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Advances in the *Xenopus* immunome: Diversification, expansion, and contraction

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ABSTRACT

Xenopus is a genus of African clawed frogs including two species, *X. tropicalis* and *X. laevis* that are extensively used in experimental biology, immunology, and biomedical studies. The availability of fully sequenced and annotated *Xenopus* genomes is strengthening genome-wide analyses of gene families and transgenesis to model human diseases. However, inaccuracies in genome annotation for genes involved in the immune system (i.e., immunome) hamper immunogenetic studies. Furthermore, advanced genome technologies (e.g., single-cell and RNA-Seq) rely on well-annotated genomes. The annotation problems of *Xenopus* immunome include a lack of established orthology across taxa, merged gene models, poor representation in gene pages on Xenbase, misannotated genes and missing gene IDs. The *Xenopus* Research Resource for Immunobiology in collaboration with Xenbase and a group of investigators are working to resolve these issues in the latest versions of genome browsers. In this review, we summarize the current problems of previously misannotated gene families that we have recently resolved. We also highlight the expansion, contraction, and diversification of previously misannotated gene families.

1. Introduction

1.1. *Xenopus* species as model organisms

Xenopus, commonly known as African clawed frogs, is a genus of aquatic frogs that are native to sub-Saharan Africa (Blackburn et al., 2019). The two best-known species of this genus are *Xenopus laevis* and *X. tropicalis,* which are commonly used as model organisms to study human diseases and their potential treatments, fundamental biological processes, small molecule screens to develop novel therapies, embryogenesis, developmental biology, cell biology, toxicology, neuroscience and immunology (Harland and Grainger, 2011; Nenni et al., 2019; Wallingford et al., 2010). *Xenopus* offers a variety of experimental advantages over other amphibians, including the abundance of large and robust eggs and embryos that are easily accessible at all developmental stages. This is important especially in the study of embryogenesis and developmental biology. *X. tropicalis* was introduced as a model system in the early 1990's for genetics, and then genomic research. This complemented the work on the widely used model organism *X. laevis,* for which the immune system is extensively characterized (reviewed in Du Pasquier et al., 1989). Comparing the two species, *X. tropicalis'* shorter generation time (growing to adult in 4–6 months) and its diploid

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genome (10 diploid chromosomes) has enabled many multigenerational experiments such as the generation and preparation of transgenic and mutant lines (Chesneau et al., 2008; Grainger, 2012a; Horb et al., 2019; Hirsch et al., 2002; Offield et al., 2000) as compared to *X. laevis*, which has a longer generation time (averaging between 6 and 12 months to sexual maturity for males, and over 12 months for females). Furthermore, cytogenetic mapping of the allotetraploid *X. laevis* genome has identified 9 quartets of homologous chromosomes in two sub-genomes, whose genetic linkage has been highly conserved in X. tropicalis (Matsuda et al., 2015). These *X. laevis* chromosomes co-orthologous to the corresponding *X. tropicalis* chromosomes are defined as pair of homeologs that are distinguished based on their size into a long (L) and a short (S) homeolog (Matsuda et al., 2015; Session et al., 2016). Thus, an additional challenge in working with *X. laevis* lies in the four copy numbers for many genes, requiring a more laborious procedure to analyze potential gene-multiplication of most gene families. At the same time, the duplicated genome in *X. laevis* affords a unique opportunity to study genome evolution and sub-functionalization of duplicated genes (Session et al., 2016). Sequencing technology has contributed to dissecting the evolutionary events in the genome. However, the timeline of genome annotation and the recurring challenges (see Section 1.2) merely enabled the partial analysis of some gene families, especially those that are involved in immune functions, using readily available tools such as those on Xenbase (www.xenbase.org), the NCBI and other servers. Despite the inaccuracies in *Xenopus* sequenced genomes, many labs have been able to dissect comparative and developmental aspects of *Xenopus* immune system (Ohta et al., 2006; Flajnik et al., 1993; Edholm et al., 2014). The release of new genome browsers for *Xenopus*, has motivated us to establish a community-driven genome annotation process, the "*Xenopus* annotation jamboree", which was initiated by the *Xenopus laevis* Research Resource for Immunobiology in collaboration with Xenbase and multiple investigators. Our goal is to update, re-evaluate, refine, and expand the curation of *Xenopus* genome mostly focusing on immune genes. This coordinated project will in turn improve representation of Xenbase, the *Xenopus* genomics and bioinformatic database that collates this information for the research community. The aim of manually annotating *Xenopus* genomes is to correctly identify and characterize functional and regulatory elements across the *Xenopus* genomes, which is particularly important to characterize *Xenopus* immune gene families, hence enhancing the efficient use of *Xenopus* as an animal model in immunology research. The current review goal is to: 1) Clarify recent advances pertaining the *Xenopus* immunome; 2) Update, re-evaluate, refine, and expand the curation of the *Xenopus* immunome; 3) Focus on the diversification, expansion, and contraction of the *Xenopus* immunome. Here, we have chosen to discuss a few examples of problematic gene families that we have resolved to a sufficient degree and should be useful for comparative immunologists.

