paralogues also contain RBCC-B30.2 genes in another fish species that possesses fewer Class IV trim genes than zebrafish: in stickleback, notch1.1, notch1.2 and notch3 are associated to 7 genes belonging to the trim Class IV grouping. Since in humans, notch4 is found within the MHC and other notch genes in paralogous loci [46], this was the first indication that the linkage might be older than tetrapods. We therefore performed a systematic survey of the distribution of TRIM genes and MHC markers in the zebrafish genome, looking for a co-localization pattern. We searched the homologues of a set of classical MHC markers and their paralogues described in other vertebrates [48,49,50,51,52,53] (Fig. S9). Since it was not always possible to attribute the zebrafish homologues to one given member of the tetrad of MHC paralogues in humans [54], we defined "MHC neighbourhoods" as regions extending 5 Mb (size of the MHC proper) upstream and downstream from each MHC or paralogue marker. The MHC neighbourhood represented 376 megabases in zebrafish containing 7884 genes compared to 1187 megabases and 16072 genes for the rest of the genome. We then compared the numbers of TRIM genes that were located in these MHC neighbourhoods versus that of the rest of the genome (Fig. S9); counting each cluster of tandemly duplicated trim genes as a single occurrence to avoid skewing of the analysis. Interestingly, the Class IV TRIM genes were significantly enriched in the MHC neighbourhoods (Independence χ^2-test, p-value = 0.0035), while no bias could be detected for the other TRIM genes (Table 3).

Discussion

TRIMs are widely distributed in metazoans, and these intracellular proteins are involved in the regulation of multiple pathways. In this report, a systematic survey of TRIM genes was performed in zebrafish and pufferfish to examine the characteristics of this family in two fish species with different genome dynamics. The zebrafish genome is large (about 1600 Mb/24000 genes) and contains an abundance of repeated DNA elements [55] as well as many highly expanded gene families. In contrast, the pufferfish genome is compact (about 350 Mb/15000 genes) and the multigenic families are smaller than found in zebrafish, at least those involved in the immune system. The pufferfish belongs to *Tetraodontidae* in the vast group of percomorphs, and it is phylogenetically distinct from zebrafish, with an estimated 300 My of divergence [22]. We therefore attempted a comparison of an extensive versus a minimal repertoire of TRIM genes in teleosts to better understand their evolutionary histories.

We retrieved a large number of TRIM genes in both species, representing almost all the classes defined by Short and Cox [3] in human. Our data indicate that the main TRIM classes were already defined in the common ancestor of fishes and tetrapods. However, a few genes show specific features that illustrate the evolutionary pathways leading to the generation of new TRIM classes. There is only one class III TRIM gene

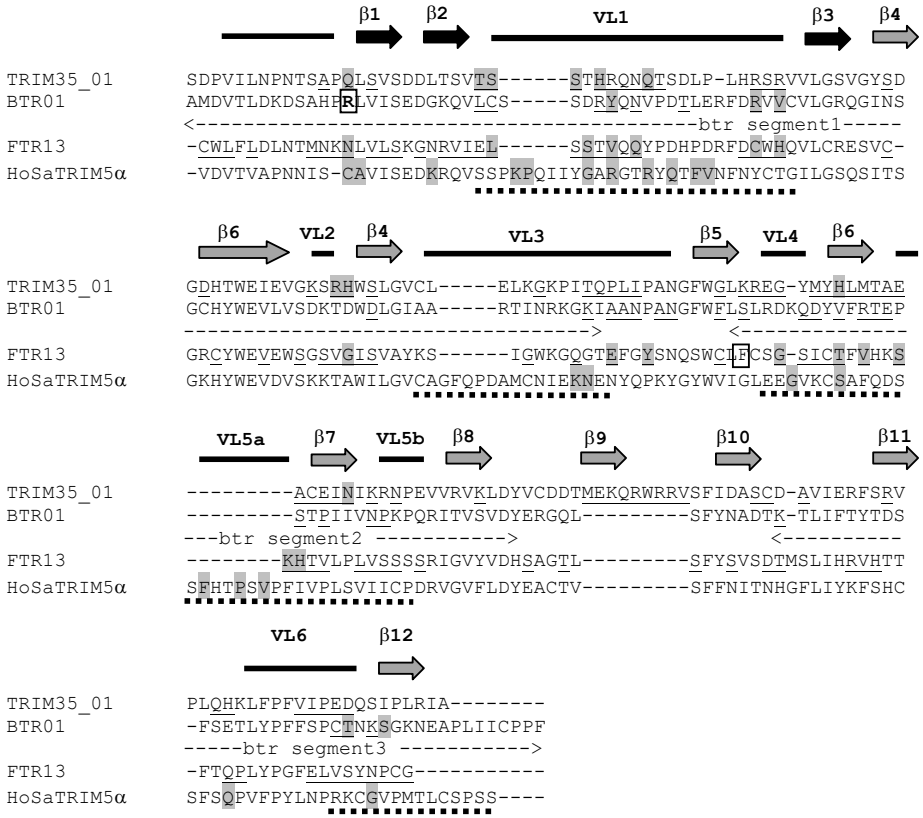

Figure 8. Positive selection in the B30.2 domain of BTR and TRIM35/HLTR. Distribution of hypervariable and positively selected residues in a multiple alignment of B30.2 domains from representative zebrafish BTR and TRIM35/HLTR, compared with a typical FTR sequence (Dareftr13: [GenBank: XM_695031]), and with TRIM5α (HosaTRIM5a). Hypervariable sites (shannon entropy >2) are underlined. Hypervariable sites previously described [21] are underlined in the FTR13 sequence. The four hypervariable regions of the TRIM5α B30.2 domain are indicated by dotted lines. The variable loop-connecting strands of the domain are named VL1–VL6. β-strands of the B30.2 domain are indicated by black (PRY region) or grey (SPRY region) arrows from [36]. Segments 1, 2 and 3 determined by the recombination GARD analysis in the BTR multiple alignment are shown under the BTR01 sequence. Positively selected sites (among zebrafish TRIM35/HLTR & BTRs: this study; among FTRs : [21] and among primate TRIM5α: [40] are boxed in grey when detected under models 2a and 8. Sites positive under M8 but not under M2 are boxed in white. In TRIM35/HLTR, Q (β-strand 1) was detected under M2 not M8. In BTR, R (β-strand 1) has been detected in the BTR analysis only under M8 with complete domain, not in segment 1. In BTR, S (β-strand 12) has been detected only under M2a and M8 of BTR segment 3, not in the analysis using the complete domain. Detailed PAML results for each position under positive selection are available in additional data file S8.

in humans (*TRIM42*) with orthologues in amniotes but not in fish. However, if class III genes are defined by domain organization alone (RBCC-FN3), fish do possess a class III trim gene, which is one of the two co-orthologues of *TRIM46*, a class I gene (RBCC-FN3-B30.2). One can then hypothesize that the human *TRIM42* itself derives from a class I TRIM gene through an ancient event involving the loss of the B30.2 domain. In the same line, the frequent loss of various C-terminal domains led to the birth of new

Table 2. PAML results.

Region[1]	**n**[2]	**c**[3]	**Parameters in ω distribution under M2a4**		**Parameters in ω distribution under M85**	
TRIM35 complete B30.2	38	122	$\omega_{>1}$ = 5.68162	$p_{>1}$ = 0.09952	$\omega_{>1}$ = 4.13995	p_1 = 0.10057
			ω_1 = 1,000	p_1 = 0.43562		p_0 = 0.89943
			$\omega_{<1}$ = 0.19870	$p_{<1}$ = 0.46485	p = 1.13670	q = 1.81193
TRIM39 complete B30.2	25	171	$\omega_{>1}$ = 3.33719	$p_{>1}$ = 0.04501	$\omega_{>1}$ = 1.99982	p_1 = 0.08529
			ω_1 = 1,000	p_1 = 0.31535		p_0 = 0.91471
			$\omega_{<1}$ = 0.19082	$p_{<1}$ = 0.63964	p = 0.88108	q = 1.86498
TRIM39 B30.2 Fragment 1-252	25	84	$\omega_{>1}$ = 3.96823	$p_{>1}$ = 0.08146	$\omega_{>1}$ = 2.74731	p_1= 0.08977
			ω_1 = 1.000	p_1= 0.39488		p_0 = 0.91023
			$\omega_{<1}$ = 0.14824	$p_{<1}$= 0.52366	p = 0.89035	q = 1.87745
TRIM39 B30.2 fragment 291-393	25	30	$\omega_{>1}$ = 1.000	$p_{>1}$ = 0.13938	$\omega_{>1}$ = 1.00000	p_1= 0.09928
			ω_1 = 1.000	p_1= 0.32559		p_0 = 0.90072
			$\omega_{<1}$ = 0.19144	$p_{<1}$= 0.5350	p = 1.18020	q = 2.35230
TRIM39 B30.2 Fragment 430-557	25	39	$\omega_{>1}$ = 2.21718	$p_{>1}$ = 0.08741	$\omega_{>1}$ = 1.90649	p_1= 0.12627
			ω_1 = 1.000	p_1= 0.12322		p_0 = 0.87373
			$\omega_{<1}$ = 0.19452	$p_{<1}$= 0.78936	p = 1.42717	q = 4.37468

[1] for sequence fragments, the numbers correspond with the position of first and last nucleotides in the alignment with excluded gaps

[2] n, the number of sequences in the alignment and tree

[3] c, the number of codons

[4] parameters determined under M2a with ω the ratio of non-synonymous rates (*dN*) and synonymous rates (*dS*) and p the corresponding proportion of sites for each ω-class

[5] parameters determined under M8 with ω the ratio *dN/dS*, the corresponding proportion (p_1=1-p_0) of sites and *p*- and *q*-estimates in the β(*p,q*)-distribution

Table 3. Class IV trims genes are concentrated in the MHC and MHC paralogues.

	MHC neighbourhood	Rest of the genome	Total	Chi square test
Length (megabase)	376[1]	1187	1563	
Number of genes (total)	7884	16263	24147	
Number of Class IV TRIM[2]	31	31	62	p = 0.0035
Number of other TRIM	10	23	33	NS

[1] The results are based on the genome assemblies available at http://www.ensembl.org (release 58). The detailed calculations and a map with MHC and MHC paralogues considered in the analysis are available in Suppl. Mat. 8.

[2] To avoid skewing the analysis by the numerous TRIM genes recently duplicated, we counted each clusters of TRIM genes as only one event From the Zv8 assembly of the zebrafish genome.

class V *TRIMs* found in zebrafish (e.g. *TRIM25*-like, *TRIM54*-like, several *ftrs*). This is also likely to be the case for some human class V *TRIM* genes; a relatively recent origin by such a mechanism would explain why few human members of this class have fish counterparts. At the N terminus, loss of the RING domain is also observed in several instances (*TRIM24*-like, *TRIM32*, *TRIM1* and *TRIM2*-like). As described above, this event results in truncated *TRIM*-like genes, but could not result from the deletion of an entire exon, which suggests that the loss of the RING was positively selected. In fact, such events can be sometimes dated before the split between the pufferfish and zebrafish lineages, or some are even much older such as *TRIM*16 that is retrieved in fish and tetrapods. The loss of B-Boxes and Coiled Coil is also sometimes observed, mainly in Class IV genes, but does not seem to be fixed as easily.

In contrast, insertions of single- or multi-exon domains downstream of a RBCC module were found to generate new *TRIM* configuration in the zebrafish genome (i.e. *ftr06*, *ftr52*, *btr31*). When this occurred is unknown, but the insertion of the chromodomain in the *ftr06* gene appears to be recent, considering the dynamics of the ftr family and the absence of inactivating mutations in the B30.2 exon displaced by the "usurper" exon. Whether this change has a functional consequence for the encoded gene remains to be tested experimentally; a detailed phylogenetic reconstruction in close relatives of the zebrafish would therefore be informative. As chromodomains are involved in chromatin remodelling such a protein would be expected to regulate gene expression; similar functions have been described for TRIM proteins with a C-terminal bromodomain, structurally distinct from chromodomains but with comparable functions. In contrast, the replacement of the B30.2 exon of *ftr52* by exons encoding a cyclophilin A (CypA) domain could have given rise to a TRIM protein with affinity to different viral proteins, because most remarkably, TRIM5-cypA proteins have also appeared at least twice independently (by retrotransposition of a cypA sequence in the TRIM5 locus) in the primate lineage, leading to proteins with demonstrated anti-retroviral activity involving capsid binding by CypA [56-61]. In spite of this, no *TRIM* gene with a CypA domain has been reported in humans or in non primate species with a

fully sequenced genome. The early stop codon found in the zebrafish *ftr52* gene leads us to speculate that although such a domain combination may provide a transient benefit against some viral infections, it may have some drawbacks that impairs its definitive fixation in a lineage. For *btr31*, the recombination events led to a unique configuration RING-TM-B30.2 where a membrane separates a RING and a B30.2 domain, which has completely unknown functional consequences.

Domain organization, sequence similarity and phylogenetic analyses indicate that one or two orthologues of multiple human TRIM genes that belong to classes I, II, V, VI, VII, VIII, IX i.e. to the "group 1" defined by Sardiello et al. [6] are present in both zebrafish and pufferfish. Often, when two co-orthologues are found for such genes, one of these has lost the C-terminal domain, while the other has retained the complete domain organisation and thus probably constitutes a true functional counterpart. This notion is also supported by similar expression patterns that were observed for genes selected in this category in vertebrates. The correspondence between the repertoires of "group 1" TRIM genes in zebrafish, pufferfish, and mammals indicates that strong purifying selection pressures were exerted to keep one (or few) copy(ies) of these genes in vertebrate genomes, illustrating their key functions in the basic biology of the cell. This is in sharp contrast with the evolutionary pathway of the Class IV RBCC-B30.2 trim genes. The RBCC-B30.2 TRIM genes from Class IV represent unique sets in the different species of mammals and other tetrapods examined in detail by Sardiello *et. al.* [6]. Our survey of zebrafish and pufferfish TRIM genes generally confirms and extends this conclusion. Most human ClassIV TRIM genes have no counterpart in the zebrafish or the pufferfish, and fish possess many ClassIV TRIM genes that do not exist in human nor in the mouse. Another feature of Class IV TRIM genes that was well exemplified in our previous report on fintrim [21] is their propensity to expand into multigenic subsets. In this study we demonstrated that fish possess in fact three multigenic subsets of TRIM genes all belonging to the Class IV: *ftr* (finTRIM), *btr* (bloodthirsty-related TRIM/TRIM39) and *TRIM35/hltr*. The number of *ftr*, *btr* and *trim35/hltr* is different between fish species belonging to distant families, indicating different degrees of expansion. This is particularly striking from the comparison of zebrafish with expanded subsets and pufferfish with a "minimal" repertoire. This contrast likely reflects the high level of genomic rearrangement of the zebrafish genome, as indicated by short conserved synteny blocks compared to other fish versus mammals, and the strong compaction of the tetraodon/fugu genome [26,62]. However, functional data would provide a better understanding of these sharp differences of class IV among teleosts. Besides, this underlines the strong constraints that maintained the conservation of TRIM belonging to the other TRIM classes (the group 1 defined by Sardiello *et al.*) in different lineages. Some members of *ftr, btr* and *TRIM35/hltr* are part of conserved synteny groups conserved among teleosts, showing that their initial emergence and subsequent diversification is ancient in the evolution of teleosts and predates the differentiation of the main fish lineages. Consistently, these genes involved in syntenies appear to be the most ancient genes in their subset. They branch close to the basis of their multigenic subset in phylogenetic trees and generally do not belong to large genomic clusters. This is the case for *ftr 82/83*, *btr1* and *btr33*, *TRIM35-12* and *-28*. In contrast, the genes composing large

genomic clusters such as zebrafish *ftr* on chromosome 2, are not included in conserved synteny groups and probably represent more recent, lineage-specific diversification events. The mechanisms for the amplification of TRIM are likely different for *ftr, btr* and *TRIM35/hltr* within a species: for example in medaka the *TRIM35/hltr* expansion occurred by duplication, while ftr expansion involved retrotransposition. Interestingly, only three sets of class IV genes are retrieved as multigenic groups in any fish species for which a complete genome assembly is available. The diversification of ftr, btr and *TRIM35/hltr* therefore appears to be rooted in ancient duplication events, followed by parallel diversification processes, which reflects similar functional constraints in different fish lineages. Multiplication of some class I TRIM genes has also occurred in mammals, albeit to a smaller scale; thus, human TRIM5, TRIM6, TRIM22 and TRIM34 likely result from such a duplication event, while in cow, theTRIM5 gene has further expanded into eight tandem copies, five of which encode a functional protein [40].

To date, functions of the multiple Class IV fish trims are still largely unknown and do not provide an obvious explanation for their extensive expansion. A non-redundant role in erythropoiesis has been reported for bloodthirsty (bty) [35], which is quite difficult to understand in the context of the large multigene btr family. It is noteworthy that this role is deduced from morpholino-based transient inactivation in embryos and the original bty gene is not found in the current zebrafish assembly Zv9 (www.ensembl.org; Tübingen background). The closest Zv9 gene is *btr18*, and it remains to be established if *bty* is unique to the original genetic background used by Yergeau *et al.* or is an allelic variant of a Zv9 gene. At least some *finTRIMs* are induced by IFN and virus infection in rainbow trout [21,63], and a *btr* is upregulated by poly I:C in Atlantic cod [64]. In fact, these TRIM genes were not only duplicated many times, but also diversified after gene expansion with an accumulation of non synonymous changes. Thus, apparent signatures of diversifying selection were found in the β2-β3 loop in the B30.2 domain of btr, TRIM35/hltr (this study, Table 2) as previously reported for ftr [21] in zebrafish. Interestingly, the B30.2 domain, especially the β2-β3 loop, was subjected to a strong diversification in primates and accounts for the species-dependent retrovirus restriction of TRIM5α in the different species [18,19,65]. Moreover, several copies of *TRIM5* can be found in the genome of certain species such as cow [40]. Considering the importance of TRIM proteins in antiviral immunity [66,67] and the role of the B30.2 domain, we believe that virus sensing/restriction may be the driving force in the diversification of the fish TRIM multigene subsets under positive selection. However, the approach we followed to find sites under positive selection may lead to false positive, and accumulation of non synonymous changes does not necessarily imply functional changes [68]. Experimental evidence, for example of multiple B30.2/virus binding, would be required for a definitive proof of the functional impact of B30.2 diversification.

Our simple analysis of localization of TRIM genes relatively to genes of the MHC and MHC paralogues would have to be complemented by a detailed phylogenomic analysis of these regions through the whole vertebrate evolution from lamprey and sharks to fish and mammals. This will become possible with the publication of good quality genomes. However, the co-location pattern that we report suggests that TRIM and B30.2 are associated with the MHC and MHC paralogues in fish, as well as already

reported in mammals and birds [43,44,45]. Could it be for the benefit of immunity? An interesting question then would be to determine if the B30.2 domain was first associated to the ancestral MHC as a part of a pre-existing Class IV TRIM molecule. The existence of TRIM-like genes with canonical B30.2 domains in *Branchiostoma* (Cephalochordates), *Drosophila* (Arthropods), *C. elegans* (Nematods) *Nematostella* (Cnidarians) and *Trichoplax* (Placozoa) ([20] and unpublished observations) indicates that genes resembling Class IV TRIMs are probably very ancient and could have been inherited from a common ancestor to vertebrates and these different groups of invertebrates. Considering the role of the B30.2 domains in mammals, we propose that ancestral Class IV TRIMs participated in defence and were part of a gene complex, the proto MHC, equipped in genes selected for processing (and later presenting) viral peptides. Indeed, intense duplication is typical of genes families involved in immunity. Such genes belonging to different families have diverged rapidly and independently within different classes of organisms in function of the pressures exerted by the pathogenic environment [69-74]. Besides, TRIM genes may have been kept in a genomic cluster with proteasome components because they were involved in targeting virus particles to the ubiquitin-dependent proteasome system in a manner analogous to LMP/TAP genes that form a tight cosegregating unit in practically all vertebrates. This simple antiviral axis might have been very ancient and could have participated in the establishment of a proto-MHC selected for proteasome-mediated destruction of virus proteins and therefore production of peptides to which the antigen presenting machinery would be added later in evolution. In support of this hypothesis, one can remark that several class IV TRIM such as *TRIM11* and *TRIM17*, are located in human MHC paralogous regions and participate in the ubiquitin-proteasome system [75,76]. The binding of TRIM5α to the retroviral capsid induces a rapid degradation of TRIM5α by the proteasome, providing an additional link between TRIM-dependent virus restriction and proteasome activity [77]. Another class IV TRIM involved in the ubiquitin-proteasome axis is TRIM21 that binds Ig constant region with very high affinity and targets viral particles coated with antibodies to the proteasome [78].Given the abundance of viruses in the aquatic environment where early metazoa developed, the necessity for diverse protective measures against viruses certainly played a major role in shaping the immune system. The recruitment and diversification of IgSF TCR-like antigen receptors from proteins used by viruses to enter cells would be a good example of the consequences of such measures [79]. Similarly, the TRIM connection with the MHC could be a remnant of the early steps towards the construction of an adaptive immune system with associative recognition (TCR, MHC-peptide) by recruitment of antiviral primary defence systems. In the genomes of modern species, the trim family provides a good model to study the evolution of multigene families and functional diversification. The identification of the ligands and functions of such diversified subsets should provide new insights on the molecular pathways developed in the main vertebrate lineages.

Our survey of fish TRIM genes in two fish species identifies subsets with very different evolutionary dynamics. Thus, TRIM genes encoding RBCC-B30.2 proteins show the same evolutionary trends in fish and tetrapods: they are fast evolving, often under apparent positive selection, and they duplicate to create multigenic families that can

be very large such as zebrafish *ftrs*. Among these multigenic subsets, we could identify several new combinations of domains, which epitomize how new trim classes appear by domain insertion or exon shuffling. Finally, TRIM genes encoding RBCC-B30.2 proteins are preferentially located in the MHC and in MHC paralogues, which suggests that such TRIM genes with a B30.2 exon may have been part of the ancestral MHC.

Acknowledgements

We are grateful to C. Caron from the CNRS/UPMC ABIMS bioinformatics platform (http://abims.sb-roscoff.fr) for providing help and support for the recombination site computing. We thank John D Hansen for discussions and proofreading of the manuscript, Olivier Chabrol, Anthony Levasseur and Manuela Royer-Carenzi for support and helpul discussions about gene distributions and positive selection analysis, and Philippe Herbomel for discussions and support.

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On ne trouve pas la solution,
on la fait.

- Marguerite Duras -

Chapter 4

FinTRIMs, fish virus-inducible proteins with E3 ubiquitin ligase activity

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Developmental and Comparative Immunology
2012 Feb; 36(2):433-41

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Abstract
TRIM proteins recently emerged as novel players in antiviral defense. They contain a tri-partite motif, composed of a RING zinc finger, one or two B-boxes and a coiled-coil domain. Many members of this large protein family of E3 ubiquitin ligases catalyze the attachment of ubiquitin molecules to a substrate protein, an activity dependent on the RING domain. We earlier made a full description of the TRIM gene family in zebrafish and pufferfish and identified three multigene TRIM subsets, a feature unique to fish. To determine their biological role, we further characterized members of the finTRIM subset. FinTRIM gene expression was studied during development and in multiple tissues in adult rainbow trout. Upregulation of a large number of finTRIM genes upon viral stimulation suggests they are involved in antiviral immunity. Finally, we demonstrate E3 ubiquitin ligase activity for two finTRIM members. This indicates that finTRIMs could regulate antiviral signaling through ubiquitination.

Introduction
Proteins belonging to the TRIM family have recently emerged as important mediators in antiviral immunity. TRIM proteins are characterized by a tri-partite motif that consists of a RING zinc finger domain, one or two B-box domains and a coiled coil domain [1-3]. This TRIM/RBCC motif is located in this order at the N-terminal side of the protein. Multiple TRIM proteins contain an additional domain at the C-terminus [4]. Over 70 TRIM genes are described on the human genome. Additionally, multiple TRIM transcripts can be generated by alternative splicing from a TRIM gene, which together form a high number of TRIM variants [3, 5]. Interest for TRIM proteins has grown extensively in the last few years as these proteins have been demonstrated to act as potent antiviral proteins, whereby TRIM5α has gained most attention due to its restriction activity against HIV-1 and other retroviruses [6, 7]. Several studies have now shown that TRIM proteins act as antiviral factors against a wide range of viruses [7-9]. A role in antiviral immunity is suggested for many TRIM proteins, as multiple TRIM genes are inducible by interferons (IFNs), key cytokines for the onset of the antiviral immune response [10, 11]. Moreover, TRIM proteins seem to play essential roles in regulating the interferon-mediated immune response, as TRIM25 regulates the activity of one of the viral pattern-recognition receptor (PRR), RIG-I [12] and other TRIM proteins regulate the PRR-IFN-associated signaling pathways that lead to expression of IFN and IFN-stimulated genes (ISGs) (reviewed by [13]).

One biochemical mechanisms underlying TRIM's function is that they possess an E3 ubiquitin ligase activity, attributed to the RING-domain [14, 15]. Protein ubiquitination is a multi-step process, mediated by three enzymes, whereby single or multiple ubiquitin molecules are covalently bound to a substrate protein. The ubiquitin-activating E1 enzyme activates ubiquitin by the formation of a thioester-bond between the C-terminal glycine-residue of ubiquitin and a cysteine-residue in the active site of the E1. This is a high-energy process mediated by ATP. E2 is the ubiquitin-conjugating enzyme that conjugates the activated ubiquitin, whereby ubiquitin is transferred from the cysteine-residue of E1 to a cysteine-residue of E2. E3 is the ubiquitin-ligase that

transfers activated ubiquitin from E2 to a substrate. Hereby, E3 mediates the formation of an isopeptide bond between the activated ubiquitin conjugated to E2, and a lysine-residue of the substrate [16, 17]. There are different types of ubiquitin linkages: a substrate protein can be modified by single or multiple ubiquitin molecules, or by chains of ubiquitin. Ubiquitin itself has seven lysine residues that can be further conjugated by another ubiquitin molecule to form polyubiquitin chains. Polyubiquitin chains can be formed by forked chains of ubiquitins that are linked either via similar, or different lysine residues [16]. In addition, linear polyubiquitin chains can be formed by linkages between the N- and C-terminus of ubiquitin [18]. Protein modification by ubiquitination has various outcomes, depending on the type of ubiquitin linkage, leading to either degradation by the 26S-proteolytic pathways, or altering protein-protein interactions [19]. TRIM proteins for which E3 ligase activity has been described, are implicated in both protein degradation, as modulation of protein-protein interactions [12, 14, 20].

TRIM genes and proteins are widely distributed in metazoans [5], but functional studies on non-mammalian TRIM proteins are rather limited. We have recently started the characterization of TRIM proteins in teleost fish and provided a full description of TRIM genes on the genome of zebrafish (*Danio rerio*) and pufferfish (*Tetraodon nigroviridis*) [21, 22]. This description showed that a high number of TRIM genes are conserved among teleosts and mammals, and interestingly, we identified three multigene subsets of TRIM genes, an unique feature for fish. Two of the fish multigene subsets were related to the genes encoding TRIM25 and TRIM39 in mammals, but beyond fish, no orthologs were identified for the third multigene subset. Therefore, genes belonging to this subset were named finTRIMs, for fish novel TRIM genes [22]. On the zebrafish genome, 84 finTRIM genes were identified, and in rainbow trout, for which the whole genome sequence is not available yet, a high number of transcripts were identified by 3'RACE-PCR. Interestingly, both genes and transcripts are highly variable in length and hereby vary in the number of domains they contain. Long transcripts encode for finTRIM proteins that contain a B30.2 domain next to the RBCC-motif. We moreover demonstrated that the B30.2 domain has evolved under positive selection pressure, as was demonstrated for the retrovirus restriction factor TRIM5α [23].

To further characterize finTRIMs and elucidate a possible implication in the antiviral immune response, finTRIM gene expression was studied during development and upon viral induction and the intracellular localization of these proteins was determined. We more specifically investigated rainbow trout finTRIM members that are induced during viral infection using deep sequencing. Finally, an *in vitro* ubiquitination assay was set up, whereby we demonstrated that trout finTRIM proteins display E3 ubiquitin ligase activity. These results provide a basis to further establish the functions of finTRIMs and the mechanism by which finTRIMs may act during viral infection.

Materials and Methods

Animals

Rainbow trout (*Oncorhynchus mykiss*) were bred and reared at 10-12°C in re-circulating UV-treated water at the fish facilities of INRA in Jouy-en-Josas, France.

Fish from the homozygous clonal fish line B57 [24] were used in all experiments. For collection of organs, adult fish (2-3 years old) were sacrificed by overexposure to 1% 2-phenoxyethanol (2-PE), blood was collected by puncture of the caudal vein and organs (spleen, posterior head kidney, anterior kidney, hindgut, liver, muscle, gills, skin, atrium, ventricle and brain) were surgically removed and stored in 1 ml RNA later stabilization reagent (Qiagen) at -80°C until RNA extraction. Fish were sacrificed during different life stages of developing trout; eyed-egg stage (20 days post fertilization, p.f.), hatching (30 days p.f.), first feeding (50 days p.f.) and three weeks after first feeding (70 days p.f.). All experiments were conducted according to the protocols approved by the local ethical committee.

Cells and viral hemorrhagic septicemia virus (VHSV) strain

Fathead minnow EPC cell line was used for virus production and titration. EPC cells were cultured in BHK-21 medium (Invitrogen-Gibco), supplemented with 10% (V/V) fetal calf serum (FCS, Eurobio, Les Ulis, France), 10% (V/V) tryptose broth, streptomycin (50 µg/ml) and penicillin (50 units/ml). Human Embryonic Kidney 293T cells (HEK293T) were cultured in Dulbecco's Modified Eagle's Medium (Eurobio, Les Ulis, France), supplemented with 10% (V/V) fetal calf serum, streptomycin (µ mg/ml) and penicillin (50 units/ml).

A fibroblast-like cell line (called B57 thereafter) was derived from a trout homozygous clonal fish line B57 generated by a gynogenesis based strategy [22] following the same procedure as for the RTG-2 cell line. B57 cells were stimulated with inactivated VHSV strain 07-71 [25], leading to a typical type I interferon response (personal observations). Virus was prepared as cell supernatant (10^8 pfu/ml) and inactivated by 1 hr incubation at RT with diluted (1:4000) β-propiolactone (BPL), followed by 48 hrs incubation at 4°C.

RNA extraction and first strand cDNA synthesis

Trout organs were thawed on ice and transferred to one ml TRIzol. Tissues were homogenized with ceramic beads (1-1.2 mm; N1100; Mineralex, France) and two rounds of centrifugation (6000 g; 15 s; Precyllis 24). After homogenization, 200 µl chloroform was added per 1 ml TRIzol, incubated two min at RT and centrifuged (12 min; 13,000 g, 4°C). The aqueous phase was transferred to a new tube and RNA was precipitated with 500 µl isopropanol, and centrifuged (10 min, 13,000 g, 4°C). The RNA pellet was washed with cold 70% ethanol, dried to the air and redissolved in 350 µl RLT buffer. RNA was further purified on column according to the manufacturer's protocol with an on-column DNase treatment included (RNeasy Mini kit, Qiagen). RNA concentrations were measured by spectrophotometry (Nanodrop, Thermo Scientific) and integrity was ensured by analysis on a 1% agarose gel before proceeding with cDNA synthesis. To each reaction, 2.5 µg RNA, 2 µl 10 mM poly(dT)25 primers and 1 µl 10 mM dNTP mix were added and incubated for 5 min at 65°C, followed by 1 min incubation on ice. After incubation, to each sample 4 µl 5x first strand buffer, 2 µl 0.1 M DTT and 1 µl RNase inhibitor was added, incubated 2 min at 42°C and 1 µl Superscript II Reverse Transcriptase (RT, Invitrogen) was added. The reaction was incubated for 50 min at

42°C, followed by an enzyme-inactivation step of 15 min at 70°C.

Real-time quantitative PCR (RQ-PCR)
Universal primers for trout finTRIMs were used to quantify finTRIM gene expression (B144, see Table 1). ELF1α gene expression served as an internal standard. For RQ-PCR, 5 µl cDNA, 5 µl mix of forward and reverse primers (300 nM each) were added to 10 µl Power SYBR® Green Master mix (AB Gene). RQ-PCR (10 min at 95 °C, 40 cycles of 15 s at 95 °C and 60 s at 60 °C) was carried out with the Mastercycler®ep Realplex (Eppendorf). After each run, melting curves were produced by detecting fluorescence from 60 to 95°C at 1°C intervals.

Constitutive expression of finTRIMs was determined in various organs and tissues of five individual adult trout, and rendered as a ratio of target gene vs. reference gene (ELF1α) calculated with the Pfaffl method [26], according to the following equation:

$$\text{Ratio} = (E_{\text{reference}})^{\text{Ct reference}} / (E_{\text{target}})^{\text{Ct target}}$$

where E is the amplification efficiency and Ct is the number of PCR cycles needed for the signal to exceed a predetermined threshold value.

Expression following stimulation was rendered as a ratio of target gene vs. reference gene relative to expression in unstimulated control samples according to the following equation:

$$\text{Ratio} = (E_{\text{target}})^{\Delta Ct\,\text{target}\,(\text{control-sample})} / (E_{\text{reference}})^{\Delta Ct\,\text{reference}\,(\text{control-sample})}$$

Differential expression in TRIM gene expression was determined by a Student *t*-test.

RT-PCR and purification of PCR-product
PCR-products for high-throughput sequencing were prepared as follows. RT-PCR reactions were carried out with 1 µl cDNA, 10 mM dNTPs, 1 µl pfu DNA polymerase (Promega), 1x pfu DNA polymerase buffer (Promega), 8 pmol forward primer and 8 pmol reverse primer. Primers contained both a common TRIM sequence and a barcode-sequence (MID1-6) to distinguish between templates (see Table 1). The following PCR-program was used: 2 min at 94°C, 40 cycles of 30 s at 94°C, 30 s at 60°C and 90 s at 74°C, followed by 10 min at 74°C. Three RT-PCR reactions were carried out per template, pooled and purified on Qiaquick spin columns (Qiagen) and eluted in 30 µl. A second purification step was carried out by running the purified PCR products on an agarose gel, followed by gel extraction according the manufacturer`s protocol (Qiagen Gel extraction kit, Qiagen). Purified PCR-products were quantified spectrophotometry (Nanodrop, Thermo Scientific).

High-throughput screening and bio-informatics
Expressed fintrim transcripts were analyzed by the high-throughput Genome Sequencer FLX Titanium system (Roche-454 Life Sciences). RT-PCR-products were prepared according to the GS FLX Titanium Method manual at the Genotoul genomic platform,

Table 1. Primers

primer	5'-3' sequence	Step
oliB32_B21_Attb1	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCACCGTC-CAAATGGCTCAACAGGGAG	molecular cloning
Attb2TRIM37rv	GGGGACCACTTTGTACAAGAAAGCTGGGTCCAGTTTAAC-CAGCTCAGCAGTACCA	
AttB2TRIMLV50rv	GGGGACCACTTTGTACAAGAAAGCTGGGTCCTGTTTCT-GTTTCTCAGTCCTCTCT	
oliLV50s	GGGGCGGCCGCAACCATGGCTCAACAGGGAGTTCTGCTGGA	
oliLV50as	GGGCGCCGGCGCTGTTTCTGTTTCTCAGTCCTCTCT	
oliLRTG37s	ACTGCGGCCGCGAAATGGCTCAACAGGGAGATCTG	
oliLRTG37as	CGAGCGGCCGCCCCAGTTTAACCAGCTCAGCAGTACCA	
ELFalpha_fw	GCTGATCGTTGGAGTCAACA	qRT-PCR
ELFalpha_rv	ACAGACTTGACCTCAGTGGT	
On_b144r	AGGACATGAGGGCTTTCTGCTT	
On_b144f	AAGCAGAAAGCCCTCATGTCCT	
Mid1omFTR_454_Fw	ACGAGTGCGTGTCTA(T/C)(A/T)GCTGTCCTCAGTGCAG	454 sequencing
Mid1omFTR_454_Rv	ACGAGTGCGTGCTGACACTGTATCATGGCCTTTATG	
Mid2omFTR_454_Fw	ACGCTCGACAGTCTA(T/C)(A/T)GCTGTCCTCAGTGCAG	
Mid2omFTR_454_Rv	ACGCTCGACAGCTGACACTGTATCATGGCCTTTATG	
Mid3omFTR_454_Fw	AGACGCACTCGTCTA(T/C)(A/T)GCTGTCCTCAGTGCAG	
Mid3omFTR_454_Rv	AGACGCACTCGCTGACACTGTATCATGGCCTTTATG	
Mid4omFTR_454_Fw	AGCACTGTAGGTCTA(T/C)(A/T)GCTGTCCTCAGTGCAG	
Mid4omFTR_454_Rv	AGCACTGTAGGCTGACACTGTATCATGGCCTTTATG	
Mid5omFTR_454_Fw	ATCAGACACGGTCTA(T/C)(A/T)GCTGTCCTCAGTGCAG	
Mid5omFTR_454_Rv	ATCAGACACGGCTGACACTGTATCATGGCCTTTATG	
Mid6omFTR_454_Fw	ATATCGCGAGGTCTA(T/C)(A/T)GCTGTCCTCAGTGCAG	
Mid6omFTR_454_Rv	ATATCGCGAGGCTGACACTGTATCATGGCCTTTATG	

Toulouse, France. A dedicated script was developed for the data-analysis. Briefly, among all sequences (all from a single region of the same run), TRIM protein encoding sequences were identified by the barcode (Multiplex Identifier, MID) of primer-specific sequences. The script searched MID-tag sequences in each read, identified their location, and checked that they were in the expected configuration. The amplified sequence was then extracted, MID and primer sequences removed, and a MID-tag added to its ID. Non-consistent sequences were filtered. 46603 TRIM sequences were extracted, of which 36334 with a workable size. Sequences were corrected for repetitive nucleic acid sequences introduced during the sequencing reaction, whereby the script replaced stretches of three or more contiguous identical nucleotides "N" (N= A, C, T or G) by the

motif NNR in the sequence. This step avoids counting sequencing artifacts as additional new fintrim sequences. Corrected sequences were further processed by an iterative similarity-based clustering approach using the algorithm UCLUST (http://drive5.com/uclust_userguide_3_0.pdf; page 6). The clustering was repeated in a bootstrap process 50 times, and sequences were considered to belong to a given cluster when grouped together in more than 80% of cases. Sequences were grouped among 128 clusters whereby sequences belonging to one cluster shared over 95% sequence similarity. A table describing clusters content (sorted by tag, MID, sequence and cluster size) was created and the distributions of cluster sizes were compared using a non-parametric two-sample Kolmogorov-Smirnov test for each pair of samples. The heatmap R package (cran.r-project.org/web/packages/pheatmap) was employed for hierarchical clustering of the frequencies of fintrim sets, and to produce the heatmap (distance: correlation/linkage: ward). Relative frequencies were converted into sequence counts by multiplying with the average depth of sequencing. The DESeq R package [27] estimates variance mean dependence in count data and tests for differential expression based on a model using the negative binomial distribution.

Cloning of trout fintrim ftr_RTG37 and ftr_LV50

Trout ftr_RTG37 (Acc. nr. AM887820) and ftr_LV50 (Acc. nr. AM887860) transcripts were among the 3'RACE-PCR clones that we described earlier [22]. Both ftr_RTG37 and ftr_LV50 were cloned from the vector pCR2.1-TOPO (TOPO TA, Invitrogen) into the mammalian expression vectors pcDNA-DEST47_GFP and pcDNA-DEST6.2_V5 (invitrogen), which encode for proteins with either GFP or the viral epitope V5 as tag at the C-terminus. The manufacturer's protocol (Gateway system, Invitrogen) was followed, whereby a PCR-reaction was performed with a common forward primer (oliB32_B21_Attb1) and reverse primer specific for the 3' region of ftr_RTG37 or ftr_LV50 and recombination site Attb1 sequence (see Table 1). PCR-products were cloned into the entry vector pDONRTM221 (Invitrogen) by a first recombination reaction (BP), and subsequently into the destination vector by a second recombination reaction (LR). Mcherry-tagged constructs were generated by cloning ftr_RTG37 and ftr_LV50 directly into the expression vector pcDNA3_MCherry. This vector is a derivative from the vector pcDNA3 (Invitrogen) and encodes for the C-terminal tag Mcherry. PCR products were generated using pCR2.1-ftr_RTG37 or pCR2.1-LV50 as template, with primers containing Hind III and EcoR1 restriction sites and cloned into the multiple cloning site of pcDNA3_MCherry by a ligation reaction with T4 DNA ligase. Maxipreps were prepared using the endotoxin-free maxiprep kit (Qiagen).

Immunohistochemistry

EPC cells were transfected with the vectors pDEST47, pDEST6.2 and pcDNA3_MCherry expressing ftr_RTG37 and ftr_LV50 using 3 µg plasmid and the Nucleofector (Lonza) and the nucleofector kit cell line T (Lonza). Cells were cultured in µ-slides with 8 wells (Ibidi, France) and expression was examined two or three days after transfection. Mcherry- and GFP-constructs were examined in vivo, upon counterstaining of nuclei with Hoechst. To examine expression of V5-constructs, cells were fixed with 4%

paraformaldehyde in phosphate buffered saline (PBS), permeabilized with 0.1% Triton X-100 in PBS, blocked with 1% bovine serum albumin in PBS. V5-tagged proteins were detected with a mouse monoclonal antibody against V5 (1:200, Invitrogen), followed by a goat-anti-mouse antibody conjugated with Alexa-Fluor-594 (1:1000, Invitrogen) as secondary antibody. Nuclei were counterstained with DAPI. Expression was examined using a Zeiss ApoTome microscope. Images were analyzed with ImageJ software.

Protein preparation and in vitro ubiquitination assay
HEK293T cells ($1*10^5$ cells per transfection) were transfected with 2 µg pDEST6.2_V5 plasmid expressing ftr_RTG37, ftr_LV50, or 2 µg pCMV-FLAG-TRAF6 with 3 µl of Fugene-HD (Invitrogen) and after 3 days, cells were lysed in 250 µl lysis buffer (100 mM Tris-HCl, 150 mM NaCl, 1% NP-40, protease inhibitors (Roche)). V5-tagged proteins were purified from 750 µl cell lysate by incubation with 30 µl of goat-anti-V5-antibody immobilized on agarose beads (Bethyl Biolabs) and overnight incubation under rotation at 4°C. FLAG-tagged TRAF6 proteins were purified from 1 ml cell lysate by overnight incubation with 120 µl anti-FLAG M2 agarose affinity gel (Sigma-Aldrich). Beads were washed with wash buffer (50 mM Tris-HCl, 150 mM NaCl, protease inhibitors) and finally resuspended in wash buffer. The purification was verified by Western Blot. Therefore, samples were mixed with sample buffer (4% SDS, 2% DTT, 0.1% blue bromophenol, 30% glycerol). After migration on 12% Tris-Buffered PAGE gel (NuPAGE, Invitrogen) proteins were transferred to nitrocellulose membranes (BioRad). Membranes were hybridized either with a monoclonal mouse-anti-V5 antibody (1:5000 in 5% milk in TBS-T, Invitrogen) or a monoclonal mouse anti-FLAG M2 antibody (1:1000, in 5% milk in TBS-T, Sigma-Aldrich) as primary antibodies, and a polyclonal goat-anti-mouse antibody conjugated to HRP (1:5000, PARIS) as secondary antibody and visualized by chemiluminescence (ECL, Amersham).