1.2. Timeline, progress, and current challenges in Xenopus genome annotation

The progress of genetic studies in *Xenopus* and the generation of disease models are linked to the availability of reliable genome sequencing and post-genomic analysis. Each genome annotation release is a major step towards a more accurate representation of the vastly complex genetic code packed onto this organism's chromosomes. We fully understand that this is a laborious and complex task, and that the end results represent a hypothetical version of the encoded DNA, which is subject to change with future genome assemblies. The *X. tropicalis* genome sequencing was completed in 2010 (Hellsten et al., 2010) followed in 2016 by the sequencing and assembly of the related allotetraploid *X. laevis* species (Session et al., 2016). Annotation of the *X. tropicalis* (v7.0) and *X. laevis* (v7.1, v8.1) genome assemblies resulted into considerable coverage of the genomes (almost 50%). However, information regarding potential gene inversions were sometimes inaccurate, since these assembly releases were not contiguous (Riadi et al., 2016). In general, draft genomes impeded software abilities to connect adjacent genes, implying that the number of identified genes was underestimated (Salzberg, 2019). The reliability of each of these two genomes has been improved by multiple rounds of sequence assemblies at the chromosomal level and the construction of genome maps, and thus, new genome versions for both *X. tropicalis* (v7.1, v8.0, v9.0, v9.1) and *X. laevis* (v9.1, v9.2) were released. The contribution of RNA-Seq and other next-generation sequencing (NGS) techniques facilitated the construction of new genetic maps. Many genes that presented high homology with human counterparts were sequenced, and disease phenotypes were generated and analyzed in *Xenopus* (Blum et al., 2009; Grainger, 2012; Naert and Vleminckx, 2018). Despite the progress of sequencing, previous genome assemblies still contained scaffolds that were incomplete or inaccurate, particularly for immune genes. Session et al. (Session et al. (2016) analyzed many developmental genes and some key immune genes such as antigen receptors, antigen receptor signaling, major histocompatibility complex (MHC), cytokines, and innate immune genes (Session et al., 2016). The innate pattern recognition receptors (PRRs) for both *X. laevis* and *X. tropicalis* were particularly challenging. Many of *PRRs* were not identified in one or both species and, thus, their homology inference was not possible. Furthermore, some genes that encode short transcripts were poorly detected in these assemblies (e.g., many cytokines and *tlr4*) or were in scaffolds unnamed and/or uncharacterized, rendering it difficult to identify potential orthologous genes. Many genes that were designated "uncharacterized gene" could be annotated manually. One characteristic example is *mhc1b-uba10.2.L* (formerly *XCN10.2*) (Edholm et al., 2015). Even though *mhc1b-uba10.2.L* and the respective amino acid sequences were published (Edholm et al., 2014), the respective gene in *X. laevis v9.2* assembly did not include information regarding the protein sequence. In 2021 a new genome version was released for *X. tropicalis* (v10) followed by *X. laevis* (v10.1) in 2022 at NCBI (www.ncbi.nlm.nih.gov). We decided to assess the annotations and database representation of a range of immune gene families by following an *in silico* procedure, as summarized in (Fig. 1). The new v10 genome assemblies have resolved many issues encountered in previous annotations. For example, many gene models thought to be pseudogenes are now identified as *bona fide* protein coding genes, and new models have been identified on the short (S) chromosomes of *X. laevis*. Subsequently, we were able to identify new gene models that existed in the latest genome assemblies, or were identified by examining the genome sequence as well as, to create or update gene pages on Xenbase (see Section 2). The *Xenopus* nomenclature convention, introduced by Session (Session et al., 2016), states that *X. laevis* homoeologous chromosomes that are co-orthologous to the corresponding *X. tropicalis* chromosomes are distinguished by appending an "L" or "S", referring to the long and short chromosomes respectively, and respective genes should carry an ".L" or ".S" suffix. In our annotation, we were able to discern if a gene is duplicated or previously existed but not identified (e.g., *mhc1-uba6.1.L - mhc1-uba6.3.L*, intronless interferons *ifnx1-36*, and three interleukins *il17a* genes) (Matsuda et al., 2015). Most of the genes with annotation inaccuracies that we resolved are summarized in **Supplementary *Table S1***. Nevertheless, there are still some problems that will need to be addressed, thus a multi-faceted bioinformatic approach was used throughout this review. Our approach included assessing synteny (GENOMICUS, JBrowse), sequence alignment (BLAST), multi-species sequence alignment and phylogenetic trees (NCBI/COBALT, MEGA), and/or searching protein sequence in homology groups (DIOPT, EggNog, HomoloGene). Details of these bioinformatic tools/analyses are given in Supplementary Table S2. For example, in the new assembly there are some genes that overlap each other and create a 'merged' model in JBrowse (e.g., *mhc1-uba10.1.L and mhc1-uba10.2.L*), although we are well aware that these are two separate protein coding genes (Edholm et al., 2014). We also identified uncharacterized sequences that need further bioinformatic and syntenic analysis. In addition, there are still a few genes that need identification numbers (gene ID), or their nomenclature needs to be updated in both

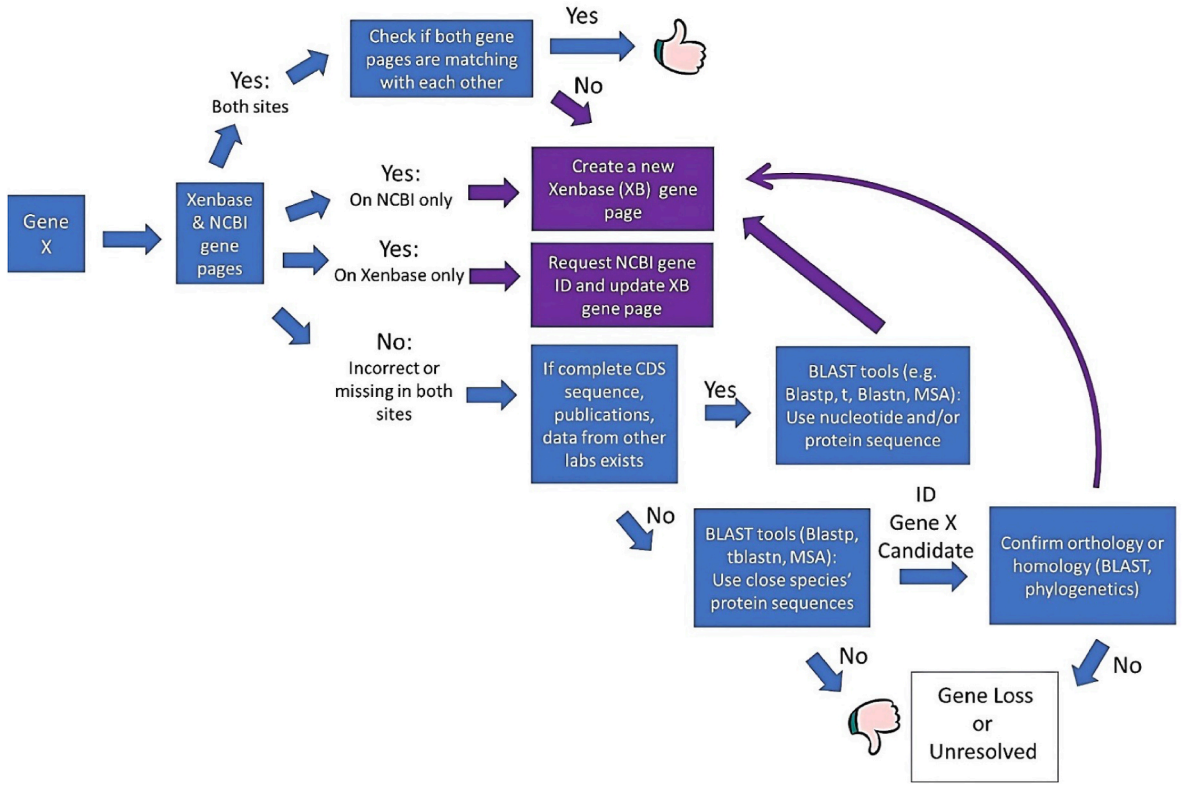

Fig. 1. Schematic of the methodology used by the jamboree team to identify, correct, and validate gene annotation in new genome browser (v.10). MSA: multi-sequence alignments.

Xenopus species (e.g., *mhc1-uba* homologs/paralogs in *X. tropicalis* and *X. laevis*, see Section 2.2). Our team's work is ongoing, and part of this work is involved in this publication.

Comparative immunology combined with expanded sequencing data has revealed the evolutionary forces that shaped *Xenopus* genome by gene expansion or contractions as well as gene silencing Flajnik et al. (2001). Multiple *Xenopus* gene families that are involved in innate and adaptive immunity have undergone expansion, contraction or differential silencing in expression compared to their mammalian counterparts (see Section 2. *Updates in immune genes annotation*). Furthermore, some immune genes and their role in immune responses has been extensively investigated in *X. laevis* leading to interesting connections between innate and adaptive immunity (Edholm et al., 2014; Banach et al., 2017; Flajnik et al., 1993). It is noteworthy that genome editing tools (CRISPR/Cas9) were successfully used to generate transgenic models for several diseases, malignancies, and immune defenses against pathogens based on previous genome assemblies (Grainger, 2012; Naert et al., 2017; Van Nieuwenhuysen et al., 2015; Blum and Ott, 2018). In recent years, NGS-based sequencing technologies were expanded to boost the release of new genome assemblies. Thus, a new era is rising for *Xenopus* due to easier and detailed analysis of immune genes during early ontogeny, as well as their importance for experimental and biomedical research.

2. Xenopus immune gene families

2.1. Toll-like receptors (TLRs)

During evolution, amphibians had to develop an effective immunity against a wide variety of pathogens in both aquatic and terrestrial habitats (Zhang et al., 2022; Roach et al., 2005). The evolved complex of amphibian Toll-like receptor (*tlr)* genes has likely contributed to the survival of ancestral species that were first exposed to land-based ecosystems.

TLRs are a group of pattern recognition receptors (PRRs) that recognize molecular patterns of microbial components (e.g., microbial nucleic acids, proteins, glycans, etc.) and play important roles in innate immunity (Nie et al., 2018; Liu et al., 2019). Most TLR structures consists of one or many leucine-rich repeat (LRR) domains, a short transmembrane domain, and an intracellular Toll/interleukin-1 receptor (TIR) domain (Ishii et al., 2007; Liu et al., 2019). Overall, six TLR families have been identified across vertebrates (Supplementary Fig. S1): TLR1 (TLR1,2,14), TLR3 (TLR3), TLR4 (TLR4), TLR5 (TLR5), TLR7 (TLR7,8,9), and TLR11 (TLR11,12,13,19,21,22) (Roach et al., 2005; Liu et al., 2019; Nie et al., 2018). The respective TLR ligands and TLR genes for humans, *Xenopus,* and several representative bony fish species (*Danio rerio, Oncorhynchus mykiss, Fugu rubripes, Ictalurus punctatus*) are depicted in Table 1. The number of TLRs vary considerably across species, from 10 in human to 16 genes in bony fish and *Xenopus* (Chen et al., 2021; Liu et al., 2019; Nie et al., 2018; Roach et al., 2005). The expansion of *tlr* genes in *Xenopus* involves tandem gene duplication within some subfamilies (e.g., TLR1 subfamily: *tlr14.2* and *tlr14.3*) or emergence of additional novel subfamilies (e.g., TLR11). Even though TLRs have been annotated in the previous genome assemblies, the latest version indicated some inaccuracies and mis-annotations. In the new genome assemblies (v10/v10.1), we verified and re-evaluated which genes have been expanded between *Xenopus* and humans and we summarized them in Table 1, as well as in Supplementary Table S1 and Fig. S1 (Ishii et al., 2007; Roach et al., 2005; Zhang et al., 2022). Among all TLRs, *tlr4* was an important gene to annotate accurately. Although *tlr4* was initially identified in the *X. tropicalis* genome (Roach et al., 2005), it was not accurately annotated in previous assemblies (lack of protein sequence) or was identified in non-coding areas and possibly truncated (Ishii et al., 2007). Furthermore, *tlr4* could not be retrieved in the latest *X. tropicalis* genome assembly (v10). However, a *tlr4* gene model is now present in the updated *X. laevis* v10 genome assembly (Supplementary Fig. S2), which underlines the significant improvement of this assembly (see Supplementary Table S1). Notably, *tlr4* has been

Table 1
TLRs genes in vertebrates and their ligands.

Ligand	Human	*X. tropicalis*	*X. laevis*	Bony fish[a]
triacylated lipopeptide	*TLR1*	*tlr1*	*tlr1.L*	*tlr1*
lipopeptides, peptidoglycan, and lipoteichoic acids	*TLR2*	*tlr2*	*tlr2.L, tlr2.S*	*tlr2*
dsRNA molecules	*TLR3*	*tlr3*	*tlr3.L*	*tlr3*
LPS, cd14	*TLR4*	*tlr4*	*tlr4.S*	*tlr4*[b]
Flagelin	*TLR5*	*tlr5*	*tlr5.L*	*tlr5*
Lipoteichoic acid, MALP2,	*TLR6*	*tlr6*	*tlr6.L*	*tlr6*[a]
ssRNA	*TLR7*	*tlr7*	*tlr7.L*	*tlr7*
GU-rich single-stranded RNA, G-rich oligonucleotides	*TLR8*	*tlr8*	*tlr8.L*	*tlr8*
Unmethylated CpG motifs	*TLR9*	*tlr9*	*tlr9.L*	*tlr9*
Pam3Cys and FSL-1,	*TLR10*	*Not present*	*Not present*	*Not present*
Uropathogenic bacteria-derived protein (e.g., T. gondii profilin)	*Not present*	*Not present*	*Not present*	*tlr11*[a]
Apicomplexan profilin	*Not present*	*tlr12*	*tlr12.L*	*Not present*
23S rRNA	*Not present*	*tlr13*	*tlr13.L*	*Not present*
Undefined		*tlr14.2, tlr14.3*	*tlr14.2.L tlr14.3.L*	*tlr14*[a]
CpG oligos in bony fish and chicken only, undefined for the rest	*Not present*	*Not present*	*Not present*	*tlr19*
CpG oligos in zebrafish only.	*Not present*	*Undefined*	*Undefined*	*tlr21*
TLR22 (bacterial RNA)	*Not present*	*tlr22*	*tlr22.L*	*tlr22*
TLR23 MyD88 and TRIF	*Not present*	*Not present*	*Not present*	*Not present*

→As a general comment here we mention that this Table is subject to changes in the future due to new entries in NCBI gene pages of bony fish.

[a] (zebrafish (*Danio rerio*), fugu (*Takifugu rubripes*), rainbow trout *(Oncorhynchus mykiss*), catfish (*Ictalurus punctatus*).

[b] *tlr4* is present only in *Ictalurus punctutatus* and *Danio rerio*.