For the in vitro ubiquitination assay, 10 µl beads suspension was incubated with 0,5 µg biotinylated ubiquitin, 100 ng his-tagged Ub E1, 200 ng his-tagged E2 (BioMol) in 50 mM Tris-HCl pH=7.4, 1 mM DTT, 2.5 mM ATP, 2.5 mM MgCl2 for 90 min at 37°C. After centrifugation (1 min; 8000 rpm; 4 °C), supernatant was removed and beads were washed twice in wash buffer. Beads and supernatants were analyzed by western blot following the same procedure as described above. Hybridization was carried out either with an anti-hUb antibody-conjugated to HRP (1:2500, P4D1, Santa-Cruz) in 5% milk in TBS-T, or with streptavidin conjugated to HRP (1:20,000, Pierce) in 5% BSA in TBS-T.

Results

FinTRIM genes are constitutively expressed during development and in adult fish
Beside functions in the antiviral immune response, TRIM proteins also play roles in cell differentiation, growth and development. Expression of trout finTRIMs was investigated at four different stages of development: the ‘eyed-egg’-stage whereby the black spots of the eyes are visible through the egg envelope, hatching, first feeding and 3 weeks post first feeding. Expression was measured with primers located in the RING/B-

box-encoding region (Fig. 1A), which are conserved in most of known trout finTRIMs. Analysis by quantitative RT-PCR showed that finTRIM gene expression is low during early stages of development, but had increased significantly at 3 weeks post first feeding ($p < 0.05$) (Fig. 1B). In adult fish, constitutive finTRIM expression was determined in various organs (spleen, head kidney, spleen, kidney, gills, skin, liver, ventricle and brain) by quantitative RT- PCR. Overall, finTRIM genes were constitutively expressed in all organs at comparable levels (Fig. 1C).

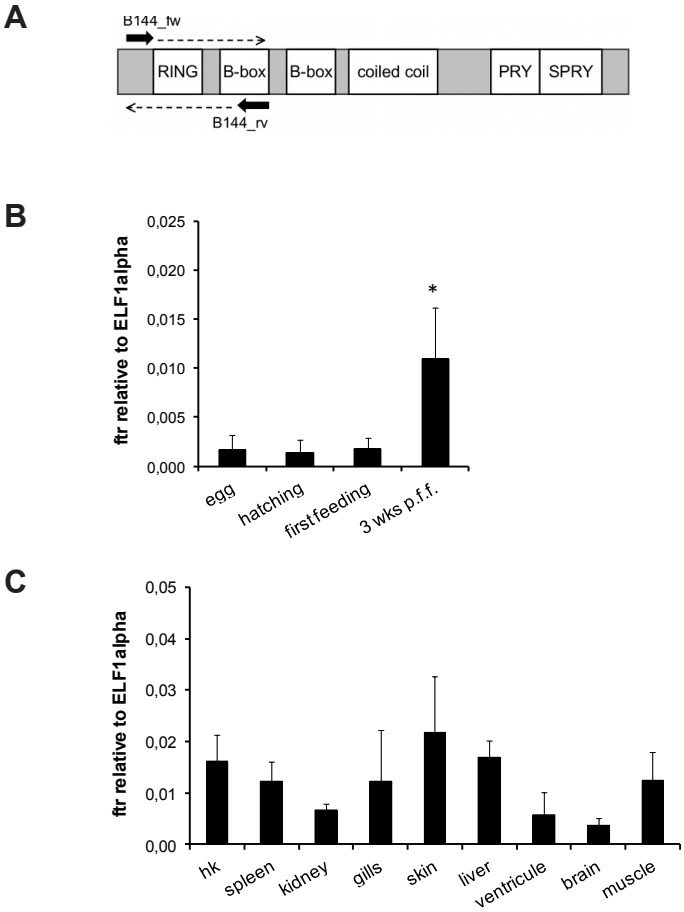


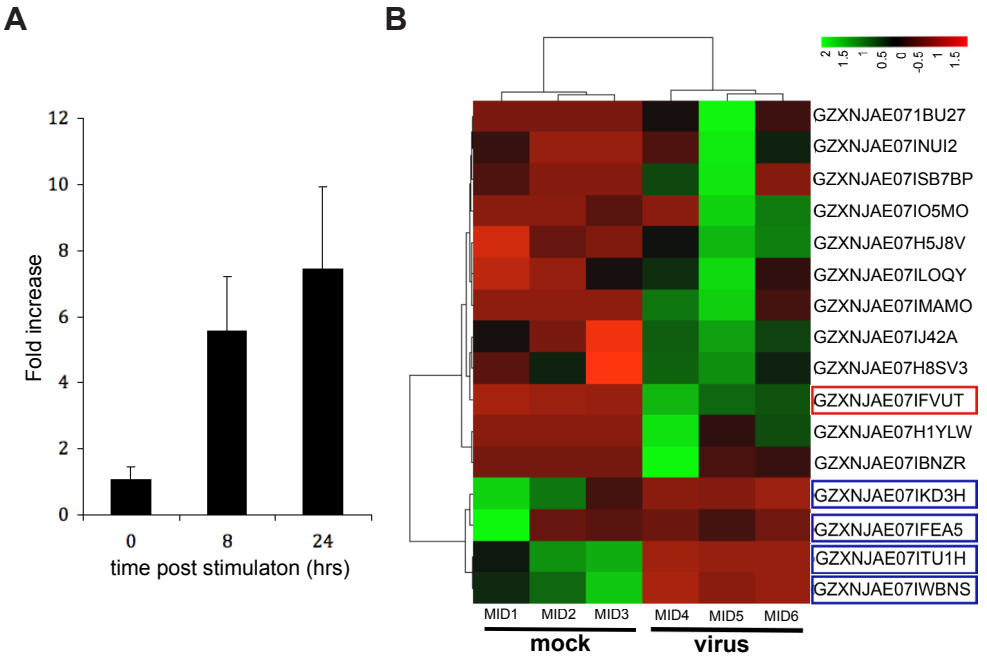
Fig 1. Constitutive finTRIM gene expression in developing fish and adult trout. A Universal primers (B144 fw/rv) were used that amplify the finTRIM (ftr) region encoding for the RING zinc finger domain and first B-box. **B** Expression at four different stages in developing fish: egg with eye developed, hatching, first feeding, three weeks post first feeding (p.f.f.). Averages (n=4-5 animals) and SD are given, * $p<0.05$, as determined by a student *t*-test. C Expression in various organs of adult fish. FinTRIM gene expression is indicated as the ratio finTRIM relative to the housekeeping gene ELF1alpha. Averages (n=4-5 animals) and SD are given.

Fig 2. FinTRIM gene expression upon viral stimulation. (right page, above) **A** FinTRIM gene expression upon stimulation of the B57 fibroblastic cell line with inactivated virus (VHSV) for 8 hrs or 24 hrs. Expression is indicated as the ratio finTRIM relative to the housekeeping gene ELF1alpha and relative to non-stimulated cells (0 hrs). Averages (n=3 wells) are shown and SD is given. **B** Heatmap of finTRIM clusters overrepresented (clusters 1-12) or underrepresented (clusters 13-16) upon viral stimulation. MID1-3 represent control cells and MID4-6 represent virus-stimulated cells. Clusters with significant difference of frequency between mock and virus-stimulated cells are boxed in orange (overrepresented) and blue (underrepresented).

Trout finTRIM gene expression is induced upon viral stimulation, but upregulation is not designated to a selection of genes

The diversity of trout finTRIM transcripts was previously determined by 3`RACE-PCR from cDNA of the trout cell line RTG stimulated with polyI:C, and from cDNA of leukocytes stimulated with the trout rhabdovirus VHSV [22]. Leukocytes were isolated from fish of a trout homozygous clonal trout line (B57); these fish individuals are homozygous at all loci, which ruled out diversity by allelic variation, indicating that the identified transcripts were derived from different genes. A cell line with fibroblast-like morphology established from this homozygous clone was used to determine if the virus induced up-regulation of finTRIM genes is a general feature, or if it is restricted to a few inducible genes. Cells were incubated with virus inactivated by β-propiolactone treatment, and finTRIM gene expression was determined by quantitative RT-PCR. Compared to mock, a 5.6-fold induction of finTRIM gene expression was observed at 8 hrs post stimulation, and a 7.5-fold induction at 24 hrs post stimulation (Fig. 2a).

The primers that were used in the above quantitative RT-PCR (B144fw/rv) are located in conserved sequences and amplify the majority of finTRIM transcripts. As the high sequence similarity among finTRIM genes renders difficult the design of primers for individual transcripts, a high-throughput sequencing approach was employed to quantify expression of individual transcripts. New primers were designed (ftr454fw/rv) that amplify a larger region (~400 bp) of a large diversity of finTRIM transcripts. While these primers may amplify a set of finTRIM sequences slightly different than the first primer set, it was verified by quantitative RT-PCR that the new primers (ftr454fw/rv) led to a consistent upregulation (data not shown). FinTRIM transcripts were amplified by RT-PCR from cDNA from the above experiment, either from mock (n=3) or after 24 hr stimulation with inactivated virus (n=3). Primers with specific tag sequences (MID1-6) were used to enable later identification of sequences derived from each mock, or virus-stimulated sample, allowing to operate the sequencing reaction in a single 454 region. Similar quantities of PCR product were used for each sample in the sequencing reaction. Sequences of the whole dataset were clustered by similarity (see Material and methods), which led to 128 finTRIM sets corresponding to individual, or highly similar genes. Counts of reads per set were distributed over the six samples, i.e. three mocks and three virus-infected samples, using the MID tags. The frequencies of the finTRIM sets were computed for each sample (Table S1). The most frequent cluster represented more than 10% of sequences in each sample (11 to 23%), corresponding to the level of expression of a given genes (or a subset of genes highly similar to each other). All



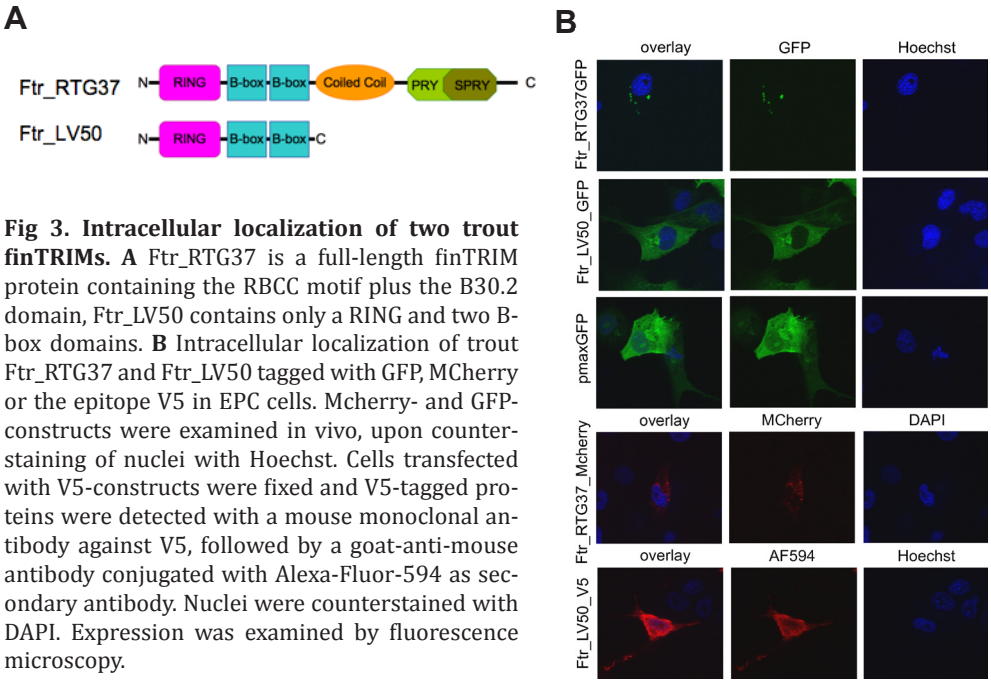


Fig 3. Intracellular localization of two trout finTRIMs. A Ftr_RTG37 is a full-length finTRIM protein containing the RBCC motif plus the B30.2 domain, Ftr_LV50 contains only a RING and two B-box domains. **B** Intracellular localization of trout Ftr_RTG37 and Ftr_LV50 tagged with GFP, MCherry or the epitope V5 in EPC cells. Mcherry- and GFP-constructs were examined in vivo, upon counterstaining of nuclei with Hoechst. Cells transfected with V5-constructs were fixed and V5-tagged proteins were detected with a mouse monoclonal antibody against V5, followed by a goat-anti-mouse antibody conjugated with Alexa-Fluor-594 as secondary antibody. Nuclei were counterstained with DAPI. Expression was examined by fluorescence microscopy.

combinations of frequency distributions were compared using a non-parametric two-sample Kolmogorov-Smirnov test (Table S2). This test did not reveal significant difference between the distributions of finTRIM sets in control versus infected samples, indicating that no drastic modification of the relative frequency distribution of finTRIM mRNAs was induced upon the virus treatment. However, while distributions in the control condition appeared heterogeneous, those in the virus treated condition were not significantly different which may suggest a partial convergence (Table S2).

To get a more detailed insight into the distributions, the frequency table was subjected to a hierarchical clustering analysis. Sixteen finTRIM clusters were identified from the clustering analysis, which were under-, or over- represented, after viral stimulation (Fig. 2B). A specific statistical test was performed using the DESeq R package to validate the differential representations of these clusters in mock and virus infected samples (Table S2). The relative frequency of only one finTRIM cluster (GZXNJAE07IFVUT) was found significantly increased upon viral stimulation. Four clusters were decreased upon viral stimulation: GZXNJAE07IKD3H_2, GZXNJAE07IFEA5_1, GZXNJAE07ITU1H_5, GZXNJAE07IWBNS_3. The finTRIM cluster with increased frequency represents a very small proportion of the amplified finTRIM sequences (Table S2). Therefore, the global up-regulation measured by quantitative RT-PCR cannot be explained by a strong induction of these sequences only. Rather, the finTRIM induction must be attributed to a global up-regulation of finTRIM genes.

Trout finTRIM proteins are cytosolic proteins but located in different cell compartments

To investigate intracellular localization, two trout finTRIM transcripts, ftr_RTG37 and ftr_LV50, were cloned into mammalian expression vectors. Ftr_RTG37 is a full-length finTRIM protein containing the RBCC motif plus the B30.2 domain, while ftr_LV50 contains only a RING and two B-box domains (Fig. 3A). These two proteins share 95 % similarity in amino acid in the N-terminal RING/B-box region. Intracellular localization was investigated by comparison of three different constructs whereby the finTRIM proteins were expressed in EPC cells with either GFP, Mcherry or V5 as C–terminal tag. The analyses with different tags led to consistent results. Both ftr_RTG37 and ftr_LV50 were expressed in the cell cytoplasm (Fig 3B), however, differentially, as ftr_RTG37 was expressed in granula-like structures and ftr_LV50 was expressed in a diffuse-manner.

FinTRIMs display E3 Ubiquitin ligase activity

We next investigated whether finTRIMs auto-ubiquitinate and display E3 ubiquitin ligase activity by performing cell-free in vitro ubiquitination assays. V5-tagged ftr_RTG37 and ftr_LV50 were expressed in HEK293T cells, purified by immunoprecipitation using beads conjugated with an anti-V5 antibody (Fig 4A) and beads with the bound TRIM proteins were subsequently used in an in vitro ubiquitination assay. After the reaction, beads were washed to remove the majority of non-attached ubiquitin, E1 and E2 enzymes and auto-ubiquitination of ftr_RTG37 and ftr_LV50 was analyzed by Western blot. Biotinylated ubiquitin was used in the assay and polyubiquitin chain formation was revealed with either streptavidin conjugated with HRP, or an anti-ubiquitin antibody conjugated with HRP. The results showed that ftr_RTG37 and ftr_LV50 were auto-ubiquitinated by poly-

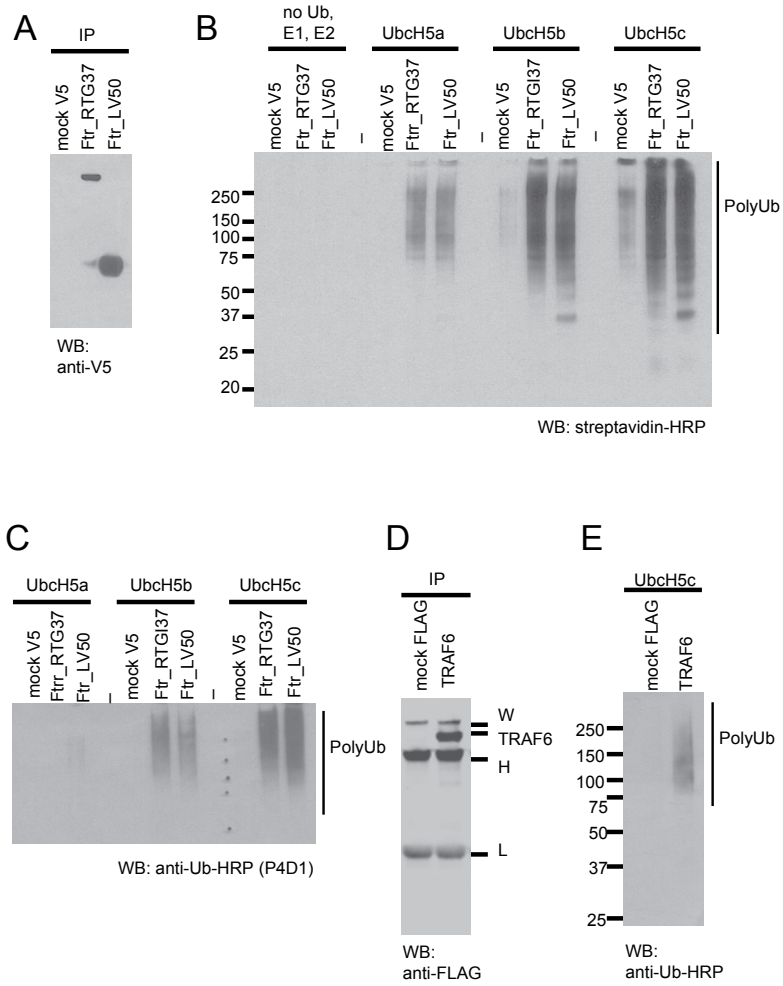


Fig 4. In vitro ubiquitination assay. A Immunoprecipitation of ftr_RTG37_V5, ftr_LV50_V5 and mock with anti-V5 antibody-conjugated agarose beads. V5-tagged proteins were revealed with an anti-V5 antibody. The molecular weights of ftr_RTG37 and ftr_LV50 are 66.8 and 26.2 kDa, respectively. **B** and **C** Ftr_RTG37_V5, ftr_LV50_V5 and mock antibody-immobilized on agarose beads were assayed by co-incubation with either UbCH5a, UbcH5b, UbcH5c, or in absence of ubiquitin (Ub) E1 and E2. Poly-ubiquitin chain formation was revealed with either Streptavidin-HRP (**B**) or an anti-ubiquitin antibody conjugated with HRP (**C**). **D** TRAF6-FLAG was included as a positive control. Immunoprecipitation of TRAF6_FLAG or mock with anti-FLAG antibody conjugated agarose beads. TRAF6_FLAG was revealed with an anti-FLAG antibody, followed by a secondary goat-anti-mouse-HRP antibody. The goat-anti-mouse antibody also reveals the heavy (H) and light (L) chains and intact (W) anti-FLAG antibody conjugated to the beads. **E** TRAF6-FLAG antibody-immobilized on agarose beads and mock were assayed by co-incubation with UbcH5c. Poly-ubiquitin chain formation was revealed with an anti-ubiquitin antibody conjugated with HRP.

ubiquitin chains upon reaction with the E2 enzymes UbcH5a, UbcH5b and UbcH5c (Fig 4B and 4C). In absence of TRIM proteins (mock transfected cells), poly-ubiquitin chain synthesis was insignificant. The poly-ubiquitin chain synthesis was specific for the in vitro reaction with biotinylated ubiquitin, as the analysis with streptavidin conjugated with HRP also showed auto-ubiquitination of the ftr_RTG37 and ftr_LV50. Ftr_RTG37 and ftr_LV50 were not already mono-or polyubiquitinated in vivo, as was verified by analysis of immunoprecipitated ftr_RTG37 and ftr_LV50, not used in the assay, with an anti-ubiquitin antibody (data not shown).

Purified FLAG-TRAF6 prepared in a similar way in HEK293T cells was used as a positive control (Fig 4D). TRAF6 conjugates poly-ubiquitin chains in complex with UbcH5c and FLAG-TRAF6 was auto-ubiquitinated by poly-ubiquitin chains upon reaction with UbcH5c. In absence of FLAG-TRAF6 (mock transfected cells) no poly-ubiquitin chain synthesis was observed (Fig 5E). Auto-ubiquitination was not observed upon reaction of ftr_RTGT37 and ftr_LV50 with E2 enzyme UbcH8 (Fig. S1).

Discussion

In this study we further characterized finTRIMs, a subset of TRIM genes that form a multigene family unique for fish [22]. As the expression of finTRIM genes is low during the early stages of development of rainbow trout, finTRIMs are less likely to play a role in development, a function that is attributed to several other TRIM proteins (see for examples [28-30]). The wide distribution of finTRIM constitutive expression in both immune-related and non-immune related organs of adult fish, does not suggest a function restricted to lymphoid tissues, but rather a role in intracellular processes that are common to many types of somatic cells. FinTRIM gene upregulation upon viral infection and IFN stimulation suggests that finTRIMs are involved in the antiviral immune response. In fact, one trout finTRIM transcript was initially revealed by a SSH screening for virus-induced genes, whereby gene expression in mock leukocytes and in fish rhabdovirus VHSV inoculated leukocytes was compared [31]. A first characterization of finTRIM gene expression revealed that they were overall induced in trout fibroblasts by polyI:C and in leukocytes upon stimulation in vitro with VHSV and IFN, but not with LPS [22]. In this initial study, primers were used that amplified most finTRIMs transcripts. Hereby it was not clear whether the observed induction is attributed to few genes that were strongly upregulated, or whether the entire set of finTRIM genes was upregulated in a more modest manner. We here extended the analysis of the viral induction of finTRIMs to the gene level, by using a high-throughput strategy to quantify expression of individual transcripts (or set of quasi-identical transcripts) upon viral stimulation. As we observed that the induction concerns a large diversity of finTRIM genes, rather than a few specific genes, it appears that expression of most individual finTRIM genes is regulated by similar signaling pathways. A few finTRIM sequences found among rainbow trout ESTs did not match with the primers used in this study, which represent divergent members of the family as observed in zebrafish [26] and may have another expression pattern. The trout genome is currently being sequenced and future analysis of the promoter regions for the individual genes will provide more insight. Simultaneous expression may imply that finTRIM proteins work in concert

with each other. Although we currently do not know which are the binding partners of finTRIM proteins, we observed intracellular localization within the cytosol for the two finTRIMs investigated in this study, allowing interaction with other cytosolic proteins. The presence of a coiled-coil domain is a requirement for the formation of granula-like bodies, in accordance with previous reports on other TRIM proteins [3]. The coiled coil domain allows oligomerization and formation of protein complexes. The presence or absence of a coiled coil domain contributes to the diversity of finTRIMs, as it allows finTRIM proteins to act as a single molecule and as multimers, likely expanding the range of interacting partners.

TRIM proteins form a large subfamily of E3 ubiquitin ligases in mammals [14]. Here we showed that at least two finTRIM members display E3 ubiquitin ligase activity in rainbow trout. A first biological function of protein modification by ubiquitination was the targeting to the ubiquitin-dependent proteolytic pathways [32, 33]. It is now well established that the outcome of protein ubiquitination is not only degradation, but also represents an important type of post-translational modification that directs the biological activity of proteins. As ubiquitin chains can be disassembled by deubiquitination enzymes (DUBs), modification by ubiquitin is a reversible type of protein modification and allows control of many conserved cellular processes such as gene expression, cell cycle progression, DNA damage repair and cell signaling [34]. Ubiquitination plays also a significant role in immunity [35, 36]. For example, signaling pathways leading to activation of NF-κB, a major transcription factor for cytokine expression, are highly controlled by ubiquitination [37]. For example, the E3 ubiquitin ligase TRAF6 that was included as a control in our assay, functions with an E2 complex composed of Ubc13 and Uev1a (Mms2) to catalyze Lys63-linked polyubiquitination that activates the kinase complex TAK1/TAB1/TAB2 [38, 39]. In addition, unanchored Lys63-polyubiquitin chains synthesized by TRAF6 in complex with UbcH13/Uev1a, activate TAK1 by binding the ubiquitin receptor TAB2. Also, in complex with UbcH5, TRAF6 synthesizes unanchored Lys63 polyubiquitin chains that activate directly the IKK complex, consisting of IKKα/IKKβ/NEMO [40]. Activation of IKK leads to IκBα phosphorylation, followed by degradation by the ubiquitin-proteasome pathways, upon which NF-κB is liberated and translocated to the nucleus (see for review [35]).

Polyubiquitin chain formation was observed for two trout finTRIMs in conjunction with E2 enzymes UbcH5a, UbcH5b and UbcH5c, which are closely related E2 enzymes [41]. UbcH5 is a promiscuous E2 enzyme that acts in concert with multiple E3 enzymes and promotes synthesis of ubiquitin chains of various linkages [42]. Biotinylated ubiquitin was visualized both with streptavidin-avidin and with an antibody against ubiquitin, P4D1. This antibody recognizes mono-ubiquitin and poly-ubiquitin chains, including both Lys48- and Lys63-linked chains. Lys48-linked chains target proteins for degradation by the 26S proteasome, while Lys63-linked chains usually mediate the recruitment of binding partners and are implicated in cell signaling [16]. In order to better understand possible outcomes of ubiquitination by finTRIMs, further biochemical studies will be needed to specify the type of ubiquitin-linkages that are targeted. Notably, ftr_LV50, that lacks a coiled-coil domain, could synthesize ubiquitin chains, indicating the oligomerization is not requirement for the E3 ubiquitin

ligase activity for finTRIM, as for instance is for TRAF6 [39].

Next to ubiquitin, ubiquitin-like modifiers (UBLs) that include ISG15, SUMO, NEDD8, Atg8, are targeted to a substrate protein by a similar cascade of enzymes as ubiquitin, activated by specific E1 enzymes and partial sharing of E2 and E3 enzymes [43]. For TRIM proteins, E3 ligase activity has been demonstrated both for SUMO and ISG15 [44, 45]. Human TRIM25 is an E3 ligase for ISG15 in conjunction with UbcH8 [46], an E2 enzyme that is also involved in ubiquitination [47]. TRIM25 is not the ortholog of finTRIMs, but it is one of the closest finTRIM relative found in mammals. However, we could not demonstrate poly-ubiquitin chain formation for the two finTRIMs when used in this study in conjunction with UbcH8.

It is not yet clear whether TRIM proteins for which a function in immunity and E3 ligase activity has been demonstrated, restrict viruses by ubiquitination and subsequent proteolytic degradation of viral proteins. One study showed that TRIM22 interaction with encephalomyocarditis virus (ECMV) protease 3C ($3C^{pro}$), and co-expression of TRIM22 and $3C^{pro}$ in HeLa cells leads to enhanced ubiquitination of $3C^{pro}$. This is not yet validated by an *in vitro* ubiquitination assay and does not rule out the involvement of other E3 ligases [48]. However, it is becoming apparent that TRIM proteins restrict viruses indirectly through their ubiquitin E3 ligase activity by targeting proteins in the viral PRR signaling pathways that lead to IFN production. Hereby, TRIM proteins either positively, or negatively regulate the antiviral immune response. A first finding of such a role was demonstrated by the discovery that activation and signaling of RIG-I, a cytosolic receptor for viral RNA, is highly dependent on ubiquitination of RIG-I by TRIM25 [12]. Ubiquitination of NEMO by TRIM23 promotes NF-κB signaling and IFN-β production upon polyI:C treatment and Sendai virus infection [49]. TRIM21 promotes stabilization of IRF3 and IFN-β production. Hereby, TRIM21 interferes with the interaction between IRF3 and peptide-prolyl isomerase (Pin1) that promotes degradation of IRF3 [50]. Although another group found an opposite role for TRIM21, stating that in presence of TRIM21, ubiquitination of IRF3 is enhanced, which promotes degradation of IRF3 and hereby negatively regulates IFN-γ production [51]. Overall, evidence is increasing that TRIM proteins control viral PRR signaling pathways at various levels, partly mediated by their E3 ubiquitin ligase activity (recently reviewed by [13]).

In conclusion, here we demonstrated that the induction of finTRIM gene expression upon viral stimulation is attributed to a global upregulation of finTRIM genes. Further, by setting up an *in vitro* ubiquitination assay, we demonstrated that trout finTRIMs display E3 ubiquitin ligase activity. The in here established *in vitro* ubiquitination assay will be beneficial to further establish the biological role of finTRIMs in cell signaling and antiviral immune responses.

Acknowledgements

We thank Corinne Thory for generating the trout B57-derived cell line, Dr. Rosario Castro and Eloi Verrier for providing trout cDNA samples, Emilie Lauret for help with molecular cloning, and Dr. Edwige Quillet for providing trout clones. We acknowledge the microscopy platform MIMA at INRA, Jouy-en-Josas for assistance with the Apotome. This work was supported by the Institut National Recherche Agronomique and by the

Wageningen University.

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Lorsque tu ne sais pas ou tu vas,
regarde d'ou tu viens.

- proverbe africain -

Chapter 5

CXCL8 chemokines in teleost fish: two lineages with distinct expression profiles during early phases of inflammation

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PLoS One 2010; 5(8):e12384

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Abstract

During the inflammatory process, chemokine CXCL8 plays a pivotal role in recruitment of human neutrophilic granulocytes. A diversity of sequences similar to CXCL8 was reported in fish, but their evolutionary relationships and functional homology with their human homolog remain unclear. We screened fish genomes to seek for sequences related to CXCL8. A first lineage was retrieved in all teleosts, while a second CXCL8 lineage was found in zebrafish and carp only. An early inflammatory function for both lineages was indicated by several lines of evidence. The induction of carp CXCL8s, CXCb, and CXC receptor-1 and -2 was analyzed after *in vitro* stimulation of leukocyte subpopulations and in two *in vivo* inflammation models. Recombinant proteins of carp CXCL8 proteins were produced and showed significant chemotactic activity for carp leukocytes. While both carp CXCL8s appear to be functional homologs of mammalian CXCL8, their different induction requirements and kinetics evoke a gene-specific subfunctionalization.

Introduction

Chemokines are specialized cytokines with chemotactic activity and orchestrate mobilization and migration of specific subsets of cells along a gradient. Although initially discovered to be involved in leukocyte recruitment during early inflammation, they are now known to regulate various steps of the immune response, and to direct cell migration during growth and development. Moreover, chemokines are implicated in cancer, by regulating the development of metastasis (reviewed by [1]). CXCL8, also known as IL-8 for interleukin-8, or NAP-1 for neutrophil-activating peptide, was the first chemokine discovered. It was initially purified from LPS-stimulated human blood monocytes [2,3] and it is now recognized that CXCL8 is produced by a wide range of cell types including non-immune cells, like fibroblasts and endothelial cells [4,5]. Besides its potent chemotactic activity for neutrophils, basophils, resting T cells, and stimulated eosinophils, CXCL8 also activates cells by induction of respiratory burst, exocytosis and degranulation of storage proteins and production of lipid mediators [6,7,8]. Moreover, CXCL8 regulates growth of endothelial cells and myeloid progenitor division [9,10].

CXCL8 is classified into the CXC subfamily, based on the presence of the CXC cysteine-motif at the N-terminus. In human, sixteen CXC ligands are identified, of which the majority is located in mini-clusters on chromosome four [11,12]. The human CXC proteins CXCL1, -2, -3, -5, -6, -7 and -8 contain an ELR (Glu-Leu-Arg) signature upstream of the CXC motif which is important for receptor affinity. In general, human CXC chemokines with the ELR signature recruit polymorphonuclear leukocytes (PMN, e.g. neutrophils, basophils and eosinophils) during inflammation and promote angiogenesis. In contrast, CXC chemokines lacking the ELR signature specifically attract lymphocytes and monocytes, not neutrophils, and they inhibit angiogenesis [13]. The receptors for CXCL8 in humans, CXCR1 and CXCR2, are promiscuous and also bind other chemokines including CXCL1, -4 and -7 [14,15]. Both receptors are highly expressed on human neutrophils [16], but CXCR1 is also well expressed on many other cell types.

Orthologs for human CXCL8 are identified in other mammals as monkeys, cow,

dog, cat, but not in mouse and rat. Outside mammals, CXCL8-related genes have been described in chicken [17,18] and in multiple teleost fish [19-27]. Orthology of fish CXCL8-related sequences with mammalian CXCL8 has currently not been firmly shown. The chemokine family of ligands and receptors has evolved in a distinctive way in fish, compared to tetrapods [12,18,26,28]. For example, the CC chemokine family has expanded and diversified extensively in zebrafish and in total 111 chemokine genes have been identified in the zebrafish genome, against 44 genes in humans [26]. Moreover, a fifth chemokine subgroup with a CX signature exists in zebrafish [26]. While some of the mammalian CXC ligands have unambiguous orthologs in fish, such as CXCL12 and CXCL14 [29], other CXC genes form fish-specific lineages [26,28,30]. Since these proteins are short and evolve quickly, it is often difficult to show more than a tendency in distance or phylogenetic analysis, especially when only a few sequences are available. Subsequently, CXCa was proposed as an alternatively name for fish CXCL8-like genes, together with CXCb that designated chemokines from the fish lineage most similar, but probably not orthologous, to human CXCL9,-10 and -11 [21].

A specific feature of teleost CXCL8-related proteins is the absence of a conserved ELR signature, with the exception of haddock [22]. Nevertheless, first studies with recombinant proteins demonstrate a chemotactic activity for neutrophils and macrophages [25,31,32]. Furthermore, gene expression studies indicate that fish CXCL8s are pro-inflammatory cytokines, suggesting that they may fulfill similar functions in inflammation as mammalian CXCL8 [24,33,34].

Recently, a second CXCL8-related sequence was identified from a carp EST database that was named CaIL-8 [35]. CaIL-8 was slightly more similar to the human CXCL8 than the carp CXCa previously known, but shared only low sequence similarity with the CXCa [21], indicating that CaIL-8 is a second CXCL8-like gene. Together with two other carp sequences and a zebrafish EST, the CaIL8 therefore constituted a second fish CXCL8-lineage (CXCL8_L2). The earlier described teleost CXCL8-like genes, including CXCa of carp, form the first lineage and will now be referred to as CXCL8_L1.

In this study we further mined available databases for CXCL8_L1 and CXCL8_L2-like sequences in teleost fish and studied their phylogenetic relationships and synteny groups. To establish the functions of different carp chemokines during inflammation, we performed an extensive analysis of expression profiles of carp CXCL8_L2, CXCa_L1, CXCb and the receptors CXCR1 and CXCR2 after in vitro stimulation of leukocyte subpopulations. We moreover looked at selective expression of carp chemokines and receptors in two in vivo models of inflammation: a zymosan-induced peritonitis model and a model of hyperosmotic shock by immersion vaccination to *Aeromonas salmonicida*. Finally, we established that both the CXCL8-L1 and -L2 chemokines truly represent functional chemotactic peptides using recombinant proteins.

Methods

Bioinformatics, phylogeny and synteny

Carp CXCL8_L2 (GenBank accession number AB470924) and zebrafish CXCL8_L1_chr1 (GenBank accession number XM_001342570) sequences were used as query in BLAST

searches. CXCL8_L1 genomic sequences were retrieved for zebrafish (*Danio rerio*), medaka (*Oryzias latipes*), tetraodon (*Tetraodon nigroviridis*), stickleback (*Gasterosteus aculeatus*) and fugu (*Takifugu rubripes*) from the EMSEMBL website (http://www.ensembl.org/index.html). CXCL8_L1 genes corresponded with Ensembl gene identifiers ENSTNIG00000017810 for tetraodon, ENSGACG00000001729 for stickleback, ENSORLG00000005096 for medaka and fugu Genbank accession number AB125645.1. BLAST searches against EST databases were performed on the website of NCBI (http://www.ncbi.nlm.nih.gov/). Zebrafish EST sequences corresponding to CXCL8_L2_chr17 were EH557944, EH536693, EH441857 and EH977746. Multiple sequence alignments were made with ClustalW within the MEGA4 software. Phylogeny trees were constructed with MEGA4 using Neighbour Joining (NJ; [36]) and Maximum Likelihood (ML; [37]). The online tool *Genomicus* (http://www.dyogen.ens.fr/genomicus-56.02/cgi-bin/search.pl) was employed to study conserved synteny for fish CXCL8 genes. *Genomicus* allows analysis of genes with approved gene symbols published on the ENSEMBL website. Since zebrafish CXCL8 genes are not yet annotated by ENSEMBL, annotated genes in close proximity to the genes of interest were used as reference genes in the analysis. Camk2d2 was taken as a reference gene for zebrafish CXCL8_L1_chr1, asb7 was used as a reference for zebrafish CXCL8_L2_chr7 and *C14orf104/kintoun* was used as a reference for zebrafish CXCL8_Chr17.

Animals

Young individuals (6-9 months) of common carp Cyprinus carpio L (50-60 g b.w), from the Department of Immunology, Polish Academy of Science, Golysz, Poland (R7xW) and "De Haar Vissen" facility in Wageningen (R3xR8) were reared at 23°C in recirculating tap water [38]. Fish were fed pelleted dry food (Trouvit, Nutreco). All animals were handled in strict accordance with good animal practice as defined by the relevant national and/or local animal welfare bodies, and all animal work was approved by the appropriate committee (license numbers: 3019b/2003 and 013b/2010, Wageningen University ethical committee; 16/OP/2001, Jagiellonian University ethical committee).

Tissue and section preparation

Fish were anaesthetised with 0.2 g/l tricaine methane sulphonate (TMS, Cresent Research Chemicals, Phoenix, AZ, USA) buffered with 0.4 g/l NaHCO3 (Merck, Darmstadt, Germany). Organs and tissues for RNA extraction were carefully removed, snap frozen in solid CO2 or liquid N2 and stored at -80°C.

In vivo study: hyperosmotic shock experiment

Fish were immersed in 4.5% (w/v) NaCl (1450 mOsm/kg aerated overnight before use) for 2 min and immediately net transferred to vaccine solution (LPS–DTAF (0.2% (w/v)), *A. Salmonicida* bacterin-FITC (2.4×107 bacteria/ml) or BSA-FITC (2% (w/v)) for 10 min (HI, hyperosmotic immersion, fish). The high salinity of the hyperosmotic solution caused the fish to passively float to the surface. DI (direct immersion) vaccinated fish were immersed in vaccine solution only for 10 min. Control fish were not exposed to NaCl and vaccin immersion. After vaccination fish were returned to their tanks. At

selected time points, animals were sacrificed and their gills were isolated by carefully excising whole gill arches [39].

In vivo study: zymosan-induced peritonitis
The animals were i.p. injected with freshly prepared zymosan A (2 mg/ml, 1 ml /50 g b.w., Sigma, Z) in sterile PBS (270 mOsM) or with sterile PBS only (control group). At selected time points animals were sacrificed and their peritoneal cavities were lavaged with 1 ml of ice cold PBS. Peritoneal leukocytes were centrifuged for 10 min at 800 g at 4°C and frozen in liquid nitrogen and stored at -80 °C [33].

Cell isolation and in vitro culture
Animals were anaesthetized with 0.2 g/l TMS. Fish were bled through puncture of the caudal vein using a heparinised syringe. Blood was centrifuged 5 min at 100 g and afterwards 10 min at 800 g and 4°C. The buffy coat and a small amount of serum were mixed and loaded on 3 ml Ficoll (density 1.077g/ml, Amersham Bioscience, Uppsala, Sweden). Following subsequent centrifugation at 800 g at 4°C for 25 min with the break disengaged, leukocytes at the interface were collected and washed twice with carp RPMI medium (cRPMI) (RPMI 1640, Invitrogen, Carlsbad, CA); adjusted to carp osmolarity (270 mOsm/kg) and containing 10 IU/ml heparin (Leo Pharmaceutical Products Ltd., Weesp, The Netherlands) and once with $cRPMI^{++}$ (cRPMI supplemented with 0.5% (v/v) pooled carp serum, 1% L-glutamine (Merck, Whitehouse Station, NJ), 200 nM 2-mercaptoethanol (Bio-Rad, Hercules, CA), 1% (v/v) penicillin G (Sigma–Aldrich, St. Louis, MO), and 1% (v/v) streptomycin sulphate (Sigma–Aldrich, St. Louis, MO).

Isolation of head kidney leukocytes
Head kidney cell suspensions were obtained by passing the tissue through a 50 μm nylon mesh with cRPMI and washed once. This cell suspension was layered on a discontinuous Percoll (Amersham Biosciences, Piscataway, NJ) gradient (1.02, 1.060, 1.070, and 1.083 g/cm^3) and centrifuged for 30 min at 800 g with the brake disengaged.

Isolation of head kidney monocytes/lymphocytes and phagocyte populations
Cells from the density range of 1.020 – 1.060 g/cm^3 (predominantly, >80% monocytes/lymphocytes), the range of 1.060 - 1.070 g/cm^3 (predominantly macrophages, but also monocytes, lymphocytes and some (~10%) granulocytes), the range from 1.070 to 1.083 g/cm^3 (~80% neutrophilic granulocytes) [40] or combined 1.060 – 1.083 g/cm^3 fractions (enriched phagocytes) were collected, washed, and seeded at 5×10^6 cells per well (in a volume of 900 μl) in a 24-well cell culture plate at 27°C, 5% CO2 in $cRPMI^{++}$.

In vitro stimulation of cells
Cells were incubated 1-12 h with lipopolysaccharide (LPS, 10-50 μg/ml, E. coli serotype O55: B5, Sigma–Aldrich, St. Louis, MO), or with poly-inosinic poly cytidiylic (Poly I:C, 50 μg/ml, Sigma–Aldrich, St. Louis, MO), phytohemagglutinin (PHA, 10 μg/ml, Sigma–Aldrich, St. Louis, MO). Control cells (c) received medium only.

RNA isolation and first strand cDNA synthesis
RNA was isolated using an RNeasy Mini Kit (Qiagen, Valencia, CA) according the manufacturer's protocol. Final elution was carried out in 30 µl of nuclease-free water, to maximize the concentration of RNA. RNA concentrations were measured by spectrophotometry and integrity was ensured by analysis on a 1% agarose gel before proceeding with cDNA synthesis. For each sample a non–RT (non-reverse transcriptase) control was included. Two µl of 10x DNase I reaction buffer and 2 µl DNase I (Invitrogen) was added to 2 µg total RNA and incubated for 15 min at room temperature. DNase I was inactivated with 25mM EDTA (2 µl, 65°C, 10 min). To each sample, 2 µl random primers and 2 µl 10mM dNTP mix were added, and the mix was incubated for 5 min at 65°C and then 1 min on ice. After incubation, to each sample 8 µl 5x First Strand buffer 2 µl 0.1 M dithiothreitol (DTT) and 2 µl RNase inhibitor were added. To 19 µl from each sample (but not to the non-RT controls) 1 µl Superscript RNase H-Reverse Transcriptase (RT, Invitrogen) was added and reagents were incubated for 5 min at 25°C, then spun briefly and incubated 60 min at 50°C. Reactions were inactivated 15 min at 70°C. Samples were set at 100 µl with demineralized water and stored at -20°C until future used.

Real-time quantitative PCR
PRIMER EXPRESS software (Applied Biosystems) was used to design primers for use in real-time quantitative PCR. Carp-specific primers (5' to 3') for chemokines: CXCL8_L2, CXCa_L1, CXCb and for chemokine receptors: CXCR1 and CXCR2 were used. The 40S ribosomal protein s11 gene served as an internal standard (accession numbers and primer sequences are listed in Table 1). For RQ-PCR, 5 µl cDNA and forward and reverse primers (4.2 µM each) were added to 7 µl Absolute QPCR SYBR Green Mixes (ABgene). RQ-PCR (15 min at 95°C, 40 cycles of 15 s at 94°C, 30 s at 60°C, and 30 s at 72 °C, followed by 1 min at 60°C) was carried out with a Rotorgene 2000 realtime cycler (Corbett Research, Sydney, Australia). Following each run, melt curves were collected by detecting fluorescence from 60°C to 90°C at 1°C intervals.

Constitutive expression of chemokines was determined in various organs and tissues of four individual adult carp, and rendered as a ratio of target gene vs. reference gene (40S ribosomal protein s11 gene) calculated with the Pfaffl method [41], according to the following equation:

$$\text{Ratio} = (E_{\text{reference}})^{\text{Ct reference}} / (E_{\text{target}})^{\text{Ct target}}$$

where E is the amplification efficiency and Ct is the number of PCR cycles needed for the signal to exceed a predetermined threshold value.

Expression following stimulation was rendered as a ratio of target gene vs. reference gene (40S ribosomal protein s11 gene) relative to expression in unstimulated control samples according to the following equation:

$$\text{Ratio} = (E_{\text{target}})^{\Delta Ct}\text{target}\,(^{\text{control-sample}}) / (E_{\text{reference}})^{\Delta Ct}\text{reference}^{(\text{control-sample})}$$

Table 1. Primers

Gene	Sense (5'-3')	Antisense (5'-3')	Acc. no
40S	CCGTGGGTGACATCGTTACA	TCAGGACATTGAACCTCACTGTCT	AB012087
CXCL8_L2	TCACTTCACTGGTGTTGCTC	GGAATTGCTGGCTCTGAATG	AB470924
CXCa_L1	CTGGGATTCCTGACCATTGGT	GTTGGCTCTCTGTTTCAATGCA	AJ421443
CXCb	GGGCAGGTGTTTTTGTGTTGA	AAGAGCGACTTGCGGGTATG	AB082985
CXCR1	GCAAATTGGTTAGCCTGGTGA	AGGCGACTCCACTGCACAA	AB010468
CXCR2	TATGTGCAAACTGATTTCAGGCTTAC	GCACACACTATACCAACCAGATGG	AB010713

Cloning carp CXCa_L1 and carp CXCL8_L2

Synthetic genes encoding the mature carp CXCa_L1 (Genbank accession no. AJ550164) and carp CXCL8_L2 (Genbank accession no. AB470924), codon optimized for *E. coli* expression, were ordered at Mr. Gene (Regensburg, Germany). Restriction sites for *BamHI* and *HindIII* were included at respectively the 5`and 3`end of the coding sequences to enable cloning in the corresponding restriction sites of expression vector pET15new. This vector is a derivative from the vector pET15 (Novagen), that encodes for N terminal tag containing 6 histidine residues under the control of a Lac operon and T7 promotor. The protein sequence for recombinant carp CXCa_L1 was:

MSYYHHHHHHLESGSMSLRGLGVDPRCRCIETESQRIGKLIESVELFPPSPHCKDTEIIATLKVSRKEICLDPTAPWVK KVIEKIIANKTPAA

The protein sequence for recombinant carp CXCL8_L2 was:

MSYYHHHHHHLESGSRPKSQQLSCRCPRMHSEPAIPANKVLSLRVIPAGPICKNENIIATMKKGQVCLDPTKDWVISL NEEIKKRNLKSQP

whereby 6x-His-tag and LESGS epitope are indicated in bold.

Expression and purification of recombinant carp CXCa_L1 and carp CXCL8_L2

Vectors pET15new_carpCXCa_L1 and pET15new_carpCXCL8_L2 were used to transform *E. coli* BL21_CodonPlus(DE3)_RIL (Agilent Technologies). A single colony was picked from an overnight plate, grown in LB containing chloramphenicol, ampicillin and 1% glucose. The culture was spread on a LB agar plate and grown overnight at 37°C. Bacteria were harvested with an inoculation loop and resuspended in 10 ml LB and subsequently in 500 ml LB containing ampicillin and chloramphenicol. The culture was grown at 37°C until an optimal density at 600 nm (OD_{600}) of 0.6-0.8 was reached. Gene expression was induced by addition of 1 mM IPTG and bacteria were incubated for 3 h while shaking. Bacteria were spun down by centrifugation at 16912 g, 4°C for 15 min (Sorvall Instruments, DuPont, RC5C, GS-3 rotor). The bacterial pellet was resuspended in 40 ml buffer B (20 mM Tris, 500 mM NaCl) containing 0.1 mg/ml lysozyme (Merck). The bacteria suspension was transferred to a 50 ml tube and incubated under rotation for 30 min at RT. Five ml of buffer C (100 mM DTT, 50 mM EDTA, 10% Triton X-100) was added, mixed and stored overnight at -20°C, thawn, refrozen for 30 min, thawn/freeze cycles were repeated three times. After the last thawing step, 3 ml 0.5 M $MgCl_2$ and 75 units benzonase nuclease (Novagen) was added and incubated for 15 min. Total protein lysate was centrifugated at 10000 g for 15 min at 4°C. The pellet, containing

CXCa_L1 or CXCL8_L2 inclusion bodies, was washed in buffer I (50 mM Tris-HCL, 500 mM NaCl) with 1% triton-X100, centrifuged at 10,000 x g for 10 min at 4°C. Pellet was dissolved in 3 ml buffer II (50 mM Tris-HCL, 500 mM NaCl, 10 M Urea, 15 mM imidazole) and centrifuged at 10,000 g for 10 min to remove insoluble material. The 6xHis-tagged protein was purified by immobilized metal affinity chromatography (IMAC) on chelating sepharose fast flow (Amersham-Biosciences) charged with Ni^{2+} according to the manufacterer`s protocol. The column was equilibrated with buffer IV (20 mM Tris-HCl, 500 mM HCl, 8 M urea, 25 mM imidazole) and dissolved inclusion bodies were applied to the column. The bound protein was washed with buffer IV (20 mM Tris-HCl, 500 mM HCl, 8 M urea, 25 mM imidazole) containing 1% Triton-X100, followed by buffer IV (20 mM Tris-HCl, 500 mM HCl, 8 M urea, 25 mM imidazole) containing 1% Triton-X114. Triton X-114 was removed by a wash step with buffer VI (20 mM Tris-HCl, 500 mM HCl, 6 M urea) containing 40% (W/V) isopropanol, followed by buffer IV (20 mM Tris-HCl, 500 mM HCl, 8 M urea, 25 mM imidazole) to restore the concentration of urea. Protein was eluted by adding elution buffer (50 mM Tris-HCl, 500 mM HCl, 8 M urea, 250 mM imidazole) and refolded by diluting the eluted fractions 10x in refolding buffer (50 mM Tris-HCl, 0.5 M L-arginine, 0.1 mM oxidized glutathione and 0.5 mM reduced glutathione) and incubation overnight at 4°C. The protein was dialyzed against 1x PBS and centrifuged at 4000 g for 30 min at 4°C to remove any precipitation, filtered with a sterile 0.2 µM filter and stored at -80°C. Protein concentrations were determined by the Micro BCATM Protein assay kit (Pierce). Protein samples were verified on a Bis-Tris-HCl buffered polyacrylamide gel (NuPAGE, Invitrogen) and protein bands were analyzed by matrix-assisted laser desorption ionization- time of flight (MALDI-TOF) as described earlier [42].

In vitro chemotaxis assay

Chemotactic activity for recombinant carp CXCa_L1 and CXCL8_L2 was analyzed in vitro in a 48-well microchemotaxis chamber (Neuro Probe Inc., Maryland, USA) as described earlier. Briefly, lower compartments were filled with either negative controls (serum-free RPMI for random cell migration or recombinant carp IFN-γ-2 (20 ng/ml) (personnal observation), or positive control (zymosan-activated carp serum (ZAS); [43]) for the chemotaxis assay. Recombinant carp CXCa_L1 and CXCL8_L2 were tested at 2, 20, 200, 400 and 2000 ng/ml in serum-free RPMI. The lower compartment was covered with a 5 µm pore nitrocellulose filter (Nucleopore membrane, Neuro Probe Inc. Maryland, US) and wells of the upper compartment were loaded with phagocyte suspensions ($2x10^6$ cells/ml), prepared as described in paragraph 5.2.1. Chambers were incubated for 3 hours at 27°C and after incubation, filters were fixed and hematoxylin-stained as described earlier [43]. Cells that had migrated into the filter were counted in three high-power fields (400x) using a light microscope the average number of cells per field was determined.

Statistical analysis

Data were expressed as mean ± standard deviation (SD) and significance of differences was determined using student *t*-test.

Results

Identification of two new putative CXCL8-like genes in zebrafish

A second CXCL8-like gene was recently described in carp, that was clearly distinct from the one earlier described in this species (CXCa) and in other fish, indicating that two CXCL8-lineages exist in fish. The first set of CXCL8 like genes in fish, including carp CXCa, will be referred as lineage one (L1) and the new carp CXCL8 and related genes will be referred to as lineage two (L2). We screened the zebrafish genome to identify counterparts for carp CXCL8 of the second lineage (CXCL8_L2). By tBLASTn we retrieved reliable hits on chromosome seven and seventeen, corresponding with partial gene models with three exons (chromosome 7) and two exons (chromosome 17). The new putative CXCL8 genes were named *CXCL8_L2_chr7* and *CXCL8_L2_chr17* and share 89% nucleotide sequence similarity to each other, indicating that they are not duplication artifacts of the genome assembly, but rather distinct genes. No matching EST sequences were retrieved for the gene model located on chromosome seven, but the one from chromosome 17 was 100% similar to four identical ESTs indicating that this gene is expressed. These ESTs contain a full-length CXC ORF including the exons missing on chromosome 17, which could not be retrieved in the genome assembly using BLAT and tBLASTn (Fig. 1). The exon/intron structure of *CXCL8_L2_chr7* and *CXCL8_L2_chr17* is rather similar to the one of CXCL8 genes from lineage 1 such as the one present on the zebrafish chromosome 1 (*CXCL8_L1_chr1*) (Fig. S1a). BLASTp search with both gene sequences retrieved mammalian CXCL8 as the most similar CXC sequence. *CXCL8_L2_chr7* and *CXCL8_L2_chr17* share less about 40% protein sequence similarity with zebrafish *CXCL8_L1_Chr1*, corresponding to a distant homology (Fig. S1b-d).

The second CXCL8 lineage is specific to cyprinids

To search for CXCL8 genes from the second CXCL8 lineage in other fish, we analyzed EST databases and the full genome assembly of tetraodon, fugu, stickleback and medaka. TBLASTn search was performed with CXCL8 protein sequences from both lineages: zebrafish CXCL8_L1_chr1, carp CXCL8_L2, and zebrafish CXCL8_L2_chr17. While tBLASTn and BLASTp analyses identified several sequences belonging to the first CXCL8 lineage with highly significant scores (table S1) in various fish, no counterpart of CXCL8 from the second lineage could be retrieved outside cyprinids.

An analysis for conserved synteny groups including the three zebrafish CXCL8 genes showed that fish CXCL8_L1 and neighboring genes are part of a synteny block that is well conserved in zebrafish, medaka, tetraodon and fugu. This indicates that fish CXCL8 of the first lineage are true orthologs (Fig. S2a). Genes located in close proximity to zebrafish CXCL8_L2_chr7 form a conserved synteny group in teleosts, mammals and birds (Fig. S2b), but no CXCL8 or other CXC genes are described on corresponding chromosomes. Genes located in close proximity to CXCL8_L2_chr17 form a conserved synteny group only with mammals and birds (Fig. S2c), which also lacks CXC genes.

Phylogenetic trees constructed for fish and tetrapod CXCL8 using NJ and ML methods shows two distinct clusters for teleost CXCL8 (Fig. 2). One cluster corresponds with the fish CXCL8_L1 and includes zebrafish CXCL8_L1_chr1 and carp CXCa_L1. The

second clusters consists of carp CXCL8_L2, zebrafish CXCL8_L2_chr7 and zebrafish CXCL_L2_chr17. This indicates that two CXCL8-lineages are present in fish, of which the first is conserved among fish and the second is specific for cyprinids. Instability of tree nodes at the split of reptile, mammalian and fish CXCL8_L2 clusters, using NJ or ML as tree construction method, render the respective evolutionary distances of fish CXCL8_L1 and fish CXCL8_L2 to mammalian CXCL8 difficult to assess precisely.

Constitutive expression of carp CXCL8_L2 gene in immune organs and brain

The expression of carp CXCL8_L2 was determined in various organs and tissues. CXCL8_L2 showed high constitutive expression in immune organs (spleen, head kidney). Highest constitutive expression was observed in the periphery, in gills, skin and to a lesser extent gut. In whole brain, a much lower constitutive expression of CXCL8_L2 was observed and also in the nucleus preopticus region of the hypothalamus (NPO), the brain area of specific interest for the stress response, as well as in the pituitary pars distalis (PD) and pars intermedia (PI) the constitutive expression of CXCL8_L2 gene was

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EST_EH557944     ATGAAGTTGAGCGTTTCAGCCTTCATGCTTCTGATCTGCACGACTGCACTGCTGTGCGCC 60
genomic_chr17    ATGAAGTTGAGCGTTTCAGCCTTCATGCTTCTGATCTGCACGACTGCACTGCTGTGCGCC
genomic_chr7     ATGAAGTTGAGCATTTCAGCCTTCATGCTTCTGATCTGCACAACTGCACTGCAGTGCACC 60
CXCL8_L2_chr17   M  K  L  S  V  S  A  F  M  L  L  I  C  T  T  A  L  L  C  A
CXCL8_L2_chr7    M  K  L  S  I  S  A  F  M  L  L  I  C  T  T  A  L  Q  C  T

EST_EH557944     AATGAGGGTGAAGCTCTACCTCCACCGCAGCGCTGTCAGTGCATTAAAACTCATTCAAAA 120
genomic_chr17    AATGAGGGTGAAGCTCTACCTCCACCGCAGCGCTGTCAGTGCATTAAAACTCATTCAAAA
genomic_chr7     AATGAGGGTCAACCTCCACCTCCACCGCTGCGCTGTCAGTGTGTTAAAATTTATTCACAA 120
CXCL8_L2_chr17   N  E  G  E  A  L  P  P  P  Q  R  C  Q  C  I  K  T  H  S  K
CXCL8_L2_chr7    N  E  G  Q  P  P  P  P  P  L  R  C  Q  C  V  K  I  Y  S  Q

EST_EH557944     CCACCAATTCCTAAACGACAAGTACTCGGACTGAAGGTGACTCCTGCTGGATCACACTGC 180
genomic_chr17    CCACCAATTCCTAAACGACAAGTACTCGGACTGAAGGTGACTCCTGCTGGATCACACTGC
genomic_chr7     CCACCGATTCCTAGGCGACAAGTACTCGCACTGAAGGTGAATTCTGCTGGACCACACTGC 180
CXCL8_L2_chr17   P  P  I  P  K  R  Q  V  L  G  L  K  V  T  P  A  G  S  H  C
CXCL8_L2_chr7    P  P  I  P  R  R  Q  V  L  A  L  K  V  N  S  A  G  P  H  C

EST_EH557944     AGAAACGAGGAGATCATCGCCACACTTAAGAAAGGACAGATTTGTCTCAATCCTACCGAG 240
genomic_chr17    AGAAACGAGGAGATCAT/
genomic_chr7     AGAAATGAGGAGATCATGGCCACACTGAAGAACGGACAGACTTGTCTCAATCCTACAGAG 240
CXCL8_L2_chr17   R  N  E  E  I  I  A  T  L  K  K  G  Q  I  C  L  N  P  T  E
CXCL8_L2_chr7    R  N  E  E  I  M  A  T  L  K  N  G  Q  T  C  L  N  P  T  E

EST_EH557944     ACGTGGGTGATTTCCCTCAAGGAAAAGTTTGCAGCATCAGCTACTAAATTAGCAGCAACA 300
genomic_chr7     AACTGGGTGATGTCACTCAAG/---------------------------------- 270
CXCL8_L2_chr17   T  W  V  I  S  L  K  E  K  F  A  A  S  A  T  K  L  A  A
CXCL8_L2_chr7    N  W  V  M  S  L  K/

EST_EH55794      GCAGCACCAGCACAGACAACAACAACATTTTCAACAATAATGACCACGAATTAA 354
CXCL8_L2_chr17   T  A  A  P  A  Q  T  T  T  T  F  S  T  I  M  T  T  N
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Fig. 1. Multiple nucleotide alignment of zebrafish EST EH557944, zebrafish CXCL8_L2_chr7 (3 exons) and zebrafish CXCL8_L2_chr17 (2 exons). EST EH557944 and CXCL8_L2_chr17 are 100% similar. Putative splicing sites are underlined. The translation for both putative genes is presented and differences are boxed.

low (Fig. 3a). Moreover, after prolonged restraint, (24 h netting) no significant changes of expression of the CXCL8_L2 gene were observed, neither in the hypothalamic brain areas, nor in the head kidney (data not shown).

Constitutive expression of chemokine and chemokine receptor genes in leukocyte populations.

Both in HK phagocytes and PBLs, constitutive expression of CXCL8_L2 and CXCa_L1 genes was higher than expression of CXCb. Furthermore the expression of CXCR1 was higher than CXCR2. Expression of all studied genes was higher in HK phagocytes then in PBLs (Fig. 3b). In density-separated fractions of HK leukocytes, the highest expression of the CXCL8_L2 gene was measured in the monocyte/lymphocyte fraction. Granulocyte enriched fractions showed high basal expression of the genes for CXCa_L1, CXCb and CXCR1 (Fig. 3c). Macrophage enriched fractions showed intermediate levels of expression of CXCL8_L2, CXCa_L1 and CXCR1. Expression of the CXCR2 gene was low in all studied HK fractions.

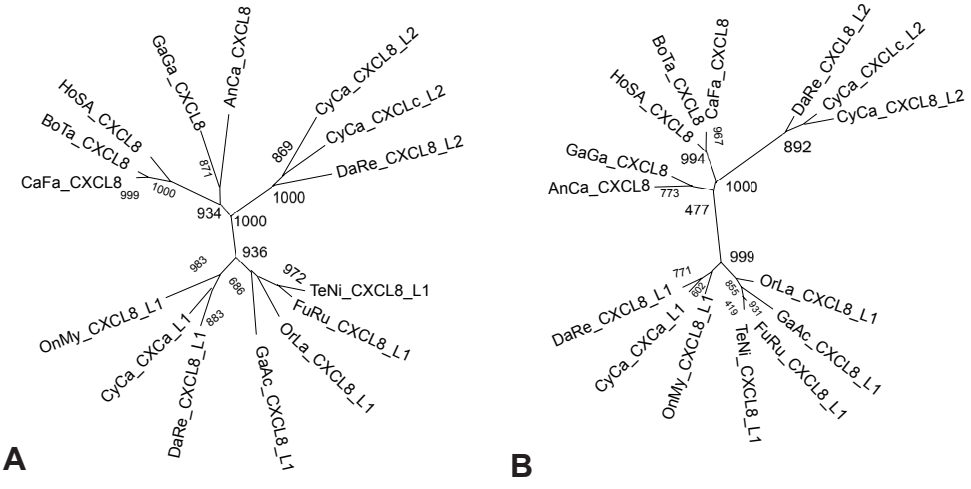
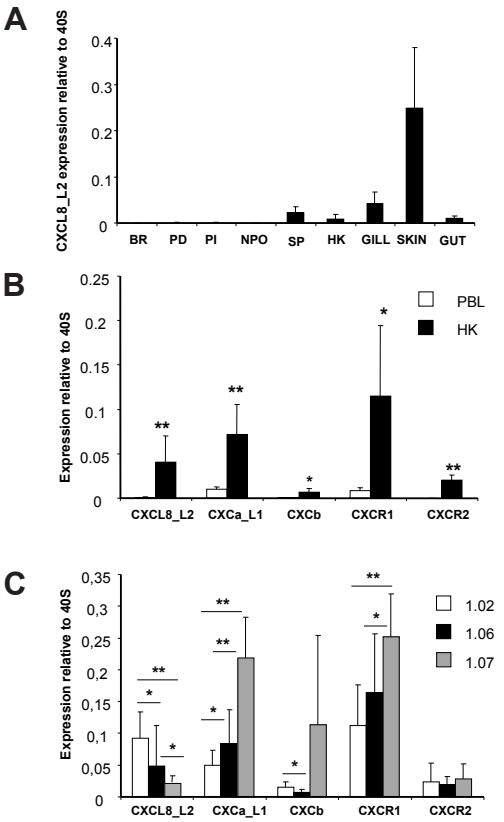


Fig. 2. CXCL8 phylogeny trees constructed by Neighbour Joining (**A**) or Maximum Likelyhood (**B**) with complete deletion of gaps from the alignment. Relevant bootstrap (N=1000) values are indicated in red. CXCL8 accession numbers are as follows: anolis AnCa_CXCL8, ENSACAG00000011382; chicken GaGa_CXCL8, P08317; common carp lineage1 CyCa_CXCa_L1, CAD13189; common carp lineage2 CyCaCXCL8_L2 BAH98111; common carp lineage2 CyCaCXCc_L2 EC394283; cow BoTa_CXCL8, P79255; dog CafaCXCL8, P41324; fugu lineage1 FuRuCXCL8_L1, 49532767; human HoSaCXCL8, P10145; medaka lineage1 OrLaCXCL8_L1, ENSORLG00000005096; rainbow trout lineage1 OnMyCXCL8, CAC33585; stickleback lineage1 GaAcCXCL8_L1, ENSGACG00000001729; tetraodon lineage1 TeNiCXCL8_L1, ENSTNIG00000017810; zebrafish lineage1 DaReCXCL8_L1, XP_001342606; zebrafish lineage2 DaReXCL8_L2, EH441857;

Fig. 3. Constitutive expression of chemokine (CXCL8_L2, CXCa_L1, CXCb) and chemokine receptor (CXCR1 and CXCR2) genes in brain areas and immune-related organs (**A**), in peripheral blood leukocytes- or head kidney phagocytes (**B**), in monocyte/lymphocyte- (1.020 – 1.060 g/cm3), macrophage- (1.060 - 1.070 g/cm3) and granulocyte- (1.070 to 1.083 g/cm3) enriched fractions from head kidney (**C**). Expression was determined by quantitative real time PCR and plotted relative to the expression of 40S ribosomal protein s11 gene. Bars represent the average expression ± SD in organs or tissues obtained from four individual carp. (n=4-5 for fig3A and n=5-9 for fig3Bb and 3C). BR, brain, PD, pars distalis; PI, pars intermedia, NPO, nucleus pro-opiticus of hypothalamus; SP, spleen; HK, head kidney, PBL, peripheral blood leukocytes. * ,p<0.05, **, p<0.01.



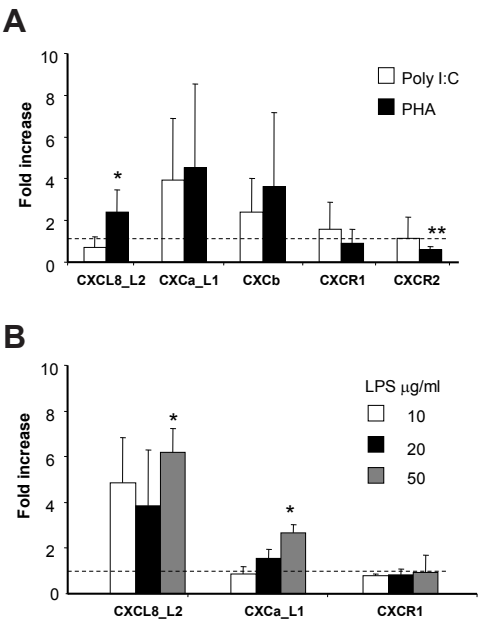


Fig. 4. Expression of chemokine (CXCL8_L2, CXCa_L1, CXCb) and chemokine receptor (CXCR1 and CXCR2) genes in peripheral blood leukocytes stimulated with poly-inosinic poly cytidiylic (Poly I:C, 50 μg/ml) or phytohemagglutinin (PHA, 10 μg/ml) (**A**) or with different concentration of lipopolysaccharide (LPS, **B**) Data are shown as x-fold increase of mRNA expression compared to non-stimulated control cells standardized for the housekeeping gene 40S ribosomal protein s11. Averages (n=4 for fig 4A and n=6 for 4B) and SD are given. *, p<0.05.

Expression of chemokine and chemokine receptor genes after in vitro stimulation of carp leukocytes

While their constitutive expression is low, chemokines are inducible in PBLs. Significant increase of CXCL8_L2 gene expression was observed 4 h after PHA (10 μg/ml, Fig. 4a), or LPS (50 μg/ml, Fig. 4b), but not polyI:C (50 μg/ml , Fig. 4a), stimulation. The same dose of LPS also induced upregulation of CXCa_L1 expression (Fig. 4b). Expression of the CXCR1 gene in PBLs was unchanged upon stimulation (Fig.4a, b), while expression of CXCR2 was downregulated upon PHA treatment (Fig. 4a). Both CXCb and CXCR2 gene expression was not stimulated upon LPS treatment (data not shown).

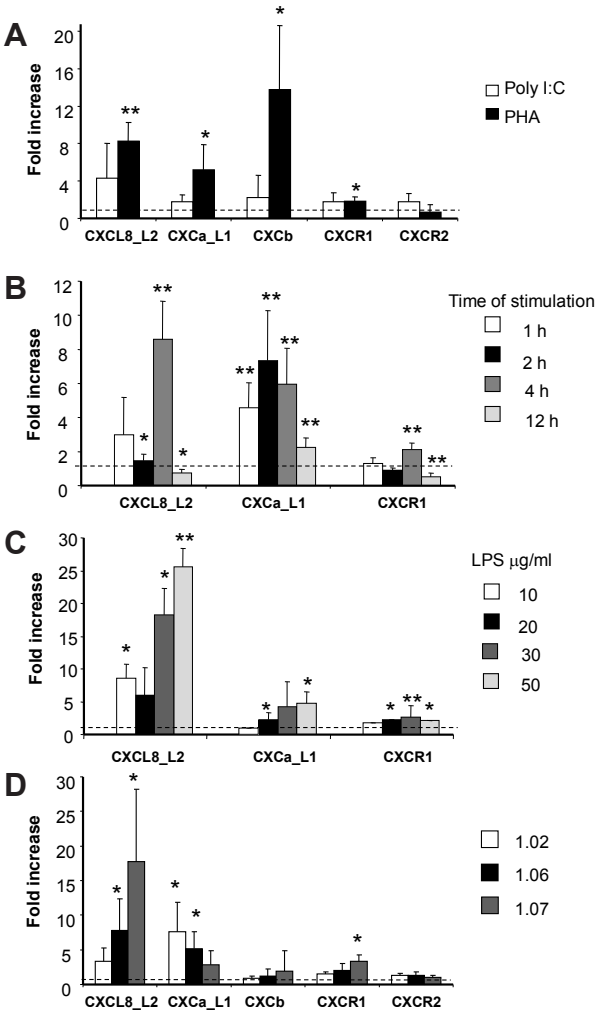


Fig. 5. Expression of chemokine (CXCL8_L2, CXCa_L1, CXCb) and chemokine receptor (CXCR1 and CXCR2) genes in head kidney phagocytes (**A-C**) or in monocyte/lymphocyte- (1.020 – 1.060 g/cm3), macrophage (1.060 - 1.070 g/cm3) and granulocyte- (1.070 to 1.083 g/cm3) enriched fractions from head kidney (**D**). Cells were stimulated with poly-inosinic poly cytidiylic (Poly I:C, 50 μg/ml) or phytohemagglutinin (PHA, 10 μg/ml) (**A**) or with lipopolysaccharide (LPS) (**B-D**). Data are shown as x-fold increase of mRNA expression compared to non-stimulated control cells standardized for the housekeeping gene 40S ribosomal protein s11. Averages (n=4-6 for fig 5A-C and n=9 for fig 5D) and SD are given. *, $p<0.05$, **, $p<0.01$.

Significant upregulation of CXCL8_L2, CXCa_L1 and CXCb gene expression was observed in HK phagocytes at 4 h of PHA (10 µg/ml) stimulation. Stimulation levels ranged from 5-fold for CXCa_L1 to 14-fold for CXCb (Fig. 5a). Expression of the CXCL8_L2 gene started to increase in head kidney phagocytes at 2 h reaching a 9-fold increase at 4 h and a subsequent downregulation at 12 h after in vitro LPS (50 µg/ml). Gene expression of CXCa_L1 rapidly increased after stimulation (1 h), remained higher till 4 h of stimulation and was less but still significantly increased at 12 h. Also upon LPS treatment, significant increase of expression of the CXCR1 gene was observed in HK phagocytes at 4 h of stimulation, followed by a decrease at 12 h (Fig. 5b). The dose-response relationship for CXCa_L1 and CXCL8_L2 responses to LPS differed. At 4 h after stimulation, doses of 10, 30 and 50 µg/ml of LPS stimulated expression of the CXCL8_L2 gene with a maximum

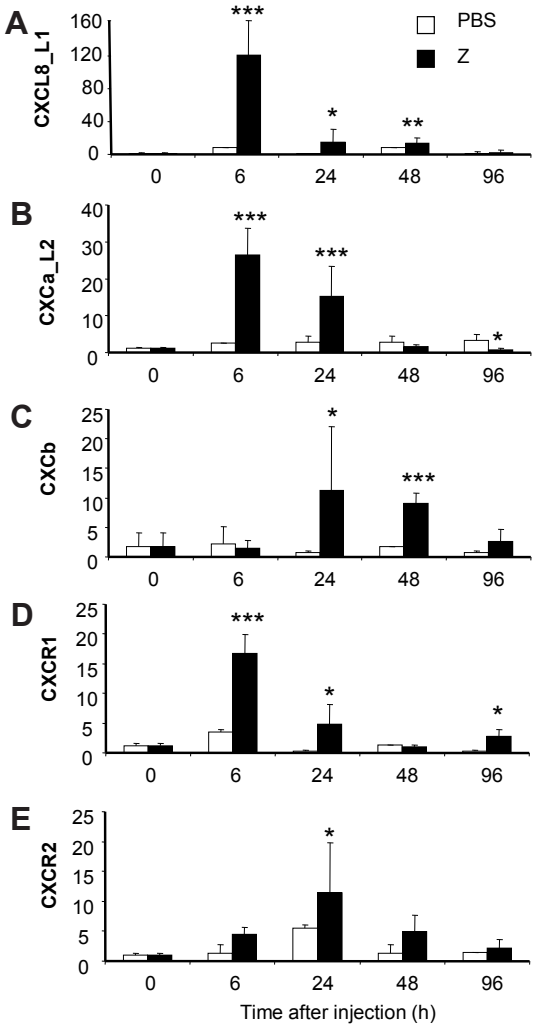


Fig. 6. Expression of chemokine (CXCL8_L2, CXCa_L1, CXCb) and chemokine receptor (CXCR1 and CXCR2) genes in peritoneal leukocytes 0, 6, 24, 48, 96 h after zymosan (Z, 2 mg/ml ie 0.5ml/50g b.w.) induced peritonitis. cDNA of n=4-9 fish was used as template for quantitative real time PCR. Messenger RNA expression is shown as x-fold increase compared to control saline-treated animals at time 0 (PBS) standardized for the housekeeping gene 40S ribosomal protein s11. Averages (n=4-9, data combined from 3 separate experiments) and SD are given. *, $p<0.05$, **, $p< 0.01$, ***, $p< 0.001$

response at 50 µg/ml, whereas CXCa_L1 gene expression was upregulated by doses of 20, 30 and 50 µg/ml of LPS (Fig. 5c). CXCb and CXCR2 expression were not responsive to LPS treatment (data not shown).

Compared to lymphocyte/monocytes fractions and macrophage fractions the expression of CXCL8_L2 in the HK granulocytes could be stimulated with LPS to a much higher extent, both in fold expression and even more in absolute terms. For CXCa_L1 the reverse was found (Fig. 5d). CXCR1 gene expression was upregulated in the HK granulocyte fraction, while all leukocyte subpopulations did not show changes in expression of CXCb and CXCR2 genes upon LPS-stimulation (Fig. 5d).

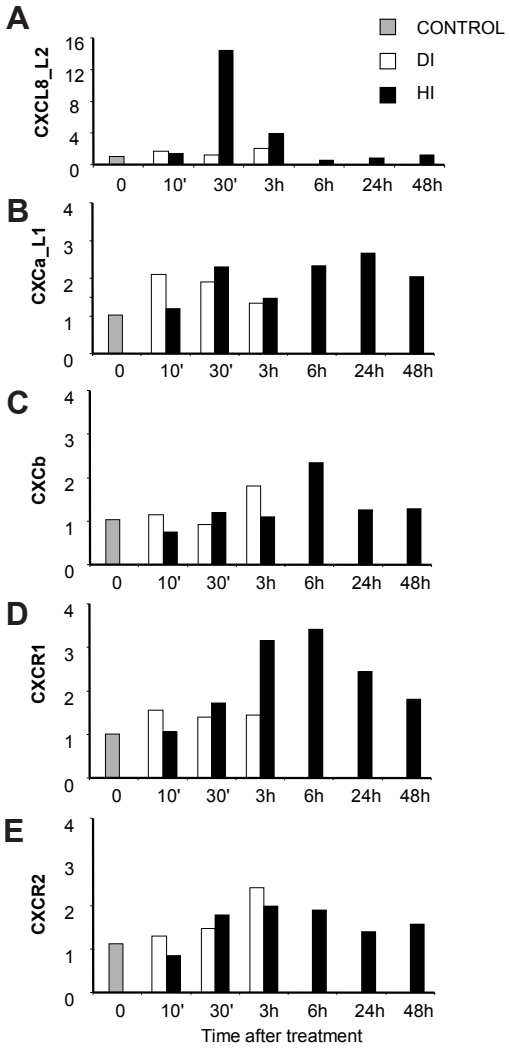


Fig. 7. Expression of chemokine (CXCL8_L2, CXCa_L1, CXCb) and chemokine receptor (CXCR1 and CXCR2) genes in gills upon hyperosmotic immersion (HI) or direct immersion (DI) with A. Salmonicida bacterin-FITC (2.4×107 bacteria/ml). Gene expression is shown at 0, 10 and 30 min and 3, 6, 24, 48 hours after vaccination. Messenger RNA expression is shown as x-fold increase compared to control non-treated animals at time 0 and standardized for the housekeeping gene 40S ribosomal protein s11. Averages (n=2-3) are given.

In vivo expression of chemokine and chemokine receptor genes in peritoneal leukocytes during zymosan-induced peritonitis
Intraperitoneal injection of zymosan induced acute inflammation in the peritoneal cavity, manifested by massive influx of phagocytes into the focus of inflammation and changes in expression of pro- and anti-inflammatory mediator genes [33]. Expression of the CXCL8_L2 gene was quickly and significantly upregulated in peritoneal leukocytes at 6-48 h after zymosan injection, with a maximal increase at 6 h of inflammation (Fig. 6a). Increased expression of the CXCa_L1 gene was observed at 6 and 24 h of peritonitis, while 96 h after zymosan injection CXCa_L1 gene expression was downregulated (Fig. 6b). A significant increase of expression of the CXCb gene in the peritoneal leukocytes was recorded during later stages, 24 and 48 h after the onset of the inflammatory reaction (Fig. 6c). Also the level of expression of the CXCR1 gene was upregulated at 6, 24 and 96 h of inflammation with an early peak at 6h (Fig. 6d). Upregulation of expression of the CXCR2 gene was only statistically significant at 24 h after zymosan stimulation (Fig. 6e).

Expression of chemokine and chemokine receptor genes in gills after hyperosmotic shock.
Hyperosmotic treatment induced mild disruption of the integrity of the gill epithelia. After immersion vaccination with LPS-DTAF, granulocytes were quickly leaving the head kidney and appeared in PBL. An inflammatory reaction was observed in the gills with an IL-1β and iNOS peak at 3h after immersion vaccination [39]. Gene expression of CXCL8_L2 increased 14-fold in HI-treated fish within 30 min following the immersion vaccination with LPS-DTAF, which decreased to 4 fold after 3 h to finally return to baseline levels within 6 h (Fig. 7a). While expression of CXCR1 was upregulated in the HI-group 3-24 h after treatment (Fig. 7d), only small changes in gene expression of CXCa_L1 (2-3 fold) (Fig. 7b) and CXCb (2-fold) (Fig. 7c,) were detected between HI- and DI- treated animals. Again, expression of CXCR2 remained unaltered (Fig. 7e).

Recombinant carp CXCa_L1 and CXCL8_L2 are both chemotactic for carp phagocytes
To investigate whether CXCL8 chemokines from both lineages are chemotactic, recombinant proteins were prepared for carp CXCa_L1 and carp CXCL8_L2 (Fig. S3). N-terminal His-tagged recombinant proteins were expressed in *E. coli* and purified on a nickel-column; analyses on NuPAGE gel confirmed protein purity. MALDI-TOF analysis validated protein sequences. Chemotactic activity was assessed in vitro with chemotaxis chambers for carp head kidney phagocytes. Compared to RPMI or recombinant IFN-γ-2 prepared in parallel to chemokines, both recombinant carp CXCa_L1 and CXCL8_L2 showed a strong chemotactic activity, with a maximum response matching the one induced by zymosan-activated carp serum, at 200 ng/ml (Fig. 8).

Discussion

In contrast to mammals, at least two CXCL8-like lineages are present in teleosts. Lineage 1, CXCL8_L1 consists of chemokine genes that are conserved among multiple fish species and include carp *CXCa_L1* [21] and zebrafish *CXCL8_chr1* [26], as well as IL-8 of flounder [19], trout [20], haddock [22], fugu [23], atlantic cod [24] black seabream [25]

and three striped trumpeter [27]. Sequence similarity, phylogeny and synteny analyses show that fish *CXCL8_L1* genes are orthologues. Although zebrafish *CXCL8_L1_chr1* is located in a synteny group that is partly conserved in mammals (our results, [34]), orthology with mammalian CXCL8 could not be validated by phylogeny, probably due to the low phylogenetic signal of these short sequences subjected to fast evolution. On the other hand, CXCL8 sequences from the second lineage appeared to be slightly closer to tetrapod CXCL8 [35], suggesting that they may represent their true counterpart. Surprisingly, these fish CXCL8_L2 genes, including the recently identified carp CXCL8 [35] and the newly described putative genes in zebrafish on chromosome 7 and 17 (this report) were found only in cyprinids. Since common carp has a tetraploid genome, two or four CXC8_L2 genes may be present, depending on when the duplication event has occurred that has led to the two CXCL8_L2 genes in zebrafish. However, gene loss occurs frequently after duplications, and the determination of the number CXCL8_L2 genes in carp will have to wait the complete genome of this species. Synteny analysis did not provide any clue about their origin. Zebrafish *CXCL8_L2_chr7* and *CXCL8_L2_chr17* are located in gene clusters that are conserved in other vertebrates. In other vertebrates

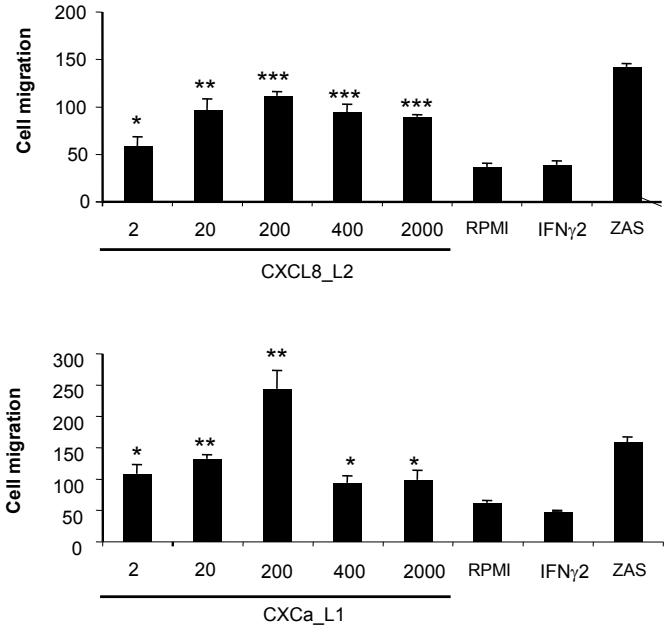


Fig. 8. In vitro chemotaxis of carp phagocytes to (A) recombinant carp CXCL8_L2 (2; 20; 200; 400; 2000 ng/ml) and (B) CXCa_L1 (2; 20; 200; 400; 2000 ng/ml) after 3 hr incubation. Serum-free RPMI and recombinant carp IFN-γ-2 (20 ng/ml) were included as negative controls, zymosan-activated serum (ZAS, non-diluted) as positive control. Cell migration is indicated as average cell number of three fields per well. Averages (n=3) and SD are indicated. *, $p<0.05$, **, $p< 0.01$, ***, $p< 0.001$.

however these clusters do not contain CXC genes located in the neighborhood or even on the corresponding scaffolds or chromosomes. CXCL8 genes have been identified in species that predate the tetrapod-fish split and include lamprey (LFCA-1, [44]), hagfish (BJ653776) and elasmobranchi (AB063299, [26]). However, it is difficult to establish unambiguous and stable phylogeny for CXC genes, due to their small size and high divergence rate (personal observation, [12,18,26]. Multiple species-specific CXCL(8)-lineages have probably arisen, as for example the multiple CXCL8 that have been cloned in catfish, or identified by screening of current sequence databases, and do not show clear phylogenetic clustering ([45], personal observations). Thus, it is not clear whether the duplication leading to the two fish CXCL8-lineages occurred before the divergence of fishes and tetrapods, and the similarity of CXC8_L2 with tetrapod CXCL8 has most probably arisen by convergence.

In this context it is interesting to emphasize that no true CXCL8 orthologue has been found in mouse and rat either. In humans, members of the growth-related gene product (GROα, -β and -γ, CXCL1, -2 and -3 resp) family also mediate, but in a lesser extent than CXCL8, neutrophil chemotaxis [46]. Mouse and rat orthologs of the GRO family (mouse: KC/CXCL1, MIP-2/CXCL2 and rat CINC-1-3), are major mediators of neutrophil chemotaxis in these species [47-52].

To investigate the respective functions of the two CXCL8 lineages identified in cyprinids, we performed an extensive *in vitro* and *in vivo* study to characterize their expression in different tissues and in different conditions of cell stimulation. For this purpose, we focused on the carp model, which allows easier sampling and ex vivo functional assays. First indications that both CXCa_L1 and CXCL8_L2 are involved in immunity are provided by their high constitutive gene expression in gills, skin, gut and in classical fish lymphoid organs like spleen and head kidney ([21,29], this article). This is in contrast to the non-immune chemokine CXCL12 lineage that is highly expressed in brain tissue. Gills, skin and gut form a first line of defense against pathogens and local CXCa_L1 and CXCL8_L2 expression may be attributed to resident leukocytes in these organs. The head kidney in fish, where heamatopoiesis and leukocyte maturation take place [53,54], is considered as a functional homolog for human bone marrow. CXCa_L1 and CXCL8_L2 mRNA that we observe in head kidney leukocytes may function as a reservoir for fast protein synthesis after head kidney egress. Head kidney granulocytes express constitutively high levels of CXCa_L1 but lower levels of CXCL8_L2 mRNA. Granulocytes have high constitutive expression of the CXCR1 gene, which presumably allows rapid mobilization of these cells in response to infection. In contrast, the expression of CXCR1 by leukocytes that are already in circulation (PBLs) is low. Carp CXCR1 is a candidate receptor for both CXCL8 proteins, due to high sequence similarity between carp and mammalian CXCR1 [55], but receptor-ligand interaction has not been demonstrated yet experimentally.

The *in vitro* induction of both CXCL8–like genes early after stimulation indicates functional homology to mammalian CXCL8. This observation is further corroborated by the early peaks of CXCa_L1 and CXCL8_L2 expression *in vivo*, within 6 h after the onset of zymosan-induced peritonitis and 30 min after the fast recruitment of granulocytes to the gill tissue after HI-induced damage of epithelial surface of the gills. Yet distinct

differences in expression profiles in organs, e.g. a relatively low constitutive expression of CXCL8_L2 compared to CXCL8_L1 in gut [29], or in different cell populations as well as differences in timing could be observed, corresponding to specific subfunctionalization.