[c] not present in fugu (*Takifugu rubripes*) rainbow trout *(Oncorhynchus mykiss*).

identified only in Anuran amphibians to date and appears to be absent in Caudata (newts and salamanders), suggesting gene loss in a common ancestor (Zhang et al., 2022). In the bony fish, *tlr4* has been identified only in a few species (e.g., *Danio rerio, Cyprinus carpio, Astyanax mexicanus*) (Supplementary Fig. S2) (Palti, 2011). TLR4 ligands in mammals including LPS and the TLR4- CD14-myeloid differentiation protein 2 (MD2)-LPS complex recruits MyD88 and MyD88-adapter-like (MAL) adaptors and sequentially activate NF-κB and mitogen-activated protein kinase (MAPK) (Kawasaki and Kawai, 2014; Sakai et al., 2017). Alternatively, endocytosis of TLR4-MD2–LPS complex and sequential binding to the TRIF and TIR domain-containing adapter molecule 2 (TRAM) adaptors triggers production of IFN1, IRF7 and NF-κB resulting in the activation or suppression of inflammatory cytokines genes (Kawasaki and Kawai, 2014). Interestingly for both fish and amphibians, there is no clear evidence that LPS ligand is recognized by TLR4, and molecules like MD2 and CD14 have not been identified in bony fish and *Xenopus* (Nie et al., 2018). Thus, the exact role of *tlr4* gene in *Xenopus* immunity needs to be further evaluated. The expansion of TLR genes in aquatic vertebrates is thought to represent an evolutionary adjustment to pathogen-rich habitats.

2.2. Major histocompatibility class I (mhcI)

The Major Histocompatibility Complex (MHC) genomic region consists of a plethora of genes (around 500) (Klein et al., 1993). MHC molecules are categorized into 2 groups based on their structural and functional features, MHC class I that interact $CD8^+$ T-cells, and MHC class II that interact with $CD4^+$ T-cells (Flajnik et al., 1993; Iwabuchi and Van Kaer, 2019). Both MHC class I and MCH class II encoding genes are highly polymorphic genes (Rodgers and Cook, 2005). Besides the classical MHC class I, there is a group of nonclassical MHC class I genes that are located either inside or outside the MHC locus, are typically oligomorphic, and bind to different conserved types of ligands from peptides to lipids or other small molecules (Allen et al., 2013). Non-classical MHC class I molecules interact with a subset of T cells that express invariant or semi-invariant T Cell Receptor (TCR) re-arrangement, the innate-like T-cells (iT) (Iwabuchi and Van Kaer, 2019; Allen et al., 2018; Edholm et al., 2014). In human and mice, nonclassical MHC class I molecules including the cluster of differentiation 1d (CD1d) and MHC related protein 1 (MR1), bind non-protein ligands such as glycolipids and vitamin B products, respectively, and present these ligands to iT cells such as invariant natural killer cells (iNKT) for CD1d and mucosal-associated invariant T-cells (MAIT) for MR1 (Edholm et al., 2014; Iwabuchi and Van Kaer, 2019; Rodgers and Cook, 2005). While CD1d orthologs are present across jawed vertebrates, MR1 appears to be evolutionary more recent, although both genes have been lost in different taxa including in mammals (Harly et al., 2022). There are other nonclassical MHC class I genes in mammals (e.g., HLA-E, HFE, HLA-G, FcRn) whose evolutionary origins are currently unknown. These mammalian nonclassical MHC class I genes could be located outside of the MHC locus (Banach et al., 2017; Edholm et al., 2014; Goyos et al., 2011; Ohtsuka et al., 2008). Even though the role of iT cells has been investigated in inflammatory responses and several malignancies (Gleimer and Parham, 2003), the exact role of iT cells in aquatic vertebrates like amphibians, especially for the innate and adaptive immunity, needs further research.

X. laevis and *X. tropicalis* contain a quite a large number of nonclassical MHC class I genes compared to mammals, which are located outside the MHC locus in the telomeric region of chromosome 8 (Du Pasquier and Flajnik, 1990). These genes, initially named XNCs in *X. laevis* and SNCs in *X. tropicalis*, are clustered in close proximity to SLAM (signaling lymphocyte activation molecule) family members (Edholm et al., 2014; Goyos et al., 2011). Because no true orthologs of these genes could be identified in the human genome or other mammals, we assigned them a new name, *mhc1-uba,* following the nomenclature system for non-mammalian MHC genes, and in consultation with MHC nomenclature experts (Ballingall et al., 2018) (Supplementary Table S1). We specifically used the "u lineage as for MHC class I" following the nomenclature used for ectothermic vertebrates: having classical MHC class I alpha chain referred as "uaa", sequentially followed by other families or groups: uba, uca, uda, uea, etc. Therefore, for *Xenopus* "uba" stands for MHC class I (u), family or group (b), alpha chain (a). In total, we have confirmed the presence 29 *mhc1-uba* genes for *X. tropicalis* and 23 *mhc1-uba* genes and 3 pseudogenes for *X. laevis* (Edholm et al., 2014, 2018; Goyos et al., 2011; Banach et al., 2017; Flajnik et al., 1993, 2001). *Xenopus* nonclassical MHC class I or uba genes share some similarities with the respective mammalian genes including restricted tissue expression and limited to no polymorphism (Goyos et al., 2011; Rodgers and Cook 2005). Interestingly, the *mhc1-uba* genes are highly conserved between *X. laevis* and *X. tropicalis,* as well as across the other *Xenopoidae* species, and they have been categorized into subfamilies based on the amino acid sequences of the α1 and α2 domains (Goyos et al., 2011; Flajnik et al., 1993, 2001; Edholm et al., 2014). Annotation for the *mhc1-uba* genes (formerly *XNCs/SNCs*) was initially conducted in v9.0/v9.1 genomes assemblies. Thus, to ensure accuracy for future research, we re-assessed the annotation, nomenclature, and representation of these genes in the latest release (v10.1 and v10). A major task was, and still is, for us to ensure that as many immune genes as possible are represented on Xenbase gene pages with informative and stable gene symbols and gene names, and with as much supporting data as possible. (i.e., gene ID, mRNA and protein accessions, literature, synonyms). Applying gene nomenclature guidelines, we have proposed new gene symbols to replace generic LOC-identifiers (e.g., *mhc1-uba3.L* for LOC108699128), which we have submitted to the HGNC (human gene nomenclature committee) and

others for approval. We are currently generating these new pages as well as applying new nomenclature with proper aliases. The updated annotation for most of these genes is summarized in Supplementary Table 1 and information about expanded subfamilies members and their potential function is summarized in Table 2.