While the constitutive expression of CXCa_L1 is higher than that of CXCL8_L2 in neutrophilic granulocytes, only the CXCL8_L2 gene showed a considerable upregulation of expression after LPS stimulation. As we could also observe a differential stimulation with regard to time and dose-response of LPS in the total head kidney phagocyte fractions, we conclude that CXCa_L1 and CXCL8_L2 gene expression is subject to distinct regulatory pathways. The promotor region of mammalian CXCL8 contains binding elements for NF-κB and AP-1 (reviewed by [56]) and expression of the CXCL8 gene is inducible directly by pathogens upon recognition of pathogen associated molecular patterns (PAMPS) by the specific pattern recognition receptors (PRRs) as well as by cytokines and growth factors that are activated after detection of an infectious agent (see for reviews [56] [57]). Further analyses for enhancer elements in the promotor region of CXCa_L1 and CXCL8_L2 will provide more clues on how different transcription factors regulate the expression of both genes in fish, but remain highly difficult to date since the LPS activation pathway is currently not understood in fish. Neither CXCa_L1 nor CXCL8_L2 are induced in vitro by Poly I:C in PBLs or head kidney phagocytes, in contrast to earlier observations for CXCL8_L1 in other fish species [20,23,24,25].

For two in vivo models of inflammation, the zymosan induced peritonitis ([33]) and the HI-induced damage of gill epithelia [39], a massive influx of mainly neutrophilic granulocytes is observed during the early phase of reaction. In accordance to the above mentioned in vitro results this correlated to a fast and powerful upregulation of predominantly CXCL8_L2. In both models this coincides with the early peak of expression of the pro-inflammatory cytokine IL-1β, followed by a peak of expression of the pro-inflammatory mediator iNOS. The observation that in vivo the upregulation of CXCL8_L2 and also CXCa_L1 correlate in time with the expression of CXCR1, may support our earlier hypothesis that their chemotactic activity is mediated through this receptor.

In contrast to the early CXCL8-like expression, both in vitro and during in vivo peritonitis or immunization, the expression of CXCb, which is more closely related but not orthologous to CXCL9-11, appears at later time points. In mammals, IFN-γ, together with LPS, are principal inducers for expression of CXCL9, -10 and -11 in neutrophils, thereby mediating T-lymphocyte recruitment via the CXCR3 (for review [57]). In carp, we earlier identified two IFN-γ genes, IFN-γ-1 and IFN-γ-2, of which IFN-γ-2 expression is significantly induced in PHA-stimulated lymphocytes [58]. Results with recombinant carp IFN-γ-2, have demonstrated that carp CXCb is inducible by IFN-γ-2 and is highly synergistic in combination with LPS, thus indicating that carp CXCb expression is regulated in a similar way as mammalian CXCL9, -10 and -11 [59]. Expression of CXCb during the late phase of inflammation suggests that it recruits cells other than neutrophils. Although two CXCR3 genes have been identified for zebrafish [18], we could not find a carp CXCR3 sequence in available databases (personal observations).

Thus, the CXCL8-like genes appear to be at least partial functional counterparts of the human CXCL8. This is corroborated by the finding that in vivo expression is

correlated with massive influx of phagocytes to the site of infection [33]. As expression and induction patterns of both CXCL8 genes do not coincide, a sub-functionalization is indicated. Sub-functionalization of duplicated genes of common origin is a classical pattern in teleost fish due their additional genome duplication round(s) [60]. Teleost CXCa_L1 and CXCL8_L2 lack an ELR motif upstream the CXC-motif, a motif that is associated with a functional role in leukocyte chemotaxis in mammals [13], but may not be a prerequisite for chemotaxis of teleost leukocytes [25,31]. Further characterization of the functional diversity of fish CXCL8s therefore required a direct analysis of their chemotactic activity. For that reason the recombinant CXCL8_L2 and CXCa_L1 proteins were made for which we could subsequently demonstrate a clear chemotactic activity of both chemokines towards phagocytes *in vitro*. We now show that despite the lack of an ELR-motif both carp CXCLa_L1 and CXCL8_L2 are potent chemoattractants for head kidney leukocytes. Both recombinant proteins showed a similar optimum dose response, with a decrease in chemotaxis at higher concentrations, probably due to receptor desensitization, as commonly observed for chemokines [13,31,46].

In conclusion, although phylogeny and synteny analysis could not confirm true orthology of carp CXC8_L1 and CXCL8_L2 with mammalian CXCL8, based on chemokine gene expression and their chemotactic activity, we hypothesize that these ligands are functional homologs of mammalian CXCL8. We now showed that apart from the carp chemokine CXCa_L1, the newly identified CXCL8_L2 has a crucial biological role in recruitment of neutrophilic granulocytes during the early phase of inflammation.

Acknowledgments

We like to thank Dr. Joop Arts and Wouter Derks for their contribution to the in vitro stimulation experiments, Dr. Mark Huising for HI-treatments, Maria de Boer for the help with the in vitro chemotaxis assay, Pierre Pontarroti for helpful discussions, and Didier Chevret for the analysis of the recombinant CXCa_L1 and CXCL8_L2 by MALDI-TOF.

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Chaque fois que la science avance d'un pas, c'est qu'un imbecile la pousse, sans le faire expres.

- Emile Zola -

Chapter 6

Pro-inflammatory functions of carp CXCL8-like and CXCb chemokines

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Developmental and Comparative Immunology
doi: 10.1016/j.dci.2011.11.011

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Abstract

Numerous CXC chemokines have been identified in fish, however, their role in inflammation is not well established. Here, CXC chemokines of the CXCL8-like (CXCa_L1 and CXCL8_L2) and CXCL9/10/11-like (CXCb) subset were investigated in carp. Recombinant CXCa_L1, CXCL8_L2 and CXCb all stimulated chemotaxis of macrophages and granulocytes *in vitro*. CXCb also attracted lymphocytes. Distinct effects on phagocyte activation were observed: the CXCL8-like chemokines increase in respiratory burst activity, but not nitrite production. The three chemokines differentially induced a moderate increase in IL-1β, CXCa_L1 and CXCL8_L2 gene expression. Intracellular calcium mobilization in granulocytes upon CXCa_L1 stimulation implies signal transduction through G-protein coupled CXC receptors. Notably, upon intraperitoneal administration, carp CXCL8-like chemokines strongly induced *in vivo* leukocyte recruitment, including neutrophils and monocytes/macrophages, in contrast to CXCb, for which the number of recruited leukocytes was low. The results indicate functional homology for carp CXCL8-like and CXCb chemokines with mammalian CXCL8 and CXCL9-11, respectively.

Introduction

Attraction of leukocytes towards inflamed tissues is a multi-step process that is tightly regulated by cytokines. Chemokines are chemotactic cytokines that play an important role in inflammation as they orchestrate leukocyte recruitment and retention at the inflammatory foci. They thereby determine the specificity of cell subsets that are recruited and mediate in cell activation. Based on a distinct cysteine-motif at the N-terminus, chemokines are classified in different subgroups, namely CXC, CC, CX3C, C and CX [1, 2]. Chemokines attract cells that express specific chemokine receptors, which belong to the G-protein coupled receptor family (GPCRs). Chemokine receptor activation triggers intracellular signaling pathways that lead to calcium mobilization in the cell, and ultimately rearrangement of the cytoskeleton and chemotaxis [3].

CXC chemokines of particular interest in inflammation are CXCL8 and the CXCL9, CXCL10 and CXCL11 chemokines. As is well established in mammals, CXCL8 is among the first chemokines expressed during inflammation. The pro-inflammatory cytokines IL-1β and TNF-α induce CXCL8 expression in endothelial cells, fibroblasts and local immune cells [4, 5]. Cells expressing CXCR1 and CXCR2 are responsive to CXCL8 and CXCL8 is a major chemoattractant for the recruitment of neutrophilic granulocytes, which are first cells to arrive at the site of inflammation [6-8]. The IFN-γ inducible CXCL9-11 chemokines participate in the onset of the adaptive immune response, by orchestrating recruitment of type 1 helper T (Th1) CD4+ cells and effector CD8+ T cells (see for a recent review [9]). When local macrophages and dendritic cells (DCs) are activated, they produce IL-12, which stimulates natural killer (NK) cells to produce IFN-γ [10]. Together with IL-1β and/or TNF-α, IFN-γ induces production of CXCL9, -10, and -11 in various cell types, including granulocytes, macrophages and fibroblasts [11]. Activated T cells that express the CXCL9-11 receptor CXCR3 are then recruited [12]. As activated T cells produce IFN-γ themselves, infiltrated T cells act in a positive amplification loop to further drive T-cell recruitment [13]. Anti-inflammatory cytokines

like IL-10 inhibit IFN-γ synthesis, and hereby down regulate T-cell recruitment [14, 15].

The chemokine family is evolutionary old and originated at the emergence of vertebrates [16]. The chemokine family already highly expanded before the divergence of fish and tetrapod lineages, as was demonstrated by a complete description of the chemokine gene family on the genome of zebrafish [2, 16], indicating a functional diversification for fish chemokines [17]. Numerous chemokine ligand and receptor genes have been cloned from various fish species. Gene expression studies indicate for multiple fish chemokines a role in inflammation (see for a recent review [18]). Which leukocyte subsets are attracted by the individual teleost chemokines and their function during the different phases of the inflammatory response still await better clarification. Several genes have been identified in teleost fish that phylogenetically resemble the mammalian CXCL8 [2, 19-28] and CXCL9, -10 and -11 [29-32] most in gene sequences. Due to the rapid evolution of chemokine genes and their small size, these phylogeny studies however have not firmly established true orthology to their mammalian counterparts. To underscore this, an alternative nomenclature was earlier proposed to name fish CXCL8-like genes CXCa, and CXCL9-11-like genes CXCb [21]. In two fish species that belong to the cyprinid lineage, common carp and zebrafish, we and others earlier reported that two CXCL8-like lineages evolved: CXCa_L1 (lineage 1) and CXCL8_L2 (lineage 2) [33, 34]. The CXCa_L1 gene is orthologous to IL-8-like genes described in other teleost species, while the CXCL8_L2 gene so far only has been identified in cyprinids [34]. Both genes are expressed during the early phase of inflammation in carp, therefore, an active role in early leukocyte recruitment is expected [21, 34, 35]. We earlier produced recombinant proteins for both carp CXCa_L1 and CXCL8_L2 and demonstrated by an in vitro assay that both chemokines are indeed chemotactic for carp phagocytes [34]. For CXCb, one carp gene has been identified, which is expressed during the second phase of leukocyte recruitment towards the site of inflammation [29, 34, 35]. Moreover, as recombinant carp IFN-γ2 stimulates induction of CXCb expression in carp phagocytes in synergy with LPS, CXCb possibly has functional homology with mammalian CXCL9-11 chemokines [36, 37].

To further characterize the role of fish CXC chemokines from different subsets during the inflammatory response, recombinant protein was prepared for carp CXCb, in addition to the earlier prepared recombinant proteins for carp CXCa_L1 and CXCL8_L2 [34]. By an *in vitro* and *in vivo* approach, migration of different leukocyte populations towards these chemokines was determined. Moreover, activation of the pro-inflammatory phagocyte activity was investigated, to reveal an important, but differential role of the three CXC chemokines in analogy with mammalian CXCL8 and CXCL9-11.

Material and methods

Animals

Common carp (Cyprinus carpio L, 50 g body weight) were bred and reared at 23°C in recirculating UV-treated tap water at the fish facilities `De Haar Vissen` at the Wageningen University, The Netherlands. R3xR8 fish were used, which are the offspring

of a cross between fish of Polish (R3 strain) and Hungarian origin (R8 strain) [38]. All in vivo experiments were conducted according to license no. 2009124 from the local ethical committee.

Preparation recombinant chemokines
Preparation of recombinant proteins for carp CXCa_L1, CXCL8_L2 was described earlier [34]. Recombinant carp CXCb was prepared by a similar approach. Briefly, the synthetic gene encoding the mature carp CXCb (Genbank accession no. AB082985), codon-optimized for *E. coli* expression, was ordered at Mr. Gene (Regensburg, Germany). Restriction sites for BamHI and HindIII were included at respectively the 5`and 3`end of the coding sequences to enable cloning in the corresponding restriction sites of expression vector pET15new. This vector is a derivative from the vector pET15 (Novagen) that encodes for N-terminal tag containing 6-histidine residues under the control of a Lac operon and T7 promotor. The protein sequence for recombinant carp CXCb was: **MSYYHHHHHHLESGS**QARAPKGRCFCVDKGVNMVPPKQIEKVEIIPASRSCKTQEIVVTLKNSTEQK CLNPESKFTQKYIMKAVEKRSLQKK, whereby 6x-His-tag and LESGS epitope are indicated in bold. Expression and purification of recombinant carp CXCb in *E. coli* BL21_CodonPlus(DE3)_RIL (Agilent Technologies) was performed as described earlier [34]. Purity was verified on a NuPAGE protein gel (Fig. S1) and the protein sequence was validated by MALDI-TOF analysis. Potential glycosylation sites were determined with the software tool NetNglyc 1.0 and NetOglyc 1.0. All three recombinant chemokines are devoid of N-glycosylation sites, while only 10-14 potential O-glycosylation sites were found for the individual proteins (Fig. S2) .

In vivo study: in vivo peritonitis
Fish were either untreated (intact fish), or i.p. injected (500 µl per individual) with sterile PBS (negative control), zymosan (positive control; 2 mg/ml in PBS), recombinant chemokine (200 ng/ml or 20 ng/ml in PBS) or heat-inactivated recombinant chemokine (negative control, 200 ng/ml). Heat-inactivated proteins were prepared by heat-treatment (30 min; 94°C). At 6, 24 or 48 hours post injection, animals were sacrificed and blood was collected by puncture of the caudal vessel and peritoneal cavities were lavaged with 2 ml of ice-cold PBS. Total numbers of peritoneal leukocytes (PTL) and phagocytes were determined by Turk-staining [39] and counting in a hemocymeter. A small fraction of the PTLs was taken to produce cytospins. The remaining PTLs were centrifuged (2000 g; 10 min; RT, table-top centrifuge) and resuspended in RLT buffer (Qiagen) and stored at -80°C until RNA processing.

In vivo study: myeloperoxidase (MPO) staining
PTLs (100 µl of $1x10^6$ cells/ml suspension) were mounted on poly-lysine coated slides by two rounds of cytospin (5 min; 500 g; 4°C and 1 min; 500 g; 4°C) and air-dried. MPO staining was performed according to Kaplow [40]. Cells were fixed 60 s with 4% formaldehyde in 100% ethanol, rinsed in tap water for 15-30 s and incubated 30 s with MPO incubation medium (3 mg/ml benzidine dihydrochloride (Sigma); 1.32 mM $ZnSO_4$; 0.123 M sodium acetate; 0.0146 M sodium hydroxide and 0.02% H_2O_2; 0.1% safranin

(w/v); in 30% ethanol). Slides were briefly rinsed with tap water and dried to the air and embedded in Depex (BDH Laboratory Supplies, Poole, England). All steps were carried out at RT.

Head-kidney leukocyte isolation

Head kidney cell suspensions were obtained by passing the tissue through a 50 µm nylon mesh with cRPMI (RPMI 1640 (Invitrogen, Carlsbad, CA); containing 10 IU/ml heparin (Leo Pharmaceutical Products Ltd., Weesp, The Netherlands); adjusted to carp osmolarity (270 mOsm/kg)) and washed once. The cell suspension was layered on a discontinuous Percoll (Amersham Biosciences, Piscataway, NJ) gradient (1.02, 1.060, 1.070, and 1.083 g/cm^3) to obtain enriched fractions for different leukocyte subsets. After centrifugation for 30 min at 800 g with the brake disengaged, cells from the density range of 1.020 – 1.060 g/cm^3 (predominantly, >80% monocytes/lymphocytes), the range of 1.060 - 1.070 g/cm^3 (predominantly macrophages, but also monocytes, lymphocytes and some (~10%) granulocytes), the range from 1.070 to 1.083 g/cm^3 (~80% neutrophilic granulocytes) [41] or combined 1.060 – 1.083 g/cm^3 fractions (enriched phagocytes) were collected, washed, and resuspended in cRPMI^{++} (cRPMI supplemented with 0.5% (v/v) pooled carp serum, 1% L-glutamine (Merck, Whitehouse Station, NJ), 200 nM 2-mercaptoethanol (Bio-Rad, Hercules, CA), 1% (v/v) penicillin G (Sigma–Aldrich, St. Louis, MO), and 1% (v/v) streptomycin sulphate (Sigma–Aldrich, St. Louis, MO).

In vitro chemotaxis assay

Chemotactic activity for recombinant carp CXCa_L1, CXCL8_L2 and CXCb was analyzed in vitro in a 48-well microchemotaxis chamber (Neuro Probe Inc., Maryland, USA) as described earlier [42]. Briefly, lower compartments were filled with either negative controls (serum-free cRPMI) for measurement of random cell migration, or recombinant carp CXCa_L1, CXCL8_L2, CXCb proteins (20, 50, 100, 200 and/or 2000 ng/ml) in serum-free cRPMI. The lower compartment was covered with a 5 µm pore nitrocellulose filter (Nucleopore membrane, Neuro Probe Inc. Maryland, US) and wells of the upper compartment were loaded with cell suspensions (2x10^6 cells/ml), prepared as described above. Chambers were incubated for 3 hours at 27°C and after incubation, filters were fixed and hematoxylin-stained as described earlier [42]. Cells that had migrated into the filter were counted in three high-power fields (400x) using a light microscope the average number of cells per field was determined.

Respiratory burst

The respiratory burst activity of phagocytes was measured with the nitroblue tetrazolium (NBT) as described earlier [41]. Head kidney phagocytes were first pre-treated with different concentrations of CXCa_L1, CXCL8_L2 or CXCb (20-2000 ng/ml) for 20 hrs and then stimulated with phorbol 12-myristate 13-acetate (PMA, 0.1 µg/ml, Sigma–Aldrich, St. Louis, MO) in the presence of NBT (1 mg/ml, Sigma–Aldrich, St. Louis, MO). Following 2 hours incubation the reaction was stopped with methanol. The plates were air-dried and 120 µl of 2 N potassium hydroxide and 140 µl of dimethyl sulphoxide

were added to each well. The OD was recorded in an ELISA reader at 630 nm.

Nitric oxide (NO) level
Nitrite/nitrate production, an indicator of nitric oxide synthesis, was measured in cell culture supernatants as described previously [42]. Head kidney phagocytes were treated with different concentration of CXCa_L1, CXCL8_L2 or CXCb (20-200 ng/ml) and stimulated with lipopolysaccharide (LPS, 30 ug/ml, E. coli serotype O55: B5, Sigma–Aldrich, St. Louis, MO) for 24 hrs. Culture supernatants (75 µl) were added to 75 µl Griess reagent (1% (w/v) sulphanilamide; 0.1% (w/v) N-naphthyl-ethylenediamine in 2.5% (w/v) phosphoric acid) in a 96-well flat-bottom plate. The absorbance reading at 540 nm (with 690 nm as a reference) was taken using cRPMI^{++} medium as blank. Cytokine gene expression Macrophage-enriched fractions from carp head kidney were seeded in a 24-well cell culture plate (5 × 10^6 cells per well) and cultured at 27°C, 5% CO_2. Cells were stimulated for 4 or 20 hrs with recombinant CXCa_L1, CXCL8_L2 or CXCb (20; 200; 2000 ng/ml) or LPS (30 µg/ml). After stimulation, cells were harvested, centrifuged (10,000 rpm for 1 min) and immediately frozen in liquid nitrogen. Samples were stored at -80°C until RNA processing.

Calcium mobilization assay
Granulocyte-enriched head kidney cell fractions (1.25 x 10^6 cells/ml) were incubated overnight in RPMI^{++} culture medium. They were subsequently incubated for 45 min at 27°C (5% CO2) with Fluo-4 dye loading solution (Fluo-4 NW Calcium assay kit, Molecular Probes, Invitrogen), prepared according to the manufacturer's instructions, but with HBSS osmolarity adjusted to 280-290). Cells were stabilized at RT for 15 min and loaded cells were stimulated with 200, 2000, or 4000 ng/ml recombinant CXCa_L1, calcium ionophore III (Cat. nr. 21186, Sigma, 1.25 ng/ml) or PBS. The calcium response was measured with the SpectraMax® M5 Microplate Reader (Molecular Devices). Green fluorescent signal was excited with a 495 nm Xenon laser and detected using a long pass filter (516 nm) every 2 seconds.

RNA isolation, DNase treatment and first strand cDNA synthesis
RNA was isolated using an RNeasy Mini Kit (Qiagen, Valencia, CA) according the manufacturer's protocol. Final elution was carried out in 30 µl of nuclease-free water, to maximize the concentration of RNA. RNA concentrations were measured by spectrophotometry and integrity was ensured by analysis on a 1% agarose gel before proceeding with cDNA synthesis. For each sample a non–RT (non-reverse transcriptase) control was included. Two µl of 10x DNase I reaction buffer and 2 µl DNase I (Invitrogen) was added to 2 µg total RNA and incubated for 15 min at room temperature. DNase I was inactivated with 25mM EDTA (2 µl, 65°C, 10 min). To each sample, 2 µl random primers and 2 µl 10mM dNTP mix were added, and the mix was incubated for 5 min at 65°C and then 1 min on ice. After incubation, to each sample 8 µl 5x First Strand buffer 2 µl 0.1 M dithiothreitol (DTT) and 2 µl RNase inhibitor were added. To 19 µl from each sample (but not to the non-RT controls) 1 µl Superscript RNase H-Reverse Transcriptase (RT, Invitrogen) was added and reagents were incubated for 5 min at 25°C, then spun briefly

and incubated 60 min at 50°C. Reactions were inactivated 15 min at 70°C. Samples were set at 100 µl with demineralized water and stored at -20°C until future used.

Real-time quantitative PCR
PRIMER EXPRESS software (Applied Biosystems) was used to design primers for use in real-time quantitative PCR. Carp-specific primers (5' to 3') for cytokines were used, the 40S ribosomal protein s11 gene served as an internal standard (accession numbers and primer sequences are listed in Table 1). For RQ-PCR 5 µl cDNA and forward and reverse primers (4.2 µM each) were added to 7 µl Absolute QPCR SYBR Green Mixes (ABgene). RQ-PCR (15 min at 95°C, 40 cycles of 15 s at 94°C, 30 s at 60°C, and 30 s at 72°C, followed by 1 min at 60°C) was carried out with a Rotorgene 2000 realtime cycler (Corbett Research, Sydney, Australia). Following each run, melt curves were collected by detecting fluorescence from 60 to 90°C at 1 °C intervals. Expression following stimulation was rendered as a ratio of target gene vs. reference gene (40S ribosomal protein s11 gene) relative to expression in unstimulated control samples according to the following equation [43]:

$$\text{ratio} = (E_{\text{target}})^{\Delta Ct\,\text{target}\,(\text{control-sample})} / (E_{\text{reference}})^{\Delta Ct\,\text{reference}\,(\text{control-sample})}$$

where E is the amplification efficiency and Ct is the number of PCR cycles needed for the signal to exceed a predetermined threshold value.

Statistical analysis
Data were expressed as mean ± standard deviation (SD) and significance of differences was determined using a student t-test.

Table 1. Primers

Gene	Sense (5'-3')	Antisense (5'-3')	Acc. No
40S	CCGTGGGTGACATCGTTACA	TCAGGACATTGAACCTCACTGTCT	AB012087
IL-1β	AAGGAGGCCAGTGGCTCTGT	CCTGAAGAAGAGGAGGCTGTCA	AJ245635
TNF-α	CTGTCTGCTTCACGCTCAA	CCTTGGAAGTGACATTTGCTTTT	AJ311800
iNOS	AACAGGTCTGAAAGGGAATCCA	CATTATCTCTCATGTCCA-GAGTCTCTTCT	AJ242906
CXCa_L1	CTGGGATTCCTGACCATTGGT	GTTGGCTCTCTGTTTCAATGCA	AJ421443
CXCL8_L2	TCACTTCACTGGTGTTGCTC	GGAATTGCTGGCTCTGAATG	AB470924
CXCb	GGGCAGGTGTTTTTGTGTTGA	AAGAGCGACTTGCGGGTATG	AB082985
CXCR1	GCAAATTGGTTAGCCTGGTGA	AGGCGACTCCACTGCACAA	AB010468
CXCR2	TATGTGCAAACTGATTTCAGGCTTAC	GCACACACTATACCAACCAGATGG	AB010713
IL-10	CGCCAGCATAAAGAACTCGT	TGCCAAATACTGCTCGATGT	AB110780

Results

Recombinant carp CXCa_L1, CXCL8_L2 and CXCb are chemotactic for carp head kidney granulocytes and macrophages in vitro

Chemotaxis was assessed with cells from enriched fractions of carp head kidney lymphocytes/monocytes, granulocytes and macrophages. Both macrophage- and granulocyte-enriched fractions migrated towards CXCa_L1 (200 and 2000 ng/ml for the macrophage fraction and 50, 200 and 2000 ng/ml for the granulocyte fraction), CXCL8_L2 (200 and 2000 ng/ml for the macrophage fraction and 50, 200 and 2000 ng/ml for the granulocyte fraction) and CXCb (20, 50, 100 and 2000 ng/ml for the macrophage- and granulocyte fraction) (Fig. 1a-c). The lymphocyte/monocyte-enriched fraction migrated only towards 50 ng/ml of CXCb, but not to CXCa_L1 and CXCL8_L2.

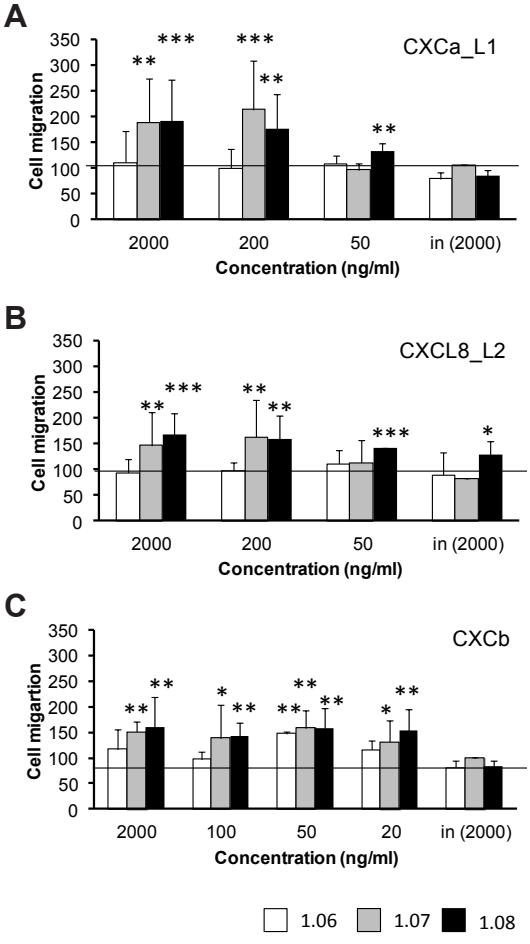

Fig. 1. In vitro chemotaxis of carp leukocytes to (**A**) recombinant carp CXCa_L1, (**B**) CXCL8_L2 and (**C**) CXCb. Chemotactic activity towards the different chemokines was determined for head kidney enriched fractions for lymphocytes/monocytes (1.06; white bars), macrophages (1.07; grey bars) and granulocytes (1.08; black bars). Heat-inactivated (in) recombinant proteins (2000 ng/ml) were included as a negative control. Cell migration is indicated as a percentage relative to random migration towards control medium. Average and SD are indicated. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

In vitro activation of phagocytes by recombinant CXCa_L1, CXCL8_L2 and CXCb
Pre-treatment (24 h) with recombinant CXCa_L1 and CXCL8_L2 but not CXCb significantly enhanced the oxidative burst in response to PMA stimulation (Fig. 2a). Without PMA stimulation, a slight decrease in respiratory burst activity was observed for all studied chemokines. No significant difference in nitrite production was observed upon treatment with recombinant CXCa_L1, CXCL8_L2 and CXCb compared to control cells stimulated with LPS only (Fig. 2b). Potential chemokine-induced changes in expression of pro- or anti-inflammatory genes in macrophage-enriched head kidney cells were determined by quantitative RT-PCR. Only the highest concentration of recombinant chemokines (2000

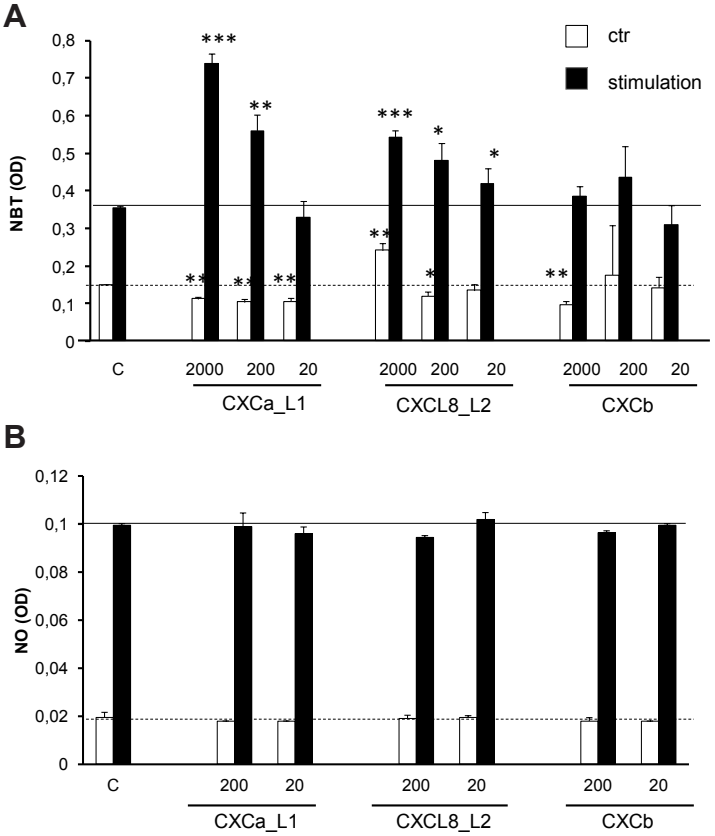

Fig. 2. Oxygen radical production and nitrite production in carp head kidney phagocytes. **A** Phagocytes were pre-treated with recombinant carp CXCa_L1, CXCL8_L2 and CXCb or medium only (c) for 24 hrs and stimulated with PMA (0.1 μg/ml) for 2 hrs or not stimulated (ctr). **B** Phagocytes were treated with recombinant carp CXCa_L1, CXCL8_L2 and CXCb or medium only (C), or co-stimulated with LPS (30 μg/ml) for 24 hrs. NO was determined in culture supernatant. White bars represent cells treated only with chemokines or medium, black bars represents cells co-stimulated with PMA or LPS. Average and SD are indicated, the graphs are representative for three experiments *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

ng/ml) induced moderate changes in expression of particular inflammatory-related genes. The pro-inflammatory cytokine IL-1β was slightly, but significantly increased 3.3-fold upon stimulation (20 hrs) with CXCa_L1 and 1.3-fold upon stimulation with CXCL8_L2, but not with CXCb. CXCa_L1 showed a moderate, but significantly, increased expression upon 20 h of stimulation with recombinant CXCa_L1 (2.1-fold), CXCL8_L2 (1.4-fold) and CXCb (1.3-fold) (Fig. 3b). CXCL8_L2 gene expression was induced at 4 and 20 hrs by recombinant CXCa_L1 (2.3-fold and 3.1-fold, respectively), but not by recombinant CXCL8_L2 and CXCb (Fig. 3c). CXCb gene expression was induced 4.2-fold at 20 hrs upon CXCL8_L2 stimulation. The recombinant chemokines did not alter expression of TNF-α, IL-10, CXCR1 and CXCR2. LPS was included as a positive control for cell activation and induced a significant increase in gene expression of IL-1β and CXCL8_L2 at both 4 and 20 hrs post stimulation and of CXCa gene at 20 hrs (table 2).

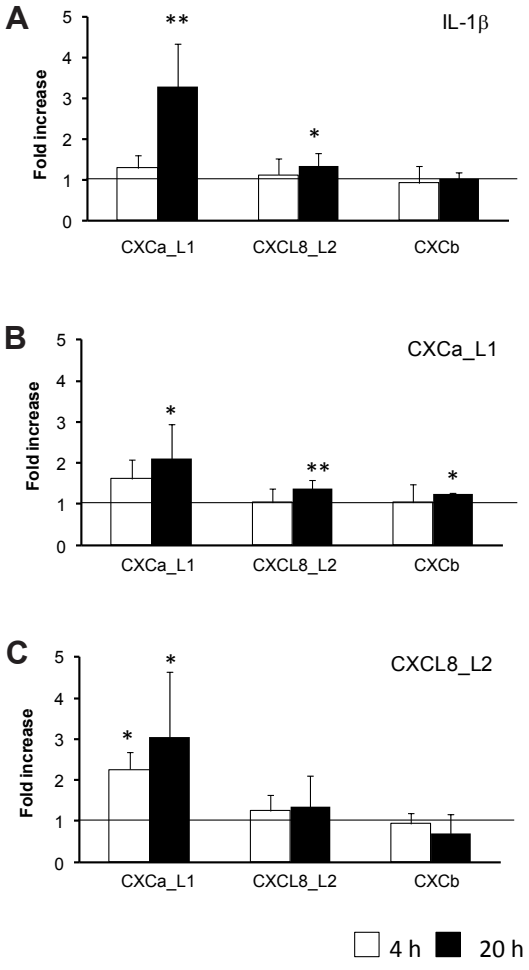

Fig. 3. Gene expression in carp head kidney macrophages upon chemokine stimulation. Macrophages were stimulated for 4 hrs (white bars) and 20 hrs (black bars) with 2000 ng/ml CXCa_L1, CXCL8_L2, CXCb, or non-stimulated. Cytokine gene-expression (IL-1β (**A**), CXCa_L1 (**B**) and CXCL8_L2 (**C**)) was determined by quantitative RT-PCR. Messenger RNA expression is shown as x-fold increase compared to control non-stimulated cells (1; indicated by the horizontal line) and standardized for the housekeeping gene 40S ribosomal protein s11. Significance determined by a student t-test. Average and SD are indicated. *, $p < 0.05$; **, $p < 0.01$. Replicates were CXCa_L1 2 (n = 6); CXCL8-L2 (n=5), CXCb (n=2) for 20 hrs and n=8 for LPS for 4 hrs; CXCa_L1 (n = 3), CXCL8-L2 (n=5), CXCb (n=3), LPS (n=5) for 4 hrs.

Table 2. Gene expression in carp head kidney macrophages upon chemokine stimulation.

Stimulation	CXCa_L1		CXCL8_L2		CXCb		LPS	
	4 hrs (n=3)	20 hrs (n=6)	4 hrs (n=5)	20 hrs (n=5)	4 hrs (n=3)	20 hrs (n=2)	4 hrs (n=5)	20 hrs (n=8)
pro-inflammatory cytokines								
IL-1β	1.29 ± 0.32	**3.28 ± 1.06****	1.12 ± 0.42	**1.34 ± 0.32***	0.92 ± 0.41	1.01 ± 0.16	**12.94 ± 10.57***	**17.84 ± 6.03****
TNF-α	1.09 ± 0.38	1.65 ± 1.39	1.22 ± 0.55	5.79 ± 4.53	0.89 ± 0.44	2.83 ± 1.71	3.38 ± 3.03	6.23 ± 8.33
chemokines								
CXCa_L1	1.62 ± 0.46	**2.12 ± 0.83***	1.04 ± 0.33	**1.38 ± 0.20****	1.04 ± 0.43	**1.26 ± 0.01***	2.54 ± 2.15	**4.86 ± 4.11***
CXCL8_L2	**2.26 ± 0.42***	**3.06 ± 1.59***	1.25 ± 0.38	1.34 ± 0.78	0.94 ± 0.25	0.70 ± 0.48	**5.18 ± 2.11****	**2.33 ± 1.09****
CXCb	0.82 ± 0.38	2.24 ± 1.95	1.01 ± 0.54	**4.24 ± 2.37***	0.82 ± 0.38	4.03 ± 0.98	1.38 ± 1.53	1.13 ± 0.54
chemokine receptors								
CXCR1	1.12 ± 0.35	2.16 ± 2.47	0.95 ± 0.29	1.34 ± 0.78	0.89 ± 0.21	2.49 ± 1.73	0.88 ± 0.07	1.51 ± 1.12
CXCR2	0.92 ± 0.21	2.20 ± 2.69	0.95 ± 0.32	2.28 ± 2.37	0.95 ± 0.38	3.71 ± 2.91	1.11 ± 0.55	2.46 ± 3.01
anti-inflammatory cytokine								
IL-10	1.09 ± 0.55	1.28 ± 1.03	0.82 ± 0.30	3.18 ± 2.91	1.00 ± 0.44	1.93 ± 1.13	3.06 ± 2.43	3.61 ± 3.84

Macrophages were stimulated for 4 hrs and 20 hrs with 2000 ng/ml CXCa_L1, CXCL8_L2, CXCb, LPS (30 µg/ml), or non-stimulated. Cytokine gene-expression was determined by quantitative RT-PCR and fold increase was determined relative to non-stimulated cells, standardized for the housekeeping gene 40S ribosomal protein s11. Average ± SD are indicated, significance was determined by a student t-test and significant fold increases are indicated in bold. *, $p<0.05$, **, $p<0.01$.

In vivo i.p injection of recombinant carp CXCa_L1, CXCL8_L2 and CXCb recruits leukocytes to the peritoneal cavity

Carp individuals were i.p injected with recombinant carp chemokines. A statistically significant influx of total PTLs was observed at 24 hrs and 48 hrs, in animals injected with the high dose (100 ng) of CXCa_L1 or CXCL8_L2 but not CXCb (Fig. 4a). Administration of heat-inactivated proteins did not increase PTL influx. CXCa_L1 (10 ng) significantly increased the number of PTLs at 48 hrs. Zymosan injection resulted in significant leukocyte recruitment already at 6 hrs, and was significant at 48 hrs p.i.

To further determine the cellular subsets migrating to the peritoneal cavity upon chemokine injection, numbers of morphologically distinct phagocytes were determined. Both doses of CXCa_L1 induced increased phagocyte numbers at 24 and 48 h p.i. However the 100 ng heat-treated protein also induced phagocyte influx (Fig. 4b). Statistically significant increase of phagocyte number was also observed in fish treated with CXCL8_L2, but not in animals injected with heat-inactivated protein. CXCb injection induced slight influx of phagocytes. Values were significant at 24 hrs (100 ng) and at 6 and 48 hrs (10 ng) Administration of zymosan resulted in significantly increased phagocyte numbers at 24 and 48 h.p.i.

Determination of the percentage of MPO+ cells showed for all three chemokines an early neutrophil recruitment (Fig. 4c). Again in comparison to the effect of CXCb, the two CXCL8-like proteins were most potent. The percentage of neutrophilic granulocytes increased at 6 h.p.i. with both doses of CXCa_L1 and in carp treated with the lower dose of CXCa_L1 it remained elevated at 24 hrs. Significant increase of neutrophils was also observed at 6 and 24 hrs upon CXCL8_L2 injection (100 ng) and at 6 hrs upon

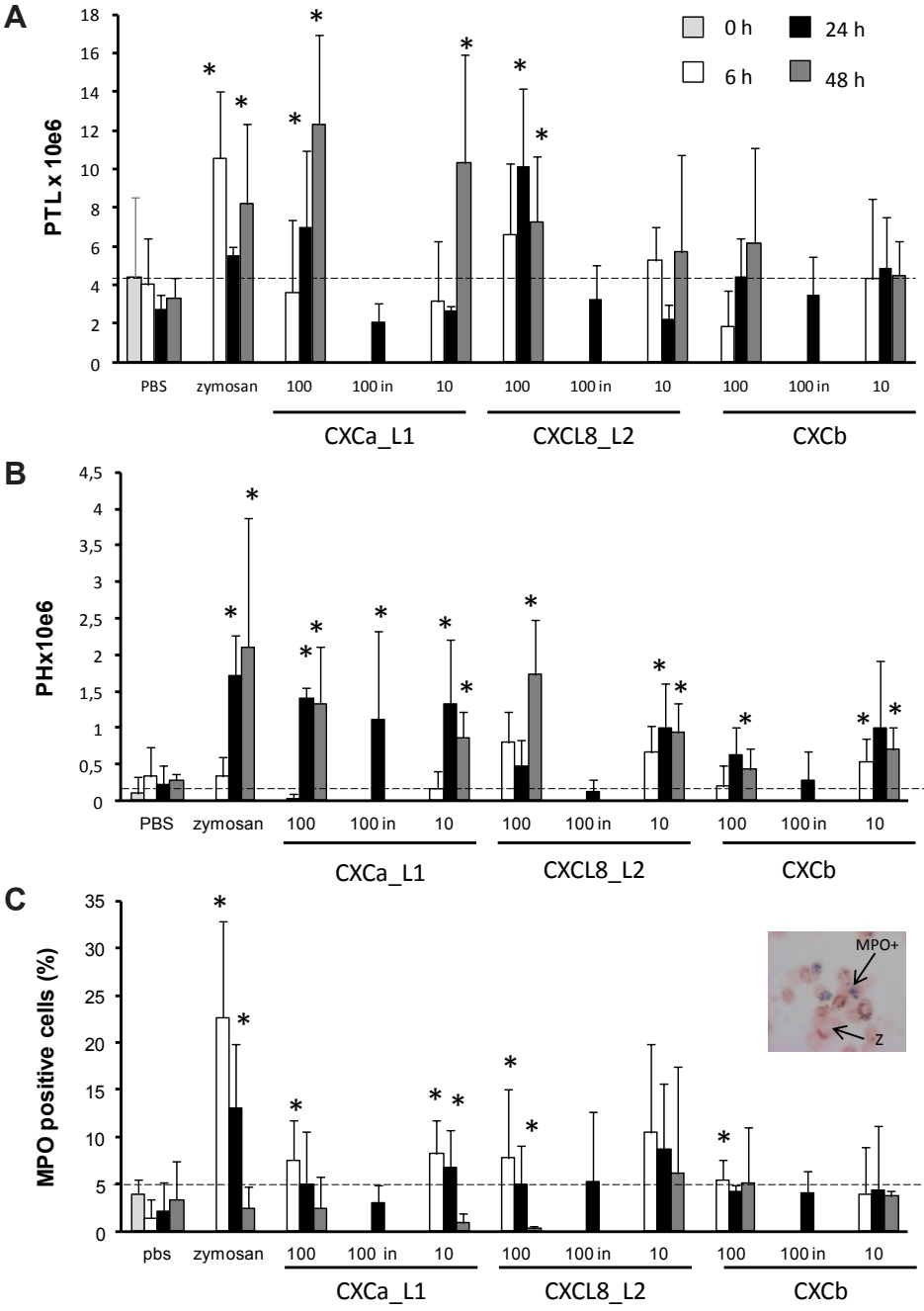
A
PTL x 10e6
0 h
24 h
6 h
48 h
PBS
zymosan
100
100 in
10
CXCa_L1
CXCL8_L2
CXCb
B
PHx10e6
C
MPO positive cells (%)
pbs
MPO+
Z

Fig. 4 (left page). Kinetics of peritoneal leukocyte influx after *in vivo* i.p. injection of recombinant carp chemokines: CXCa_L1 CXCL8_L2 and CXCb and heat-inactivated chemokines (in, 100 ng), PBS or zymosan (1 mg). Cell numbers were determined for total peritoneal leukocytes (PTL) (**A**), phagocytes (PH) (**B**), or myeloperoxidase-positive neutrophillic granulocytes (MPO) (**C**). Insert in (**C**): Neutrophils were identified as cells containing MPO positive stained granulas (MPO+), for zymosan injected fish, phagocytosed zymosan particles were observed (Z). Average (n=5) and SD are indicated, significance was determined by a student t-test by comparison with PBS injected fish. *, $p < 0.05$.

administration of 100 ng CXCb. Heat-inactivated protein did not change the proportion of neutrophilic granulocytes among PTL's, while zymosan injection significantly stimulated neutrophil migration to the peritoneum at 6 and 24 hrs p.i.

To confirm the actual inflammatory response induced by the chemokine attracted phagocytes, expression profiles for the pro- and anti-inflammatory genes in the attracted leukocytes were determined for the pro-inflammatory mediators (IL-1β, TNF-α, iNOS), CXC chemokines (CXCa_L1, CXCL8_L2 and CXCb), chemokine receptors (CXCR1 and CXCR2), and the anti-inflammatory cytokine (IL-10) (table 3). Although RNA yields were low for certain samples, changes in gene expression could be observed. Administration of CXCa_L1 (100 ng/ml) increased the relative expression of CXCL8_L2 at 24 h. CXCb expression increased later, at 48 h post-injection. Injection of CXCL8_ L2 stimulated gene expression of TNF-α at 24 h (5.5-fold increase) and 48 h (3-fold increase) as well as CXCL8_L2 (30-fold increase) at 24 h. At 48 h p.i. of CXCb, increased gene expression of IL-1β and CXCL8_L2 was observed.

Table 3. Gene expression in total peritoneal leukocytes (PTLs) upon i.p. administration of 100 ng of recombinant CXCa_L1, CXCL8_L2, CXCb, or zymosan in adult carp.

administration	CXCa_L1		CXCL8_L2		CXCb		zymosan	
	24 hrs (n=4)	48 hrs (n=2)	24 hrs (n=2)	48 hrs (n=3)	24 hrs (n=2-3)	48 hrs (n=2)	24 hrs (n=3-4)	48 hrs (n=2)
pro-inflammatory cytokines								
IL-1β	2.51 ± 2.77	1.49 ± 0.59	14.98 ± 8.39	1.54 ± 0.76	0.88 ± 0.36	5.90 ± 1.15	4.26 ± 1.97	3.79 ± 2.11
TNF-α	1.50 ± 0.99	3.68 ± 1.31	5.52 ± 0.78	3.03 ± 1.02	2.09 ± 0.92	4.53 ± 1.92	10.19 ± 9.11	7.18 ± 8.70
iNOS	1.08 ± 0.42	0.86 ± 0.11	2.95 ± 1.31	0.86 ± 0.24	2.77 ± 0.88	1.83 ± 0.79	15.03 ± 13.5	6.50 ± 6.99
chemokines								
CXCa_L1	2.30 ± 1.23	0.98 ± 0.24	7.14 ± 2.96	1.35 ± 0.57	1.52 ± 0.50	2.80 ± 0.66	3.50 ± 1.74	2.29 ± 0.09
CXCL8_L2	5.63 ± 2.37	5.90 ± 4.33	30.20 ± 2.75	4.83 ± 2.67	1.91 ± 0.16	17.68 ± 0.87	16.57 ± 7.30	15.59 ± 7.16
CXCb	1.27 ± 0.47	4.96 ± 0.97	1.78 ± 0.53	1.08 ± 0.77	3.32 ± 3.11	8.23 ± 6.01	3.80 ± 2.15	8.56 ± 8.87
chemokine receptors								
CXCR1	0.74 ± 0.12	0.71 ± 0.22	1.12 ± 0.52	0.89 ± 0.45	1.92 ± 1.29	1.24 ± 0.47	2.39 ± 1.46	2.35 ± 1.57
CXCR2	2.26 ± 1.08	4.56 ± 1.44	1.84 ± 0.32	1.71 ± 0.18	3.51 ± 2.92	2.61 ± 2.25	4.77 ± 2.60	3.45 ± 0.41
anti-inflammatory cytokine								
IL-10	0.31 ± 0.11	0.74 ± 0.33	0.41 ± 0.15	0.45 ± 0.08	1.00 ± 0.57	0.43 ± 0.03	1.02 ± 0.18	3.31 ± 1.64

Cytokine gene-expression was determined by quantitative RT-PCR for PTLs collected at 24 and 48 h.p.i. Fold increase was determined relative to non-injected fish, standardized for the housekeeping gene 40S ribosomal protein s11. Average ± SD are indicated.

Stimulation with recombinant carp CXCa_L1 triggers intracellular calcium mobilization
In carp head kidney granulocytes In view of the potent pro-inflammatory activity of CXCa_L1 it was further investigated whether CXCa_L1 signals via G-protein coupled receptors that typically trigger intracellular calcium pathways. CXCLa_L1 induced a rapid, dose-dependent and transient increase in cytosolic calcium levels in granulocyte-enriched head kidney cell fractions (Fig. 5). The positive control with calcium ionophore induced a strong calcium mobilization response in granulocytes, and no increase in cytosolic calcium levels was detected upon PBS administration.

Discussion

The production of carp recombinant CXCb, combined with the earlier synthesized recombinant molecules for CXCa_L1 and CXCL8_L2 [34] offered us the unique possibility to now apply an integrated in vitro and in vivo approach to elucidate the physiological role of these CXC chemokine lineages in the innate immune response of teleost fish. We found that both CXCL8-like and CXCb proteins induced chemotaxis of macrophages and granulocytes in vitro. In addition, in vitro, CXCb also attracts cells from the lymphocyte/monocyte fraction. Interestingly CXCL8-like and CXCb chemokines also display differential capability to attract cells towards the focus of inflammation. Intraperitoneal administration of both CXCa_L1 and CXCL8_L2 induced influx of peritoneal leukocytes of which a significant proportion were myeloperoxidase-positive neutrophilic granulocytes. Both CXCL8-like chemokines also elevated *in vitro*

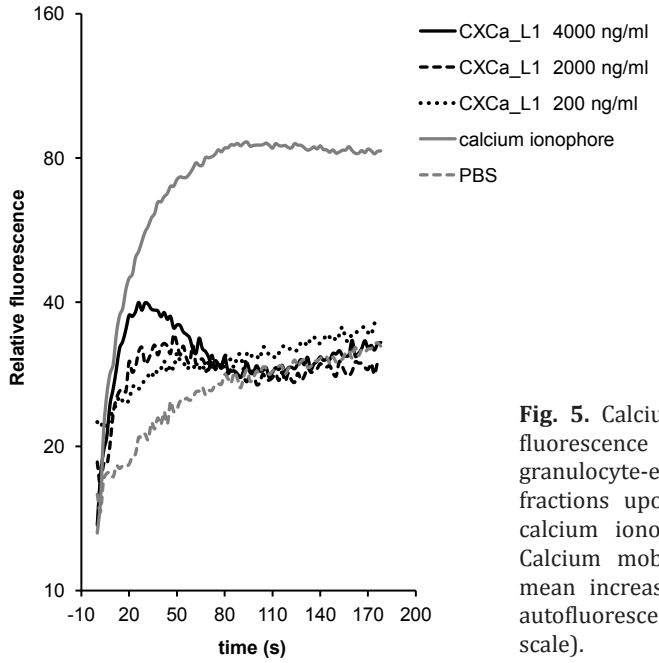

Fig. 5. Calcium mobilization, measured as fluorescence (516 nm) in Fluo-4 loaded granulocyte-enriched head kidney cell fractions upon stimulation with CXCa_L1, calcium ionophore (1.25 ng/µl) or PBS. Calcium mobilization is indicated as the mean increase in fluorescence relative to autofluorescence prior stimulation (log scale).

respiratory burst activity in carp phagocytes and slightly up-regulated the expression of pro-inflammatory cytokines and chemokines. In contrast, CXCb protein did not influence leukocyte respiratory burst in vitro. Also *in vivo*, CXCb was less potent to recruit leukocytes towards the peritoneal cavity. However, at later time points CXCb stimulated the expression of pro-inflammatory molecules. These timely differential expression and activity profiles indicate a different physiological role for chemokines that belong to the fish CXCL8-like and CXCb subsets.

CXC chemokines undoubtedly play a pivotal role in the regulation of leukocyte migration from the blood into the infected/damaged tissue during the inflammatory response. CXCL8 is synthesized during the early phase of inflammation and mainly attracts CXCR1 and/or CXCR2 expressing neutrophilic granulocytes [44-46], while CXCL9-11 chemokines are ligands for CXCR3. For carp, we already showed that during the early phase of inflammation, when a massive influx of mainly neutrophilic granulocytes is observed, both CXCa_L1 and CXCL8_L2 and CXCR1 are highly expressed at the inflammatory foci [34]. Moreover, granulocytes display high constitutive expression of the CXCR1 gene, which can be even upregulated upon LPS stimulation, presumably allowing rapid mobilization of these cells in response to infection [34]. The present data demonstrating early migration of MPO-positive cells after i.p. administration of CXCa_L1 or CXCL8_L2 protein corroborate the evidence for functional homology of the carp CXCL8-like chemokines to mammalian CXCL8. Furthermore, now we demonstrated for the first time for fish that, similar to mammalian chemokines [6-8, 44], also carp CXCa_L1 induces dose-dependent calcium mobilization in granulocytes in accordance to a specific signal transduction mediated via G-protein coupled CXC receptors.

In contrast to the early CXCL8-like expression, the expression of CXCb appears at later time points during in vivo inflammation, which suggests that CXCb recruits cells other than neutrophils [34]. This is supported by our present *in vivo* results, which show that although CXCb did not induce a substantial influx of total leukocytes, it did increase the ratio of monocytes/macrophages over neutrophilic granulocytes, as the CXCb attracted phagocytes are mainly MPO negative. Although in mammals it has been well established that the CXCL9-11 chemokines recruit mainly activated T cells expressing CXCR3 [13], expression of CXCR3 is also reported for monocytes. Moreover, mammalian CXCL9-11 chemokines, as for example CXCL10/IP-10, promote monocyte/macrophages recruitment [47]. Interestingly, the CXCR3 genes of zebrafish (CXCR3.2) and medaka (CXCR3a) are specifically expressed by macrophages [48, 49] and in rainbow trout, CXCR3 is expressed in head kidney macrophages upon stimulation with macrophage-colony stimulating factor 1 (MCSF1) [50]. Recently, a CXCR3 gene has been identified for common carp which showed constitutive expression in different leukocyte populations, but among them macrophages (in preparation). This could explain the responsiveness of fish monocytes/macrophages towards CXCb although signaling of CXCb through CXCR3 has not yet been established.

Macrophages are involved in all steps of the inflammatory response: detection of pathogens and tissue damage, cell activation and migration, pathogen eradication and resolution of inflammation. Different macrophage M1 and M2 subsets, activated by different stimuli, mediate the different steps of the inflammatory response (reviewed

by [51]). The M1 subset produces pro-inflammatory mediators (IL-1β, TNF-α, IL-6, iNOS) and reactive oxygen species, while the M2 subsets express anti-inflammatory cytokines (IL-10, TGF-β, arginase 2) and factors that stimulate tissue repair. Also for carp macrophages, a similar polarization profile has been observed [52]. In addition, both CXCL9-11 expression in mammalian macrophages and CXCb expression in carp phagocytes, are inducible by IFN-γ in synergy with LPS, typical stimuli for M1 polarization of macrophages [11] [36]. Therefore we hypothesize that M1 macrophages are the main source of CXCb. During zymosan-induced peritonitis, the peak of CXCb gene expression (at 48 hrs post zymosan administration) indeed coincides with the peak of expression of iNOS [35]. From this moment onward, also gene expression of IL-10 is increasing (48-168 hrs). Interestingly, our preliminary data for CXCR3 showed high gene expression during the late phase of inflammation (96 hrs) (in preparation). Altogether this suggests that CXCb is involved in the attraction of, or polarization to M2 macrophages.

At present, functional data on fish CXC chemokines are limited, but chemotactic activity has been reported for several fish CXCL8-like chemokines, orthologs of the cyprinid CXCa_L1 chemokine [25, 27, 34, 53, 54]. For example, recombinant trout IL-8 specifically attracted the monocyte-like, but not the macrophage-like subpopulation of RTS11, a rainbow trout monocyte/macrophage-like cell line. Moreover, trout IL-8 slightly induced gene expression of chemokines CK-6 and IL-8 and the pro-inflammatory cytokines IL-1β and TNF-α [54]. Similar effects of recombinant IL-8 on IL-1β and IL-8 gene expression were observed in half-smooth tongue sole (Cynoglossus semilaevis) [27]. Surprisingly, while trout IL-8 had no effect on the respiratory burst of RTS11, it did induce superoxide production in trout head kidney cells [53, 54]. This is corroborated by our data; CXCL8-like chemokines stimulated production of reactive oxygen species and slightly up-regulated expression of IL-1β and CXCL8-like chemokine genes in carp head kidney phagocytes. In vivo, the chemoattractant capacity of trout IL-8 was first demonstrated indirectly by showing massive neutrophil infiltration upon intraperitoneal injection of a trout IL-8 encoding expression vector [55]. Direct evidence of an in vivo function of IL-8 was provided by Omaima Harun *et al.* [53]. They showed that intraperitoneal administration of recombinant trout IL-8 had a clear dose-dependent effect on total leukocyte number in the peritoneal cavity. Neutrophils were the dominant cell type recruited, especially at higher IL-8 concentrations. It thus appears that the pro-inflammatory chemokinetic and chemotactic activity observed for the carp CXCL8 lineages is probably common for the fish CXCL8-like chemokines.

In conclusion, although previous phylogeny studies did not confirm true orthology of the carp CXCL8 lineages and CXCb with mammalian CXCL8 and CXCL9/10/11, respectively, our data presented here strongly indicate that they are functional homologs. We showed a pro-inflammatory activity for the CXCL8 lineages with strong chemotactic activity on phagocyte recruitment. We hypothesize that CXCb has a physiological role in inflammation, enabling the polarization of macrophages from the M1 towards the M2 subset. Although receptors for fish chemokines can be predicted based on known mammalian ligand-receptor relationships and fish expression data, future research should focus on functional studies to demonstrate chemokine–chemokine receptor

specificity.

Acknowledgements

We like to thank Edwin Tijhaar for his guidance in recombinant protein production, Anja Taverne-Thiele, Maria de Boer, Marleen Scheer and Loes Wiersma for technical assistance. This work was supported by Wageningen University, the Institut National de la Recherche Agronomique (LA), by ZONMW grant 85500007 (LG) and by FP7-People-ERG-2008-228583 and MNiSW/999/7.PR/UE/2009/7 (MC).

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La science décrit la nature,
la poésie la peint et l'embellit.

- Buffon -

Chapter 7

Diversification of IFN-γ inducible CXCb chemokines in cyprinid fish

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Abstract

We earlier identified two CXCL8-like lineages in cyprinid fish, which are functional homologues of the mammalian CXCL8, but with diverged functions. We here investigated whether the carp IFN-γ-inducible CXCb gene, that is related to the mammalian CXCL9, CXCL10 and CXCL11 chemokines, has undergone a similar diversification. On the zebrafish genome, a cluster of seven CXCb genes was found on chromosome five. Analysis of the promoter of the zebrafish CXCb genes suggests a partially shared, but differential induction. A second CXCb gene, CXCb2, was identified in common carp by homology cloning. CXCb2 is constitutively expressed in immune-related tissues, predominantly by head kidney lymphocytes/monocytes. Interestingly, a synergistic induction of CXCb2 gene expression with recombinant carp IFN-γ2 and LPS was observed in lymphocytes, macrophages and granulocytes. Finally, difference in sensitivity to LPS, and kinetics of CXCb1 and CXCb2 gene expression during zymosan-induced peritonitis, was observed. These results indicate a functional diversification for cyprinid CXCb chemokines, with functional homology to mammalian CXCL9-11.

Introduction

Chemokines are low molecular weight proteins and belong to a large protein family that regulates cell trafficking. Based on a cysteine-motif at the N-terminal side of the protein, chemokines are classified into four groups in mammals, CXC, CX3C, CC and C, whereby in zebrafish an additional fifth group, CX, is found [1, 2]. Chemotaxis is induced upon binding of chemokines to chemokine receptors, CXCR, CX3CR, CCR, CR respectively. These are seven-transmembrane G-protein coupled receptors (GPCRs) that upon activation induce intracellular calcium mobilization and subsequent reorganization of the cytoskeleton [3]. A characteristic of chemokines is their promiscuity and redundancy: one ligand may bind multiple receptors and one receptor may share multiple ligands [4]. This is reflected by their genomic organization: many chemokine and chemokine receptor genes are localized in (mini)clusters on the genome, a result of repeated (tandem) duplication events. Ligands located in one cluster often share receptors. Next to multiple duplication events, chemokines are subject to high selection pressures towards diversification and have evolved to a family that can direct migration of multiple cell types [4, 5]. Chemokines have multiple biological roles and are implicated in development, immunity and cancer [6-10].

In immunity, chemokines play an important role in the induction and regulation of inflammation, whereby they orchestrate trafficking of specific subsets of leukocytes to the site of inflammation in a spatial and temporal manner. For CXC chemokines, in total sixteen genes are described in humans [4]. CXCL8, or IL-8, was the first chemokine to be identified and directs migration of neutrophils and monocytes [11-13]. A second group of CXC chemokines implicated in inflammation is formed by CXCL9, -10 and 11. Human CXCL9-11 genes are localized in a mini-cluster on chromosome four [1, 4]. These three chemokines are also known by their synonyms: monokine-induced by interferon-gamma (IFN-γ) (MIG/CXCL9), IFN-γ-inducible protein 10 (IP-10/CXCL10) and IFN-inducible T-cell alpha chemoattractant (I-TAC/CXCL11) [14-16]. As their names

indicate, the expression of all three genes is induced by IFN-γ, which is a type II IFN produced by natural killer (NK) and effector T cells. CXCL9-11 share the same receptor, CXCR3, which is expressed on activated T helper 1 CD4+ cells, effector CD8+ T cells, NK, NKT, plasmacytoid dendritic cells (pDCs) and monocytes/macrophages [17-20]. Spatial and timely differential expression patterns of CXCL9-11 fine-tune T-cell trafficking over the course of an immune response and all three chemokines are implicated in acute and chronic inflammation [17, 21, 22].

The chemokine family is estimated to have arisen around 650 million years ago with the emergence of vertebrates and lamprey is the first species in which chemokine genes have been identified [5, 23, 24]. Chemokines are fast evolving genes and few chemokine genes show true orthology between fish and mammals [25]. Among CXC chemokines, only CXCL12 and CXCL14, which both have evolutionarily conserved roles in development, are well conserved [9, 26]. In zebrafish (*Danio rerio*), for which the whole genome has been sequenced, a detailed screening enabled identification of the entire chemokine family of ligands and receptors [2, 5]. In common carp (*Cyprinus carpio*), like zebrafish belonging to the cyprinid lineage, but whose whole genome is not yet sequenced, currently six CXC chemokine genes are identified: two CXCL8-like genes, one CXCL9-11-like gene, two CXCL12 genes and one CXCL14 gene [26-30]. In addition, three CXC receptors were reported for common carp: CXCR1, CXCR2, and CXCR4 [25, 31] and we recently cloned CXCR3 (in preparation). In another cyprinid fish, grass carp (*Ctenopharyngodon idella*), CXCR3 and CXCR5 genes have been cloned [32, 33]. We and others earlier reported that the CXCL8-like genes in cyprinids form two distinct lineages: CXCLa_L1 and CXCL8_L2 [27, 28]. In carp, two CXCL8-like genes were cloned and in zebrafish we identified three CXCL8-like genes. As cyprinid CXCL8-like genes are no clear orthologs of mammalian CXCL8 genes, we earlier proposed to name fish CXCL8-like genes CXCa [30]. Despite not being clear orthologs, the carp CXCL8 lineages do appear functional homologs of mammalian CXCL8, as both genes are expressed early in inflammation and recombinant proteins are chemotactic for carp neutrophils and monocytes/macrophages both in vitro and in vivo. Moreover, we demonstrated that G-protein coupled CXCRs are involved in CXCa_L1 signal transduction, as it induced intracellular calcium mobilization. As the individual carp CXCL8 genes have distinct expression profiles in inflammation, they probably have distinct subfunctions in inflammation. [27, 34].

The carp CXCL9-11-like gene is the closest homolog of the mammalian CXCL9-11 genes, but in analogy to results earlier shown for CXCa and mammalian CXCL8, it could not be validated as a true ortholog of mammalian CXCL9-11 by phylogenetic analysis. We therefore named this gene CXCb [25]. We earlier showed that CXCb expression is inducible by recombinant carp IFN-γ2, that CXCb is expressed during the inflammatory response and demonstrated that recombinant CXCb is chemotactic for carp granulocytes, macrophages and lymphocytes/monocytes [27, 34, 35]. In search for a possible diversification of CXCb chemokines in cyprinids, we screened the zebrafish genome for CXCb related genes and found a cluster of CXCb genes on chromosome five. A second CXCb gene was cloned in carp that was named CXCb2. Based on an analysis of the zebrafish CXCb promoter regions and *in vitro* and *in vivo* gene expression studies of

carp CXCb1 and CXCb2 genes, we hypothesize that cyprinid CXCb genes have undergone functional diversification and display functional homology with mammalian CXCL9-11.

Material and methods

Bioinformatics, phylogeny and promoter in silico analysis
Carp CXCb1 (Acc nr. AB082985) was used as a query in BLAST search on the zebrafish genome assembly (Zv9, ENSEMBL www.ensembl.org). Zebrafish CXCb genes located on chromosome five corresponded with Ensembl gene identifiers ENSDARG00000095747 (CXCL-chr5b), ENSDARG00000092423 (CXCL-chr5g), ENSDARG00000094706 (CXCL-chr5h) and ENSDARG00000093779 (CXCL-chr5i); three genes (CXCL-chr5d/e/f) and two pseudogenes (CXCL-psi1-2) are not yet referenced in Ensembl. The Ensembl gene identifier for the non-CXCb related CXC chemokine CXCL-chr5c is ENSDARG00000075163,while CXCL-chr5a has not yet an Ensembl ID. BLAST searches against EST databases were performed on the website of NCBI (www.ncbi.nlm.nih.gov). Multiple sequence alignments were made with ClustalW within the MEGA5 software. Phylogeny trees were constructed with MEGA5 using Neighbor Joining (NJ; [36]).

The software tool Genomatix MatInspector Matrix library version 8.0 (www.genomatix.de) was employed for promoter analysis [37]. One kb DNA sequence upstream the predicted start codon was extracted for each studied CXC gene from the zebrafish or human genome assembly (ENSEMBL). DNA sequences were analyzed using the matrixes for general core promoter elements for vertebrates and enhancer elements were used specifically for nuclear factor kappa B/c rel (NF-κB), signal transducer and activators for transcription (STAT), interferon regulatory factors (IRFs) and activating protein-1 (AP-1). Matrix matches with a similarity score > 0.80 were accepted and relevant elements were selected.

CXCL accession numbers used for the tree construction or promoter analysis were as follows: carp Cyca_CXCa_L1 (CAD13189), Cyca_CXCL8_L2 (AB470924), Cyca_CXCb1 (AB08298), Cyca_CXCb2 (AB08298); CXCL8_L1_Chr1 (XM_001342570), CXCL8_chr17 (EST EH441857); channel catfish CXCL10 (AY335949); rainbow trout Onmy_vig7 (AF483527), Onmy_vig8 (AF483528), Onmy_gIP-10 (AJ417078.); mouse CXCL9 (NM_008599), CXCL10 (NM_021274), CXCL11 (AF179872) ; human CXCL1 (NM_001511), CXCL2 (NM_002089), CXCL3 (NM_002090), CXCL4 (NM_002619), CXCL5 (NM_002994), CXCL6 (NM_002993), CXCL7 (NM_002704), CXCL8 (P10145), CXCL9 (NM_002416), CXCL10 (NM_001565), CXCL11 (NM_005409), CXCL12 (NM_199168), CXCL13 (NM_006419) and CXCL14 (NM_004887); Zebrafish CXCL8_L1_Chr1 (XM_001342570), CXCL8_chr17 (EST EH441857). Zebrafish CXCb protein sequences were initially obtained from the supplementary data of Nomiyama *et al.* [2] derived from the Zv6 assembly, but were checked for congruence with the Zv9 assembly; three of these sequences are also available from GenBank: CXCL-chr5b (NP_001119885.1), CXCL-chr5d (XP_001339307), and CXCL-chr5f (XP_696046).

Cloning carp CXCb2: 5'RACE-PCR and 3'RACE-PCR
Two oligonucleotide primers, zfccCXC4fw1 and zfccCXC4rv1 (table 1) were designed

based on the zebrafish CXCL-chr5g sequence. As template for PCR, cDNA from total head kidney leukocytes stimulated with carp rIFN-γ2 [38] was used. A PCR reaction mixture was prepared with 5 µl of 10x Goldstar buffer (Eurogentec), 1.5 mM $MgCl_2$, 200 µM dNTPs, 1.25 units of Goldstar DNA Polymerase (Eurogentec), 200 nM forward primer, 200 nM reverse primer and filled up to 50 µl with H_2O. PCR, 30 cycles, were carried out under the following conditions: 94°C for 2 min, 94°C for 30s and 55°C for 30s and 72°C for 1 min, followed by 72°C for 10 min. A fragment of 152 bp was amplified and cloned into a pGEM-T easy vector (Promega) according the manufacturer's protocol and sequenced using the ABI prismBigDye Terminator Cycle Sequencing Ready Reaction kit and analysed using 3700 DNA analyser. Based on this sequence, new primers were designed for 5`RACE-PCR and 3`RACE-PCR, that were carried out with the Gene Racer™ RACE Ready cDNA kit with Superscript III RT (L1502-01, Invitrogen). For the 3`RACE-PCR reaction, RACE-ready cDNA template was prepared from carp kidney RNA according to the manufacturer's protocol. A first round of 3'RACE-PCR was carried out with the gene specific primer fwGSPcarpCXC4. A reaction mixture was prepared containing 5 µl of 10x Goldstar buffer, 1.5 mM MgCl2, 200 µM dNTPs, 1.25 units Goldstar DNA Polymerase (Eurogentec), 200 nM fwGSPcarpCXC4, 600 nM GeneRacer™ 3`Primer, filled to 50 µl with sterile H20. PCR conditions were: Hot start of 94°C for 2 min, 94°C for 30s and 60°C for 30s and 72°C for 60s, for 40 cycles, followed by 72°C for 10 min. The obtained 3'RACE-PCR product was subsequently amplified by a nested 3'RACE-PCR reaction. A similar 3'RACE-PCR reaction mixture was prepared as in the first round, containing fwGSPcarpCXC4nested as forward primer and GeneRacerTM 3' Nested Primer as reverse primer. For nested 3'RACE-PCR, conditions were: Hot start of 94°C for 2 min, 94°C for 30s and 65°C for 30s and 68°C for 30s for 30 cycles, followed by 68°C for 10 min. 3'RACE-PCR product was isolated from gel and cloned for sequencing.

For the 5`RACE-PCR reaction, RACE-ready cDNA template was prepared from spleen that was collected from a carp three weeks upon infection with the parasite *Trypanoplasma carassi*. RACE-ready cDNA was prepared according to the manufacter`s protocol with random primers. A first round of 5'RACE-PCR was carried out with the gene specific primer cycaCXCb2_rv1. A reaction mixture was prepared containing 5 µl of 10x Expand High Fidelity buffer, 1.5 mM $MgCl_2$, 200 µM dNTPs, 2.6 units Expand High Fidelity polymerase mix (Roche), 300 nM cycaCXCb2_rv1, 300 nM GeneRacer™ 5`Primer, 2 µl template, filled to 50 µl with sterile H20. PCR conditions were: Hot start of 94 °C for 2 min, 94 °C for 15s and 55 °C for 30s and 72 °C for 45s, for 35 cycles, followed by 72°C for 7 min. The obtained 5'RACE-PCR product was cloned for sequencing. The full length sequence was validated by an additional PCR reaction with high fidelity DNA polymerase (Expand high fidelity PCR system, Roche) and primers (see table 1) located on the start and stop codons.

Animals

Young individuals (6-9 months) of common carp Cyprinus carpio L (50-60 g b.w), from the "De Haar Vissen" facility in Wageningen (R3xR8) were reared at 23 oC in recirculating tap water [39]. Fish were fed pelleted dry food (Trouvit, Nutreco). All animals were handled in strict accordance with good animal practice as defined by the

Table 1. Primers used for cloning carp CXCb2

primer	Sense (5'-3') or antisense (5'-3') sequence	Step
zfccCXC4_fw1	5'-GTTTTTCTGGCGTGTCTGCT-'3	homology cloning
zfccCXC4_rv1	5'-TTCCACAAGATGGACTTGGA-3'	
Fw_GSPcarpCXC4	5'-GGAGTGGATGTGGTTTTACTGAAGAACGTTGA GAAGTTTGAAATCATCCC-'3	3'RACE-PCR
Fw_GSPcarpCXC4nested	5'-GGAGTGGATGTGGTTTTACTGA-'3	
cycaCXCb2_rv1.1	5'-CGTTCTTCAGTAAAACCACA-3	5'RACE-PCR
cycaCXCb2_fw1	5'- CAGTCTTAGACAATCATCAACACTT -3'	validation
cycaCXCbrvc_rv1.2	5'- ACACTACTCTGATTTCATGAGC -3'	

relevant national and/or local animal welfare bodies, and all animal work was approved by the appropriate committee.

Tissue and section preparation
Fish were anaesthetised with 0.2 g/l tricaine methane sulphonate (TMS, Cresent Research Chemicals, Phoenix, AZ, USA) buffered with 0.4 g/l NaHCO3 (Merck, Darmstadt, Germany). Organs and tissues for RNA extraction were carefully removed, snap frozen in solid CO_2 or liquid N2 and stored at -80°C. Isolation of head kidney leukocytes Head kidney cell suspensions were obtained by passing the tissue through a 50 µm nylon mesh with cRPMI (RPMI 1640, Lonza, Belgium, adjusted to carp osmolarity of 270 mOsm/kg with distilled water) containing 10 IU/ml heparin (Leo Pharmaceutical Products Ltd., Weesp, the Netherlands) and washed once. This cell suspension was layered on a discontinuous Percoll (Amersham Biosciences, Piscataway, NJ) gradient (1.02, 1.060, 1.070, and 1.083 g/cm^3) and centrifuged for 30 min at 800 g with the brake disengaged. Cells from the density range of 1.020 – 1.060 g/cm^3 (predominantly, >80% monocytes/lymphocytes), the range of 1.060 - 1.070 g/cm^3 (predominantly macrophages, but also monocytes, lymphocytes and some (~10%) granulocytes), the range from 1.070 to 1.083 g/cm^3 (~80% neutrophilic granulocytes) [40] or combined 1.060 – 1.083 g/cm^3 fractions (enriched phagocytes) were collected, washed, and seeded at 5 × 10^6 cells per well (in a volume of 900 µl) in a 24-well cell culture plate at 27°C, 5% CO_2 in cRPMI^{++} (cRPMI supplemented with 0.5% (v/v) pooled carp serum, 1% L-glutamine (Merck, Whitehouse Station, NJ), 200 nM b-mercaptoethanol (Bio-Rad, Hercules, CA), 1% (v/v) penicillin G (Sigma–Aldrich, St. Louis, MO), and 1 % (v/v) streptomycin sulphate (Sigma–Aldrich, St. Louis, MO).

In vitro cell stimulation
Leukocytes were incubated 4 hrs with lipopolysaccharide (LPS, 30 µg/ml, *E. coli* serotype O55: B5, Sigma–Aldrich, St. Louis, MO), recombinant IFN-γ2 (100 ng/ml [35]), or co-incubated with LPS and IFN-γ2. Control cells received RPMI only. Cells were collected in RLT buffer and stored at -80°C before proceeding with RNA isolation.

Zymosan-induced peritonitis
The animals were i.p. injected with freshly prepared zymosan A (2 mg/ml, 1 ml /50 g b.w., Sigma, Z) in sterile PBS (270 mOsM) or with sterile PBS only (control group). At selected time points animals were sacrificed and their peritoneal cavities were washed with 1 ml of ice cold PBS. Peritoneal leukocytes were centrifuged for 10 min at 800 g at 4°C and frozen in liquid nitrogen and stored at -80°C [41].

RNA isolation and first strand cDNA synthesis
RNA was isolated using an RNeasy Mini Kit (Qiagen, Valencia, CA) according the manufacturer's protocol. Final elution was carried out in 30 µl of nuclease-free water, to maximize the concentration of RNA. RNA concentrations were measured by spectrophotometry and integrity was ensured by analysis on a 1% agarose gel before proceeding with cDNA synthesis. For each sample a non–RT (non-reverse transcriptase) control was included. Two µl of 10xDNase I reaction buffer and 2 µl DNase I (Invitrogen) was added to 2 µg total RNA and incubated for 15 min at room temperature. DNase I was inactivated with 25mM EDTA (2 µl, 65°C, 10 min). To each sample, 2 µl random primers and 2 µl 10mM dNTP mix were added, and the mix was incubated for 5 min at 65°C and then 1 min on ice. After incubation, to each sample 8 µl 5x First Strand buffer 2 µl 0.1 M dithiothreitol (DTT) and 2 µl RNase inhibitor were added. To 19 µl from each sample (but not to the non-RT controls) 1 µl Superscript RNase H-Reverse Transcriptase (RT, Invitrogen) was added and reagents were incubated for 5 min at 25°C, then spun briefly and incubated 60 min at 50°C. Reactions were inactivated 15 min at 70°C. Samples were set at 100 µl with demineralized water and stored at -20°C until future used.

Real-time quantitative PCR
PRIMER EXPRESS software (Applied Biosystems) was used to design primers for use in real-time quantitative PCR. Carp-specific primers (5' to 3') were designed for chemokines CXCb1 and CXCb2. The 40S ribosomal protein s11 gene served as an internal standard (accession numbers and primer sequences are listed in Table 1). For RQ-PCR 5 µl cDNA and forward and reverse primers (4.2 µM each) were added to 7 µl Absolute QPCR SYBR Green Mixes (ABgene). RQ-PCR (15 min at 95°C, 40 cycles of 15 s at 94°C, 30 s at 60°C, and 30 s at 72°C, followed by 1 min at 60°C) was carried out with a Rotorgene 2000 realtime cycler (Corbett Research, Sydney, Australia). Following each run, melt curves were collected by detecting fluorescence from 60 to 90°C at 1°C intervals.

Constitutive expression of CXCb1 and CXCb2 genes was determined in various organs and tissues of four individual adult carp, and rendered as a ratio of target gene

Table 2. Primers used for gene expression studies

Gene	Sense (5'-3')	Antisense (5'-3')	Acc. no
40S	CCGTGGGTGACATCGTTACA	TCAGGACATTGAACCTCACTGTCT	AB012087
CXCb1	GGGCAGGTGTTTTTGTGTTGA	AAGAGCGACTTGCGGGTATG	AB08298
CXCb2	AGGCAGGTGCTTCTGTGCTGACA	TTCATGCATTTCCGCTCTGCGCT	JN104598

vs. reference gene (40S ribosomal protein s11 gene) calculated with the Pfaffl method [42], according to the following equation:

$$\text{ratio} = (E_{\text{reference}})^{\text{Ct reference}} / (E_{\text{target}})^{\text{Ct target}}$$

where E is the amplification efficiency and Ct is the number of PCR cycles needed for the signal to exceed a predetermined threshold value.

Expression following stimulation was rendered as a ratio of target gene vs. reference gene (40S ribosomal protein s11 gene) relative to expression in unstimulated control samples according to the following equation:

$$\text{ratio} = (\text{Etarget})^{\Delta \text{Ct target}\,(\text{control-sample})} / (E_{\text{reference}})^{\Delta \text{Ct reference}(\text{control-sample})}$$

Statistical analysis

Data were expressed as mean ± standard deviation (SD) and significance of differences was determined using student *t*-test.

Results

Identification of six zebrafish CXC genes in zebrafish related to carp CXCb

To investigate whether the CXCb subgroup has expanded in fish as found earlier for CXCL8-like genes, the zebrafish genome was screened for counterparts of carp CXCb. Using tBLASTn search, reliable hits were retrieved on chromosome five (see Fig 1. and table S1). Seven genes corresponded with genes earlier described by Nomiyama et al. [2] and that had been named CXCL5-chr5b, -d, -e, -f, -g, -h and –i. Three genes were earlier cloned by Chen *et al.* [43] and alternatively named: CXC-64 corresponding to CXCL-chr5b, CXC-66 corresponding to CXCL-chr5e and CXC-56 corresponding to CXCL-chr5f. In addition, two hits corresponded with pseudogenes that we named CXCL-chr5φ1 and CXCL-chr5φ2. No first exon could be identified for pseudogene CXCL5φ1, CXCL5φ2 contained a frameshift in the first exon and for both genes, no fourth exon could be identified. Six genes are located in two mini-clusters spanning a region of ~830 kb, and zCXCL5-chr5b is located ~3.300 kb upstream from this cluster. The amino acid sequences of these seven zebrafish CXCb genes were 32-99% similar (see Table S2a). Two other CXC genes were located within the CXC cluster on chromosome five that were also earlier described by Nomiyama *et al.* [2] and named CXCL-Chr5a and CXCL-Chr5c. Both CXCL-Chr5a and CXCL-Chr5c had low similarity (16-17% and 22-26% resp.) with the zebrafish CXCb related genes. BLASTp search with CXCL-Chr5a and CXCL-Chr5c, either with or without the leader peptide sequence, retrieved human CXCL13 and CXCL6 as most similar sequences for CXCL-Chr5a and human CXCL8, CXCL10 and CXCL11 were first hits for CXCL-Chr5c (see Table S2b-c).

Cloning of a second carp CXCb gene: CXCb2

To determine whether in carp multiple CXCb genes are present, primer sets were designed based on the zebrafish CXCb-related gene sequences and a PCR-product was

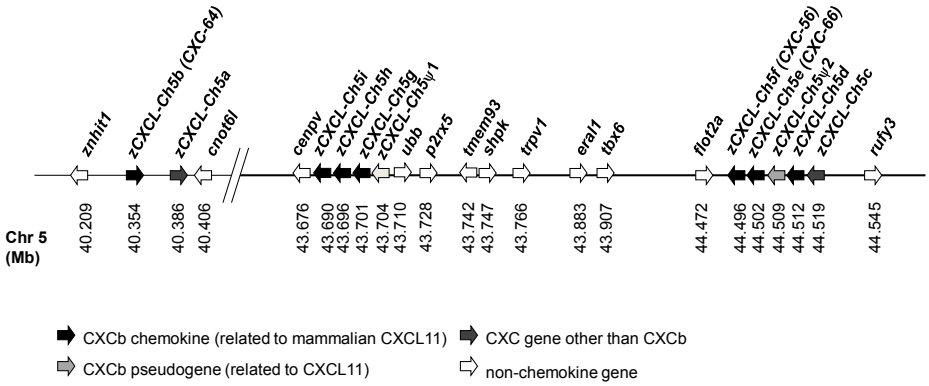


Figure 1. Genomic organization of zebrafish genes similar to carp CXCb1. Zebrafish genes related to carp CXCb1 were identified by tBLASTn and are all localized on chromosome five (Zv9 assembly) in a gene cluster earlier described by Nomiyama *et al.* [2]. The nomenclature provided by Chen *et al.* [43] in indicated in between brackets. Carp CXCb1 related genes are indicated by black arrows, the two CXCb-related pseudogenes in dark grey, and two non-related CXCb1 related chemokines in the gene cluster by light grey arrows. Non-chemokine genes located in the gene cluster are indicated in white.

```
CXCb1       ATGAAAATCATTACCGCTGTGGTTATTCTCGGCTGCCTGCTTGTTGTAGAGGTGAAAGGC 60
CXCb2       ATGAAGACTGCCACAGTTTTTGTTTTTCTTGCCTGTCTGCTTGCAACAGAAGTACAAGGG 60
CXCb1        M  K  I  I  T  A  V  V  I  L  G  C  L  L  V  V  E  V  K  G
CXCb2        M  K  T  A  T  V  F  V  F  L  A  C  L  L  A  T  E  V  Q  G

CXCb1       CAGGCTAGAGCTCCAAAGGGCAGGTGTTTTTGTGTTGACAAAGGTGTGAACATGGTTCCT 120
CXCb2       CAGAACAAGTTTCACAAAGGCAGGTGCTTCTGTGCTGACAAAGGAGTGGATGTGGTTTTA 120
CXCb1        Q  A  R  A  P  K  G  R  C  F  C  V  D  K  G  V  N  M  V  P
CXCb2        Q  N  K  F  H  K  G  R  C  F  C  A  D  K  G  V  D  V  V  L

CXCb1       CCAAAACAGATTGAGAAGGTCGAAATCATACCCGCAAGTCGCTCTTGTAAAACCCAGGAG 180
CXCb2       CTGAAGAACGTTGAGAAGTTTGAAATCATCCCTCCAAGTCCATCTTGTGGAAAACAGGAG 180
CXCb1        P  K  Q  I  E  K  V  E  I  I  P  A  S  R  S  C  K  T  Q  E
CXCb2        L  K  N  V  E  K  F  E  I  I  P  P  S  P  S  C  G  K  Q  E

CXCb1       GATTGTTGTTACTCTGAAAAATAGTACAGAGCAGAAATGCTTGAATCCAGAATCTAAATT 240
CXCb2       GATTATTGTCACTCTTAA---GAGCGCAGAGCGGAAATGCATGAATCCAGAGTCTAAATT 237
CXCb1        I  V  V  T  L  K  N  S  T  E  Q  K  C  L  N  P  E  S  K  F
CXCb2        I  I  V  T  L  K  -  S  A  E  R  K  C  M  N  P  E  S  K  F

CXCb1       CACGCAGAAATACATCATGAAGGCTGTTGAAAAGAGGAGCCTGCAGAAGAAATAA     294
CXCb2       CACCCAGAATTTAATCAAGAGGGCTCTTGAAAAGATTGCCCACCAGTAG------     285
CXCb1         T  Q  K  Y  I  M  K  A  V  E  K  R  S  L  Q  K  K
CXCb2         T  Q  N  L  I  K  R  A  L  E  K  I  A  H  Q
```

Figure 2. Alignment of carp CXCb1 and CXCb2 sequences. Alignment of nucleotide and deduced amino acid (underlined) sequences of carp CXCb1 and CXCb2 are 71% similar in nucleotide and 59% similar in amino acid sequence. Differences are boxed in black.