In addition to *mhc1-uba* genes, there are three additional Nonclassical MHC class I genes located elsewhere in the *Xenopus* genomes (Ohta et al., 2019): *mhc1-uca* (class I 145), *mhc1-uda* (class I 16004), *mhc1-uea* (class I112). Based on a phylogenetic analysis, *mhc1-uca* is most closely related to the *MHC class Ia* and *mhc1-uba* (Ohta et al., 2019). Indeed, the *mhc1-uca* gene maps between the MHC locus and the *mhc1-uba* cluster in Chr8 of the diploid *X. tropicalis*. In *X. laevis*, *mhc1-uca* was mapped in the corresponding region of Chr8L but was lost from Chr8S. The genomic region containing *mhc1-uba* genes is predicted to be originally duplicated and translocated from the ancestral MHC locus found across jawed vertebrates (Ohta et al., 2019). Thus, *mhc1-uca* may have moved out of the MHC locus during the duplication of the *mhc1-uba* cluster from MHC locus. We predict that *mhc1-uca* fulfils functions similar to some *mhc1-uba genes*. The *mhc1-uda* gene (class I 16,004) is found in the *X. tropicalis* Chr3 in the vicinity of genes that were mapped to the human chromosome 19p13. This human chromosomal region has been identified as one of the MHC paralogs regions that were generated by genome-wide duplication during vertebrate evolution (Ohno et al., 1968; Simakov et al., 2020). The presence of nonclassical MHC class I genes in this MHC paralogs region in *Xenopus* suggests that MHC class I may have been present prior to the genome duplications in a vertebrate ancestor. BLAST searches using *mhc1-uda* as query against vertebrates "refseq_protein" database matched at one time with the mammalian Nonclassical class I *Fcgrt (*gene encodes FcRn*)*, which maps to the human chromosome 19q12. However, phylogenetic analysis did not support the orthology of *mhc1-uda* to *Fcgrt* (Ohta et al., 2019) and the search result could not be repeated. Unlike *mhc1-uca*, the *mhc1-uda* genes are not diploidized in the allotetraploid *X. laevis* genome as it is present on both the Long (L) and Short (S) chromosomes. Finally, *mhc1-uea* (class I 112) is the most evolutionarily conserved gene among all *Xenopus* nonclassical MHC class I genes. Orthologs of *mhc1-uea* are present in reptiles such as alligators and turtles. The *mhc1-uea* was mapped near immunoglobulin heavy (*IgH*) and *TCRαβ* loci on *Xenopus* chromosome Chr1. This synteny is presumably ancestral and reflects the close linkage of antigen receptor genes to the MHC in the primordial genome.

Table 2
mhc1-uba genes and their known role.

mhc1b-uba	*X. tropicalis*	*X. laevis* (Known or putative function)	References
1	*mhc1-uba1.1-1.2*	*mhc1-uba1.L (Putative role during larval development)*	Banach et al. (2017)
2	*mhc1-uba2.1-2.2*	*mhc1-uba2.S*	
3	*mhc1-uba3*	*mhc1-uba3.L*	
4	*mhc1-uba4*	*mhc1-uba4.L (Anti-mycobacterial immunity)*	(E. S. Edholm et al., 2018; Rhoo et al., 2019)
5	*mhc1-uba5*	*mhc1-uba5.L*	
6	*mhc1-uba6.1-6.2*	*mhc1-uba6.1-6.3L, mhc1b-uba6.4S*	
7	*mhc1-uba7.1-7.6 (Ubiquitous expression)*	*mhc1-uba7.L (Ubiquitous expression including thymic stroma)*	*Goyos* et al. *(unpublished data)* Goyos et al. (2011)
8	*unidentified*	*mhc1-uba8.1-8.4L (Strict expression in lungs - mucosal immunity?*	*Goyos unpublished data*
9	*mhc1-uba9*	*mhc1-uba9.L*	
10	*mhc1-uba10*	*mhc1-uba10.1 (Anti-ranaviral immunity) mhc1-uba10.2*	(E.-S. Edholm et al., 2015)
11	*mhc1-uba11*	*mhc1-uba11.L (Cancer biology)*	(Haynes-Gilmore et al., 2014)
12	*mhc1-uba12*	*unidentified*	
13	*mhc1-uba13.1-13.5*	*mhc1-uba13.1, mhc1b-uba13.5.L*	
14	*mhc1-uba14*	*mhc1-uba14 (Strict expression in the intestine - mucosal immunity?)*	*Edholm* et al. *(unpublished data)*
16	*mhc1-uba16.1-16.4L*	*unidentified*	
17	*mhc1-uba17.L*	*unidentified*	

2.3. Major histocompatibility complex class II (mhc II)

Three MHC class II beta (DAB, DBB, & DCB) sequences were previously published (Sato et al., 1993; Liu et al., 2002), but only two MHC class II alpha (DAA & DBA) sequences were identified (Liu et al., 2002). Although Liu et al. predicted the presence of the third alpha gene (DCA), they were not able to sequence it and thus, the status of DCA remained unconfirmed. Similar to MHC, these names are consistent with the nomenclature (Ballingall et al., 2018). Here, the first letter D designates MHC class II, followed by different gene (A, B, or C), then alpha or beta chain (A or B). During the examination of v10 *Xenopus* genomes, we found all three genes for both MHC class II alpha and beta chains in the *X. laevis* genome. All alpha and beta gene sets are syntenic in a head-to-head (DAA/DAB; DBA/DBB) or head-to-tail (DCA/DCB) orientation.

DBA and DBB are somewhat separated with non-coding RNA and other hypothetical protein gene models. Interestingly, all three gene sets are mapped to Chr8L, but none are mapped to Chr8S. This is consistent with early studies showing diploidization of the MHC class II genes (Session et al., 2016). In the *X. tropicalis* genome, there is only one set of alpha and beta genes, which we have provisionally named *mhc2-daa* and *mhc2a-dab*. We infer that the MHC class II genes in *X. laevis* and X. *tropicalis* are related and likely paralogues derived from a common ancestor by duplication. However, we were not able to establish immediate orthology between three *X. laevis* genes and one *X. tropicalis* gene. Thus, *mhc2-daa.L* from *X. laevis* and *mhc2-daa* from *X. tropicalis* are not orthologues. A similar case is also true for *mhc2-dab* from both species. We also assessed the non-classical MHC class II genes, which we provisionally named *mhc2-dma* and *mhc2-dmb*. Although we have previously identified these genes in the earlier versions of genome assemblies (Ohta et al., 2006; Session et al., 2016), we found that the gene models for *mhc2-dma.S* and *mhc2-dmb.S* are erroneously fused into a single gene model in the v10 *X. laevis* genome annotation (Supplementary Table S1).

3. Example of immune genes duplication

3.1. IL-2 and IL-2R

In mice and humans, IL-2 is an *αα*-helical bundle cytokine (Taniguchi et al., 1983; Shaw et al., 1978), which is a key regulator of T cell proliferation, development, helper and cytotoxic activities (Reviewed in Malek, 2008; Ross and Cantrell, 2018). IL-2 activity is mediated through binding to its high affinity cytokine receptor expressed at the membrane of activated T cells (Cantrell and Smith, 1983). The high affinity IL-2 receptor (IL-2R) is formed by three transmembrane subunits referred as IL-2R*αα*, β, *γγ* chains also known as CD25, CD122 and CD132, respectively (Sharon et al., 1986; Stauber et al., 2006; Sugamura et al., 1992; Wang et al., 2005). CD122 and CD132 confer the IL-2 cell signal transduction (Malek, 2008; Ross and Cantrell, 2018). CD122 is shared with IL-15, and that CD132 is shared with other *αα*-helical bundle cytokines including: IL-4, 7, 9, 15 and 21 (Reviewed in (Leonard et al., 2019; Rochman et al., 2009). CD122 and CD132 have β-sheet structure and are prototypical members of the class I cytokine receptor superfamily (Wang et al., 2005). In contrast, CD25 contains two "sushi'' domains (Wang et al., 2005; Stauber et al., 2006). Importantly, IL-15 also use a receptor with "sushi" domain. CD25 and IL-15R*αα* chain genes are closely associated in gene tandem in humans and mice (Anderson et al., 1995; Olsen et al., 2007).

In non-mammalian vertebrates including *X. laevis,* IL2-like factor has been functionally characterized upon in *in vitro* T cell proliferation (Haynes and Cohen 1993; Watkins and Cohen 1987). Accordingly, *il2* genes, distinct from *il-15* genes were found on both homolog chromosomes 1 in *X. laevis* (Supplementary Table S1). Similarly, highly conserved *il2* gene synteny across all jawed vertebrates has allowed the identification of *il2* gene orthologs (Wang et al., 2021; Venkatesh et al., 2014). In different ray-fined fish species *il2* orthologs have been shown to stimulate *in vitro* and *in vivo* T cell differentiation, proliferation, and effector function as in mammals (Buonocore et al., 2020; Díaz-Rosales et al., 2009; Mu et al., 2018; Wang et al., 2021). Considering the IL-2R in *X. laevis*, gene orthologs encoding CD132 and CD25 chains (distinct from IL-15R*αα*) were found as singleton on the chromosome 8L and 3S, respectively (Supplementary Table S1). In contrast, gene orthologs encoding CD122 and IL-15R*αα* chains were found on both homoeolog chromosomes (Supplementary Table S1). Interestingly, lobe-finned, ray-finned and cartilaginous fish have a single copy of the structurally closely related IL-15R*αα* and CD25 genes, which were referred to as IL-2/15R*αα* (Mu et al., 2021; Wang et al., 2021). It is postulated that fish IL2-transduction is mediated by the heterotrimer composed of IL-2/15R*αα*, CD122 and CD123 (Mu et al., 2022; Wang et al., 2021). Together, these data suggest that IL2/IL2R signaling and function are evolutionarily conserved in jawed vertebrates. Nevertheless, the significance of a tetrapod specific CD25 and the tandem duplication of IL-2R*αα* remain to be investigated.

3.2. Interleukin 9 (il9)

First identified in mice as P40, stimulator for T cell growth (Van Snick et al., 1989), and as a hematopoietic growth factor in humans (Van Snick et al., 1989; Yang et al., 1989), interleukin 9 (*il9*) is involved in regulation of hematopoietic cells through the activation of the JAK/-STAT pathway (Noelle and Nowak, 2010). Despite having a gene page in NCBI for *X. tropicalis*, and a page for a *X. laevis il9*-like gene, Xenbase lacked pages for any *il9*-related gene. A comparative analysis of the synteny, as well as protein blast (BLASTp) searches using the RefSeq *X. tropicalis* sequences confirmed the *X. tropicalis* gene model as an *il9* ortholog. A Xenbase page was generated for this gene using the NCBI gene data.

The *X. laevis* interleukin-9-like gene found in NCBI (LOC121401224) is a singleton on the 3L chromosome and despite being highly syntenic with the *il9* genes in *X. tropicalis*, *Homo sapiens* and other amniotes, the five-exon structure of *il9*, found in all other species examined, was not observed in *X. laevis*. Instead, the first two exons appear to have been lost, and the gene begins with an extended version of the third exon. The loss of these exons was confirmed by nucleotide blast (BLASTn) searches of the *X. laevis* genome using the RefSeq *X. tropicalis* mRNA sequences, as well as by using the RefSeq *X. tropicalis* protein sequences to search a translated *X. laevis* genome nucleotide database (tBLASTn). These searches found no similarity in the *X. laevis* genome to the first two exons of the *X. tropicalis* gene at either the nucleotide or amino acid level. Despite these differences, the *X. laevis* gene is considered the ortholog of *X. tropicalis il9*, and was included on the *il9* Xenbase gene page XB-GENEPAGE-25874677, with its former gene symbol il9l recorded as a synonym.

3.3. Interleukin 18 (il18)

The interleukin 18 gene is found on chromosome 7 in *X. tropicalis* and both chromosome 7L and chromosome 7S in *X. laevis*. The *il18.L* homeolog (GeneID: 121,395,553) is most similar to *X. tropicalis il18*, however, was named interleukin 18–like. On chromosome 7S we found tandem gene models for two versions of the gene, both named 'interleukin 18'. These two '*il18.S*' genes are very similar to each other, suggesting a recent duplication on this chromosome, and interestingly, show greater divergence from the *X. tropicalis* gene than the *X. laevis il18.L* gene (Supplemental Fig. S3). Our phylogenetic analysis also supports this. Applying *Xenopus* gene nomenclature rules, the longest gene model (GeneID: 108697612) was placed on the *il18* gene page (XB-GENEPAGE-876700), and the shorter duplicated gene (GeneID: 108697611) will be provisionally named 'interleukin18, gene 2S homeolog' with gene symbol *il18.2.S* and placed on a unique Xenbase gene page.

4. Example of immune gene sub-functionalization

4.1. CXCL8 of chemokines

CXCL8 (interleukin-8, IL-8) is an important inflammatory CXC chemokine, first discovered in mammals for its role in the chemotaxis of neutrophils (Baggiolini et al., 1989). CXCL8-mediated neutrophil recruitment is conferred through CXCL8 binding to the G protein-coupled CXC chemokine receptor 1 (CXCR1, CXCL8Rα) or CXCR2 (CXCL8Rβ) (Kulbe et al., 2004). Most mammals encode single CXCL8 (IL-8) chemokine genes, which belong to a group of chemokines possessing the Glu-Leu-Arg (ELR) motif (Rot and Von Andrian, 2004). Like CXCL8, the other members of this chemokine group also facilitate the recruitment of neutrophils through binding to the CXCR1 (IL-8 and CXCL6/GCP-2) and/or CXCR2 (all ELR + chemokines) chemokine receptors (Rot and Von Andrian, 2004). Notably, rodents lack a direct homolog of CXCL8, but encode CXCL1/KC, CXCL2/MIP-2 and CXCL5-6/LIX, which are thought to be functional paralogs to CXCL8 (Souza et al., 2004; Sekido et al., 1993; Belperio et al., 2005). While none of these rodent chemokines are direct homologs of CXCL8, they belong to the same cluster of closely related chemokines associated with neutrophil recruitment (Zlotnik and Yoshie, 2000). Conversely, except for Gadiformes (cod, haddock), bony fish CXC chemokines generally lack an ELR motif, instead possessing X (other residue)-Leu-Arg (XLR, DLR in salmonids) motifs (Hebert et al., 1991). CXCL8 genes have been identified across a range of bony fish species, with many species encoding multiple CXCL8 isoforms (de Oliveira et al., 2013).