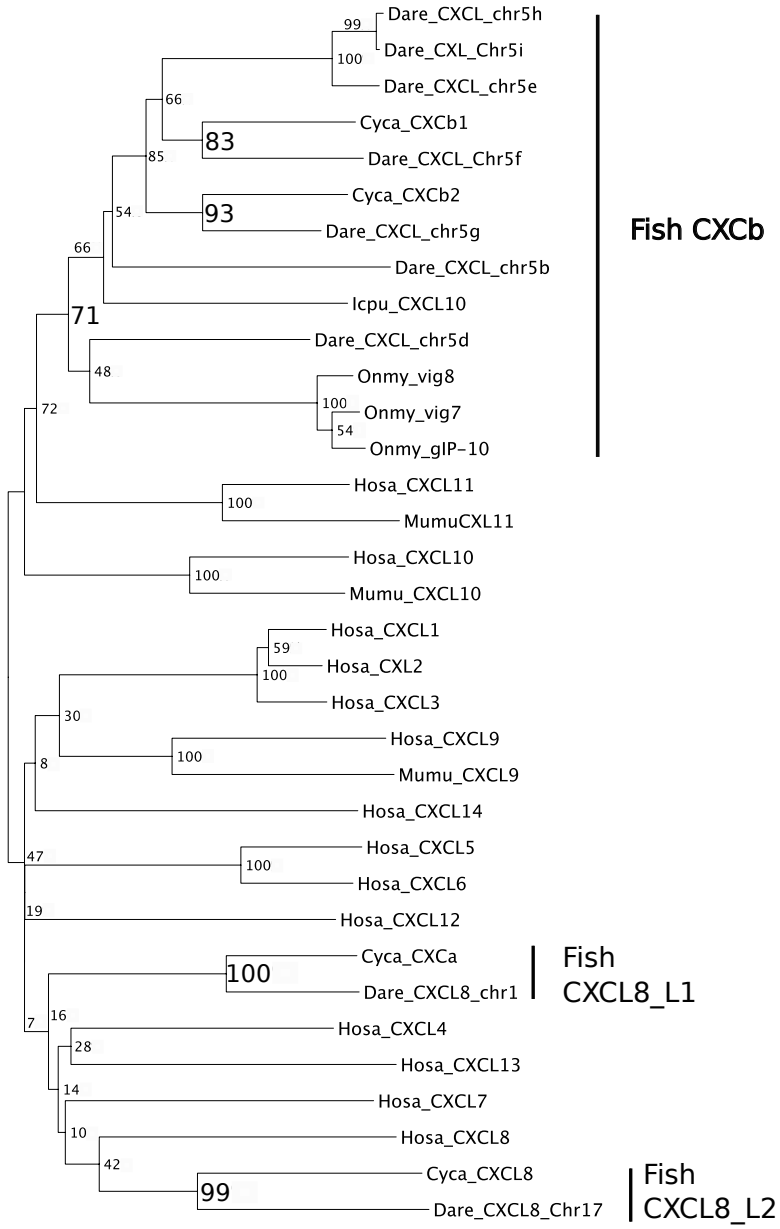


Fig 3. Fish CXCa and CXCb phylogeny. Phylogeny tree constructed from CXCL protein sequences constructed by Neighbor Joining (NJ) with partial deletion of gaps from the alignment. Relevant bootstrap (N=1000) values are indicated. Fish CXCb and CXCL8-like sequences are indicated by vertical lines.

obtained by RT-PCR with the primer set corresponding to CXCL5-chr5g. Followed by 5'RACE-PCR and 3'RACE-PCR, a second carp CXCb gene, CXCb2, was cloned. Carp CXCb1 and CXCb2 share 71% similarity in nucleotide and 59% in amino acid sequence (Fig 2). Phylogeny trees were constructed for fish and mammalian CXCL genes using the Neighbor Joining method (Fig. 3). The distance tree showed that both carp CXCb1 and CXCb2, and the seven zebrafish CXCb-related sequences, cluster in a fish CXCb branch with a bootstrap value of 71%, together with trout and channel catfish CXCL9-11 related sequences. Carp CXCb1 and zebrafish CXCL-chr5f cluster with a bootstrap value of 83 % and carp CXCb2 and zebrafish CXCL-chr5g cluster with a bootstrap value of 93%. The fish CXCb-related sequences are most similar to human and mouse CXCL11, with a bootstrap of 72%. Inclusion of CXC-chr5a and CXC-chr5c sequences, resulted in a tree with a low bootstrap value for the fish CXCb clusters (see Fig. S1). However, it could be observed that zebrafish CXC-chr5a clustered with human CXCL13, albeit with a low bootstrap value of 69%. CXC-chr5c did not cluster with any of the other sequences.

Promoter regions of zebrafish related CXCb genes contain binding elements for NF-κB, IRFs, AP-1 and STATs

Carp has two genes for IFN-γ and we previously demonstrated that carp CXCb1 gene expression is inducible in head kidney phagocytes by recombinant carp IFN-γ2, which is synergistic to LPS co-stimulation [35, 44]. To gain insight in the regulation of cyprinid CXCb gene expression, we performed an in silico analysis of the promoter regions of the seven zebrafish CXCb genes to search for transcription factor binding elements. We particularly searched for binding elements of transcription factors that are known to be activated upon IFN type I and II receptor binding, including interferon-regulatory factors (IRFs and ISRE), activator protein-1 (AP-1), nuclear factor κB (NF-κB) and activators of transcription (STATs). The software Matinspector was employed and matrix scores above 0.80 were accepted, to ensure inclusion of sites that might be not well conserved between fish and mammals. In fig. 4a results are shown for 1 kb regions upstream the start codon of each zebrafish CXCb-gene. Binding sites for IRFs (specific for IRF1, -2, -3, -4, -6, -7, or not IRF-type specified and ISRE,) were identified for all seven zebrafish genes, but differed in number and subtype. For AP-1, binding sites were identified for six genes, but not CXCL-chr5e. NF-κB binding sites were identified for all six genes, except CXCL-chr5g. STAT (STAT1, -5, -6 and not-STAT-type specified) binding sites were identified for all seven genes, with CXCL-chr5i containing six STAT binding sites. Interestingly, although the genes CXCL-chr5h and CXCL-chr5i encode proteins that are 99% identical, they have very different promoters. The promoter of the human CXCL9,-10 and -11 genes were included in the analysis for comparison (Fig 4b) and showed a different distribution of transcription factor binding sites among the three genes. Further, the outcome of the analysis was in agreement with the description of the promoter made by others [45].

The promoter of the chemokine non-related to CXCb, CXC-chr5a and CXC-chr5c, were also analyzed and compared with the promoter of human CXCL13 (Fig 4c). The promoter of CXCL-chr5a contained binding sites for STAT, IRFs and AP-1, but not NF-κB. CXCL5-chrc did not contained one binding site for NF-κB, and binding sites for STAT and

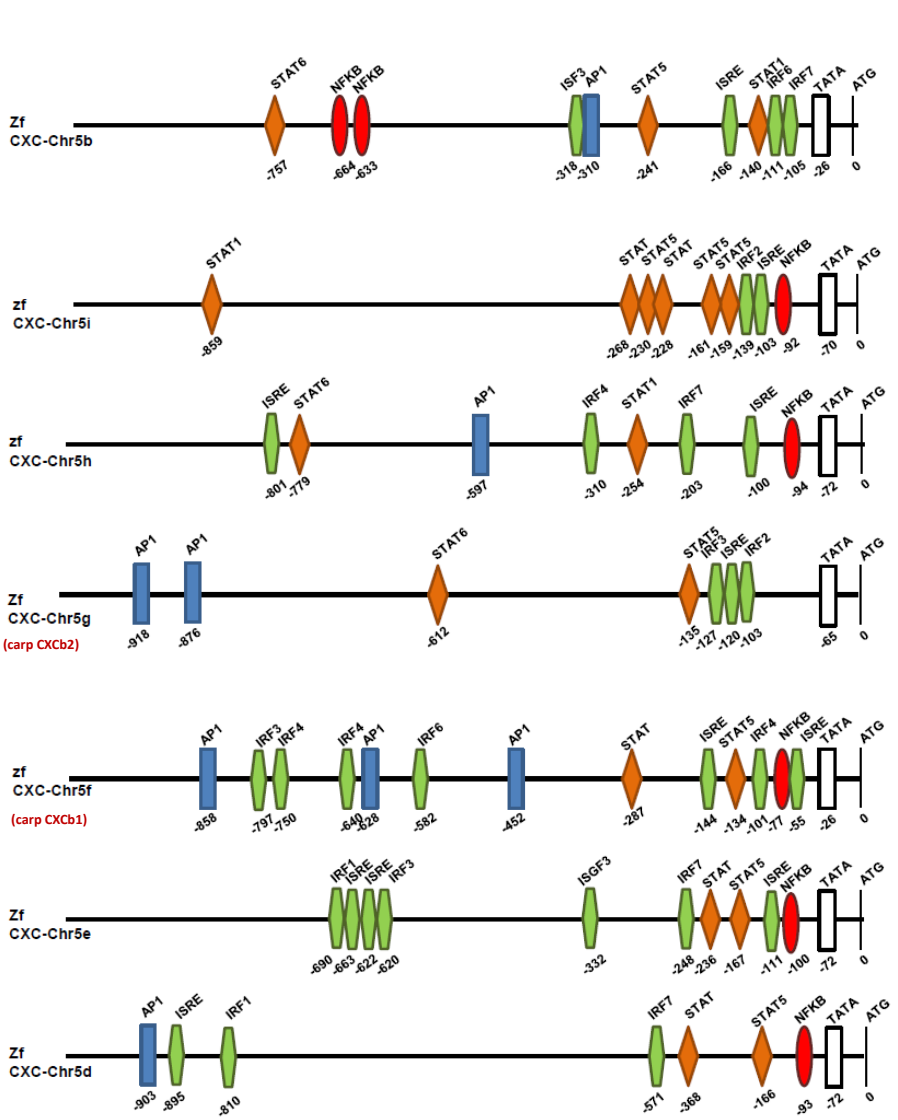
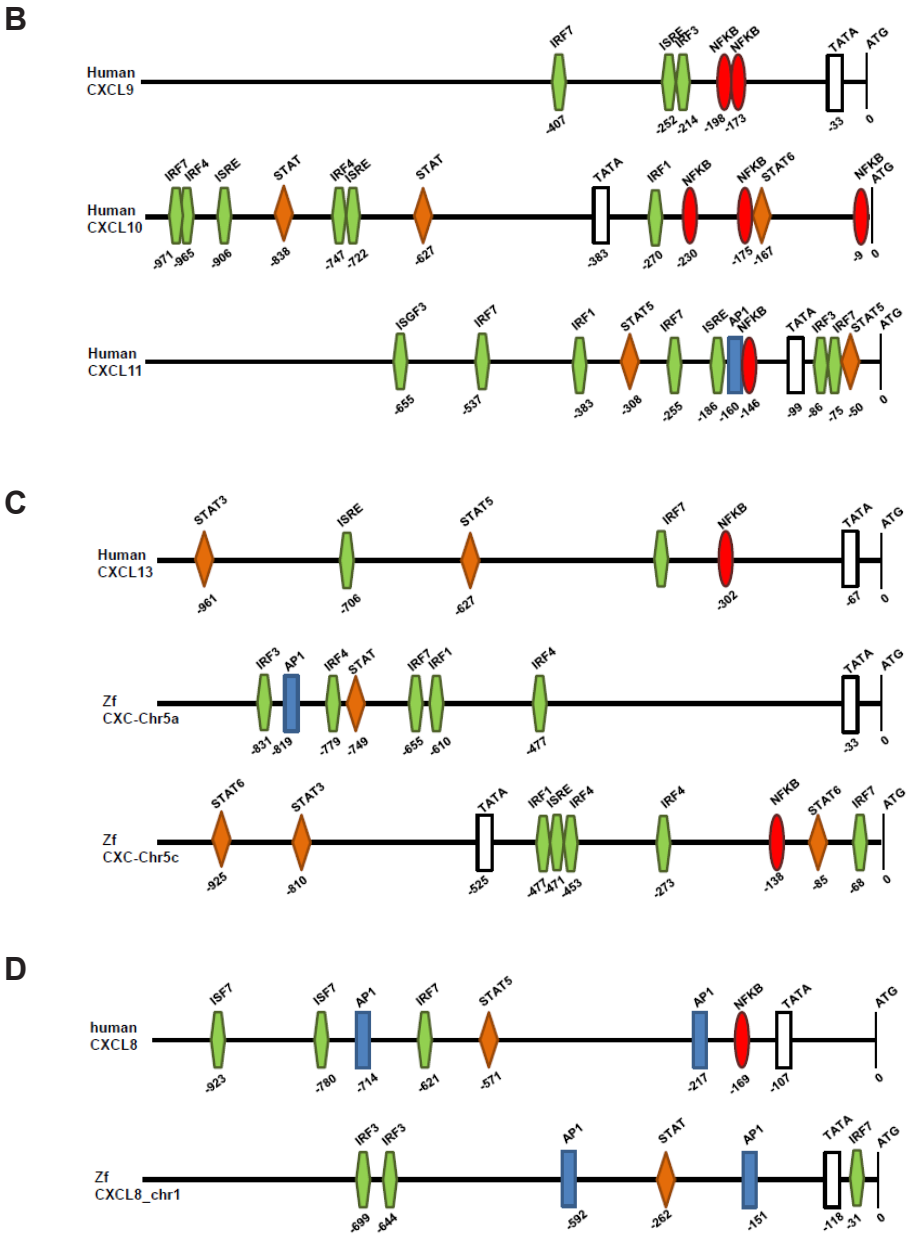


Figure 4. Promoter analysis of zebrafish CXC genes related to carp CXCb. Representation of transcription factor binding sites on the promoter region of (**A**) the individual zebrafish CXCb genes, (**B**) human CXCL9, CXL10 and CXCL1, (**C**) human CXCL13 and zebrafish CXC-chr5a and CXC-chr5c and (**D**) human CXCL8 and zebrafish CXCL8_L1_Chr1. Relevant enhancer elements were identified by Matinspector. Numbers indicate the distance from the last nucleotide position of each element relative to the start codon (position 0). When multiple TATA boxes were identified, only the one most proximate to the start codon is shown. ISRE: interferon response element;



AP1, activator protein 1; IRF, interferon regulatory factor; NF-κB, nuclear factor κ B; STAT, signal transducer and activator of transcription. As a reminder, it is indicated in between brackets that carp CXCb1 is most similar to zebrafish CXC-chr5f and carp CXCb1 is most similar to zebrafish CXC-chr5g.

and brain. Expression was high for both CXCb1 and CXCb2 in immune related tissues, but low in skin, PBL, heart, ovaria and testis. Constitutive gene expression of CXCb1 and CXCb2 was determined in different leukocyte cell fractions of head kidney (Fig. 4b). High expression was observed for both genes in monocyte/lymphocyte-enriched fractions. The expression of CXCb2 was low in the macrophage-enriched fraction, but higher for CXCb1. Both CXCb1 and CXCb2 are low expressed in the granulocyte-enriched fractions.

IFN-γ2, in synergistic co-stimulation with LPS, induces gene expression of carp CXCb2
Gene expression of CXCb2 was determined in cultured head kidney phagocytes upon stimulation with LPS, IFN-γ2 and LPS and IFN-γ2 combined. CXCb2 gene expression is induced at 4 hrs, upon stimulation with LPS and IFN-γ2 alone at similar levels and

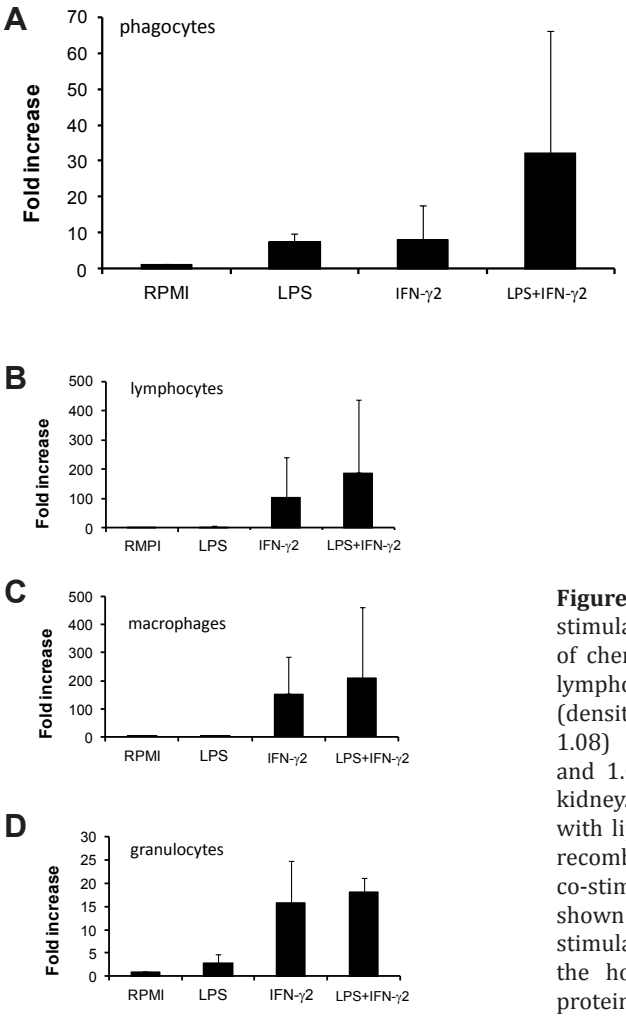


Figure 6. Gene expression of CXCb2 in stimulated HK leukocytes. Expression of chemokine CXCb2 gene in monocyte/lymphocyte- (density 1.02), macrophage- (density 1.06), granulocyte- (density 1.08) and phagocyte- (density1.06 and 1.08) enriched fractions from head kidney. Cells were stimulated for 4 hrs with lipopolysaccharide (LPS, 30 μg/ml), recombinant carp IFN-γ2 (100 ng/ml) or co-stimulated with LPS and IFN-γ2. Data shown is x-fold increase compared to non-stimulated cells (RPMI), standardized for the housekeeping gene 40S ribosomal protein s11. N=2 fish and SD is given.

IRFs, but not AP-1. A TATA-box was only identified 525 bp upstream the start codon. No obviously similarity was observed, as the promoter of CXC-chr5a did not contained a NF-κB binding site, but did contain an AP-1 site, in contrast to human CXCL13. Also the promoter regions of human CXCL8 and zebrafish CXCL8 found on chromosome one were analyzed (Fig 4d). Identified transcription factor binding sites for human CXCL8 were consistent with what was described by others [46]. For zebrafish CXCL8_chr1, binding sites were identified for IRF3, IRF7, STAT and AP-1. One binding site was identified for NF-κB 1785 bp upstream the start codon (not shown). Together, these results suggests a differential induction of zebrafish CXCb gene expression by type I and II IFNs, with variable sensitivity to the different transcription factors.

CXCb1 and CXCb2 constitutive gene expression in carp organs

Constitutive gene expression of carp CXCb1 and CXCb2 was determined in various organs and tissues of adult carp (Fig. 5a). Overall, a similar pattern of expression was observed for CXCb1 and CXCb2, with the exception of spleen, thymus and brain. CXCb1 gene expression was higher than CXCb2 in spleen, and CXCb1 is lower than CXCb2 in thymus

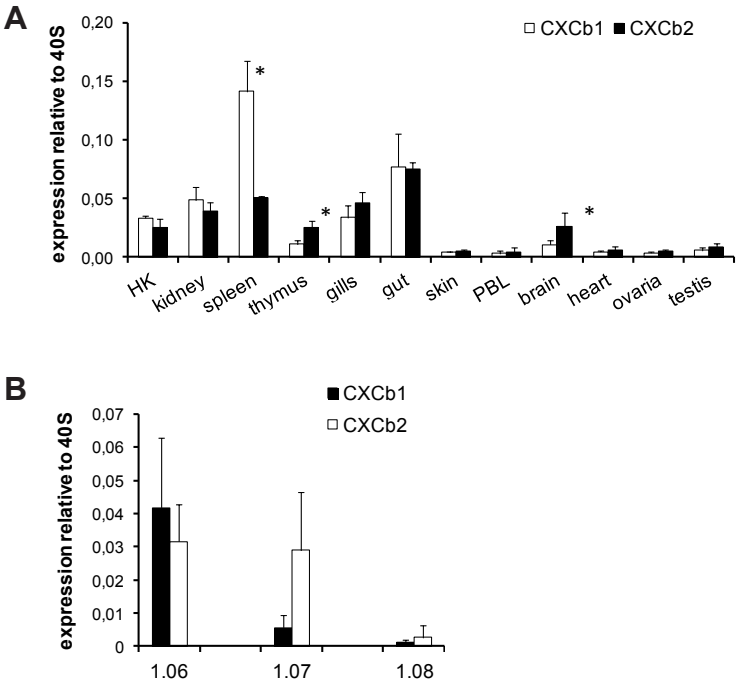


Figure 5. Constitutive expression of carp CXCb1 and CXCb2 genes. **A** Constitutive gene expression of CXCb1 and CXCb2 in various organs of adult carp. **B** Constitutive gene expression of CXCb1 and CXCb2 in monocyte/lymphocyte- (density 1.02), macrophage- (density 1.06) and granulocyte- (density 1.08) enriched fractions from head kidney. Expression was determined by quantitative RT-PCR and plotted relative to the expression of the 40S ribosomal protein s11 gene. Bars represented the average expression ± SD, n = 4 fish (A) or n=2-9 fish (B).

enhanced when co-stimulated with LPS and IFN-γ2 (Fig. 6a). In addition, in head kidney cell fractions, CXCb2 gene expression was determined upon stimulation with LPS, IFN-γ2 and LPS and IFN-γ2 combined. In lymphocyte-, macrophage- and granulocyte-enriched cell fractions, CXCb2 gene expression was induced 4 hrs upon stimulation with IFN-γ2 and co-stimulation with LPS and IFN-γ2 (Fig. 6b-d).

Carp CXCb1 and CXCb2 are differentially expressed in peritoneal leukocytes during zymosan-induced peritonitis

A zymosan-induced peritonitis model was used, by which i.p. injection of zymosan leads to a massive influx of leukocytes into the peritoneal cavity and to changes in expression of pro-and anti-inflammatory genes [41]. Expression of CXCb1 and CXCb2 genes in accumulating leukocytes was determined in this model (Fig 7). Expression of CXCb1 and CXCb2 genes was significantly increased upon peritoneal inflammation with a peak response at 24 – 48 hrs after zymosan injection. CXCb1 kinetics however showed a sharper peak in comparison to CXCb2. Already 6 hrs after zymosan injection CXCb2 gene expression was significantly higher and remained significantly elevated at 96 hrs after injection.

Discussion

We here identified multiple CXCb genes in cyprinids that are most similar in sequence to the mammalian CXCL9-11 chemokines. While carp is a tetraploid fish, carp CXCb1 and CXCb2 share 71% similarity in nucleotide sequence, indicating that these are most probably two distinct genes, rather than encoded by different alleles. The present study moreover identified a cluster of seven genes on chromosome five of the zebrafish

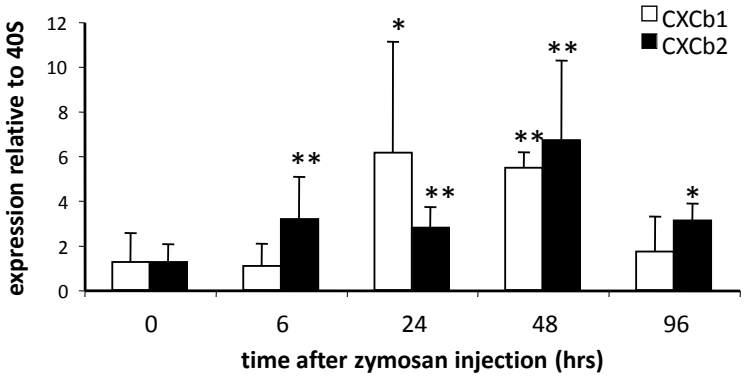


Figure 7. Gene expression of carp CXCb1 and CXCb2 in zymosan-induced peritonitis. Expression of carp CXCb1 and CXCb2 genes in peritoneal leukocytes 0, 6, 24, 48, 96 h after zymosan (Z, 2 mg/ml, 0.5 ml/50 g b.w.) induced peritonitis. cDNA of n=4-9 fish was used as template for quantitative real time PCR. Messenger RNA expression is shown as x-fold compared to control saline-treated animals, relative to time 0 (PBS) standardized for the housekeeping gene 40S ribosomal protein s11. Averages (n=4-9 fish) and SD are given. *, $p<0.05$, **, $p< 0.01$.

genome that are related to carp CXCb, indicating that duplications and diversification of CXCb genes is common at least to fish species of the cyprinid lineage. Analysis of the promoter of zebrafish CXCb genes, together with the expression of both carp CXCb1 and CXCb2 genes during inflammation and upregulation upon stimulation with IFN-γ2, indicates a function for cyprinid CXCb in immunity, related to the function of mammalian CXCL9-11.

While constitutive expression of carp CXCb1 and CXCb2 transcripts is abundant in all organs of adult fish, highest expression was observed in most of the organs and tissues of the immune system, supporting its predominantly immune-related function. Further, in head kidney cell fractions, the highest constitutive gene expression of both CXCb1 and CXCb2 was observed in the lymphocyte-enriched cell fraction. In contrast, CXCL9-11 expression can be induced in many mammalian cell types in response to IFN-γ. Among fish interferons, type II interferons appear to be conserved [35, 44, 47, 48]. In both carp and zebrafish, two IFN-γ genes have been identified, IFN-γrel and IFN-γ2, of which IFN-γ2 shares the strongest structural and functional similarities with mammalian IFN-γ [35, 44, 48-50]. In carp head kidney leukocytes, both CXCb1 and CXCb2 gene expression is inducible by recombinant carp IFN-γ2, and this induction is synergistic with LPS ([35] and this study). This synergy is attributed to distinct intracellular pathways that are triggered upon IFN-γ, or LPS stimulation. The mammalian IFN-γ receptor consists of two chains, IFNgR1 and R2, which upon ligand binding trigger the Jak/STAT pathway. This leads to phosphorylation and formation of a homodimer of STAT1, also known as IFN-γ-activated-factor (GAF). Upon translocation to the nucleus, GAF binds DNA with a specific motif, gamma activated sequences (GAS), resulting in induction of gene expression (reviewed by [51]). The signaling pathways induced by IFN-γ appear to be conserved in fish, as crucian carp IFN-γ1 and IFN-γ2 signaling via the IFNGR1-2 and IFNγR1-1 respectively, induces STAT1 phosphorylation and binding to GAS elements [52] and also in Atlantic salmon it has been shown that STAT1is phosphorylated upon both IFN-a1 and IFN-γ stimulation and that upon IFN-γ stimulation STAT1 is translocated to the nucleus [53]. In mammals, LPS is recognized by toll-like receptor 4 (TLR4), which ultimately leads to the activation of the transcription factor NF-κB [54, 55]. The signaling pathways for LPS activation are not well understood in fish, TLR4 has been identified in fish, but not the MD-2 protein required for TLR4 dimerization. Further, LPS binding proteins (LBPs) have been found that have a strong similarity to mammalian bacterial/permeability-increasing protein (BPI) and it is not clear whether fish LBP/BPI proteins are involved in TLR4 signaling, or rather display antimicrobial activity. Fish TLR4 homologs do not recognize LPS, although TLR4 and LBP/BPI gene expression can be upregulated upon bacterial infection [56-58]. Simultaneous binding of multiple transcription factors, for which canonical sites in the promoter seem to be fairly conserved between fish and mammals, leads to synergistic enhancement of gene expression. The carp genome has not been sequenced yet, but analysis of the promoter of CXCb-related genes in zebrafish showed that binding elements for both STAT and NF-κB are present. Whether zebrafish CXCb genes are inducible by IFN-γ, or by type I IFNs, has not yet been tested. Interestingly, all CXCb-related zebrafish genes contained one to three ISRE sites in the promoter, indicating

that these genes could also be inducible by type I IFNs, which upon stimulation of the IFN-α/β receptor induce the formation of STAT1/STAT2/IRF-9-complex that binds to ISREs [51]. It has been demonstrated for human CXCL10/IP-10 and CXC11/ITAC that these genes can be induced upon stimulation by IFN-α alone [16, 59].

CXCL9-11-related genes have also been described in other teleost fish, like channel catfish and rainbow trout [47, 60, 61]. The phylogeny analysis showed that trout and channel catfish CXCL9-11-related genes cluster with the cyprinid CXCb genes. Recombinant trout IFN-γ induces gIP-10 gene expression in the trout monocyte/macrophage cell line RTS11 and analysis of the promoter of trout gIP-10 has shown that it also contains sequences corresponding with GAS and ISRE elements that are both required for gene expression [47, 62]. Thus, the induction of fish CXCb genes by IFN-γ is likely conserved in fish species outside the cyprinid lineage.

The promoter analysis also included human CXCL8 and the zebrafish CXCL8-like gene located on chromosome 1. For zebrafish CXCL8_chr1, two AP-1 sites in the promoter were identified, but no NF-κB binding site was found in proximity of the start codon. However, induction of zebrafish CXCL8_chr1 gene expression can be upregulated upon bacterial infection [63]. For carp, we earlier compared gene expression profiles for CXCLa_L1 (ortholog of zebrafish CXCL8_chr1 [27]) and CXCL8_L2 upon stimulation by LPS alone, or rIFN-γ2 alone, or co-stimulation, in head kidney phagocytes [35] . We observed that stimulation with LPS alone enhances gene expression for both carp CXCLa_L1 and CXCL8_L2, but not upon stimulation with rIFN-γ2 alone. Further, co-stimulation with LPS and rIFN-2 did not lead to a synergistic increase in gene expression for CXCLa_L1 and CXCL8_L2. Thus, the absence of a STAT binding site in the promoter region of zebrafish CXCL8_chr1 is in line with experimental data in carp.

We observed two CXC genes located in the zebrafish CXCb genomic cluster that were not related to the CXCL9-11 chemokines. Human CXCL13, or B cell-attractant chemokine 1 (BCA-1), was among the most similar sequences to zebrafish CXC-chr5a and phylogeny studies showed that CXC-chr5a clustered with CXCL13, albeit not with a reliable bootstrap value. In addition, a synteny was observed for zebrafish CXC-chr5a with human CXCL13, as both are immediate neighbors of the *cnot6l* gene (data not shown); CXC-chr5a may thus be considered an ortholog of CXCL13. Notably, on the genome of human and mice, the CXCL13 gene is located less than 3 Mb from the CXCL9-11 mini-cluster [4], just as zebrafish CXC-chr5a is located within the CXCb cluster. Interestingly, it has been demonstrated that human CXCL13 has not only affinity for CXCR5, but also for CXCR3, the receptor of CXCL9-11 [64]. A possible evolutionary scenario could be the following: at the time of the fish/tetrapod split, CXCR3-binding chemokines had already divided into two genes, duplicated in tandem. One of these ligand genes, the ancestor of CXCL13/CXC-Chr5a, did not diversify further, perhaps because it has a homeostatic function and because it has to be able to bind, in addition to CXCR3, CXCR5, restraining a further diversification. By contrast, the second gene, with affinity for only CXCR3, was more directly involved in response to pathogens and has been subject to stronger diversifying pressures. This gave rise to CXCL9-11 in the mammalian lineage and the various CXCbs in cyprinids, whereby CXCL-Ch5b has retained the original location, while other members drifted a few megabases away as a

consequence of repeated gene duplications and losses.

The observed gene expression of both CXCb1 and CXCb2 upon peritoneal inflammation, underlines the physiological importance of both genes for the inflammatory response. Although differentially, both genes are expressed at 24 hrs and CXCb2 already at 6 hrs, simultaneous to a peak expression of the pro-inflammatory cytokines IL-1β and TNF-α [41]. Differential constitutive expression profiles for carp CXCb1 and CXCb2 in spleen, thymus and brain and differential kinetics of expression during zymosan-induced peritonitis furthermore suggest distinct functions for both chemokines. Further, a different sensitivity was observed for LPS, as a moderate induction of CXCb2 gene expression was observed upon LPS stimulation, while this was previously not shown for CXCb1 [35]. Redundant, collaborative and antagonistic subfunctions have been proposed for mammalian CXCL9-11 as well (as reviewed in [17]). First, CXCL9-11 differ in affinity for CXCR3 [16, 65]. Secondly, they differ in regulation of gene expression, observed both on promoter level, as assayed upon IFN-γ and TNF-α, IL-1β, or LPS co-stimulation [16, 45, 59]. Thirdly, CXCL9-11 are anatomically differentially expressed in tissues. For example in the skin, CXCL10 and -11 are expressed at higher levels in the epidermis and have stronger affinity for CXCR3-bearing T cells than CXCL9, which is expressed at lower levels in the dermis [45, 66].

We previously showed by a combined in vitro and in vivo approach with recombinant proteins for the carp CXCL8 lineages and CXCb1 that all three chemokines are chemotactic for monocytes/macrophages and neutrophilic granulocytes. Moreover, CXCb1 was also chemotactic for lymphocytes [34]. This correlates with the distribution of the CXCR3 in mammals, which is expressed by effector and memory T cells upon activation, but also by monocytes/macrophages [20, 22, 67]. Fish have two genes for CXCR3 [23]. We recently cloned a CXCR3 gene in common carp that is expressed in different leukocyte populations, including macrophages (in preparation). In grass carp, one CXCR3 was cloned, that appears to be expressed by cells with monocyte-, macrophage- and lymphocyte-morphology [33]. Two CXCR3 genes are described in zebrafish [2, 33] and CXCR3.2 is expressed on macrophages in zebrafish larvae [68], but whether this receptor is expressed by T cells is still unclear. In medaka, it was also reported that CXCR3a is expressed by mononuclear phagocytes in larvae. In this study, the authors also looked at the expression of CXCR3a in adult fish, and expression correlated with cells expressing myeloid-associated markers, but not lymphoid-associated markers and immature lymphocytes did not express CXCR3a [69]. The chemokine ligands for fish CXCR3.2 have not yet been identified by biochemical studies, but as fish CXCbs are most similar in sequence to mammalian CXCL9-11, in addition to the indirect synteny discussed above, it makes them putative candidates.

In conclusion, we demonstrated that multiple CXCb-like genes exist in cyprinid fish that are most similar, but not true orthologs to mammalian CXCL9-11. These chemokines probably are functional homologs, as carp CXCb1 and CXCb2 are both IFN-γ2 inducible and are expressed in vivo during peritoneal inflammation. Identification of the CXCb receptor by biochemical studies will be required to further elucidate the biological function for the individual cyprinid CXCb genes.

Acknowledgments
We like to thank Dr. Carla Ribeiro and Loes Wiersma for technical assistance.

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J'aime la nuit, j'ai les idées plus claires dans le noir.

- Serge Gainsbourg -

Chapter 8

General Discussion

Two large protein families were the subject in this thesis, which both have roles in the immune system, at two different levels. In **part one** of the thesis, TRIM proteins were studied, which are part of an ancient intracellular immune system and were recently identified to play an important role in the antiviral immune response. A detailed description of the entire TRIM gene family in fish was made. It was discovered that certain TRIM genes have undergone a radical expansion that gave rise to three multigene families, a feature unique for fish. Experimental studies indicated a role for trout finTRIMs in antiviral immunity and demonstrated that they have E3 ubiquitin ligase activity. In **part two** of this thesis, inflammatory CXC chemokines were studied. CXC chemokines are cytokines involved in chemotaxis of leukocytes during inflammation, for which currently limited functional data is available in fish. Two lineages were identified for fish chemokines belonging to the CXCL8-like subset: CXCa_L1 and CXCL8_L2. A similar diversification was observed for chemokines belonging to the CXCb subset. A characterization of the functions for cyprinid CXCL8-like (CXCa_L1 and CXCL8_L2) and CXCL9-11-like (CXCb) chemokines was made by in vitro and in vivo gene expression studies. By preparation of recombinant proteins it was shown that carp chemokines of the CXCL8-like and CXCb subsets are both chemotactic for fish leukocytes. Chemokines of the CXCL8-like and CXCb subsets appeared to be functionally distinct, as they have a different effect on phagocyte activation and act during different phases of the inflammatory response.

In this chapter will be discussed how (multi)gene families evolve and co-evolve with viruses, how research on TRIM proteins has provided new insights on intracellular immunity, and how E3 ubiquitin ligases control intracellular signalling in antiviral immunity. Based on the functional data obtained on fish chemokines in this thesis, a model is proposed for the role of fish chemokines during the inflammatory response.

Evolution of multigene families

TRIM proteins and chemokines both belong to large gene families, and in this thesis it was discovered that in fish, three multigene TRIM families have evolved (**Chapters 2 and 3**). TRIM proteins are rather large proteins (25-75 kDa) consisting of multiple domains. Chemokines are small cytokines (8-14 kDa) that have one structural 'domain' that relies on disulfide-bond stabilization by its cysteine-motif. The evolution of (multi) gene families is characterized by gene duplication events, diversification and domain shuffling; events that were also described in this thesis for TRIM and chemokine genes.

Evolution by gene duplication

Gene duplications arise mainly by tandem duplications and from polyploidization (whole genome duplication events). Gene duplication is a common event whereby chemokines evolved, evidenced by the multiple gene clusters in which chemokine ligand and receptor genes are located on the genome. For example, zebrafish CXCb genes are located in a gene cluster on chromosome five (**Chapter 7**). The three fish multigene TRIM families arose by repeated duplication events and the majority of the genes are located in multiple clusters, spread over the fish genome (**Chapters 2 and 3**). A classical theory developed by Susumu Ohno [1] states that a gene duplication event

can have two different outcomes. One outcome is that the function of the ancestor gene is retained for one gene, while this function is lost for the other gene by deleterious mutations and becomes a non-functional pseudogene. A second outcome is that both genes are retained, whereby one gene retains its original function, while the other gene acquires a new function. The acquisition of a novel function is dependent on beneficial mutations in this gene. This seems to be a rare event, therefore, the first outcome (gene loss) was presumed to happen more often. However, another, later developed, model argues that there is a higher chance that both genes are preserved. According to this duplication-degeneration-complementation (DDC) model, upon duplication of a gene, the derived genes evolve either by non-functionalization, subfunctionalization, or neofunctionalization, depending on subsequent mutations in the two gene copies [2]. Subfuntionalization is common in chemokine gene evolution and the lineages belonging to either the fish CXCL8-like subset, or the CXCb subset, seem to have diverged and acquired subfunctions (partial redundancy). For example, recombinant CXCa_L1 and CXCL8_L2 are chemotactic for similar cell types, but differ in kinetics of gene expression (**Chapters 5 and 6**). FinTRIMs possibly evolve by neofunctionalization, as phylogeny analysis and the distinction of three gene clusters suggest that these genes are in transition (**Chapter 2**). FinTRIM genes belonging to an ancestral branch (group C) are more conserved, as genes of this group are most similar to TRIM16 and TRIM25, and consist of only a few members. In contrast, genes belonging to the A group are more distant to TRIM16 and TRIM25 and are highly diversified. Group C would represent genes that have maintained an ancient function, while group A genes may have acquired a new function. Group B genes would then represent genes with both functions, or lost, or gained, one of the functions.

Diversification by positive selection

Gene duplication events are often followed by diversification. Different amino acid sites of a protein evolve under different selection pressures: neutral, negative and positive selection, whereby positive selection leads to diversification. Whether genes evolve under positive selection can be determined for a set of closely related genes (multigene families) of one species, or for an orthologous gene of different species. Hereby, the rate of synonymous (silent) and non-synonymous (amino acid altering) substitutions is determined for each codon within the sequence. A higher rate of non-synonymous than synonymous substitutions indicates that this site evolves under positive selection [3, 4]. It was demonstrated that all three multigene TRIM families in fish evolve under positive selection (**Chapters 2 and 3**). This indicates the presence of an evolutionary pressure that drives fish multigene TRIM genes to diversify. As pathogens are a major driving force for the immune system to diversify and it was demonstrated for finTRIM gene that the expression is induced by viruses, the fish multigene TRIM genes possibly all have evolved under viral diversifying pressure, as was previously shown for mammalian TRIM5α and TRIM22 [5, 6].

Diversification by domain shuffling

Domain shuffling allows modular assembly of novel genes from existing genes. An

exon encoding for one domain can integrate within an intron of another gene by recombination. Novel domains can be acquired by a juxtaposition event, whereby one exon is integrated within another exon. This is for example how the B30.2 domain originated, which is one of the C-terminal domains that associate with TRIM proteins. The B30.2 domain is encoded by a single exon and arose by the incorporation of the PRY sequence into the more ancient SPRY domain [7]. Domain shuffling is considered an important event in evolution that has contributed to the increasing complexity of higher organisms, as genomes of higher organisms have a higher content of multi-domain proteins with various domain architectures. Moreover, as genomes of higher metazoan are less "gene-dense" and more "intron-rich", this allows easier exon shuffling via intronic recombination (reviewed by [8]). While the RBCC-module of TRIM proteins is highly conserved, TRIM proteins associate with different domains at their C-terminus [9]. The formation of multi-domain proteins allows more complex protein interactions, by which each individual domain possess specific binding specificities, or catalytic specificities [8]. Thus, for TRIM proteins, the RING domain catalyzes ubiquitination, while the other domains are involved in substrate binding. The coiled-coil domain in addition allows multimerization to form protein complexes. The acquisition of different additional domains at the C-terminus has probably driven TRIM proteins to establish novel protein-protein interactions and to expand their range of functions in the cell.

B30.2 domain: a mobile domain of the immune system

As was described in **Chapter 2**, the exon encoding for the B30.2 domain of group A finTRIMs has shuffled between finTRIMs and a subtype of zebrafish NOD-like receptors (NLRs) that contain a B30.2 domain [10, 11]. Domain shuffling is an important event by which at least many of the innate immune receptors have evolved. PRRs and corresponding adapter molecules are made up by nine major domains, which are the Ig (immunoglobulin), LRR (leucine-rich repeat), C-type lectin domain (CTLD), ITAM (immunoreceptor tyrosine-based activation motif), TIR (Toll/IL-1 receptor), NBS (nucleotide-binding site), Death domain family (CARD, caspase-recruitment domain, and PYD, Pyrin domain) and helicase domain [12]. These domains are identified in PRRs and adapter molecules under different protein architectures. The B30.2 domain, which is most frequently found to associate with TRIM proteins, and is also present in the three TRIM multigene families in fish (**Chapters 2 and 3**), might be considered as a tenth domain to be added to this list. The B30.2 domain is a typical mobile domain, as it is associated with proteins of different families, which are intracellular, membrane-bound and secreted. Moreover, these proteins have (putative) implications in immunity. Protein families in which the B30.2 domain has been identified so far include TRIM proteins, NLRs in zebrafish, butyrophilins, enterocytin, enterophylin and fish stonus toxins [13-15]. The function for zebrafish NLRs with a B30.2 domain is not yet known, but as other members of the NLR family are involved in pathogen recognition [16, 17], NLRs with a B30.2 domain may have a similar function. In NLRs (and TLRs), the LRR domain is responsible for pathogen recognition. Therefore, it will be interesting to investigate what the role is of the B30.2 domain and whether is also implicated in direct pathogen sensing and interaction. Butyrophilins (BTNs) share structural homology with

the B7 family. These are co-stimulatory molecules expressed by antigen-presenting cells (APCs) that interact with CD28 or CTLA on T cells to stimulate, or inhibit respectively, T cell proliferation. BTNs contain next to an IgV-like, IgC-like and transmembrane (TM) domain, the B30.2 domain at the C-terminus. Interestingly, BTNs and BTN-like genes have undergone species-specific diversification and are differentially expressed by immune and non-immune cellular subtypes, indicating specialized functions for the individual members (reviewed by [18]). BTNs have been demonstrated to act as both inhibitory and stimulatory molecules for T cells and are associated with the immune disorder sarcoidosis, cancer, gut inflammation and inflammatory bowel disease (IBD) [19-22]. Two other B30.2-domain containing proteins associated with the gut are enterocytin and enterophilin. In addition to the B30.2-domain, enterocytin contains a coiled-coil domain, whereas enterophilin contains a leucine-zipper [13, 14]. Both proteins are expressed by enterocytes and may have a function in enterocyte differentiation, but their exact functions remain to be determined. Stonefish stonustoxin (STNX) is the lethal factor from the Synanceja horrida venom that has a strong haemolytic activity and is related to toxins from other stonefish species (reviewed by [23]). The STNX protein contains a B30.2 domain that is associated with a sequence of unknown domain identity [15].

One notably characteristics of most of the proteins with a B30.2 domain is that they are highly diversified (TRIM, NLR and BTNs). As mentioned previously, the B30.2 domain originated by the incorporation of the PRY sequence into the SPRY domain. The B30.2 domain has only been described in vertebrates and invertebrates, but the SPRY domain is an ancient domain. The SPRY domain is found in all eukaryote branches, including plants, but also in prokaryotes [7, 24]. TRIM proteins with a B30.2 domain are less conserved than those without an additional domain, or that are associated with one of the other C-terminal domains (**Chapter 3** and [25]). Further, almost half of the mammalian TRIM proteins and the three fish multigene families contain a B30.2 domain and this domain evolves under positive selection in the three fish multigene TRIM families, as in some primate TRIM genes (**Chapters 2 and 3**, [5, 6]). As it is demonstrated for several mammalian TRIM proteins that the B30.2 is involved in direct binding to viruses, it could be hypothesized that the direct interaction of viruses with the B30.2 domain has contributed to the diversification of the corresponding genes.