X. laevis encodes multiple CXCL8 isoforms (Supplementary Table S1), with one/some forms possessing an ELR motif (*cxcl8a*), while others (*cxcl8b*) lacking it (Hauser et al., 2020; Koubourli et al., 2018) (see CXCL8 alignment in Fig. 2). Functional studies suggest that while the *X. laevis* CXCL8a is involved in inflammatory responses, the CXCL8b may have additional roles in the recruitment of neutrophil/granulocyte subset(s) with immunosuppressive/repairing functions (Hauser et al., 2020; Koubourli et al., 2018). Both the *X. laevis* CXCL8a and CXCL8b appear to utilize the CXCR1/2 receptors, although the CXCR8b may depend more heavily on CXCR2, at least for some of its functions (Koubourli et al., 2018). It appears that in addition to the functionally characterized CXCL8a and CXCL8b chemokines, *X. laevis* encodes multiple forms of what are presently denoted as *cxcl8a* (*cxcl8a1* and *cxcl8a2*) and *cxcl8b* (*cxcl81* and *cxcl8b2*) (Fukui and Matsunami, 2021). We anticipate that these respective *X. laevis cxcl8* gene products are functionally reminiscent of the mammalian CXCR1/CXCR2 ligands, wherein they may each have distinct receptor binding preference and potentially have at least partially non-overlapping roles in amphibian immunity and physiology.

A detailed annotation of *cxcl8 Xenopus* genes and further research into the functional roles of these different isoforms will grant new perspectives into the evolution of vertebrate neutrophil biology and leukocyte chemotaxis.

4.2. Interferons (IFNs) among cytokines

The interferon (IFN) system is one of the cytokine complexes that has evolved and diversified in tetrapod when compared to bony and cartilaginous fishes. The functional and molecular diversity in the amphibian IFN system makes it an ideal model to study its role in immunity, especially in terms of immune regulation against intracellular pathogens such as viruses. Previous analyses of the *Xenopus* genomes revealed that

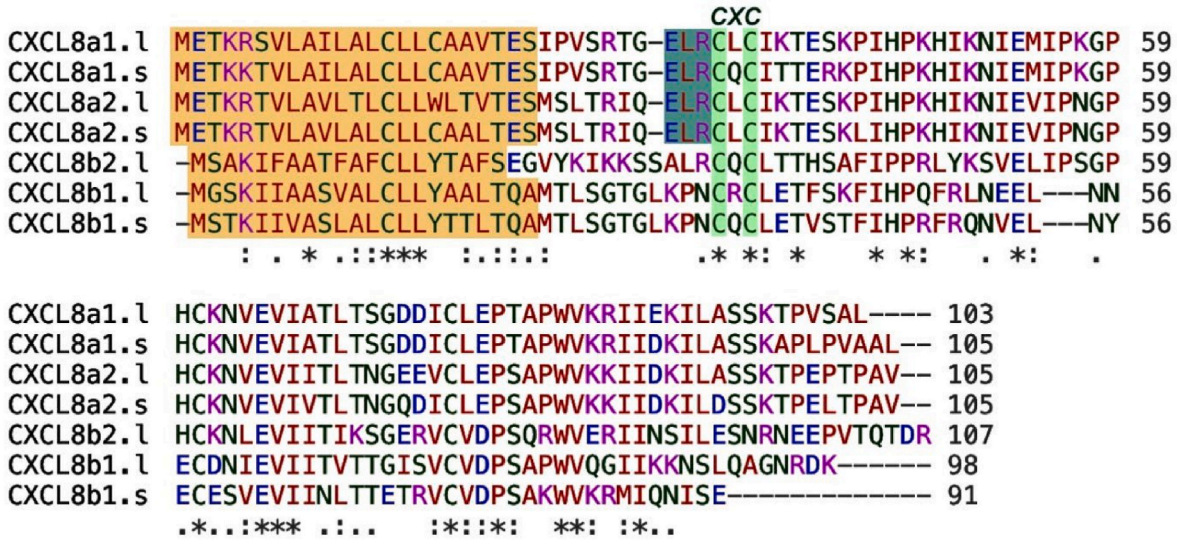

Fig. 2. CXCL8 protein alignment. The protein alignment was performed using ClustalW2 server. Fully conserved residues are indicated by an asterisk (*), partially conserved and semi-conserved substitutions are represented by ":" and ".", respectively. Putative signal peptides are highlighted in beige, the ELR motif is boxed in blue and the conserved CXC motif is highlighted in light green. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

the *Xenopus* IFN complex has 37 intronless IFN-like genes (including 4 near intronless IFN-like genes retaining a single <50 bp short intron) and 3 pseudogenes in *X. tropicalis*, while 26 intronless IFN-I genes (including 8 with a short remaining intron and 4 pseudogenes) were identified in the *X. laevis* genome. The v10 *Xenopus* genome assemblies contain intronless IFNs as well as four types of novel intron-containing *ifn* genes (IFN-I to -IV) not revealed in previous studies (Sang et al., 2016). The current repertoire of *ifn* gene loci in *X. tropicalis* has been updated including the co-existence of 14 intron-containing IFNs (7 *ifn*-I, 1 *ifn*-II, and 6 *ifn*-III) and the expansion of 37 intronless *ifn*-like genes (36 *ifn*-I and 1 *ifn*-III). In *X. laevis,* the system includes 18 intron-containing (7 *ifn*-I, 1 *ifn*-II, 9 *ifn*-III and 1 *ifn*-VI) and 24 intronless *ifn*-coding genes (22 *ifn*-I and 2 *ifn*-III) (Tian et al., 2019).

A recent comparison of IFN gene families across 120 vertebrate genomes demonstrated that after originating in fish, IFN genes diversified, including the emergence and deletion of specific IFN subtypes across distinct vertebrate classes and species (Sang et al., 2016; Shields et al., 2019). Our analysis supports the hypothesis that there was an early emergence of intronless IFN-I and IFN-III genes in amphibian species but not reptiles (Tian et al., 2019) s. Mammals have well-characterized IFN-I gene subtypes, which includes *ifn*-α, *ifn*-β, *ifn*-δ, *ifn*-ε,-κ, IFN-τ, *ifn*-ω, and ζ, of which *ifn*-α, *ifn*-β, *ifn*-ε and *ifn*-κ are encoded by all mammals, while *ifn*-δ, *ifn*- κ, *ifn*-τ, *ifn*-ω, and *ifn*-ζ occur in a species-specific manner (Ivashkiv and Donlin, 2014; Levy et al., 2011; Tian et al., 2019). Even greater *inf* diversity is found in some amphibians. Our analyses of 19 amphibian species indicate that more than half carry encoding intronless *ifn-coding* genes of either *ifn*-I and/or *ifn*-III types (Supplementary Table S1). Moreover, 10 species harbor as many as 36 intronless *inf*-I genes, and up to 14 *ifn*-III genes (Gan et al. ,2017, 2018; Sang et al., 2016; Tian et al., 2019; Adeyemi et al., 2022 and this issue).

Previous phylogenetic analysis of 10 intronless *Xenopus ifn*-I genes supported the hypothesis that amphibian IFN genes are closely related to intronless *ifn*-I genes of tetrapod, yet represent the earliest intronless genes of tetrapod *ifn*-I/III genes that have been identified since they clustered close to mammals and reptiles. The other *Xenopus* intronless *ifn*-I genes clustered close to fish intron-containing *ifn* genes, highlighting their phylogenetic proximity to these primitive ancestor genes (Sang et al., 2016; Shields et al., 2019; Tian et al., 2019). These analyses also revealed several *Xenopus ifn*-I genes that were found to contain a single short intron (<50 bp). These genes are predicted to be potential intermediates as *ifn* genes in the process of becoming intronless (Shields et al., 2019; Tian et al., 2019). In this annotation jamboree, we found that the *X. laevis* v10.1 genome harbors 18 intron-containing gene models (7 *ifn*-I, 1 *ifn*-II, 9 *ifn*-III, and 1 *ifn*-IV) and 18 intronless *ifn*-coding gene models (16 *ifn*- I and 2 *ifn*-III).

4.3. IFN regulatory factors (IRFs)

Interferon regulatory factors (IRFs) are a family of transcription factors that regulate several facets of innate and adaptive immune responses, hematopoietic differentiation, and immunomodulation. Vertebrate IRFs play vital roles in the regulation of IFNs and IFN-stimulated gene expression (Harada et al., 1989). As revealed in studies of humans and mice, IRFs are critical in mediation of IFN signaling and other immune responses. However, the molecular function of most of the IRFs has not been well established in non-mammalian vertebrates such as fish, amphibians, and reptiles (Hu et al., 2022). Structurally, IRFs contain a conserved N-terminal region of about 120 amino acids, which when folded binds specifically to the IRF-element (IRF-E) motifs within the promoter region of IFN responsive genes (Weisz et al., 1992).

There is a total of eleven IRF genes/proteins in *Xenopus* (*irf1* to *irf11*), which fall into four subfamilies based on molecular architecture and phylogenetic relationships: the IRF1 group (*irf*1, 2, and 11); the IRF3 group (*irf*3 and 7); the IRF4 group (*irf*4, 8, 9, and 10); and the IRF5 group (*irf*5 and 6). In the human genome, nine IRF genes/proteins (IRF1 to IRF9) have been identified, and orthology between *Xenopus* and human was assessed in this study.

Previous genomic and phylogenetic analyses suggest that the IRF transcription factors appeared and rapidly diversified early in vertebrate evolution, with all IRF subfamilies present in *X. laevis* and *X. tropicalis* (Huang et al., 2010). Our syntenic and phylogenetic analyses support the orthology between human and *Xenopus irf1-irf9* genes. Our annotation updates to the *Xenopus irfs* include new Xenbase gene pages with corresponding gene IDs provided by NCBI (Supplementary Table S1).

4.4. FLT3 and its ligand FLT3LG

The *fms related* tyrosine kinase (FLT3), also referred as CD135 or Fltk2, is a class III receptor tyrosine kinases (RTK-III). RTK-III members include KIT - KIT proto-oncogene, receptor tyrosine kinase, colony-

stimulating factor-1 receptor (CSF1R), and platelet-derived growth factor receptors (PDGFR) alpha and beta (Verstraete and Savvides, 2012). RTK-III molecules are characterized by an extracellular, ligand-binding region that is divided into 5 Ig-like domains, a single transmembrane domain, and a cytoplasmic region formed by the regulatory juxtamembrane, and the tyrosine kinase domains (Verstraete and Savvides, 2012). CSF1R, KIT, and their ligands (which are dimeric short-chain α-helical bundles) are all critically involved in hematopoiesis. FLT3 activation by the binding of its ligand FLT3lg (also known as FL, which is recognized as the only known ligand for FLT3) regulates the proliferation, differentiation, and survival of lymphoid and myeloid progenitors (Kazi and Rönnstrand, 2019; Tsapogas et al., 2017). FLT3 and FLT3lg also control the development and homeostasis of dendritic cells (DC), which are major innate immune cells in mammals (Liu et al., 2021). In addition to the importance of FLT3 in pro-B cell maturation, FLT3 has been shown to be involved in B-cell activation including class-switch recombination (Svensson et al., 2015).

RTK-III members arose from the two round of whole genome duplications that occurred after the lamprey/Gnathostomata split (Brunet et al., 2016). *Flt3* orthologs are, therefore, found across all jawed vertebrates. In a recent study (Paiola et al., 2022), we have shown that in *X. laevis* both gene homologs have been conserved; whereas, Teleostei conserved only one *flt3* gene ortholog despite a third whole genome duplication. *Flt3lg* gene orthologs, based on blast-, and synteny-based searches were found across all jawed vertebrates except *Actinopterygii*. The two *flt3lg* genes were also conserved on *X. laevis* homolog chromosomes. Predicted 3D structure and tissue expression during ontogenesis suggest that *flt3/flt3lg* gene homologs have sub-functionalized. We produced tagged recombinant FLT3lg.S and.L, demonstrating that these ligands bind to FLT3.S and.L *in vitro* and can trigger a transient phosphorylation of Erk1/2. Notably, we found that FLT3 is expressed at the surface of DC-like cells and IgM + B-cells in the spleen of *X. laevis.* This is similar with the DC-like cells of chickens, the Atlantic cod, and human cDC (Guslund et al., 2020; Wu et al., 2022). Investigating *flt3/flt3lg* homologs sub-functionalization in primordial *X. laevis* dendritic cells will contribute to untangle the complex biological role of FLT3/FLT3lg.

5. Concluding remarks and future perspectives

Xenopus immunogenomics analysis is important for comparative immunology as well as for modelling human diseases linked to mutations of those genes. In this review, we summarized the results of combined work from different research laboratories that are working on the field of comparative immunology. Furthermore, to date we managed to improve the representation for hundreds of immune genes on Xenbase (Supplementary Table S1). Even though sequencing technology has rapidly grown, hindrances are still encountered. Indeed, there are still immune gene models that need to be validated by a more detailed bioinformatic analysis. Newly developed sequencing technology known as direct RNA-Seq has the ability to sequence RNA sequences, especially transcripts without the need to convert them to DNA as former technologies (Alfonzo et al., 2021). Sequencing of RNA will give us information regarding alternative splice sites, isoforms as well as some transcriptional modifications that either promote or inhibit translation of transcripts. This approach will assist comparative immunology studies as well as disease phenotype analysis linked to mutated genes whose transcript sequences are not available or incomplete. Besides the genomic analysis of *Xenopus* species and its importance in dissecting evolutionary events, immune genes analyses could contribute to preservation and well-being of amphibians. Amphibian populations worldwide are undergoing major declines and infectious diseases such as chytridiomycosis and ranavirosis are critically contributing to this decline (Lips et al., 2006; Robert et al., 2017). Immunogenomics holds promise to be elucidating why frogs are particularly sensitive to these pathogens.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.dci.2023.104734.

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