Diversification by viral hijacking of the chemokine system

Viruses have been, and are still, one of the major driving forces for the immune system to diversify. The co-existence of viruses and their hosts is often described as an evolutionary battle, whereby viruses continuously evolve new strategies to evade and modulate host immune responses, which promotes the host immune system to further adapt to the virus. One successful viral strategy is molecular mimicry. Hereby, viruses acquire, by host-virus gene transfer, genes that encode for cytokines and receptors. Also the host chemokine network is hijacked by the viruses (reviewed by [26, 27]). Viral chemokines and receptors act by different mechanisms. Certain viral chemokines are agonists that mediate leukocyte recruitment, promoting viral pathogenesis. For example, the human cytomegalovirus (HCMV) ORF UL146 encodes for the viral

chemokine vCXCL-1, which is a functional agonist for CXCR1 and CXCR2 and induces neutrophil migration [28, 29]. It is hypothesized that neutrophils recruited by vCXCL1, expressed by HCMV-infected endothelial cells, are subsequently infected and may serve as a vehicle for viral dissemination by transporting the virus to endothelial cells at other sites [28]. Other viral chemokines act as antagonists, as for example the chemokine-like protein MC148R1 of Molluscum contagiosum poxvirus (MCV), which is a CC homolog that interferes with CXCL12a signaling via CXCR4 [30].

Viruses can further deregulate the host immune responses by the expression of seven transmembrane chemokine receptors (vGPCRs). Certain vGPCRs act as scavengers for host chemokines, but as some viruses also use chemokine receptors for cell entry, viruses may utilize vGPCRs to promote viral dissemination (reviewed by [26]). Interestingly, vGPCRs are also used by the virus to deregulate intracellular signaling pathways. For example, signaling via the Kaposi's sarcoma-associated herpesvirus (KSHV) chemokine receptor ORF74, subverts cell signaling pathways that stimulate angiogenesis and contributes to oncogenic transformation [31]. In addition, viruses encode for viral chemokine binding proteins (vCBPs). CBPs are structurally unrelated to chemokine receptors and act as scavengers for host chemokines. Hereby, vCBPs prevent interaction of host chemokines with corresponding receptors, or glycosaminoglycans (GAGs). For example, the monkeypox virus (MPV) viral chemokine inhibitor (vCCI) binds macrophage-inflammatory protein 1 (MIP-1) and inhibits MIP-1 mediated chemotaxis [32].

It has been proposed that viral hijacking of the chemokine network contributed to the rapid evolution and expansion of the chemokine family [33] and positive selection has indeed been demonstrated for chemokine receptors [34]. Interestingly, a high diversification of viral chemokines has been noted on viral genomes [35, 36] and it has been demonstrated that certain viral chemokines evolve under positive selection pressure [37]. This could indicate a possible bi-directional diversifying pressure between host and viral chemokine genes.

Interestingly, the chemokine network is also modulated by other pathogens, as CBPs were recently identified in ticks [38, 39]. Tick CBPs, named evasins, found in the saliva, selectively bind different CC chemokines and have anti-inflammatory activities [39]. Further, next to their well described chemotactic activity, chemokines also have defensin-like anti-microbial activities. Possibly this anti-microbial activity has contributed to the diversification of chemokines. The CXCL9, CXCL10 and CXCL11 chemokines in mouse for example, each differentially restrict *Bacillus anthracis* [40]. With CC chemokines being the most diverse chemokine group in teleost fish [41, 42], fish CC chemokines would be interesting candidates to investigate for such antimicrobial activity.

Neofunctionalization: from development to immunity

Diversification allows the acquisition of new and specialized functions. Both chemokine and TRIM gene families are characterized by a limited set of conserved genes and genes that have undergone species-specific diversifications (**this thesis** and [25, 43]). For conserved chemokines, an implication in development and homeostasis is described

[44], while those that have diversified, are involved in immunity, as was for example observed for the cyprinid CXCL8-like and CXCb chemokines (**Chapters 5 and 7**). Although a full description of TRIM genes in invertebrates has not been made yet, TRIM genes are identified in sea anemone, drosophila and worm, with seven TRIM genes identified in drosophila and six genes in *C. elegans* [24, 25]. Functional studies on drosophila and *C. elegans* TRIM proteins showed that these are implicated in development and interestingly, these functions appear to be conserved. For example, the C. elegans MADD-2 protein and mammalian MID1/TRIM18 both function in embryonic midline development [45, 46]. The less conserved TRIM proteins, are more likely to play a role in immunity. This is demonstrated for mammalian TRIM proteins with a B30.2 domain, which are not well conserved among vertebrates and have undergone species-specific duplications [25]. As TRIM proteins in fish are highly diversified (**Chapters 2 and 3**), this argues further for a role in immunity for the fish multigene TRIM proteins.

Host-virus co-evolution of TRIM proteins

The evolutionary characteristics of TRIM genes are a good example of the host-virus co-evolution. This is best described for TRIM5α, which is an important restriction factor in retrovirus infection. TRIM5α of rhesus monkey was initially identified as a HIV-1 post-entry restriction factor, which only slightly restricts simian immunodeficiency virus (SIV). In contrast, human TRIM5α only has a minor restriction activity against HIV-1 and SIV [47]. Subsequent studies showed that TRIM5α is a species-specific restriction factor for retroviruses: TRIM5α of different primate species has restriction activity for different retroviruses [48-51]. TRIM5α binds the capsid of incoming viral particles, whereby it accelerates uncoating of the viral core and interferes with reverse transcription [52]. The species-specific restriction activity of TRIM5α is attributed to the B30.2 domain [48]. The TRIM5α B30.2 domain contains four hypervariable regions, which have evolved by lineage-specific expansions, deletions and mutations. The retroviral capsid binding sites are located within these hypervariable regions [53, 54]. Further, the B30.2 domain has evolved by retroviral diversification pressure. This is evidenced by the identification of sites that evolved under positive selection pressure, located within the hypervariable regions [5, 53].

While the B30.2 domain is thus important for binding the viral nucleocapsid, it was discovered that in owl monkey and in macaques, primates belonging to the New and Old World monkeys respectively, independent events have occurred, the B30.2 domain of TRIM5α is replaced by cyclophilin A (CypA) [55-60]. Interaction of CypA with the viral capsid is required for efficient virion assembly during HIV infection. When fused to TRIM5α, TRIM5α-CypA interferes with the interaction of CypA (not fused to TRIM5α) to the nucleocapsid and hereby with viral replication. The TRIM5 gene is absent on the fish genome, but interestingly, a finTRIM gene was identified on the zebrafish genome that also associates with CypA (**Chapter 3**). However, this gene is a pseudogene that contains a stop codon in the first exon.

CypA is a member of the immunophilin family, which are peptidyl-prolyl cis-trans isomerases that catalyze protein folding. Several viruses make use of host cyclophilin A and B in their viral life cycle. For example, hepatis C virus requires binding

of NS5B to CypB for proper replication [61]. But also for other flaviviruses, SARS, and human papillomavirus 16 (HPV16), it is reported that interaction of viral proteins with immunophilins promotes viral replication [62-64]. Thus in the case of TRIM5α-CypA, the host efficiently turns one of its own proteins, that was initially a benefit for the virus, against it. Interesting to note is that another TRIM protein interferes with the activity of another immunophilin, namely TRIM21 with Pin1. Pin1 interacts with the transcription factor IRF3. Whether the interaction of TRIM21 with Pin1 has a positive or negative effect on the activity of IRF3, is still controversial [65, 66].

Intrinsic immunity

TRIM proteins as TRIM5α are virus-specific restriction factors. A new concept in immunology, intrinsic immunity, was recently introduced by Paul Bieniasz. It refers to an intracellular immune system that is present in all types of somatic cells [67]. This intrinsic immune system acts in viral infection before innate and adaptive immune responses are initiated and consists of a diverse set of virus-specific cellular restriction factors. These viral restriction factors act autonomously and are constitutively expressed, although some are also upregulated by type I IFNs, once a virus is sensed by neighbouring cells. A growing number of proteins are identified to belong to this intrinsic immune system. Best studied are retroviral restriction factors. TRIM proteins belong to the intrinsic immune system, as well as tetherins and APOBEC cytidine deaminases. Tetherin is a type I IFN-inducible protein that prevents the release of enveloped viruses (reviewed by [68]). It is expressed on the plasma surface as a dimer, of which single molecules consist of a transmembrane domain, a coiled coil domain and GPI-anchor. Tetherin literally tethers, upon budding, nascent HIV-1virions to the plasma membrane, which accumulate on the extracellular side of the cell membrane. Hereby, tetherin prevents virion spreading to new cells [69]. Viruses of different families encode viral proteins that antagonize tetherins [68]. Interestingly, HIV-1 Vpu protein antagonizes tetherins by a species-specific manner and it is reported that tetherin genes evolve under positive selection [70, 71].

APOBEC cytidine deaminases are DNA editing enzymes that remove the amino group from cytidine, to turn it into uridine. APOBECs edit both single and double stranded DNA of viral, mitochondrial, or nuclear origin. The APOBEC family consists of AID, APOBEC1, APOBEC2, APOBEC4 and seven APOBEC3s (A3s) are reported in humans: A3A, A3B, A3C, A3DE, A3F, A3G and A3H (reviewed by [72, 73]). Although all APOBEC3s are IFN-inducible, A3A is most strongly induced by IFN and is expressed in phagocytic cells, where it targets foreign dsDNA [74]. Members of the APOBEC3 family introduce hypermutations in DNA of positive RNA viruses with a reverse transcription step and also DNA viruses, which include retroviruses, hepatitis B virus and recently, herpesvirus ([75-78] and reviewed by [79]). As uridine base-pairs with adenine, editing of minus-strand DNA (during the reverse-transcription step) results in a G-to-A mutation in the coding strand. Moreover, it was demonstrated for HIV-1 that APOBEC3G and A3F are packaged in HIV-1 virions, resulting in non-infectious virions. This activity is counteracted by HIV-1 Vif protein that interferes with the incorporation of APOBEC3G and A3F in virions and additionally targets A3G and A3F to the proteasome (reviewed

by [73, 80]). Interestingly, primate APOBEC3G has evolved under positive selection [81].

Thus, factors belonging to the intrinsic immune system display several characteristics. First, they act autonomously with both a viral sensing and restriction activity. Secondly, they are constitutively expressed, but inducible by type I IFNs. Thirdly, they co-evolved with viruses, as is demonstrated by the evolution under positive selection for several restriction factors and the discovery hat viruses have evolved mechanisms that counteract the activity of intrinsic immune factors. In **Chapter 2 and 4** it was described for finTRIM genes that they are constitutively expressed, the expression is induced by IFN and upon virus infection, and have evolved under positive selection. Therefore, finTRIMs are possibly also part of the intrinsic immune system.

Control of cell signaling by ubiquitination

It was demonstrated in **Chapter 4** that trout finTRIMs display E3 ubiquitin ligase activity, an activity that is described for multiple mammalian TRIM proteins [82]. The insight that protein ubiquitination does not only lead to protein degradation by the 26S proteasome, but in addition, has non-proteolytic regulatory mechanisms, is still relatively unknown by biologists. Evidence is growing that protein modification by ubiquitination is essential for control of protein activity, protein-protein interactions and intracellular signaling pathways [83]. The outcome of protein modification by ubiquitin depends on the type of ubiquitin-linkage, as depicted in figure 1 [84]. Protein-modification by ubiquitin affects protein-protein interactions in several ways. First, many proteins contain a ubiquitin-binding domain (UBD) and ubiquitination of one protein, provides an additional binding site to another protein for interaction [85]. Secondly, ubiquitination may cause a conformational change that enhances, or inhibits protein-protein interaction. Thirdly, ubiquitination at a protein-protein interaction site, prevents interaction (reviewed by [86]). As phosphorylation, ubiquitination is a reversible type of protein modification. Deubiquitinating proteases (DUBs), specialized for different types of ubiquitin-linkages, remove ubiquitin from a modified protein. There are around hundred DUBs reported in humans. Some DUBs associate with the 26S proteasome and specifically remove ubiquitin molecules from ubiquitinated proteins upon degradation, facilitating ubiquitin re-usage. Others DUBs are located in the cytoplasm and nucleus of the cell and remove ubiquitin from proteins that are ubiquitinated in the non-canonical pathways. This reversible modification of proteins allows tight control of the activation and inactivation of signaling pathways (for on overview of all DUBs, see [87, 88]). Moreover, it was recently discovered that, next to covalent attachment of ubiquitin to a lysine residue on a substrate protein, unanchored ubiquitin chains are a novel mechanism of signal transduction, which serve as a ligand for proteins with an ubiquitin-binding domain (UBD) [89].

Activation of PRRs and downstream signaling pathways heavily relies on ubiquitination, as is already established for phosphorylation (reviewed by [83, 90-92]). The importance of ubiquitination in intracellular signaling of immune pathways, was demonstrated recently by the finding that the NF-κB inhibitor A20, which is both an ubiquitin E3 ligase and a DUB, antagonizes the activity of three major E3 ubiquitin ligases (TRAF2, TRAF6 and cIAP1) in the NF-κB signaling pathways [93, 94]. The importance

of A20, which thus acts in the negative feedback loop of NF-κB signaling, is reflected by the observation that a certain haplotype in the locus TNFAIP, that encodes for A20, is associated with systemic lupus erythematosus (SLE) and correlated with a significant decrease in A20 mRNA expression [95]. Loss of A20 function is associated with an increasing list of immunopathologies that are characterized by chronic activation of NF-κB signaling, including rheumatoid arthritis, Crohn's disease and B-cell lymphomas ([96, 97] and reviewed by [98]).

For many members of the TRIM family, evidence is accruing for a regulatory role of signaling pathways that are activated upon viral detection by PRRs and that lead to IFN production (reviewed by [99, 100]). An overview of the current knowledge on PRR/IFN signaling control by TRIM proteins is depicted in figure 2. This figure illustrates

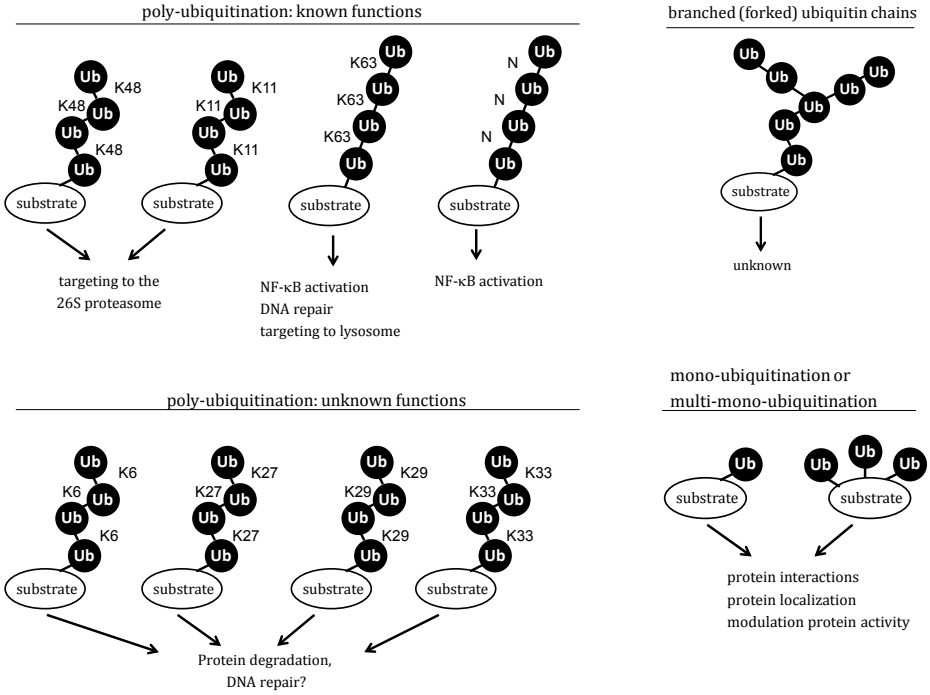


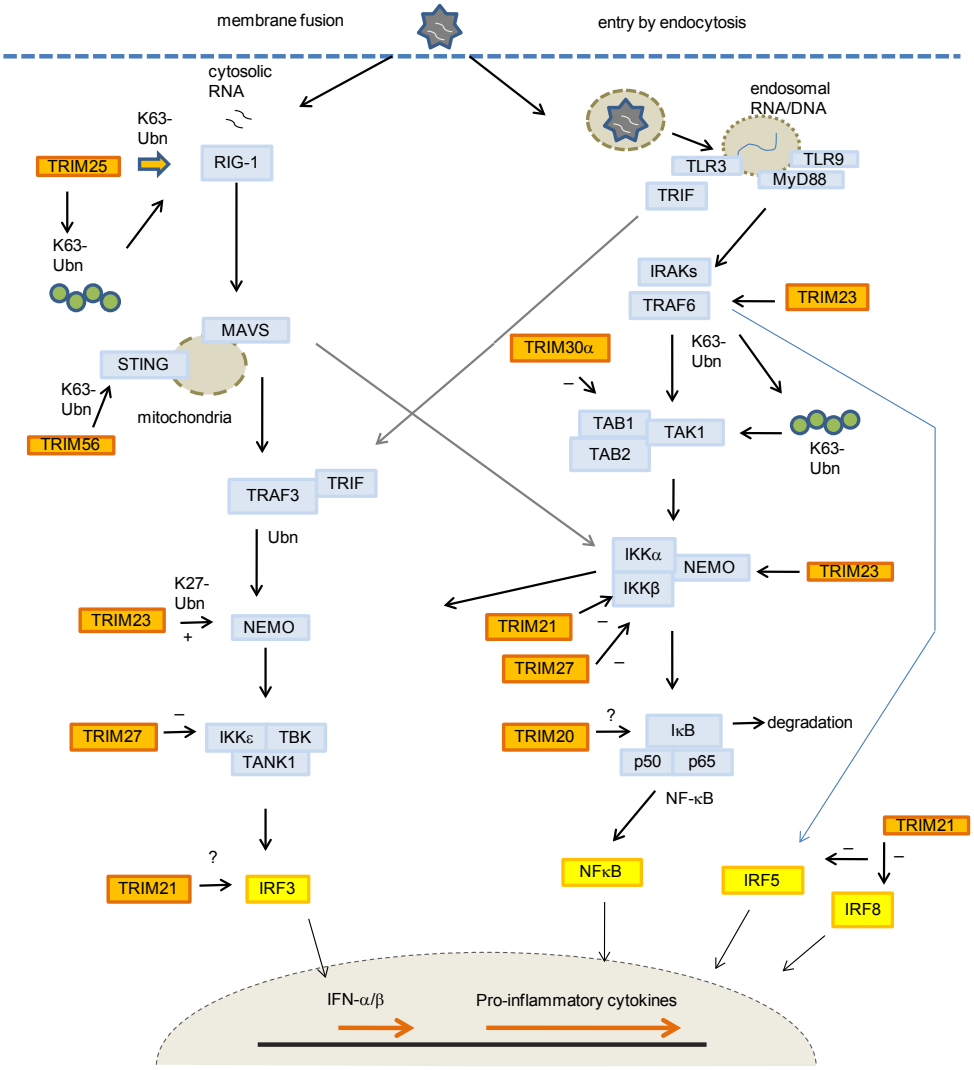
Figure 1. Different outcomes upon ubiquitination. Modification of proteins by ubiquitination leads to both protein activation or degradation, dependent on the type of ubiquitin-linkage. Ubiquitin itself contains seven lysine residues, which allows the formation of ubiquitin-chains, linked by the different lysine residues. Ubiquitination by Lys48- and Lys-11 linked chains typically results in degradation of the modified protein by the proteasome. The other type of linkages affect protein-protein interactions and the biological activity of the protein that is modified. Recently it was found that also linear ubiquitin-chains and unanchored ubiquitin-chains are processed by the ubiquitin enzymes, of which the latter acts as a signal transduction in signaling pathways. Adapted from Ye *et al.* [84].

that the viral PRR/IFN signaling pathways heavily rely on regulation by TRIM proteins. Moreover, the activity of many kinases is controlled by ubiquitination. With only few TRIM members being investigated up to date, future research on the complete TRIM family will certainly identify more TRIM members involved in cell signaling upon pathogen sensing.

Ubiquitin and ISG15 crosstalk

E3 ligases modify proteins not only by ubiquitin, but also by ubiquitin-like modifiers (Ubls). These Ubls include ISG15, SUMO and NEDD8 (reviewed by [86]). Ubls are targeted to a substrate by a similar cascade of E1, E2 and E3 enzymes, whereby ubiquitin and each Ubl are activated by distinct E1 enzymes, but E2 and E3 enzymes are shared. Ubiquitin-dependent proteasome pathways are responsible for 80-90% of protein turnover in cells [101] and although ISG15 conjugation has also been linked with the proteasome-dependent degradation, Ubls rather appear to modify protein-protein interactions [102]. Ubiquitin is encoded as a polyprotein with tandemly repeated precursors that are processed by proteolytic enzymes into monomeric proteins [103]. ISG15 resembles ubiquitin in structure, as it consists of a tandem diubiquitin sequence; ISG15 probably derived from ubiquitin after it lost the cleavage site in between the two ubiquitin subdomains [104].

ISG15 is among the genes most strongly induced by type I IFNs and it restricts numerous viruses [105]. However, the biochemical effect of ISG15 conjugation to a target protein is poorly understood (reviewed by [106]). ISG15 exerts its antiviral activity by direct targeting of viral proteins. Importantly, ISG15 also targets proteins of the host that are part of the antiviral immune system. It appears that modification by ubiquitin and ISG15 of proteins acting in the PRR signaling pathways, together determines whether these pathways are activated, or downregulated. For example, the activity of RIG-I is highly controlled by both ubiquitination and ISGylation. TRIM25 was the first identified E3 ligase required for activation of RIG-I upon sensing of viral RNA. Upon silencing of TRIM25, RIG-I does not interact with the adapter molecule MAVS, and subsequent activation of the transcription factor IRF3 and IFN-β production is severely reduced [107]. By the E3 ubiquitin ligase activity of TRIM25, one of the CARD domains of RIG-I is ubiquitinated, allowing the other CARD domain to interact with MAVS [107]. It was subsequently demonstrated that RIG-I contains an ubiquitin-binding domain (UBD), which serves as a receptor for unanchored Lys63-ubiquitin chains [108]. A second E3 Ub ligase that positively regulates RIG-I is riplet, which is indispensable for RIG-I mediated expresson of IFN [109]. A third E3 ubiquitin ligase, RNF125, negatively regulates RIG-I, by targeting it to the proteasome. In addition, RNF125 also ubiquitinates MDA5 and MAVS [110]. Further, conjugation of linear ubiquitin chains on TRIM25 by the E3 ligase complex HOIL-1L and HOIP, results in Lys48-linked ubiquitination of TRIM25 and subsequent degradation. In addition, by direct binding to RIG-I, HOIL-1L/HOIP inhibits the interaction of TRIM25 with RIG-I [111]. RIG-I is also directly modified by ISG15, whereby ISG15 acts in a negative feedback loop on type I IFN production [112]. Herc5 and TRIM25 are both E3 ligases for ISG15, but from the latter study it is not clear whether RIG-I ISGylation is dependent on TRIM25. TRIM25 may have a dual


membrane fusion
entry by endocytosis
cytosolic RNA
endosomal RNA/DNA
K63-Ubn
TRIM25
RIG-1
TLR3
TLR9
MyD88
TRIF
K63-Ubn
IRAKs
TRAF6
TRIM23
MAVS
STING
TRIM30α
K63-Ubn
K63-Ubn
mitochondria
TRIM56
TAB1
TAB2
TAK1
K63-Ubn
TRAF3
TRIF
Ubn
IKKα
IKKβ
NEMO
TRIM23
K27-Ubn
TRIM23
NEMO
TRIM21
TRIM27
TRIM27
IKKε
TBK
TANK1
TRIM20
IκB
degradation
p50
p65
NF-κB
TRIM21
?
TRIM21
IRF3
NFκB
IRF5
IRF8
IFN-α/β
Pro-inflammatory cytokines

function in both activating and inhibiting RIG-I activity, depending on the abundance of ISG15 in the cell [113-115].

Interestingly, the activity of TRAF6 is also co-regulated by ubiquitin and ISG15. TRAF6, an E3 ubiquitin ligase in the NF-κB signaling pathway, synthesizes unanchored Lys63-linked ubiquitin chains in conjunction with UbcH5c, which activates the kinase complex consisting of TAK1/TAB1/TAB2. TAK1 contains a ubiquitin binding domain for Lys63-Ub chains, which upon ubiquitin recognition, becomes activated and subsequently phosphorylates the IKKα/IKKβ/NEMO complex [89]. In addition, TRAF6 directly ubiquitinates and activates TAK1/TAB1/TAB2 in conjunction with the E2 complex Ubch13/Msm2 [116]. Interesting, ISG15 binds E2 UbcH13 and hereby suppresses the activity of UbcH13 and TRAF6, and subsequently NF-κB activation [117, 118]. However, it was also shown that ISG15 targets the protein phosphatase 2Cβ (PP2Cβ), which dephosphorylates and suppresses TAK1 and IKK and hereby NF-κB activation. Hereby, ISGylation of PP2Cβ leads to a slight increase of NF-κB activation [119].

The above data indicate that the activity of proteins acting in PRR signaling pathways is tightly regulated by multiple E3 ligases, either by the conjunction of ubiquitin or ISG15. Notably, next to RIG-I, protein kinase R (PKR), which acts both as a viral sensor and restriction factor, is also a target of ISG15 [120]. Whether ISGylation, possibly together with ubiquitination, also controls the activity of PKR is not yet investigated.

ISG15 enigma

Only two E3 ISG15 ligases have been identified so far, which are TRIM25 and the HECT-domain containing ligase Herc5 [113-115]. As protein modification by ubiquitin highly depends on specific E3 ligases, for modification of a large set of viral proteins one would expect ISGylation to be mediated by a diverse set of E3 ligases as well. However, it appears that Herc5 is the major E3 ligase for ISG15 that mediates conjunction for most

Figure 2. Control of viral PRR signaling pathways by TRIM proteins.
TRIM proteins control the activity of many proteins that act in the signaling pathways of viral pattern-recognition receptors (PRRs). Viruses release their genome into the cytosol either upon fusion of the viral envelope with the cell membrane, or upon uptake by the endosomal route and escape from the endosomes. The PRR RIG-I recognizes viral 3`ppp-dsRNA in the cytosol, TLRs recognize viruses in the endosomal compartments. Both PRRs activate downstream signaling pathways that lead to type I IFN (IFN-α/β) and pro-inflammatory cytokine expression. Different TRIM proteins regulate the activities of different proteins in these signaling cascades. TRIM25 positively regulates the activation of RIG-I by ubiquitination. TRIM56 ubiquitinates STING, which is important for TBK-1 recruitment and induction of IFN-β expression.TRIM23 activates NEMO to enhance IRF3 and NF-κB signaling. TRIM21 interacts with IRF3, for which the outcome is not clear. The TLR3 signaling pathway integrates with the pathway of RIG-I via its adapter molecule TRIF. TRL9 activates another signaling pathway via its adapter molecule MyD88. TRIM23 also activates TRAF6 TRIM21 and TRIM27 both inhibit IKKβ and TRIM21 also inhibits IRF5 and IRF8 activation. Adapted from McNab *et al.* [100] and Kawai *et al.*[99].

of the 300 ISG15 targets (reviewed by [106]), as silencing for Herc5 drastically reduces ISG15-conjugates [115]. It was demonstrated by Durfee *et al*. that upon overexpression of ISG15, together with the ISG15 E1 activating enzyme Ube1L, E2 conjugating enzyme UbcH8 and E3 ligase Herc5, only de novo synthesized proteins are ISGylated [121]. Further, the authors demonstrated that Herc5 is associated with polyribosomes and proposed that ISG15 modifies proteins in a co-translational matter. These data would suggest that also proteins of the host are ISGylated in a non-specific manner. It can be expected that this should have a major impact on the physiological state of the cell. The authors here hypothesized that, as ISGylation is an inefficient process and not all newly synthesized proteins are ISGylated, the majority of proteins that are non-specifically modified during viral infection, will be viral proteins [121]. Further, specific DUBs have been identified for ISG15, supporting the argument that ISGylation is also a reversible type of protein modification and can be controlled [122]. However, with 100 DUB encoding genes on the human genome, and only five DUB proteins characterized so far that remove ISG15 from ISGylated proteins, this may indicate that ISGylation plays a less significant role in protein regulation than ubiquitin. This is presumably ascribed to the relatively recent origin of ISG15, which has only been reported in vertebrates, in contrast to the highly conserved ubiquitin, distributed in all eukaryotes. ISG15 may not have evolved yet a sophisticated set of E2, E3 and DUBs like ubiquitin has and its biological role is therefore probably more restrained. As the expression of ISG15 is dependent on type I IFNs, its functions might be limited to immunity. Interestingly, it was reported that ISG15 can also be secreted, whereby it stimulates natural killer cells and IFN-γ production [123]. This observation, together with the finding that ISG15 downregulates IFN production by suppressing the activity of both RIG-1 and TRAF6, indicates that ISG15 may mediate in both the downregulation of the early innate immune response and in the induction of the adaptive immune response. The results described in this thesis on fish TRIM proteins and ubiquitination, paves the way for further comparative investigation of the implication of ubiquitination and ISGylation in antiviral immunity in fish.

A model for fish inflammation

It is well described in mammals that inflammation is a multistep process, driven by numerous cytokines, chemokines and enzymes. Pro-inflammatory cytokines on one hand induce inflammation and mediate recruitment and activation of immune cells, which eliminate infectious agents and clear infected cells. On the other hand, anti-inflammatory cytokines orchestrate the resolution of inflammation and tissue repair. These are overlapping processes that require tight regulation, as prolonged inflammation results in irreversible tissue damage. At specific time points during inflammation, leukocytes of different cell populations with specialized functions are recruited, which are required at different phases in inflammation. Neutrophilic granulocytes and macrophages are important actors in inflammation, as these are not only effector cells that eliminate microbes, but also are sources of pro- and anti-inflammatory mediators. Neutrophils are the most abundantly circulating leukocytes in mammals and typically the first cells to arrive at the site of inflammation. They have

a primary role in pathogen clearing and act during the first hours of the inflammatory response. However, recent data indicate that neutrophils also drive the resolution of inflammation [124]. Monocytes infiltrate after the initial neutrophil recruitment, but still early during inflammation, upon which they differentiate into macrophages. Due to the macrophage plasticity and functional polarization, they play key roles during the entire inflammatory response. First, resident macrophages initially recognize infectious microbes and initiate the immune response by the expression of pro-inflammatory cytokines. Secondly, upon monocyte recruitment, macrophages polarize into different subsets, directed by local stimuli present at that time of the inflammatory response [125]. Classically activated M1 macrophages are induced by IFN-γ alone, or together with microbial stimuli or pro-inflammatory cytokines. They are high producers of IL-12p70, nitric oxide (NO) and reactive oxygen intermediates (ROI), and low producers of IL-10. M1 macrophages play a pre-dominant role in promoting inflammation and pathogen clearing. M2 polarized macrophages are divided in three subsets which mediate the resolution of inflammation and promote tissue repair and remodeling. Alternatively activated M2a macrophages are induced by the Th2 cytokines IL-4 and/or IL-13 and have low antimicrobial activity. M2a macrophages deregulate the activity of IL-1β by the expression of the decoy receptor IL-1RII and the IL-1 receptor agonist (IL-1Ra). Further, they have a high arginase activity and produce polyamines that promote cell growth. M2b macrophages are stimulated by TLR ligands and immune complexes, while M2c macrophages are driven by IL-10. Both M2b and M2c polarized macrophages produce IL-10 and M2c also produces TGF-β and factors for remodeling of the extracellular matrix, e.g. versican and PTX3 (reviewed by [125]).

With the results presented in the second part of this thesis, together with previous obtained data ([126, 127], unpublished work), a model for the inflammatory response in carp is depicted in figure 3. It is based on gene expression of pro-inflammatory and anti-inflammatory mediators (e.g. cytokines, chemokines and enzymes) and chemokine receptors, determined at selected time points in a carp zymosan-induced peritonitis model. This model enabled to delineate the inflammatory response and shows that fish already possess a sophisticated set of cytokines that drive inflammation, with high similarity to the mammalian inflammatory response. Importantly, a similar macrophage heterogeneity appears in fish ([128], reviewed by [129]). This is also indicated in the carp inflammation model [126], that shows a dominant expression of IL-12p35 at day 1 and for iNOS at day 2, while arginase expression has a most prominent peak at 7 days after zymosan injection. Interestingly it also shows that arginase is already modestly expressed at the start of the inflammatory response, indicating that tissue repair/remodeling is initiated simultaneously with pathogen elimination.

At the start of the thesis project, it was hypothesized that fish CXC chemokines that resemble mammalian CXCL8 and CXCL9-11 most in sequence similarity, are possibly functional homologs, despite not being true orthologs. In this thesis, the in vitro and in vivo studies for CXCL8-like chemokines of both lineages (CXCa_L1 and CXCL8_L2) indeed show functional homology to mammalian CXCL8. First, they are expressed early in inflammation and their expression coincides with the gene expression of pro-inflammatory cytokines IL-1β and TNF-α and iNOS, indicative for the

presence of activated M1 macrophages (**Chapter 5**, [126]). Secondly, both CXCL8-like chemokines mediate a rapid influx of neutrophilic granulocytes upon administration in vivo (**Chapter 6**). Thirdly, both chemokines have a stimulatory effect on phagocyte activation *in vitro*. Together, this strongly suggests a pro-inflammatory function for fish CXCL8-like chemokines of both lineages, mediating predominantly neutrophil recruitment and activation.

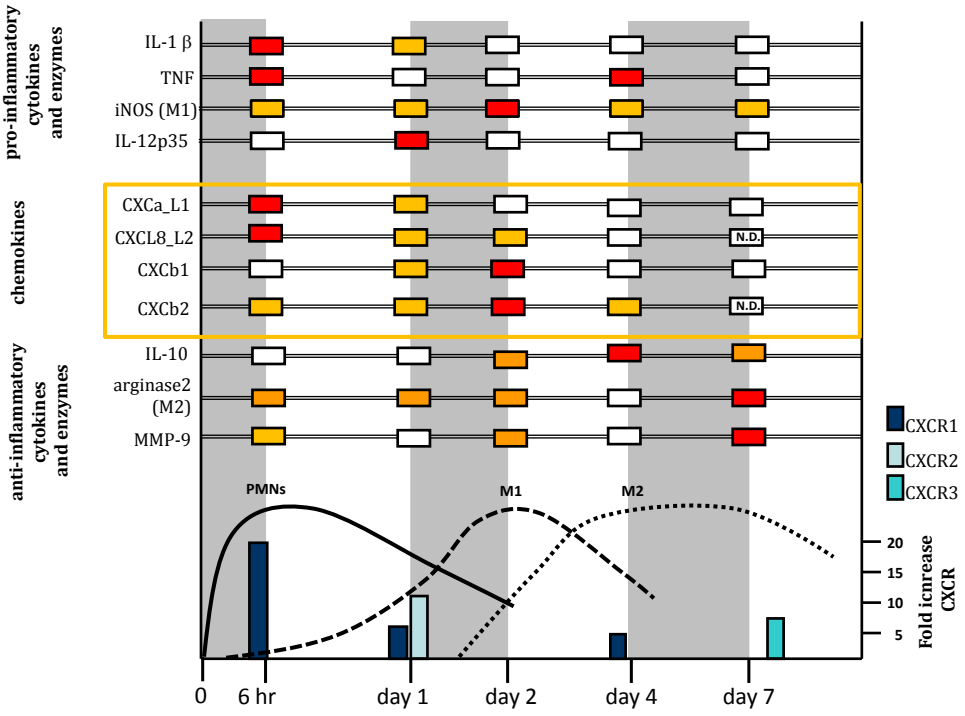


Figure 3. A carp zymosan-induced peritonitis model The model indicates cytokine and enzyme gene expression determined in peritoneal leukocytes, leukocyte recruitment and expression of chemokine receptors upon intraperitoneal administration of zymosan. The gene expression of pro-inflammatory cytokines (IL-1β, TNF and IL-12p35) and enzymes (iNOS), chemokines (CXCLa_L1, CXCL8_L2, CXCb1, CXCb2), and anti-inflammatory cytokines (IL-10) and enzymes (arginase2, MMP-9) is represented by boxes at selected time-points (0 hrs, 6 hrs, day 1, 2, 4, 7). Red boxes represent the highest detected significant increase in expression, orange boxes represent significantly induced expression, white boxes represents time-points at which the expression was not significantly induced. For CXCL8_L2 and CXCb2, the expression was not determined at day 7 (N.D.).The influx of polymorphonuclear cells (PMNs, e.g. neutrophilic granulocytes), M1 polarized macrophages and M2 polarized macrophages are indicated by curves. Gene expression of chemokine receptors CXCR1, CXCR2 and CXCR3 are indicated by blue bars, the fold-increase is indicate at the right vertical axis. Data is derived from part two of this thesis and [126, 127].

For chemokine lineages of the CXCb subset, different expression patterns where observed, whereby the CXCb2 gene is also expressed at the beginning of the inflammatory reaction, while CXCb1 gene expression increased after 1 day of zymosan administration (**Chapter 7**, [126]). Importantly, expression of both genes peaks at 1 and 2 days after the induction of inflammation, which coincides with monocyte/macrophage recruitment. Moreover, it was shown that, although CXCb1 is chemotactic for both granulocytes and macrophages in vitro, it only modestly recruits neutrophils *in vivo* (**Chapter 6**). It might be postulated that CXCb chemokines moreover mediate monocyte recruitment during the stage of the inflammatory response when stimuli are present that drive M2 polarization and mediate the resolution phase. Moreover, peak expression of both CXCb genes coincides with first detection of gene expression of the anti-inflammatory cytokine IL-10, whereas IL-1β, TNF-α and CXCLa_L1 genes are already downregulated at this stage. The mammalian CXCL9-11 chemokines typically recruit activated T-cells. Attraction of lymphocytes was also demonstrated *in vitro* for CXCb1 and it was further shown that both CXCb1 and CXCb2 gene expression are inducible by IFN-γ *in vitro* (**this thesis**, [130]). This further advocates for functional similarity of fish CXCb genes with mammalian CXCL9-11 chemokines.

The different kinetics in gene expression of chemokine lineages belonging to either the CXCL8-like or the CXCb subsets, indicates functional diversification for these chemokines, which will allow to fine-tune the inflammatory response. It has to be noted that the inflammation model is much more complex than the data presented here and neutrophils and monocyte/macrophage recruitment is orchestrated by multiple chemokines and other chemoattractants (e.g. bacterial products, complement factors, TNF-α), as described in mammals [131]. This is also becoming more evident in fish, as a high number of chemokine genes are expressed during inflammation and microbial infection (recently reviewed by [132]). Biological functions have been established for only a few fish chemokines, but for CXCL8-like chemokines of lineage 1, it is shown in other fish that these have pro-inflammatory effects and mediate recruitment of neutrophilic granulocytes [133-135], indicating that the carp chemokine model is applicable to other fish species.

Future research should aim for the identification of corresponding receptors for the individual CXC chemokines. Four CXC receptors are identified so far in common carp ([44, 136], unpublished work), with different kinetics in gene expression over the course of the inflammatory response, as was determined for CXCR1, CXCR2 and CXCR3 (**this thesis**, [126], unpublished work). The model shows that expression of CXCR1 coincides with early recruited leukocytes (predominantly neutrophilic granulocytes), CXCR2 with later recruited leukocytes and CXCR3 with cells present during the resolution phase. This correlates with the data obtained in vitro (**Chapter 5**), which showed that constitutive CXCR1 expression is high for granulocytes and low for macrophages, whereas 6-day cultured macrophages highly express CXCR3 (unpublished data). CXCR2 constitutive expression is low for all cell populations. Further, LPS enhances CXCR1 expression in granulocytes (**Chapter 5**). This indicates that the early expressed CXCa_L1 and CXCL8_L2 both recruit CXCR1 expressing cells, whereas CXCb chemokines presumably recruit CXCR3 positive cells. However, as chemokine receptors are promiscuous, CXCR2

positive cells might be recruited by both the CXCL8-like, as well as CXC chemokines. It has to be noted that the determination of CXC receptor expression is based on mRNA expression and may not truly represent the levels of CXCR expression on the membrane, as chemokine receptor expression, internalization, recycling and synthesis are differentially regulated for different chemokine receptors [137]. Cells that express CXCR3 might correspond to M2 polarized macrophages acting in the resolution phase. Although the biological function of CXCR3 positive monocytes is not yet well described, it is reported in mice that CXCR3 expressing monocytes/macrophages are implicated in wound repair and vascular remodeling [138, 139]. This strengthens the hypothesis that CXCb chemokines mediate tissue repair and the resolution of inflammation by recruiting CXCR3 positive monocytes, which may polarize towards the M2 subset.

Outlook

It was recently discovered that TRIM5α is a viral pattern-recognition receptor. A requirement for a PRR is that it recognizes a specific pathogen-associated molecular pattern (PAMP) and, via an adapter molecule, subsequently activates signaling pathways that induce an immune response. TRIM5α was shown to be a sensor for the retroviral capsid lattice, upon which it generates unanchored Lys63-chains that are subsequently recognized by RIG-I. In here, RIG-I acts as a receptor for unanchored Lys63-chains and activates downstream signaling pathways leading to type I IFN production [140]. This is a first proof for a TRIM protein to be a PRR. With the various characteristics for TRIM proteins that were described in this thesis, it is presumed that more TRIM proteins have a function in pathogen recognition [141]. The finTRIMs that were described in this thesis, and probably members of fish TRIM35 and TRIM39/bloodthirsty multigene families, possibly are PRRs.

Ubiquitination, ubiquitin-like modification, and unanchored ubiquitin chains serving as a ligand for ubiquitin-binding proteins, indicate an important mode of regulation and activation of intracellular pathways. It is estimated that the human genome encodes for over 600 E3 ligases. Taken into consideration that the human TRIM family consists of seventy genes, which in addition encode for alternative spliced isoforms, TRIM proteins make up roughly fifteen percent of the total E3 ligase genes encoded by the cell. TRIM proteins are therefore suspected to play a key role in the regulation and control of cellular systems. While in this thesis the focus was on TRIM proteins and their roles in antiviral immunity, evidence is increasing that functions of TRIM proteins reach beyond antiviral immunity. Deregulated expression of TRIM proteins, mutations, or genomic aberrations in TRIM genes are linked with auto-immune diseases and cancer (reviewed by [142, 143]). Future research into the ubiquitin(-like) protein-modifying activity by TRIM proteins in molecular pathways will certainly contribute to a better understanding of the cellular defense mechanisms that combat threats for cellular homeostasis. An exciting future lies ahead for TRIM research.

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Le bonheur n'est pas le but, mais la moyen de la vie.

- Paul Claudel -

Summary & Acknowledgements

Summary

Pathogens are a major driving force for the immune system to diversify. The battle between pathogens and their hosts is often referred as an evolutionary race. Pathogens on one hand continuously evolve new strategies to evade host immune responses, while the immune system on the other hand constantly evolves new mechanisms to combat invading pathogens. Diversification of the host immune system offers an advantage at two levels. First, the immune system is in the possession of a large and diverse set of receptors that allows recognition of a wide range of pathogens. Pathogen recognition is a key step for the immune system to initiate the immune response. Secondly, diversification of intracellular and extracellular signaling molecules has generated specialized molecules to finely regulate immune responses. Tight regulation of the immune response is essential, as uncontrolled immune responses are detrimental to the host.

Two protein families of the immune system that typically co-evolved with pathogens are **TRIM proteins** and **chemokines**. These families highly expanded in gene numbers and are implicated in multiple biological processes. TRIM proteins are named after a conserved tri-partite motif at the N-terminal side of the protein: a RING zinc finger domain, one or two B-boxes and coiled coil domain. TRIM proteins are intracellular proteins that restrict viruses. Chemokines are secreted cytokines that are specialized in orchestrating cell migration. Leukocytes recruitment to the site of infection is essential during the immune response, as leukocytes are specialized in eliminating infectious agents and clearing of infected cells. The recruitment of leukocytes is preceded by vasodilation and increased vascular permeability; these processes together are named inflammation. The diversification of the chemokine family enables the immune system to direct the sequential recruitment of distinct leukocyte subsets, required during different phases of the inflammatory response.

A higher degree of diversification and specialization of the immune system is observed with increasing complexity of multicellular organisms. Teleost fish emerged early during vertebrate evolution and already have a fully functional immune system. Its characterization improved our insight in the evolution of this complex system. In this thesis, the evolutionary dynamics of TRIM proteins and chemokines were studied in fish and a characterization of their function was made.

In **part one** of this thesis, a full description was made of the TRIM gene family in zebrafish and tetraodon. These are two fish species for which the whole genome sequence is available. It was first found that numerous TRIM genes are conserved between fish and mammals. Further, a high number of TRIM genes are not well conserved between fish and mammals contain an additional domain at the C-terminus that is named B30.2. Interestingly, it was discovered that among TRIM genes containing a B30.2 domain, certain genes have undergone a dramatic expansion that generated three multigene TRIM families, a feature unique to fish. Orthologous genes were found in mammals for two multigene families and named TRIM35 and TRIM39/bloodthirsty. No orthologs could be found for the third multigene family, which was therefore named finTRIM,

for fish novel TRIM. The finTRIM gene family showed a high degree of diversification: diversity in length and number of protein domains for which they encode, diversification in sequence and in gene numbers among different fish species. The finding of sites that evolve under positive selection in the B30.2 domain of all three multigene families, indicates that they evolve under pathogen diversifying pressure (**Chapters 2 and 3**).

In order to find a function for fish TRIM proteins in antiviral immunity, finTRIMs were further investigated in rainbow trout, a well-established model to study viral infection. Also in trout, a high number of finTRIM transcripts of diverse lengths were identified. The induction in finTRIM gene expression by the fish rhabdovirus VHSV and polyI:C indeed indicated a role of trout finTRIMs in antiviral immunity (**Chapter 2**). This induction of finTRIM gene expression was attributed to the whole set of finTRIM genes, demonstrated by a high-throughput sequencing approach. Two finTRIM proteins were found to localize in the cytosol, which allows interaction with other cytosolic factors (**Chapter 4**).

In addition to their role as direct viral restriction factors, an E3 ubiquitin ligase activity is demonstrated for multiple mammalian TRIM proteins. E3 ubiquitin ligases catalyze the conjugation of ubiquitin to a substrate protein and recent data indicate an important role for E3 ubiquitin ligases in regulating signaling pathways (discussed in **Chapter 8**). For two trout finTRIMs it was found that they display E3 ubiquitin ligase activity, implying that they can control the activity of other proteins, possibly those involved in signaling pathways associated with antiviral immunity (**Chapter 4**).

In **part two** of this thesis, inflammatory CXC chemokines were investigated. Zebrafish and common carp, which both belong to the cyprinid lineage, were used as a model. Two CXC chemokines were earlier identified in carp, of which their sequence most resembles mammalian CXCL8 and CXCL9/10/11 chemokines, but true orthology could not be demonstrated by phylogeny studies. These two chemokines were alternatively named CXCa and CXCb, for the CXCL8-like and CXCL9/10/11-like chemokines, respectively. It was found that chemokines belonging to both the CXCa and CXCb subset diversified in cyprinid fish (**Chapters 5 and 7**). Two CXCL8-like lineages were identified, and named CXCa_L1 and CXCL8_L2. CXCa_L1 has orthologous genes in other fish lineages, while for CXCL8_L2 no orthologs were identified in fish outside the cyprinid lineage. For CXCb, a similar diversification was observed. A second CXCb gene was cloned in carp that was named CXCb2. In zebrafish, a cluster of seven CXCb-related genes was identified on chromosome five.

In vitro studies demonstrated that chemokines within the CXCL8-like subset, or the CXCb subset, respond differently towards microbial stimuli (**Chapters 5 and 7**). Further, it was observed that the promoter regions of the individual zebrafish CXCb genes contain different transcription factor binding sites, indicating that these genes are regulated by different stimuli. Moreover, in two carp in vivo inflammation models, a more potent induction was observed in gene expression for CXCL8_L2 over CXCa_L1. Based on these results, it is speculated that the multiple lineages belonging to either the CXCL8-like subset, or the CXCb subset, have diverged functions.

The fish CXC chemokines behave as “true” chemokines, as chemotactic activity

of carp leukocytes towards recombinant proteins prepared for CXCa_L1, CXCL8_L2 and CXCb was demonstrated in vitro and in vivo (**Chapters 5 and 6**). Further, intracellular calcium mobilization was observed upon stimulation of granulocytes with CXCa_L1, indicating signaling via typical chemokine G-protein coupled receptors (**Chapter 6**).

Based on the kinetics of CXCa_L1, CXCL8_L2 and CXCb1 gene expression in a carp zymosan-induced peritonitis model, it was hypothesized that these chemokines are functional homologs of their mammalian counterparts (**Chapter 5**). By an in vivo study in which the recombinant proteins were administrated intraperitoneally, it was observed that both CXCa_L1 and CXCL8_L2 strongly recruit leukocytes, predominantly of the neutrophilic granulocyte population (**Chapter 6**). Together with a stimulatory effect on carp phagocytes in vitro, a pro-inflammatory function was attributed to both CXCL8-like lineages. CXCb was not potent in leukocyte recruitment in vivo. It was speculated that CXCb recruits monocytes and enables macrophage polarization towards the M2 subset, which acts in tissue repair and remodeling. In addition, the two carp CXCb chemokines both resemble the mammalian IFN-γ inducible CXCL9-11 chemokines, as CXCb1 and CXCb2 genes were both inducible by IFN-γ2, moreover, CXCb1 was also chemotactic for lymphocytes (**Chapters 6 and 7**).

In conclusion, the results obtained in this thesis indicate that specialized functions of TRIM proteins and CXC chemokines in immunity were already present in early vertebrate evolution. Based on the features whereby TRIM genes evolved in fish, it is hypothesized that fish multigene TRIM proteins are implicated in pathogen sensing and possibly represent a novel class of pattern-recognition receptors. Future work on chemokines should aim at the identification of receptors, to further delineate their role during inflammation.

Samenvatting

De grote diversiteit aan ziekteverwekkers die aanwezig zijn, en waren, heeft bijgedragen aan de ontwikkeling van een goed ontwikkeld immuunsysteem, dat in staat is om veel van deze ziekteverwekkers te herkennen en te bestrijden. Naast hun grote diversiteit, hebben ziekteverwekkers als virussen en bacteriën, allerlei strategieën ontwikkeld om aan het immuunsysteem te ontsnappen. Bovendien hebben virussen en bacteriën een snelle ontwikkeling en ontstaan er voortdurend nieuwe soorten. Deze eigenschappen van ziekteverwekkers: diversiteit, ontsnappingstrategieën en snelle ontwikkeling, maken het dat ziekteverwekkers een belangrijke aandrijvingkracht zijn achter de evolutie van het immuunsysteem. Het immuunsysteem moet namelijk nieuwe manieren blijven ontwikkelen om ziekteverwekkers toch uit te kunnen schakelen.

Hogere organismes zoals de mens hebben een zeer goed ontwikkeld immuunsysteem. Maar ook vissen zijn heel goed in staat om zich te weren tegen ziekteverwekkers. Dit blijkt vooral uit het feit dat vissen een zeer succesvolle groep vormen binnen het dierenrijk: meer dan de helft van alle gewervelde dieren, zijn vissen. Vissen zijn meer dan 450 miljoen jaar geleden ontstaan, kort na het ontstaan van gewervelde dieren. Vissen zijn daarom een interessante groep dieren voor de studie aan het immuunsysteem. Begrip van het immuunsysteem van de vis, leidt namelijk tot een beter inzicht van de ontwikkeling van het immuunsysteem van meer primitieve diersoorten, naar hogere organismen. In dit proefschrift is de evolutie van het immuunsysteem bestudeerd aan de hand van de de vis. We hebben hierbij specifiek gekeken naar twee grote families van eiwitten die een belangrijk rol spelen in de immuunreactie: de familie van **TRIM eiwitten** en **chemokines**.

Virussen zijn ziekteverwekkers die cellen binnendringen en gebruik maken van systemen aanwezig in de cel voor hun vermenigvuldiging. Er zijn meerdere factoren die bepalend zijn of een bepaald type virus, in staat is om in een bepaalde type cel te kunnen vermenigvuldigen. Het is onlangs ontdekt dat eiwitten die tot de **TRIM familie** behoren, een belangrijke rol hebben hierbij. Veel leden van de TRIM familie kunnen namelijk virussen remmen. Op het genoom van de mens zijn zeventig TRIM genen geïdentificeerd die behoren tot de TRIM familie. Dit maakt het een grote familie van eiwitten. TRIM eiwitten zijn vernoemd naar een geconserveerd driedelig motief (in het Engels: *tri-partite motif*). Wat een interessante ontdekking was, is dat bepaalde virussen, alleen geremd worden door een bepaald TRIM eiwit. In de resusaap is bijvoorbeeld een TRIM eiwit gevonden, TRIM5a, dat het HIV virus kan remmen. Mensen hebben ook het gen dat codeert voor het TRIM5a eiwit, maar zijn niet beschermd tegen HIV. Dit komt door een klein verschil in de DNA sequentie van het TRIM5 gen van de mens en de resusaap. Er zijn meer TRIM eiwitten gevonden die alleen een bepaald type virus kunnen remmen. Deze bevinding is een belangrijke aanwijzing dat de individuele TRIM genen van verschillende diersoorten, samen geëvolueerd zijn met virussen.

Aan de start van dit onderzoek was ontdekt dat ook in de vis, een TRIM eiwit gemaakt wordt tijdens infectie met virus. De functie van dit TRIM eiwit was onbekend. We hebben daarom de evolutie en functies van de TRIM familie nader onderzocht en de

vis gebruikt als model. De sequentie van het genoom is bekend voor een aantal vissen, onder meer voor de zebravis en de kogelvis. Eerst hebben we een beschrijving gemaakt van alle TRIM genen die we konden identificeren op het genoom van deze twee vissen (**Hoofdstukken 2** en **3**). Een interessante bevinding was dat in vissen, TRIM genen sterk zijn vermenigvuldigd en een nog grotere familie vormen dan in de mens. We ontdekten dat in de vis drie multigene TRIM subfamilies zijn gevormd. Deze multigene families bestaan uit een groot aantal genen die sterk overeenkomen in DNA sequentie. In zoogdieren zijn multigene TRIM families niet beschreven; het lijkt dat de vorming van deze multigene families uniek is voor de vis. Voor twee multigene TRIM families konden we een vergelijkbaar gen vinden op het genoom van andere groepen dieren, dit zijn de TRIM35 en TRIM39 genen. Voor de derde multigene familie, konden we geen overeenkomende genen vinden in andere diergroepen. Deze familie hebben we daarom finTRIM genoemd, voor 'nieuwe vis TRIM genen' (*fish novel TRIM*). Nadere bestudering van deze multigene TRIM families in de vis toonde aan dat deze families erg divers zijn. De genen binnen een familie verschillen in lengte en coderen voor eiwitten van verschillende groottes. Daarnaast zijn de genen op bepaalde plaatsen verschillend in sequentie. Dit is een aanwijzing dat deze TRIM eiwitten kunnen binden aan iets wat ook heel divers is. Bovendien konden we aantonen dat de drie multigene TRIM familie geëvolueerd zijn onder positieve selectiedruk. Op basis van deze resultaten, hebben we de hypothese opgesteld dat leden van de TRIM multigene families in de vis, mogelijk betrokken zijn in de herkenning en bestrijding van ziekteverwekkers. Om te bepalen of de multigene TRIM families inderdaad belangrijk zijn voor het immuunsysteem, hebben we de functie van de finTRIM familie nader onderzocht. Hiervoor hebben we de regenboog forel gebruikt, een vis die veel gebruikt wordt als model om virusinfecties in de vis te bestuderen. We vonden dat tijdens infectie met virus, de finTRIM genen tot expressie komen en dat dit geldt voor het merendeel van de finTRIM genen (**Hoofdstukken 2** en **4**). Hieruit concludeerden we dat alle finTRIM genen mogelijk een rol spelen in virale infectie.

Voor TRIM eiwitten in zoogdieren is beschreven dat zij niet alleen belangrijk zijn voor de directe herkenning en remming van virussen, maar dat zij ook E3 ubiquitine ligase activiteit bezitten. Dit betekent dat TRIM eiwitten een proces in de cel katalyseren waarbij kleine eiwitten, de ubiquitine eiwitten, geplakt worden aan andere eiwitten in de cel. Dit proces wordt ook wel ubiquitinering genoemd. Aanvankelijk werd gevonden dat ubiquitinering van eiwitten resulteert in afbraak van deze eiwitten. Meer recent onderzoek heeft aangetoond dat ubiquitinering ook eiwitten kan activeren. In zoogdieren is ontdekt dat TRIM eiwitten andere eiwitten ubiquitineren die belangrijk zijn voor het immuunsysteem (**Hoofdstuk 8**). Hiermee spelen TRIM eiwitten een belangrijke rol in het activeren en reguleren van de immuunreactie. Om te onderzoeken of TRIM eiwitten in de vis ook andere eiwitten kunnen ubiquitineren, hebben we een techniek ontwikkeld om dit te kunnen bepalen (**Hoofstuk 4**). We vonden voor twee finTRIM eiwitten van de regenboog forel, dat ook zij deze E3 ubiquitine ligase activiteit hebben. Op basis hiervan hebben we de hypothese gemaakt dat de multigene TRIM eiwitten in de vis, mogelijk ook de immuunreactie na infectie met virus kunnen reguleren door middel van ubiquitinering. Toekomstig onderzoek zal dit moeten aantonen.

De TRIM eiwitten maken deel van een cellulair, intrinsiek, immuunsysteem (**Hoofdstuk 8**). Het immuunsysteem is verder opgebouwd uit meerdere organen en gespecialiseerde cellen, die gezamenlijk ziekteverwekkers bestrijden. Voor deze samenwerking is goede communicatie tussen cellen onderling belangrijk. Eiwitten die de communicatie binnen het immuunsysteem verzorgen worden cytokines genoemd. Sommige cytokines zijn gespecialiseerd in het aantrekken van immuuncellen naar de plaats in het lichaam waar een virus of bacterie zich bevindt. Deze cytokines worden **chemokines** genoemd. Het proces van rekrutering van immuuncellen naar de plaats waar een infectie gaande is, wordt ontsteking genoemd. Meerdere celtypes van het immuunsysteem zijn betrokken bij het ontstekingsproces. Omdat een ongecontroleerde ontsteking kan leiden tot ernstige schade aan cellen van het lichaam, is het belangrijk dat het ontstekingsproces goed gereguleerd wordt. Doordat het immuunsysteem in het bezit is van veel verschillende chemokines, kan het immuunsysteem het ontstekingsproces heel precies reguleren. De chemokines vormen dus ook een grote familie van eiwitten.

In zoogdieren is bekend dat verschillende chemokines een rol spelen tijdens de verschillende fasen van de ontstekingsreactie. Onder meer de chemokines **CXCL8**, **CXCL9**, **CXCL10** en **CXCL11** zijn belangrijk in de ontstekingsreactie. CXCL9, CXCL10 en CXCL11 worden vaak samen gegroepeerd, omdat ze dezelfde cellen aantrekken. Aan de start van dit onderzoek waren een aantal chemokine genen beschreven in de vis, waarvan de DNA sequentie sterk lijkt op de genen die coderen voor CXCL8, CXCL9, CXCL10 en CXCL11 in zoogdieren. Of deze chemokines ook een actieve rol spelen in de ontstekingsreactie van de vis, was nog niet goed bekend aan het begin van dit onderzoek. In **deel Twee** van dit proefschrift is de evolutie van chemokines bestudeerd in de vis en zijn hun functies nader onderzocht. Een eerste interessante ontdekking was dat in de vis, bepaalde chemokine genen zijn gedupliceerd. Op het genoom van de zebravis werden drie genen geïdentificeerd die lijken op CXCL8 van zoogdieren. Zeven genen werden gevonden die coderen voor genen die sterk lijken op de CXCL9, CXCL10 en CXCL11 chemokines. Ook in de karper vonden we dat een duplicatie van deze genen heeft plaats gevonden, voor beide groepen chemokines werden twee genen geïdentificeerd (**Hoofdstukken 5 en 7**). De vis chemokines die, of op CXCL8, of op CXCL9-11 lijken, verschillen onderling in hun sequentie. Dit duidt aan dat zij misschien een specialistische functie hebben. Om dit te onderzoeken hebben we gekeken naar de activering van deze genen tijdens de ontstekingsreactie. Voor de karper was al eerder een model opgezet waarbij een stofje wordt ingespoten in de buikholte van de vis, wat een infectie nabootst. Hiermee kan een ontstekingsreactie worden opgewekt, waarbij immuuncellen migreren naar de buikholte van de vis. De activiteit van de verschillende chemokine genen was bepaald in de immuuncellen gemigreerd naar de buikholte (**Hoofdstuk 5**). Een interessant bevinding was dat de twee CXCL8-achtige chemokine genen beiden al kort na injectie, binnen zes uur, actief zijn. De twee CXCL9-11-achtige genen zijn pas actief één tot twee dagen na injectie. Dit komt overeen met wat beschreven is voor de CXCL8 en CXCL9-11 chemokines in zoogdieren. CXCL8 in zoogdieren is erg belangrijk bij het inzetten van de ontstekingsreactie. CXCL8 rekruteert de eerste immuuncellen die in de bloedbaan circuleren, naar de plaats van infectie. Het zijn met name de neutrofiele granulocyten, bepaalde type cellen van het aangeboren

immuunsysteem, die als eerste gerekruteerd worden. Granulocyten zijn heel effectief in het opruimen van ziekteverwekkers. CXCL9-11 chemokines rekruteren onder meer T-cellen, cellen van het aangeworven immuunsysteem. T-cellen zijn pas later in de immuunreactie belangrijk.

Het patroon in activering van de CXCL8- en CXCL9-11-achtige genen komt dus overeen tussen vis en zoogdier. Om te bepalen of hun functies ook overeenkomen, hebben we vervolgens experimenten gedaan waarbij we onderzochten welke celtypes worden aangetrokken door de verschillende chemokines (**Hoofdstukken 5 en 6**). Immuuncellen werden geïsoleerd uit de vis en de verschillende celtypes van elkaar gescheiden. We vonden dat beide CXCL8-achtige chemokines heel sterk de neutrofiele granulocyten aantrekken, dus overeenkomend met zoogdieren. Voor CXCL9-11 chemokines vonden we dit ook, hierin lijken de CXCL8- en CXCL9-11-achtige chemokines niet van elkaar te verschillen. We vonden daarnaast dat CXCL9-11 ook monocyten en lymfocyten aantrekken. Monocyten zijn cellen die differentiëren in macrofagen, een ander celtype van het immuunsysteem. De functie van CXCL9-11-achtige chemokines is misschien toch verschillend van de CXCL8-achtige chemokines. We hebben daarom onderzocht of de verschillende chemokines immuuncellen ook kunnen activeren. Hiervoor werden granulocyten en macrofagen gebruikt. Deze cellen worden ook wel fagocyten genoemd, omdat ze ziekteverwekkers kunnen fagocyteren, 'opeten'. We vonden in dit experiment een verschil: de CXCL8-achtige chemokines activeren fagocyten, terwijl we dit niet zagen voor de CXCL9-11-achtige chemokines. Vervolgens hebben we gekeken of de vis chemokines ook immuuncellen aantrekken, en dus een ontsteking inzetten, wanneer de vis chemokines direct geïnjecteerd worden in de buikholte van de vis (**Hoofdstuk 6**). We zagen dat al kort na injectie, binnen 6 uur, de CXCL8-achtige chemokines allebei sterk immuuncellen aantrekken naar de buikholte. Met name neutrofiele granulocyten en ook macrofagen werden gerekruteerd. Injectie met de CXCL9-11-achtige chemokine resulteerde in de aantrekking van weinig immuuncellen naar de buikholte.

Op basis van de bevinding dat de genen van de CXCL8-chemokines actief zijn aan het begin van de ontstekingsreactie, de CXCL8-achttige chemokines neutrofielen aantrekken zowel *in vitro* als *in vivo*, en fagocyten activeren, concluderen we dat CXCL8-achtige chemokines een belangrijke rol spelen bij het inzetten van de ontsteking. De CXCL9-11-achtige chemokines lijken een rol te spelen tijdens een latere fase van de ontsteking, omdat de genen pas actief zijn als de ontstekingsreactie al is ingezet. Daarnaast activeren de CXCL9-11-achtige chemokines fagocyten niet. Toch kunnen de CXCL9-11-achtige chemokines immuuncellen aantrekken, en lijken dus wel actief betrokken bij de ontstekingsreactie. Mogelijk zijn de CXCL9-11-achtige chemokines betrokken bij het opklaren van de ontsteking, omdat we *in vitro* zagen dat zij ook monocyten aantrekken. Monocyten differentiëren in macrofagen, die verder opgesplitst kunnen worden in verschillende celtypes. De verschillende type macrofagen zijn betrokken bij of het opruimen van ziekteverwekkers, of bij het herstel van het weefsel van de gastheer.. Een hypothese is dat CXCL9-11-achtige chemokines de monocyten/macrofagen aantrekken om de resolutie op te klaren. Verder onderzoek is nodig om dit vast te stellen.

Meer onderzoek is nodig om aan te tonen wat de precieze functie is van de multigene TRIM eiwitten. Mogelijk zijn zij betrokken in het direct herkennen en remmen van virussen. De bevinding dat TRIM eiwitten E3 ubiquitine activiteit hebben, toont aan dat TRIM eiwitten andere eiwitten kunnen reguleren, mogelijk eiwitten die belangrijk zijn voor de immuunreactie. Identificatie van de eiwitten die door TRIM eiwitten geubiquitineerd worden, zal dit verder uitwijzen. De bevinding dat chemokine genen zijn vermenigvuldigd in de vis, samen met het kunnen aantonen dat zij een actieve rol spelen in de ontstekingsreactie, geeft aan dat het immuunsysteem van de vis in staat is om de ontstekingsreactie heel precies te reguleren. De resultaten beschreven in dit proefschrift duiden aan dat de specialistische functies van TRIM eiwitten en CXC chemokines in het immuunsysteem, al verkregen zijn tijdens het ontstaan van de gewervelde dieren. Vervolgonderzoek in de vis zal zeker bijdragen aan een beter begrip van de evolutie van het immuunsysteem.

Acknowledgements

By writing this chapter of my thesis I will now also finalize a chapter in my life during which I learned a bit of French, had the opportunity to live as a *Parisienne*, had to deal with unexpected situations, and made new friends that came from all over the world. I hope we will somehow stay in touch, either in science, or for social activities. I would like to take the opportunity here to thank everybody that has supported me during my French-Dutch thesis.

Beste **Huub**, toen jullie mij het aanbod deden om onderzoek te doen aan TRIMs, waren deze eiwitten mij nog geheel onbekend. De combinatie van werken aan iets nieuws, de extra uitdaging van het leren van een vreemde taal, de al eerder positieve ervaring bij CBI tijdens mijn afstuderen, en uiteraard ook het mogen wonen in Parijs vier jaar, maakten dat ik uiteindelijk ja zei. Ik vond het heel leuk om aan het begin van de TRIM-hype te hebben gestaan. Met het later bijgekomen CXC-project kwam de Wageningse expertise ook meer aan bod en begon immuniteit als `systeem` meer duidelijk te worden. Ik wens je veel succes met het verder op de kaart zetten van het Wageningse allergie, vis en veterinair immunologisch onderzoek.
Beste **Lidy**, Ik denk dat het ons goed gelukt is om de chemokines weer opnieuw de aandacht te geven die ze verdienen en vond het erg leuk om hieraan bij te dragen. Ook al was het in eerste instantie niet mijn hoofdproject, we hebben er in korte tijd veel uitgehaald en ik ben heel blij met de artikelen en natuurlijk heel benieuwd naar het vervolg. Zonder jouw goede ideeën en organisatie van helpende handen was het zeker niet gelukt om de tweede helft van mijn boekje rond te krijgen. Ik wens je veel plezier met de volgende CXC-etappe.
Cher **Pierre**, j'ai beaucoup aimé travailler sur les TRIMs; peu connu au début, la communauté scientifique a gagnė beaucoup d'intérêt pour ce domaine pendant ma thèse. Je te remercie pour toute l'organisation autour de ma thèse et de m'avoir fait profiter des diverses techniques. Je te souhaite une bonne continuation avec tes projets. **Abdenour**, je voudrais te remercier de m'avoir accueilli à l'INRA.
Dear **Magda**, It was very pleasant to work with you and valuable for me to benefit from your inflammation knowledge. Thank you for replying always so quickly to my e-mails, all contribution to the experiments and for your feedback on the papers. I'm very happy with the articles that we managed to write together. I wish you good luck with leading your research in Krakow.
Cher **Jean-Pierre**, je voudrais te remercier pour toute la contribution pendant ma thèse. Il était très valable d'avoir un expert du poisson zèbre dans ce projet. Merci pour le partage des séquences, matériel et des idées pendant ce projet. Je suis curieuse de la fonction des TRIMs et je vous souhaite un bon avenir à les éclaircir. **Philippe**, je te remercie pour les discussions qu'on a eues autour de ce projet. **Milka**, you worked so hard on dealing with the *petite* zebrafish! I hope the chemokines will soon bring satisfying results. **Nuno**, thanks for all the fun we had in Viterbo and in Paris. I wish you good luck with your *thèse*.

Chers collègues de l`IIP, je vous remercie de m`avoir introduit dans le labo, à votre pays, à votre langue et de m`avoir fait goûter aux délicatesses françaises. Votre patience éternelle à m`expliquer la grammaire française et la signification des mots a sûrement

facilité mon intégration en France. Chère **Emilie,** merci pour tous les moments sympas qu'on a passés ensemble dans et en-dehors du labo. J'ai beaucoup aimé les discussions sur le but de la vie avec toi, en français! Merci pour ton sens de la perfection au niveau des manips, pour m'avoir laissé me plaindre auprès de toi, toute contribution dans ce projet et la super visite de 'ton' Alsace. Chère **Cathy**, il était vachement ;-) sympa de t'avoir comme collègue! J'ai beaucoup aimé ma visite de ta ville rose et je te souhaite tout pour le mieux dans le Sud. **Stefan**, j'ai beaucoup aimé nos discussions sur l`immunologie et les conversations sur la culture française pendant les trajets Vaubouyen-labo. J`espère que tu profites bien de ta retraite. **Charo**, thank you very much for being my roommate during the second half of my thesis. I very much enjoyed our conversations and thank you for all of your wise advice. I'm very happy for you that you now found your Spanish post-doc. **Corinne**, merci pour ton assistance au niveau des cellules et pour avoir `soigné' mes cellules pendant mes absences. **Brigitte,** merci d'avoir continué à corriger mes fautes grammaticales! J'espère qu'il n'y en a pas trop dans ce chapitre ;-) (et merci à **Florence** pour le vérifier!) **Fabienne** ton bonheur était une merveille pour le labo. **Malika**, merci pour ta contribution au premier article des TRIMs et le partage de l'addiction pour les bons gâteaux aux framboises. Merci **Luc** pour les analyses de bio-informatique. **Annie, Armel, Céline**, **Christian**, **Eloi**, **Éric**, **Erina**, **Jean-François**, **Mar, Marjolaine**, **Nicolas**, **Noé**, **Paul** et **Pierre de K**, je vous souhaite de bonnes manipulations et une bonne continuation de vos projets.
Vous êtes trop nombreux pour pouvoir tous vous mentionner, mais je voudrais également remercier les collègues de la VIM au premier étage pour leur assistance pendant ce projet. **Khalid**, **Paola** et **Stéphanie**, merci pour les moments sympas en-dehors du labo. Merci mesdames du secrétariat: **Isabelle**, **Marylise**, **Caroline** et **Corinne**, pour toute l'organisation autour de mon projet. **Damien**, **Dimitri, Eric**, **Gerard**, merci d'avoir nourri et pêché les truites pour mes manips. **Didier,** merci pour les analyses de MALDI-TOF et LC-MS/MS de mes CXCs, **Pierre A**, **Renaud**, **Gaëtan**, merci pour tout l'assistance avec l`Apotome. **Luis** pour toute l'assistance au nouveau IT, les conservations sympas et avoir transporté mes meubles pendant mes déménagements multiples! **Emmanuel**, merci pour l`assistance avec les manips d'ubiquitinylation, je suis très contente d'avoir réussi ces manips et l`article.
Autres étrangers trouvés à l`INRA: **Ewa**, **Fabrizio**, **Liliane**, **Raffael**, **Oriana**, 'The Brazilians' **Luis M**, **Dani**, **Gustavo**, **Clarissa**, **Marcella** : merci pour toutes les soirées super à Jouy et Paris. Mes colocs de la rue d`Ouessant: **Roland**, **Rachel**, **Patricia**, merci pour les soirées et sorties sympas et Patrica merci pour les petites vacances chez toi en Alsace. **Betty**, merci pour les sorties et avoir fait la fête ensemble à Paris! J'ai adoré les montagnes dans ton pays et ma visite chez toi, Bertram et ta famille était super!

Mijn Hollandse collega's, dankjulliewel voor de gezellige sfeer in Wageningen. Al was ik maar part-time jullie collega, jullie gaven me altijd het gevoel er echt bij te horen. Met jullie hulp kwam alles altijd voor elkaar tijdens mijn korte en hectische bezoeken aan Wageningen. Lieve **Nathalie**, dankjewel voor alle gesprekken *sur la vie*, de vele avondjes uit in Wageningen en vele keren mogen slapen in je huis. Wat fijn dat je mijn paranymf bent op deze o-zo belangrijke dag. **Edwin**, bedankt voor alle hulp en het met veel plezier maken van recombinante eiwitten, en het thuisbrengen van de CBI-feestjes als deze weer eens laat eindigden. **Joop,** bedankt voor alle hulp tijdens Wouters Nederlandse AV en discussies omtrent de IFN-γ en chemokines. **Wouter**, mijn beste student! Ik heb

met heel veel plezier met je samengewerkt in Jouy en in Wageningen, bedankt voor je grote enthousiasme en inzet tijdens het CXC werk, **Loes**, je was dan niet officieel `mijn` student, je hebt me heel goed geholpen, bedankt voor het bij elkaar pipetteren van de vele RQ-data! **Lieke G**, graag draag ik het CXC-stokje over aan een naamgenoot! Dankjewel voor je bijdrage aan de calciumproeven met onze geliefde, doch variabele vissen. **Carla**, dankjewel voor al je harde werk om ons tweede CXC paper te voltooien en fijn om vis-AIO ervaringen te kunnen delen. **Maria de B**, wat jammer dat je zo snel weg ging! Je harde inzet voor de chemotaxis proeven was zeer waardevol. **Marleen**, bedankt voor het met veel enthousiasme doorkloneren aan chemokines. Dankjewel **Anja** voor de celisolaties en vele goede adviezen, **Trudy en Sophie**, bedankt voor het regelen van alle bestellingen. **Hilda** voor alle administratieve hulp en gezellig kletsen tussen het pipeteren en schrijven door. **Tosca**, bedankt voor het gevraagd en ongevraagd mogen `lenen` van je werkplek. **Hans**, bedankt voor de gevraagde en ongevraagde afleiding tijdens het schrijven, goede adviezen en de lekkere broodjes zalm. **Yvonne**, ik vond het gezellig borrelen met je tijdens mijn Utrechtse zomer. We moeten deze traditie maar voortzetten in Antwerpen en Laussane denk ik zo. Andere AIO`s en post-docs, **Mark, Joeri, Adriaan, Anders, Christine, Danilo, Gerco, Jacobien, Inge**, **Maria F**. bedankt voor de gezelligheid op het lab en de avondjes uit in good-old Wageningen! **Geert, Jan, Harry, Ruth,** bedankt voor de gezellige praatjes op CBI en goede adviezen. Beste collega's van EZO en HMI: bedankt voor het mogen gebruiken van apparatuur, alle adviezen en gezelligheid. De 'Haar' vissen, bedankt voor de organisatie van de *in vivo* proeven.

Lieve ladies: **Annemarie, Gabry**, **Jo**, **Linnea**, **Margot**, **Tessa**, dankjulliewel voor het altijd gezelig kunnen theeën, lachen, klagen, de wijze raad, logeren, de geweldige Amerika-trip en vele uitjes. Jullie support was heel belangrijk voor me de afgelopen jaren en ik hoop dat we al deze leuke dingen nog steeds doen op `hoge` leeftijd. Lieve paranymf **Jo**, ik vind het heel prettig te weten dat ik altijd bij je terecht kan en fijn dat je mee het podum op gaat! Ook `de mannen` **Wienus, Chris en Merijn**, bedankt voor het mogen logeren en gezelligheid. **Eileen**, **Elise**, **Roos**, dankjulliewel voor de vele theetjes in Utrecht, goede gesprekken en de (soms ongevraagde ;-) afleiding tijdens het schrijven. **Kirsten**, ook jij bedankt voor de logeerpartijen en waardevolle adviezen van een experienced AIO. **Dolf** en **Annelies**, wat fijn dat ik altijd welkom was, ik zie jullie snel in Antwerpen!

Jeroen en **Sylvia**, dankjulliewel dat ik altijd welkom was op de Fijnjekade en later in jullie mooie huis! Ik voelde me altijd heel erg welkom bij jullie en heb genoten van de lekkere maaltijden, de goede gesprekken en de uitjes in Den Haag. En Jeroen, bedankt voor de mooie omslag! Bedankt **Marieke** en **Dirk** voor het altijd beschikbaar staan met auto tijdens mijn grootste hobby, verhuizen! **Pap** en **mam**, bedankt voor jullie eeuwige vertrouwen en steun in mijn doen en laten.

La vie devient une chose délicieuse, aussitôt qu'on décide de ne plus la prendre sérieux.

- Henry de Montherlant -

About the author

Curriculum Vitae
List of Publications
Overview of completed training activities

Curriculum Vitae

Lieke Maria van der Aa was born in Hapert, the Netherlands, on May 15, 1981. After graduating from the Rythovius College (VWO) in Eersel, she started her study Biology in 1999 at Wageningen University. During her MSc thesis, she carried out three research projects in immunology and virology. A first MSc thesis project she carried out at the Cell Biology and Immunology group of Wageningen University, in collaboration with the Department of Animal Physiology at the Radboud University in Nijmegen. In this project she investigated the evolutionary conserved adrenal Corticotrophin-Releasing Hormone (CRH) system in fish. A second MSc project was carried out at the Faculty of Veterinarian Medicine at Utrecht University, in collaboration with the Gene Therapy Group at the VU Medical Centre in Amsterdam. In this project, the application of a mouse coronovirus (MHV) in virotherapy, in the treatment of cancer, was studied. She went to Australia to work on a third MSc project at the School of Molecular and Microbial Sciences at the University of Queensland in Brisbane. Here she investigated the production of small non-coding RNA by flaviviruses. She graduated in 2006 and started her PhD thesis in 2007, of which the results are described in this thesis. Her thesis project was a collaboration between the Cell Biology and Immunology group of Wageningen University and the Virologie et Immunologie Moléculaires unit at the French Institute INRA in Jouy-en-Josas, and carried out under the supervision of Prof. Dr. Ir. Huub Savelkoul, Dr. Lidy Verburg-Van Kemenade and Dr. Pierre Boudinot. She also collaborated with Dr. Jean-Pierre Levraud and Dr. Philippe Herbomel at the Pasteur Institute in Paris and Dr. Magdalena Chadzinska at the Jagiellonian University in Krakow, Poland. In 2011, she started her post-doc at Tibotec (Janssen Pharmaceuticals, Johnson & Johnson) in Beerse, Belgium. She is enrolled in the Marie Curie International Training Network EUVIRNA, which aims at the development of novel antiviral therapies against (+)RNA viruses.

List of Publications

Van Der Aa LM, Chadzinska M, Derks W, Scheer M, Levraud JP, Boudinot P, Verburg-van Kemenade BML. Diversification of IFN-γ inducible CXCb chemokines in cyprinid fish. *Submitted.*

Van Der Aa LM, Chadzinska M, Golbach LA, Ribeiro CM, Verburg-van Kemenade BML. *Developmental and Comparative Immunology, doi:10.1016/j.dci.2011.11.011.* Pro-inflammatory functions of carp CXCL8-like and CXCb chemokines.

Van Der Aa LM, Jouneau L, LaPlantine E, Bouchez O, Verburg-van Kemenade BML, Boudinot P. *Developmental and Comparative Immunology. 2012 Feb; 36(2):433-41.* Virus-induced TRIM proteins in rainbow trout display E3 ubiquitin ligase activity.

Boudinot P, Van Der Aa LM, Jouneau L, Du Pasquier L, Pontarotti P, Briolat V, Herbomel P, Benmansour A, Levraud JP. *PLoSOne 2011;6(7):e22022* Origin and evolution of TRIM proteins: new insights from the complete TRIM repertoire of zebrafish and pufferfish.

Van Der Aa LM, Chadzinska M, Tijhaar E, Boudinot P, Verburg-van Kemenade BM. *PLoS One. 2010 Aug 26;5(8):e12384.* CXCL8 chemokines in teleost fish: two lineages with distinct expression profiles during early phases of inflammation.

Van Der Aa LM, Levraud JP, Yahmi M, Lauret E, Briolat V, Herbomel P, Benmansour A, Boudinot P. *BMC Biology. 2009 Feb 5;7:7.* A large new subset of TRIM genes highly diversified by duplication and positive selection in teleost fish.

Pijlman GP, Funk A, Kondratieva N, Leung J, Torres S, Van Der Aa L, Liu WJ, Palmenberg AC, Shi PY, Hall RA, Khromykh AA. *Cell Host Microbe. 2008 Dec 11;4(6):579-91.* A highly structured, nuclease-resistant, noncoding RNA produced by flaviviruses is required for pathogenicity.

Huising MO, van der Aa LM, Metz JR, de Fátima Mazon A, Kemenade BM, Flik G. Journal of *Endocrinoly. 2007 Jun;193(3):349-57.* Corticotropin-releasing factor (CRF) and CRF-binding protein expression in and release from the head kidney of common carp: evolutionary conservation of the adrenal CRF system.

Würdinger T, Verheije MH, Van Der Aa LM, Bosch BJ, de Haan CA, van Beusechem VW, Gerritsen WR, Rottier PJ. *Leukemia. 2006 Dec;20(12):2182-4.* Antibody-mediated targeting of viral vectors to the Fc receptor expressed on acute myeloid leukemia cells.

Training and Supervision Plan

The Basic Package

WIAS Introduction Course, Wageningen, the Netherlands	2007
Courses on philosophy of science and ethics , Universitė Paris-Sud 11, France	2008
Subtotal Basic Package	***3 credits***

Scientific Exposure

International conferences

Journées Francophones de Virologie, Paris, France	2007
Virology Congress, Early steps of the viral life cycle, Paris, France	2007
Nederlandse Verening voor de Immunologie (NVVI), annual congress, Noordwijkerhout, the Netherlands	2007
Journées Francophones de Virologie, Paris, France	2008
Journées Francophones de Virologie, Paris, France	2009
European Zebrafish meeting: Zebrafish as model for immunology and cancer, Spoleto, Italy	2009
International Society of Developmental and Comparative Immunology (ISDCI), Prague Czech	2009
International symposium of fish Immunology, European society of fish Immunology (EOFFI), Viterbo, Italy	2010

Seminars and workshops

Animation on fish transgenesis of the PHASE department, Paris, France	2007
Animation on fish transgenesis of the PHASE department: Les poissons comme modèles en physio-pathologie, Paris, France	2008
Noah`s Ark and the immune system, Collège de France, symposium, Paris, France	2008
Bernard Halpern Immunology Symposium, Collège de France, Paris, France	2008
Journée Méthodologique Imagerie at INRA, Jouy-en-Josas, France	2008
Immunomodulation and Allergy, seminar,Wageningen, the Netherlands	2008
Journée PTR, INRA and Pasteur Institute, Paris, France	2008
Colloque Doc`J, PhD day at INRA, Jouy-en-Josas, France	2009
JAS2009, meeting of INRA Département Santé Animale, Port d`Albret, France	2009
Animation on fish transgenesis of the PHASE department: Immunologie des poissons-modèles, Paris, France	2009
Of fish and men: curiosities of the immune system, seminar, Wageningen, the Netherlands	2009
Workshop: Role of various types of ubiquitin chains in membrane trafficking, European network Rubicon, Paris, France	2010
Knowing your enemy makes sense, seminar, Wageningen, the Netherlands	2010

Presentations

Journées Francophones de Virologie, Paris, France (poster)	2008
Journées Francophones de Virologie, Paris, France (oral)	2009
Colloque Doc'J, PhD-day at INRA, Jouy-en-Josas, France (oral)	2009
JAS2009, national meeting of INRA Département Santé Animale, Port d`Albret, France (poster)	2009
Fish Workshop, Wageningen, the Netherlands (oral)	2010
International Society of Developmental and Comparative Immunology (ISDCI), Prague, Czech (poster)	2009
European Zebrafish Meeting, Spoleto, Italy (poster)	2009
Rubicon Workshop: Role of various types of ubiquitin chains in membrane trafficking, Paris France (poster)	2010
European International symposium of fish Immunology, (EOFFI), Viterbo, Italy (oral)	2010
WIAS Science Day, Wageningen, the Netherlands (oral)	2011
Subtotal Scientific Exposure	***22,2 credits***

In-Depth Studies

Disciplinary and interdisciplinary courses

Fish Workshop, Wageningen, The Netherlands	2007
Atelier Phylogenomique, Marseille, France	2007
Nederlandse Verening voor de Immunologie (NVVI), workshop: Vaccination Immunology, prevention and beyond, Lunteren, the Netherlands	2008
Nederlandse Verening voor de Immunologie (NVVI), workshop: Sensing and Signalling Lunteren, the Netherlands	2009
Advanced visualisation, integration and biological interpretation of -omics data, Wageningen, the Netherlands	2009
Introduction Course Immunology, Utrecht University, the Netherlands	2010
Subtotal In-Depth Studies	***6,6 credits***

Professional Skills Support Courses

French Course, INRA, Jouy-en-Josas, France	2007
How to develop an European Career, INRA, France	2007
Techniques for writing and presenting a scientific paper, Wageningen, the Netherlands	2008
Course Supervising MSc thesis work, Wageningen, the Netherlands	2008
Interpersonal Communication for PhD student, Wageningen, the Netherlands,	2008
Career Orientation course, Wageningen, the Netherlands	2010
Subtotal Professional Skills Support Courses	***7,2 credits***

Didactic Skills Training

Lecturing

presentation for visiting master students Agro-Paris-Tech at INRA, Jouy-en-Josas, France	2008

Supervising theses

1 Msc major	2008
Subtotal Didactic Skills Training	***2,3 credits***
Total number of credit points	***41,3 credits***

Herewith the WIAS Graduate School declares that the PhD candidate has complied with the educational requirements set by the Educational Committee of WIAS.

**** one ECTS credit equals a study load of approximately 28 hours***

The research described in this thesis was financially supported by Wageningen University and l`Institut National de la Recherche Agronomique (INRA)

Cover design: Jeroen van der Aa
Design and layout: Lieke M. van der Aa
Figures on chapter pages are derived from *toile de Jouy* (Jouy fabric), Jouy-en-Josas, France

The thesis was printed by Wöhrman Print Service

Financial support from Wageningen University and INRA for printing this thesis is gratefully acknowledged.